Specific Gene Products of *Fusarium:* Analysis of Ribosomal Proteins

J. E. Partridge

University of Nebraska, Lincoln 68583.
This manuscript has been assigned Journal Series 9401, Agricultural Research Division, University of Nebraska.
Accepted for publication 27 March 1991 (submitted for electronic processing).

The purpose of my paper in this discussion session is to provide a modicum of background on the use of ribosomal proteins as tools in the search for an understanding of the systematics of *Fusarium*. In light of the number of mitochondrial DNA (mtDNA) papers being presented at this meeting, I have opted not to address mtDNA. There are no magic solutions that will provide the definitive answer to systematics, but I hope my participation will cause you to think, to agree, and to disagree. If this occurs, my participation will have been successful.

In my laboratory, our approach to systematics is, and has been, to look for commonalities among species of *Fusarium* and in particular within the group *Liseola*. Philosophically, this approach is conceptual degrees from the more popular orientations. This approach also results in a more conservative opinion as to the basis for describing new species, and is intended to be somewhere between the "lumpers" and the "splitters."

Probably the greatest error extant in systematics research is the use of very small numbers of isolates and/or isolates from a single geographical area. Of course, natural questions follow. How many isolates are enough? What constitutes a single geographical area? The first question is easily answered. Without being flippant, one cannot have too many isolates. While 12 may be sufficient, a minimum of 20 isolates is better. Our experience has indicated that there is more variation between isolates of some species than others as reflected in their ribosomal proteins (15); consequently, the greater the number of isolates, the greater the potential for finding both the outliers and the normals of each species. We have determined a lower limit of 20 for our current endeavors. The definition of a single geographical area is more problematic. *Fusarium* are quite easily disseminated by wind, water, soil, seed, and debris; therefore, one must use caution and judgment when deciding whether any two isolates are actually from different geographical areas. It is best to use isolates that represent a global distribution unless, of course, a species is limited in its distribution. Our analysis of *F. crookwellense* Burgess, Nelson & Toussoun supports the contention that bona fide species present the same electrophoretogram of ribosomal proteins irrespective of the diversity of their geographic origin. *F. episphaeria* (Tode) Snyd. et Hans. is an example that possesses considerable variation irrespective of geographic origin (15).

Our examination of *Fusarium* has focused on the constituent proteins of the 80S ribosome. The decision to use ribosomal proteins was based on the knowledge that all living entities have ribosomes, ribosomes are produced in sufficient quantities to make the work feasible, and ribosomes are discrete subcellular particles that can be purified much as one would purify a virus. One complicating factor not often recognized by researchers is that there are also 70S ribosomes in the mitochondria, and precautions must be taken against cross-contamination. As an aside, the concomitant nucleic acid 70S and 80S counterparts are also produced.

Analysis of the 80S ribosomal proteins of a number of fungal species by one-dimensional polyacrylamide gel electrophoresis (PAGE) revealed significant differences in the proteins by location and intensity (8,14) (Fig. 1). Differences in ribosomal proteins were found to occur between a host plant (corn, *Zea mays* L.) and some of its fungal pathogens (13). In fact, sufficient differences were found to allow a quantitative estimate of a fungus in the host using ribosomes isolated from diseased tissue and assayed serologically using polyclonal antibodies raised against 80S ribosomes of host and/or parasite (9,10).

As a continuation of our initial study, we assessed the diversity of *Fusarium*. Previous comparative electrophoretic studies of the ribosomal proteins of various eukaryotic organisms of the same genus or closely related genera had been reported (1,3,5–7). These studies primarily indicated large conservation of structural ribosomal proteins; however, these authors also reported some proteins that may be unique to a given species or even certain mutants (3).

In an extensive comparative study of *Saccharomyces* and *Kluyveromyces* by one-dimensional PAGE, Adouette-Panvier et al (1) suggested that the relatedness of the structural ribosomal proteins within a genus may have a high degree of correlation with that based on DNA/DNA homology (2).

At this juncture we began to investigate the 80S ribosomal proteins of 145 isolates of *Fusarium* encompassing all of the putatively bona fide species (12,15). This investigation was based on one-dimensional gradient PAGE in SDS and enabled us to visualize up to 40 bands from each ribosomal protein sample. Because a ribosome is composed of approximately 70 structural proteins, some with varying degrees of phosphorylation, this meant that our system did not completely resolve all of the proteins. But the system had sufficient fidelity to draw limited conclusions. Among the isolates of some species there was, essentially, identity in the protein bands observed, e.g., *F. graminearum* Schwabe and *F. crookwellense*. However, among isolates of other species there was considerable diversity, e.g., *F. merismoides* Corda syn. *F. episphaeria* (Tode) Snyd. & Hans. pro parte (15).

At that time we published an electrophoretogram (15) of a representative isolate of each species and concluded that there were proteins apparently common to all *Fusarium* species and that those species with a common sexual stage had more commonly shared bands than comparative species of unlike sexual stages. Recent reanalysis of the isolates and gels of the one-dimensional PAGE study have been made by machine vision image analysis using the standard image capture and analysis programs of BioImage (A Millipore Company, Ann Arbor, MI) and software we have written specifically for the purpose. The results of the reanalysis can be depicted as a computer generated representation for the ribosomal proteins of 20 species of *Fusarium* (Fig. 2). By referencing all bands on all gels to the same protein standards, very close estimates of the molecular weight of each band can be determined. These molecular weights are then used by the software program to determine like and unlike bands within a tolerance of 1% of the molecular weight. These programs also provide similarly matching values between each pair of isolates. While is it almost obligatory to present a table of pairwise comparisons in a discussion of systematics, these pairwise comparison values seek to indicate a relationship between two individuals using the formula $f = PC/((P1 + P2)/2)$ where PC = proteins common to both individuals, P1 = total number of proteins from individual one, and P2 = total protein from individual two (sensu

11). While we have generated the values, we have chosen not to present them because we feel that these values from one-dimensional PAGE gels are of very little value in determining the actual relatedness of species. This conclusion is based on the fact that one-dimensional PAGE simply does not resolve all proteins in a lane. Therefore it is not possible to know with any degree of certainty the number of proteins in a single band, and accordingly it is not possible to determine the total number of proteins in a sample. Therefore, no degree of mathematical analysis will suffice to generate accurate values. This inherent lack of resolution by one-dimensional PAGE does not negate the value of these experiments, because it provided the preliminary indications that differences do exist between species of *Fusarium*.

While we found it interesting that one could approach species identification by one-dimensional PAGE of 80S ribosomal proteins, we were not satisfied that this approach was sufficient in and of itself to unequivocally identify an unknown isolate or address systematic questions. Accordingly, the study was expanded to include monoclonal antibody and two-dimensional PAGE analytical approaches.

Monoclonal antibodies made against *F. moniliforme* Sheldon and *F. graminearum* were of sufficient fidelity that homologous reactions occurred 100 times more often than any heterologous combination. This high degree of specificity provided strong support for the hypothesis that species-specific differences could be found among the ribosomal proteins. However, when antibodies were used as tools to study the in planta movement and growth of these species in corn, inefficiency of quantitatively extracting fungal ribosomes from plant tissue caused us to abandon this approach.

Two-dimensional gradient PAGE currently provides the best opportunity to visualize, identify, and quantitate all of the proteins of an 80S ribosome. Using this method coupled with machine vision image analysis of the gels, we are beginning to identify 80S ribosomal proteins common among diverse organisms as well as those that are unique to more closely related taxa. The two-dimensional gradient PAGE system uses a urea-based first dimensional 4% acrylamide gel. The second dimension is a modified (15) Laemmli system using a 12–20% acrylamide gel. After electrophoresis, the gels are silver stained (16).

Gels are analyzed on a BioImage Visage110 machine vision image analyzer using their EQ and two-dimensional programs. These programs capture the image from a 512 × 512 matrix diode camera, locate the protein spots, and provide data for each spot, e.g., molecular weight, optical density, quantitative value, and percentage of total sample. The two-dimensional program com-

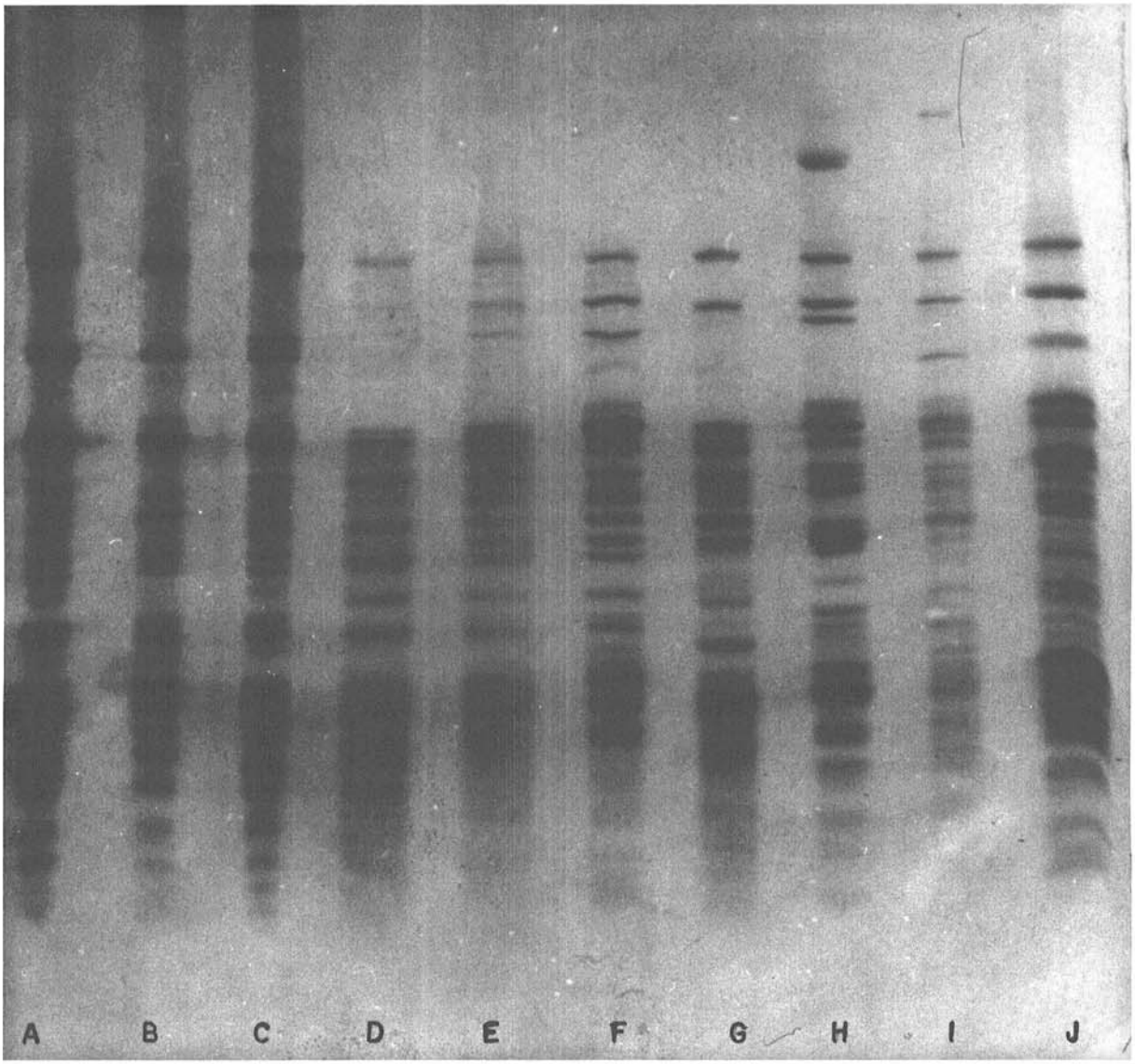

Fig. 1. Polyacrylamide gel electrophoresis of ribosomal proteins of single isolates of fungi visualized by Coomassie blue stain on an SDS-15%-25% polyacrylamide gradient slab gel. Fungal ribosomes (80S) examined were: **A,** *Fusarium equiseti;* **B,** *F. graminearum;* **C,** *F. moniliforme;* **D,** *Helminthosporium maydis;* **E,** *H. sorghicola;* **F,** *H. turcicum;* **G,** *Periconia circinata;* **H,** *Macrophomina phaseolina;* **I,** *Diplodia maydis;* **J,** *Pythium ultimum.*

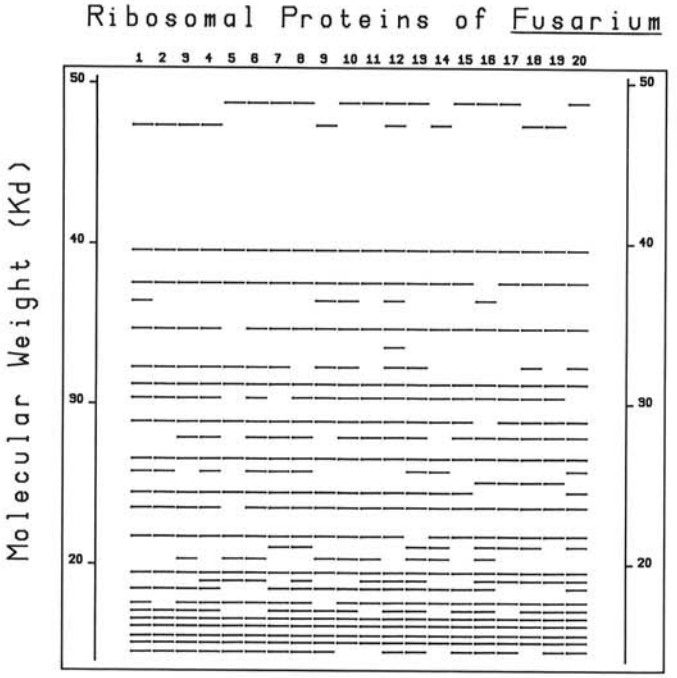

Fig. 2. Computer generated representation of one-dimensional polyacrylamide gel electrophoresis of twenty species of *Fusarium*. Species represented are: *F. avenaceum, F. semitectum, F. equiseti, F. scirpi* var. *acuminatum, F. scirpi* var. *filiferum, F. scirpi* var. *longipes, F. graminearum, F. culmorum, F. sambucinum, F. crookwellense, F. moniliforme, F. subglutinans, F. anthophilum, F. lateritium, F. solani, F. episphaeria, F. oxysporum, F. sporotrichoides, F. trincinctum, F. poae.*

pares two gels and identifies spots common and not common between the sample and a reference. By this routine one is able, through analysis of the spatial relationships of the spots, to identify like spots on any two gels. We have developed subsequent programs (4) that allow us to compare up to 50 gels and perform pairwise analysis for each gel and spot. For this presentation, we have synthesized plots that are the result of four to six gels of several isolates of a single species. This procedure smooths out the electrophoresis run differences and allows for better comparisons.

Figure 3A–C presents a computer representation of two-dimensional PAGE electrophoretograms of *F. graminearum, F. moniliforme,* and rabbit reticulocytes, respectively. Figure 3D is a graphic comparison of *F. moniliforme* and *F. graminearum.* As an indication of the commonalities across a widely divergent species, Figure 3E shows the common proteins between *F. moniliforme* and rabbit ribosomes. Even the most cursory appraisal reveals there are fewer common proteins between rabbit and *F. moniliforme* 80S ribosomes than there are between the two *Fusarium* species. However, one cannot help but recognize the existence of select ribosomal proteins common to all three (Fig. 3F). In fact, polyclonal antibodies raised against the 80S ribosomes of any of these will show significant and specific binding to the others.

As we continue our research, 123 isolates of seven *Liseola* group *Fusarium* species are under investigation. Preliminary investigation with only a few isolates indicates that there are a set of possibly six to nine proteins that are representative of the group and may provide the basis for a systematic separation within the genus of *Fusarium.*

It must be reiterated that when looking at systematics of *Fusarium,* and probably any genus, conclusions that are not based on a number (12–20) of isolates of each species should not be accepted, irrespective of the elegance of the methodologies. As we progress into increasingly sophisticated and sensitive techniques, we must be vigilant to adhere to the scientific method, a cardinal tenet of which demands replication—replication that includes numbers of isolates as well as experiments and analyses.

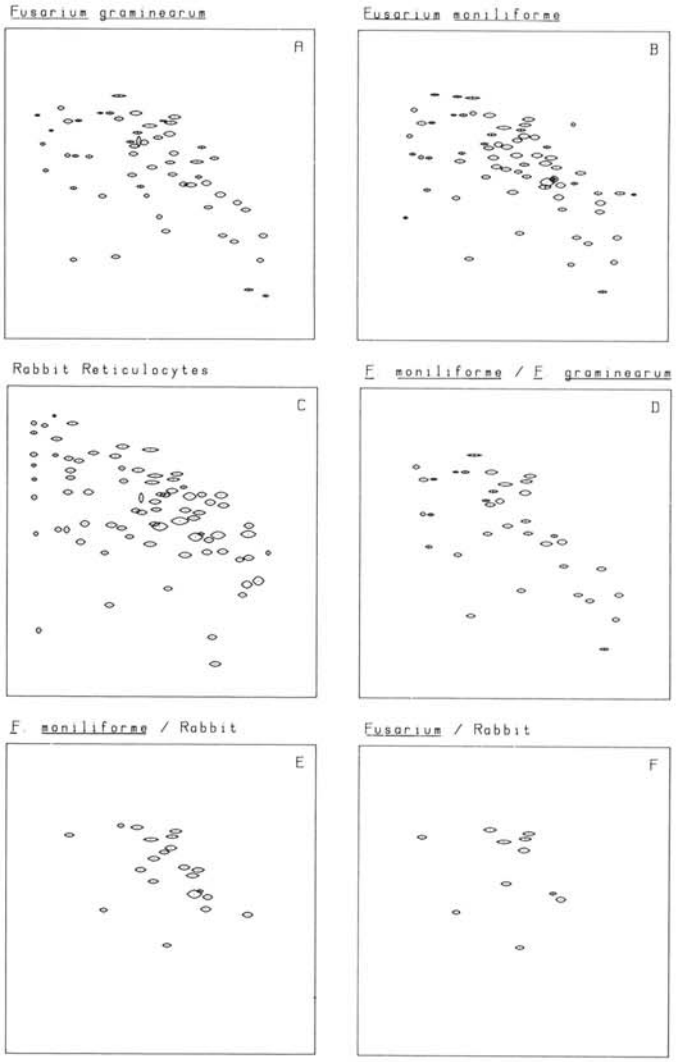

Fig. 3. Computer representations of ribosomal proteins of: **A,** *Fusarium graminearum;* **B,** *F. moniliforme;* **C,** rabbit reticulocytes; and rRibosomal proteins common to: **D,** *F. graminearum* and *F. moniliforme;* **E,** *F. moniliforme* and rabbit reticulocytes; **F,** *F. moniliforme, F. graminearum,* and rabbit reticulocytes.

LITERATURE CITED

1. Adouette-Panvier, A., Davies, J. E., Grits, L. R., and Littlewood, B. A. 1980. Studies of ribosomal proteins of yeast species and their hybrids by gel electrophoresis and immunochemical cross-reactions. Mol. Gen. Genet. 179:273-282.
2. Bicknell, J. N., and Douglas, H. C. 1970. Nucleic acid homologies among species of *Saccharomyces.* J. Bacteriol. 101:505-512.
3. Delaunay, J., Creusot, F., and Schapira, G. 1973. Evolution of ribosomal proteins. Eur. J. Biol. 39:305-312.
4. Dougherty, D. A., Zeece, M. G., Wehling, R. L., and Partridge, J. E. 1989. High-resolution two-dimensional electrophoresis of wheat proteins. J. Chromatogr. 480:359-369.
5. Fujisawa, T., and Eliceiri, G. L. 1975. Ribosomal proteins of hamster, mouse and hybrid cells. Biochim. Biophys. Acta 402:238-243.
6. Gotz, H., and Arnold, C.-G. 1980. Analysis of ribosomal proteins from various species of algae: Comparative electrophoretic study of proteins from chloroplast ribosomes. Biochem. Physiol. Pflanz. 175:108.
7. Gotz, H., and Arnold, C.-G. 1980. Comparative electrophoretic study of ribosomal proteins from algae. Planta 49:19-26.
8. Marshall, M. R. 1980. Detection of *Fusarium moniliforme* in corn (*Zea mays* L.) stalk tissue based on differences in ribosomal proteins of host and pathogen. Ph.D. dissertation. University of Nebraska, Lincoln. 92 pp.
9. Marshall, M. R., and Partridge, J. E. 1981. *Fusarium moniliforme* detection corn (*Zea mays* L.) stalk tissue: Identification of specific ribosomal proteins by polyacrylamide gel electrophoresis. Physiol.

Plant Pathol. 18:133-144.
10. Marshall, M. R., and Partridge, J. E. 1981. Immunochemical identification of *Fusarium moniliforme* ribosomes from diseased corn (*Zea mays* L.) stalk tissue. Physiol. Plant Pathol. 18:277-288.
11. Nei, M. 1987. Molecular and Evolutionary Genetics. Columbia University Press, New York. 443 pp.
12. Nelson, P. E., Toussoun, T. A, and Marasas, W. F. O. 1983. *Fusarium* Species: An Illustrated Manual for Identification. Pennsylvania State University Press, University Park. 193 pp.
13. Partridge, J. E. 1981. Electrophoretic comparison of ribosomal proteins of fungal pathogens of corn and sorghum. (Abstr.) Phytopathology 71:248.
14. Partridge, J. E., and Marshall, J. E. 1981. Non-homology of antibodies produced against ribosomal proteins of *Fusarium moniliforme* and *Fusarium roseum* 'graminearum'. (Abstr.) Phytopathology 71:248.
15. Partridge, J. E., Nelson, P. E., and Toussoun, T. A. 1984. Ribosomal proteins of the genus *Fusarium*. Mycologia 76:533-544.
16. Wozniak, C. A., and Partridge, J. E. 1988. Analysis of growth in sorghum callus cultures and association with a 27 KD peptide. Plant Sci. 57:235-246.6