POLYACRYLAMIDE GEL ELECTROPHORETIC STUDIES OF HOMOTHALLIC SPECIES OF NEUROSPORA

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CHAPTER I

INTRODUCTION

In the present research, soluble protein profiles of homothallic species of Neurospora, one of the best known and most intensively studied genera in the class Ascomycetes, were investigated. Members of this genus have assumed a fundamental role in biological studies. A review by Moreau-Froment (1956), which covers the taxonomy, ecology, morphology, cytology, physiology and the economic importance of this genus, as well as historical items, summarizes in an excellent fashion the growth of knowledge concerning these fungi up to the last decade.

Two types of Neurospora, namely, heterothallic and homothallic forms, exist in nature (Alexopoulos, 1962). Heterothallic types have two kinds of mycelia, both of which are required for sexual reproduction. The two types of mycelia are morphologically indistinguishable but are physiologically distinct as shown by their mating reactions. Homothallic types have only one type of mycelium which by itself is capable of reproducing sexually. Dodge (1927), showed that this condition arises in the secondarily homothallic species, N. tetrasperma, because of the presence within individual hyphae of nuclei of the two sexes.

The primary objective of the present investigation was to delineate taxonomic differences between five recently described Neurosporas on the basis of similarities and/or dissimilarities in

their soluble protein profiles. The five species studied were Neurospora terricola, Gochenaur & Backus, (Gochenaur and Backus, 1962); N. dodgei, Nelson & Novak, (Nelson et al., 1964); N. galapagosensis, Mahoney & Backus, and N. africana, Huang & Backus, (Mahoney et al., 1969); N. lineolata, Frederick & Uecker, (Frederick et al., 1969). The morphological characteristics of these species are well documented and clearly differentiated (Frederick et al., 1969; Austin et al., 1974).

A search of the literature has failed to reveal a comparative electrophoretic investigation of the five homothallic species listed by Frederick et al., (1969). Because of the importance of Neurospora species in genetic studies, a study of the type reported here seemed warranted in order to further assess the taxonomic standing of the homothallic species. It is hoped that results obtained from this study will serve as an additional basis for the accurate delineation of these species and also provide for a further evaluation of the usefulness of soluble protein profiles as a taxonomic criterion.

CHAPTER II

REVIEW OF LITERATURE

History and Discovery of *Neurospora* Species

Neurospora, known in its imperfect or non-sexual stage as *Monilia*, was called to the attention of science about two hundred years ago as a pest in bakeries. Before the days of mold inhibitors in commercial bread, a bakery infested with *Neurospora* was in for serious difficulties. On account of this it was the subject of several scientific inquiries in the last century (Shear and Dodge, 1927). Shear and Dodge were the first to publish an account of the life histories of members of this genus, including the sexual stages. They proposed the generic name *Neurospora* and described the heterothallic species, *N. sitopila* and *N. crassa*, and the secondarily homothallic species *N. tetrasperma*. *N. erythraea* (Moller) Shear and Dodge, which had previously been described as a *Melanospora* in 1901 by Moller, was also included in their report (Shear and Dodge, 1927). Living cultures of *N. erythraea* were not available to Shear and Dodge in 1927 and apparently are not available now for the species is not listed in the catalogues of any fungal culture collections. It is assumed that *N. erythraea* has not been encountered since its isolation during the early part of this century in Brazil.

Dodge recognized the advantages of *Neurospora* in genetics and undertook, therefore, a series of investigations using species of *Neurospora* (Dodge, 1927, 1928, 1930, 1931, 1935, 1942). Dodge

determined the manner of sex inheritance, demonstrating that segregation for the gene pair occurred at either the first or second meiotic divisions. He also showed that "albinism" (the failure to form conidia) in N. sitophila was inherited in a simple fashion with genetic segregation in either the first or second meiotic divisions.

Eight years after the initial work of Shear and Dodge was published, two additional species, namely, N. toroi Tai and N. intermedia Tai were described (Tai, 1935). Since 1962 five new species have been reported. All of these new species were found in soil and are truly homothallic, non-conidial, eight-spored forms exhibiting characteristics previously unknown in the genus. The isolates of the five new species came from four different continents, namely North America, South America, Africa, and Asia (Mahoney et al., 1969; Frederick et al., 1969). N. dodgei, N. terricola, and N. galapagosensis, were obtained from soil samples collected from the Luquillo Experimental Forest in the Luquillo Mountains of Puerto Rico, on Wisconsin lowland, and on the Galapagos Islands, respectively. N. africana was obtained from soil collected in Eastern Nigeria and N. lineolata was obtained from soil collected in the vicinity of Lahore, West Pakistan.

Present Taxonomic Status of Non-conidial Homothallic Neurosporas

As mentioned previously, the features of the Neurospora species used in this study are well documented and clearly differentiated. The non-conidial homothallic Neurosporas are primarily distinguished on the basis of ascospore morphology (Frederick et al., 1969;

Austin et al., 1974). N. terricola, the first described and the only one known in the temperate zone, is easily distinguished from all the others. It has the smallest ascospores and ascocarps and it is the only species that has spores with a single germ pore (Gochenaur and Backus, 1962). The tropical species (N. africana, N. dodgei, N. galapagosensis and N. lineolata) apparently have closer affinities to one another, from a morphological standpoint, than they have to N. terricola. For example, their ascospores have two germ pores and the spores are ellipsodial instead of round. N. dodgei, resembles the heterothallic species N. crassa in perithecial dimensions, as well as in the size, shape, and germ pore pattern of its ascospores. However, the ascospores of N. dodgei have fewer and coarser ribs that tend to anastomose more extensively than those of N. crassa (Nelson et al., 1964). The spores of N. dodgei are large and the ribs are broad and mostly continuous. Together with the intercostal veins they form the prominent sculpturing pattern of the spore wall.

N. galapagosensis resembles N. dodgei in both morphological and cultural features, but their patterns of ascospore sculpturing are quite different (Mahoney et al., 1969). The ribs of N. galapagosensis are narrow, occasionally branched or anastomosed, and form prominent sculpturing together with the intercostal veins on the spore wall.

In cultural appearance N. africana is essentially indistinguishable from N. dodgei and N. galapagosensis. It is readily separated, however, from the latter species by the size and sculpturing patterns of its ascospores. It differs from N. dodgei and N. galapagosensis mainly in

the smaller size of spores and perithecia (Mahoney et al., 1969). The spores of N. africana are small but broad with distinct veins.

N. lineolata closely resembles N. africana (Frederick et al., 1969). Both species have perithecia of similar dimensions and ascospores with a similar number of ribs on the spore wall. They differ from one another in that the intercostal veins of N. lineolata are distinct, the ribs are lower, and the spores are narrower. These morphological differences have recently been confirmed by Austin et al., (1974) in their report on scanning electron microscope observations of ascospores of homothallic Neurosporas.

Taxonomic Criteria Used in Establishing Species

Taxonomy, according to Talbot (1971), is both the method and practice of systematic classification. Classification in most cases are hypotheses of biological relationships among organisms, and these may change as more is known about the organisms. However, a good character on which classification is based is usually one with little variation among a large number of specimens and therefore mostly called a constant character. But, as Nelson (1965) pointed out, there is no single criterion which can be used universally to assess biological relationships. An understanding of the degree of similarities and the magnitudes of differences among organisms will be derived from a variety of assessments. Such a composite knowledge will involve studies from the disciplines of all the biological sciences.

Morphological characteristics as taxonomic criteria.

Until recently the taxonomy of fungi has been largely based on the morphology of the reproductive structures, this being the only criterion available to early taxonomists, and it is likely to continue to be one of the major bases for classification (Glynn and Reid, 1969). In most situations, morphological features of members of this group are sufficiently recognizable, uniform, stable and distinctive to allow assessment of genetic relationships among taxa. However, the identification of some fungi, both in culture and on natural substrates, has frequently been difficult because of the absence of these reproductive structures or controversy about their formation. Hence, for various reasons, many workers have considered the possibility of using other characters as taxonomic criteria. Johnson and Hall (1965) postulated that morphological characteristics reflect genetic differences and collectively provide a broad base for inference regarding genome relationships. However, a number of limitations are encountered when morphological characteristics are used for the purpose of inferring gene homology. Firstly, morphological characteristics represent the maximum number of genes and therefore cannot be readily equated to gene number. Because of these morphological features genetic diversity is often masked by form simplicity and if morphology is complex it may not reflect genetic similarity among taxa. Also, it is not possible from a gross morphological character to distinguish homology of genes from analogy of genes (Anfinsen, 1959).

Protein and isoenzyme profiles as taxonomic criteria.

Due to the limitation of the determination of gene homology by morphological characteristics, electrophoresis applied to fungal proteins has received much attention recently (Dessauer and Fox, 1964; Durbin, 1966; Gill and Powell, 1968; Hall, 1971; Tyrell, 1971). Representatives of every major group of fungi have been studied electrophoretically and favorable (Franke and Berry, 1972; Shechter et al., 1966) and unfavorable (Meyer, 1969; Shipton and MacDonald, 1970) results have been reported. However, a general conclusion could be drawn from the results of these various studies that indicates that protein profiles probably provide a more direct measure of gene homology than do morphological features. Protein profiles have a number of advantages as taxonomic criteria, since they can be done fairly easily, using any fungus, preferably from pure culture. These profiles are reproducible when prepared from the same isolates under standard conditions. In most instances different profiles are obtained from different taxa (Hall, 1973). Such protein profiles may be easily analyzed quantitatively and qualitatively. Investigators also report that in contrast to morphological characters protein or isoenzyme profiles are not highly responsive to environmental conditions. Such results have led workers to suggest that the understanding of speciation must be sought in the structure of proteins.

Ingram (1955), employing electrophoresis, showed that the abnormal sickle cell hemoglobin (known to be due to a single gene) differs from the normal by a single amino acid residue. Since then

numerous examples of the same type of work have appeared in the literature. Consequently, the accumulated gene mutations which distinguish species may be identified in part through their effect on the mobility of specific fractions of the protein.

Electrophoretic Methods as a Taxonomic Tool in Fungal Studies

Zone electrophoresis, which has been determined to be the best method for protein separation, has been employed by many workers as a taxonomic tool in fungal studies (Chang et al., 1962; Clare, 1963; Hall, 1969; Reddy and Threlkeld, 1971 and 1972; Reddy, 1973). Many stabilizing media have been available for zone electrophoresis over the years including filter paper, cellulose acetate, agar, agarose, sephadex, starch gel and polyacrylamide gel (Oelshlegel and Stahmann, 1973). Each medium has its own particular advantages, disadvantages, and specific applications. However, greater separation is possible with starch and polyacrylamide gel media because the size of these media can be made to be similar to the pore size of the molecules (that is proteins) to be resolved (Shaw, 1969). Thus starch and polyacrylamide gel electrophoreses separate soluble proteins not only on the basis of charge, but on the basis of size via a sieving effect. For this reason starch and polyacrylamide gel electrophoreses are sometimes referred to as high resolution electrophoreses. For example, serum proteins can be separated into about twenty-five components in polyacrylamide gel, but only into five on filter paper (Shaw, 1969). Chang et al., (1962) separated twenty-five soluble protein fractions from

Neurospora species using polyacrylamide gel.

Smithies (1955) first used starch gel as a supporting medium in his work with serum proteins from human adults. Hunter and Markert (1957) were able to locate esterase isoenzymes by histochemical techniques after subjecting extracts of mouse organs to starch gel electrophoresis. Raymond and Weintraub (1959) and Davis and Ornstein, as quoted by Ornstein (1964) independently suggested polyacrylamide gel as a stabilizing medium for zone electrophoresis. The development of starch and polyacrylamide gel systems have played a major role in the growth in research and knowledge of isoenzymes and proteins in both biology and chemistry.

Studies using Protein and/or Isoenzyme Profiles as Taxonomic Criteria

Many workers have studied the influence of the growth conditions and developmental stages on the fungal protein pattern and zymogram in order to assess their reliability as taxonomic criteria for use in establishing biological relationships. Identical patterns for dehydrogenases were observed by Tsao (1962) when eight wild-type strains of Neurospora crassa were cultured in different media. Changes in protein and enzyme patterns have been observed during differentiation of cellular slime molds (Solomon et al., 1964). Tsao and Madley (1969) observed a single fast-moving phosphofructokinase band from an 18-hour mycelial extract of N. crassa and two additional slower bands in a 48-hour mycelial extract. Changes in fungal enzymes have been observed to vary with the physiological age of the culture, morphology,

and physical conditions for growth such as type of medium and temperature (Lloyd et al., 1971; Shannon et al., 1973).

Reddy and Threlkeld (1971) evaluated isoenzyme analysis as a means for species identification by comparing zymograms from thirty-six strains representing eight species of Neurospora. The esterase zymograms obtained from their work seemed to provide an acceptable means of distinguishing six species of Neurospora. They reported, however, that they were unable to distinguish between strains belonging to N. tetrasperma and N. intermedia, both secondarily homothallic species.

Chang and Chan (1973) working with Volvariella volvacea, observed differences among profiles from various stages of development of the basidiocarp as well as in different parts such as the volva and pileus.

The concept that strains of fungi can be distinguished by protein electrophoresis has been developed and supported by many workers (Hall, 1969, Shipton and MacDonald, 1970, Stipes, 1970; Tyrell, 1971; Reddy, 1973). In 1962, distinctly different banding patterns were obtained by Chang et al., (1962), after subjecting mycelial extracts of three strains of N. crassa to gel electrophoresis. These authors compared results obtained from extracts of a mutant strain of N. crassa with those from a wild-type and concluded that interspecific differences were greater than intraspecific differences. It was suggested from these results that gel electrophoresis might prove to be a useful taxonomic tool in fungal research. Since then considerable attention has been paid to determination of proteins and enzymes from various components of fungi as a possible aid in resolving some of the

problems encountered in fungal taxonomy.

It has been reported by several workers that gel electrophoresis might be useful in the identification of fungal species (Clare, 1963; Durbin, 1966; Whitney et al., 1968; Hall, 1969; Reddy and Threlkeld, 1971). Gottlieb and Hapden (1966), studying species of Streptomyces, obtained results which suggested that electrophoretic studies can serve in conjunction with other criteria as an aid in Streptomyces taxonomy. They concluded, however, that electrophoretic studies cannot yet be used as a major means of solving problems in taxonomy.

Electrophoresis has recently been applied to solving problems in angiosperm classification (Levin and Schaal, 1970; Johnson and Thien, 1970). In the latter study the emphasis was mainly phylogenetic rather than identification.

Clare (1963), studying Pythium, found that isolates of any species obtained from different locations were similar in their soluble protein profiles. On the basis of these results he suggested that protein patterns may be helpful in classifying Pythium species. Durbin (1966) found that the majority of the protein profiles obtained from Septoria species were electrophoretically identical or quite similar, there being, in some, variations in band position. He therefore concluded that the use of protein profiles for identifying Septoria species could become a valuable adjunct to other techniques for investigating Septorias parasitic on grasses, where the problem

is relatively restricted in scope. Hall (1969) studied protein patterns from ten isolates of Verticillium dahliae and twenty-two isolates of V. albo-atrum using polyacrylamide gel electrophoresis. He observed that isolates within a species produced essentially identical protein profiles but that V. dahliae patterns were distinctly different from those of V. albo-atrum. His results support the view that V. dahliae and V. albo-atrum should be considered as separate species. Kulik and Brooks (1970) determined a phylogenetic relationship among Aspergillus species. They reported that more protein components were shared among members of A. flavus group than between this group and other Aspergilli. Polyacrylamide gel electrophoretic studies were conducted by Sorenson et al., (1971) with soluble protein extracts from mycelium of five species of the genus Aspergillus. These authors obtained results which suggest that the technique of polyacrylamide gel electrophoresis might be of help in assessing the degree of similarity between species and/or closely related genera. However, their results did not support the concept that such protein patterns can be used in the clear identification of species. Stipes (1970), studying mycelial protein and enzyme patterns in four species of Ceratocystis on polyacrylamide gels by disc electrophoresis, observed greater similarity of protein profiles within than among isolates of the same species. Identical alkaline phosphatase patterns were observed within each species, but there was no alkaline phosphatase site common to all. His results provided

additional support for electrophoresis as a taxonomic tool. These results further demonstrated intraspecific and interspecific biochemical differences and similarities in organisms studied with polyacrylamide gel electrophoresis and enhanced the usefulness of this technique as a tool in taxonomic studies.

Several objections have been raised against the taxonomic use of the biochemical characters obtained electrophoretically as they are subject in some situations to the same limitations as are taxonomic criteria based on conventional morphological characteristics. A number of reports have been published recently which do not support disc electrophoresis as a useful tool in fungal taxonomy. Peberdy and Turner (1968) released results from their work on nine strains of Mortierella ramanniana which showed esterase profiles were of no taxonomic significance. A year later, Glynn and Reid (1969) obtained results from studying species of Fusarium that were not supportive of the concept of gel electrophoretic studies of fungal components being of taxonomic value. These authors observed that the composition of the medium and also the quantity of inoculum had an appreciable influence on the profiles obtained. They also reported an important deviation from previously published electrophoretic protein profiles of fungal components in that they observed similar profiles of species from different genera. The usefulness of protein profiles as taxonomic criteria was challenged by Shipton and Fleishmann, (1969) who expressed reservations about the reliability of R_f values published.

Shipton and MacDonald (1970) observed that there was as much difference in the profiles obtained from two different tissues of the same species as in those obtained from two different species. They obtained these results from their work with two species of Drechslera. Because of this reason they questioned the use of protein profiles in fungal taxonomy. However, due to the many advantages of the electrophoretic technique in fungal taxonomy, it seemed reasonable to undertake the present research, in order to assess its usefulness in delineating the five homothallic Neurosporas and establishing their taxonomic relationships.

CHAPTER III

MATERIALS AND METHODS

Neurospora Culture Sources

The five homothallic species of *Neurospora* (*N. africana*, *N. dodgei*, *N. galapagosensis*, *N. lineolata*, and *N. terricola*) used in this investigation were obtained from the Atlanta University Fungal Collection. They were all, with the exception of *N. lineolata*, originally obtained from the American Type Culture Collection, Rockville, Maryland. The *N. lineolata* culture was an isolate from a stock culture of that species in the department collection.

The stock cultures used to inoculate 200 ml of *Neurospora* minimal medium in 500 ml wide mouth erlenmeyer flasks were five-day-old cultures maintained at 25 C on corn meal agar, supplemented with 20 g of sucrose.

Preparation of *Neurospora* Minimal Medium

The basal or minimal medium used had the following composition in grams per liter: ammonium tartrate, 1.0; ammonium nitrate, 1.0; ammonium phosphate, 1.0; potassium phosphate, 1.0; magnesium sulfate, 0.5; sodium chloride, 0.1; calcium chloride, 0.1; biotin, 0.0005 g. These compounds were dissolved in 1 liter of distilled water and supplemented with 20 g of sucrose. In addition the medium contained the following trace elements in milligrams per liter: Mo 0.02; Fe 0.2; Mn 0.02; Cu 0.1; Zn 2.0; and B 0.1 (Beadle and Tatum, 1947; Klein and Klein, 1970). Each liter of medium was adjusted to pH 6.5 by adding a

sufficient volume of 1 N HCl. The medium was stored in glass bottles at 20-25 C.

Growth Characteristics of Cultures

Five hundred ml flasks containing 200 ml medium were inoculated with 10 mm plugs of Neurospora mycelia. These flasks were incubated at 22 C for five days on a rotary shaker at 140 rpm. Shake cultures were used instead of stationary liquid cultures because the former facilitates equal distribution of nutrients to the growing mycelia. According to Calam (1969), in a stationary liquid medium nutrients are rapidly depleted around the mycelia causing the cells to sink or float. This situation results in layers of growth in which the medium becomes exhausted and cells die. Apart from nutrient depletion, the occurrence of a stationary layer immediately surrounding the cells provides a diffusion barrier which restricts the rate at which nutrients can enter the cells. It is the object of agitation to overcome such obstacles to nutrient supply. Furthermore, according to Calam, circulation is required to overcome layering and the formation of regions of starvation, and turbulence reduces the diffusion barrier in the immediate vicinity of the cells. Agitation is also needed to facilitate transfer of oxygen to the liquid.

Harvesting of Mycelia and Extraction of Soluble Proteins

The mycelium was separated from the culture media by vacuum filtration and washed with 300 ml of distilled water. The mats were

squeezed to remove excess water, weighed, lyophilized and stored at 0 C for later use. A quantity of mycelium equivalent to 0.0100 g was weighed and homogenized in a ml of tris-citrate buffer, pH 9.0, 0.075 M, containing 50% sucrose, with a chilled mortar and pestle. A quantity of 0.0050 g of sand was added to each sampling of tissue to facilitate grinding. The homogenate was transferred to centrifuge tubes using an additional 2-4 ml of homogenizing buffer and spun for 30 min at 10,000 rpm to remove cell debris. The resulting supernatant was used for protein estimation or the electrophoretic separation of the soluble proteins.

Protein Estimation

Soluble proteins were precipitated overnight in the cold with 5% trichloroacetic acid. The resulting protein precipitate was separated from the supernatant by centrifugation for 30 min at 10,000 rpm. One ml, of N NaOH was added to the protein and heated for 20 min in a boiling water bath. The resulting protein solution was diluted to 10 ml with distilled water. The quantity of protein present was determined by the method of Lowry et al., (1951) using bovine serum albumin standards.

Separation of Soluble Proteins

Soluble proteins were separated by verticle flat bed discontinuous electrophoresis in gradient pore polyacrylamide gels using an Ortec high voltage pulsing system. The gradient pore gels were prepared by layering of acrylamide solutions of varying concentrations to form a

matrix with discoid pores of differing diameters.

The first layer cast was a 59 mm high small pore running gel, consisting of 8% acrylamide. Immediately afterwards, a 9 mm high layer of gel of intermediate pore size was layered over the 8% layer. This layer consisted of 6% acrylamide. A layer of water was immediately placed on top of the second layer and the two were allowed to polymerize for 20 min. The water layer was subsequently removed and a 5 mm high layer consisting of 4 1/2% acrylamide was cast, water-layered, and allowed to polymerize for at least 15 min. These three layers of acrylamide were buffered with tris-chloride, pH 8.4, 0.0375 M. After removal of the water, a fourth layer, the well-forming layer, was placed on layer three. This 8% acrylamide layer was buffered with tris-chloride, pH 8.4, 0.075 M. Each layer contained an amount of methylene bis-acrylamide equivalent to 2.5% of the quantity of acrylamide used in the particular layer. Twelve sample wells were formed by inserting a twelve pronged well-former into the fourth layer and allowing 20 min for polymerization. The well-former was then removed and the wells rinsed with water and wiped with Kimwipes.

For separation of soluble proteins, a volume of extract containing 300 ug protein was placed in each well. The wells were then capped with a gel of composition similar to that of the well-forming gel. After at least 20 min polymerization, cells containing gels loaded with crude protein extract were assembled in the Ortec electrophoresis buffer tank.

The electrode buffer used for the separation of soluble proteins was tris-glycine, pH 8.4, 0.0495 M. Approximately 1 hr and 15 min was allowed for the complete migration of the samples from the wells to within a distance of 10 mm from the edge of the gel. Power settings were increased at intervals of 30, 25, and 10 min increments of 75 pulses per second (pps) until the initial setting of 75 pps was increased to 300 pps. When one cell was run, a current of 325 volts was used. However, 400 volts were utilized in the running of two cells (Instructional Manual, Ortec Incorporated, 1969). After electrophoretic separations, the gels were removed from the casting cells and the soluble proteins were localized.

Localization of Soluble Proteins

Gels were put into 12% trichloroacetic acid for 30 min at room temperature. The trichloroacetic acid was then poured off and the gel slabs rinsed 5-6 times with tap water. They were then placed in 0.2% Coomassie Blue, agitated for 1 min and placed in a 37 C water for 1 hr. After 1 hr the Coomassie Blue was discarded and the gels were destained by several rinsings with 10% acetic acid. The gels were then stored in 10% acetic acid (Ortec Application Notes, 1972).

CHAPTER IV

RESULTS AND DISCUSSION

Mycelial Growth of *Neurospora* Species

Table 1 shows the relative growth of the five species used in this study. *N. dodgei* yielded the highest average amount of dry matter, 10 g, under the conditions employed. The second highest average yield, 5.8 g, was obtained from *N. africana* and *N. galapagosensis*. *N. lineolata* followed closely with an average dry weight yield of 5.5 g. The lowest average amount of dry matter, 1.2 g, was produced by *N. terricola*. The above pattern of growth for these species proved consistent after several repetitions. It was therefore postulated that the low yield of dry matter observed from *N. terricola* was regulated by factors intrinsic in its make-up and that the high yield of dry matter from the other four species (*N. dodgei*, *N. africana*, *N. galapagosensis*, and *N. lineolata*) was probably an intrinsic characteristic of these species also. This fact is implied by the consistent high yield of dry matter obtained from the latter species and the equally low yield of dry matter obtained from *N. terricola* under the same growth conditions. It was observed also that when growth of *N. terricola* on the corn meal agar plates was compared with the growth observed from the other species on that medium, its growth was sparse.

Percent of Soluble Protein of Dry Matter from Mycelial Yields

Data collected from the determination of the percent soluble protein in mycelia from the *Neurospora* species studied are presented

Table 1. Dry weights (g) of mycelia formed in five-day-old shake cultures of five species of Neurospora.

Species	Harvest Number 1	2	3	4	Average
N. africana	6.0*	5.0	7.0	5.0	5.8
N. dodgei	9.0	12.0	10.0	10.0	10.0
N. galapagosensis	6.0	6.0	5.0	6.0	5.8
N. lineolata	6.0	5.0	5.0	6.0	5.5
N. terricola	1.0	2.0	0.9	1.0	1.2

*Each figure represents the weight of pooled mycelia from five flasks after five days of growth.

in Table 2. N. dodgei, the species with the highest mycelial yield, had the lowest percent soluble protein of dry matter (24.0%). N. terricola, the species with the lowest mycelial yield, had the highest percentage soluble protein of dry matter. N. galapagosensis, with an average of 55.0% soluble protein, was next to N. terricola. N. lineolata and N. africana contained 42.0% and 25.0% soluble protein, respectively. These data show that the soluble protein content of these Neurospora species was generally very high. This is not unusual, however, as fungi are known to have a high content of soluble protein. In 1929, Takata, as quoted by Thatcher (1954), showed that the mycelium of Aspergillus oryzae contained 38% soluble protein and was a rich source of vitamins.

A peculiar correlation was observed when results from the determination of the soluble protein content of some of the Neurospora species were compared with data obtained from their dry matter. An inverse correlation is apparent from these results, that is, the higher the amount of soluble protein present, the lower the mycelial growth. Mycelial yields of N. dodgei and N. terricola clearly illustrates this feature. This finding differs from the relationship that is reported to normally exist between the amount of protein and growth (Thatcher, 1954). However, a conclusion cannot be categorically drawn from these data since determinations were made for soluble proteins only. Insoluble proteins cannot be accounted for as this fraction was not examined. Further investigation is necessary in this

Table 2. Soluble protein content of dry matter from mycelia of five species of Neurospora after shake culture for five days in Neurospora minimal medium.

	Determination Number			
Species	1	2	3	Average percent of soluble protein
N. africana	25.0*	25.0	25.0	25.0
N. dodgei	24.0	24.0	24.0	24.0
N. galapagosensis	55.0	54.0	55.0	55.0
N. lineolata	42.0	40.0	41.0	41.0
N. terricola	56.0	56.0	56.0	56.0

*Each figure represents the percent of soluble protein detected in a 1-gram of dry mycelium.

area in order to fully understand the relationship that exists between the protein content and growth in homothallic Neurosporas.

Electrophoretic Studies of Soluble Proteins

Figure 1 shows the soluble protein profiles of the Neurospora species studied. At least twelve replicate profiles of each species was obtained. Figure 2 is a duplication,from replicate cultures, of the soluble protein profiles presented in Figure 1. The latter figure shows that the banding patterns were consistently reproducible. The soluble protein profiles in Figure 3 represent patterns obtained from replicate cultures after repeating the electrophoretic process. These profiles reveal also that the banding patterns obtained from cultures grown at a different time are essentially identical to those in these profiles. Diagrams of replications of these soluble protein profiles are presented in Figures 4, 5, and 6 respectively.

A total of twenty-one soluble protein fractions were consistently detected in profiles of the five Neurospora species. Eleven of these occurred in profiles of N. dodgei and N. lineolata. Profiles of N. africana and N. galapagosensis contained thirteen bands and N. terricola contained fifteen. A comparatively large number of soluble protein bands are reported to occur in Neurospora species by workers who have studied these organisms with polyacrylamide gel electrophoresis. Chang et al., (1962), for example, resolved

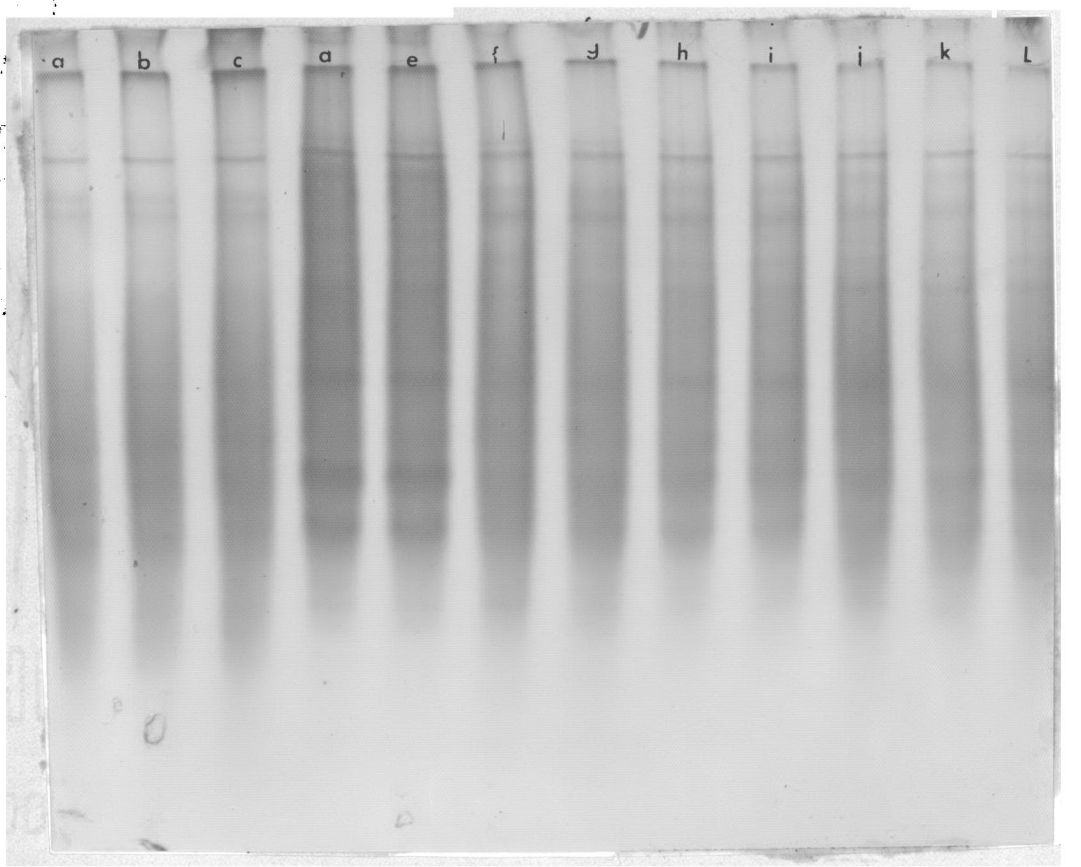

Fig. 1. Polyacrylamide gels of soluble proteins from Neurospora species. N. dodgei, a, b, and c; N. africana, d and e; N. lineolata, f and g; N. galapagosensis, h and i; N. terricola, j, k, and l.

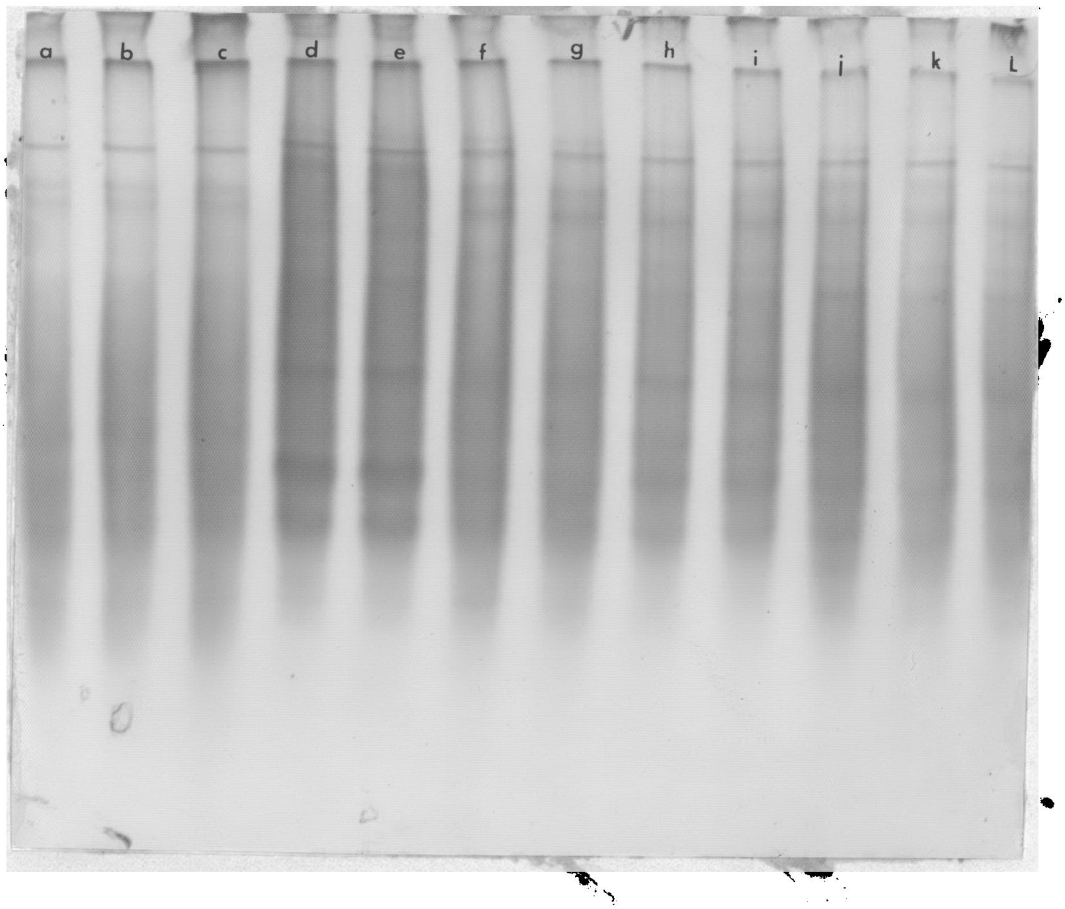

Fig. 2. Replication of the soluble protein profiles, showing that the banding patterns were consistently reproducible. N. dodgei, a, b, and c; N. africana, d and e; N. lineolata, f and g; N. galapagosensis, h and i; N. terricola, j, k, and 1.

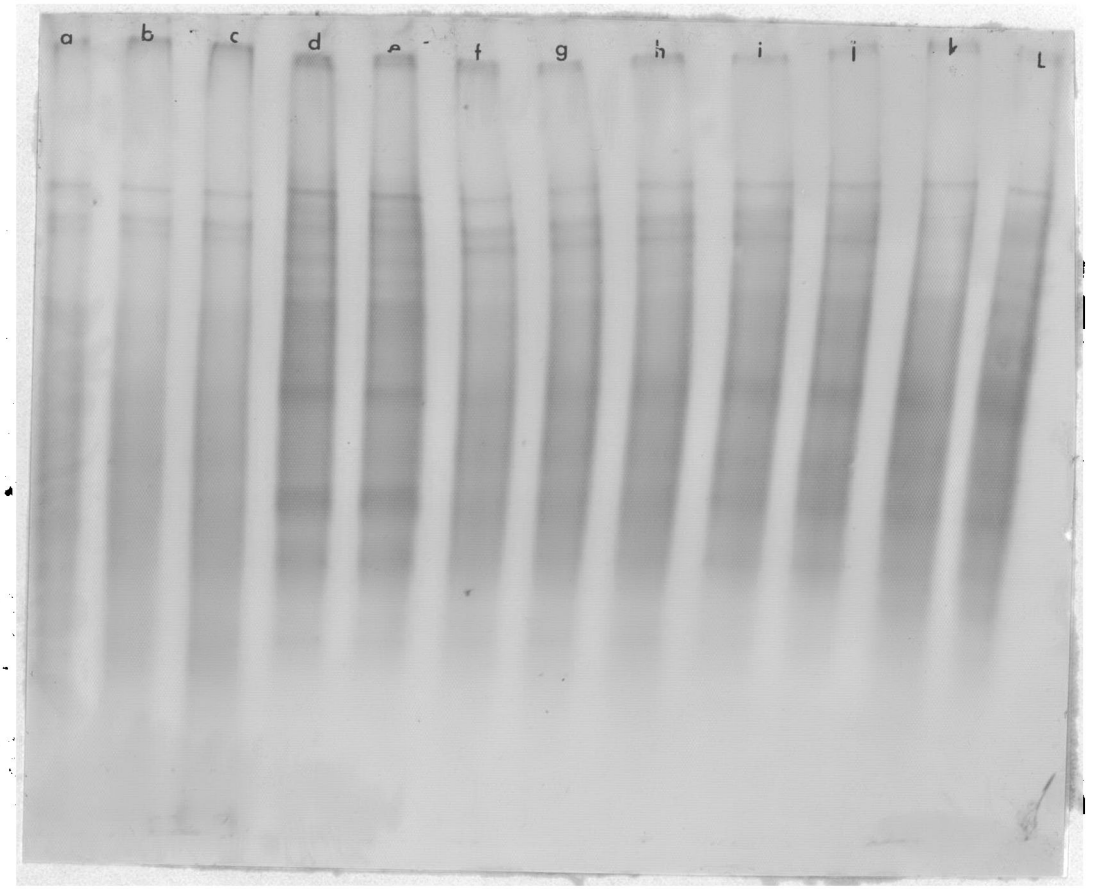

Fig. 3. Soluble protein profiles from other cultures grown at a different period of time revealing banding patterns essentially identical to those originally obtained. N. dodgei, a, b, and c; N. africana, d and e; N. lineolata, f and g; N. galapagosensis, h and i; N. terricola, j, k, and l.

Figure 4

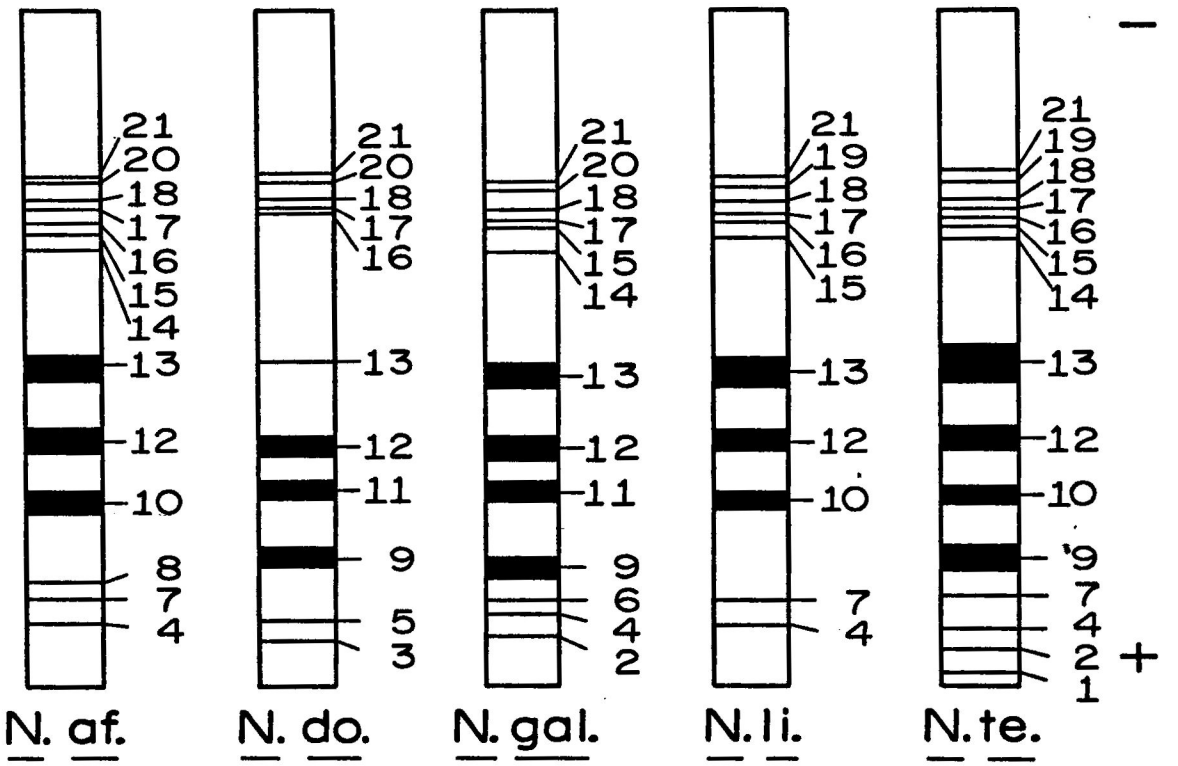

Fig. 4. Diagramatic representation of soluble protein profiles shown in Fig. 1.

1. N. af. - N. africana
2. N. do. - N. dodgei
3. N. gal. - N. galapagosensis
4. N. li. - N. lineolata
5. N. te. - N. terricola

Figure 5

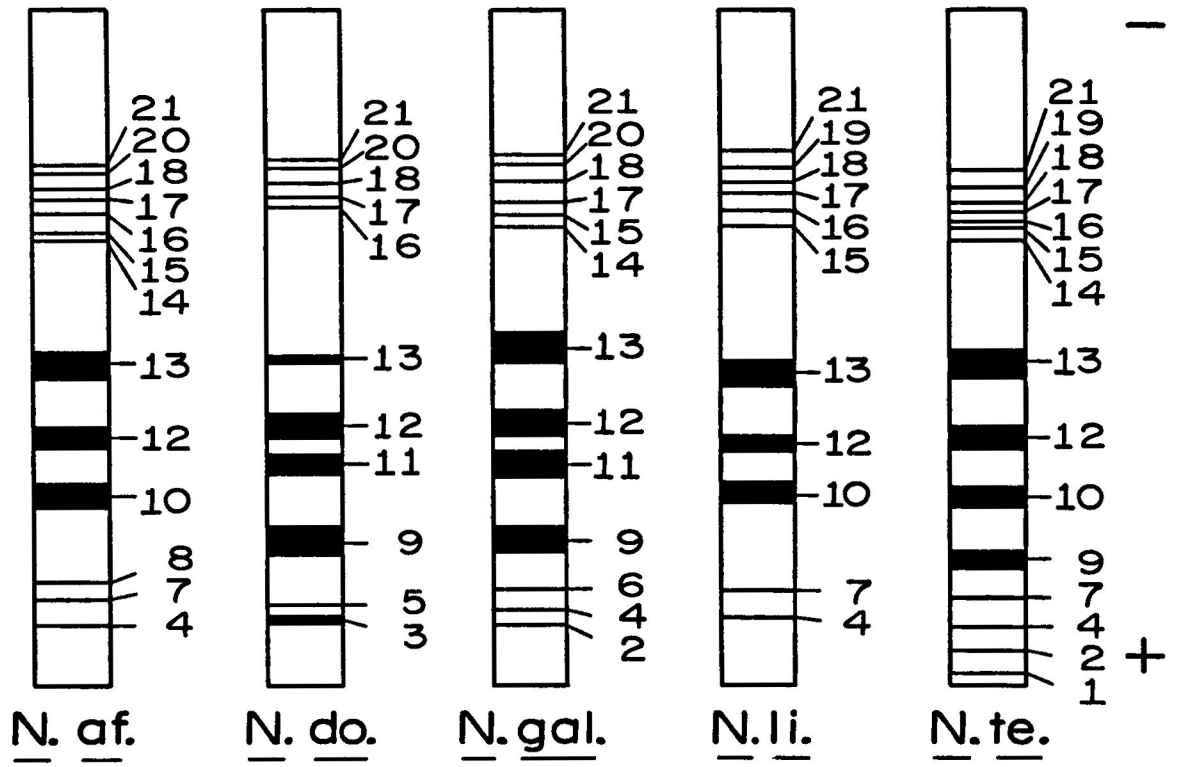

Fig. 5. Diagram of replicate soluble protein profiles of five *Neurospora* species from Fig. 2.

1. *N. af.* - *N. africana*
2. *N. do.* - *N. dodgei*
3. *N. gal.* - *N. galapagosensis*
4. *N. li.* - *N. lineolata*
5. *N. te.* - *N. terricola*

Figure 6

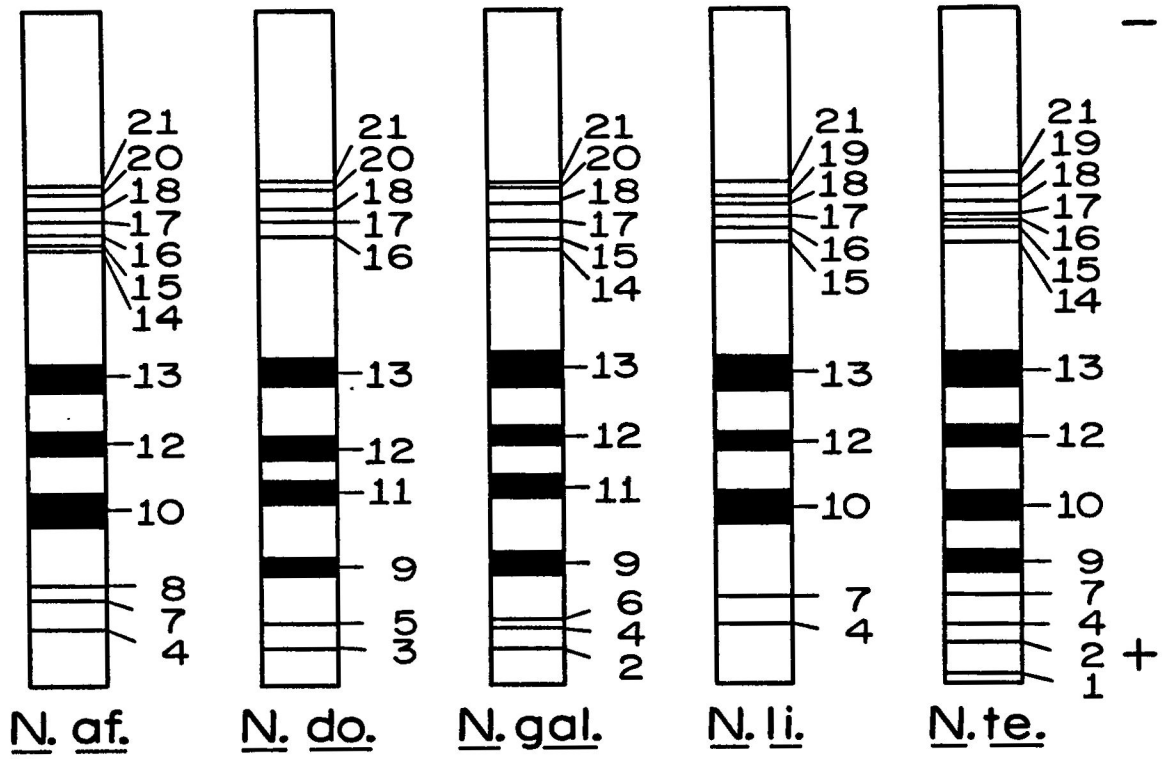

Fig. 6. Diagram of soluble protein profiles of five Neurospora species from Fig. 3.

1. N. af. - N. africana
2. N. do. - N. dodgei
3. N. gal. - N. galapagosensis
4. N. li. - N. lineolata
5. N. te. - N. terricola

twenty-five bands of soluble protein fractions from the heterothallic species Neurospora crassa, N. sitophila, and N. intermedia, as well as from a mutant strain of N. crassa.

The soluble protein fractions present in each of the five species studied and their R_{tD} values are presented in Tables 3 and 4 respectively. Five soluble protein fractions (12, 13, 17, 18, and 21 at R_{tD} 0.58, 0.47, 0.27, 0.26, and 0.23 respectively) were found in all the Neurospora species examined. The presence of these common soluble protein fractions indicates a high degree of similarity between these species of Neurospora.

Soluble Protein Profiles as Species Markers

Four of the five homothallic Neurospora species contained soluble protein fractions which distinguished each from the other species. Two soluble protein bands, 3 and 5, (Table 3) at R_{tD} 0.83 and 0.81, (Table 4) are present exclusively in N. dodgei. N. terricola can be distinguished from the others by the presence of a soluble protein band 1 at R_{tD} 0.89, while N. galapagosensis and N. africana are distinguished by the occurrence of soluble protein band 8, at R_{tD} 0.77 and band 6, at R_{tD} 0.80, respectively. These results revealed no soluble protein band either present or absent, that was unique to N. lineolata. The pattern of occurrence of these species-specific soluble proteins support the current claim that these five Neurosporas are distinct and separate species. Even though N. lineolata does not exhibit a species-specific soluble protein band,

Table 3. Soluble protein fractions obtained from mycelia of five species of Neurospora grown in shake culture for five days.

	Soluble protein fractions (bands)																				
	Band number																				
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
N. af.				x			x	x		x		x	x	x	x	x	x	x		x	x
N. do.			x		x				x		x	x	x			x	x	x		x	x
N. gal.		x		x		x			x		x	x	x	x	x		x	x		x	x
N. li.				x			x			x		x	x		x	x	x	x	x		x
N. te.	x	x		x			x		x	x		x	x	x	x	x	x	x	x		x

N. af. = N. africana
N. do. = N. dodgei
N. gal. = N. galapagosensis
N. li. = N. lineolata
N. te. = N. terricola

Table 4. R_{tD} values of soluble protein fractions from mycelia of five Neurospora species.

Soluble protein fraction	R_{tD} value
1	0.89
2	0.84
3	0.83
4	0.82
5	0.81
6	0.80
7	0.79
8	0.77
9	0.72
10	0.64
11	0.63
12	0.58
13	0.47
14	0.30
15	0.29
16	0.28
17	0.27
18	0.26
19	0.25
20	0.24
21	0.23

soluble protein fractions present in one species and absent in the other species, with which it is being compared, and the number of bands absent in a species but present in the other. Data on variant sites are presented in Table 5. The higher the number of variant sites between different taxa, the more unrelated the taxa are said to be. According to this parameter, the highest number of variant sites occur between N. dodgei and N. terricola, namely twelve. This indicates that the greatest taxonomic difference among the five species exists between N. dodgei and N. terricola. Next to N. terricola, in terms of the degree of difference from N. dodgei, is N. lineolata with ten variant sites, followed by N. africana with nine variant sites and N. galapagosensis with eight. These data suggest that N. dodgei and N. galapagosensis are the most closely related species of the group and that N. dodgei and N. terricola are the most distantly related. When these data are further analyzed to ascertain taxonomic relatedness, they indicate that N. africana and N. lineolata are closely related. Only four variant sites occur between these species. N. africana and N. lineolata vary from N. terricola at six and four sites respectively. These variant sites data (Table 5) support the conclusions of Frederick et al., (1969) that each of these five organisms represent distinctly different species of Neurospora.

Soluble protein fractions from different taxa that migrate the same distance from their points of origin are said to be homologous proteins (Kulik and Brooks, 1970). The occurrence of homologous

Table 5. Variant sites of soluble proteins between all possible combinations of five Neurosporas.

Species	*N. af.*	*N. do.*	*N. gal.*	*N. li.*	*N. te.*
N. af.	-	9	7	4	6
N. do.	9	-	8	10	12
N. gal.	7	8	-	8	6
N. li.	4	10	8	-	4
N. te.	6	12	6	4	-

N. af. = *N. africana*

N. do. = *N. dodgei*

N. gal. = *N. galapagosensis*

N. li. = *N. lineolata*

N. te. = *N. terricola*

proteins may be used therefore to determine relatedness among organisms. When this premise is employed in the present work, those species that have the highest number of common homologous soluble protein fractions are considered to be most closely related. The number of soluble protein fractions common to all possible two pair combinations of the five Neurospora species is given in Table 6. Data presented in this table indicates that N. terricola is closely related to both N. lineolata and N. africana for the former species shares eleven common sites with each of the latter two species. Utilizing this parameter, N. galapagosensis also appears to be closely related to N. terricola for they share ten common sites. N. dodgei shares fewer common sites with the other four species and would be regarded as the most distantly related of the species. N. galapagosensis appears to be the species with the closest relationship to N. dodgei. These two species share eight common sites. On the other hand, however, N. galapagosensis has closer affinities to the three other species than it has to N. dodgei for it shares ten common sites with N. terricola, and nine with N. africana and N. lineolata. The relatedness evidenced from these data agrees with that based on the variant sites data. However, the data from the homologous soluble protein are mainly dependent on the number of protein bands present in the species being compared, that is the higher the number of soluble protein bands the higher the number of homologous protein bands which will be exhibited between the two species under comparison.

Table 6. Homologous soluble protein fractions between all possible pairings of five *Neurospora* species.

Pair	Number of homologous protein fractions
N. terricola vs. *N. africana*	11
N. terricola vs. *N. lineolata*	11
N. terricola vs. *N. galapagosensis*	10
N. africana vs. *N. lineolata*	9
N. galapagosensis vs. *N. africana*	9
N. galapagosensis vs. *N. lineolata*	9
N. galapagosensis vs. *N. dodgei*	8
N. dodgei vs. *N. terricola*	7
N. dodgei vs. *N. africana*	7
N. lineolata vs. *N. dodgei*	6

Because of this feature of the homologous protein fraction, determination of the variant sites gives a better and more valid indication of the relatedness between organisms. Data from each analysis, that is variant sites and homologous protein fractions, support the claim that these five organisms are separate species.

When geographical origin of the species studied is considered these data provide no evidence that the homothallic Neurospora species from tropical soils are more like each other than to the species from temperate soils. As noted above, N. terricola, a species from temperate soils, reveals soluble protein profiles that are similar to N. africana, N. lineolata, and N. galapagosensis of tropical soils, than N. dodgei, a tropical soil form, has to these species.

Members of the genus Neurospora are traditionally delineated taxonomically by the morphology of the perfect and/or imperfect stages. Some workers, however, have cautioned against delineating fungal species on the basis of few and variable criteria such as morphology (Hall, 1973). As many factors as possible should be assessed when an understanding of the differences among isolates of species is in question. Also, other criteria should be evaluated when species within a genus are separated on the basis of closely similar morphological characters. The five homothallic Neurosporas used in this study are considered to be readily distinguishable on the basis of ascospore features (Austin et al., 1974; Frederick et al., 1969). Culturally, the general growth rate, colony habit, and other features vary only

slightly among isolates of the species. Perithecia of these species are also of similar form and size.

By comparison, soluble protein patterns obtained by repeated runs of samples are also distinctive for each species and are reproducible. Furthermore, there are striking interspecific similarities when the soluble protein profiles of N. terricola, N. lineolata, N. galapagosensis and N. africana are compared.

The results from this study are mostly in line with results reported from other studies of Neurospora species. Reddy and Threlkeld (1971) compared esterase, lactate dehydrogenase, amylase, peroxidase, and phosphatase zymograms from thirty-six strains representing eight species of Neurospora. The results obtained from their study indicated that esterase zymograms provided an acceptable means of distinguishing between the six species of Neurospora but that zymograms of the other isozymes were of limited value. Three of the species studied by Reddy and Threlkeld were used in this study, namely, N. africana, N. dodgei, and N. terricola. They found the esterase patterns of the species to be similar to each other. They furthermore distinguished between the homothallic and heterothallic Neurospora species on the activity of lactate dehydrogenases in these species. They considered it impossible, however, to distinguish between the secondarily homothallic species on the basis of esterase zymograms.

The results obtained in this study, even though they are based on the whole soluble protein component, generally corroborate the

findings of Reddy and Threlkeld (1971), Reddy (1973), and others (Chang, 1962) who have studied the biochemical features of these organisms. Also, these results agree with those reported by Austin et al. (1974), who separated the homothallic Neurospora species on the basis of topographic features of the walls of the ascospore, using the scanning electron microscope. For example, on the basis of wall topographic patterns N. dodgei and N. lineolata were regarded as the least similar of the bipolar homothallic species. N. lineolata and N. africana were considered to manifest closely similar features to each other and N. dodgei and N. galapagosensis were regarded as closely similar. In this study the differences observed between these species remained constant under the cultural conditions used and provided additional substantiation to the taxonomic conclusions based on morphological studies about these fungi.

Results from this study also shed additional light on the taxonomic relatedness and species distinctiveness of the homothallic species of Neurospora. Those species in this investigation that exhibited similar soluble protein profiles are apparently more closely related than those species with very different soluble protein profiles. Using this premise, three of the species with bipolar ascospores, viz., N. africana, N. galapagosensis, and N. lineolata, are closely related to each other. On the other hand, N. dodgei, the fourth species with bipolar ascospores appears to be more distantly related to those three species. It was concluded therefore that the taxonomic

determinations relative to the species standing of the organisms is sound.

Even though N. terricola exhibited protein profiles that differed little from those of N. africana, N. galapagosensis, and N. lineolata, the unipolar nature of ascospores and their more rounded shape suggests that this species has a more distant taxonomic relationship to the four species with bipolar ascospores. N. terricola is evidently closely similar to the three bipolar species in terms of the kinds of soluble proteins each synthesizes but differs in a more profound way by being capable of forming a germ pore in only one end of its spore wall.

CHAPTER V

SUMMARY

Soluble proteins from five homothallic Neurospora species were fractionated by polyacrylamide gel electrophoresis.

Preparations from N. terricola exhibited fifteen soluble proteins fractions, N. africana and N. galapagosensis each exhibited thirteen and eleven were exhibited by N. dodgei and N. lineolata, respectively.

Comparisons of each of these five Neurospora species with one another for homologous soluble protein fractions seemed to indicate that more protein components are shared among N. terricola, N. africana, and N. lineolata. N. galapagosensis also has a high number of soluble protein fractions in common with N. terricola. N. dodgei has the least number of protein components shared with N. terricola.

The soluble protein profiles from N. dodgei differed the most from the profiles of all the other species observed. The soluble protein profiles from N. africana and N. lineolata were observed to be highly similar to each other and also to the profiles from N. terricola.

Protein preparations from all five taxa contained five common soluble protein fractions. Preparations of N. dodgei had two soluble protein fractions that were not found in the other species. N. africana, N. galapagosensis, and N. terricola each had one soluble

protein fraction exclusive to each species. There was no soluble protein fraction unique to *N. lineolata*.

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