A COMPARATIVE ELECTROPHORETIC STUDY OF SEVERAL *ASHMUNELLA* (GASTROPODA: POLYGYRIDAE) POPULATIONS FROM THE CHIRICAHUA MOUNTAINS OF ARIZONA

by

Donna Michele Trifan

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SIGNED: Donna Michele Trifan

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Walter B. Miller — 13 May 1976

W. B. Miller
Associate Professor of Biology

Date

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ABSTRACT

Six species of terrestrial snails of the genus Ashmunella from the Chiricahua Mountains of Arizona were compared on the basis of protein and esterase banding patterns in polyacrylamide gels. Additional comparisons were made with two Ashmunella species from other mountain ranges in the state, but these seem to be more closely related to each other than to the Chiricahua species. A great deal of similarity is found among the banding patterns of all the Chiricahua species although some obvious differences exist. Further experimentation and a more refined technique would be required for future studies of this genus. Electrophoresis alone cannot prove any taxonomic relationships but must be used in conjunction with anatomical and cytological studies.

INTRODUCTION

In recent years, electrophoresis has been used increasingly as a taxonomic tool in studies of molluscan species and populations. Many of these studies have been done using starch gel electrophoresis in analyzing esterases, for example, of Littorina littorea (Matteo, Schiff, and Garfield 1975) and four species of Bulinus (Wright and File 1968). Cheng (1964) has used membrane electrophoresis to demonstrate differences between several species of marine and fresh-water gastropods. An excellent table listing electrophoretic research in malacology (particularly for those molluscs of importance in parasitology) has been compiled by Michelson (1973).

Since about 1959, a new electrophoretic technique called polyacrylamide gel electrophoresis (also known as disc or zone electrophoresis) has been developed. Disc electrophoresis has been applied in experiments using tissue samples from various parts of molluscs, such as reproductive tissue in species of the land snail Partula (Schwabl and Murray 1970) and crystalline styles of some marine molluscs (Bedford and Reid 1969). As a matter of fact, crystalline styles in those molluscs which have them are considered to be perhaps the best samples for electrophoretic studies

in that styles are less susceptible than other tissues to environmental influences. They yield consistently identical results for animals of the same species collected from different areas.

Much of the work in molluscan electrophoresis has been done by Davis in illustrating differences between species, subspecies, and populations using foot muscle proteins of Pomatiopsis lapidaria (1967), Oncomelania hupensis (1967, 1968) and Semisulcospira libertina (1972). Other studies done by Davis and Lindsay (1967) showed that while there were significant electrophoretic variations between populations of Pomatiopsis lapidaria, the species still had a characteristic banding pattern recognizable in each population.

According to Davis (1973), the best starting point for any systematic or evolutionary study is a population. For this study, populations of snails of the genus Ashmunella were collected in the Chiricahua Mountains of southeastern Arizona. Populations were considered as separate species based on prior knowledge and for the sake of convenience. In addition to the Chiricahua snails, a few specimens were obtained from the Huachuca Mountains (A. levettei) and the Blue Mountains (A. pilsbryana). Although these are definite species, they were considered to be an interesting source for additional electrophoretic

comparison. The purpose of this study was to attempt to find electrophoretic similarities and differences among Ashmunella populations and to use the data obtained to clarify taxonomic relationships within the genus. Foot muscle extract was used in this study of Ashmunella since experiments with Helix pomatia foot muscle yielded more bands than if hemolymph were used (Davis and Lindsay 1967). Foot muscle also provided a consistent pattern in spite of the variations in physiological condition one would expect when animals age or are maintained for long periods in the laboratory.

Disc electrophoresis is used to separate proteins on the basis of size, conformation, and net charge (Chrambach and Rodbard 1971). Gel lattices are carbon-carbon polymers with pendant amide groups, are relatively inert chemically, and have few or no ionic side groups (B. Davis 1964). Gels consist of three layers: a large-pore sample gel in which electrophoretic concentration of the sample proteins is initiated, a large-pore spacer gel in which concentration of the sample proteins is completed, and a small-pore separation gel through which proteins migrate at different rates resulting in a gel banding pattern visible after staining. The large-pore gels function as anti-convection media while the small-pore gel also serves as a sieving medium (B. Davis 1964). Chemical

and theoretical aspects of polyacrylamide gel electrophoresis are discussed by Chrambach and Rodbard (1971) and Maurer (1971).

Davis and Lindsay (1967) have stated several advantages of disc electrophoresis over other electrophoretic techniques. The standard 7.5% polyacrylamide gel allows greater fractionation and resolution of components, and there is greater flexibility in the technique since the pore size of the gel can easily be adjusted by altering the ratios of chemicals used. Gels can be charged with a sample as small as 300-800 micrograms, and running time is about 30 minutes in contrast to several hours for starch gels. The gels are also mechanically strong and very easy to store. Despite these advantages, disc electrophoresis is a very sensitive technique involving so many chemicals and steps that small experimental errors in gel preparation may have proportionally large negative results. Each acrylamide gel is a separate entity in contrast to starch slabs where all samples are run on the same surface. Although in theory, disc electrophoresis gels should yield identical results (even for different runs) for samples from the same organism, this is not always the case in practice. So many factors come into play, that perfectly reproducible results are difficult to obtain. This necessitates averaging of results in order to obtain characteristic R_f (relative

mobility) values. When something does go wrong in the technique, it is difficult to determine the exact nature of the cause.

MATERIALS AND METHODS

Snails collected from the Chiricahua Mountains (Fig. 1) were kept in wooden terraria until samples for electrophoresis were needed. Foot muscle of these snails was then homogenized with distilled water. Various degrees of dilution were tried, and of these, the best results were obtained using a volume of water equal to seven times the total weight of foot muscle (weighed on a torsion balance). Greater dilution yielded gels with fewer bands. Due to the small (about $\frac{1}{2}$ inch diameter) size of most Ashmunella species, tissue from several snails was usually pooled in order to have enough material to allow homogenization. Hand homogenization alone proved impractical due to the presence of mucus which hampered supernatant formation during centrifugation. Different combinations of homogenization and centrifugation times were used to determine a technique which would consistently yield a good supernatant layer. The best method found consisted of eliminating excess mucus by squeezing the tissue between two pieces of paper toweling, hand homogenizing for one minute, microhomogenizing using a Virtis 23 microhomogenizer for five minutes, and centrifuging at maximum speed (60 cycles) in an Adams Safeguard centrifuge for five minutes. The stainless steel

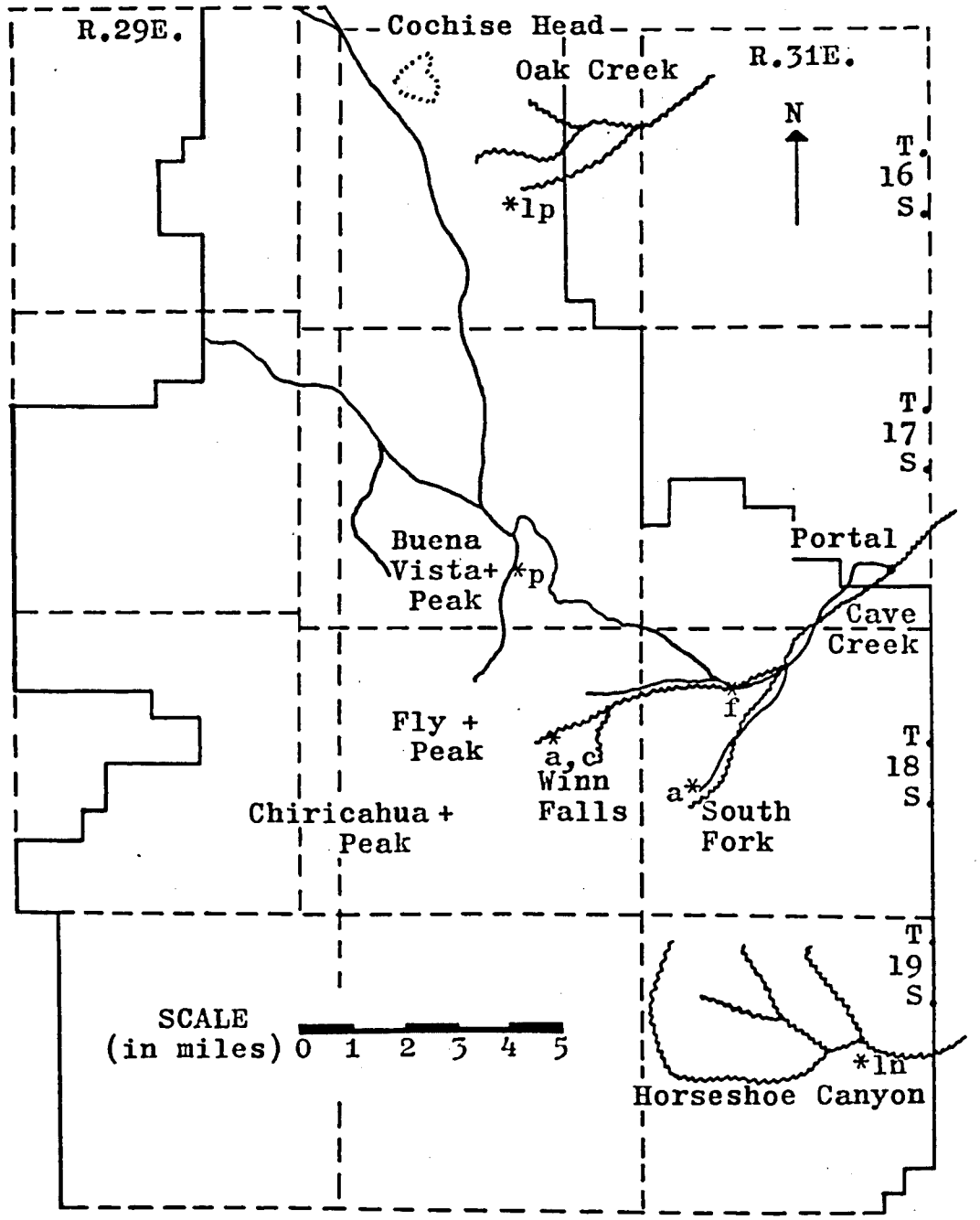

Fig. 1. Collecting sites of Ashmunella in the Chiricahua Mountains, Arizona.

—— = national forest boundary --- = section boundary
~ = road ~~~ = creek or canyon + = peak
* = collecting site a = A. angulata c = A. chiricahuana
f = A. ferrissi ln = A. lenticula lp = A. lepiderma
p = A. proxima
(A. levettei was collected from Carr Peak, Huachuca Mts. and A. pilsbryana from Clifton, Arizona).

microhomogenizer cup and the centrifuge tubes were chilled in a freezer prior to use, and the sample was kept cold using ice baths wherever possible. After centrifugation, the clear supernatant was removed with a 1 cc. hypodermic syringe and stored in a small plastic vial in the freezer. If Carriker's physiological saline (Carriker 1946) was used instead of distilled water for dilution, more bands were seen in gels stained for esterase of foot muscle from Ashmunella chiricahuana.

The electrophoretic technique used is very similar to that employed by Davis and Lindsay (1967) and B. Davis (1964). Acrylamide, tris, glycine, bis, riboflavin, temed, and all solutions except stains were kept under refrigeration (about 5° C.) in brown glass bottles. Table 1 lists chemical ingredients of all stock solutions needed for electrophoresis. (Note that bis = N,N'-methylenebisacrylamide, tris = 2-amino-2-hydroxymethyl-1,3-propanediol, temed = N,N,N',N'-tetramethylenediamine). Stock solutions were allowed to stabilize for at least one day before use although this may not be necessary as solution F was found to work just as well without this precaution. Distilled water was used in making all solutions and cleaning all glassware. Prior to doing a run (the time period during which current is run through the gels in the electrophoretic chamber), the ammonium persulfate catalyst was prepared and

Table 1. Stock Solutions

Solution A (Buffer pH=9.2) Put 24 ml. 1 N HCl in a 100 ml. graduated cylinder. Add 19.15 g. tris and enough water to make 95 ml. of solution. While stirring on a magnetic stir plate, add .23 ml. temed and enough water to make a total volume of 100 ml.

Solution B (Buffer pH=6.9) To 25.6 ml. 1 M H_3PO_4 and 5.7 g. tris in a graduated cylinder, add enough water to make 100 ml. of solution. Add .05 ml. temed and stir on a magnetic stir plate.

Solution C (Monomer) Put .8 g. bis and 30 g. acrylamide into a volumetric flask. Add water to make 100 ml. Stir.

Solution D (Monomer) Place 2.5 g. bis and 10 g. acrylamide in a volumetric flask. Add water to make 100 ml. and stir.

Solution E Dissolve .004 g. riboflavin in 100 ml. water. This solution is sensitive to ultraviolet light and must be kept in the dark.

Solution F (Upper gel concentrate) Mix one part solution B, two parts solution D, and one part solution E. Keep away from light.

Solution G (Catalyst stock) Dissolve .14 g. ammonium persulfate in 100 ml. water. This solution must be filtered (use Millipore vacuum filtering) prior to use. Fresh solution must be made each week.

vacuum filtered through a Millipore filter. Solutions A, C, and G were taken out of the refrigerator and allowed to warm up to room temperature since bubbles were more likely to form in gels made with cold solutions. When not in use, three inch long glass gel tubes ($\frac{1}{2}$ cm. diameter) were stored in a solution of .5 ml. Kodak Photo-Flo 200 and 100 ml. distilled water. This facilitates gel removal after a run. These glass tubes were dried with cotton swabs before being placed vertically in rubber caps cemented to a small wooden board. All tubes were etched with lines 6 mm., 13 mm., and 48 mm. from the top (Fig. 2). The last mark indicated the point at which the run was terminated. This short run limit was extended in later experiments to a point 66 mm. from the top of the tube since it was thought that a longer run would separate the protein bands better.

Gels are composed of three separate layers, the first of these being the lower or separation gel since it functions primarily in the final separation of proteins. Two ml. of solution A, two ml. of solution C, and four ml. of solution G were pipetted into a small beaker, briefly stirred together, and drawn into a 20 cc. syringe. After removal of the syringe needle, the syringe was shaken by hand with one finger placed over the syringe opening, and the plunger of the syringe was pulled out slowly several times. This allowed small bubbles in the solution to rise

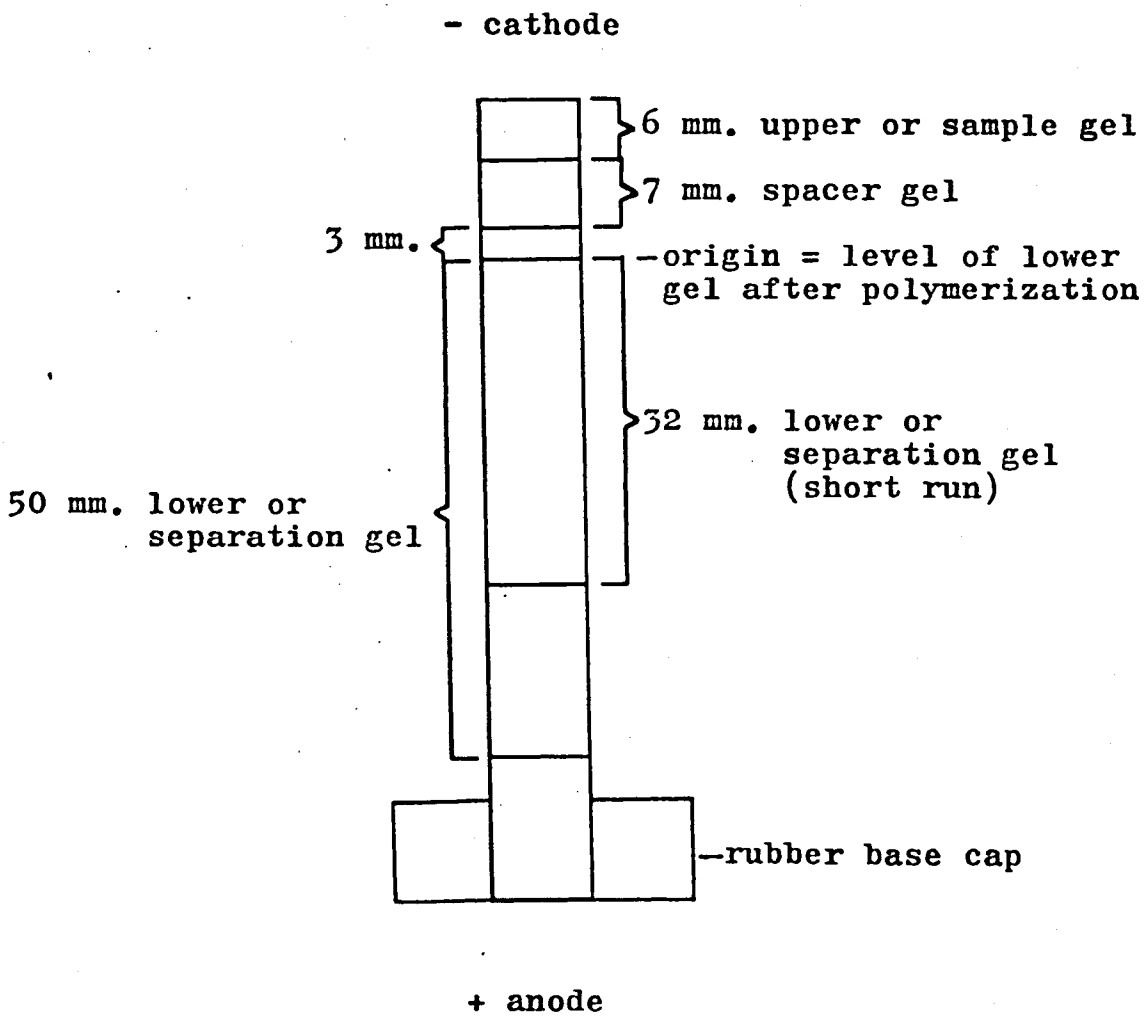

Fig. 2. Gel Layering

to the surface and lessened the chance of bubbles appearing in the gel. Six glass tubes were filled so that the top of the meniscus was in line with the middle etch mark. Using a 1 cc. syringe with a needle bent at a 45° angle, a stream of distilled water was carefully layered on top of the lower gel. Water layering was necessary to flatten the meniscus of the gel so that straight bands would be obtained after the run was completed. Any turbidity at the interface necessitated redoing of the entire gel since distortion of the gel would yield distorted bands. The above procedure was repeated for the remaining six tubes, and the gels were allowed to polymerize for fifteen minutes. Polymerization could be observed by the appearance of a new interface below the original water - gel interface. After polymerization, the tubes were inverted on top of a piece of paper toweling, and excess water was removed. When the tubes were turned upright, any remaining water was soaked up with the twisted end of a paper towel, care being taken to avoid touching the gels.

All the tubes were rinsed with spacer gel (two ml. solution F mixed with two ml. water) to wash out their open ends. This spacer gel was removed with paper toweling, and the tubes were refilled so that the bottom meniscus of the spacer gel was in line with the top etch mark. Water was layered above the spacer gel, and all the gels were placed about one inch below a 15 watt fluorescent lamp where they

were allowed to photopolymerize (turn white in color) for fifteen minutes. After polymerization, water was shaken out of the tubes as before, and this time the tubes were rinsed with solution F. The remaining space at the top of each tube was filled with the sample gel solution (one part snail supernatant to two parts solution F). Gels were placed under fluorescent light again and allowed to polymerize for 15-20 minutes. Meanwhile, the cylindrical electrophoretic chamber was set up and the lower tray filled with running buffer (Table 2). After polymerization, the gel tubes were carefully removed from their base caps by pulling them out at an angle to break the vacuum and prevent gel distortion. A rubber cap was fitted on the top of each tube and used to secure the tubes in their respective places in the chamber. After this was done, the upper tray was filled with running buffer. Both ends of each tube were thus immersed in buffer solution. The chamber was placed in the refrigerator in a level position and connected to a Heathkit power supply. The latter was not turned on until the temperature within the refrigerator dropped to at least 5°C. The run was made with a current of 5 milliamps per tube (60 milliamps for a run of twelve tubes), and it was necessary to frequently adjust the power supply since the amperage tended to drop. The run was terminated when the dye front reached the predetermined mark on the glass tubes. The upper and lower

Table 2. Running Buffer and Staining Solutions

Running Buffer (pH=8.2-8.4)	To 57.6 g. glycine and 12 g. tris, add enough water to make two liters of solution. Stir on a magnetic stir plate. Add a few grains of bromophenol blue until the solution is faintly purple. Half of this solution will be used for the lower buffer and half for the upper. The buffer is good for about 10-15 runs or as long as it remains clear.
Protein Stain	Dissolve 1 g. amido schwartz (buffalo black) in 100 ml. 7% acetic acid. The stain cannot be re-used and is good for as long as it remains blue. Gels were stained for one hour.
Esterase Stain	Weigh .04 g. alpha-naphthyl acetate and .08 g. fast blue RR salt. Add these to 100 ml. tris-HCl buffer (500 ml. water, 4.1 ml. concentrated HCl, 6.06 g. tris). This solution is very sensitive to light and heat. Gels were stained for about $2\frac{1}{2}$ hours in an incubator set at 38°C. After staining, they were incubated at the same temperature for one hour in a mixture of three parts ethanol (100%) and two parts 10% acetic acid.

buffers were poured back into bottles, each being stored separately to prevent contamination. Each gel was removed from its glass tube by using a 5 cc. syringe to inject a layer of water between the gel and the surrounding glass. The gels were then placed in a test tube of protein or esterase staining solution (Table 2). Gels stained for proteins were destained in 7% acetic acid until bands were clearly visible and then stored in corked glass tubes containing 7% acetic acid. A number of esterase staining techniques were tried, but only the methods used by Flowerdew and Crisp (1975) and Markert and Hunter (Maurer 1971) produced results. The latter was the only technique which yielded bands dark enough to remain visible after several days of storage in 10% acetic acid. The distance of all bands from the origin (Fig. 2) was measured with a metric ruler placed against the gel on a light table with a yellow or green Plexiglas filter. From these data, R_f values were calculated for all bands.

RESULTS

Average R_f values were calculated for each species. The number of gels run depended to a large extent on the availability of specimens. Figures 3 - 6 illustrate the average banding pattern for each species with solid lines indicating bands visible in more than half the gels tested and dashed lines indicating bands visible in less than half the gels. Results from short and long runs were not combined as there was, in some cases, too much difference in R_f values. A. angulata in Fig. 4 was collected from South Fork while A. angulata in Fig. 6 was collected from Winn Falls.

While the protein stain yielded many distinct bands, the esterase stain showed very few. Haites, Don, and Masters (1972) found eighteen esterases (primarily cholinesterases and some carboxylesterases) in Helix aspersa. Whether there are considerably fewer esterases in Ashmunella species or whether the esterase staining technique was for some reason inadequate is not known. The chemistry of esterase staining is explained by Shaw (1965).

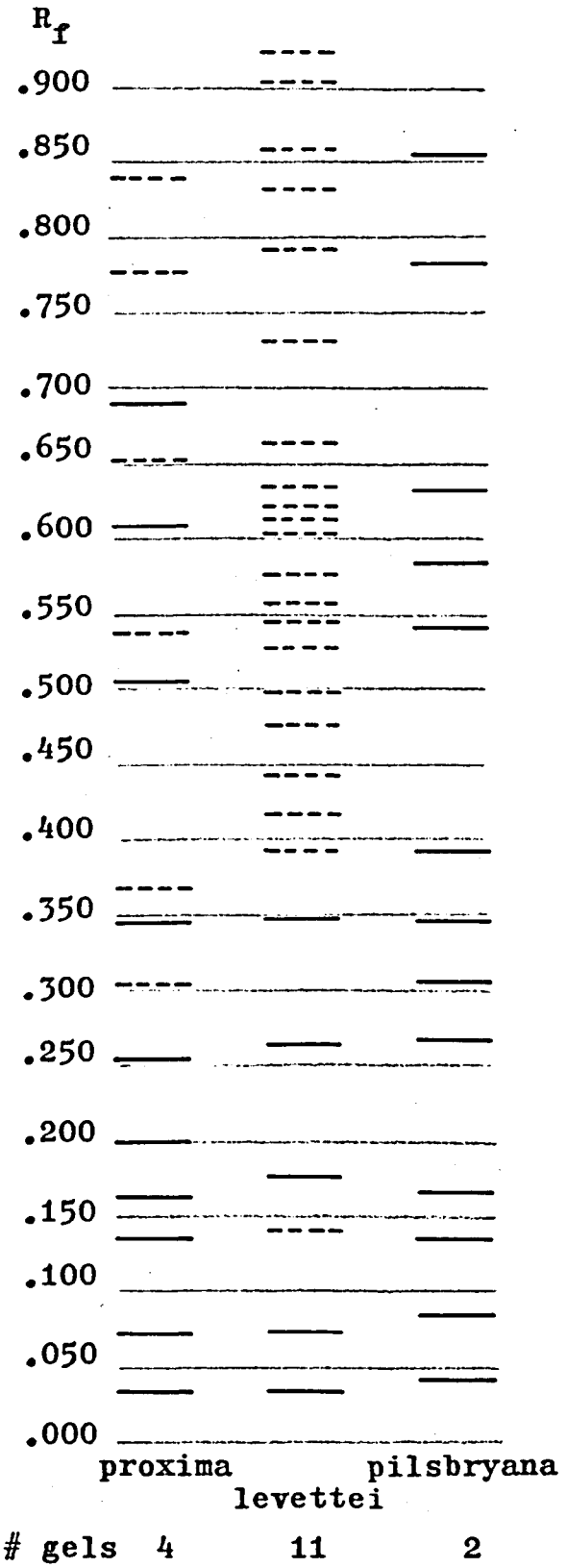

Fig. 3. Protein Banding from 32 mm. Runs

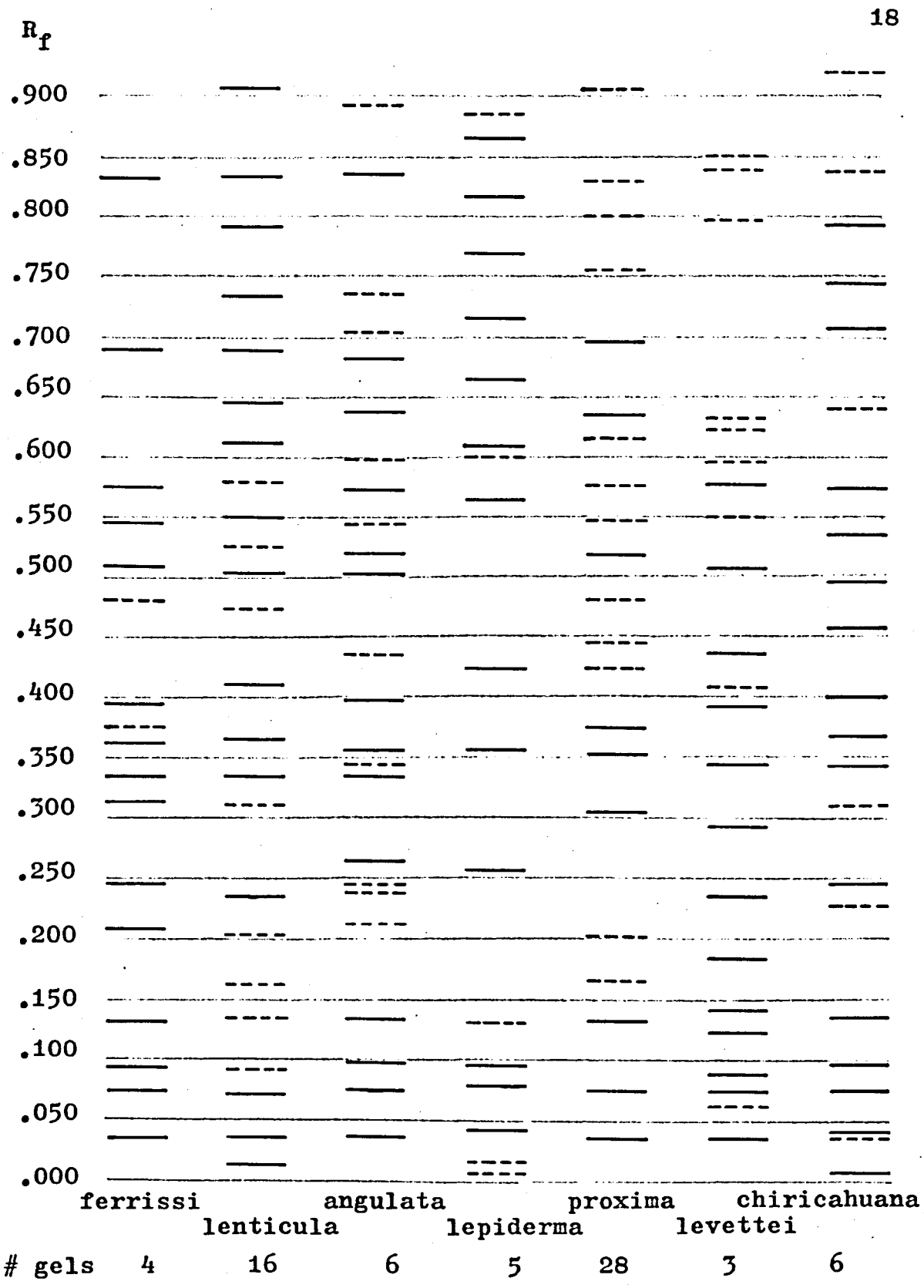

Fig. 4. Protein Banding from 50 mm. Runs

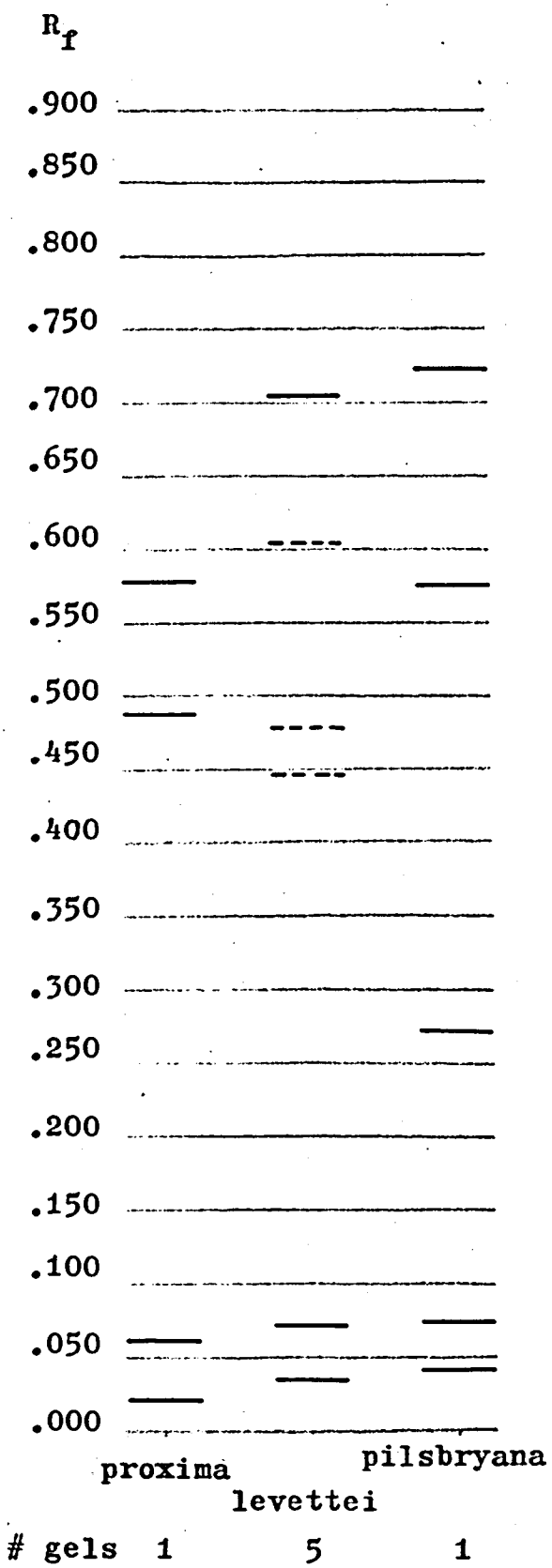

Fig. 5. Esterase Banding from 32 mm. Runs

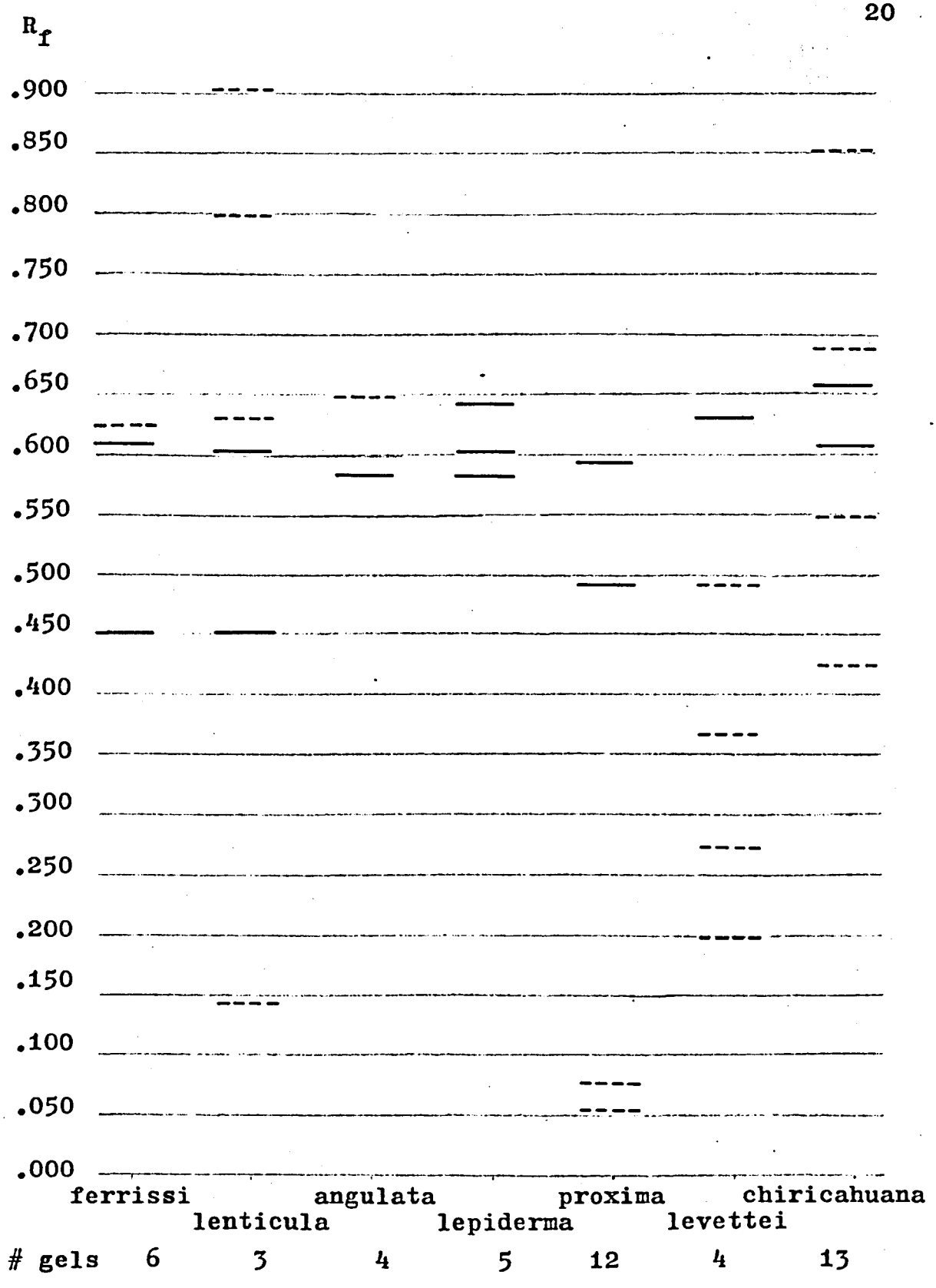

Fig. 6. Esterase Banding from 50 mm. Runs

DISCUSSION AND CONCLUSIONS

A great deal of similarity is apparent among all the species of Ashmunella tested. This is most noticeable in the protein-stained gels. Bands in the vicinity of .037, .075, .137, .170, .260, .346, .785, and .850 (all numbers noted are R_f values) appear to be common to A. proxima, A. levettei, and A. pilsbryana in short runs. In long runs, all species showed bands at about .036, .076, .134, .350, .575, and .830. The esterase bands (short run) at .037 and .072 are common to A. proxima, A. levettei, and A. pilsbryana. In the longer runs, no decisive comparison is possible, although all species have bands in the range of .583-.650.

Despite these similarities which may exist in all members of the genus, differences are present which make it possible to identify some populations. In the short run gels stained for protein, only A. proxima has a band at .200 and .690. A. levettei has no evidence of banding at .305 whereas A. proxima and A. pilsbryana do, and in the longer run, only A. levettei shows a double band near .140 and a dense band at .185. Further tests may indicate that this last value is low and corresponds to the near .200 bands of other species. In the esterase staining (short runs), only A. pilsbryana showed a dense band at .271,

but more data needs to be collected on this species. The long run protein-stained gels show that only A. proxima lacks a band at .095 and near .250. A. lepiderma has the characteristic features of lacking a band at .500, having only a single band near .565, and showing a series of bands at .663, .714, .770, .818, and .865. The latter series of bands pose an interesting problem in that, if these values were increased slightly, the banding pattern would look remarkably like that of other species. A. lepiderma also has three dense esterase bands (long run), more than the number shown by other populations. Unlike other species, A. ferrissi shows no protein bands (long run) from .600 to .675.

Based on the data acquired in these experiments, an attempt to reach some sort of conclusion with regard to taxonomic relationships is difficult. A. ferrissi is related to A. lenticula by an esterase band at .450, to A. angulata by the lack of protein banding from .750 to .825, and to A. chiricahuana by a lack of protein banding at .600. These four groups all show a similar pattern of protein banding from .400 to .450. Other studies have linked A. angulata with A. lenticula, but electrophoresis yielded a protein band at .794 and lack of one at .260 in the latter as compared to the former. A. angulata is related to A. lenticula by a pair of protein bands near .510 to .520 and to

A. lepiderma by a .575 esterase band and a lack of a .300 protein band. A. lepiderma is similar to A. proxima in lacking protein bands at .240 and .400. In the short runs, more evidence exists to link A. levettei to A. pilsbryana (lack of .200 protein band and presence of an esterase band near .710) than to A. proxima (.490 esterase band and lack of .270 esterase bands).

These results indicate a close relationship among A. ferrissi, A. lenticula, and A. angulata. A. chiricahuana is most like A. ferrissi and A. lenticula in its electrophoretic banding. Some evidence exists to link A. angulata to A. lepiderma and the latter to A. proxima.

The taxonomic relationships within the genus Ashmunella have been difficult to determine. Some differences between species are based on shell morphology, there being a very strong degree of carination in A. ferrissi with a decreasing amount in A. lepiderma, A. angulata, and A. proxima (Pilsbry 1940). Shell teeth are present in all of these but not in A. chiricahuana. However, there is a great degree of variability in the teeth of those species which have them. According to Bequaert and Miller (1973), the teeth may be well developed, obsolete, or lacking, even within the same species, subspecies, or population. Genetic studies done by Reeder (1975) have proved inconclusive in taxonomically separating some of the more

closely related groups. Bequaert and Miller (1973) consider A. lenticula to be synonymous with A. angulata, but electrophoretic results suggest that these may be separate species (due to .260 and .794 protein band differences). Bequaert and Miller (1973) also classify A. lepiderma and A. angulata as subspecies of A. proxima. Electrophoresis indicates that while these three share some bands, they also have distinct banding characteristics. Reeder (1975) found A. lenticula to be genetically distinct from A. proxima. This is additionally supported by the fact that the former has a dense protein band at .238 while the latter does not.

Pilsbry (1940) found that anatomically, all the Chiricahua species are more closely related to each other than to the Huachuca species. Snails from both ranges (A. chiricahuana and A. levettei) evolved toothless shells. This probably indicates independent parallel evolution with the Chiricahua and Huachuca snails representing two distinct phylogenetic lines. There are obvious electrophoretic differences between the two species mentioned above. In conflict with Pilsbry's findings, Reeder (1975) found that genetically, A. levettei is more closely related to some of the Chiricahua species than some of the latter are to each other.

Perhaps a better insight into these taxonomic problems could be attained by further electrophoretic

testing. With a greater number of runs and more refinements in technique, one may be able to calculate more representative average banding patterns. A quantitative analysis of bands and immunological tests with selected antigen-antibody systems may help to determine any homology of bands located in analogous positions in gels of different taxa. Electrophoresis alone cannot prove any taxonomic relationships but must be used in conjunction with anatomical and cytological studies.

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