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Seed Esterases, Leucine Aminopeptidases and Catalases of Species of the Genus *Gossypium*[1]

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Summary. Polyacrylamide and starch gel electrophoresis were used to analyze the isozyme makeup of three enzyme systems (esterases, leucine aminopeptidases and catalases) from the dormant seeds of twenty-nine species within the genus *Gossypium*.

Isozyme variation was observed for all three enzymes between the species of the different genome groups. The within species polymorphism noted for the esterases was not observed for the leucine aminopeptidase and catalase patterns. In general, only minor qualitative banding pattern differences distinguished the A and B genome species, whereas, band variations were greatest between the more distantly related species in the C, D and E genomes. *Gossypium longicalyx* (F genome) showed an overall banding pattern unique to itself. The species of the genomes (C, D, E and F) removed from the postulated area of genetic origin (Southern Africa) also exhibited greater isozyme variability than that of the wild species of the A and B genomes, both located in Southern Africa.

Synthetic mixtures of seed extracts from parent species of recently formed synthetic allopolyploids produced additive isozyme patterns for esterase, leucine aminopeptidase and catalase that were closely comparable to the zymograms produced by their hybrids. In contrast all three enzyme systems showed significant qualitative isozyme variations between the three natural allotetraploids, *G. tomentosum*, *G. barbadense* and *G. hirsutum* when compared to the zymograms of the synthetic mixtures of their alleged parental forms.

Introduction

Isozymes, as separated by the technique of electrophoresis, are forms of enzymes which catalyze similar reactions but differ in molecular composition (Markert and Moller, 1959), and are under genetic control (Scandalios, 1969). Numerous electrophoretic analyses have shown that species differ from one another in band frequencies.

The genus *Gossypium* is comprised of about 30 diploid and three natural allotetraploid species. The diploid species are divided into six genome groups, A through F. The three allotetraploid species contain genomes A and D (Beasley, 1942). Analysis of the genus *Gossypium* by means of polyacrylamide gel electrophoresis of proteins obtained from dormant seeds has been reported by Cherry et al. (1970; 1971) and Johnson and Thein (1970). Preliminary studies concerning the esterase isozyme composition of species from the genus *Gossypium* have been presented by Cherry and Katterman (1971a, b). The chemotaxonomic comparisons of the protein and esterase patterns for species within and between genomes gave supportive data to the present classification of *Gossypium* and the origin of the natural allotetraploids. In addition, the esterase studies showed that much variation in isozyme patterns existed within a species. This isozyme polymorphism suggested that intraspecific variation should be evaluated before any interspecific analyses were undertaken. A more extensive examination of the isozyme composition of the three enzyme systems, esterases, leucine aminopeptidases and catalases present in the dormant seeds will be described in this communication.

Material and Methods

The wild diploid and natural allotetraploid species analysed are listed in Figure 2. The following types were also included in this study: three varieties of *G. herbaceum*, the wild type, var. *africanum* (G304), and two cultivated types, AG152 and AG153; five varieties of *G. arboreum*, AG109, G10, G266 and G24; the synthetic allotriploid F_1 hybrid of *G. hirsutum*, var. Acala 44-10-1 × *G. sturtianum* and its colchicine produced hexaploid (Experimental 6×-3); four varieties of *G. barbadense*, Pima S × P, Pima S-2, Menoufi and Sea Island; and 15 varieties of *G. hirsutum*, Acala 44-10-1, Acala 1517A, Stoneville 7A, Mexican Big Boll, Hibred, Paymaster 54B, Kechi, Lockett 4789, CB3051 (Yugoslavian strain), Macha, Clevewilt, King 82, Contextum, Rowden and Hopi Sacaton. The geographical areas of seed collection for *G. thurberi* were described by Cherry and Katterman (1971a).

Twenty-four seeds (random samples of greenhouse and field grown plots) of each species and variety were used for the esterase analysis by polyacrylamide gel electrophoresis. Only two seeds were available for study of the species *G. robinsonii*. For the study of esterase activity of *G. thurberi*, 268 seeds were used (Cherry and Katterman, 1971a). The analysis of leucine aminopeptidase and catalase activity on starch gel electrophoresis involved 12 seeds from each species and variety.

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All procedures were carried out at 0–4 °C. Each seed was ground by means of a mortar and pestle in 0.4–0.9 ml of tris-glycine buffer (0.1 M; pH 8.3). The crude extracts were partially purified by centrifugation for 10 min at 4300 xg in a Sorvall refrigerated centrifuge. The supernatant fraction was then submitted to electrophoresis. A Canalco Model 1200 Bath electrophoretic apparatus and a Beckman Model Rd-2 Duostat power source were used. The methods of preparation of the polyacrylamide gels and conditions of electrophoresis were discussed by Steward et al. (1965) and Cherry et al. (1970). A homemade starch-gel electrophoretic apparatus was constructed following the procedures of Brewbaker et al. (1968). A Heathkit IP-32 power supply unit was used. The methods of preparation of the starch gel and the analysis of the samples were also similar to the techniques developed by these latter authors. Esterase activity was examined following the procedures outlined by Cherry and Katterman (1971a). The staining procedures for leucine aminopeptidase and catalase were those of Brewbaker et al. (1968).

The gels were photographed in bright sunlight in Petri dishes containing 50% methanol on a diffuse white background illuminated from beneath with a fluorescent lamp. The photographed gels were increased in size to 10.5 cm. The drawings in this manuscript were copied directly from these photographs. The bands of different species were paired according to the marker band and the origin. The exact position of the bands within the zymograms was determined by the method of Cherry (1971).

Results and Discussion

Analysis of the Genome Groups of the Genus Gossypium

Comparison of esterase banding patterns between the varieties of the two A genome species shows that the same group of isozymes is present (Fig. 1). The differences between varieties are based on the presence or absence of certain bands. Overall, a small increase in banding complexity is observed in the varieties of *G. arboreum* when compared to the varieties of *G. herbaceum*. The increase in banding complexity observed in the varieties of *G. arboreum* could be due to within species variability not yet detected in *G. herbaceum*. This possibility may also apply to the isozyme variation observed in the other species comparisons made in this investigation. No variations in the banding patterns of leucine aminopeptidase and catalase are observed between the varieties of the A genome species (Figs. 2 and 3). These two species have long histories of cultivation in Africa and Asia and are closely related (Hutchinson, 1954).

Comparison of the esterase isozyme patterns between the species of the B genome indicate qualitative differences of minor bands (light staining bands, Fig. 1). However, no such differences are distinguish-

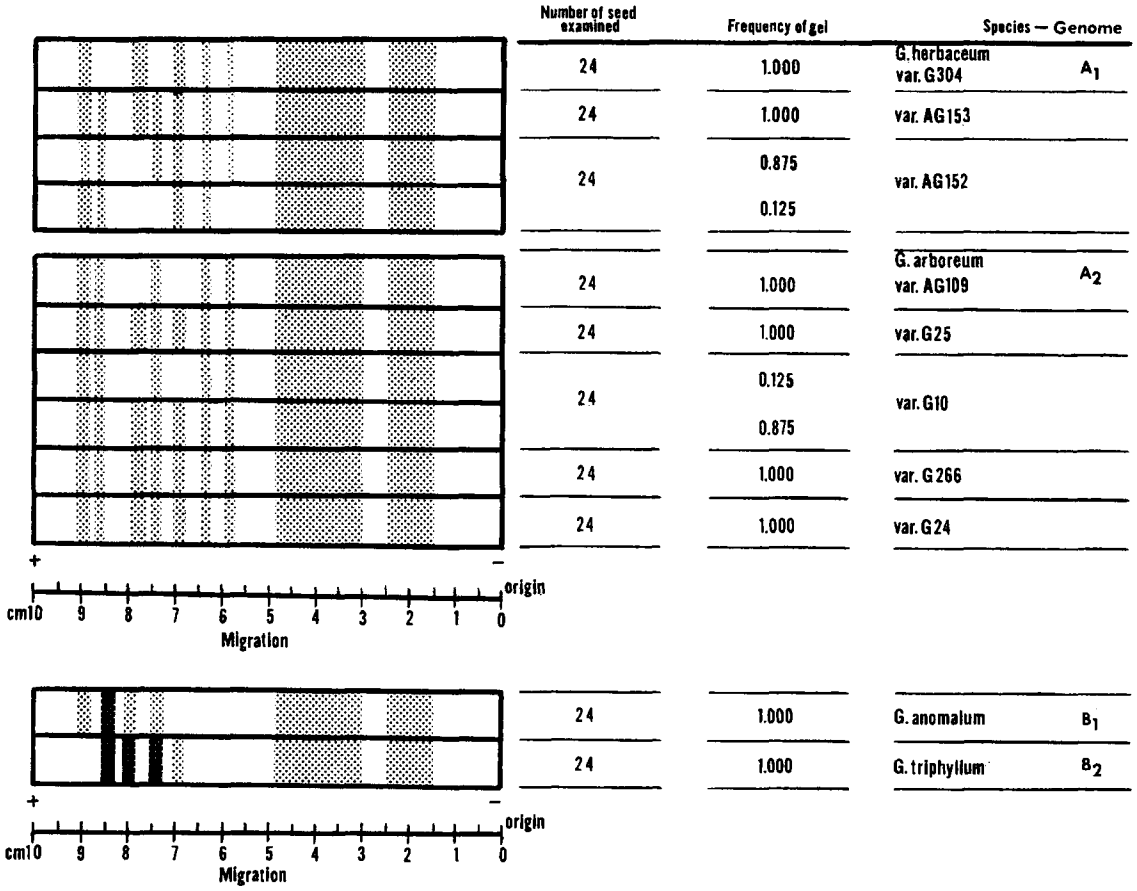

Fig. 1. Polyacrylamide gel electrophoretic esterase spectra of four and six varieties of the A_1 and A_2 genomes, respectively and of two species within the B genome.
Included are the number of seed examined and the frequencies of the banding patterns

able in the leucine aminopeptidase and catalase zymograms of these species (Fig. 2 and 3).

Fryxell (1965), using morphological, geographical and cytological analyses in a report on the species of the C genome showed that *G. robinsonii* and *G. sturtianum* were closely related. *G. bickii* and *G. australe* were also observed to be more related to each other than to the other species of the C genome. Only the absence of a minor esterase band in the lower frequency gel of *G. sturtianum* var. *nandewarense*, partially distinguishes the varieties of *G. sturtianum* from *G. robinsonii* (Fig. 4). However, due to the limited supply of seed for *G. robinsonii*, the intraspecific isozyme variation of this species could not be fully evaluated. No differences are observed in the leucine aminopeptidase and catalase activity of these two species (Fig. 2 and 3). Both *G. australe* and *G. bickii* produced a number of esterase bands present

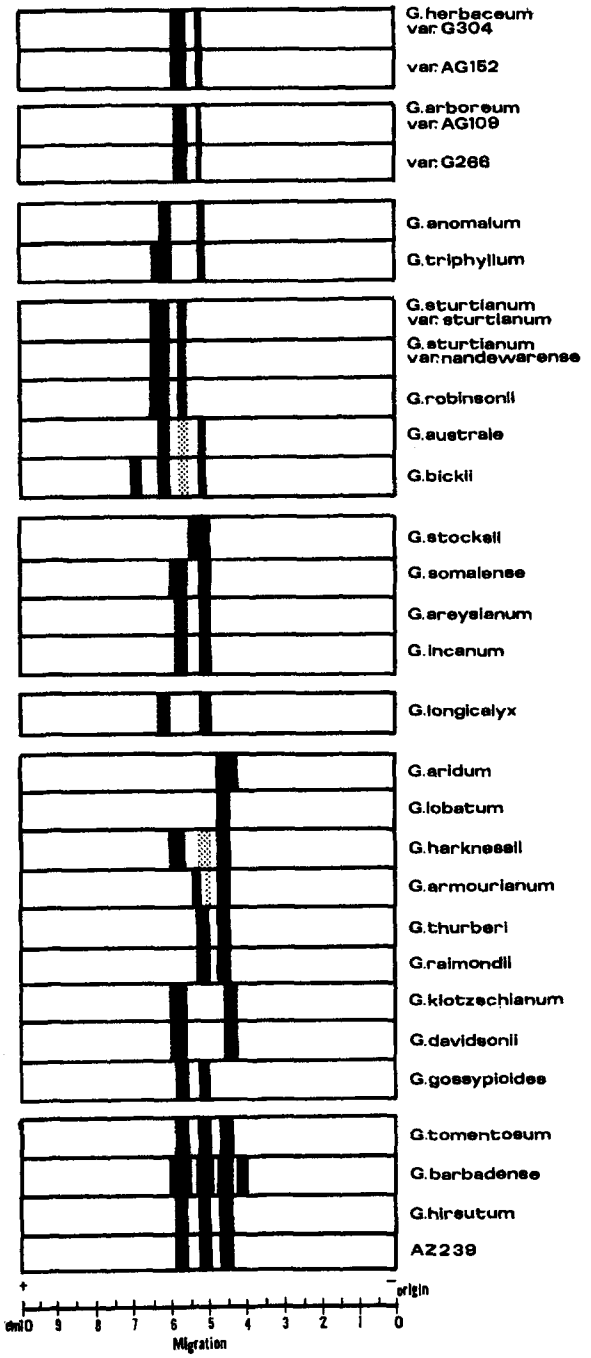

Fig. 2. Starch gel electrophoretic leucine aminopeptidase spectra of diploid and allotetraploid species of *Gossypium*

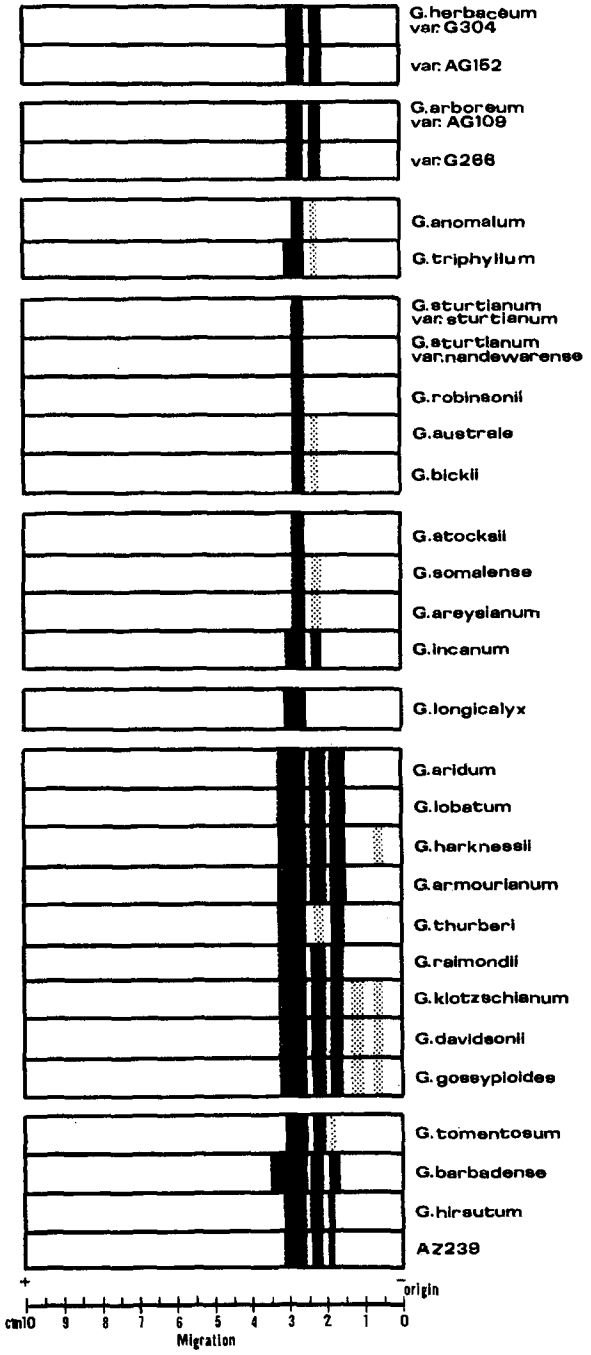

Fig. 3. Starch gel electrophoretic catalase spectra of diploid and allotetraploid species of *Gossypium*

in *G. sturtianum* and *G. robinsonii*. However, the former two species produce closely related esterase zymograms which are in general, distinct from the latter two species. Similar observations are noted for the leucine aminopeptidase and catalase zymograms.

As for the species of the E genome, the overall banding patterns of all three enzyme systems in *G. stocksii* and *G. somalense* do not match closely (Figs. 2, 3 and 4). The banding patterns of *G. areysianum* and *G. incanum* coincide more closely to each other than to the other species of the E genome. *Gossypium areysianum*, *G. incanum* and *G. somalense* in turn are more closely related in their banding patterns than to that of *G. stocksii*. This observation

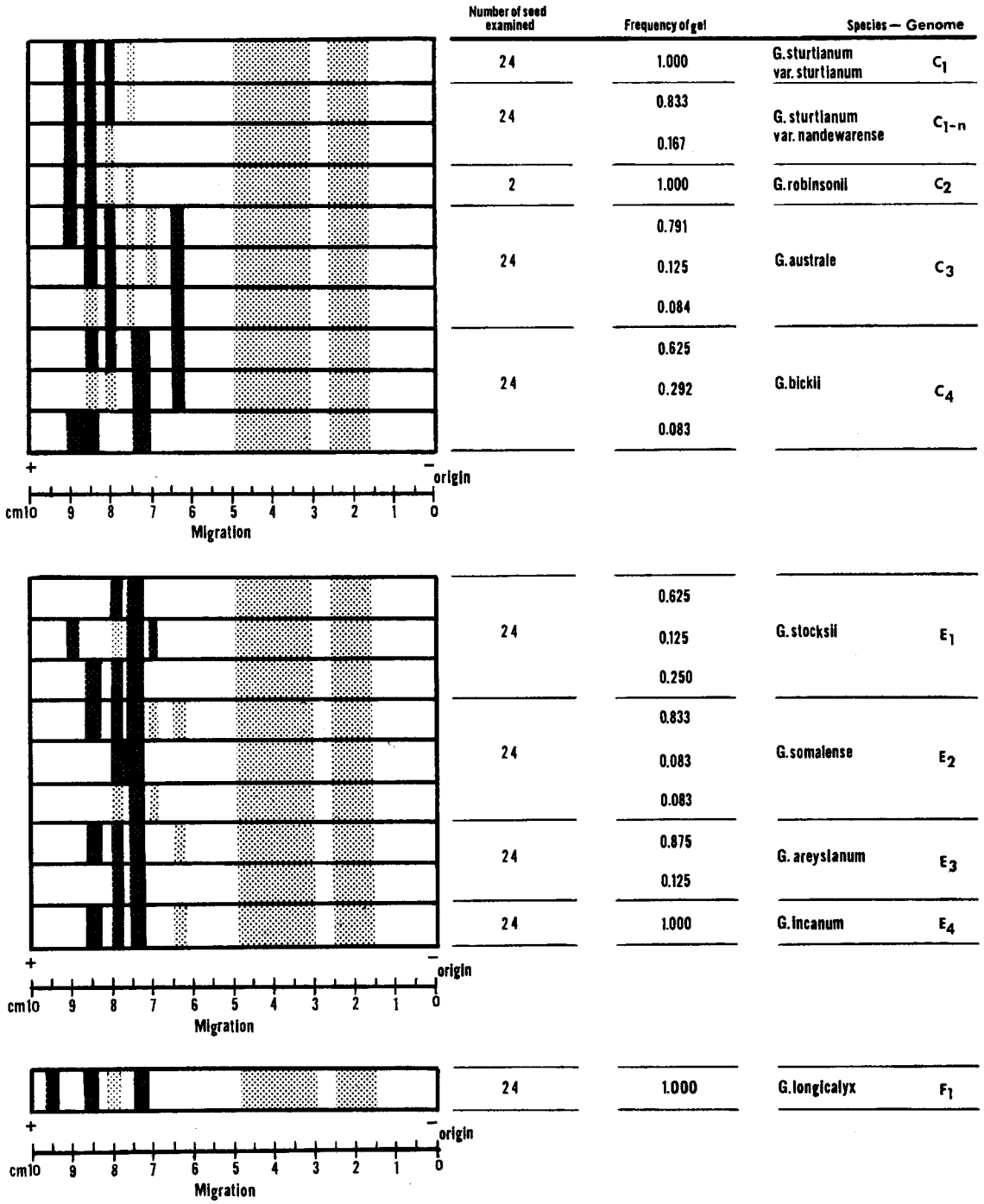

Fig. 4. Polyacrylamide gel electrophoretic esterase spectra of five species within the C genome, four species within the E genome, and one species from the F genome.
Included are the number of seeds examined and the frequencies of the banding patterns

is in agreement with the morphological data (Saunders, 1961). Overall, the isozyme patterns of the E genome are unique and more variable than those of the A, B, and C genomes.

The distinct banding patterns of *G. longicalyx* (F genome), i. e., the occurrence of major esterase activity (dark staining bands) at 9.5 cm and a major band of leucine aminopeptidase at 6.2 cm, which are not present in any species of the E genome, support the individuality of this species (Figs. 2, 3 and 4; Phillips and Strickland, 1966). Several bands of esterase, leucine aminopeptidase and catalase activity in *G. longicalyx*, however, have migration rates similar to those present within the E genome. Based on analyses of chromosome size and bivalent formation in a triploid hybrid (*G. hirsutum* × *G. longicalyx*)

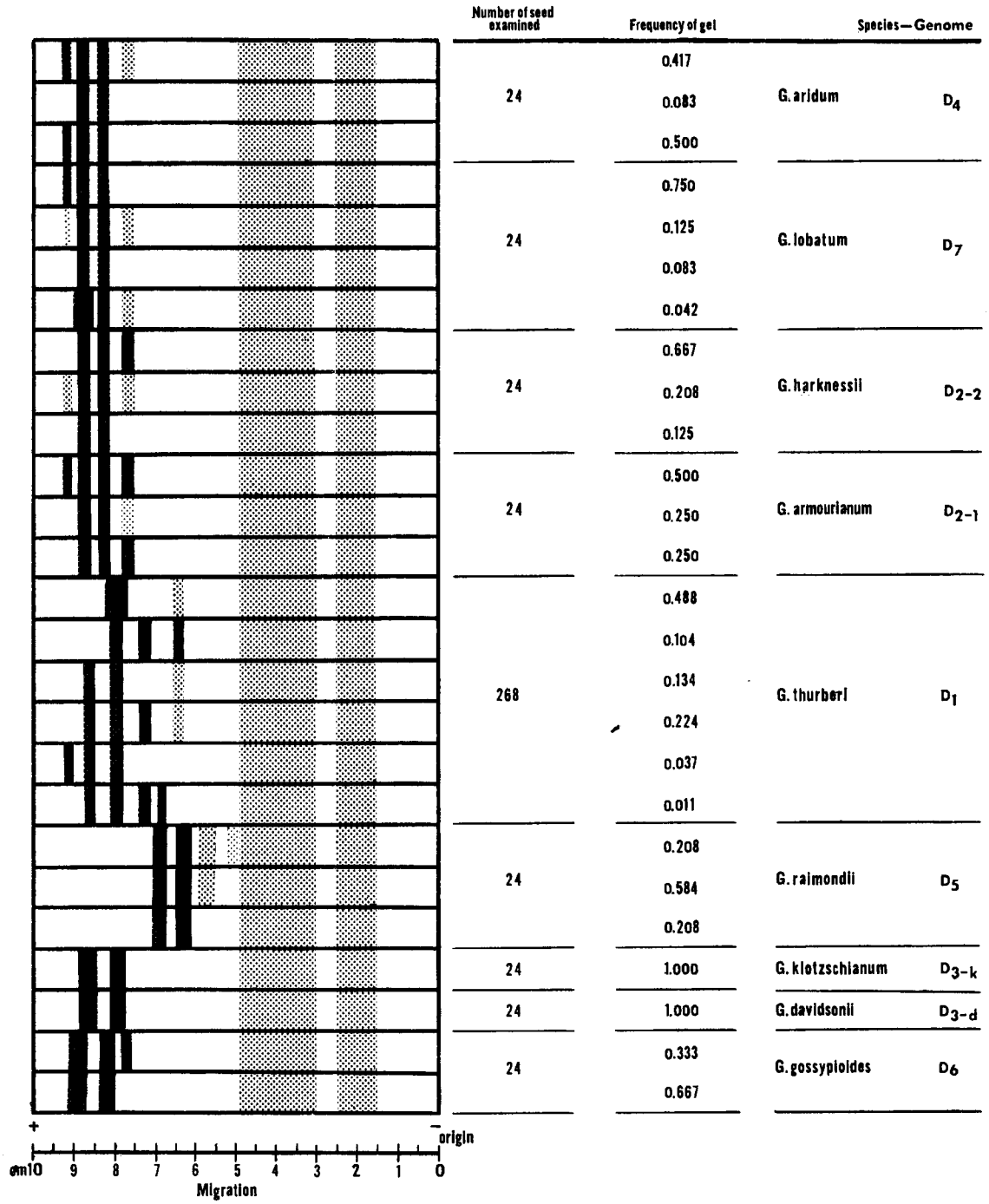

Fig. 5. Polyacrylamide gel electrophoretic esterase spectra of nine species within the D genome. Included are the number of seeds examined and the frequencies of the banding patterns

Phillips and Strickland (1966) pointed out that *G. longicalyx* was more related to the A genome than to the E genome. No such conclusions can be drawn from the enzyme data presented here.

Much variability of the esterase is observed within and between the species of the D genome (Fig. 5). Recently, Cherry and Katterman (1971a) showed the presence of six types of esterase zymograms for natural populations of *G. thurberi*. These zymograms occur at varying frequencies within and between the populations studied. This observation indicated that notable genetic variability exists within the species at the molecular level.

Examination of *G. thurberi* (268 seeds) indicates that intraspecific esterase variability would increase if more seeds were examined. However, if a large number of single seeds are examined from the genus as a whole as done in this investigation and all of these isozyme patterns are considered together, a very accurate picture of the taxonomic relationship between species can be developed (Shaw, 1965). In a number of cases, specific isozyme patterns occurred with a low frequency in one species and a high frequency in another whereas the reverse situation could be obtained with other isozyme patterns. For example, in *G. harknessii* a zymogram with three esterase bands occurs with a high frequency of 0.667. Within this species a zymogram with four esterase bands occurs in a lower frequency of 0.208. In *G. armourianum*, the former zymogram has a lower frequency of 0.250 and the latter is in highest frequency of 0.500. Also noted is that *G. aridum*, *G. lobatum* and *G. harknessii* each contain a zymogram in low frequency with two major esterase bands. This zymogram pattern however is present in higher frequency in *G. gossypioides*. A similar condition is noted with *G. stocksii* and *G. areysianum* of the E genome. These data indicated that possible different heteroallelic combinations coding for the esterases are present within the species of these genomes.

Endrizzi (1957) showed cytologically that *G. lobatum* and *G. aridum* were closely related. *Gossypium harknessii* was shown to cross freely with *G. armourianum* (Kearney, 1957). The highest chiasma frequency among the D genome species was produced in crosses between *G. klotzschianum* and *G. davidsonii* (Phillips, 1966). These comparisons are further supported by the isozyme patterns of the three enzyme systems; the more closely related the species are genetically, the more similar are their banding patterns. Hutchinson et al. (1947) indicated that *G. raimondii* was closely related to *G. thurberi*. This latter observation is supported by the leucine aminopeptidase activity and in part by the catalase isozyme patterns. However, the zymograms of esterase do not support this relationship. *Gossypium raimondii* appears to produce esterase isozymes unique from the other species of the D genome.

The above studies show that the species and/or varieties observed within each genome have banding patterns of enzyme activity that are more similar to one another than to members of other genome groups (Figs. 1–5). These results bear out the earlier conclusions of Cherry et al. (1970) as to the species relationship within and between these six genomes as developed from their electrophoretic protein data.

The Relationship between Species in Southern Africa to the Species Removed from this Postulated Center of Origin

Hutchinson et al. (1947) stated that the original ancestor of the species of the genus *Gossypium* probably arose in the southern periphery of South Africa. At the present time, species belonging to the four different genome groups, A, B, E and F, are located on the continent of Africa; species of two of these genome groups (A and E) have spread into Asia. In addition, one group of species developed in Australia (C genome) and another in America (D genome).

The species of the genomes (C, D, E and F) removed from the postulated center of origin (South Africa) tend to show an increase in the amount of major band activity over that produced by the A genome species *G. herbaceum* var. *africanum* and the B genome species, both of which occur in Southern Africa. Less catalase isozyme variability is present between the genome groups than that observed for esterase and leucine aminopeptidase. Examination of these two enzyme systems shows that the C, E and F genomes are made up of combinations of band mobilities from both the A and B genomes plus some additional bands. An increase in isozyme diversity is present within and between the species of the D genome over that of the other genomes of *Gossypium*.

Electrophoretic studies comparing the banding patterns of seed proteins showed an increase in complexity and variability in the species of those genomes located away from the postulated center of origin (Cherry et al., 1970). Similarly, the enzyme data support these observations.

Analysis of the Natural and Synthetic Allopolyploid Species and Varieties

Cherry et al. (1970) summarized the data derived from the classical techniques concerning the origin of the allotetraploids of the genus *Gossypium*. These classical data indicated that the allotetraploids were formed from a cross between species having genomes similar to *G. herbaceum* (A_1) and *G. raimondii* (D_5) followed by a spontaneous doubling of the chromosomal number.

Studies of enzyme zymograms of amphidiploids of plants showed that they compared closely to the additive isozyme patterns of the parent species when the latter were combined in a synthetic mixture (West and Garber, 1967; and Vaughn and Waite,

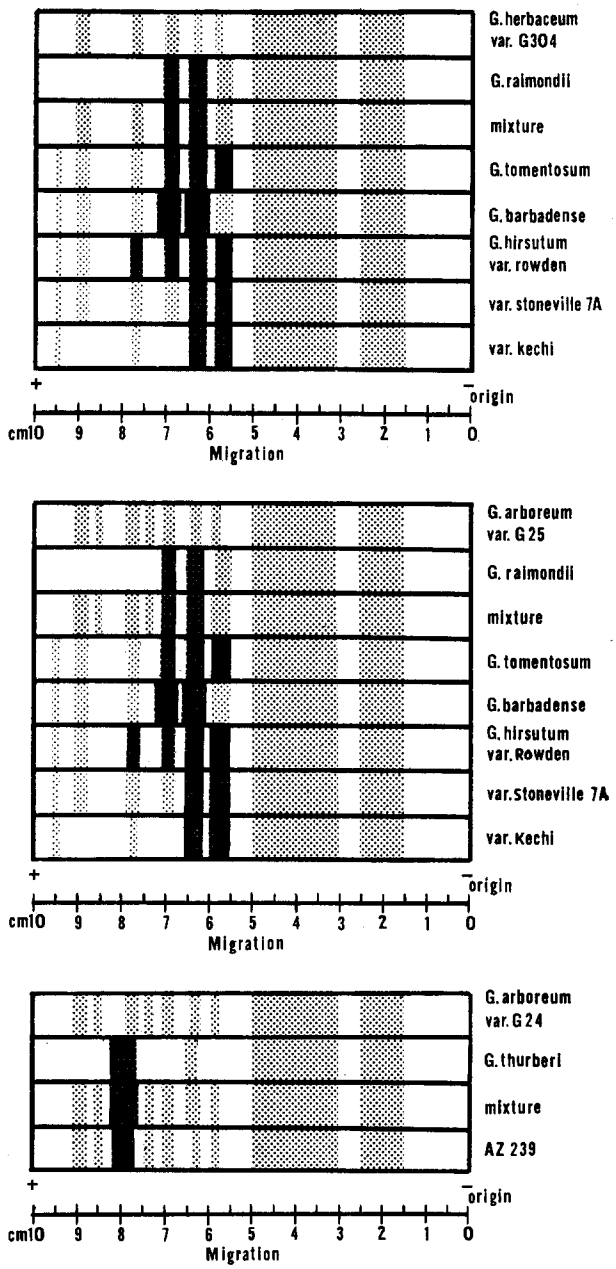

Fig. 6. Polyacrylamide gel electrophoretic esterase spectra of a comparison between the possible parentals, their synthetic mixtures and the allotetraploids.
Upper spectra: Comparison between *G. herbaceum* var. *africanum* (G304), A_1 genome, *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids: *G. tomentosum*, *G. barbadense* and *G. hirsutum*.
Middle spectra: Comparison between *G. arboreum* var. G25 (A_2 genome), *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids.
Lower spectra: Comparison between *G. arboreum* var. G24 (A_2 genome), *G. thurberi* (D_1 genome), their synthetic mixture and their synthetic allotetraploid: $2(A_2D_1)$, AZ239

1967a, b). These data indicated that such studies could be performed on the synthetic and natural allopolyploids of *Gossypium*.

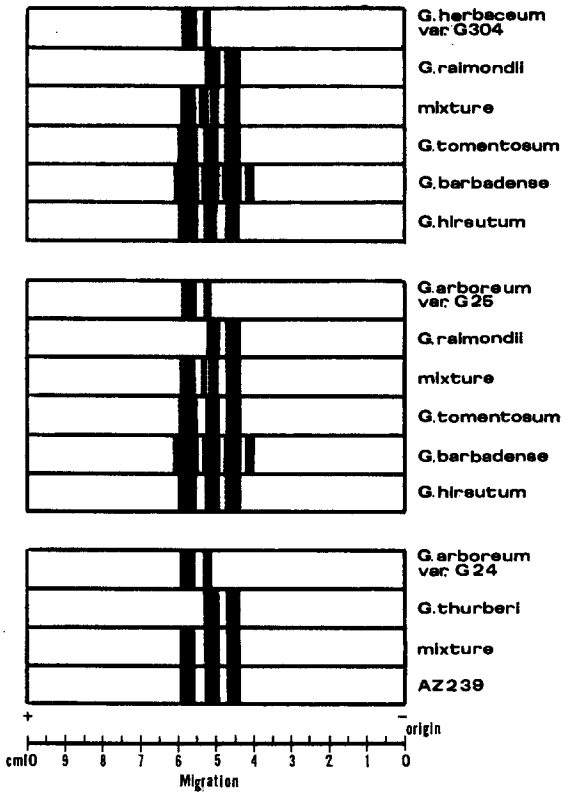

Fig. 7. Starch gel electrophoretic leucine aminopeptidase spectra of a comparison between the possible parentals, their synthetic mixtures and the allotetraploids.
Upper spectra: Comparison between *G. herbaceum* var. *africanum* (G304), A_1 genome, *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids: *G. tomentosum*, *G. barbadense* and *G. hirsutum*.
Middle spectra: Comparison between *G. arboreum* var. G25 (A_2 genome), *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids.
Lower spectra: Comparison between *G. arboreum* var. G24 (A_2 genome), *G. thurberi* (D_1 genome), their synthetic mixture, and their synthetic allotetraploid: $2(A_2D_1)$, AZ239

Seed esterase, leucine aminopeptidase and catalase from a synthetic 2 (AD) allotetraploid (AZ239), which originated in the 1930's at Raleigh, North Carolina, were examined by electrophoresis (Figs. 6—8). This allotetraploid was formed from a cross between a variety of *G. arboreum* (A_2) and *G. thurberi* (D_1). A synthetic mixture of the seed extracts was formed from *G. arboreum* var. G24 and *G. thurberi* and their isozyme patterns were compared with that of the allotetraploid AZ239. In the synthetic mixture an additive zymogram was formed for each of the three enzyme systems. These additive zymograms compared closely to the isozyme patterns of AZ239.

Recently, Muramoto (1969) synthesized a triploid from a cross between *G. hirsutum* (Experimental Acala 44-10-1) and *G. sturtianum* var. *sturtianum*. The chromosomal number of the triploid was doubled (hexaploid: Experimental 6x-3) with the aid of colchicine. As observed with the synthetic allotetra-

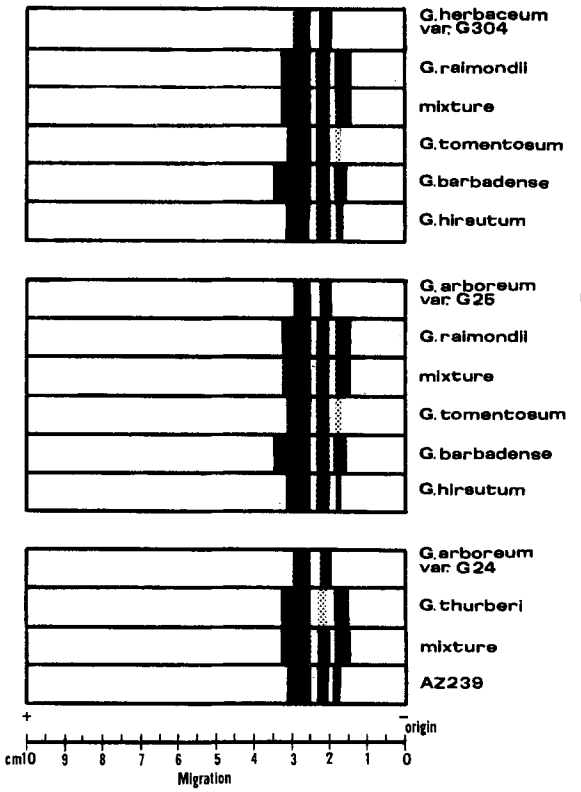

Fig. 8. Starch gel electrophoretic catalase spectra of a comparison between the possible parentals, their synthetic mixtures, and the allotetraploids.
Upper spectra: Comparison between *G. herbaceum* var. *africanum* (G304), A_1 genome, *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids: *G. tomentosum*, *G. barbadense* and *G. hirsutum*.
Middle spectra: Comparison between *G. arboreum* var. G25 (A_2 genome), *G. raimondii* (D_5 genome), their synthetic mixture, and the three natural allotetraploids.
Lower spectra: Comparison between *G. arboreum* var. G24 (A_2 genome), *G. thurberi* (D_1 genome), their synthetic mixture, and their synthetic allotetraploid: $2(A_2D_1)$, AZ239

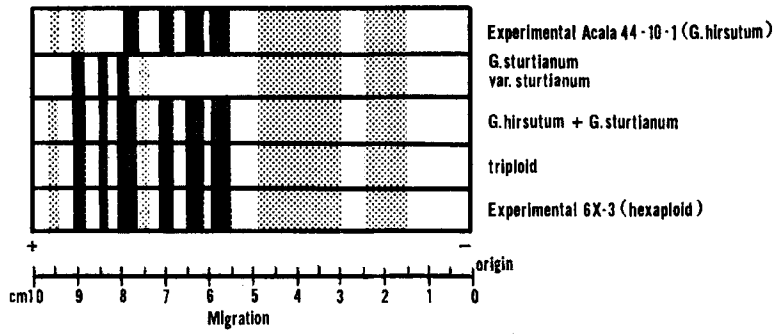

Fig. 9. Polyacrylamide gel electrophoretic esterase spectra of a comparison between *G. hirsutum* var. Acala 44-10-1, $2(AD)_1$, *G. sturtianum* var. *sturtianum* (C_1), their synthetic mixture, and the synthetic triploid and colchicine-induced hexaploid, Experimental 6x-3

ploid AZ239, the banding patterns of both the triploid and hexaploid seed esterase compared well with the additive isozyme patterns of the parents in the synthetic mixture (Fig. 9).

It should be noted that the banding pattern occurring in highest frequency within the species compared shows a good additive relationship of the parent zymograms to the synthetic mixture and to the allopolyploids. The possibility cannot be disregarded, however, that a plant containing the genetic make-up for one of the lower frequency zymograms may have contributed to the formation of the original allopolyploids.

The synthetic mixture made up of the A_1 genome (G304) and the D_5 genome (*G. raimondii*) shows an additive pattern of the enzyme activity present in the two diploids. This additive pattern is produced in all three of the enzyme systems (Figs. 6—8). *G. tomentosum*, *G. barbadense* and several of the varieties of *G. hirsutum* compared closely to the synthetic mixture of the alleged parents. However, qualitative differences do exist in some of the varieties of *G. hirsutum*. These varieties exhibited three basic types of gel patterns represented by Rowden, Stoneville 7A and Kechi (Cherry, 1971). Only one esterase zymogram pattern is observed for *G. tomentosum* as well as for the varieties of *G. barbadense* (Fig. 6). Except for an additional band at 4.2 cm for leucine aminopeptidase in *G. barbadense*, no qualitative variations in leucine aminopeptidase and catalase activity occurred within the three polyploid species (Figs. 7 and 8).

The synthetic mixture of esterase activity involving the A_2 genome (var. G25) and D_5 genome (*G. raimondii*) differs slightly from that of the $A_1 + D_5$ mixture (Fig. 6). Minor bands 7.5 cm and 8.6 cm, present in var. G25 and the synthetic mixture are absent in the allotetraploids. Since no differences in leucine aminopeptidase and catalase activity were observed between *G. herbaceum* and *G. arboreum* (Fig. 7 and 8), both species produced identical synthetic mixtures when combined with *G. raimondii*. Therefore, with regard to these enzymes no conclusions as to which A genome contributed to the allotetraploids could be made. The similarity of the esterase banding patterns of the allotetraploids to that of the *G. herbaceum*—*G. raimondii* mixture, however, provides some additional support for the hypothetical origin of the allotetraploids.

It was shown that the esterase, leucine aminopeptidase and catalase activity of the synthetic allotetraploids could be duplicated by mixing the enzyme extracts of their parents. Therefore, the synthetic mixtures of $A_1 + D_5$ can indicate with some degree of approximation the type of banding patterns for seed enzymes that the original ancestral 2(AD) allotetraploid hybrid may have contained. Examination of the three natural allotetraploids, however, revealed that several qualitative changes have taken place in their enzyme banding patterns. These changes in the natural allotetraploids would be expected to be more extensive than that of the recently

synthesized allopolyploids due to the relative lengths of time involved for the evolutionary mechanisms to operate (Cherry et al., 1970; 1971). Of interest at this point is a possible explanation for these evolutionary changes.

The theories of gene regulation in higher organisms (Britten and Davidson, 1969) and diploidization (Endrizzi, 1966) as possible mechanisms of explaining the genetic changes that have occurred in the natural allopolyploids of the genus *Gossypium* were discussed by Cherry et al. (1970; 1971). Differential quantitative and/or qualitative control by specific genes resulting from mutational changes within either one or the other homeologous genome or in both genomes of the natural allotetraploids might account for the different isozyme patterns of the three enzyme systems as shown in Figs. 6—8.

The superimposed banding patterns of seed proteins also indicated that the genomes similar to A_1 and D_5 were the most likely ancestors of the natural allotetraploids (Cherry et al., 1970). In addition, they also showed that several changes had taken place in the protein composition of the three natural allotetraploids. As observed for the enzyme data, the allotetraploids that were synthesized recently showed little variation in their protein banding patterns when compared to the additive patterns of their parent species (Cherry et al., 1971). Thus these protein differences and the differences observed for the three enzyme systems indicate that evolutionary changes have occurred in both homeologous genomes of the polyploid species.

Acknowledgement

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Literature

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