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AN ASSESSMENT OF TECHNIQUES FOR THE CHARACTERISATION OF *Cynodon* TURFGRASSES.

BY

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of M.Sc.

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ABSTRACT.

The genus *Cynodon* within the Chloridoideae of the Poaceae is a small genus. *Cynodon* are among the most highly valued warm season turfgrasses in the world, because of their good drought resistance and recuperative potential, their high density and good colour (Beard 1973).

Due to modern plant breeding efforts, cultivated plants develop towards uniform entities which are difficult to distinguish at the cultivar level. Methods that are traditionally used in cultivar identification vary depending on plant type. Currently identification of turf *Cynodon* cultivars is based on morphological, physiological and cytological charaters. Although these methods of cultivar identification are satisfactory, they are not ideal, as the tests are fairly time consuming and some of the phenotypic differences are difficult to measure.

The aim of this project was to find a means of characterising the turf *Cynodon* in South Africa at the sub species or cultivar level. It was hoped to achieve this by noting anatomy and morphology of the grasses, and to use electrophoresis in conjunction with chromosome cytology, to examine the phenotypes of the representative varieties. The goal of this project was to establish a relatively simple and time saving system where by turf *Cynodon* could be identified in the future.

In the cytological section a limited number of grass selections were examined. In those examined a constant chromosome number of 2n = 18 was found.

The data compiled in the morphology, microanatomy and electrophoretic sections was analysed using the personal computer software package NTsys. Data was analysed within the specific section, as well as the combined data. Definite patterns emerged separating the grass selections into groups. Two major groupings occurred, with the larger of the two groups breaking down into smaller groups.

From this study it was found that, by using the techniques in this project, *Cynodon* turfgrasses could be classified at a subspecies or cultivar level. More characters would have to be included, however, to make the cultivar identification valid at the level of individual selections. The original naming of the *Cynodon* selections was found to be localized, and probably inaccurate, this made the evaluation of classification results difficult.

DECLARATION.

I hereby certify that this dissertation is my own work, unless otherwise stated in the text, and that it has not been submitted to any other university for degree purposes.

Signed:

8th day of June, 1991.

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CHAPTER 1.

1. GENERAL INTRODUCTION.

The genus *Cynodon* within the Chloridoideae of the Poaceae is a small genus comprising species which are highly valued as both forage and turfgrasses. *Cynodon* species are among the most widely used warm season turfgrasses in the world. The term "turf *Cynodon* " includes various cultivars not necessarily from the same species (see pp 8 - 9). The turf *Cynodon* varieties are used extensively for sports fields and home lawns, because of their good drought resistance and good recuperative potential, their high density and good colour (Beard, 1973).

Due to modern plant breeding efforts, cultivated plants develop towards uniform entities difficult to distinguish at cultivar level. The classification of such plants should clearly reflect genealogic relationships and must be able to meet the remodelling caused by plant breeding progress (MacKey *et al.*, 1988). Cultivar as an additional rank for cultivated plants must be given a fundamental role, although in practice cultivars are grouped by open systems to give the necessary flexibility. Already, country of origin and year of release are used as an easy way to accomplish some kind of numerical taxonomy, but this does not present a very sound basis for identification. Use of authorised gene symbols in combination with classical characters should present an acceptable compromise in cultivar ranking. Methods that are traditionally used in cultivar identification vary depending on plant type. Currently the identification of *Cynodon* cultivars is based on morphological,

physiological and cytological characteristics such as leaf colour and texture, shoot density, growth rate and habit, colour retention, smog tolerance, chromosome number and low temperature hardiness (Beard, 1973).

Although these methods of cultivar identification are satisfactory, they are not ideal, as the tests are fairly time consuming and the minute phenotypic differences are, in some cases very difficult to measure. They are also often obscured by modifications induced by different environmental conditions. In order to meet qualifications suitable for cultivar identification, taxonomy of cultivated plants must be more anticipative, less hierarchical and allow open classification systems at base level. Taxonomists should consider that knowledge accumulated around cultivated plants to a large extent is provided by nontaxonomic specialists. These researchers are also the main users of the particular taxonomic schemes. They demand schemes which reflect genetic relationships and discontinuities; that is to indicate the different accessibility within a potential gene pool. An ideal scheme must be flexible enough to anticipate genetic remodelling events (Watson *et al.* 1985).

In the past three decades a new technique for laboratory cultivar characterisation has been provided by the analysis of plant proteins using various forms of electrophoresis. The electrophoresis of direct gene products allows the researcher access to the genome of the plant. Together with morphological techniques, such an identification of cultivars could be of great use to both the producer and consumer.

The aim of this project was to find a means to

characterise the turf *Cynodon* in South Africa at the sub-species or cultivar level. It was hoped to achieve this by using the classical means for cultivar identification, first by noting the anatomy and morphology of the grasses, then to use electrophoresis in conjunction with some chromosome counts, to examine the phenotypes of representative varieties.

The objective of this project is to establish a relatively simple and time saving system whereby turf *Cynodon* can be identified in the future, combining the above mentioned methods.

CHAPTER 2.

2. CYTOGENETICS.

2.1 INTRODUCTION

Cytogenetic studies include the study of the chromosome complement of an organism whether it is plant, animal or fungus. The depth of such a study can vary from merely finding and counting chromosomes, to examining the morphology of the chromosomes at various stages in the cell cycle.

Chromosome duplication occurs in the actively dividing tissues of plants. These are the meristematic regions or the reproductive organs of the plant. In grasses the meristematic regions selected for chromosome squashes are usually the root tips. In order to examine chromosomes in this region, frequent squashes must be done to ascertain the time when mitosis occurs in order to examine the chromosomes at identifiable tages in the cell cycle. In the reproductive organs within the inflorescence, anther squashes are examined to find pollen mother cells for counts of the meiotic complement of the plant. Most of the cytological work done in the past on *Cynodon* has been done in conjunction with other taxonomic studies in an attempt to reclassify the genus *Cynodon* (Tripathi *et al.* 1977).

In this study the aim is to establish the chromosome number of turf *Cynodon* varieties. Root tip squashes are used since many of the turf *Cynodon* varieties are completely sterile and reproduce vegetatively (Burton,

1969). In some cases when pollen mother cells do occur pollen is aborted; it should be noted that chromosomal variations can occur, accounting for pollen abortions and making chromosome counts unreliable. Chromosome counts are considered important since the results of the cytological study will be compared with those of other workers and will aid in the interpretation of the electrophoretic results.

2.2 LITERATURE REVIEW.

Chromosome number and basic chromosome morphology have previously been studied in *Cynodon*. Brilman *et al.* (1982), Oureckly, (1963) and Tripathi *et al.* (1977) studied chromosome morphology in *Cynodon* species. Brilman *et al.* (1982) studied diploid clones of *Cynodon dactylon* to determine their pachytene morphology. Pollen mother cells were collected and stained using standard acetocarmine squash techniques. Chromosome length and arm length ratios, relative length and number of prominent centromeres were noted. Little variability was found in chromosome morphology among clones. This was contrary to work done on other plant species, eg Ho and Kasha, (1974) and Maguire, (1974) who worked on maize.

Three species of *Cynodor* were cytologically studied by Tripathi *et al.* (1977). Three species of *Cynodon* , *Cynodon dactylon*, *C. arcualus* and *C. nlemfuensis* were studied with a view to ascertain the extent of morphological variation and the evolutionary trends in the genus. Standard acetocarmine staining procedures were used and pollen was used as a source of plant material. Hybridisation was attempted between *C. arcualus* and *C. dactylon* resulting in the germination and growth of hybrid seedlings. These hybrids were cytogenetically investigated. The triploid hybrid was male sterile and when back crossed yielded seedlings with different chromosome numbers to the parents.

Harlan *et al.* (1970), Forbes and Burton, (1962) and Hurcombe, (1945) studied chromosome numbers in various *Cynodon* species. Acetocarmine was used as the stain. Both pollen and root tip material were used as plant

material. Harlan *et al.* (1970) did a biosystematic analysis of *Cynodon* . It was found that in all crosses between diploid taxa, the most common configuration was 2n = 18 (in pollen mother cells). The most common configuration in triploids are 9 pairs and 9 univalents. Chromosome pairing in tetraploid hybrids was found to be variable. Harlan *et al.* (1970) pointed out that *Cynodon* is difficult to treat taxonomically and that no entirely satisfactory classification has yet been found.

Forbes and Burton, (1962) studied chromosome numbers in *Cynodon dactylon* using root tip smears. They found the chromosomes to be small and in order to make the chromosomes more visible, a colchicine pretreatment was used. Chromosome numbers of 2n = 18, 27 and 36 were observed. In hybrid plants, in most cases, the chromosome number was found to be 2n = 27. According to Harlan *et al.* (1970) these plants were triploid and not diploid. There seems to be little doubt that the basic chromosome number for *Cynodon* is n = 9.

One must however be careful when looking at polyploids, as there may be unequal crossing and the cells resulting will have different chromosome numbers. Moffett and Hurcombe (1943) found chromosome counts of 2n = 26, 27, and 28. No mention has been found in the literature on any changes occurring in the chromosome complement with changes in the environment.

2.3 MATERIALS AND METHODS

2.3.1 CHROMOSOME COUNTS

It was not in the scope of this project to study the chromosome numbers of all the grass selections. Thus five selections were chosen, and examined. The five plant collections that were chosen were:

Table 1. *Cynodon* selections for chromosome study.

1	Natal	Kokstad Golf Club	12-02-1988	*Cynodon dactylon var. florida*
8	Cape	Milnerton Golf Club	17-01-1988	*Cynodon dactylon var. kweek*
10	Natal	Pietermaritzburg Country Club	14-01-1988	*Cynodon transvaalensis var. kweek*
28	Transvaal	Nigel Golf Club	21-04-1988	*Cynodon dactylon var. skaaplaas fyn*
34	Cape	Graaff Reinet	26-02-1988	*Cynodon dactylon*

Two sources of chromosomes were examined, meiotic chromosomes in anthers and mitotic chromosomes in root tip meristems.

2.3.1.1 Root tip meristems.

Small vegetative sections were removed from the main sample, the roots cut away and tillers were placed in pots containing 'Geurlite' (an artificial absorbent

'soil') and in darkened plastic bowls containing water and 'chemicult' mix. The plants were grown in a controlled chamber in the phytotron under the conditions described (pp 11). After 1 month's growth, roots were harvested over a 12 h period at 15 min intervals, from 06h00 to 18h00. The roots were fixed in formalin acetic acid alcohol (FAA) (Johansen 1940) until examined (see Appendix 2). On examination root tips were placed in an aceto carmine stain, squashed, heated, examined and photographed using a Zeiss D-7082 compound microscope (see Appendix 3) with camera attachment. Ilford PAN F 50 black and white film was used.

2.3.1.2 Pollen.

Whe plant collections flowered, young anthers were harvested and fixed for 4 - 5 hours in FAA (Johansen, 1940). This was found to be quite difficult as plant collections 8 and 34 did not flower. Plant collections 1, 12 and 28 did flower and the anthers were examined. No suitable meiotic stages were found and this technique was abandoned for two reasons:-

1. Not all turf *Cynodons* flower.
2. Many turf *Cynodon* cultivars reproduce vegetatively as the pollen aborts before maturity. The pollen aborts because of chromosome abberations.

This being the case chromosome numbers from such material would be inaccurate.

After fixation pollen mother cells were dissected out using a Zeiss dissecting microscope, squashed in iron aceto carmine and examined under the Zeiss D-7082 compound microscope.

2.4 RESULTS.

It was not in the scope of this project to study the chromosome numbers of all the grass selections. thus five selections were chosen, and examined (see pp 15).

The five turf collections examined yielded the following results:

2.4.1 Meiotic counts.

Some of the collections studied did not flower. Where flowering did occur no suitabl meiotic stages were found. This method was consequently abandoned.

2.4.2 Mitotic counts.

Good results were obtained in the five collections studied. A suitable stage (metaphase) was found to occur between 06h00 and 06h20, and root tip squashes yielded consistant results, that is all plants had a somatic chromosome number compliment of 2n = 18. See Figure 1.

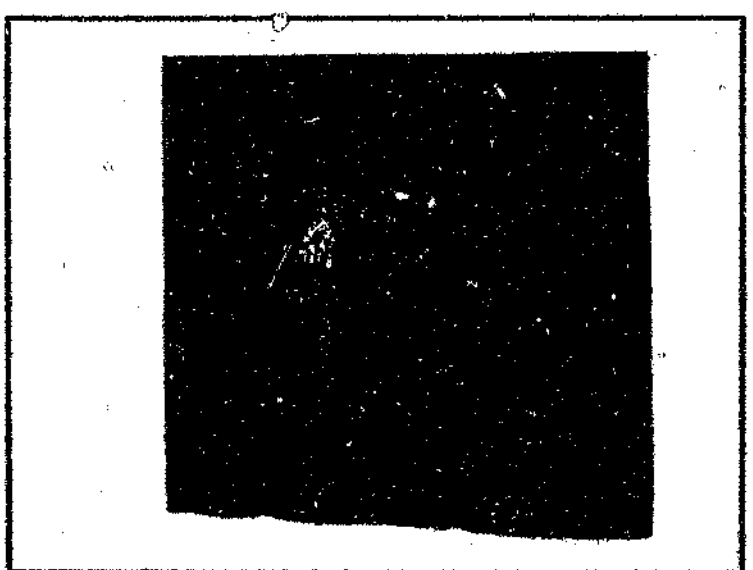


Figure 1. Metaphase chromosomes of plant collection 24 from Nigel Golf Club, Transvaal.

2.5 DISCUSSION

Five plant collections were chosen for this study. These collections were chosen both for geographical and phenotypic differences. As it was beyond the scope of this project to determine the chromosome number of each of the 36 plants collected, it was hoped that by choosing the grasses showing the most divergence in morphology and or geographical site, the cytological study would give some indication of the representative chromosomes numbers for turf *Cynodon* throughout Southern Africa, to see whether there were simple ploidy level differences.

Results were obtained using root tip smears. The mitotic metaphase chromosome numbers of these plant collections were found to be the same, namely 2n = 18. These results correspond to Forbes and Burton (1962), Harlan et al. (1969) and Harlan *et al.* (1970), who showed that *C. dactylon* and *C. transvaalensis* have a diploid chromosome number of 18. These results suggest that at least for these five collections, the chromosome number is constant and thus the results for both the morphology and electrophoresis will be based on at least a similar set of loci within the genetic material. The reason for this is that in some instances loci of a gene are duplicated and will show up in electrophoretic techniques, whereas no differences will show in the chromosome number. As the collections selected were examples showing the widest phenotypic divergence and geographic separation and since only turf *Cynodons* were examined, it is hoped, but not assumed that the same chromosome number will apply to the other plant collections.

CHAPTER 3.

3. ELECTROPHORESIS

3.1 INTRODUCTION

Electrophoresis is a fairly new technique that is used for separating mixtures of organic molecules based on their different rates of movement in an electrical field. The use of this technique to separate multiple molecular forms of enzymes, termed isozymes by Hunter and Markert (1957), followed by the histochemical staining of the isozymes has become an important part of research for plant breeders, population geneticists, cytogeneticists and developmental geneticists.

To date electrophoresis has also been the most widely applied laboratory technique for the characterisation of plant cultivars (Cooke, 1984). Much of the progress in the field of plant cultivar analysis has been made possible by the introduction of improved electrophoretic procedures using starch as the medium of separation of the various isozymes.

The popularity of using starch gel electrophoresis as a tool of taxonomic identification stems from a number of features:

1. Ease of preparation

This applies to the preparation of both the plant extract and of the starch gels. In the case of *Cynodon* , crude leaf extracts were found to give satisfactory results.

2. Visual results.

Electrophoretic histochemical staining renders gene products visible in a matter of hours in the dark, making

electrophoresis a rapid means of getting results (Cooke 1984).

3. Markers for specific cultivars.

The isozyme bands can serve as markers in the identification of specific cultivars by the absence or presence of a particular band.

The identification of cultivars is the characterisation of the genome of the different cultivars. The protein, which makes up an enzyme is the primary product of a structural gene and can thus act as a marker for that gene. For analysing cultivar relationships it is necessary to study specific proteins using electrophoresis. This can be done by examining either seed storage proteins or proteins present in the leaves in the case of vegetative growth (Cooke, 1984).

The enzymes in this study were chosen for the following reasons :

1. To cover the widest range of reactions in the biochemistry of the plant. Peroxidase, esterase, malate dehydrogenase and its NADP derivative, malic enzyme cover both cytosolic and organelle reactions. These enzymes are however very general in their activities and very abundant within plants. For example there are 24 different peroxidase enzymes present in maize at 9 loci found in the cytoplasm, microbodies and the chloroplasts (Hamill and Brewbaker 1969). These enzymes could be problematic in the sense that without more in depth studies one could not identify the source of the individual bands. Enzymes such as glutamate dehydrogenase, alcohol dehydrogenase and 6- phosphoglu dehydrogenase are more specific as to the locality of the

dehydrogenase is only found catalysing reactions in the cytoplasm (Scandalios 1977).

2. The enzymes were chosen to be as economical of chemicals as possible, as the chemicals used in both the histochemical staining and the buffer systems are expensive.

3. The enzymes were chosen according to their activity levels. A number of enzymes showed either no activity at all or were very erratic in showing any activity, and were thus of no use.

3.2 LITERATURE REVIEW.

The term isozyme refers to multiple molecular forms of an enzyme with similar or identical catalytic activities, occurring within the same organism (Scandalios, 1969). Isozyme research is currently used in a number of different fields. Plant breeding, population genetics, cytogenetics and developmental genetics being among them. A large number of electrophoretic variants of enzymes have been discovered (Shaw & Koen, 1965). With these findings came the knowledge that enzymes may exist in the same organism in more than one molecular form. Such multiple molecular forms of an enzyme have been designated isozymes (Markert and Moller, 1959). Isozymes may differ in primary structure. The primary structure may also be further modified by conjugation of molecules with reactive groups such as amino, carboxyl or hydroxyl groups of the amino acid residues on the polypeptide chain (Scandalios, 1969). This will affect the mobility of the various proteins in the supporting medium, the gel, when placed in an electrical field. This will give rise to the various bands on the gel slices.

In the 1950's experimental studies first provided evidence that enzymes existed in multiple forms. For example Mallette and Dawson, (1949) obtained 5 purified tyrosine preparations from the mushroom *Psalliota campenstris*. Roberts, (1956) found that acid phosphatase activity in leaves of wheat was caused by a group of distinct enzymes. Bowman and Westland, (1956) examined horseradish peroxidase and acid phosphatase and concluded that multiple forms may exist.

Early research workers used agar gels (Mallette and

Dawson, 1949), paper chromatography (Calib, 1952; Giri *et al.* 1952) or Dowex 50 chromatography (Buroughs, 1954). In 1955 Smithies made a major contribution to the field with the development of starch gel electrophoresis. A second contribution was made in 1957 when Hunter and Markert demonstrated that enzymes could be made visible directly on starch gels when stained with specific histochemical stains.

Controversy existed as to whether the multiple forms of enzymes did actually exist, or whether these results were due to artefacts. Refinements in the technique of electrophoresis led to conclusive evidence of the existence of isozymes (Markert and Moller 1959).

Hunter and Markert, (1957) proposed the use of the term 'zymogram' to refer to bands in which the enzyme location is observable. They found that the zymogram pattern for mushrooms, potatoes and mouse melanoma were distinct, suggesting that there were specific enzyme forms. In 1964 Scandalios showed the presence of tissue specific variants of enzymes of leucine aminopeptidase, esterase, peptidase and catalas in maize by the use of zymogram techniques. The data obtained gave evidence for the existence of multiple molecular forms of various isozymes within the same organism.

Many researchers have used isozymes for the characterisation of particular plant genotypes. Isozyme and total protein banding patterns have been used to identify species (Bingham and Yeh, 1971), cultivars (Villamil *et al.* 1982; Wehner *et al.* 1976; Wilkinson and Beard, 1972 and Weeden and Emmo, 1985), inbred lines (Stuber and Goodman, 1981 and 1982) and aneuploids

(McDaniel and Ramage, 1970 and Moore and Collins, 1982).

Two types of supporting media are popularly used. They are acrylamide and starch. Authors have used these two media for varying purposes. Cai and Chinnappa (1989) used polyacrylamide gel electrophoresis (PAGE) to note gene duplication of isozyme genes in *Stellaria longpipes*. Kelly and Adams (1977) used PAGE when testing extraction buffers in juniper leaves, as these leaves contain high concentrations of phenols and tannins. Moustakas *et al.* (1986) used PAGE and stained for general plant proteins of *Agropyron junceum*. This was used to obtain additional data for a taxonomic study. Sheen (1977) used PAGE in a phylogenetic study of *Nicotiana tabacum*.

Various authors have used PAGE for identification purposes. Ferguson and Grabe (1986) used PAGE for cultivar identification of *Lolium perenne*, staining for general proteins. Other authors have examined enzymes using PAGE in identification studies. Green *et al.* (1981) used PAGE to determine the feasibility of using isozymes for identification purposes. Wehner *et al.* (1976) and Wu *et al.* (1984) used PAGE for cultivar identification of Kentucky bluegrass, using different enzyme stains. Both authors found that this technique gave suitable results as different cultivars were identified. A number of authors have used PAGE to identify seed lots. Miller *et al.* (1982) used disc PAGE to identify hybrid seed lots of alfalfas. Villamil *et al.* (1972) used PAGE to identify fine fescue seedlots.

The chemicals required for PAGE are expensive and it is therefore not possible within the scope of this work to study large numbers of plants using this technique. It is

also not feasible to study a large number of enzymes or to replicate the results, as only a limited number of samples can be tested at a time. Another solution would be to study the total protein content, as this requires a single gel to be run and to be stained.

Hydrolysed potato starch has been frequently used as an alternative medium in electrophoretic studies. It has been used for a number of purposes in the past and is still widely used. Authors have used starch gel electrophoresis to obtain varying information about a plant or whole plant populations. Starch as an electrophoretic material is advantageous as:-

1. The starch gel can be sliced into several slices and each slice can be stained for a different isozyme.
2. A larger number of samples can be tested at one time (30 - 40) compared to acrylamide (10 - 14).
3. Starch is more economical than acrylamide.

One disadvantage with the use of starch is that the isozyme bands do not stain as clearly as they do on acrylamide.

Many studies have used electrophoretic techniques, either on their own or in conjunction with other techniques to support theories put forward either by the authors or in the literature. Smith - Huerta (1987) used starch gel electrophoresis in isozyme diversity studies of *Clarkia*. Evidence from this study supported the postulated theories of the origins of the allotetraploid species in *Clarkia*. Enzymes studied were acid phosphatase, aspartate amino transferase, esterase, glutamate dehydrogenase, leucine amino transferase and phosphoglucoisomerase. Vogelmann and Gastony (1987) used starch gel electrophoresis to assess the genetic relationships among

the seven *Agastache* species in North America. Eleven enzymes were used in this study. Doyle *et al.* (1986) studied *Glycine tomentella* and stained for phosphoglucoisomerase while Vorsa *et al.* (1988) studied *Vaccinium* staining for isocitrate dehydrogenase, malate dehydrogenase, 6 - phosphogluco dehydrogenase and phosphoglucoisomerase to aid their taxanomic studies.

Beckman *et al.* (1964), Grant *et al.* (1984), Gottlieb (1987), Kahler and Allard (1970), Kahler and Lay (1985), Nielsen (1980) and Scandalios (1963) studied the genetics of plants by noting their isozyme patterns in conjunction with some cytogenetic chromosome studies.

Gottlieb (1984), Oliver and Zapater (1984) and Staub *et al.* (1987) used starch gel electrophoresis in phylogenetic studies on *Clarkia xantiana*, *Solanum tuberosum* and *Cucumis* sp. respectively, by staining for various isozymes.

As far back as 1964 research workers found that enzymes are tissue specific. Scandalois (1964) studied the tissue specific isozyme variations of *Zea mays* staining for leucine amino transferase, once he had examined the genetics of *Zea mays*, (Scandalios 1963). More recently Gastony and Darrow (1983) used starch gel electrophoresis in finding subcellular localisation of isozymes of isocitrate dehydrogenase, malic enzyme, 6- phosphogluco dehydrogenase, phosphoglucoisomerase, shikimic dehydrogenase and in *Athyrium filifemina*. Pichersky and Gottlieb (1983) genetically analysed the mode of inheritance of multiple plastid and cytosolic enzymes of *Clarkia* staining for triose phosphate isomerase.

A number of authors have found starch a good means by which plant cultivars can be identified. Both plant and seed lots have been evaluated by various authors. Kut and Evans (1984) stained for alcohol dehydrogenase in evaluating the feasibility of seed screening of *Nicotiana tabacum*.

Bassiri (1976), Cardy and Kannenberg (1982), Mielke and Wolfe (1982) and Weeden and Emmo (1985) used starch gel electrophoresis in plant cultivar identification. Bassiri (1976) studied *Hordeum vulgare (L.)* for acid phosphatase, esterase and peroxidase. It was found that esterase alone could be used to differentiate between most cultivars. Whenever it failed to show differences, either acid phosphatase or peroxidase could be applied for complete identification. Cardy and Kannenberg (1982) surveyed the allozymic variability to determine if enough variability was present to be useful for cultivar identification of *Zea mays* noting over 10 enzymes. They concluded that such a method is useful in cultivar identification in *Zea mays*.

Gilliland *et al.* (1982), Mielke and Wolfe (1982) and Weeden and Emmo (1985) used starch gel electrophoresis for cultivar identification of *Lolium perenne, Carya illinoensis* and Kentucky bluegrass respectively with sucess. Various enzymes were studied.

3.3 MATERIALS AND METHODS

3.3.1 Plant material.

Thirty six selections of *Cynodon* were collected in South Africa from December 1987 to July 1988, as shown in Table 2.

Table 2. *Cynodon* selections according to province or country (in the case of Swaziland), and date of collection.

No	Province\ Country	Location	Date collected	Name of grass
8	Cape	Milnerton Golf Club	17-01-1988	*Cynodon dactylon var. kweek*
9	Cape	Zandvlei Bowling Club	18-02-1988	*Cynodon dactylon var. cape royal*
14	Cape	George Golf Club	21-02-1988	*Cynodon dactylon var. cape royal*
15	Cape	Zandvlei Bowling Club	18-02-1988	*Cynodon dactylon var. kweek*
16	Cape	Knysna Golf Club	19-02-1988	*Cynodon dactylon var. outenique*
17	Cape	Mossel Bay Golf Club	21-02-1988	*Cynodon dactylon var. outenique*

No	Province\ Country	Location	Date collected	Name of grass
23	Cape	Zandvlei	10-02-1988	*Cynodon dactylon var. kweek*
31	Cape	Mossel Bay	26-02-1988	Cynodon dactylon
32	Cape	Brandford	26-02-1988	*Cynodon dactylon*
33	Cape	Caledon	26-02-1988	*Cynodon dactylon*
34	Cape	Graaff Reinett	26-02-1988	*Cynodon dactylon*
35	Cape	Koekhuis	26-02-1988	*Cynodon dactylon*
36	Cape	Reddersberg	26-02-1988	*Cynodon dactylon*
1	Natal	Kokstad Golf Club	12-02-1988	*Cynodon dactylon var. kweek*
2	Natal	Matatiele Golf Club	13-02-1988	*Cynodon dactylon var. kweek*
10	Natal	Pietermaritzburg Country Club	10-01-1988	*Cynodon transvaalensis var. duffy's*

No	Province	Location	Date collected	Name of grass
12	Natal	Kloof	14-01-1988	*Cynodon dactylon var. kweek*
13	Natal	15km north of Bulwer	13-01-1988	*Cynodon dactylon var. kweek*
18	Natal	Escourt	16-02-1988	*Cynodon dactylon var. kweek*
19	Natal	Southbroom Golf Club	10-03-1988	*Cynodon transvaalensis var. mandy*
20	Natal	Underberg	15-03-1988	*Cynodon magennisii var. magennisii*
21	Natal	Matatiele	14-02-1988	*Cynodon dactylon var. florida*
25	Natal	Underberg	15-03-1988	*Cynodon dactylon var. florida*
26	Natal	Sani Pass Hotel	15-03-1988	*Cynodon dactylon var. florida*
29	Natal	Humewood 17	15-02-1988	*Cynodon dactylon var. florida*

No	Province	Location	Date collected	Name of grass
22	Swaziland	Royal Swazi Golf Club	12-12-1987	*Cynodon dactylon var. florida*
24	Swaziland	Royal Swazi Golf Club	12-12-1987	*Cynodon dactylon var. florida*
3	Transvaal	Straathofs Garden Centre	25-04-1987	*Cynodon transvaalensis var. bayview*
4	Transvaal	Top Turf Germiston	25-04-1987	*Cynodon dactylon var. florida*
5	Transvaal	Top Turf Germiston	25-04-1987	*Cynodon dactylon var. skaaplaas fyn*
6	Transvaal	Lawn Industries	25-04-1987	*Cynodon dactylon var. skaaplaas fyn*
7	Transvaal	Lawn Industries	25-04-1987	*Cynodon dactylon var. cape royal*
11	Transvaal	Wanderers Bowling Club	20-12-1987	*Cynodon dactylon var. skaaplaas fyn*
27	Transvaal	Wanderers Bowling Club	11-03-1988	*Cynodon dactylon var. skaaplaas fyn*

No	Province	Location	Date collected	Name of grass
28	Transvaal	Nigel Golf Club	21-04-1988	*Cynodon dactylon var. skaaplaas fyn*
30	Transvaal	Frankenwald Estate	28-02-1988	*Cynodon magennisii var. frankenwald*

Note: In order to achieve some sort of consistency in naming the cultivars, the cultivar initial was placed in the lower case in all instances, even when using names of people and places. There is some laxity with respect to this in the Turf industry.

The grass selections were grown under natural conditions from the time of collection until June 1987. In June 1987 the selections were transferred to a controlled environment chamber in the University of the Witwatersrand phytotron at 21/27° C with a 14 h light and 10 h dark photo period. Relative humidity was maintained at 70% atm., with a light intensity of 400 micro Einsteins (400 lux) supplied with vito tungsten strip lights. These were the conditions that were available, not necessairly the optimum conditions for the grass selections.

The grass selections were watered every third day and fertilized once a month with a hydroponic chemical mix, 'Chemicult'. Suggested amounts as directed were followed

(see Appendix 1). Problems with aphids and rust were found in certain selections. The selections were sprayed once every 3 months with Funginex and Malathion 50, both commercially available. The directions for use were followed in both the fungicide and insecticide (see Appendix 1). Maintenance of the grass selections included cutting every two to three weeks depending on the growth rate of the leaves, to a height of 3cm from the soil.

3.3.2 Analysis of the data using the computer software package NTSYS.

NTSYS (copyright 1985 by Applied Biostatistics Inc., 3 Heritage Lane, Setauket, New York 11933). This package contains licenced program materials of Megagraphics Software corporation. Copyright 1985, 1986 Megagraphics corporation, Scotts Valley CA 95066.

NTsys is a system of programs which are used to find and display structure in multivariable data. The program was originally intended for use in biology in the field of numerical taxonomy, although it has been widely used in natural science, engineering and humanities.

Initially, one needs a data matrix that contains information about the characteristics of a number of objects (OTU's). The OTU's in this study were the 36 grass selections listed on pp 8 - 9. OTU's were differentiated from each other by using characters in both microanatomy and morphology and the electrophoretic sections. NTSYS is then used to compute various measures

of similarity, or dissimilarity between all objects (OTU's). NTSYS then summarizes this information in terms of similar OTU's in a cluster analysis, or in terms of spatial arrangement along one or more axes (ordination analysis). NTSYS consists of a number of separate program modules coordinated by a menu driven main program. Each module reads one or more input files containing the matrices to be operated on and then produces one or more of the following:-

1. An output listing operation.
2. One or more output files.
3. Plot on a graphics screen.

The cluster analysis was executed using a number of program modules. The raw data matrix was placed in the OUTPUT program module. This checks that the input file, in this case the rough data matrix was correctly prepared for NTSYS. The data was standardised using the STANDARDIZATION program module, by placing the data matrix into the input file. This module was selected as it minimized the effects of different scales of measurement found in different characters in the study. The default options correspond to the usual standardisation of a matrix used in numerical taxonomy. It results in a mean of 0 and a variance of 1.

Following the standardisation the SIMINT program module is used. This module computes various similarity or dissimilarity indices for the interval measure of data. The name used for the input data matrix must follow from

the STANDARDISATION program module. The coefficient chosen was CORR which is a product-moment correlation and noted similarities. It does not compare the designated numbers, in the data matrix, to actual distant differences. The input is in the form of a retangular data matrix and the output is a symmetric matrix.

Following SIMINT the SAHN program module is used. This module performs the various clustering algorithims that Sneath and Sokal (1973) refer to as Sequential Agglomerative Hierachial and Nested clustering methods. This program searches the input matrix for the pair of OTU's that are most similar, merges them into a new cluster and then updates the matrix to reflect the deletion of the pair of similar OTU's and adds the new cluster to the input matrix. This method is repeated until all the OTU's are clustered.

A number of clustering methods were examined, eg. COMPL, complete-link method, SINGL, single-link method, UPGMA, unweighted pair-group, WPGMA, weighted pair-group arithmetic average and WPGMS, weighted pair-group method, Spearmans average. As none of the results in this study could be weighted without bias, the weighted cluster methods were discarded. UPGMA was used as the clustering method in this study, in preference to SINGL as it resulted in a more balanced tree. There was no significant difference between UPGMA and COMPL. UPGMA was selected by the author for use in this study.

A symmetrical matrix of cophenetic similarity or

dissimilarity values was examined using the programme module COPH or cophenetic values. The MXCOMP or matrix comparison program takes two symmetric similarity or dissimilarity matrices and plots one matrix against the other. It also computes the product moment correlation, r and the Mantel test statistic, Z to measure the degree of difference in the relationship shown by the two matrices. The r value can be used as a measure of goodness of fit for a cluster analysis where $r < 0.7$ is a poor fit.

3.3.3 Leaf material.

The plant material was grown as described on pp 24 -25. Plant material was harvested from the youngest and second youngest leaves for the electrophoresis. The leaves were weighed. The material was ground in the extraction buffer using a mortar and pestle. The plant material was kept on ice from the time of weighing. The extraction buffers and the mortar and pestle were also kept on ice. This was done to prevent as much protein degradation as possible.

3.3.4 Starch.

SIGMA hydrolysed starch was used (Product number S4501 Lot number 64F0779) The starch was used at three different concentrations namely 10.5%, 11% and 12.5% weight to volume.

3.3.5 Extraction buffer.

The effects of 5 different extraction buffers were examined in order to ascertain the most efficient extraction buffer. (See Appendix 10). Different concentrations of the extraction buffer were examined in

order to ascertain which gave the clearest enzyme bands. The following concentrations were examined :-

1. 1 gram plant material in 2ml extraction buffer.
2. 1 gram plant material in 3ml extraction buffer.
3. 1 gram plant material in 4ml extraction buffer.

Various techniques for electrophoresis have been found in the literature (Soltis *et al.* 1983, Shaw and Prasad 1970 and Shields *et al.* 1983). Here various percentages of starch, various extraction buffers, wick sizes and tray and gel buffers that were used for a large variety of plant species were included. As this author found no mention of electrophoresis being used on *Cynodon* it was necessary to determine a method of electrophoresis for this genus. The starch percentage, extraction buffers, wick sizes and ratios of extraction buffers to plant material were considered as variables and were examined as mentioned pp 29 to pp 31.

The extracted material was kept frozen (at -15°C) in 0.1ml aliquots in Eppendorf tubes. Each tube was thawed once only and the excess extract discarded.

3.3.6 Wicks.

Two different grades of filter paper were used as wicks, when loading the samples into the gels. They were Whatman No.1 and Whatman No.3 filter papers. Loading different numbers of wicks per sample was also examined. Another variable that was investigated with respect to wicks was the wetness of the wick. Three conditions were looked at

1. Completely air dry
2. Partially air dry
3. Completely wet

Different sized wicks were tried in an attempt to determine the best-size wick to use. Sizes examined were as follows :-

1. 1.0mm x 20mm
2. 1.5mm x 20mm
3. 2.0mm x 20mm
4. 3.0mm x 20mm
5. 4.0mm x 20mm
6. 5.0mm x 20mm

3.3.7 Tray and gel buffers.

Six different buffer systems were examined in an attempt to find one or two systems which would allow for clear staining of all the enzymes to be studied.

The buffer systems that were examined are as follows :-

1. Lithium borate pH 8.3 (Shields *et al.* 1983)
2. Tris citrate pH 7.2 (Soltis *et al.* 1983)
3. Tris EDTA borate pH 8.6 (Soltis *et al.* 1983)
4. Tris citrate pH 8.0 - 8.5 (Soltis *et al.* 1983)
5. Tris maleate pH 7.4 (Brown *et al.* 1978)
6. Histidine citrate pH 5.7 (Shields *et al.* 1983)

See Appendix 5.

3.3.8 Gel preparation.

Two means of gel preparation have been attempted, one using a microwave oven to heat the gel buffer the other using a bunsen burner. Gel preparation was carried out as follows: Decant one third of the gel buffer. Heat the remaining two thirds in a volumetric flask to boiling point. Mix the remaining third with the full amount of starch to be used, in a suction flask. Rapidly add the boiling gel buffer to the well emulsified starch solution in the suction flask. Heat the flask over a bunsen burner until starch solution begins to boil. Apply a vacuum to the suction flask to remove excess air. Immediately pour into the gel mould. See Figure 2. Any lumps or air bubbles were removed with a Pasteur pipette. The gel was allowed to cool and solidify, covered with polythene foil and left for twelve hours at room temperature to set. Gels were cooled at 4-8°C for 30 minutes prior to electrophoresis.

3.3.9 Gel loading.

After cooling the gel and removing the polythene foil the gels were trimmed and sliced transversly between 4 and 8cm from the cathodal end and wicks were inserted sequentially into the loading slice. Between 20 and 36 wicks were loaded per gel, sufficient space was left between the wick to allow for easy interpretation of the enzyme patterns. A marker wick was placed in the first loading position, to monitor front movement. The

bromophenol blue marker dye moved through the gel faster than any of the enzyme bands.

3.3.10 Running of the gel.

The gel was placed on the electrophoretic apparatus and connected to an electrical current as in Figure 2. A short electrical pulse was used to elicit movement of charged molecules from the crude extract in the wicks into the cathodal or anodal section of the gel. In this instance the gel was run at 16 watts (120V 80mA) for 15 minutes. Absorbent cloth was used to insure that good contact was made between the electrolyte and the gel at both the cathode and anode. Care was taken not to allow the gel to dry out or to heat up. The gel surface temperature must be constantly checked, if the gel heats up it can be cooled by placing ice directly onto the surface of the gel, or by lowering the current passing through the gel. To prevent the surface of the gel from drying out the gel was covered with polythene foil.



Figure 2 Electrophoresis apparatus

3.3.11 Removal of wicks from the gel.

The wicks must be removed so that proteins are not eluted into the gel at the origin continuously, causing smearing. Thus the wicks are removed after the initial electrical input.

3.3.12 Electrophoresis.

Following the removal of the wicks, spacers were placed between the gel and gel apparatus, to ensure contact between the anode and cathode portions of the gel. The gels were generally run at as high an amperage as possible without heating the gel. This was usually at 110 volts, 65mA for 4-5 hours, except for lithium borate which was run at 110 volts 65mA for 8-9 hours. Once the marker dye moved at least 8cm from the origin, thus sufficiently separating the enzyme bands (in optimum conditions), the gel was removed from the electrical

current and prepared for staining. The gel was trimmed and marked, by nicking a corner, and then sliced into 2mm thick slices (4-5 slices). These 4-5 slices were then stained simultaneously for different enzymes. Slicing was carried out with a gel slicer consisting of a piece of stainless steel wire held together by a frame. The gel was immobilized between a piece of glass and the thickness template, while the slicer was slowly drawn through the gel.

3.3.13 Staining procedures.

Two staining procedures were used in the electrophoretic study, staining trays and agar overlay.

3.3.13.1 Staining trays.

This method was used in most instances, especially where the staining chemicals were relatively inexpensive, or the activity of the enzyme was weak. In this method all chemicals are dissolved in the staining buffer directly before staining and placed in open trays in the dark. If the gel had to stain overnight, polythene foil was placed over the tray to prevent evaporation.

3.3.13.2 Agar overlay.

This method was used when the staining chemicals were expensive or the activity of the enzyme was very strong. In this method the same recipe was used as for staining trays. Ingredients were however divided in four and a 0.1% agar solution was made using 23ml of staining buffer. This solution was dissolved on a hot plate,

cooled and mixed with 2ml buffer containing all the chemical ingredients. This solution was immediately poured over the gel, which was placed in the dark.

3.3.14 Enzymes studied.

The abbreviations are from Soltis *et al.* (1983) and Shields *et al.* (1983).

Table 3.:- The following enzymes were examined.

Enzyme	Abbreviation
Acid phosphatase	ACP
Alcohol dehydrogenase	ADH
Aldolase	ALD
Catalase	CAT
Esterase	EST
Glutamate dehydrogenase	GDH
Hexokinase	HEX
Isocitrate dehydrogenase	IDH
Malate dehydrogenase	MDH
Malic enzyme	ME
Peroxidase	PER
6 Phosphogluco dehydrogenase	6PGD
Phosphogluco mutase	PGM

Enzyme	Abbreviation
Phosphogluco isomerase	PGI
Shikimate dehydrogenase	SDH

For recipes of stains see Appendix 6.

3.4 RESULTS.

Following the methods described on pp 29 - 37 starch gels were prepared, run and stained for the enzymes named. The grass selections were run in batches one batch per province in ascending order of collection number. Once banding patterns emerged the gels were photographed and sketches (zymograms) were made of the gels. Eleven out of the fifteen enzymes gave usable results. These results were then used in the analyses. The zymograms shown in Figures 3 to 13 were produced using Harvard Graphics.

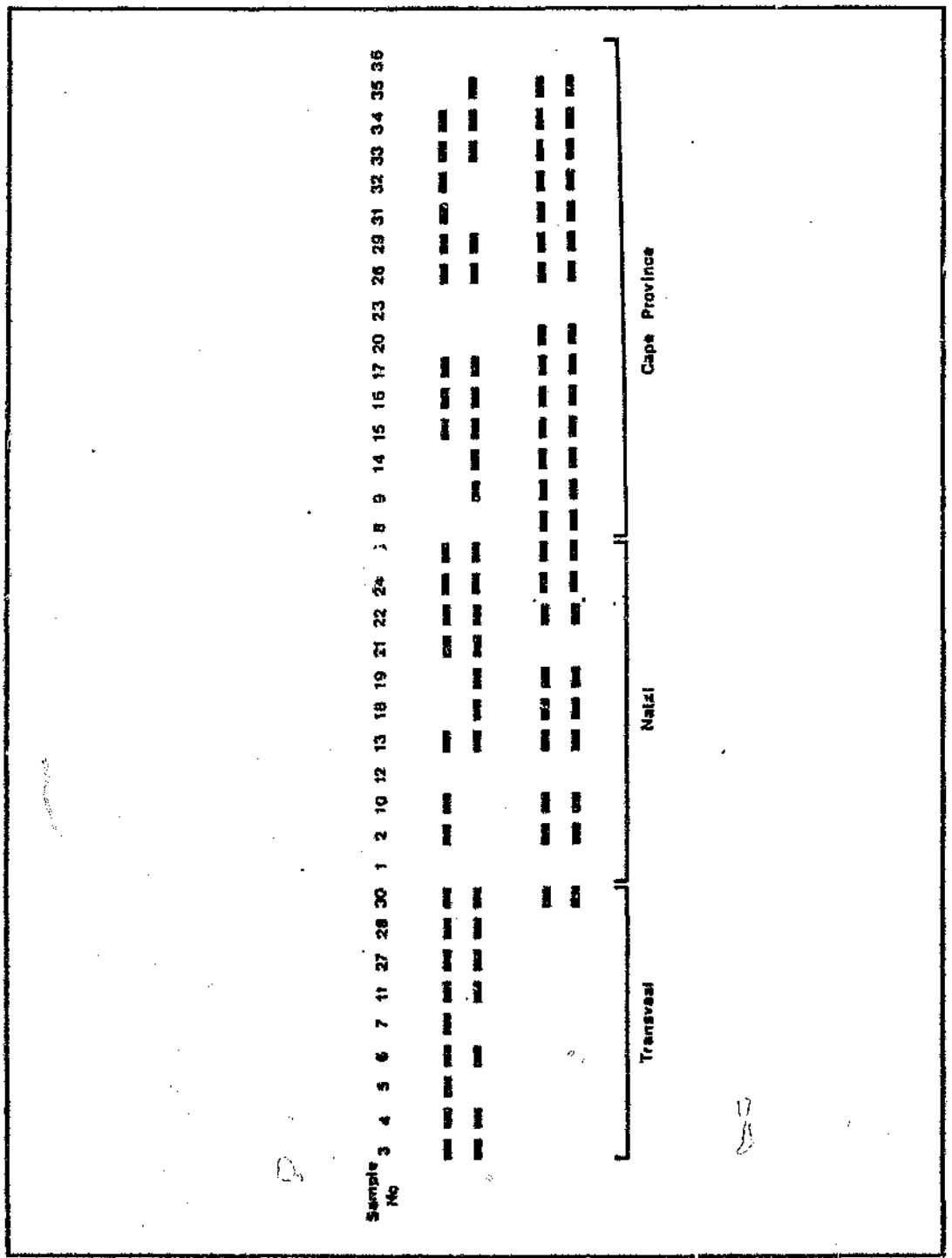


Figure 3 Zymogram of Acid phosphatase (ACP).

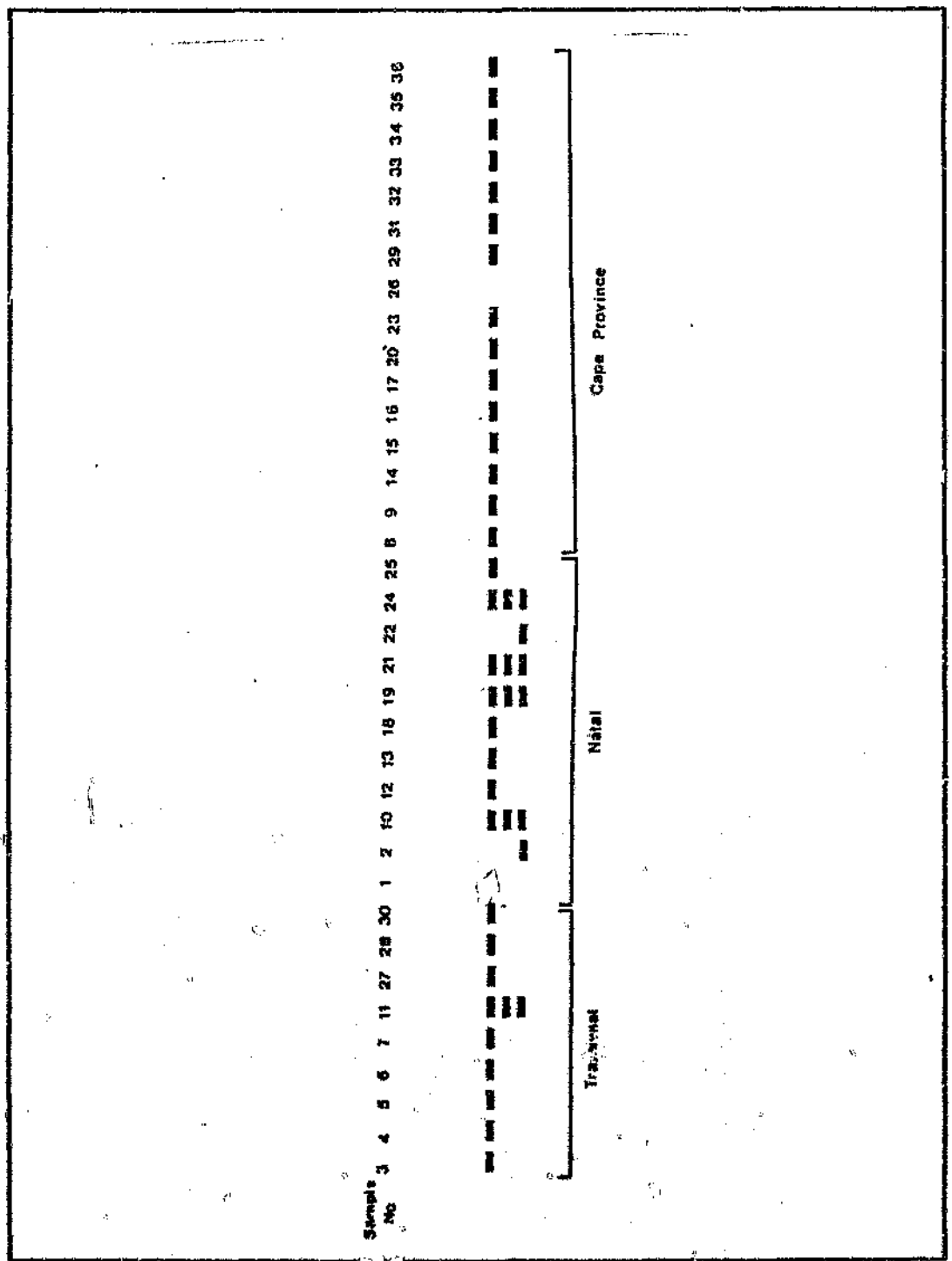


Figure 4 Zymogram of [illegible]hol dehydrogenase (ADH).

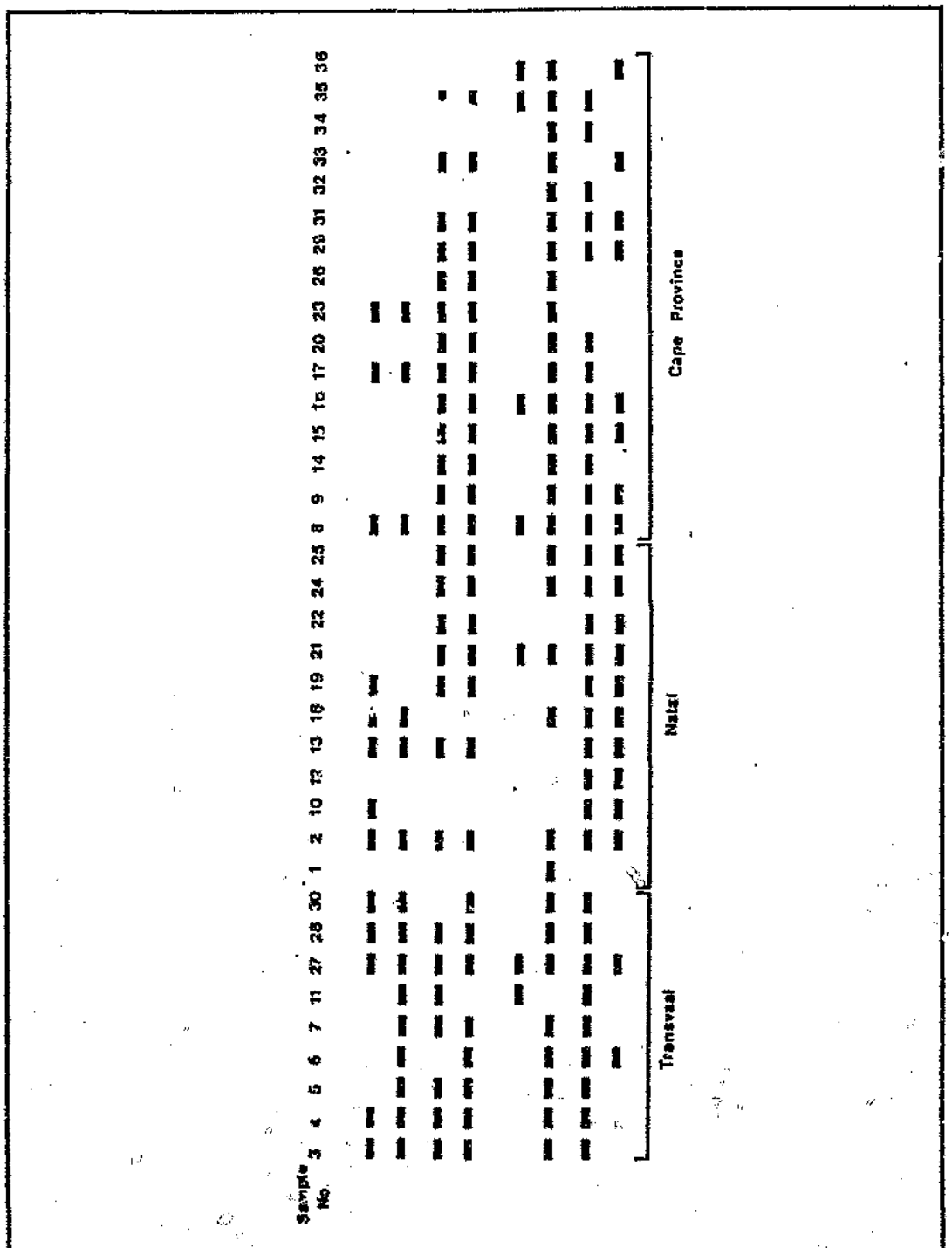


Figure 5 Zymogram of Esterase (EST).

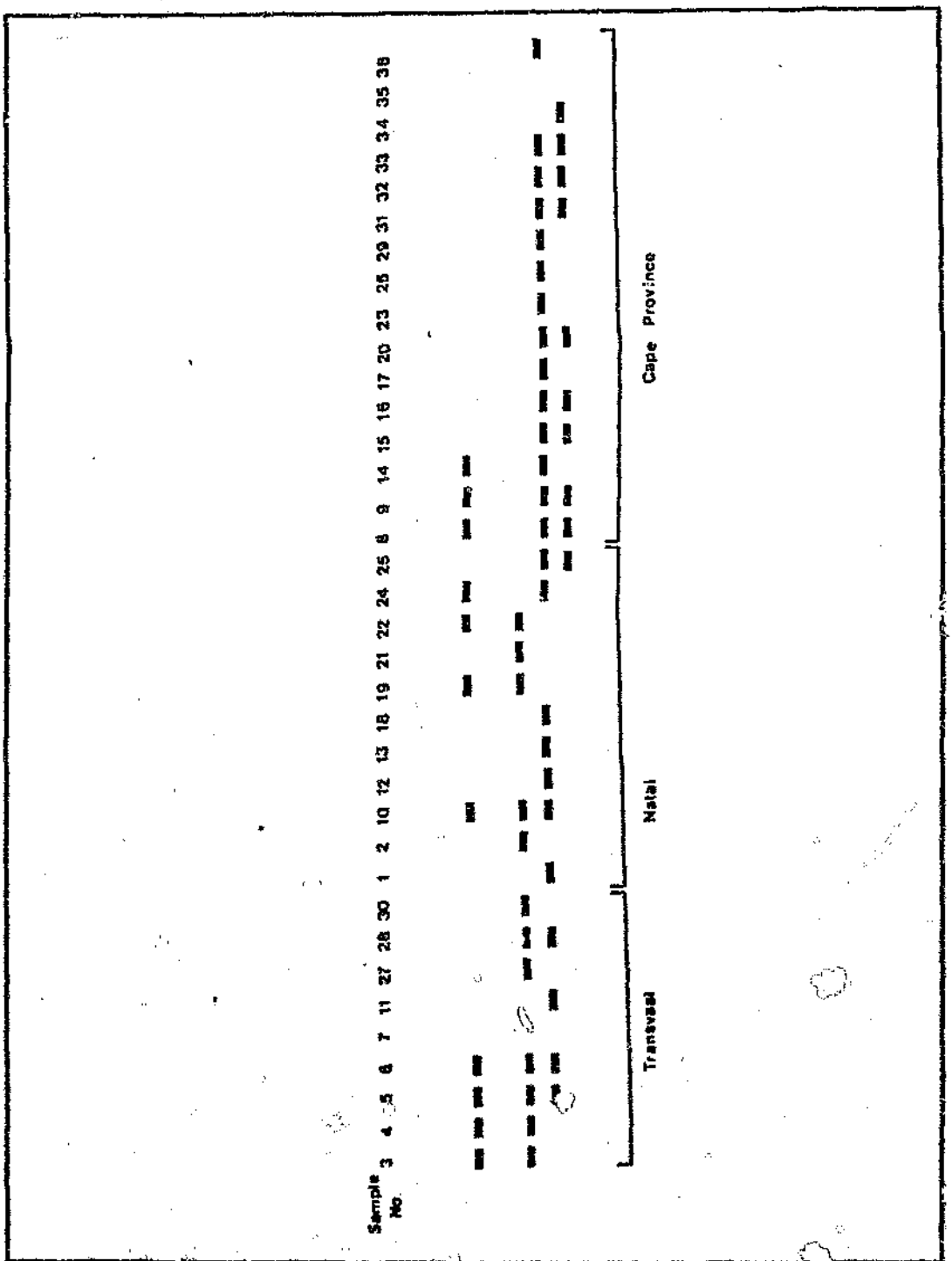


Figure 6 Zymogram of Glutamate dehydrogenase (GDH).

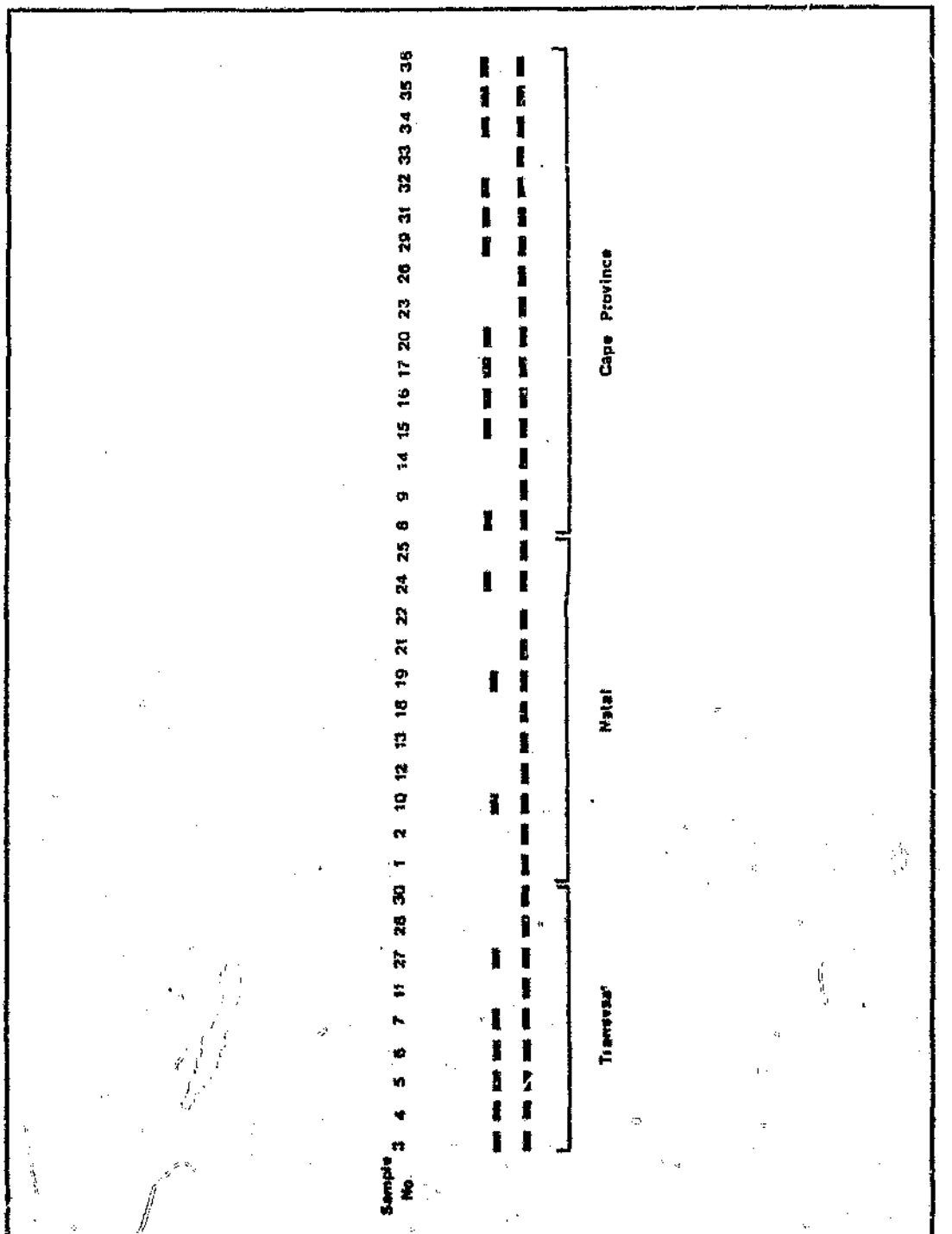


Figure 7 Zymogram of Isocitrate dehydrogenase (IDH).

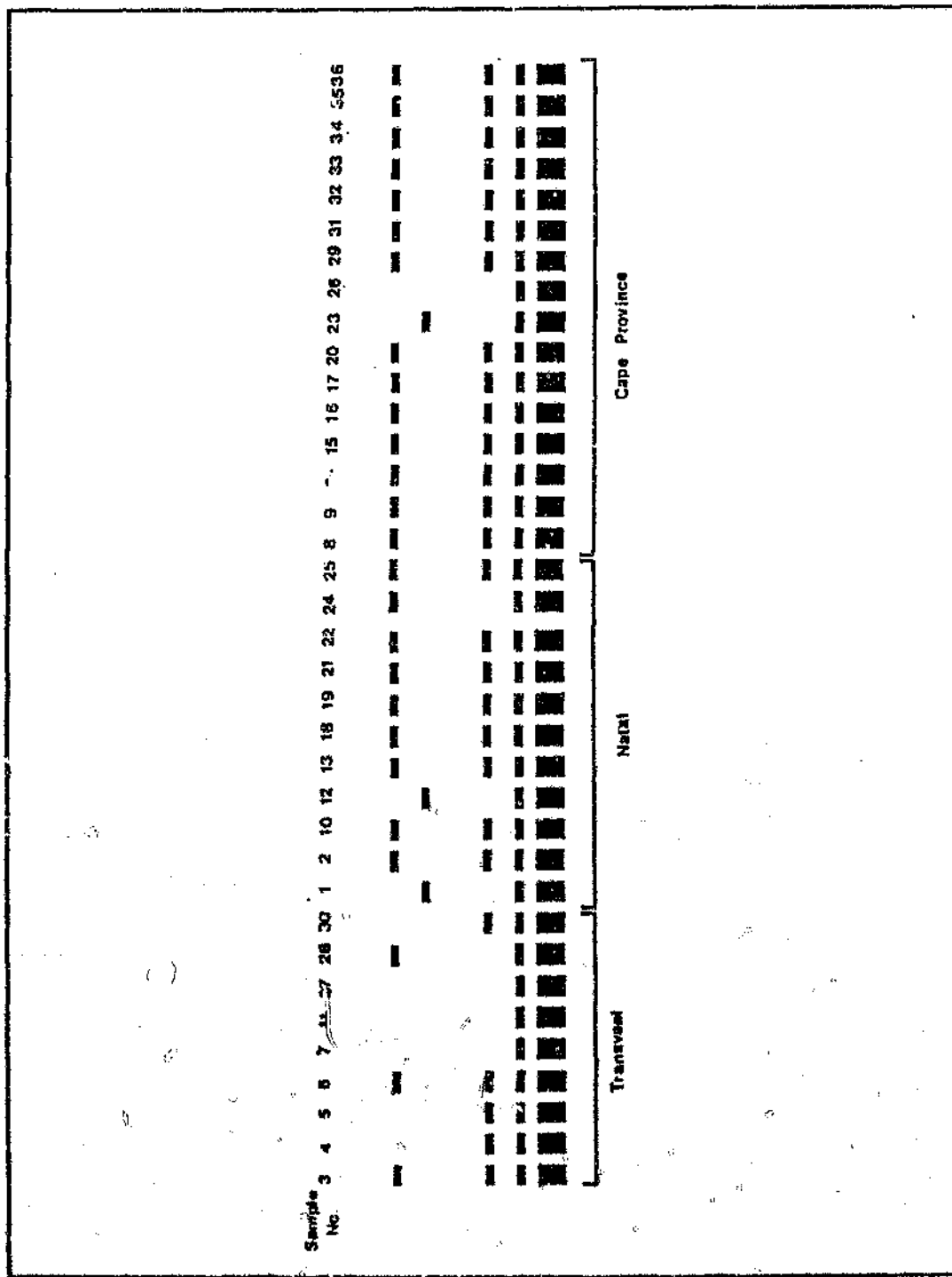


Figure 8 Zymogram of Malate dehydrogenase (MDH).

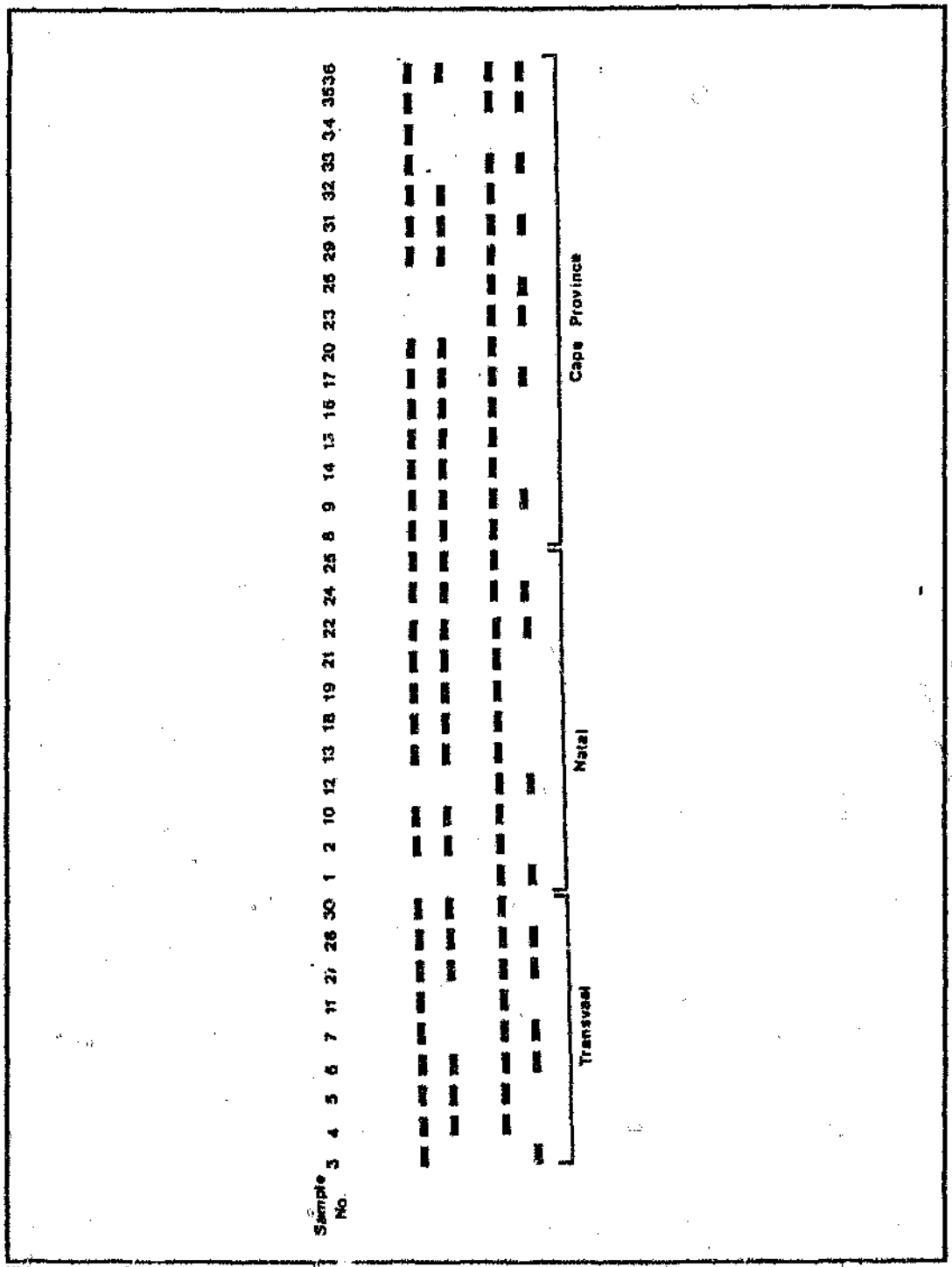


Figure 9 Zymogram of Malic enzyme (ME).

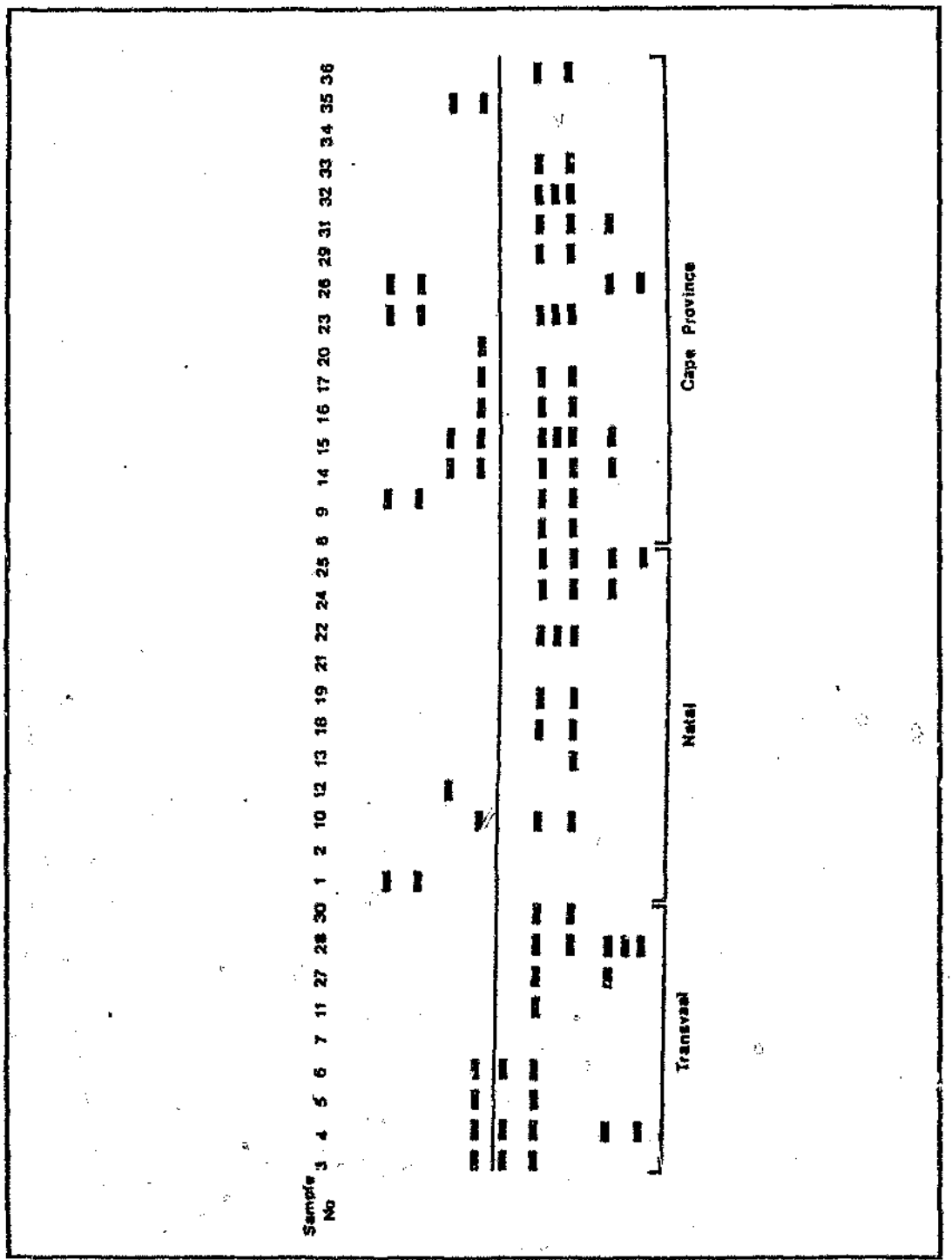


Figure 10 Zymogram of Peroxidase (PER).

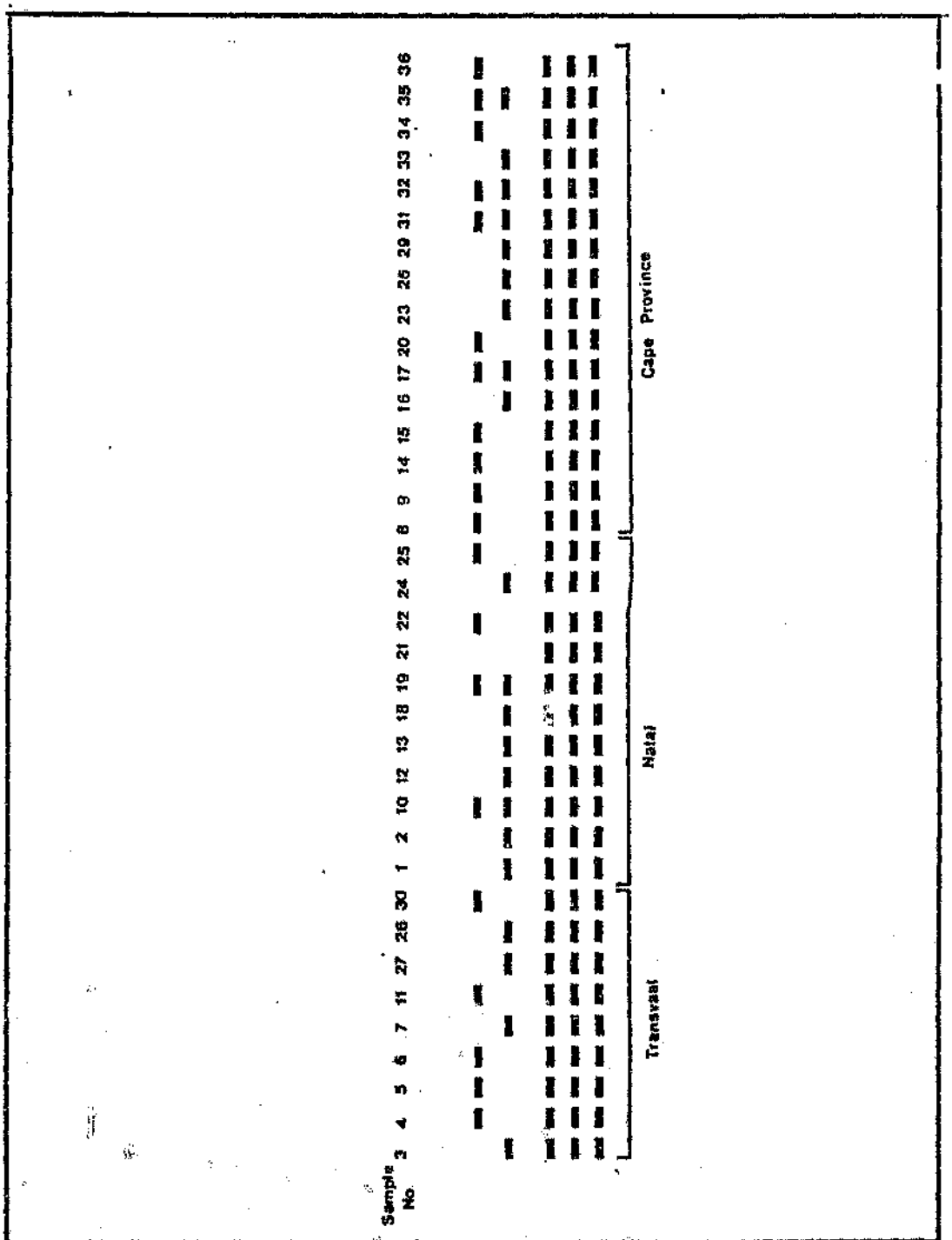


Figure 11 Zymogram of 6 Phosphogluco dehydrogenase (6PGD).

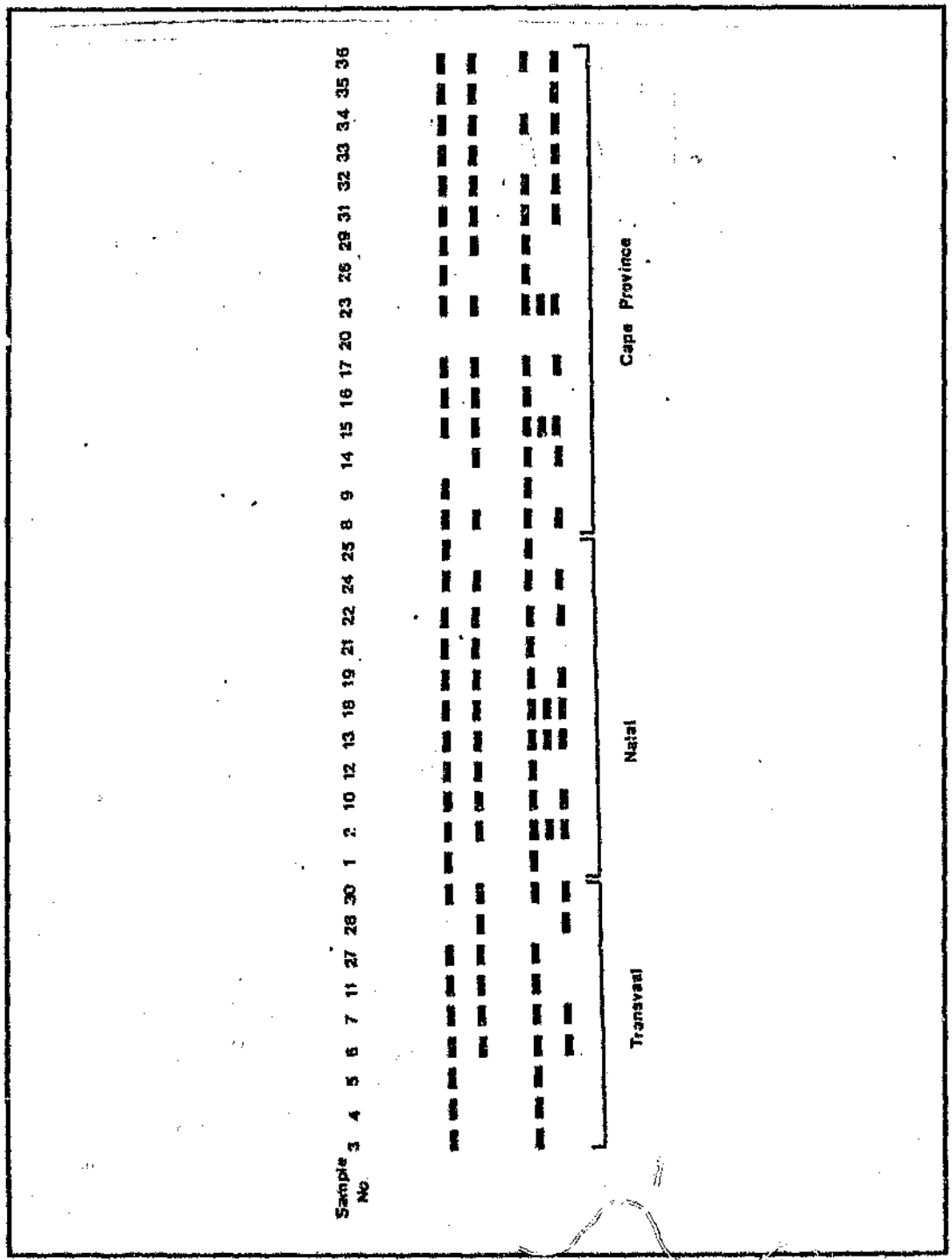


Figure 12 Zymogram of Phosphogluco isomerase (PGI).

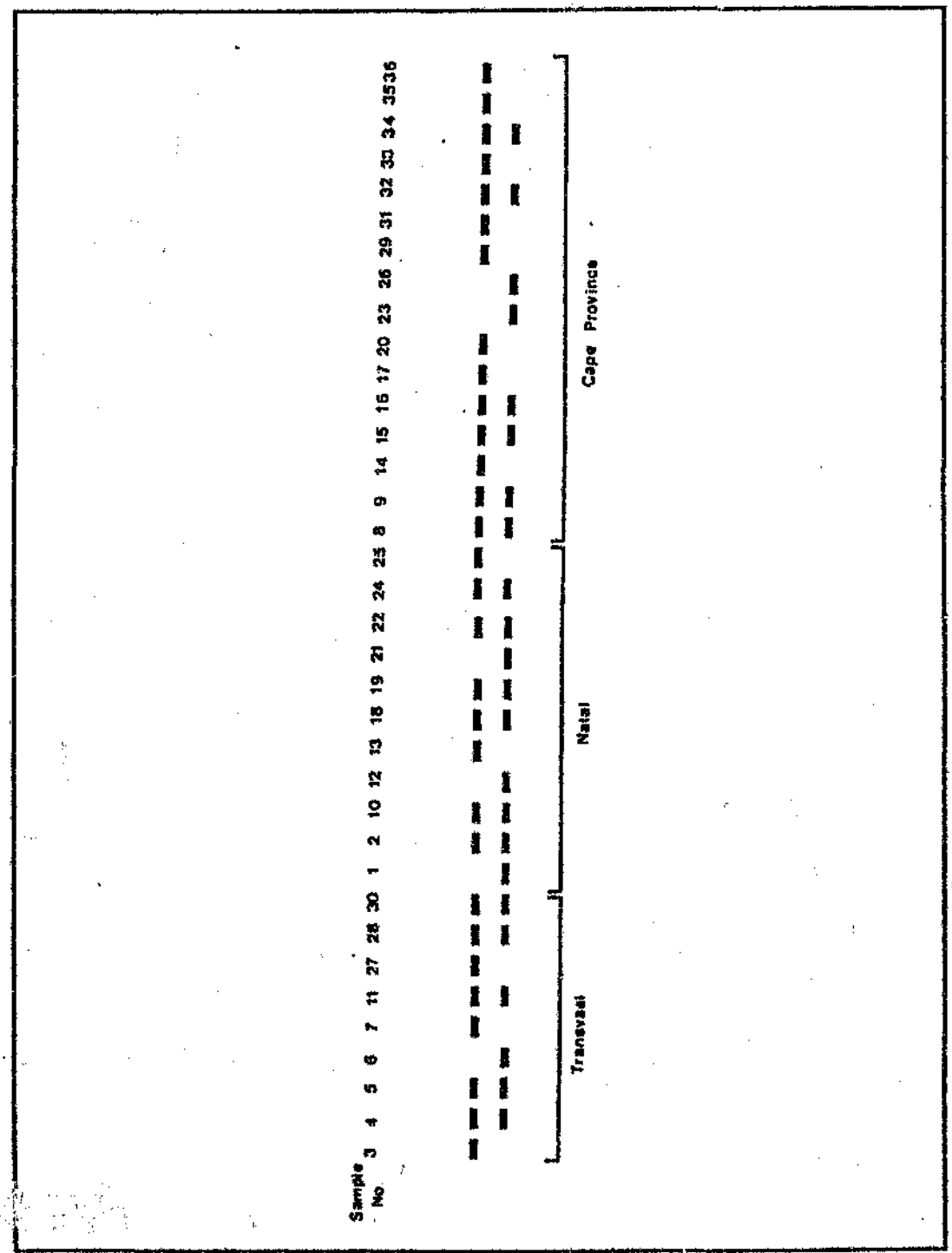


Figure 13 Zymogram of Shikimate dehydrogenase (SDH).

The electrophoretic study was repeated twice, once when all the field collecting had been completed and once again after a three month interval. Duplicates of each sample were run. The results were found to be identical in each case.

The results were placed into a matrix by assigning numbers to each enzyme pattern found in each specific enzyme. See Tables 4 to 14.
A Cluster analysis and a Principal components analysis were produced using the personal computer program NTsys. See Materials and Methods. See Figures 15 and 16.

Tables 4 to 14 show a graphical representation of how the enzymes were number coded for the data matrix.

Table 4. Acid phosphatase (ACP)

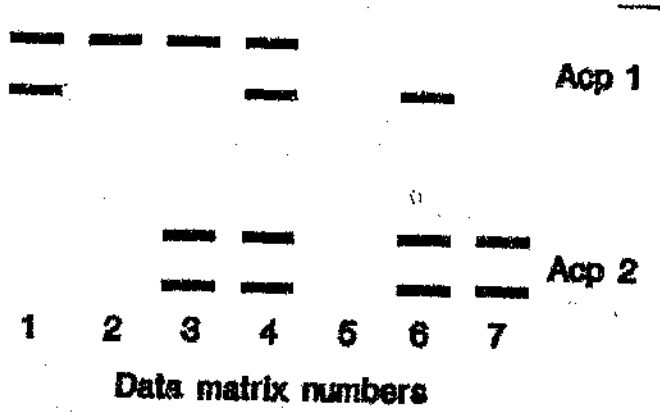


Table 5. Alcohol dehydrogenase (ADH).

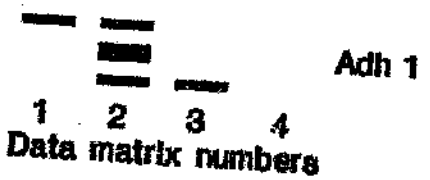


Table 6. Esterase (EST).

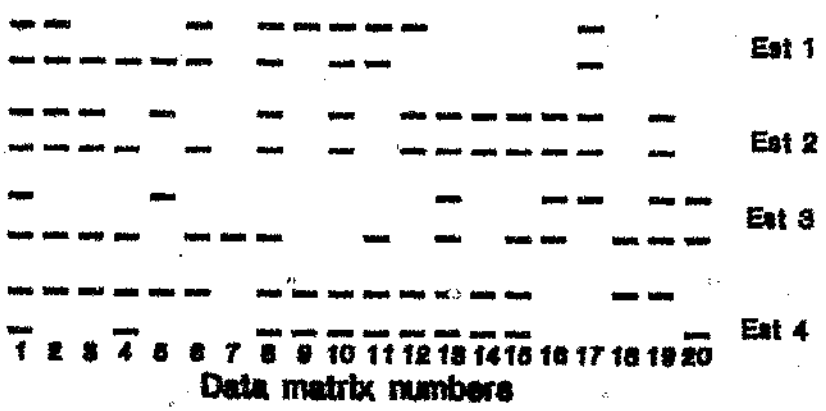


Table 7. Glutamate dehydrogenase (GDH).

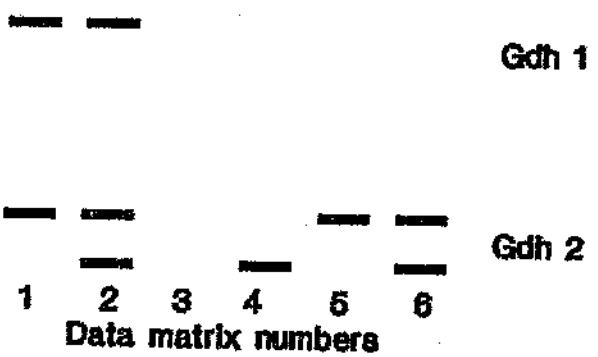


Table 8. Isocitrate dehydrogenase (IDH).

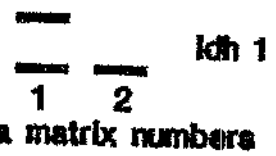


Table 9. Malate dehydrogenase (MDH).

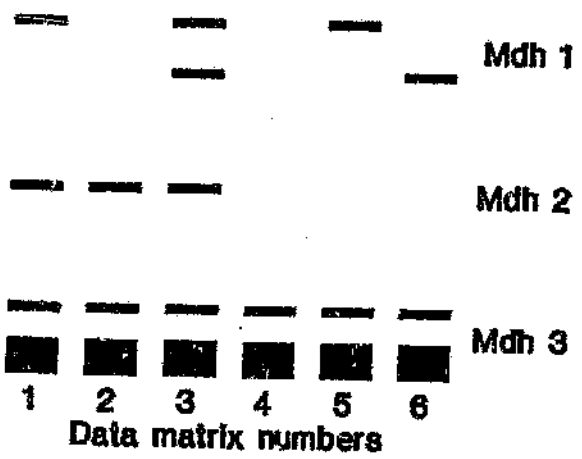


Table 10. Malic enzyme (ME).

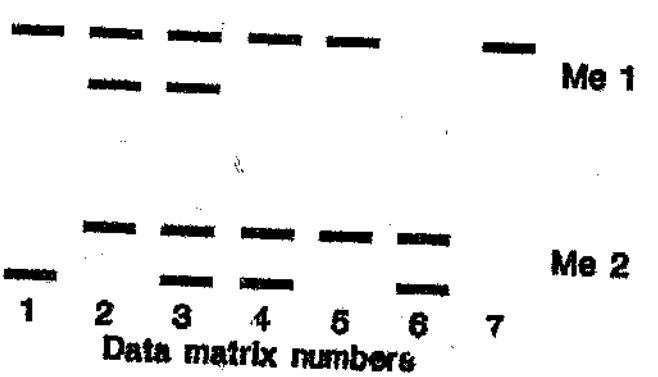


Table 11. Peroxidase (PER).

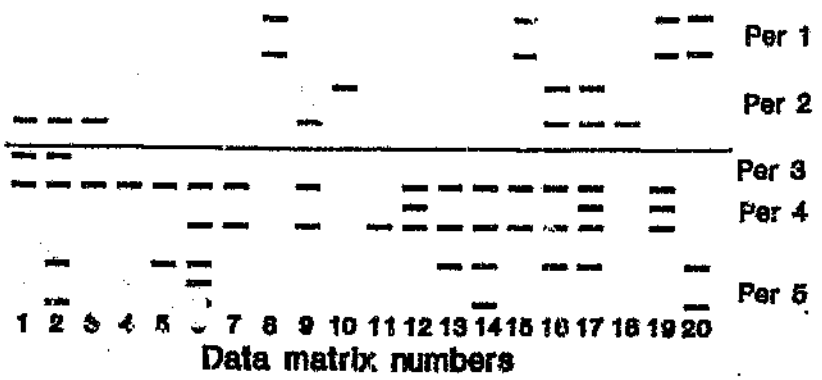


Table 12. 6- phosphoglucose dehydrogenase (6-PDG).

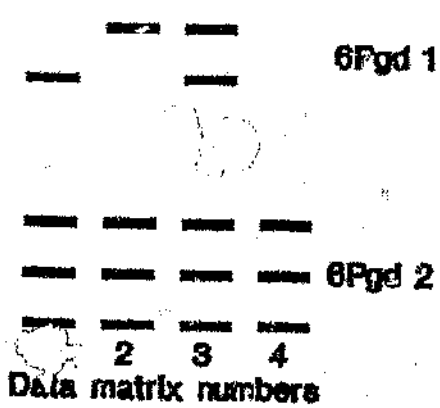


Table 13. Phosphogluco isomerase (PGI).

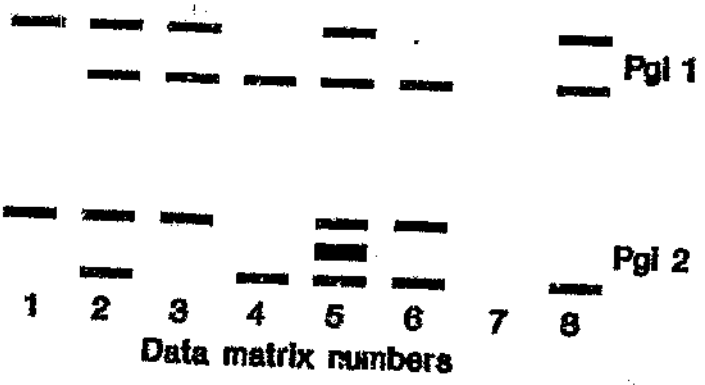
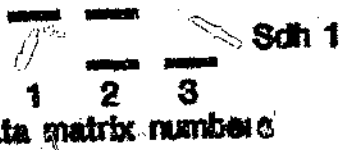


Table 14. Shikimic dehydrogenase (SDH).



Different isozyme patterns were identified within each isozyme examined, numbered accordingly, and placed into a matrix, called the data matrix. See Appendix ii. The matrix was set up using an editing program found in MS Dos. Using the personal computer software program NTSYS (Numerical taxonomy systems), cluster and principal component analyses were carried out. The grass collections were called Operational Taxonomic Units (OTU's) and listed 1-36 as per Table 1, in columns, while the enzymes were listed 1-11, in rows.

Following the procedure, a cluster analysis was produced. Two distinct groupings were found on examining the results. The first group consisted of the OTU's 1, 12 and 23. these OTU's were not closely linked (1.45). They were however more closely linked to each other than to the rest of the OTU's (1.62). The second grouping consisted of the rest of the OTU's, in this large group a number of sub-groups were recognised, see Table 16. A matrix comparison test was carried out on the matrix data to determine whether the cluster analysis results gave a true representation of the raw data. The matrix correlation for the electrophoretic results was found to be r = 0.70299, this number is in a medium range and suggests that the raw data is not entirely represented by the cluster analysis.
Figure 4 shows a graphical representation of the data presented in the form of a histogram.

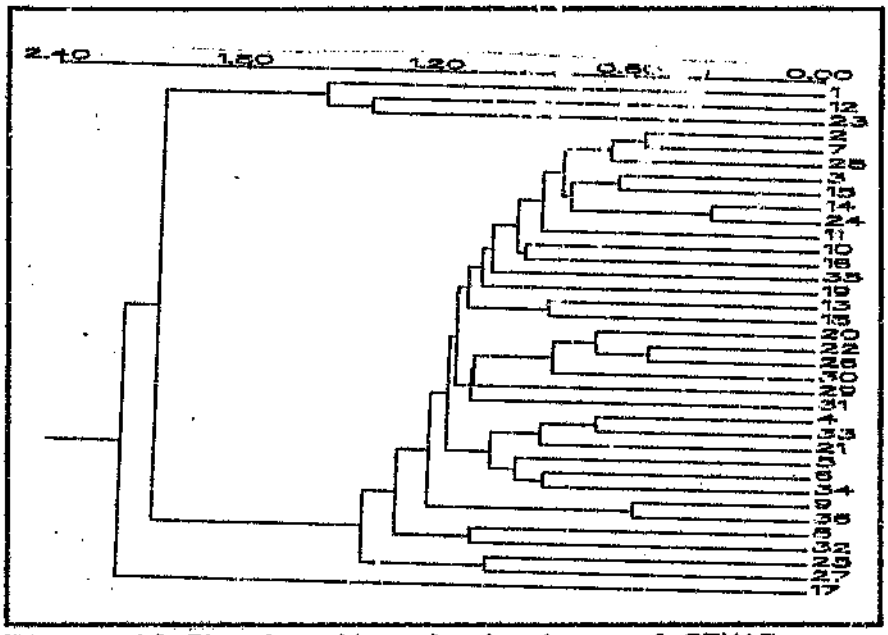


Figure 14 Showing the clustering of OTU'S once the cluster analysis had been performed.

Table 15. Table showing OTU groupings using the Cluster Analysis Test.

Group	Sub-group	OTU Number
1	i	1,12,23
2	i	14,24
	ii	9,36
	iii	2,7,28,11
	iv	3,15
	v	10,16
	vi	13,18

Group	Sub-group	OTU Number
	vii	20,22,26,29,31
	viii	4,21,33
	ix	5,6,34
	x	8,32
	xi	25,27
	xii	19,35
	xiii	17

As OTU's 1, 12 and 23 were found to be in a completely separate category to the main group of OTU's, these could have interfered with the positioning of the other OTU's. Thus these OTU's were removed from the matrix and the cluster analysis rerun see Figure 15. Noting the sub-groupings of the OTU's it can be seen that few differences occurred. OTU's 2, 9, 22, and 33 did change their sub-grouping, however the principle components test showed that these changes were slight. Therefore OTU's 1, 12 and 23 did not affect the overall groupings of the entire OTU group. The matrix correlation coefficient was found to vary from x = 0,69946 when OTU 23 was removed to x = 0.78267 when OTU's 1, 12 and 23 were removed. The low correlation coefficients again suggest that the raw data is not being truly reflected by the cluster analysis.

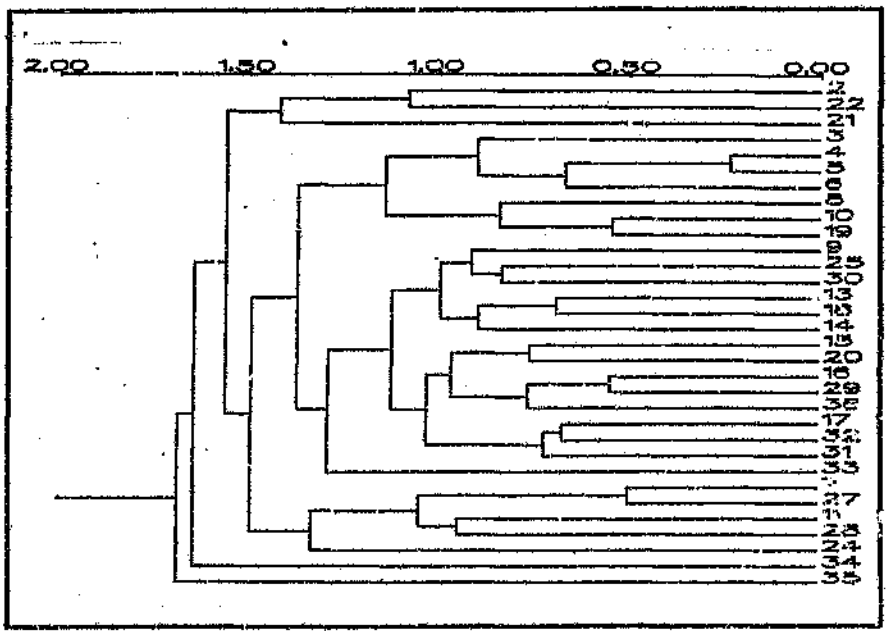


Figure 15 Result of cluster analysis once OTU'S 1,12 and 23 were removed.

O 's 1, 12 and 23 were found to form an entirely separate group from the rest of the OTU's. In figure 15 these three OTU's were removed to determine if this would affect the groupings of the larger OTU group. From figure 15 distinct groupings were noted. When these groupings were compared to figure 14 it can be seen that the groupings are very similar. The only OTU's that differ in the groupings are OTU's 22 and 24. OTU 24 moves from the group of OTU's 8, 10 and 19, to the group of OTU's 2 and 22 when OTU's 1, 12 and 23 were removed. There were no other differences in the OTU groupings.

3.5 DISCUSSION.

A wide range of enzymes were examined in the electrophoretic section. Their names and their reliability are noted in Table 16.

Table 16. Enzymes examined in the electrophoresis section.

Enzyme	Abbreviation	Used	Reliability
Acid phophatase	ACP	Yes	***
Alcohol dehydrogenase	ADH	Yes	***
Aldolase	ALD	No	*
Catalase	CAT	No	*
Esterase	EST	Yes	**
Glutamate dehydrogenase	GDH	Yes	***
Hexokinase	HEX	No	*
Isocitrate dehydrogenase	IDH	Yes	***
Malate dehyorogenase	MDH	Yes	***
Malic enzyme	ME	Yes	***
Peroxidase	PER	Yes	**
6-Phosphogluco dehydrogenase	6PGD	Yes	***
Phosphogluco mutase	PGM	No	*
Phosphogluco isomerase	PGI	Yes	***
Shikimate dehydrogenase	SDH	Yes	***

Key:- * = poor ** = fair *** = good

The following enzymes :- Aldolase, catalase, hexokinase and phosphogluco mutase were given a rating of poor and were not used in this study. Aldolase was found to produce a single monomorphic zone of activity. No variation was found within the plant collection. It was thus discarded. Catalase and phosphogluco mutase only showed activity sporadically and were thus discarded as reliable activity was desired. Hexokinase showed activity when using the wash technique for staining, but this was extremely costly and hexokinase was discarded.

Esterase, peroxidase, glutamate dehydrogenase and phosphogluco isomerase were given a reliability rating of fair for the reasons discussed below.

Glutamate dehydrogenase (GDH) functions in the tricarboxylic acid cycle in hydrolysing hydrogen bonds. In the literature Oliver and Zapater (1983), studying *Solanum tuberosum*, found a single zone of polymorphic activity when staining for GDH. No results were found for monocotyledons in the literature. In this study 2 zones of polymorphic activity were found, this being different to that of *Solanum tuberosum*, a dicotyledon. Not many plant collections could be conclusively classified down to cultivar level using the GDH banding patterns alone. For example, plant collections 5 and 6 showed similar banding patterns, both being *C. dactylon var. skaaplaas fyn* Other *C. dactylon var. skaaplaas fyn* selections were found to have different banding patterns, for example plant collections 11, 13, 18 and 28 (all *C. dactylon var. skaaplaas*) all had similar banding patterns to each other but not to plant collections 5 and 6.
Peroxidase utilizes hydrogen peroxide to oxidize a very large number of hydrogen bonds including phenolic

substances, cytochrome C, nitrate, ascorbic acid, indole and certain organic ions such as iodides (Liv 1975). All higher plants examined to date have a very large number of peroxidase enzymes. For example maize has 24 peroxidase enzymes coded by at least 9 gene loci (Hamill and Brewbaker, 1969), barley has between 8 and 12 peroxidase enzymes (Felder, 1976).

The very complex electrophoretic patterns obtained plus the occurrence of peroxidase in a number of different organelles means that the electrophoretic patterns cannot be considered "good" in reliability, thus the rating of fair.

The same is evident for esterase. Esterase hydrolyses a large number of compounds having different ester linkages *in vitro* (Gottlieb, 1982). Plant species also contain high numbers of esterase isozymes in many different tissues, for example maize contains 16 genetically distinct forms (MacDonald and Brewbaker, 1974), and barley has at least 10 genetically separable esterase isozymes, extractable from different tissues (Kahler and Allard, 1970). Again, the diversity and origin is very complex and thus esterase was rated fair in reliability.

Malate dehydrogenase (MDH) and its NADP derivative malic enzyme (ME) were rated good in reliability, even though different MDH enzymes have been found localised in distinct cellular compartments eg. mitochondria, microbodies and cytoplasm (Gottlieb, 1981). The reason for a good rating being given is that only 2 loci were used in the genetic analysis of MDH and ME, and that they were found to be monomorphic at both these loci.

The other enzymes that were rated good in their reliability, namely ACP, ADH, 6PGD, and SDH, gave similar results to those recorded in the literature, where genetics of the enzymes were studied and explained.

Acid phosphatase (ACP) enzymes hydrolyse phosphomonoester bands in a number of different molecules. Plants may have a dozen or more ACP isozymes, but they frequently differ in intensity and colour when staining (Gottlieb, 1981). Electrophoresis studies usually show 1-3 isozymes. Both dimeric and monomeric forms have been reported. Studies in maize by Efron, (1970); sunflower by Torres and Diedenhofen, (1976); *Eucalyptus obliqua* by Brown *et al.*, (1975); and *Avena barbata* by Marshall and Allard, (1969) showed dimeric forms of ACP. *Hordeum spontaneum* was studied by Brown *et al.* 1978, showed both monomeric and dimeric forms. However monomeric forms of ACP were described by Roose and Gottlieb (1976), using *Tragopogon* and Gottlieb (1975) studying *Stepanomeria exigua*. In this study 2 regions of polymorphic activity were found, in both cases monomorphic isozymes were present, the results being similar to those of other monocotyledons mentioned in the literature, for example Roose and Gottlieb 1976. A number of grass selections can be classified down to cultivar level, for example plant collections 1, 12 and 23 which are *C. dactylon var. kwaek* . Other plant collections however cannot be classified down to cultivar level, for example plant collections 3, 4, 5 and 6 show the same ACP patterning and are all *C. dactylon*, but are different cultivars.

Alcohol dehydrogenase (ADH) enzymes appear to be cytoplasmic as no ADH enzymes are associated with any subcellular organelles (Scandalios 1977). Freeling

confirmed the 2 gene dimer model for maize ADH by dissociating the SET II isozyme into its component polypeptide subunits, and reassociating them (Freeling 1974). Results in this study show the presence of a single polymorphic zone of activity, which appears to be similar to that of SET I in Gottlieb's explanation of ADH (Gottlieb, (1981). All but eight of the plant collections showed a single band of ADH activity. The double bands could have represented a duplication in loci of the gene, this however could not be proved in this project, as it was beyond the scope of the project. The two *C. transvaalensis* grass collections could be identified by their banding pattern. Other studies on plants have shown similar results. Kut and Evans (1985) used ADH for a genetic identification of parental species and hybrid lines of *Nicotiana*. Torres (1974) working on maize, and Zapater (1983) using *Solanum tuberosum* in phylogenetic studies found that ADH followed the two gene dimer model.

Isocitrate dehydrogenase (IDH) catalyses the oxidation of isocitrate to α-ketoglutarate and is generally found in diploid plants as two dimeric isozymes one located in the plastids and the other, more abundant form in the cytosol (Randall and Givan, 1981). In this study IDH showed a single zone of polymorphic activity. The banding patterns resembled a monomeric isozyme pattern showing no 3 banded results. Only two banding patterns exist, both patterns present in *C. dactylon* and only one present in both *C. magenrisii* and *C. transvaalensis*. Thus IDH would have to be used in conjunction with other isozymes to conclusively classify the plant collections to a species or cultivar level. In the literature there is mention of monomeric IDH isozymes, by Guries and Ledig (1978) working on *Pinus rigida*, and Kahler and Lay (1985)

working on *Pinus rigida*, and Kahler and Lay (1985) studying *Helianthus annuus (L.)*.

6- Phosphoglucose dehydrogenase (6PGD) is a glycolytic enzyme. In plants for each enzyme in the glycolytic pathway there are normally 2 isozymes present, one in the chloroplast and one in the cytoplasm. The literature shows 6- PGD to have similar results in both monocotyledons and dicotyledons. Two 6PGD enzymes have been reported by Guries and Ledig (1978) studying *Pinus ponderosa*. These were both dimeric which correlates with Torres (1974a) discussing maize. In this study there were a number of regions of activity which were both monomorphic and polymorphic. Only the polymorphic region was used in this study. Three banding patterns exist, and although the banding patterns are similar in most instances when classifying down to cultivar level, 6PGD would have to be used in conjunction with other isozymes to conclusively classify the plant collections.

In the present study shikimate dehydrogenase (SDH) was found to have one region of polymorphic activity, while the banding pattern was similar to that of a monomeric isozyme. Staub *et al.* (1987), studying *Cucumis*, a dicotyledon, found SDH to be conditioned by 1 locus and 3 alleles. Isozyme patterns were found to be both polymorphic and monomorphic.

From the electrophoretic data used in this study and by incorporating the data into NTSYS it can be noted that the plant collections divided up into 2 main groups, 1 major and 1 minor, the major group breaking up into smaller sub-groups. Although the raw data may not be as truly represented as it could be, the OTU groupings

From the above discussion it can be seen that to be able to utilise the data in an effective way, a number of enzymes would have to be used. ACP, ADH, 6PGD and SDH together with GDH could be effectively used, together, but would not give sufficient data if used on their own as in this study. This data however, in conjunction with morphological and micromatomical data may be a means of classifying the plant collections down to cultivar level.

CHAPTER 4.

4. MORPHOLOGY AND MICROANATOMY.

4.1 INTRODUCTION.

Until approximately 60 years ago classifications of grasses were based mainly on gross morphology of inflorescences and spikelets. Attention then turned to the inclusion of the anatomy and cytology of grasses. All new classifications subsequent to Avdulov in 1931 incorporate characters from these sources (Watson *et al.* 1985).

In 1960 Prat outlined the history of grass classification and provided a comprehensive list of characters recognised at that date to have high level classificatory significance, but the list has continued to grow (Ellis 1976, 1979; Clifford and Watson, 1977; Watson and Dallwitz 1980; Watson *et al.* 1985). Watson *et al.* 1985 reported that the list now contains 728 generic descriptions of grasses which contain information on morphology, anatomy, physiology, cytology, ecology and geography.

In turf grasses, the characteristics of importance include persistence under close mowing, colour, low growth, wear tolerance, drought resistance and cold tolerance. Due to time and space limitations such characteristics could not be examined.

In this section some easily accessible anatomical and morphological characersistics are used, which can be

combined with the electrophoretic and cytological evidence in order to find a convenient means of characterising South African turf *Cynodon* to the cultivar level.

4.2 LITERATURE REVIEW.

Morphological and cytological characteristics, together with hybridisation studies were used by Harlan and de Wet, (1969) in an attempt to reclassify the genus *Cynodon*. Hybrids were identified on morphological grounds and a number of characteristics were noted. They included winter hardiness, noted especially in hybrids. The overall structure of the inflorescence, stolon structure and colour, leaf profile and colour and the overall growth habit of the plant. The number of seeds produced and the percentage of seed producing plants was also examined. These studies showed that this genus could be fairly easily divided up into taxonomic groups using morphological characteristics. They found however, that distinct and reasonably consistent morphological discontinuities were not reflected in the cytological studies. Harlan and de Wet, (1969) stressed that morphological evidence as a tool for classification must be carefully used. They concluded that the genus *Cynodon* is difficult to treat taxonomically and that no entirely satisfactory classification is possible, when using morphological and cytological evidence.

Stiff and Powell (1974) used the stem anatomy of 20 cultivars of turfgrasses, including *Cynodon dactylon* , as a means of selecting specific cultivars. They looked at vascular bundle size composition and location in both internodes and nodes. They also looked at the usual tissue systems, which included epidermal, fundamental and vascular systems. Three types of stems were distinguished :- Single ring, multiple rings and complex rings. Two types of nodes were observed :- simple and compound.
Stiff and Powell (1974) found that the comparison of

internodes and nodes revealed a consistency of arrangement of parts and similarity of structural features at corresponding regions. They concluded that stem anatomy shows turfgrasses to be a heterogeneous group of grasses with varying evolutionary and developmental histories. They suggest that these anatomical differences must be considered in vegetative propagation techniques and selection of improved cultivars.

Metcalf (1960) regards the study of sections of culms to have limited taxonomic value, because of differences in the appearance of sections at different levels and because the similarity of structure does not always indicate affinities.

Nittler and Kenny, (1976) investigated tiller, rhizome and crown characteristics in Kentucky bluegrass cultivars, using their own classification system of the above mentioned characteristics. Using this technique they could distinguish between a number of cultivars. In this work they concluded that even though the techniques used were not perfect, they could be useful in evaluating cultivar purity in seed lots.

Rogers *et al.* (1976) used SEM to note ultrastructural differences in the storage organs of *Cynodon dactylon L.* The study was undertaken applying scanning electron microscopy in order to show differences in tissue components of rhizomes and stolons.

Morphological characteristics are to a large extent determined by the environmental conditions in which the plant grows. They also depend on the nutrients which the

the plant may be subjected. It is thus important to quantify the above conditions when looking at morphological characteristics. It is also important to recognise that morphological characteristics should be regarded as being of secondary importance when attempting to characterise grass types. Morphology that is not environmentally plastic should be considered first, that is, any morphological characters that remain stable and are independent of the environment in which the plant is grown.

4.3 MATERIALS AND METHODS.

4.3.1 SCANNING ELECTRON MICROSCOPY.

Care was taken to choose leaf material that was of a similar age. This was done by sampling the 2nd youngest leaf in all grass selections. A 1 cm long section of the mid lamina section was cut from a point one third of the way down.

The leaf material was prepared for Scanning Electron Microscopy (SEM) as follows :- The material was fixed for 24 hours in 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 6.5, followed by 2 rinses in the same buffer and dehydrated in a graded series of ethyl alcohol (60%, 70%, 80%, 90%, 95%, 100%). The specimens were stored in 100% alcohol for critical point drying.

Critical point dried specimens were trimmed and mounted on large stubbs using ACRO 17651 glue (Witcomb 1985). The glue and specimens set overnight, were gold coated in a sputter coater and examined using a Cambridge JS20.

4.3.2 LIGHT MICROSCOPY.

4.3.2.1 Frozen sections.

Fresh plant material was used for frozen sections. One centimetre long sections were taken from one third of the way from the tip of the leaf. The second youngest leaf in each grass selection was used.

The material was mounted on the microtome chuck using 'Tissue Tek,' sectioned at 20-50µm and the sections were floated on distilled water in watchglasses. The sections

were mounted on slides, stained in toluidine blue and viewed as temporary mounts, using a Z iss D-7082 light microscope with camera attachment using Ilford PAN F 50 film to take photographs of the slides.

4.3.2.2 Epidermal peels.

Two methods were used to obtain epidermal peels of the lower surface of the grass selections.

1. A razor blade was used to remove all the leaf tissue above the lower epidermis, with the aid of a dissecting microscope. The leaf section was kept moist with water to ensure preservation of the epidermis. The epidermis was mounted on a slide using water and viewed as a temporary mount.

2. The lower epidermis of the leaf was coated with clear nail varnish, once the varnish had dried it was peeled from the leaf surface and viewed as a temporary mount.

4.3.2.3 Wax embedding.

Fresh plant material was fixed using formalin-acetic acid alcohol (FAA) (Johansen 1940 see Appendix 2) for 18 h. Some samples did not fix due to buoyancy. In such cases 1% Tween 20 was used as a wetting agent. Once samples were fixed they were rinsed 4 times with distilled water.

Samples were placed in a 0.1% hydrofluoric acid solution for 15 minutes to dissolve silicon crystals. The presence of silicon crystals when sectioning caused the leaf to tear. This was found to occur in some instances when frozen sections were examined (Johansen 1940).

4.3.2.4 Dehydration.

A dehydration series using tertiary butyl alcohol was used (see Appendix 4). In the 100% step enough dry erythrosin dye was added to stain samples superficially, to allow for easier orientation of samples during embedding and microtoming.

4.3.2.5 Wax infiltration.

The gradual infiltration of the wax into the plant material gave the best results. The plant tissues were tranferred into a mixture of equal parts paraffin oil and tertiary butyl alcohol for 2 hours. The plant material was placed on top of congealing Parawax and placed into an oven at 60°C for 3 hours, until the sample became fully infiltrated with the wax, and sank to the bottom of the tube. This process was repeated in fresh wax and after the final infiltration plant tissues were embedded in paraplast and aligned for transverse sectioning.

4.3.2.6 Microtoming.

Material was sectioned at 10µm - 20µm using a rotary microtome. Sections were floated onto subbed slides and warmed slightly for sections to adhere to the slide.

4.3.2.7 Staining and viewing.

Sections were stained in Safranin and Fast green and permanently mounted in DPX.

Both wax and frozen sections were examined. The frozen sections were used as a quick temporary means of noting the morphology of the plant collections before they were all acclimatized to the same conditions. The wax embedding technique was used in the final assessment of the internal anatomy of the plant collections.

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4.4 RESULTS.

Results are given for the following characters:

4.4.1 LIGHT MICROSCOPY.

4.4.1.1 Leaf profile.

Two main leaf profiles were found, the first with a definite midrib and main vein giving rise to a V - shaped leaf. The second with a rounded midrib, which gives rise to a more U - shaped leaf. See Figure 16. These categories were subdivided and six leaf profiles were identified depending on the steepness of the V or U shape and on the shape of the ends of the leaf arms. The leaf profile was examined twice, when the plants were first brought in from the field, that is, before the environmental conditions had been stabilised and six months later, when the plant collections had been grown under constant environmental conditions. The leaves were found to exhibit constant leaf profiles. Leaf profile was thus considered as a valid identifying character.

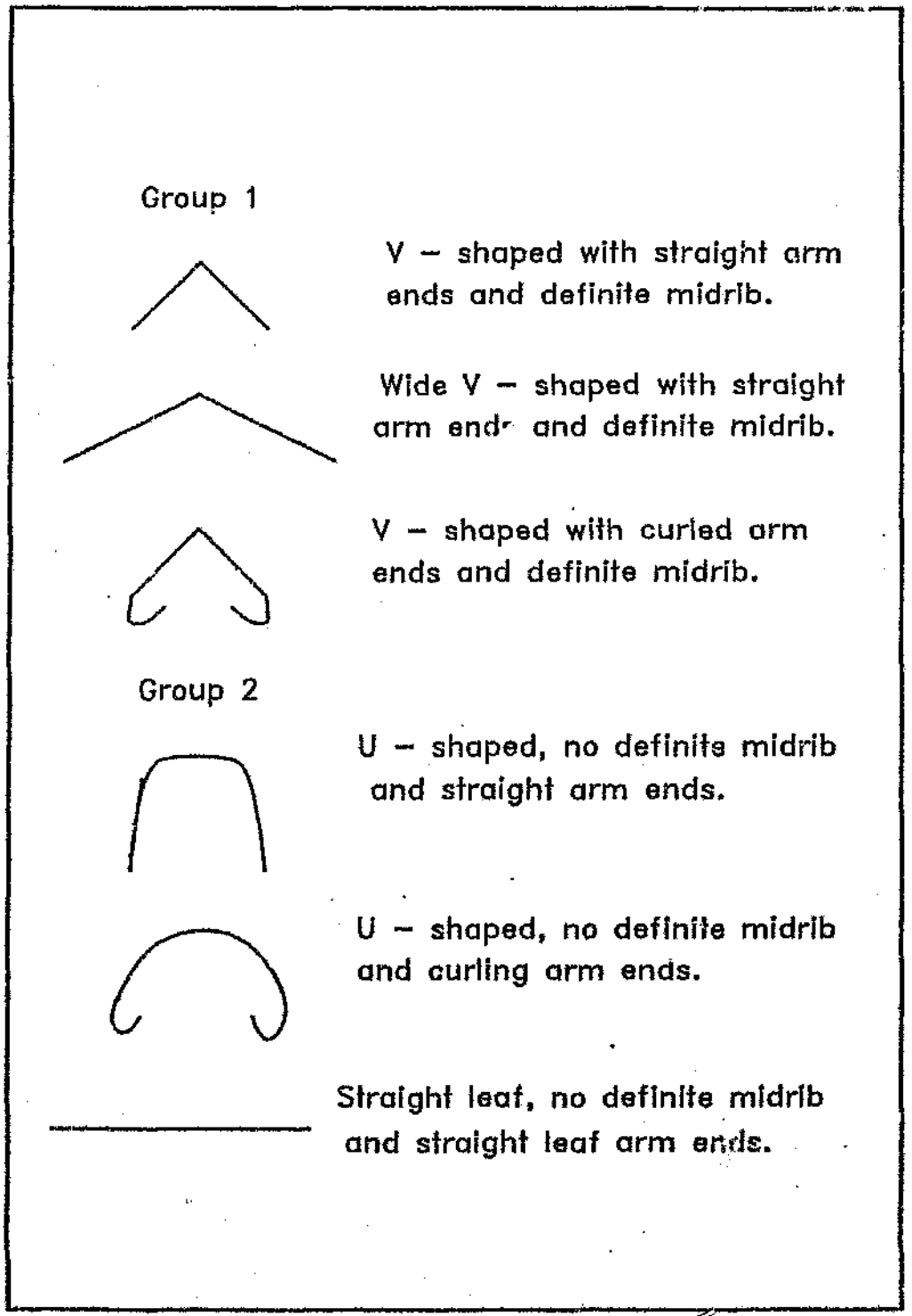

Figure 16 Diagrammatic sketches of leaf shapes found in transverse sections of *Cynodon* turf selections. See Appendix page 150 for micrographs.

4.4.1.2 Vascular bundle distribution and number.

The number of, minor, intermediate and major vascular bundles were counted. The distribution types of the bundle types were noted and each pattern designated a number. See Figure 17. In cases where intermediate vascular bundles were found, one group was designated as differing in this respect as presence of intermediate vascular bundles was the exception rather than the rule.

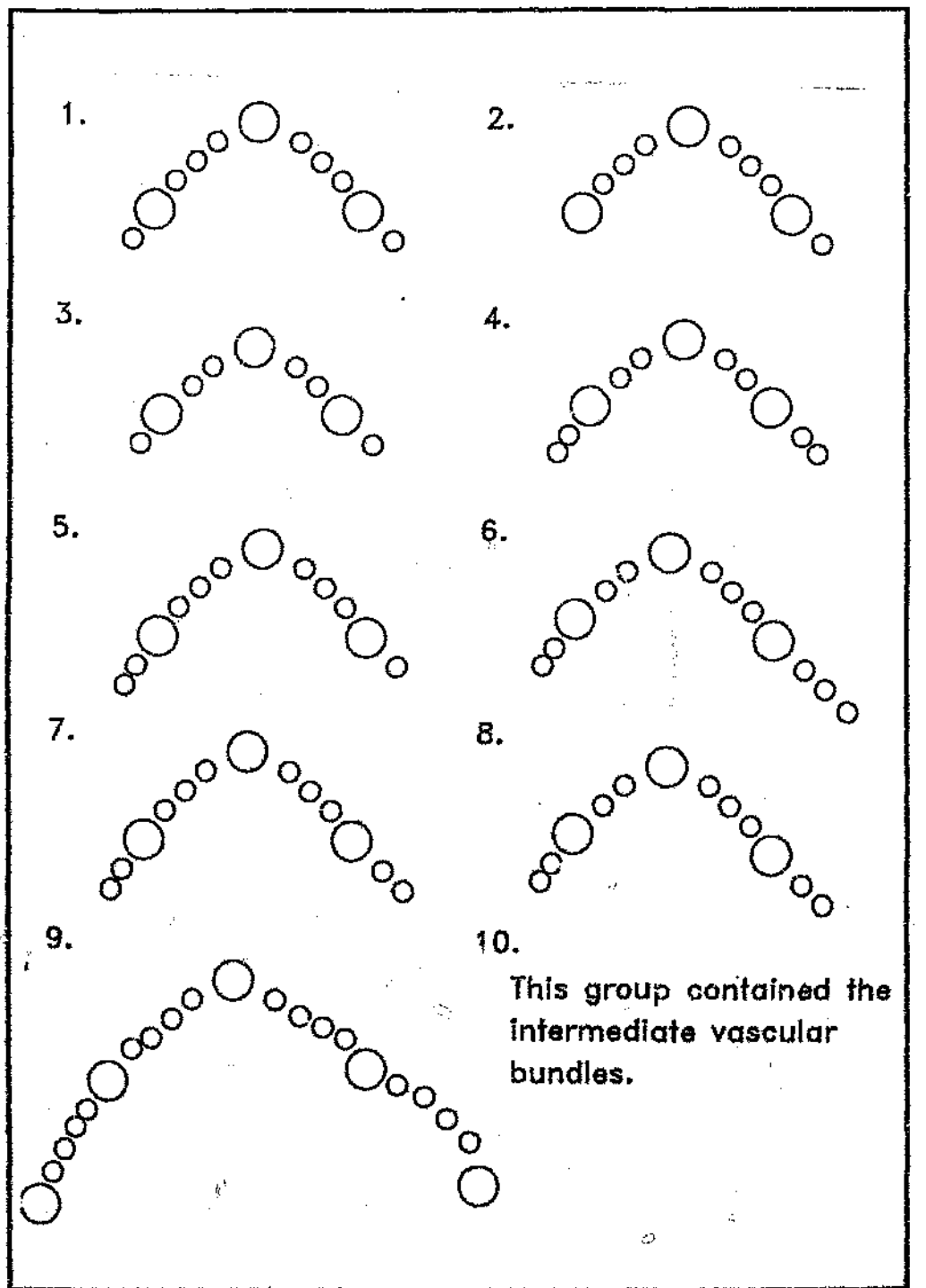

Figure 17 Diagrammatic sketches showing both positioning and numbers of various vascular bundles of the plant collections.

4.4.1.3 Bundle sheath number and bulliform cell number and shape.

The number of bundle sheath cells were counted around lateral major vascular bundles. These numbers were found to vary, even within leaves. The numbers were also found to vary in both counts, the one done when the plants arrived from the field and the second when the plants had been grown in the phytotron for six months. Thus five counts were done on each plant and averages taken. See Appendix 8.

The same was found when the number of bulliform cells were counted and the same precautions were taken. Bulliform cells were counted from the same place in each case.

4.4.1.3 Bundle sheath number and bulliform cell number and shape.

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The same was found when the number of bulliform cells were counted and the same precautions were taken. Bulliform cells were counted from the same place in each case.

Plate 1.

Shows examples of spickle shaped hairs, see micrographs A to D. Note the two shapes of spickle hairs, bulbous in micrographs A, C and D, and elongated in micrograph C.

Micrographs A and B show examples of long hairs. Note that long hairs can originate from either a collar like structure which disrupts the papillae slightly (micrograph B) or straight out of the epidermis not disrupting the papillae at all (micrograph A).

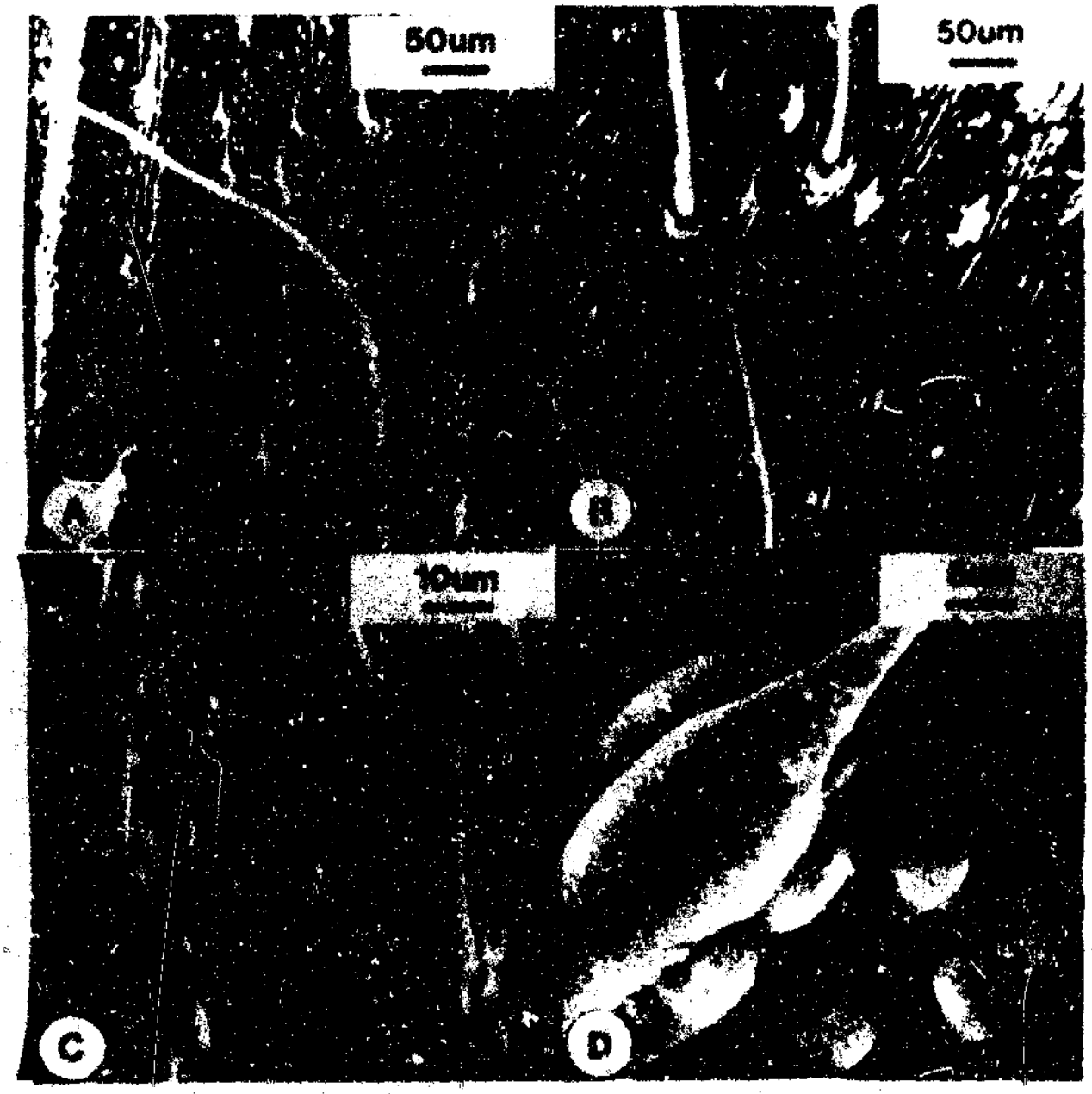
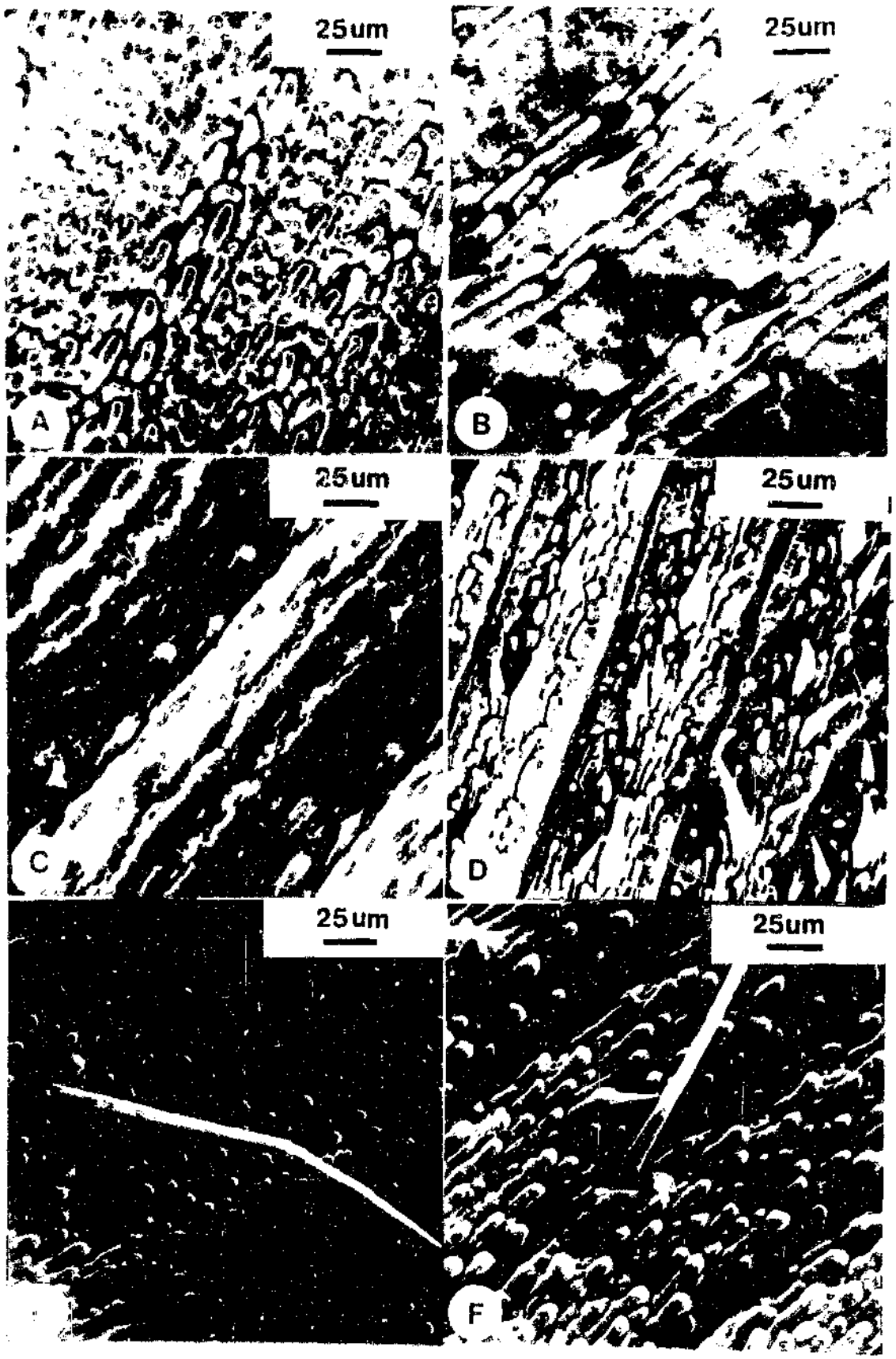

50um
50um
A
B
10um
C
D

Plate 2

Micrographs showing the variation in number and shape of papillae present on the leaf surface, and the amount of ribbing on the leaf surface.
Micrograph A shows distinct ribbing with two papilla types, which totally cover the stomata and guard cells.
Micrograph B shows distinct ribbing with very few papillae in between. Spickle hairs are present.
Micrograph C shows ribbing, but with few to no papillae present.
Micrograph D shows ribbing present with two distinct types of papilla present, and an abundance of spickle hairs.
Micrograph E shows ribbing with two distinct papilla types. Long hairs are present.
Micrograph F shows ribbing with two types of papilla present. both spickle and long hairs present.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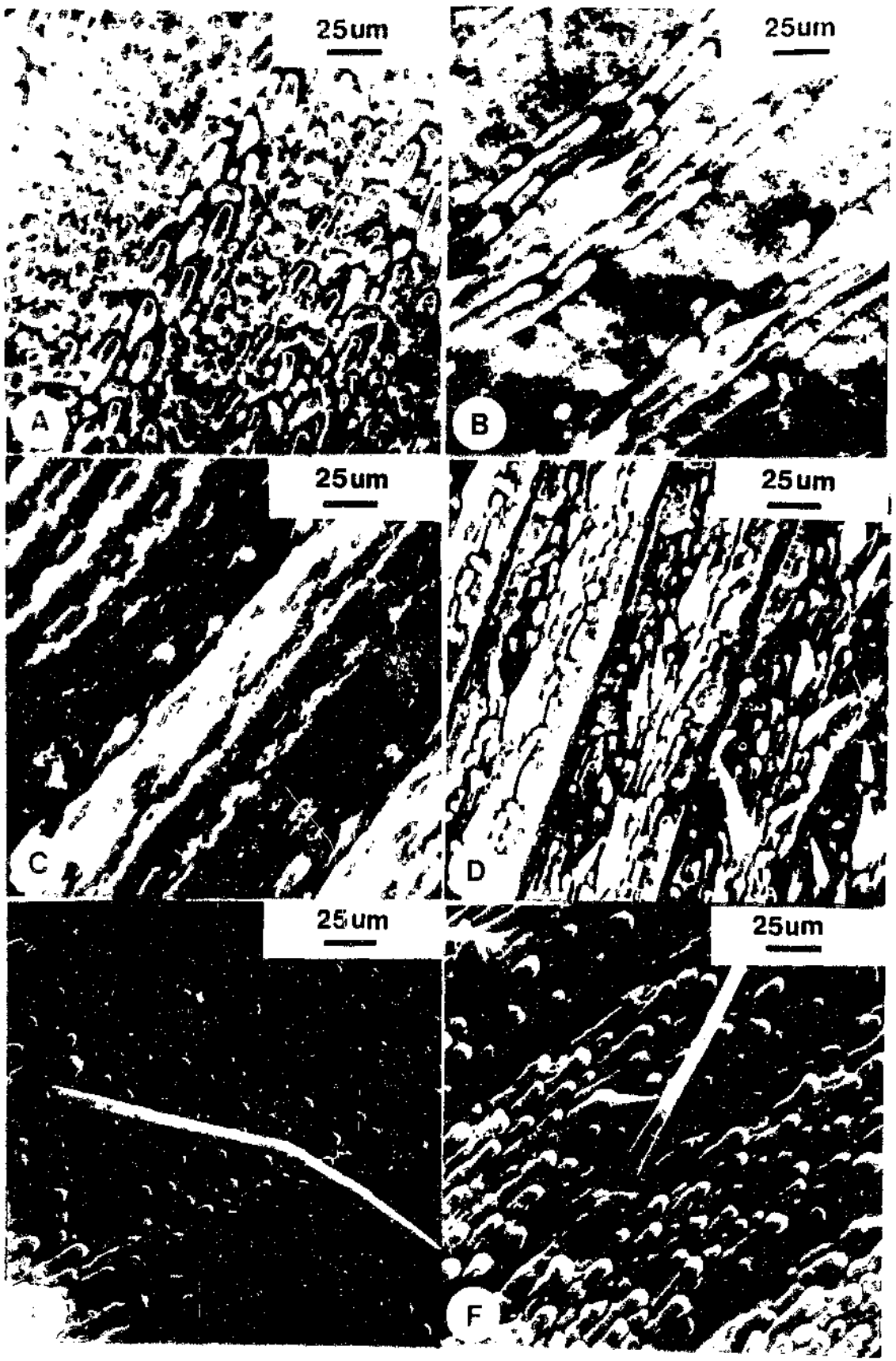
F


25um
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D
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E
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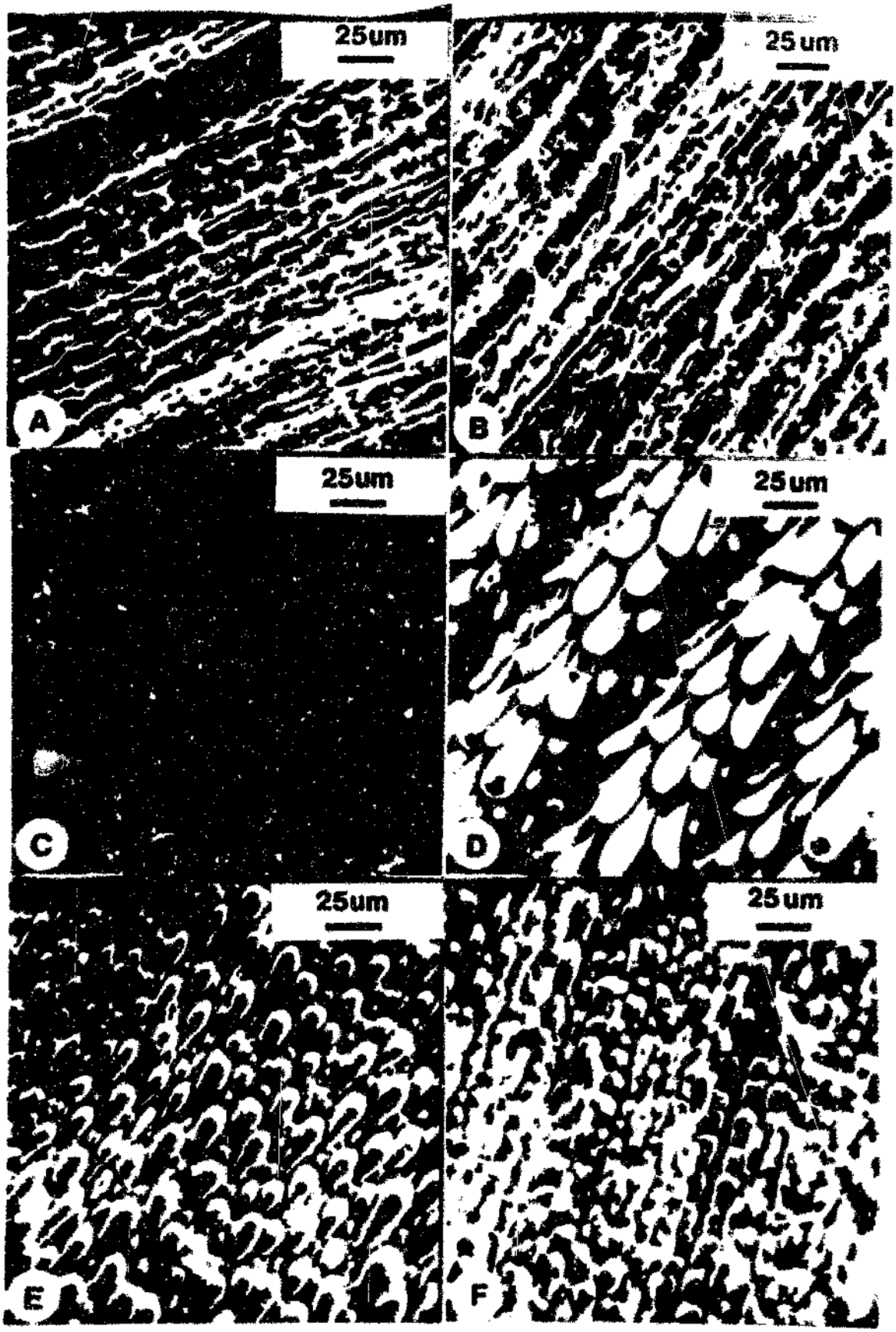
F

Plate 3

Showing papilla-patterning on the leaf surfaces.
Group 1 (see pp 89) is represented by micrographs A and B, lower and upper surfaces respectively.
Group 2 (see pp 90) is represented by micrographs C and D, lower and upper surfaces respectively.
Group 3 (see pp 90) is represented by micrographs E and F, lower and upper surfaces respectively.


25um
A
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B
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C
25um
D
25um
E
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F

Plate 4.

Showing papilla-patterning on the leaf surfaces.
Group 4 (see pp 90) is represented by micrographs A and B, lower and upper surfaces respectively.
Group 5 (see pp 90) is represented by micrographs C and D, lower and upper surfaces respectively.
Group 6 (see pp 90) is represented by micrographs E and F, lower and upper surfaces respectively.

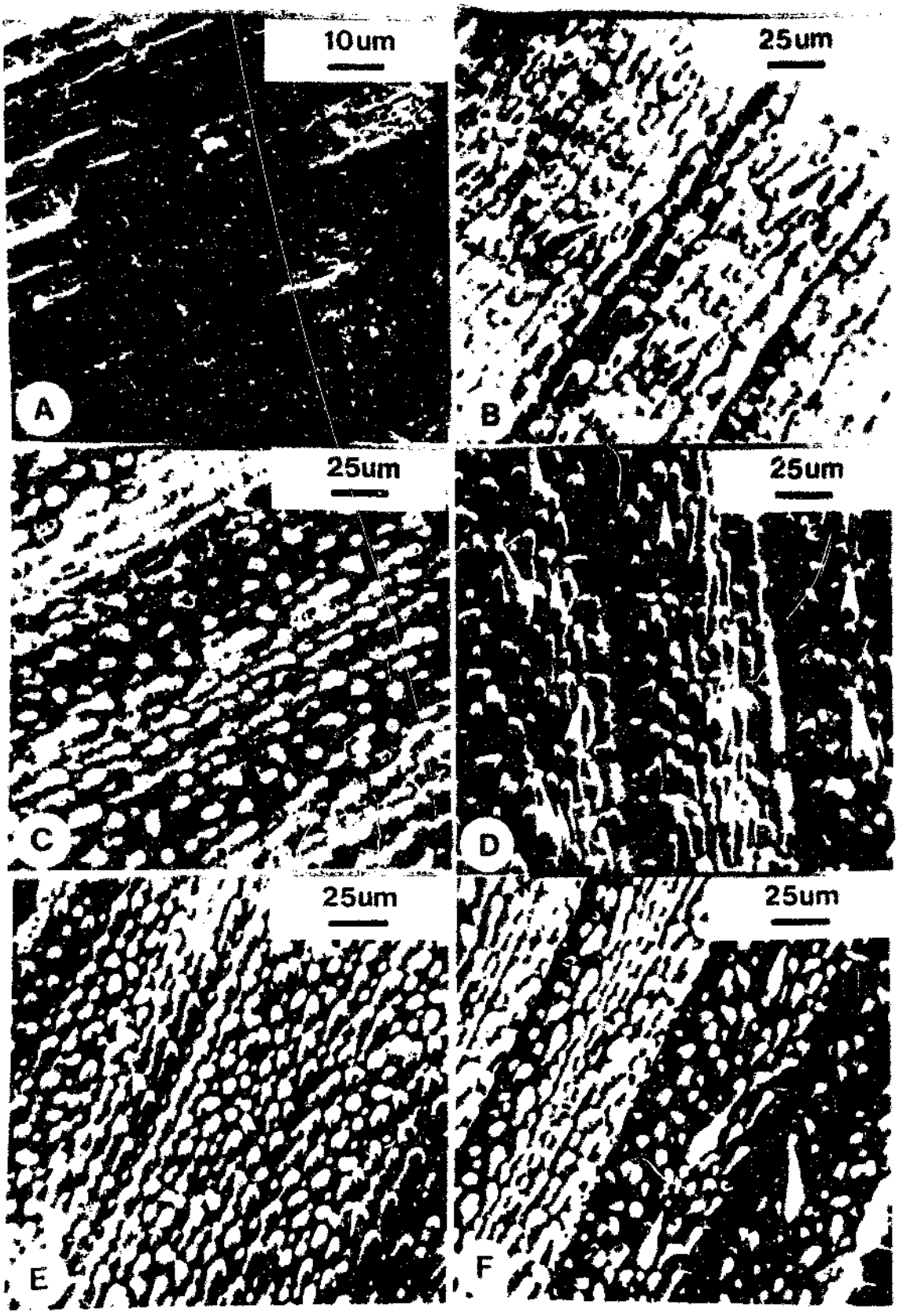
10um
A
25um
B
25um
C
25um
D
25um
E
25um
F

Plate 5.

Showing papilla-patterning on the leaf surfaces.
Group 7 (see pp 90) is represented by micrographs A and B, lower and upper surfaces respectively.
Group 8 (see pp 90) is represented by micrographs C and D, lower and upper surfaces respectively.
Group 9 (see pp 90) is represented by micrographs E and F, lower and upper surfaces respectively.

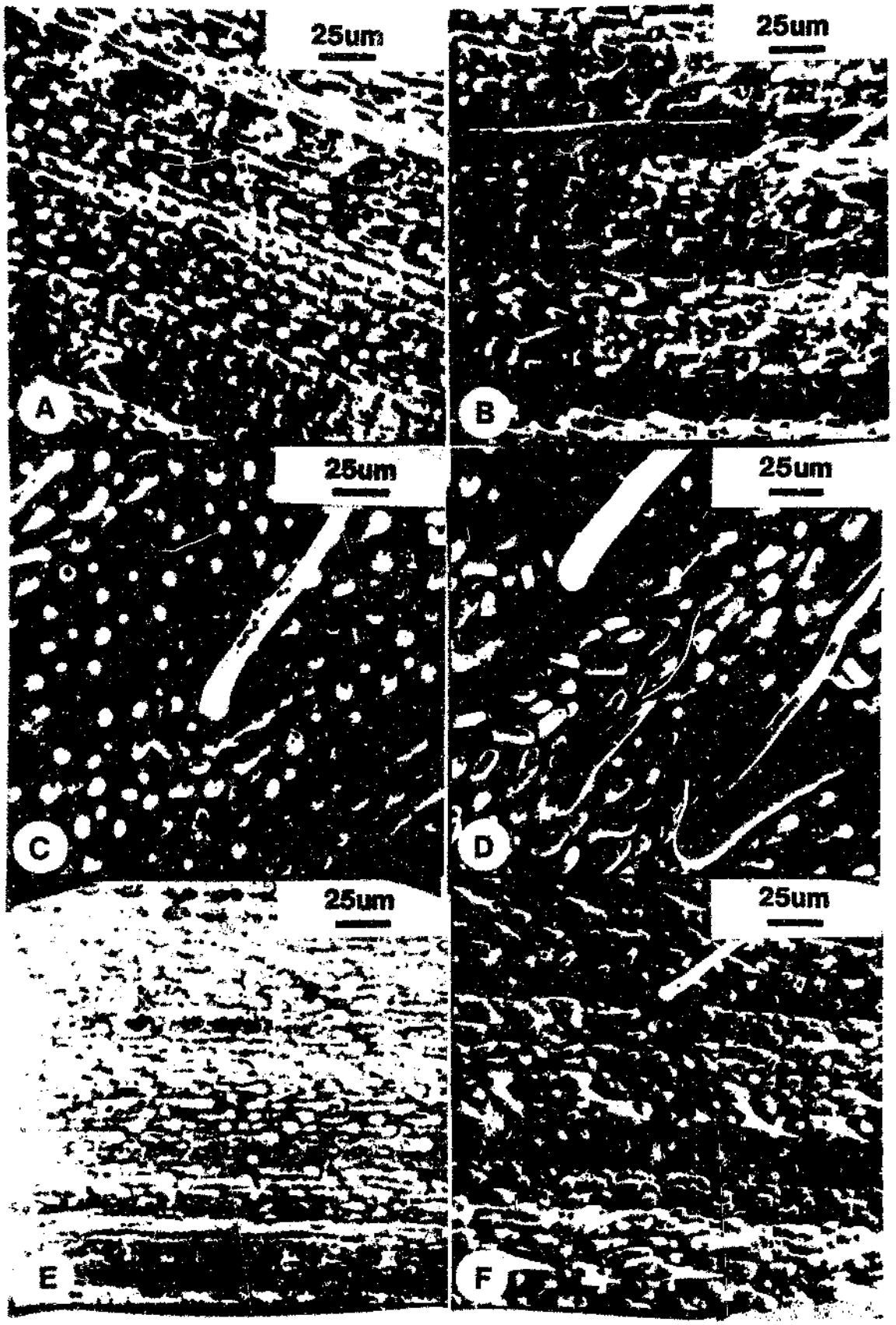
25um
A
25um
B
25um
C
25um
D
25um
E
25um
F

Plate 6.

Showing papilla-patterning on the leaf surfaces. Group 10 (see pp 90) is represented by micrographs A and B, lower and upper surfaces respectively.

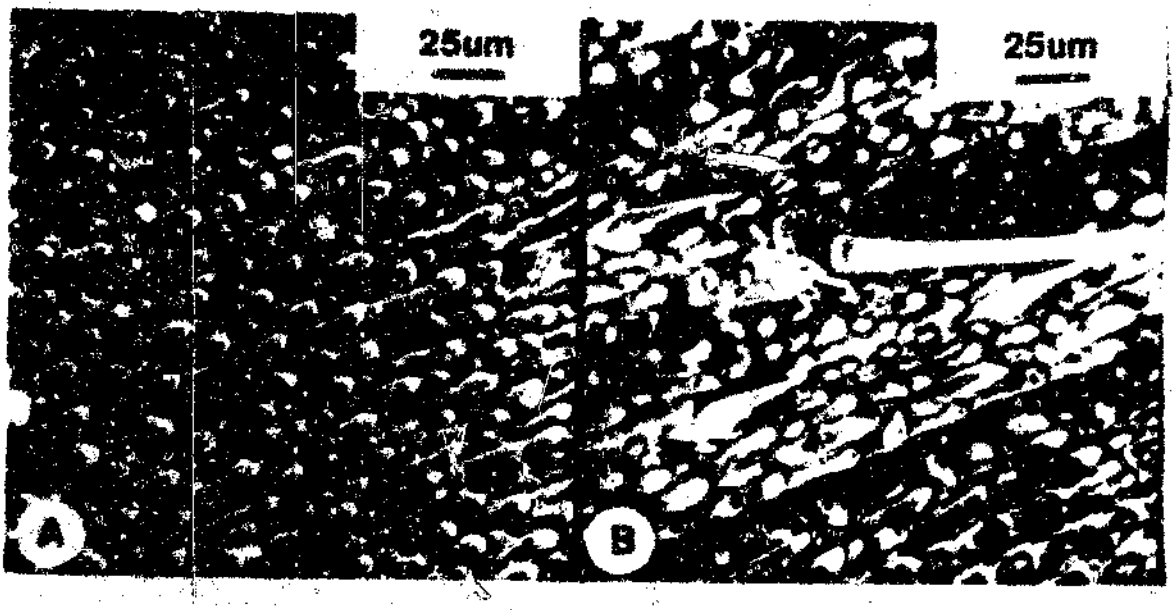
25um
25um
A
B

Plate 7.

Showing papillae arrangement around stomata and guard cells.
Group 1 having no papillae present around stomata and guard cells, micrograph A.
Group 2 having papillae surrounding stomata, but not overlapping the stomatal pore, micrographs B and E.
Group 3 having papillae surrounding and overlapping the stomatal pore and guard cells, but both are still clearly visible, micrographs C and F.
Group 4 having papillae overlapping the stomatal pore and guard cells, making identification difficult, micrograph D.


5um
A
10um
B
10um
C
10um
D
2um
E
2um
F

4.4.1.4 Epidermal cell wall structure.

The cell wall indentations of the epidermis were studied by examining the epidermal layer under the light microscope. The epidermal cells were found to have varying levels of indentations on their walls. These indentations were divided into five categories depending on the number of indentations found in the space of 10um. See Figure 18.

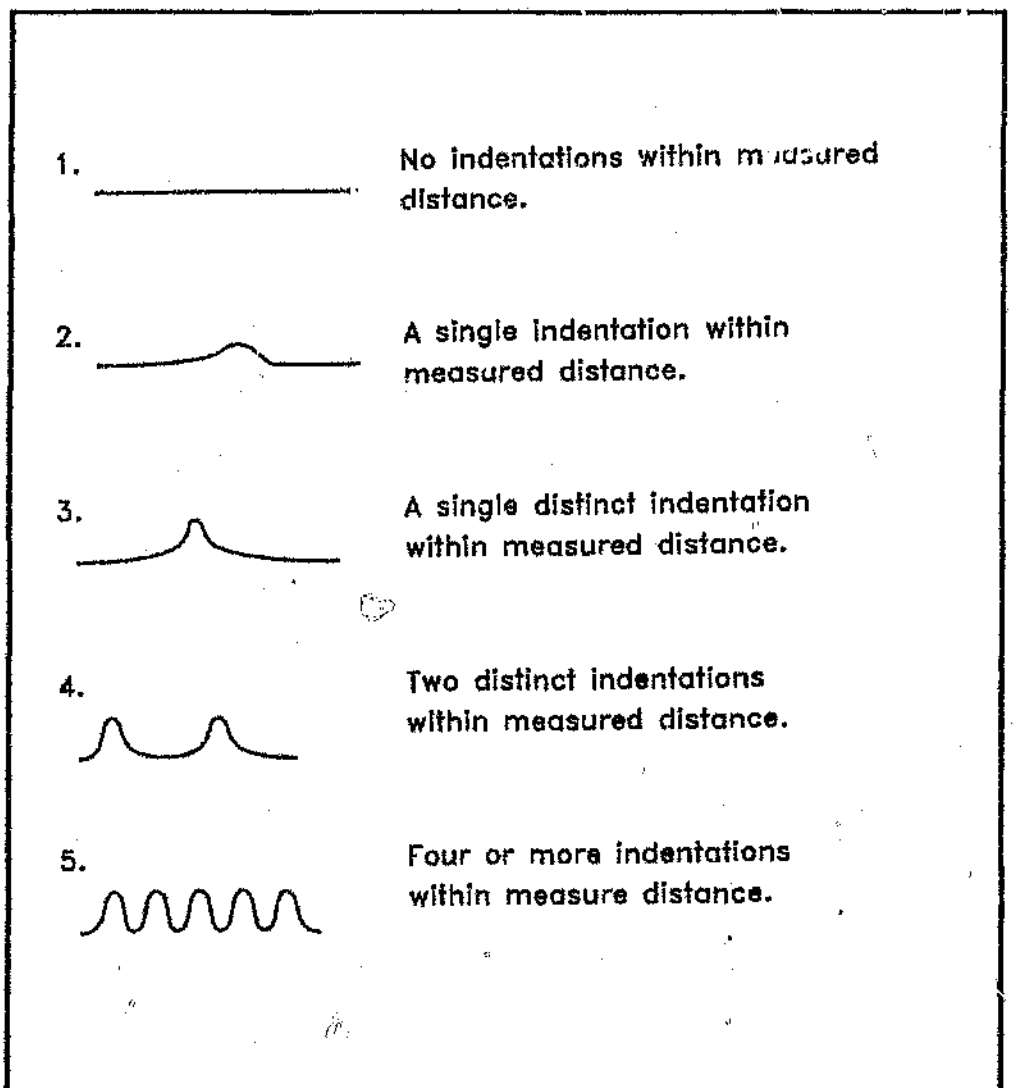

Figure 18 Showing the various wall indentations found in the epidermis studied by light microscopy.

4.4.1.5 Cuticle thickness.

The cuticle thickness was measured with a micrometer eyepiece. When cuticle was noticeably present it was measured in various places on the adaxial surface. Five measurements were made in each selection and the figures averaged. See Appendix 7.

4.4.1.6 Saltglands and hair types.

Neither of these characters was easily identified using the light microscope and could not be used here as a distinguishing character.

4.4.2 SCANNING ELECTRON MICROSCOPY.

Results are given for the following characters:

4.4.2.1 Papillae: presence and patterning.

Papillae on the leaf surfaces were found to exist in various patterns, and grouped as follows:-

Group 1 is characterised by a lack of papillae on both upper and lower surfaces.

Group 2 is characterised by a lack of papillae on the lower surface and papillae on the upper surface.

Groups 3 to 10 were found to have papillae on both surfaces. Here differences in the shape and the patterns formed by the papillae are compared.

Group 3 is characterised by papillae on the lower surface with no ribbing. The papillae are very rounded. On the upper surface both large and smaller rounded papillae were present. Spickle shaped hairs were present.

Group 4 Few papillae are found on the lower surface with an observably larger number on the upper surface and little or no ribbing.

Group 5 showed exactly the opposite situation to Group 4. That is few papillae on the upper leaf surface and considerably more on the lower with little or no ribbing.

Group 6 The papillae on both lower and upper surfaces lie very flat against the leaf surface, all sloping strongly towards the tip of the leaf.

Group 7 The papillae on both the lower and upper surfaces are smaller than those of Group 3 and have distinctive shapes. There is definite ribbing.

Group 8 The papillae are oval in shape compared to Group 3. Only one size was observed and distinct ribbing was characteristic.

Group 9 Two types of papillae exist on both leaf surfaces characterised by distinctive ribbing in each case. Two types of hairs are present, long and spickle.

Group 10 papillae present on the lower surface are characteristically H - shaped. Papillae on the upper surface show a definite ribbing pattern. Both long and spickle hairs are present.

Table 17. Designation of plant collections to papilla-patterning groups.

Group	Plant collection number.
1	17,20,25
2	1,12,23,32
3	3,5,10,13,18,19,20
4	2,11,15,35
5	8
6	4,14,16,21,27,28,31,34
7	6,26,29
8	3,22,30,33
9	7,24,25
10	9,10,36

4.4.2.2 Papillae: arrangement aroung stomata and Guard cells.

Four characteristic groups were found:-

Group 1 no papillae present around stomata and guard cells.

Group 2 papillae surrounding stomata but not overlapping the stomatal pore.

Group 3 papillae surronding and overlapping the stomatal pores and guard cells, but both still clearly visible.

Group 4 papillae overlapping the stomata and guard cells making identification difficult.

Table 18. Assignment of plant collections to papilla-arrangement around the stomata and Guard cells.

Group	Plant collection Number	
	Lower Surface	Upper Surface
1	17	1,8,12,17,23,32
2	3,6,8,11,14,15,18 19,24,28,36	10,11,14,18,19,20, 24,28,29,33,36
3	1,2,4,7,9,12,13,16, 21,23,27,34,35	2,4,7,9,13,21,27, 34
4	5,10,20,22,25,26,29 30,31,32,33	3,5,6,15,22,25,26, 30,31,35

4.4.2.3 Hair presence and types.

Two types of hairs were found on the leaf surfaces, both spickle and long hairs. In some plant collections hairs were found on one surface and not the other (see Table 19). Normally they were found more abundantly on the upper surface. The spickle hair was more commonly found than the long hair type on both the upper and lower surfaces (See Plate 1 & 2).

Table 19. Plant collection groupings based on hair type and presence.

Plant collection Number	Upper Leaf Surface		Lower Leaf Surface	
	Spickle hair	Long hair	Spickle hair	Long hair
1	A	A	A	A
2	P	P	A	A
3	P	A	A	A
4	P	P	P	A
5	P	A	P	A
6	P	A	A	P
7	P	P	A	A
8	P	A	A	A
9	P	P	A	A
10	P	A	A	A
11	A	P	A	A
12	A	A	A	A
13	P	A	P	A
14	P	A	A	A
15	A	P	A	A
16	P	P	A	A
17	A	A	A	A

Plant collection Number	Upper Leaf Surface		Lower Leaf Surface	
	Spickle hair	Long hair	Spickle hair	Long hair
18	P	A	A	A
19	P	A	A	A
20	P	A	A	A
21	P	P	A	A
22	P	A	A	A
23	A	A	A	A
24	A	A	A	A
25	P	P	A	P
26	P	P	A	A
27	P	P	P	P
28	P	P	A	A
29	P	P	P	A
30	P	P	A	A
31	P	P	A	A
32	P	P	A	A
33	P	P	A	A
34	P	A	P	A
35	P	A	A	A
36	P	P	A	A

Keys:- P = presence A = absence.

4.4.2.4 Salt glands.

It was virtually impossible to distinguish salt glands from the papillae. Thus salt glands were excluded from this study.

4.4.2.5 Cuticle.

Although cuticle presence was noted, this feature was observed more accurately in the light microscope studies and was excluded from the scanning electron microscope study.

The numbered results of the light and scanning electron microscopy studies were placed into a matrix and a Cluster Analysis test was executed. See Figure 19.

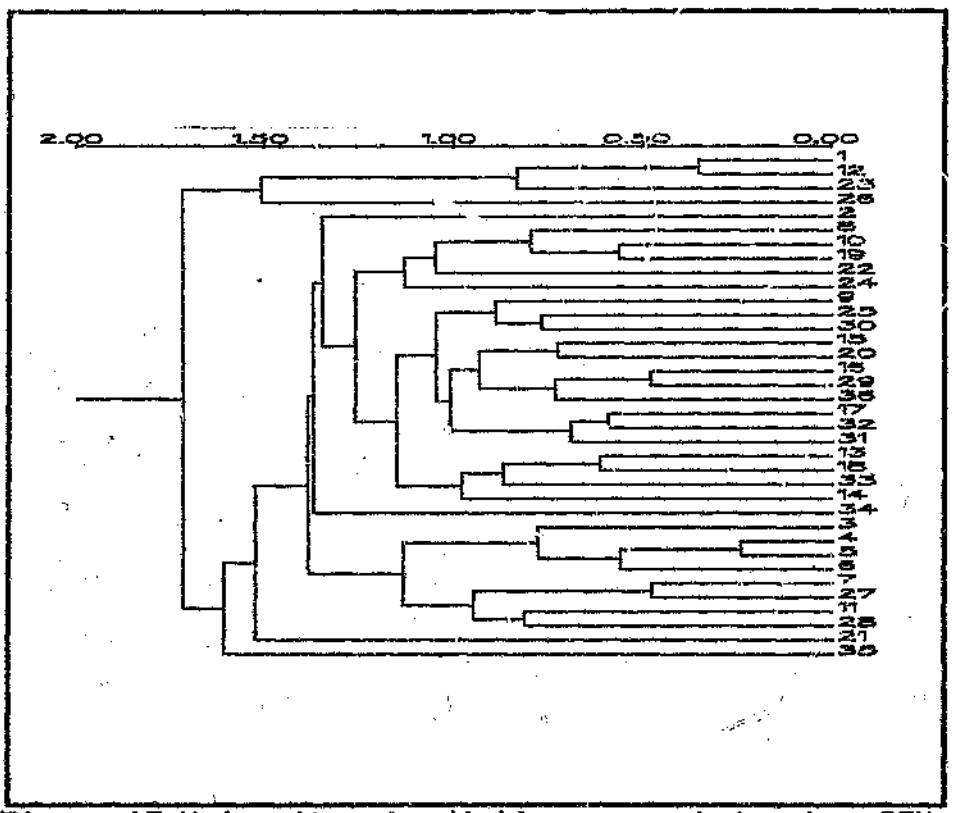

Figure 19 Using the claudistic approach to show OTU groupings.

Two main groupings were found, one large and the other consisting only of three OTU's. They were OTU's 1, 12 and 23.

4.5 DISCUSSION.

The characters th" were examined are listed in Table 20, with scoring fo ;iability.

Table 20. Morphological data.

	Light Microscopy	Scanning Electron Microscopy	Reliability	Used
1.	Leaf profile		***	Yes
2.	Vascular Bundle distribution		***	Yes
3.	Bundle sheath cell number		**	Yes
4.	Bulliform cell number		**	Yes
5.	Bulliform cell shape		*	No
6.	Epidermal cell wall structure		***	Yes
7.	Vascular bundle number		***	Yes
8.	Cuticle thickness		**	Yes

	Light Microscopy	Scanning Electron Microscopy	Reliability	Used
9.	Salt glands		*	No
10.	Hair types		*	No
11.		Papilla-presence	***	Yes
12.		Papilla-patterning	***	Yes
13.		Papilla-structure around stomata	***	Yes
14.		Hair presence	***	Yes
15.		Hair types	***	Yes
16.		Salt glands	*	No
17.		Cuticle presence	*	No

Key :- *** = Good repeatability
** = Fair repeatability
* = poor repeatability

4.5.1 LIGHT MICROSCOPY.

In the light microscopy section the following characters were found to be reliable, leaf profile, vascular bundle distribution, epidermal wall structure and vascular bundle number. These characters remained constant throughout the study. They were examined first using non-permanent frozen sections and later using permanent mounts.

It was concluded that these characters were constant and

not affected by environment especially as the first examination was carried out shortly after the plant collections were brought in for study. This examination was found to give the same results as the later study that was carried out once all the plants were subjected to the same environment for a number of months. No mention of these features were found in the literature for the use of classification in *Cynodon*. *C. dactylon* was however compared to other turf grasses examined by Stiff and Powell (1974). They noted the stem anatomy in 20 turf grass selections and found three distinguishable groupings. *Cynodon dactylon* was found to have multiple rings compared to single rings or no rings.

The following characters; number of bulliform cells, number of bundle sheath cells and cuticle thickness were rated fair in reliability. The reason for this was that there was some variation of cell number or cuticle thickness within the specified plant collections. Although this variation was not very noticeable, it was large enough to warrant a 'fair repeatability' classification. Replicates of these results were noted and averages were used (see Appendix 7, 8 and 9).

A specific region within the transverse leaf section was used when counting both the number of bulliform cells and the vascular bundle numbers. This was between two minor vascular bundles, see Figure 16. The same applied when counting the number of bundle sheath cells. These were counted only on a lateral main vein, see Figure 16.

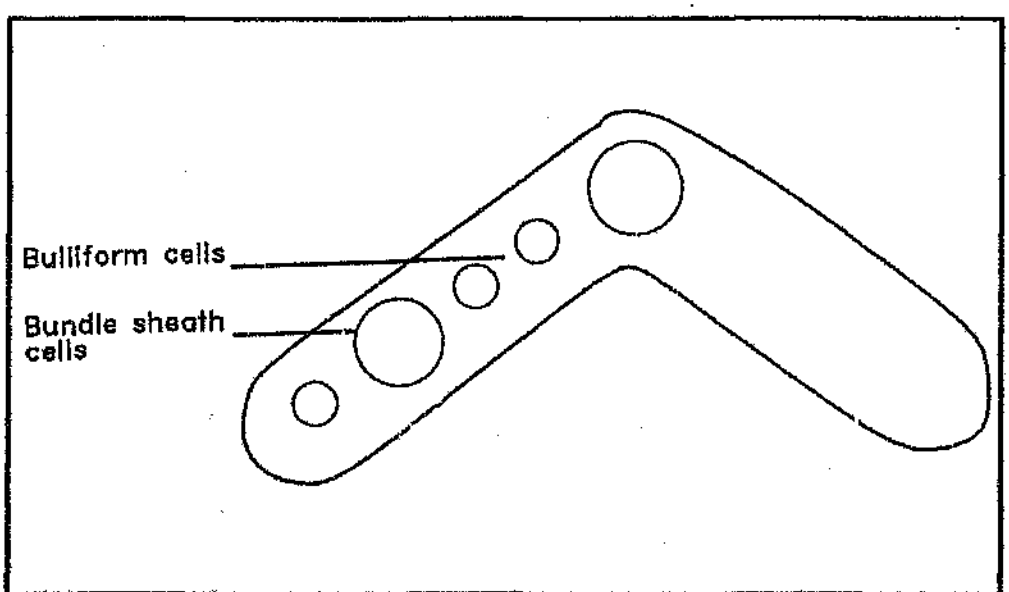

Figure 20 A diagrammmatic sketch of a hypothetical leaf showing where the bulliform and bundle sheath cells were counted.

The salt glands and hair types were given a poor reliability classification and were not used as characters in the light microscope study. This was because the salt glands were often difficult to identify and the hairs were often removed in the preparation of the section. Other authors have however examined salt glands in *C. dactylon* with success. Dross and Thompson (1982) identified salt glands using the scanning electron microscope, and in 1984 the same authors investigated the ultrastructure of salt glands in *C. dactylon* using the transmission electron microscope. Bulliform cell shape

microscope, and in 1984 the same authors investigated the ultrastructure of salt glands in *C. dactylon* using the transmission electron microscope. Bulliform cell shape was noted in all the plant collections. These were found to be consistent throughout. All were found to be conical in shape. As there were no differences this character was eliminated. o other reference to bulliform cells in *C. dactylon* was found in the literature.

4.5.2 SCANNING ELECTRON MICROSCOPY.

The SEM study was only carried out once in this project. That was after the plant collections were grown together for six months. The reason for this was chiefly expense and the time involved. In this study the following characters were given a good rating; presence of papillae, patterning of papillae, papilla structure and arrangement around stomata; pubescence and hair types. These characters were found to be clearly characterised.

The characters; saltglands and cuticle presence were not useful at the SEM level as the salt glands were difficult to discern. Once again they were often confused with papillae. Only cuticle presence could be examined in the SEM study, while the presence and thickness were already noted in the light microscope study.

The characters were then combined and placed in a matrix and run through the NTsys computer package. Two distinct groupings were found as in the electrophoretic section.

The first group consisting of OTU's 1, 12 and 23 all being *C. dactylon var. kweek* . The second consisting of the rest of the plant collections. This group is made up of smaller groupings or subgroups. Although these groups sometimes divide up the plant collections by name, for example, plant collections 13 and 18 are found in a subgroup and are both *C. dactylon var. kweek* . Other subgroups are not as accurate, for example plant collections 2, 7 and 28 form a subgroup but looking at their varieties they are totally different. Plant collection 2 is *C. dactylon var. kweek*. Plant collection 7 is *C. dactylon var. cape royal* and plant collection 28 is *C. dactylon var. skaaplaas fyn* . It can also be noted that plant collection 2 although similar in name is totally different to the smaller main group, that is plant collections 1, 12 ans 23. These are in turn different to other *C. dactylon var. kweek* varieties such as plant collections 8 and 15.

Thus from this study it can be noted, that in using the leaf morphology as a ranking characteristic some grouping of OTU'S are obtained, but these grouping are not sufficiently different to classify the cultivars down to subspecies level. To do that the morphological and anatomical data would have to be combined with the electrophoretic data.

CHAPTER 5

APPLIED AND CLASSICAL COMBINATION.

5.1 INTRODUCTION

In this project two approaches were utilized in order to classify various turf *Cynodon* selections. The first mentioned approach, the electrophoretic approach, was examined in conjunction with the chromosome counts results as far as possible. The chromosome counts did not include all the grass selections, but results in the five selections studied showed the chromosome number to be 2n = 18.

The morphology and microanatomical section covered both light and scanning electron microscopy on the *Cynodon* turf selections. The results of the electrophoretic and morphological and microanatomical sections were placed into a data matrix and a cluster analysis was carried out on the data, using the personal computer software NTsys. Each section was looked at separately and the results from the two sections were found to give adequate correlation when the analyses were compared. The results have been discussed in each section. To fulfil the aim of this project in the use of both applied and classical approaches the data from the two sections were combined and the same tests were carried out on the combined data matrix.

The reason then for carrying out the analyses on each section separately was to double check the results. It was important to see if the various selections gave fairly similar results in both the applied and classical approaches so that when the results were combined one of the sections would not cause bias. This feature would go unnoticed if the two sections were combined and not looked at separately first.

5.2 RESULTS

Data from the electrophoretic and morphology and microanatomical sections were combined and placed into a data matrix and a cluster analysis was produced, using the NTSYS computer package. Figure 21 shows the Cluster analysis results of the combined results.

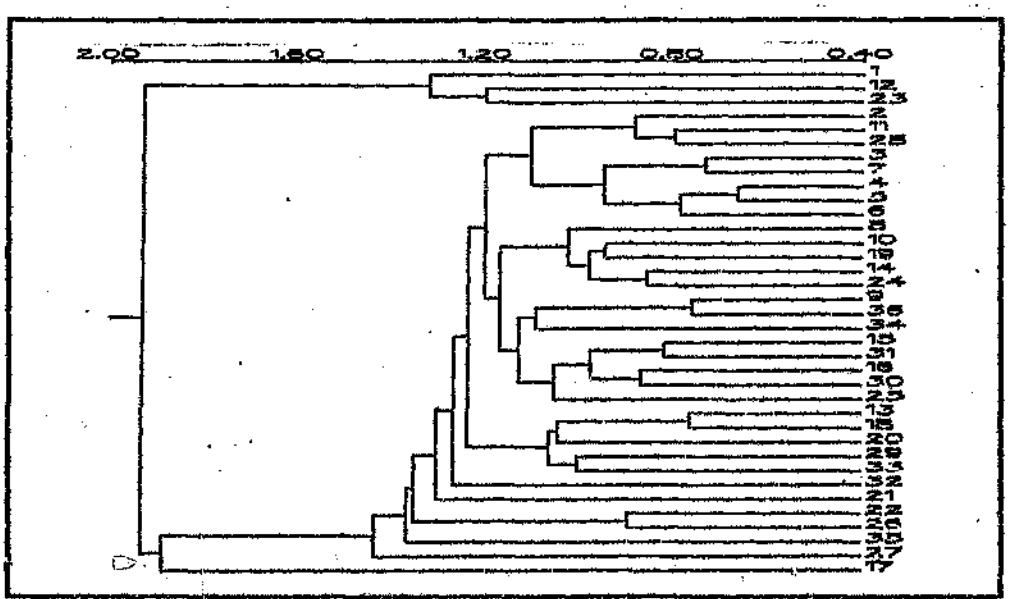

Figure 21 Cluster analysis results of OTU groupings, using the cladistic mode in NTSys.

A matrix comparison test was produced and found to give a correlation coefficient (r) of 0.9215. This number is high and suggests that combined, the raw data is well represented by the cluster analysis.

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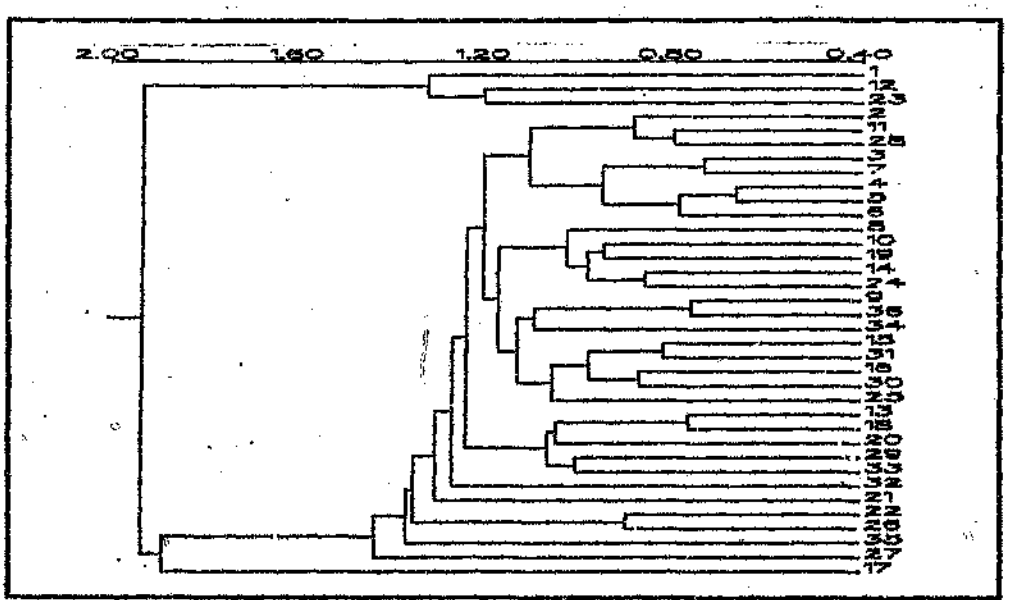

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Table 21. Showing OTU groupings using the Cluster Analysis Test.

Group	Sub-group	OTU Number
1	i	1,12,23
2	i	2,11,28
	ii	3,7
	iii	4,5,6
	iv	8,10,14,19,24
	v	9,36,34
	vi	15,16,25,30,31
	vii	13,18,20
	viii	29,33
	ix	22,26

OTU's 17, 27, 32 and 35 were found to remain separated and not to fall into any of the sub-groups. Ten distinct groups emerged as shown in Table 21. When comparing the combined cluster analysis to those performed for the electrophoretic and morphological and microanatomical sections, distinct groupings can be seen throughout. Two distinct groupings were found in all three cases, OTU's 1, 12 and 23 in a separate group and all the other OTU's in a larger group, which was subdivided into sub-groups. In the larger group similar sub-groups were noted for OTU's 3 and 6; 9 and 36; 22 and 26; 13, 18 and 20; 10 and

19; 2, 11, and 28. In all three instances certain OTU's in the large grouping, were found outside the sub-groups. Performing matrix correlation tests on the data it can be seen that all the rough data are well represented by Figures 21. Refer to Table 22 for the matrix correlation values.

Table 22. Showing matrix correlation values.

OTU('S) removed	Matrix correlation value (r^2)
none	0.89516
1	0.90146
12	0.89372
17	0.88898
23	0.90651
1,12,17 and 23	0.84972

5.3 DISCUSSION.

An extensive survey of the literature, two on line searches and constant surveillance of current literature, showed no publications dealing with turf *Cynodon* within the aims of this project. No reference was found to any electrophoretic work on *Cynodon* . This being so, no truly comparative discussion is possible with reference to the literature in the electrophoretic section.

A limited amount of literature was found, that dealt with the cytological and morphological and microanatomical sections of this project. Some comparative studies of *Cynodon dactylon* and other grasses was made by Stiff and Powell (1974), Akin and Burdick (1973) and Rogers *et al.* (1976). Stiff and Powell (1974) compared the stem anatomy of 20 selections of turfgrasses using light microscopy and found three types distinguishable. *Cynodon* was classified as having multiple rings, and was placed in this categorie. Akin and Burdick (1973) investigated warm season turfgrasses for differences in lignification sites and microanatomy. No major differences were found, and although some of the turfgrasses could be categorised the *Cynodons* were broadly grouped together with other turfgrasses. No divisions were made within the *Cynodon* genus. Rogers *et al.* (1976) used scanning electron microscopy to show differences in the tissue components of rhizomes and stolons of *Zoysia japonica* and *Cynodon dactylon* L. Here differences were found between *Zoysia japonica* and *Cynodon dactylon* , but once again no categorisation within the *Cynodon* genus itself.

A number of authors have studied the chromosome numbers of *Cynodon*. For example Brilman *et al.* (1982), Forbes and Burton (1962), Harlan *et al.* (1968), Harlan *et al.* (1970), Hurcomb (1945), Moffett and Hurcombe (1943), Oureckly (1963) and Tripathy *et al.* (1977). *Cynodon* was found to have a diploid number of 2n = 18, It was concluded by Harlan *et al.* (1970) that *Cynodon* is difficult to treat taxonomically and that no entirely satisfactory classification has yet been found. None of these studies however examined turf *Cynodon*. It thus appears that no previous work has been published on chromosome numbers in turf *Cynodon*.

In the cytogenetics section chromosomes were examined in a very limited number of grass selections. In the five plant collections that were studied, it was found that the chromosome number was a constant 2n = 18. This could give a general idea but nothing concrete. If this section were to be utilized to the maximum, a more thourough study of turf *Cynodon* genetics would have to be done. This could include chromosome counts, as well as chromosome morphology, for example by using banding stains such as Giemsa staining. This was not within the scope of this project however, but would be interesting for further study.

In the morphology and microanatomical section the number of characters used was once again limited by the aim of this project, which was to find a quick and simple means of characterising turf *Cynodon*. Features such as salt glands, together with electron diffraction analysis of x-rays would make an interesting study, but may be too specialised for mere characterisation of turf *Cynodon* varieties. Other features such as papillae may also be

too specialised for a study of this type.

In the electrophoretic section the usefulness of the enzymes should be investigated before use and only those of importance selected. It would be of further interest to discard those in this study that were given a rating of fair, and using only those with a good rating in the NTsys computer package, to see if the plant collections could be further subdivided than the two main groups that were classified in this study.

Using the personal computer software package NTsys definite patterns emerged separating the grass selections (called OTU's when using NTsys). The pattern that emerged when combining the results was the same as that of the separate results, where two main groups form, one large and one small one. The large group in all instances can be further divided into subgroupings of OTU's. The small group that forms is the same in all instances. It consisted of OTU's 1, 12 and 23, which were *Cynodon dactylon var. kweek* of which OTU's 1 and 12 were from Natal and OTU 23 from the Cape. The smaller subgroupings are not identical but have marked similarities in many instances, for example OTU's 3 and 7, being *C. dactylon* var. *bayview* and *C. dactylon var. cape royal* , are always found in a subgroup as are OTU's 4, 5 and 6, being *C. dactylon var. florida* , and var. *skaaplaas* respectively, and OTU's 10 and 19, being *C. dactylon var. duffy* , and var. *mandy* repectively, and OTU's 13 and 18.
The characters used in this study resulted in the separation of the grass selections into two main groupings one of which can be further divided into subgroups. In the scale of this project this is as far as the classification can be recommended. Any further

classification based on subgroups may not give the correct classification. This enables future workers to take a *Cynodon* turf variety, perform the necessary techniques and place the grass selections into one of the two groupings or to identify it exactly with one of the known varieties (that is one of those used in this project). In being able to do so the aim of this project is partially satisfied.

More techniques could be used to characterise a plant, not only the ones looked at in the scope of this project. Caution is advised though, in that although many more techniques may be available they are not all suitable for the turf researcher who will use such techniques. Many otherwise suitable techniques for characterisation of the turf *Cynodon* may be too time consuming or require equipment that is not available. The equipment used in this study is easily accessible, relatively cheap and the chemicals commercially available.

Sample size is of importance. In this study a homogenous sample was important in order to classify the turfgrasses. In many instances in this study small samples were collected from the place of origin, which could mean that a wide sample of that specific grass was not investigated. However, of the turf *Cynodons* that were examined (to the author's knowledge), none of the samples produced viable seeds and some did not produce any seed at all (they only reproduced vegetatively). In most cases the grass in question was removed from golf course greens where the grass was vegetatively derived from a small genetic pool, homogenous in origin. Taking these two factors into consideration, sampling as done in this work should cover all the grass types present in the selection

chosen.

Another problem that will have to be overcome is that of *Cynodon* turfgrass naming. This was found to be suspect in a number of cases. For example OTU's 1, 12 and 23, all *C. dactylon var. kweek* , were grouped in an entirely separate group to the rest of the OTU's and yet there were a number of *C. dactylon var. kweek* samples found in the larger OTU grouping.

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APPENDIX 1.

Directions for use:

1. Funginex. For ornamentals and cultivated lawns: 15ml per 10 000ml water.

2. Malathion 50. For ornamentals and cultivated plants: 15ml per 10 000ml water.

APPENDIX 2.

Formalin acetic alcohol.

70% ethyl alcohol	90ml
Glacial acetic acid	5ml
Formalin	5ml

(Johansen 1940).

APPENDIX 3.

Acetocarmine.

Glacial acetic acid	90ml
Distilled water	110ml

Heat the glacial acetic acid and the water to boiling point. Remove the solution from the heat and add 1g of certified carmin dye. Cool in an ice box and decant. Add a few drops of an aqueous solution of ferric acetate until the colour is that of dark wine red.
(Johansen 1940).

APPENDIX 4.

Tertiary butyl alcohol series.

Total % alcohol	50	70	85	95	100
Distilled water	50	30	15	-	-
95% Ethyl alcohol	40	50	50	45	-
Tertiary butyl alcohol	10	20	35	55	75
100% Ethyl alcohol	-	-	-	-	25

(Johansen 1940).

APPENDIX 5.

Gel and tray buffer recipes.

1. Lithium borate pH 8.3.

Tray buffer	Gel buffer
0.19 M boric acid	0.05 M tris
0.04 M LiOH	0.007 M citric acid
pH 8.3 with LiOH	pH 8.3 with citric acid

Gel buffer can be made up by diluting the tray buffer at a ratio 1 : 9 with distilled water. (Shields *et al.* 1983).

2. Tris citrate pH 7.2.

Tray buffer	Gel buffer
0.223 M tris	0.008 M tris

0.069 M citric acid	0.002 M citric acid
pH 7.2 with 5N HCl.	pH 7.2 with 5N HCl.

The gel buffer can be made up by diluting 35ml of the tray buffer to 1000ml of distilled water and pH 7.2 with 5N HCl (Soltis *et al.* 1983).

3. Tris EDTA borate pH 8.6.

Tray buffer	Gel buffer
0.18 M tris	0.045 M tris
0.004 M EDTA	0.001 M EDTA
0.1 M boric acid	0.025 M boric acid
pH 8.6 with boric acid.	pH 8.6 with boric acid.

The gel buffer can be made up by diluting 250ml of the tray buffer to 1000ml of distilled water and pH 8.6 with boric acid (Soltis *et al.* 1983).

4. Tris citrate pH 8.0 - 8.5.

Tray buffer	Gel buffer
0.135 M tris	0.009 M tris
0.032 M citric acid	0.002 M citric acid
pH 8.0 with 5N HCl.	pH 8.5 with 5N HCl.

The gel buffer can be made up by diluting 67ml of the tray buffer to 1000ml with distilled water and pH 8.5 with 5N HCl (Soltis *et al.* 1983).

5. Tris maleate pH 7.4.

Tray buffer	Gel buffer
0.1 M tris	0.005 M tris
0.1 M maleic acid	0.005 M maleic acid
0.01 M EDTA	0.0005 M EDTA
0.01 M $MgCl_2$	0.0005 M $MgCl_2$

pH 7.4 with NaOH	pH 7.4 with NaOH

The gel buffer can be made up by diluting the tray buffer 1 : 19 with distilled water and pH 7.4 with NaOH (Brown *et al.* 1978).

6. Histidine citrate pH 5.7.

Tray buffer	Gel buffer
0.065 M L- histidine	0.009 M L- histidine
0.02 M citric acid	0.003 M citric acid
pH 5.7 with citric acid	pH 5.7 with citric acid

The gel buffer can be made up by diluting the tray buffer 1 : 6 with distilled water and pH 5.7 with citric acid (Shields *et al.* 1983).

APPENDIX 6.

Enzyme stains.

1. Acid phosphatase (ACP).

100ml	0.05 - 0.2 M sodium acetate buffer (pH 5.0 with NaOH)
0.5 ml	1 M $MgCl_2$
5ml	acetone
100mg	α -napthyl acid phosphate
80mg	Fast Garnet GBC salt

Dissolve the α -napthyl acetate phosphate in the acetone. Store in the dark until bands appear (Soltis *et al.* 1983).

2. Alcohol dehydrogenase (ADH).

100ml	0.1 M tris (pH 8.0 with NaOH)
5ml	95% ethyl alcohol

20 mg	NAD
5mg	MTT
2mg	PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

3. Aldolase (ALD).

100ml	0.1 M tris (pH 8.0 with NaOH)
30mg	NAD
30mg	MTT
10mg	PMS
120U	Glyceraldehyde 3-phosphate dehydrogenase.

Store in the dark until the bands appear (Shields *et al.* 1983).

4. Catalase (CAT).

5ml	3% hydrogen peroxide
7ml	0.1 M phosphate buffer (pH 7.0)
78ml	distilled water

Incubate the gel slice in this solution at room temperature in the dark for 1 to 2 hours. Pour off the rinse and add the following solution, staining in the light until the bands appear.

50ml	0.09 M KI
50ml	distilled water

White bands will appear on the dark blue background. A couple of drops of glacial acetic acid will induce staining. (Soltis *et al.*1983).

5. Esterase (EST).

100ml	0.1 M phosphate buffer (pH 6.0)
5ml	acetone
40mg	α-napthyl acetate
40mg	β-napthyl acetate

100mg Fast Blue RR Salt

Dissolve the β-napthyl acetate in the acetone before adding to the staining tray. Store in the dark until bands appear (Soltis *et al.* 1983).

6. Glucose 6-phosphate dehydrogenase (G6PDH).

100ml 0.1 M tris (pH 7.5 with NaOH)
1ml 1 M $MgCl_2$
40mg Glucose 6-phosphate
15mg NADP
20mg MTT
4mg PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

7. Glutamate dehydrogenase (GDH).

100ml 0.1 M tris (pH 8.0 with NaOH)
20ml 1 M L-glutamic acid (pH 8.0 with NaOH)
20mg NAD
10mg MTT
2mg PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

8. Glutamate oxaloacetate transaminase (GOT).

100ml 1 M tris (pH 8.0 with NaOH).
100mg L-aspartic acid
100mg α-ketoglutaric acid
5mg pyridoxal 5-phosphate
100mg Fast Blue BB Salt

Store in the dark until the bands appear (Soltis *et al.* 1983).

9. Hexokinase (HEX).

100ml	0.1 M tris (pH 8.5 with NaOH)
5ml	1 M $MgCl_2$
90mg	glucose
40mg	EDTA
10mg	NADP
25mg	ATP
15mg	MTT
2mg	PMS
40U	Glucose 6-phosphate dehydrogenase

Store in the dark until the bands appear (Soltis *et al.* 1983).

10. Isocitrate dehydrogenase (IDH).

100ml	1 M tris (pH 7.5 - 8.0)
5ml	1M $MgCl_2$
100mg	isocitric acid
10mg	NADP
15mg	MTT
2mg	PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

11. Malate dehydrogenase (MDH).

90ml	1 M tris (pH 8.0 - 8.5 with NaOH).
10ml	2 M DL- malic acid (pH 8.0 with NaOH)
10mg	NAD
10mg	MTT
2mg	PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

12. Malic enzyme (ME).

90ml	1 M tris (pH 8.0 - 8.5 with NaOH)

10ml	2 M DL- malic acid (pH 8.0 with NaOH)
2ml	1 M $MgCl_2$
20mg	NADP
20mg	MTT
2mg	PMS

Store in the dark until the bands appear (Soltis *et al.* 1983).

13. Peroxidase (PER).

95ml	0.1 M sodium acetate (pH 5.0 with NaOH)
5ml	dimethyl formamide
2ml	0.1 M $CaCl_2$
2ml	3% hydrogen peroxide
65mg	3- amino 9- ethyl carbazole

Dissolve the 3- amino 9- ethyl carbazole in the 5ml dimethyl formamide before adding to the staining tray. Stain the gel in the dark until bands appear (Soltis *et al.* 1983).

14. Phosphoglucoisomerase (PGI).

100ml	0.1 M tris (pH 8.0 with NaOH)
1ml	1 M $MgCl_2$
30mg	fructose 6- phosphate
10mg	NAD
20mg	MTT
2mg	PMS
40U	glucose 6- phosphate degyhrogenase

Stain the gel in the dark until bands appear (Soltis *et al.* 1983).

15. Phosphoglucomutase (PGM).

100ml	0.1 M tris (pH 8.0 - 8.5 with NaOH)
2ml	1 M $MgCl_2$
50mg	glucose 1- phosphate

10mg	NAD
10mg	MTT
2mg	PMS
40U	glucose 1- phosphate dehydrogenase

Stain the gel in the dark until the bands appear (Soltis *et al.* 1983).

16. 6- Phosphogluconate dehydrogenase (6PGD).

100ml	0.1 M tris (pH 8.0 with NaOH)
2ml	1 M $MgCl_2$
40mg	6- phosphogluconic acid
10mg	NADP
10mg	MTT
2mg	PMS

Stain the gel in the dark until the bands appear (Soltis *et al.* 1983).

17. Shikimic dehydrogenase (SDH).

100ml	0.1 M tris (pH 8.5 with NaOH)
100mg	shikimic acid
10mg	NADP
20mg	MTT
2mg	PMS

Stain the gel in the dark until the bands appear (Soltis *et al.* 1983).

APPENDIX 7.

CUTICLE THICKNESS.

Plant collection Number	Measurem ent					Average
	1	2	3	4	5	
1	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	1.0	1.0	2.0	1.0	1.0
3	0.0	1.0	0.5	0.5	0.5	0.5
4	2.0	2.0	2.0	2.0	2.0	2.0
5	2.0	2.0	2.0	2.0	2.5	2.0
6	1.0	0.0	1.5	1.5	1.0	1.0
7	1.5	1.0	1.0	1.5	0.0	1.0
8	1.0	1.0	1.0	1.0	1.0	1.0
9	0.5	0.5	1.0	0.0	0.5	0.5
10	1.0	1.0	1.0	1.0	1.0	1.0
11	0.5	0.5	0.5	0.5	0.5	0.5
12	2.0	1.0	1.0	2.0	2.0	1.5
13	1.0	1.0	1.0	1.0	1.0	1.0
14	1.5	1.0	1.0	1.75	1.0	1.0
15	0.5	0.5	0.5	0.5	0.5	0.5
16	0.5	0.5	0.5	0.5	0.5	0.5
17	0.25	0.5	0.75	0.5	0.5	0.5
18	2.5	2.0	2.0	2.0	2.0	2.0
19	2.0	2.0	2.0	2.0	2.0	2.0

Plant collection Number	Measurem ent					Average
20	2.5	2.0	2.0	2.0	1.5	2.0
21	1.5	1.5	1.5	1.5	1.5	1.5
22	2.0	2.5	2.0	2.0	1.5	2.0
23	2.0	2.0	2.0	2.0	2.0	2.0
24	1.0	0.5	0.5	0.5	0.5	0.5
25	0.5	0.5	0.5	0.5	0.5	0.5
26	2.0	2.0	2.0	2.0	2.0	2.0
27	0.0	0.0	0.0	0.0	0.0	0.0
28	1.0	1.0	1.0	1.0	1.0	1.0
29	1.5	2.0	2.0	2.5	1.0	2.0
30	2.0	2.0	2.0	2.0	2.0	2.0
31	1.0	0.5	0.5	0.5	0.5	0.5
32	1.0	1.0	1.0	1.0	1.0	1.0
33	2.5	1.5	2.0	2.5	1.5	2.0
34	0.0	0.0	0.0	0.0	0.0	0.0
35	2.0	2.0	2.0	2.0	2.0	2.0
36	0.0	0.0	0.0	0.0	0.0	0.0

APPENDIX 8.

BUNDLE SHEATH NUMBER.

Plant collection Number	Count					Average
	1	2	3	4	5	
1	12	12	12	12	12	12.0
2	8	9	8	8	9	8.5
3	8	8	8	8	8	8.0
4	9	9	8	8	8	8.5
5	7	7	6	7	7	7.0
6	8	9	9	8	8	8.5
7	9	8	9	9	8	8.5
8	8	8	9	8	9	8.5
9	6	6	6	7	7	6.5
10	9	9	9	9	9	9.0
11	8	8	9	9	8	8.5
12	7	8	9	8	7	8.0
13	7	7	8	9	8	8.0
14	10	10	9	9	9	9.5
15	7	7	7	7	7	7.0
16	9	9	9	9	9	9.0
17	7	7	6	7	6	6.5

18	10	10	10	10	10	10.0
19	6	6	6	6	6	6.0
20	8	8	8	8	8	8.0
21	9	11	9	11	10	10.0
22	8	7	8	7	7	7.5
23	14	14	14	14	14	14.0
24	9	10	9	9	10	9.5
25	10	10	10	10	10	10.0
26	8	8	8	8	8	8.0
27	8	8	8	9	9	8.5
28	8	8	8	8	8	8.0
29	7	7	7	7	7	7.0
30	8	10	8	9	10	9.0
31	6	6	6	6	6	6.0
32	7	7	7	7	7	7.0
33	9	9	9	9	9	9.0
34	8	8	8	8	8	8.0
35	11	11	11	11	11	11.0
36	5	6	6	5	6	5.5

APPENDIX 9.

BULLIFORM CELL NUMBER.

Plant collection Number	Count					Average
	1	2	3	4	5	
1	6	6	6	6	6	6.0
2	7	7	7	6	6	6.5
3	6	6	6	6	6	6.0
4	6	6	6	6	6	6.0
5	6	6	6	6	6	6.0
6	6	6	6	6	6	6.0
7	6	7	7	7	6	6.5
8	4	4	4	4	4	4.0
9	6	6	6	6	6	6.0
10	7	4	7	5	5	6.5
11	5	5	5	5	5	5.0
12	4	6	5	5	5	5.0
13	5	4	3	4	4	4.0
14	5	5	5	5	5	5.0
15	5	5	5	5	5	5.0
16	5	5	5	5	5	5.0
17	6	6	6	6	6	6.0

Plant collection Number	Count					Average
18	4	4	3	4	5	4.0
19	5	5	4	5	6	5.0
20	5	5	5	5	5	5.0
21	5	5	5	5	5	5.0
22	6	5	5	6	5	5.5
23	4	4	4	4	4	4.0
24	6	5	6	3	3	4.5
25	4	4	4	4	4	4.0
26	5	6	6	5	5	5.5
27	6	6	6	6	6	6.0
28	6	6	6	6	6	6.0
29	3	3	3	3	3	3.0
30	5	5	5	5	5	5.0
31	3	3	3	3	3	3.0
32	6	6	6	6	6	6.0
33	6	6	6	6	6	6.0
34	6	6	6	6	6	6.0
35	6	6	6	6	6	6.0
36	6	6	6	6	6	6.0

APPENDIX 10.

EXTRACTION BUFFER RECIPES.

1. Tris - HCl grinding buffer.
   0.001 M EDTA
   0.01 M KCl
   0.01 M $MgCl_2$
   40% weight : volume PVP 40
   25ml 0.1 M Tris pH 7.5 (with HCl)

Stir into solution or allow to hydrate overnight. pH the solution with HCl. This solution can be kept in the cold room for 3 - 4 weeks.
Just before use, add 1% volume : volume 2-mercaptoethanol.
(Soltis *et al.* 1983).

2. Phosphate grinding buffer.
   0.029 M sodium tetraborate
   0.017 M sodium metabisulphate
   0.2 M L- ascorbic acid sodium salt
   0.016 M diethyldithiocarbamic acid sodium salt
   25ml 0.1 M phosphate buffer pH 7.5

Stir into solution or allow to hydrate overnight. pH the solution with HCl. This solution can be kept in the cold room for 3 - 4 weeks. Just before use, add 1% volume :

volume 2- mercaptoethanol.
(Soltis *et al.* 1983).

3. Phosphate grinding buffer II.

0.00274 M sodium metabisulphate
0.00584 M diethyldithiocarbamic acid sodium salt
0.00337 M dithiothreitol
0.005 M ascorbic acid
0.0423 M Na_2HPO_4
0.21 M sucrose
0.00155 M EDTA sodium salt
5% weight : volume PVP 40
100ml distilled water

Stir into solution or allow to hydrate overnight. pH the solution with NaOH. Just before use, add 1% volume : volume 2- mercaptoethanol.
(Soltis *et al.* 1983).

4. Tris - HCl grinding buffer.

0.1 M tris pH 7.5 (with HCl)
0.01 M KCl
0.001 M $MgCl_2$
0.01 M EDTA
0.1 M ascorbic acid
20% weight : volume PVP 40

Stir into solution. pH the solution and just before use add 1% volume : volume 2- mercaptoethanol. (Shields *et*

al. 1983).

5. Tris maleate grinding buffer.

0.2 M sodium tetraborate
0.02 M sodium metabisulphate
0.25 M L- ascorbic acid sodium salt
0.026 M diethyldithiocarbamic acid sodium salt
0.1 M maleic acid
0.1 M tris
4% weight : volume PVP 40

Stir into solution or allow to hydrate overnight. pH the solution with HCl. This solution can be kept in the cold room for 3 - 4 weeks. Just before use, add 0.1% volume : volume mercaptoethanol. (Soltis *et al.* 1983).

APPENDIX 11.

RAW DATA MATRIX

Plant collection Number	1	2	3	4	5	6	7	8	9	10	11	12
1	0.0	1.0	0.5	2.0	2.0	1.0	1.0	1.0	0.5	1.0	0.5	1.5
2	3	3	3	3	3	3	3	3	3	3	3	5
3	6	0	0	0	0	0	0	0	0	0	0	0
4	10	8	8	8	7	6	8	8	6	12	8	20
5	12.	8.5	8.0	8.5	7.0	8.5	8.5	8.5	6.5	9.0	8.5	9.0
6	6.0	6.5	6.0	6.0	6.0	6.0	6.5	4.0	6.0	5.5	5.0	5.0
7	1	1	1	1	1	1	1	1	1	1	1	1
8	0	1	1	1	1	1	1	0	1	1	1	0
9	2	4	8	6	7	9	5	10	10	4	2	3
10	3	1	2	1	2	1	2	1	2	2	1	3
11	0	1	2	1	3	3	1	0	2	1	1	0
12	3	3	2	3	4	2	3	2	3	4	2	3
13	1	3	4	3	4	4	3	1	3	2	2	1
14	0	1	1	1	1	1	1	1	1	1	1	0
15	0	0	0	1	1	1	0	0	0	0	1	0
16	0	3	1	3	1	1	3	1	3	1	2	0
17	0	0	0	1	1	2	0	0	0	0	0	0
18	3	4	3	2	2	3	3	4	13	5	4	5

Plant collection Number	1	2	3	4	5	6	7	8	9	10	11	12
19	1	1	2	3	3	1	1	1	4	2	1	3
20	0	1	1	1	2	3	1	4	3	5	1	0
21	5	3	1	1	2	1	2	7	6	3	1	5
22	1	3	1	1	1	1	1	1	1	2	2	1
23	7	8	1	1	2	3	2	5	16	9	4	10
24	4	5	1	1	2	2	3	2	2	2	4	4
25	2	2	1	1	1	1	1	1	2	1	2	2
26	6	1	1	2	2	1	4	1	1	1	4	6
27	6	2	1	2	2	3	4	2	3	2	5	6
28	9	4	1	2	3	1	4	8	16	10	5	11
29	1	1	1	2	2	2	1	2	2	3	2	1
30	1	5	1	1	1	2	2	2	1	2	3	3
31	3	2	1	2	2	3	1	2	2	2	2	3

RAW DATA MATRIX

Plant collection Number	13	14	15	16	17	18	19	20	21	22	23	24
1	1.0	1.0	0.5	0.5	0.5	2.0	2.0	2.0	1.5	2.0	2.0	0.5
2	3	3	3	3	3	3	3	3	3	3	5	3
3	0	0	0	0	0	0	0	0	0	0	7	0

Plant collection Number	13	14	15	16	17	18	19	20	21	22	23	24
4	8	8	6	9	6	10	9	6	10	8	15	8
5	8	9.5	7.0	9.0	6.5	10	6.0	6	10	7.5	14	7.5
6	4	5	5.0	5.0	6.0	4.0	5.0	5.0	5.0	5.5	4.0	4.5
7	1	1	1	1	0	1	1	1	1	1	1	1
8	1	1	1	1	0	1	1	1	1	1	0	1
9	3	6	4	6	1	3	3	3	6	8	2	9
10	1	2	1	2	0	1	2	3	1	3	3	2
11	3	2	2	3	0	2	3	3	2	3	0	2
12	3	2	2	3	1	2	2	4	3	4	3	2
13	4	2	4	3	1	2	2	2	3	4	0	2
14	1	1	1	1	0	1	1	1	1	1	0	1
15	1	0	0	0	0	0	0	0	1	0	0	0
16	1	1	2	3	0	1	1	1	3	1	0	1
17	1	0	0	0	0	0	0	0	2	0	0	0
18	5	4	2	4	3	4	3	3	4	4	4	4
19	5	1	1	4	4	5	1	5	3	5	5	1
20	1	3	1	8	3	1	5	3	7	1	0	4
21	6	10	6	6	6	10	10	11	1	6	9	6
22	1	1	1	3	1	1	2	1	2	4	1	2
23	11	17	16	14	1	12	13	17	14	15	18	16

22	1	1	1	3	1	1	2	1	2	4	1	2
23	11	17	16	14	1	12	13	17	14	15	18	16
24	4	1	6	6	5	4	2	6	5	2	5	2
25	2	2	1	1	1	2	1	1	2	2	2	1
26	1	1	1	1	1	1	1	1	3	1	6	5
27	2	2	2	2	3	2	2	2	2	3	6	3
28	12	17	18	10	10	8	8	19	4	13	20	14
29	1	2	2	1	3	1	3	2	4	2	1	1
30	5	7	5	3	2	5	2	8	3	2	5	2
31	1	1	2	2	1	2	2	1	4	2	4	2

RAW DATA MATRIX

Plant collection Number	25	26	27	28	29	30	31	32	33	34	35	36
1	0.5	2.0	0.0	1.0	2.0	2.0	0.5	1.0	2.0	0.0	2.0	0.0
2	3	3	4	3	3	3	3	3	3	3	3	3
3	0	0	0	0	0	0	0	0	0	0	0	0
4	9	6	14	6	6	6	6	6	28	10	9	7
5	10	8.0	8.5	8.0	7.0	7.0	6.0	7.0	9.0	8.0	11	5.5
6	4.0	3.5	6.0	4.0	3.0	3.0	3.0	4.0	4.0	4.0	4.0	4.0

Plant collection Number	25	26	27	28	29	30	31	32	33	34	35	36
9	9	7	6	6	7	8	6	2	8	6	4	10
10	2	3	2	1	3	2	2	2	2	2	1	3
11	2	3	1	1	1	2	2	0	2	2	1	1
12	4	4	3	2	4	4	4	4	4	3	3	2
13	4	4	3	2	2	4	4	1	2	3	4	2
14	1	1	1	1	1	1	1	1	1	1	1	1
15	1	0	1	0	1	0	0	0	1	1	0	0
16	3	3	3	3	3	3	3	3	3	1	1	3
17	2	0	3	0	1	0	0	0	2	1	0	0
18	2	3	3	4	4	4	2	4	3	1	4	1
19	1	5	0	4	4	1	1	5	5	1	1	5
20	8	3	9	3	3	3	3	3	1	6	8	3
21	6	4	3	1	6	4	5	7	6	4	8	9
22	1	5	1	3	1	1	1	1	3	1	3	1
23	16	19	5	1	16	6	16	6	21	20	22	23
24	6	5	5	6	5	5	6	6	6	4	3	5
25	2	2	1	2	1	2	1	1	2	1	1	1
26	1	4	4	5	2	2	1	1	3	1	1	1
27	2	6	3	3	2	2	3	2	4	7	4	3
28	15	21	6	7	8	8	14	13	6	4	22	8

Plant collection Number	25	26	27	28	29	30	31	32	33	34	35	36
27	2	6	3	3	2	2	3	2	4	7	4	3
28	15	21	6	7	8	8	14	13	8	4	22	8
29	2	1	1	1	1	2	3	3	1	1	3	1
30	1	1	3	4	3	2	6	2	9	2	9	2
31	1	4	1	2	1	2	1	2	1	2	1	1

In the table columns = the plant collection number and rows = data examined, see key below.

KEY: No. 1 = cuticle thickness, 2 = number of large vascular bundles, 3 = number of medium vascular bundles, 4 = number of small vascular bundles, 5 = average number of bundle sheath cell around main vein, 6 = average number of bulliform cells, 7 = papillae presence or absence on upper surface, 8 = papillae presence or absence on lower surface, 9 = papillae patterning, 10 = papille density on upper surface, 11 = papillae density on lower surface, 12 = papilla structure around stomata and guard cells on upper leaf surface, 13 = papilla structure around stomata and guard cells on lower surface, 14 = presence or absence of hairs on upper surface, 15 = presence or absence of hairs on lower surface, 16 = hair types on upper surface, 17 = hair types on lower surface, 18 = epidermal cell wall structure, 19 = leaf profile, 20 = vascular bundle distribution, 21 = ACP patterning, 22 = ADH patterning, 23 = EST patterning, 24 = GDH patterning, 25 = IDH patterning, 26 = MDH patterning, 27 = ME patterming, 28 = PER patterning, 29 = 6-PGD patterning, 30 = PGI

patterning and 31 = SDH patterning.

C
A
D
B

Group 1

1 

V – shaped with straight arm ends and definite midrib.

2 

Wide V – shaped with straight arm ends and definite midrib.

3 

V – shaped with curled arm ends and definite midrib.

Group 2

4 

U – shaped, no definite midrib and straight arm ends.

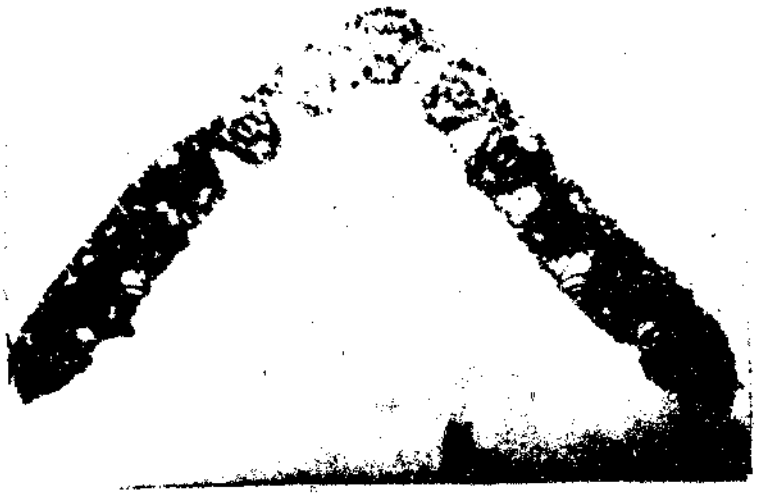
5 

U – shaped, no definite midrib and curling arm ends.

6 

Straight leaf, no definite midrib and straight leaf arm ends.

1



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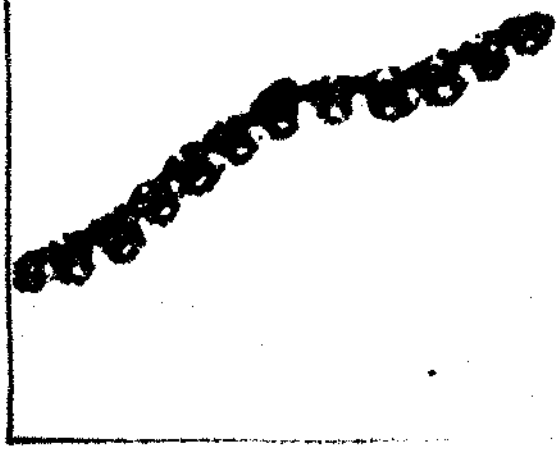


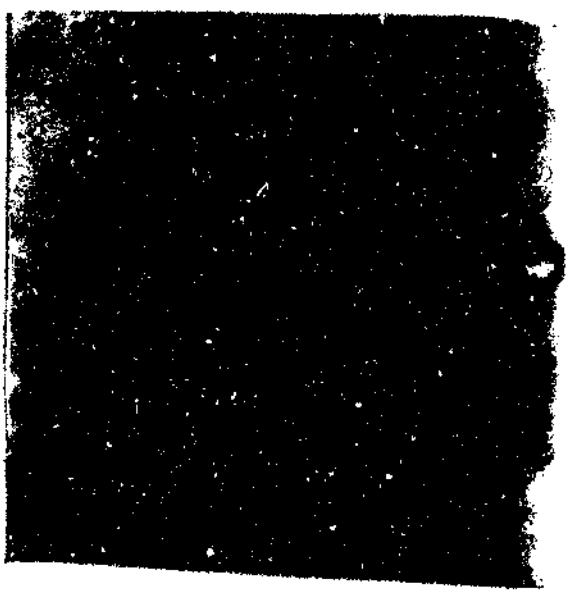
3



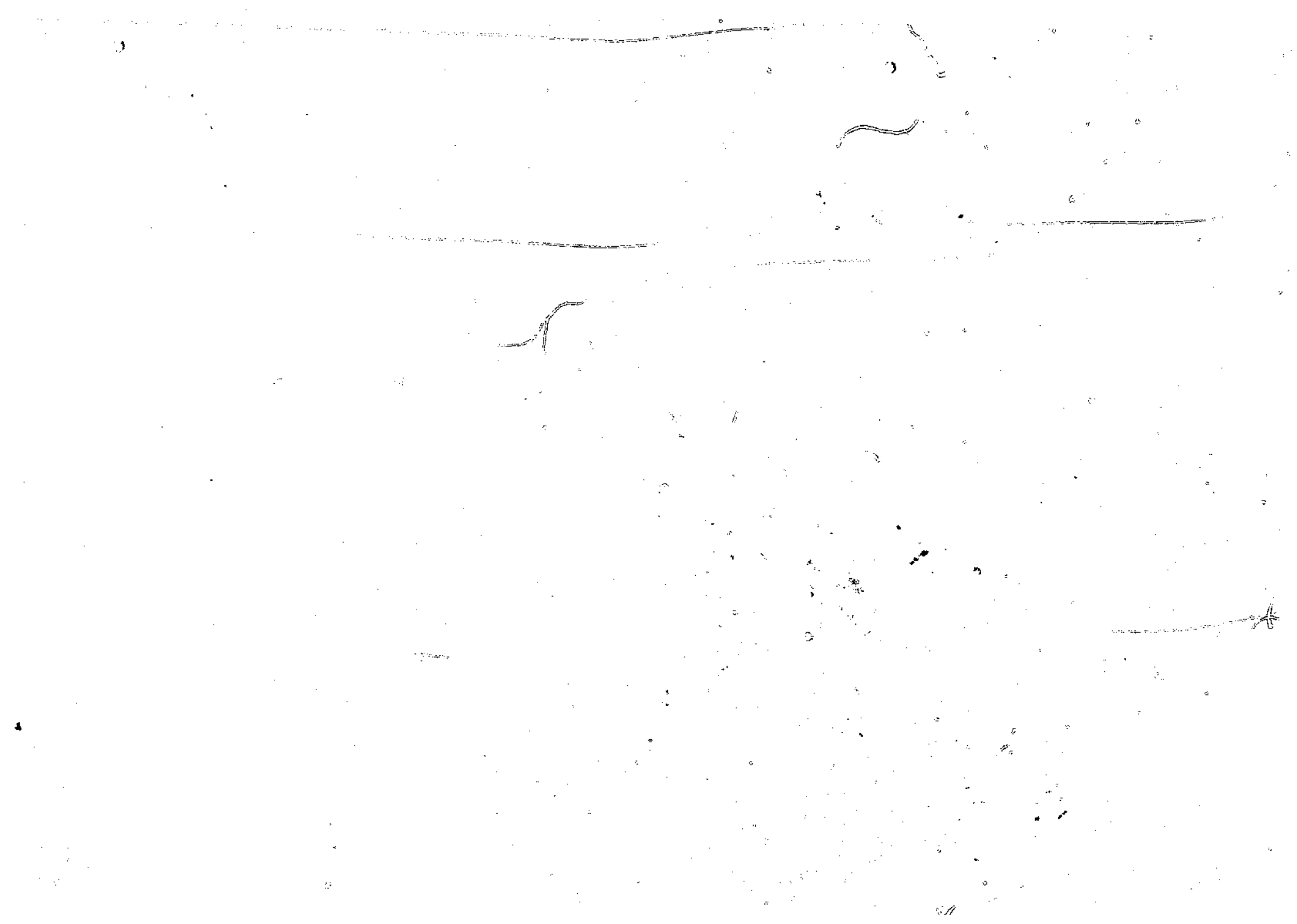


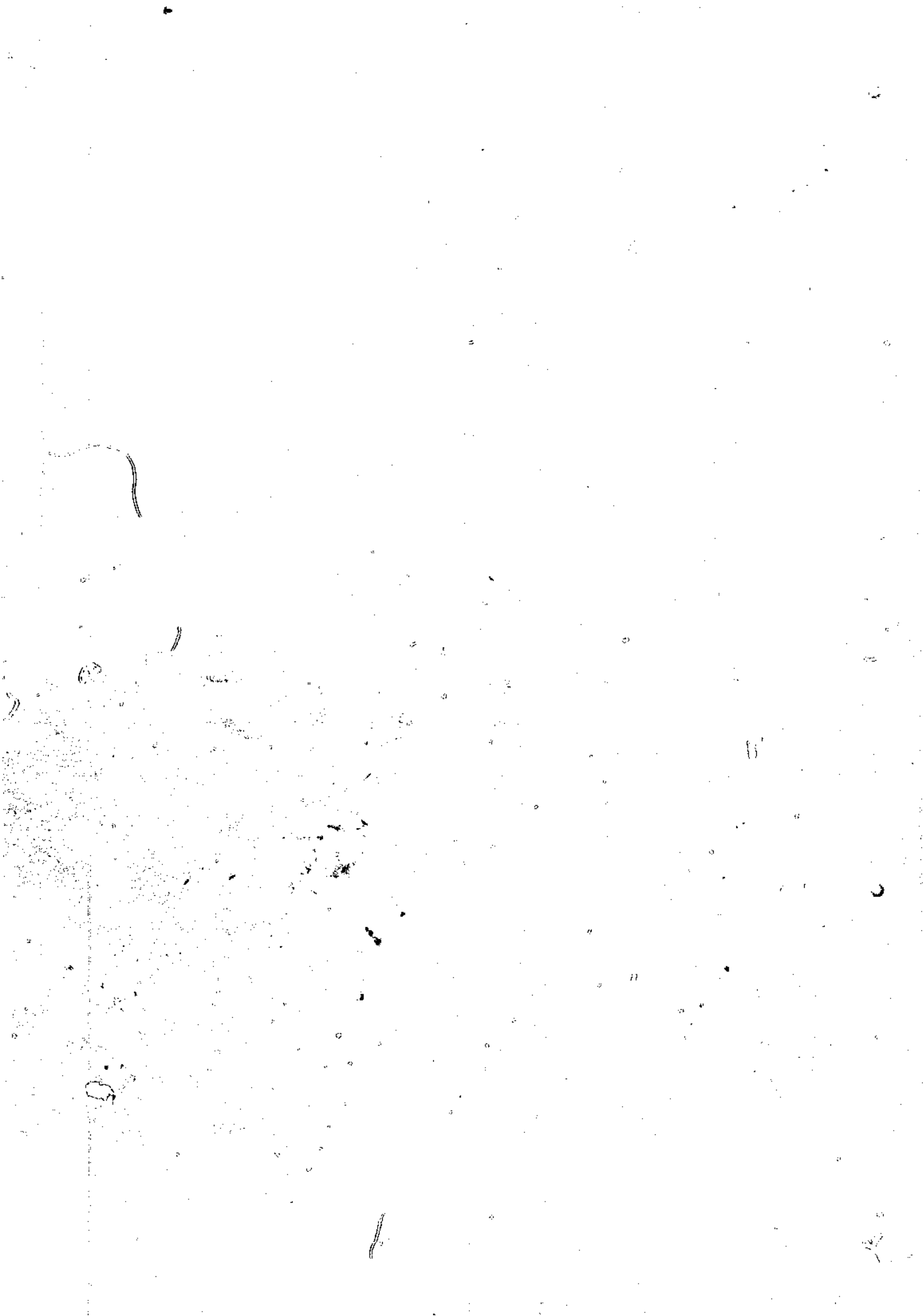
5











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