CHAPTER FIVE

LOCAL POPULATION DIFFERENCES IN *Microlaena stipoides*

5.1 GENERAL INTRODUCTION

High intraspecific variability has been observed in many species having populations that diverge spatially into various ecotypes, each uniquely adapted to a local microenvironment (Baker 1974). Different populations within a species are exposed to different aspects of the natural environment and when these effects are accumulated over a long period (Bradshaw 1984) ecotypic differentiation occurs, which is very common among plant populations (Antonovics 1976). The ecological amplitude of a species is increased if it has the ability to evolve populations adapted to different environments (Bradshaw 1984). The importance of the genotype as a functional unit of community variation has been expressed by Aarssen and Turkington (1985a). The interplay of genetic diversity and phenotypic plasticity is an important mechanism by which a population adjusts to its environment (Turkington 1989b).

There is a vast amount of literature documenting genotypic variation within species or populations in response to various abiotic environmental factors. For example, genetic specialisation of different species with respect to habitat irradiance has been observed (Gauhl 1976, Ludlow 1981, Bookman and Mack 1983, Kuiper and Smid 1985, Skálová and Krahulec 1992). Genotypic variation for many root characteristics of crop plants (Finn and Mack 1964, Mack and Finn 1970, Harris and Goebel 1976, O'Toole and Bland 1987) has been reported in response to water stress. Genetic variation in stomatal frequency and stomatal length (Wilson 1971, Walton 1974) as well as osmotic adjustment has been recorded in many crop species. Water requirements were found to vary significantly among different genotypes of *Dactylis glomerata* (Briggs and Shantz 1913, Keller 1953, Hunt 1962, Wright and Dobrenz 1970, Guobin and Kemp 1992).

The general aim of this chapter was to assess ecotypic and genotypic differences among four *M. stipoides* populations. Two experiments were conducted to determine ecotypic differences in the performance of four *M. stipoides* populations when grown under varying levels of two physical environmental factors, light intensity and

water stress. Genetic analyses were performed to examine variations in the genomic DNA sequences of the four *M. stipoides* populations.

5.2 GENERAL MATERIALS AND METHODS

5.2.1 Description of the sites where the four *M. stipoides* populations had been collected

Three of the populations were collected from the permanent pasture at 'Karuah', which was described in Chp. 4.2.1. The fourth population was collected from another permanent pasture grazed by cattle and sheep at 'Powalgarh', about 40 km north of Armidale. This property has an elevation of about 1330 m above sea level and is in the eastern part of the Northern Tablelands of New South Wales. The slope of the area is gentle, varying from 1 to 3%, and no appreciable erosion was observed in the area. Median rainfall is 75-100 mm in January, 50 mm in April and July, and 75 mm in October (Lea *et al.* 1977a). Mean daily minimum and maximum temperatures are 12° C and 28° C in January, 6° C and 20° C in April, 0° C and 12° C in July and 6° C and 22° C in October (Lea *et al.* 1977a).

The plants from 'Powalgarh' were collected from a chocolate soil. These chocolate soils, although shallow and frequently stoney are well suited to pasture production since they are usually fertile with a nearly neutral soil pH. Organic matter contents and nutrient elements are moderate and the available soil moisture range is broad (Lea *et al.* 1977b).

The sampling site was in an 8.1 ha paddock which had been cultivated and broadcast with *Phalaris aquatica* (3.37 kg/ha), *Lolium perenne* (1.12 kg/ha) and *Trifolium repens* (1.12 kg/ha) in 1960. Superphosphate fertiliser was applied annually to the pasture (125 kg/ha) from 1960 to 1980. A cluster of several trees of *Eucalyptus viminalis* subsp. *viminalis* and *E. obliqua* was situated on the north-western part of the paddock.

5.2.2 Planting materials

5.2.2.1 Propagation and multiplication of plants of four Microlaena stipoides *populations and associated grass species*

Three separate sites, each dominated by a neighbouring *M. stipoides* - dominant perennial grass pair, i.e. *M. stipoides* - *Lolium perenne*, *M. stipoides* - *Poa pratensis* and *M. stipoides* - *Dactylis glomerata*, were selected in the permanent pasture at 'Karuah'. A fourth

site was selected at 'Powalgarh' which was characterised by *M. stipoides* - *Phalaris aquatica* as the neighbouring pair. The *M. stipoides* plants were designated M (Lpe), M (Ppr), M (Dgl) and M (Paq) respectively (Fig. 5.2.1). At each site, four large plants of *M. stipoides* and the associated grass species were dug 10-cm deep together with the soil underneath to keep the root system intact. The leaves and culms were clipped to about 10% of the original above ground biomass, a small amount of water was added to moisten the soil, and were then placed immediately inside a plastic bag (Whalley and Brown 1973). The plants were shaded from direct solar radiation during transport and were left inside the plastic bags overnight. On the following day, each plant of *M. stipoides* and the associated grass species was divided into six cuttings with intact root systems using a sharp knife and planted individually in 9.5 cm diameter plastic pots using 1:1:1 sand:soil:peat moss mixture. The cuttings were propagated under glasshouse conditions, fertilised weekly with 0.05% Aquasol® solution (23% N, 4% P, 18% K, 0.05% Zn, 0.06% Cu, 0.0013% Mo, 0.04% S, 0.15% Mn) and watered regularly. Repeated subdivision and cutting of the plants was done until enough planting materials were generated for the different experiments.

5.2.2.2 Seed weight and seed germination variation among four **Microlaena stipoides** *populations*

Seed produced by cleistogamous inflorescences in the glasshouse were collected from each *M. stipoides* population. The seeds were air dried and four lots of 100 mature, plump seeds (complete with ancillary structures) weighed and the mean seed mass calculated. Mean seed weight of M (Ppr), was significantly smaller (3.55^b mg/seed) than the mean seed weights of the three other *M. stipoides* populations growing in association with *L. perenne* (M (Lpe), 5.14^a mg/seed), *D. glomerata* (M (Dgl), 5.30^a g/seed) and *P. aquatica* (M (Paq), 5.58^a mg/seed).

Four lots of 25 seeds of each *M. stipoides* population were arranged on a wet germination pad in square plastic petri plates. The seeds were incubated at 25° C and the number of germinated seeds was counted daily. A seed was considered germinated if 0.5 cm of the radicle had emerged. Seeds of M (Ppr) germinated faster than the other three bigger seeds, despite its lighter seed weight (Fig. 5.2.2). Seeds of M (Lpe) had the slowest rate of germination, while seeds of M (Dgl) and M (Paq) were intermediate (Fig. 5.2.2).

Fig. 5.2.1. The four populations of *Microlaena stipoides* (from left: M (Lpe), M (Paq), M (Dgl) and M (Ppr)) collected in the field, propagated in the glasshouse and studied in the light intensity (Chp. 5.3), water stress (Chp. 5.4), RAPD (Chp.5.5), DNA-DNA hybridisation (Chp 5.6), field competition (Chp. 6.2) and glasshouse competition (Chp. 6.3) experiments.

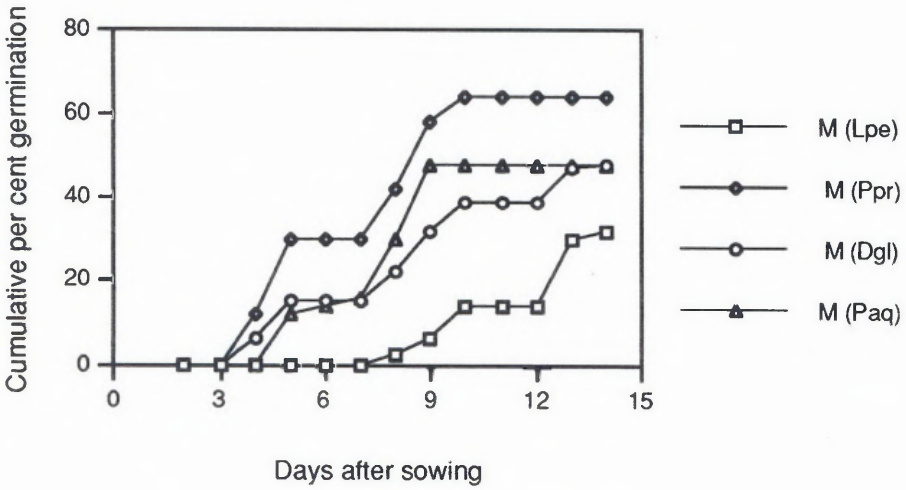


Fig. 5.2.2. Cumulative seed germination rate of four *M. stipoides* populations.

5.3 The growth of five *Microlaena stipoides* populations under four light intensities

5.3.1 Introduction

The ability to capture and utilise light efficiently is of prime importance in the survival and fitness of photoautotrophic plants as components of a community (Gauhl 1976, Bookman and Mack 1983). The growth and competitive ability of the whole plant are influenced by light intensity (Björkman 1981), geometry and dynamics of the plant's canopy and the pattern of energy allocation among the different plant organs (Givnish 1988). Plants have evolved mechanisms to acclimate to environmental fluctuations (Smith 1982), such as adjustment of the photosynthetic apparatus to the specific environment for efficient utilisation of the available light energy (Björkman and Holmgren 1963).

Both genetic and environmental factors affect the light dependence characteristics of a plant (Björkman 1981). The timing and extent of chloroplast development and gene expression vary depending on the organism's developmental strategy and use of environmental signals (Mullet 1988) such as light. Chlorophyll content per unit leaf area affects light absorption efficiency, with a greater proportion of incident light being absorbed with higher chlorophyll content (Björkman 1981). A higher chlorophyll content in plants grown under low irradiance has been observed in some plant species, including *Solanum dulcamara* (Gauhl 1976), *Plantago major* ssp. *major* (Kuiper and Smid 1985) and *Flindersia brayleyana* (Thompson *et al.* 1988).

Solanum dulcamara has a wide geographical distribution, ranging from Europe, Africa and Asia, and occurs in a great diversity of habitats. Gauhl (1976) collected clones of 30 different ecotypes of *S. dulcamara* that exhibited morphological and behavioural differences from shaded and exposed habitats. He propagated the clones vegetatively and conducted a controlled experiment where he exposed the ecotypes to two irradiance levels. He measured CO_2 exchange rate, transpiration rate, chlorophyll concentration, water-soluble protein, and RuDP-carboxylase activity. Clones originating from shaded habitats exhibited limited flexibility of photosynthetic rates, remaining low even under high light intensities, showed severe damage when exposed to strong light for an extended period of time, higher rate of CO_2 evolution of photoinhibited leaves and a marked decrease in chlorophyll content after transfer to strong light. Ecotypes from exposed habitats showed higher photosynthetic rates and higher RuDP carboxylase activity when grown under strong light and did not show damage after exposure to strong light. He concluded that different ecotypes occur within the

species *S. dulcamara* which are physiologically adapted to the light intensities prevailing in their habitats.

Kuiper and Smid (1985) reported genetic specialisation in *Plantago major* ssp. *major* with respect to habitat irradiance. They discovered that the growth of genotypes originating from a shaded habitat was less inhibited at low irradiance, had lower dark respiration rates, and higher chlorophyll (a + b) contents than individuals from exposed habitats.

Species found in grasslands are those that are able to tolerate the dense canopy as well as the thick mass of intertwined rhizomes or stolons and accumulated leaf litter (Bookman and Mack 1983). The ability of different species to absorb photosynthetically active radiation under suboptimal conditions and to colonise gaps are factors influencing the species composition of grasslands (Skálová and Krahulec 1992). Bookman and Mack (1983) observed that two grass species, *Bromus tectorum* and *Poa pratensis* occupied distinct gaps in a *Festuca/Symphoricarpos* grassland. *Bromus tectorum* dominated large scale disturbed sites, while *P. pratensis* dominated small scale disturbed sites. The successful establishment of *P. pratensis* in small scale disturbed sites was attributed to its lower light compensation point and higher light utilisation efficiency than *B. tectorum* (Ludlow 1981).

Björkman and Holmgren (1963) found that the photosynthetic rate at light saturation is higher in ecotypes of *Solidago virgaurea* from exposed habitats while the photosynthetic activity of ecotypes from shaded habitats was inhibited by strong light. Photochemical capacity of populations from shaded habitats was higher than that of populations from exposed habitats, indicating that the photosynthetic apparatus of the populations from shaded habitats can utilise weak light more efficiently. The authors concluded that the characteristic photosynthetic behaviour exhibited by the different populations indicated a genetic adaptation to the prevailing light intensities in their natural environments.

Skálová and Krahulec (1992) collected three morphologically different clones of *Festuca rubra* from three sites of different canopy density about 50 m apart in a mountain grassland in Czechoslovakia. After vegetative multiplication of the clones in a nursery bed, they conducted field and growth room experiments to investigate the effects of plant density and artificial canopy shading of the basal parts of the plants on the growth of the three *Festuca rubra* clones. Generally, the three clones had lower tiller number, lower tiller natality and higher maximum height of tillers when grown in a shaded habitat and under high density treatments. They also observed that differences among the three clones increased as the experiment progressed and proposed that the clones had a specific sensitivity to canopy shade. They inferred that the red/far red (R/FR) ratio was the primary factor involved in the

two treatments and suggested that sensitivity of morphology and growth to R/FR may be correlated with clonal origin. Clones with lower sensitivity to shading and longer leaves originated from stands with tall canopies, while clones with higher sensitivity of tillering originated from low and open stands.

The decrease of R/FR ratio in transmitted light is dependent on canopy density and structure and it is also influenced by plant species. The high sensitivity of some plants to small changes in R/FR ratio enables plants to respond to the canopy density (Holmes and Smith 1977) and to partly distinguish plant species in the close neighbourhood (Thompson and Harper 1988).

Thompson and Harper (1988) grew *Trifolium repens* plants under canopies of three grass swards, *Lolium perenne*, *Agrostis tenuis* and *Holcus lanatus*, and also under black polythene. Formation of first order branches on the main shoot of *T. repens* was greatly reduced under *Lolium* and *Agrostis* canopies and was completely inhibited under the *Holcus* canopy. Total dry weight per plant of *T. repens* was reduced to 64-67% under *Lolium* and *Agrostis* canopies and to 43% under the *Holcus* canopy. The profound effect of *Holcus* on the growth of *T. repens* was attributed to the quality of transmitted radiation under a *Holcus* canopy. Photosynthetically active radiation was reduced to about 32% of the control under all four canopies. There was no reduction in the R/FR ratio under black polythene, while it was reduced to 55% of the control under the *Agrostis* and *Lolium* canopies and to 21% of the control under the *Holcus* canopy.

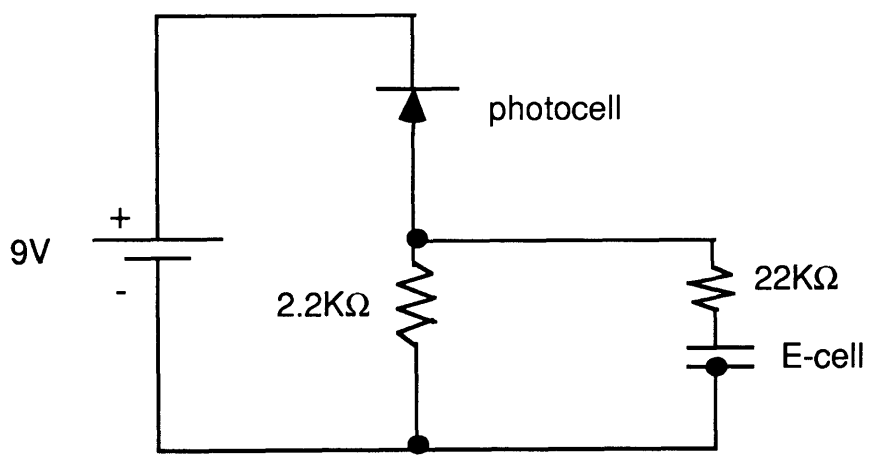
Rose (1986) reported that *Microlaena stipoides* generally showed increased plant height, longer internodes, higher chlorophyll content and increased leaf area when grown under low light intensity. Four of the five populations of *M. stipoides* used in the study reported here grew in association with different perennial grasses in a mosaic pattern in permanent pastures on the Northern Tablelands of New South Wales. Areas under the different grass canopies may have varying light intensities or qualities of transmitted light, providing varying light environments to the different *M. stipoides* populations. It could be possible that within the *M. stipoides* species, different populations represent different genotypes which have different photosynthetic and growth responses to varying light intensities. The different genotypes of *M. stipoides* may have evolved through natural selection resulting from competition with the associated perennial grasses in permanent pastures which significantly affected the quality of transmitted radiation under their canopies. This study was designed to determine differences in the growth and chlorophyll content of five *M. stipoides* populations when grown under different light intensities.

5.3.2 Materials and methods

The five populations of *M. stipoides* used in this study were the three populations collected from 'Karuah' {M (Lpe), M (Ppr), M (Dgl)}, one from 'Powalgarh' {M(Paq)} and another one collected from a paddock in a highly improved, heavily grazed pasture, on a basaltic soil at 1200 m elevation at 'Cloverdale', a property south-east of Walcha on the Nowendoc Road (Population 1). The ramets were propagated vegetatively as described in Chp. 5.2.2.

The four light treatments were full sunlight, one layer, two layers and three layers of Sarlon® shadecloth covering all four sides and top of 750 mm x 950 mm x 550 mm steel frames. The accumulated intensity of transmitted light under the steel frames was recorded every week using light integrators for the first ten weeks of the experiment. One light integrator was placed in the centre of each cage on a 300 mm high wooden pole and tied in position at a 60° angle from the horizontal facing magnetic north. Each light integrator was composed of a photocell, covered on top with a black disc with a 0.1 mm slit in the centre, mounted inside a 20 mm-diameter spherical-ended glass tube which was connected to a microcoulometer (E-cell) powered by a 9-volt alkaline battery. The otocell intercepts the incoming radiation which triggers electron flow in the circuit. The E-cell integrates the electron flow as electrical charge. At weekly intervals the accumulated electrical charge in the E-cell was read on an E-cell digital readout machine. The accumulated electrical charge from the integrators under each of the frames was expressed as a percentage of that of the integrator in the open on a weekly basis.

The connections inside each light integrator had the following design:



The intensities of transmitted light inside and outside the cages were spot read on a cloudless day on 4 October 1990 at ten o'clock in the morning using a photometer sensitive to photosynthetically active radiation and the readings were compared with the light integrator readings.

One ramet each of the five populations was transplanted into a 15 cm pot filled with a 1:1:1 sand:soil:peatmoss mixture on 26 September 1990. The ramets were maintained inside a glasshouse for two weeks to enable the plants to establish. On 11 October 1990, the field experiment was set up at Clark's farm, an experimental area adjacent to the University. There was a total of 20 pots under each light treatment, four replicates for each of the five populations, and the pots were arranged in a completely randomised design under each light treatment. Slow-release fertiliser (1.3 g Osmocote®/pot) was applied every three months and the plants were watered as necessary.

Plant height (from base of the plant to the tip of the longest leaf) was measured and the number of green and dead leaves and tillers were recorded in November 1990, January 1991, July 1991 and November 1991. The total number of leaves and tillers produced in twelve months were computed by adding the increment of the number of leaves and tillers (green and dead) from the last date of measurement. Biomass was harvested four times by cutting the shoots 5 cm from the surface of the soil during the following months: July 1992, November 1992, February 1993 and May 1993 and oven dry weights (80° C for at least 48 hours) were then determined. The number of panicles exserted between November 1991 and April 1992 was recorded weekly and the total number of panicles produced during this period was computed. In July 1991, ten fully grown leaves per ramet were selected randomly to measure leaf length and leaf width.

In November 1991, fully grown leaves were sampled randomly from the ramets for chlorophyll analysis. Chlorophyll content was determined by cutting a 0.1 g sample of fresh leaf material into small pieces and homogenising for 1 minute with 5 ml of 1:4 water:acetone solution (Arnon 1949). A further 10 ml of the water:acetone solution was added and mixed. The tubes were stoppered, covered with aluminum foil and left in the dark for 20 hours to allow extraction of chlorophyll from the leaf tissue. After chlorophyll extraction, the tubes were centrifuged at 3,000 rpm for 5 min, decanted and the absorbance at 652 nm was read against a water:acetone blank using a spectrophotometer. The amount of chlorophyll (mg chlorophyll/g of leaf fresh weight) was calculated using the following formula:

$$\text{chlorophyll concentration} = \frac{\text{absorbance at 652 nm x dilution}}{\text{specific absorption coefficient x fresh weight}} \qquad (5.3.2)$$

where the dilution was equal to 15 and the specific absorption coefficient was 34.5 (Arnon 1949).

Analyses of variance of all the data on harvested dry mass, tiller number, total number of leaves, plant height, leaf length, leaf width, total number of panicles exserted in one flowering season and chlorophyll concentration were performed using the STATVIEW statistical package. Significant differences among means were determined using Scheffé's test at the 5% significance level.

5.3.3 Results

5.3.3.1 Light intensity

The average per cent transmitted light of ten weekly light integrator readings and the spot readings of light intensity inside the cages using a photometer sensitive to photosynthetically active radiation on a cloudless day (4 October 1990) at ten o'clock in the morning gave comparable results (Fig. 5.3.1). One layer of shadecloth reduced the amount of transmitted light to 47% of the incident radiation, two layers to 16% and three layers of shade cloth to 6% (Fig. 5.3.1).

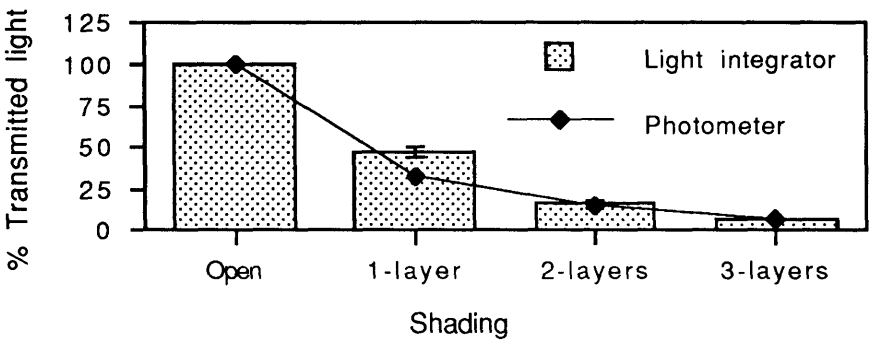


Fig. 5.3.1. The amount of transmitted light under 1, 2 and 3 layers of Sarlon® shadecloth measured by light integrators and a photometer. Vertical bars indicate ± SE. Where no error bar appears, the SE was smaller than the size of the symbol.

5.3.3.2 Total dry mass

The average total dry mass of the five *M. stipoides* populations was significantly different when grown under the four light intensity treatments. The plants yielded significantly higher biomass when grown under 47% transmitted light, followed by 16%

transmitted light, and full sunlight (Fig. 5.3.2). Mean dry mass was significantly lower when grown under severe shading (6% transmitted light) compared with full sunlight (Fig. 5.3.2). The mean total dry mass averaged over all light intensity treatments of M (Ppr) (18.78 g/pot[a]) and M (Dgl) (18.69 g/pot[a]) was significantly higher than the mean total dry mass of Population 1 (14.06 g/pot[b]), while M (Lpe) (15.00 g/pot[ab]) and M (Paq) (14.68 g/pot[ab]) were intermediate.

The biomass production of M (Lpe) was highest when grown under 47% transmitted light, was reduced by 38% (NS) when grown under 16% transmitted light, by 40% (NS) when grown under full light and was reduced significantly by 78% ($P < 0.05$) when grown under severe shading (6% transmitted light) (Fig. 5.3.2). *Microlaena stipoides* (Ppr) exhibited shade tolerance, having only a 16% yield reduction (NS) when grown under 16% transmitted light, 42% reduction (NS) when grown under full light and 72% reduction ($P < 0.05$) under severe shading compared with its dry mass yield under 47% transmitted light (Fig. 5.3.2). *Microlaena stipoides* (Dgl) demonstrated light tolerance, having the least reduction in yield, though non significant, when grown under full light in proportion to its highest yield (when grown under 47% transmitted light) among the five populations (Fig. 5.3.2). The dry mass yield of M (Dgl) was highest when grown under 47% transmitted light, was reduced by 18% (NS) when grown under full light, 30% reduced (NS) under 16% transmitted light and 76% reduced ($P < 0.05$) when grown under severe shading (Fig. 5.3.2). It is only M (Dgl) which exhibited the following trend in dry mass production: 47% transmitted light > full sunlight > 16% transmitted light > 6% transmitted light. The trend of dry mass yields of the other four populations under the different light intensities can be summarised as follows: 47% transmitted light > 16% transmitted light > full sunlight > 6% transmitted light. Population 1 showed sensitivity to high light conditions and shading, having 79% and 88% reductions ($P < 0.05$) in dry mass yield, compared with its yield at 47% transmitted light, when grown under full light and severe shading, respectively (Fig. 5.3.2).

5.3.3.3 Tiller production

Tiller production was significantly different among the four light treatments when averaged over all the five *M. stipoides* populations. The trend of tiller production per pot can be summarised as follows: 47% transmitted light > 16% transmitted light > full sunlight > 6% transmitted light (Fig. 5.3.3). There were no significant differences in the mean tiller number among the five populations averaged over all light intensities.

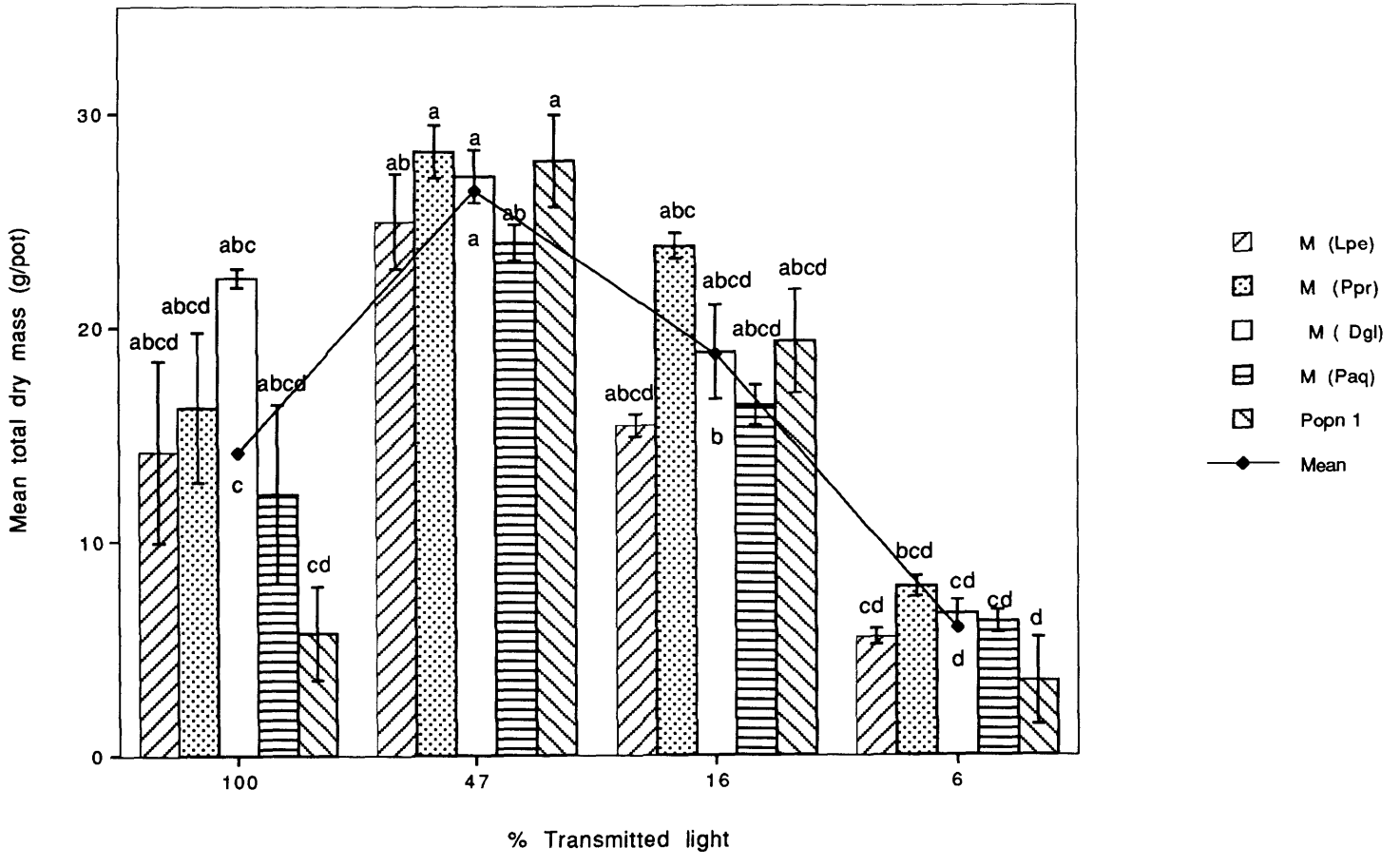


Fig. 5.3.2. Mean total harvested dry mass of five *M. stipoides* populations grown under varying light intensities. Columns which share the same letter(s) are not significantly different at P > 0.05. Vertical bars indicate ± SE.

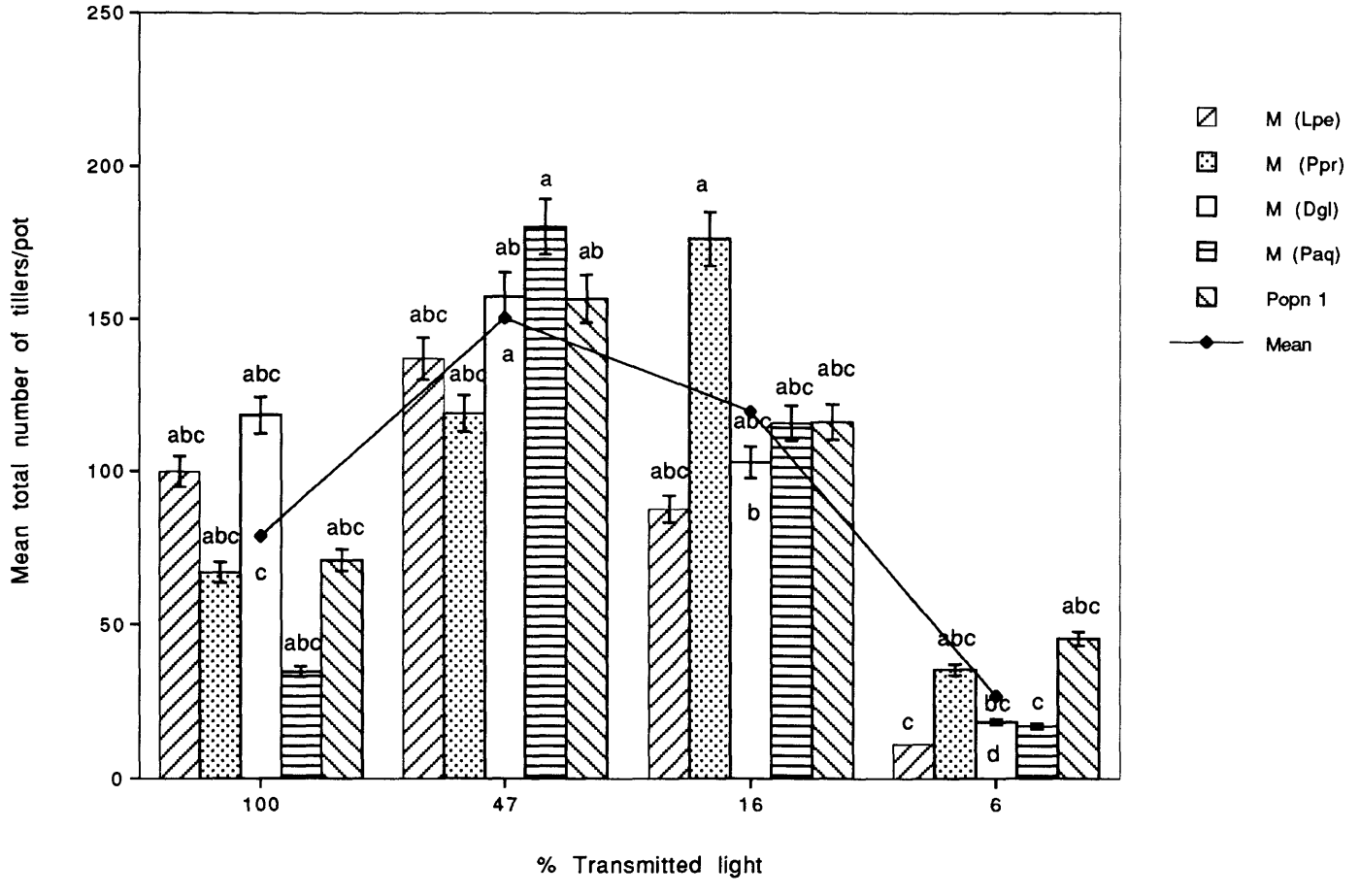


Fig. 5.3.3. Mean total number of tillers produced by five *M. stipoides* populations grown under varying light intensities. Columns which share the same letter(s) are not significantly different at P > 0.05. Vertical bars indicate ± SE.

Microlaena stipoides (Ppr) produced its largest number of tillers when grown under 16% transmitted light, while all the other four populations produced the largest number of tillers under 47% transmitted light. The trend in tiller production of M (Ppr) under the different light intensities can be summarised as follows: 16% transmitted light > 47% transmitted light > 100% light > 6 % transmitted light. Conversely, the trend in tiller production of M (Lpe) and M (Dgl) was: 47 % transmitted light > 100% light > 16% transmitted light > 6% transmitted light. Tiller production of M (Paq) and Population 1 under the different light intensities was: 47% transmitted light > 16% transmitted light > 100% light > 6% transmitted light. *M. stipoides* (Lpe) and M (Dgl) produced a larger number (NS) of tillers under 100% light than 16% transmitted light, while M (Paq) and Population 1 produced a larger number of tillers (NS) under 16% transmitted light than 100% light (Fig. 5.3.3). Significant reduction in tiller production ($P < 0.05$) was observed when M (Paq) was grown under severe shading compared with its tiller production under 47% transmitted light (Fig. 5.3.3). Similarly, the other four populations exhibited non-significant reductions in tiller production when grown under severe shading compared with their tiller production under 47% transmitted light.

5.3.3.4 Leaf production

The average number of leaves produced by the five populations under 47% transmitted light was significantly higher than when the plants were grown under 16% transmitted light (Fig. 5.3.4). Leaf production under full light and severe shading (6% transmitted light) did not differ significantly but were both significantly lower than at the other two light intensities. There were no significant differences in the total number of leaves produced by the five populations averaged over all light intensity levels.

Among the five *M. stipoides* populations, M (Ppr) showed significantly higher reduction ($P < 0.05$) in leaf production when grown under full light, compared with its leaf production under 47% transmitted light. It exhibited a 40% reduction in leaf production when grown under 16% transmitted light ($P > 0.05$), 65% reduction ($P > 0.05$) when grown under severe shading and 74% reduction ($P < 0.05$) when grown under full light compared with its leaf production under 47% transmitted light. There were no significant differences in the leaf production of M (Lpe), M (Dgl), M (Paq) and Population 1 grown under the four light intensity levels. The number of leaves produced by M (Lpe), M (Dgl) and Population 1 declined following this general trend: 47% transmitted light > 16% transmitted light > full light > 6 % transmitted light (Fig. 5.3.4).

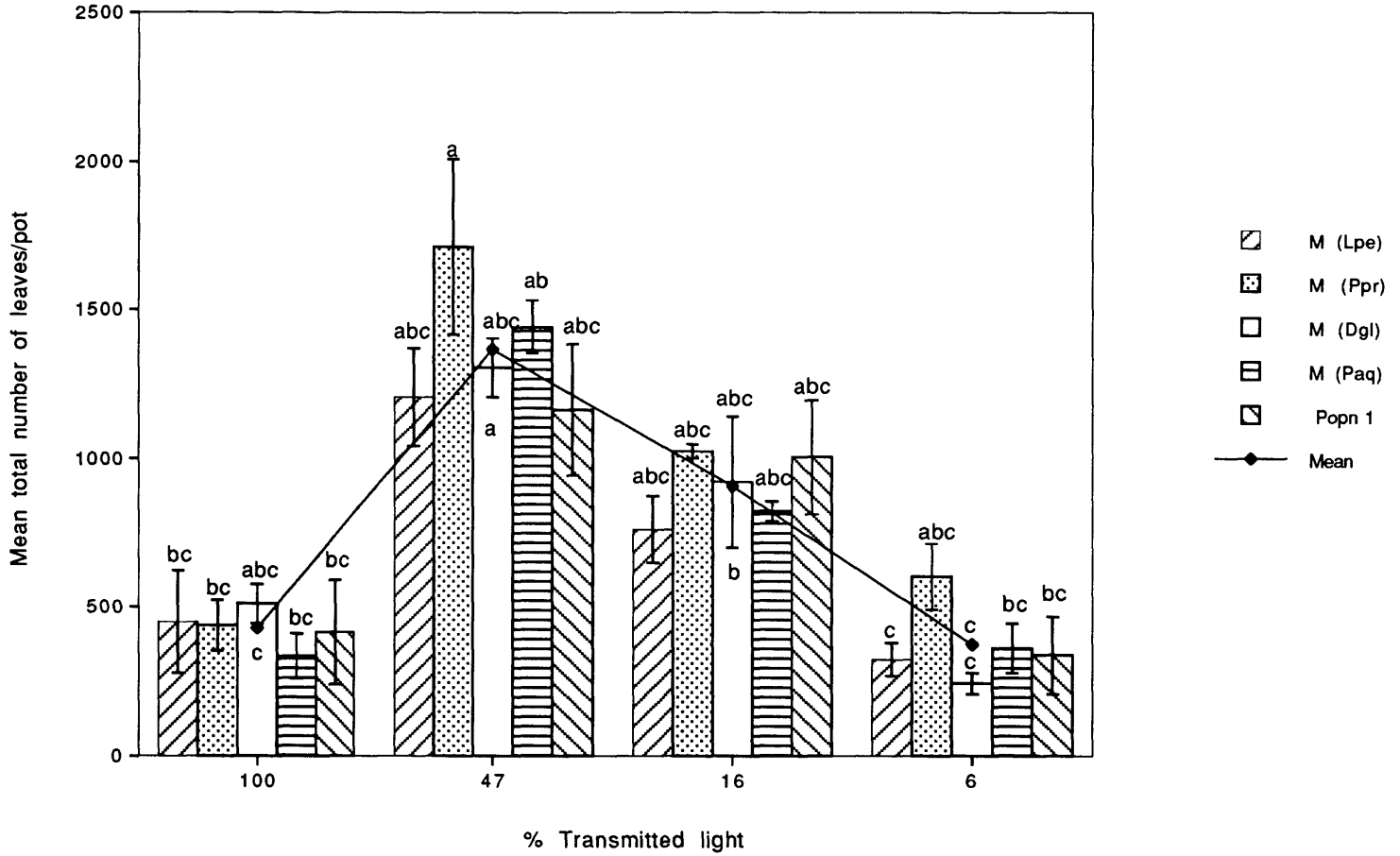


Fig. 5.3.4. Mean total number of leaves produced by five *M. stipoides* populations grown under varying light intensities. Columns which share the same letter(s) are not significantly different at P > 0.05. Vertical bars indicate ± SE.

5.3.3.5 Chlorophyll concentration

The average leaf chlorophyll concentrations (mg/g fresh weight) of the five *M. stipoides* populations grown under shade were significantly higher than those grown under full light (Table 5.3.1). There were no significant differences in the mean chlorophyll contents of leaves of the five *M. stipoides* populations grown under the three light intensities under shade (Table 5.3.1). Mean leaf chlorophyll contents of the five *M. stipoides* populations averaged over all light intensity levels differed significantly. The average chlorophyll content of M (Ppr) and Population 1 were significantly higher than M (Lpe), M (Dgl), and M (Paq) (Table 5.3.1). There were no significant differences in leaf chlorophyll content resulting from light treatment x population interaction.

Table 5.3.1. Mean leaf chlorophyll (mg/g fresh weight) contents of five *M. stipoides* populations grown under varying light intensity levels. Within each group, means which share the same letter are not significantly different at P > 0.05.

	M. stipoides population					
Transmitted light (%)	M (Lpe)	M (Ppr)	M (Dgl)	M (Paq)	Population 1	Mean
	Mean chlorophyll content (mg/g fresh wt)					
100	1.88	2.33	2.03	2.01	-	2.06[b]
47	2.52	3.35	2.83	2.73	3.08	2.90[a]
16	2.60	2.92	2.95	2.93	3.04	2.89[a]
6	2.62	3.13	2.77	2.55	3.22	2.86[a]
Mean	2.40[b]	2.93[a]	2.64[b]	2.56[b]	3.11[a]	

5.3.3.6 Leaf length

The mean leaf length (mm) of the five *M. stipoides* populations was significantly higher when grown under 47% and 16% transmitted light than when grown under full light and severe shading (Fig. 5.3.5). There were no significant differences in mean leaf lengths of the five *M. stipoides* populations averaged over all light intensity treatments. Interaction between light intensity treatments and population had no significant effect on leaf lengths.

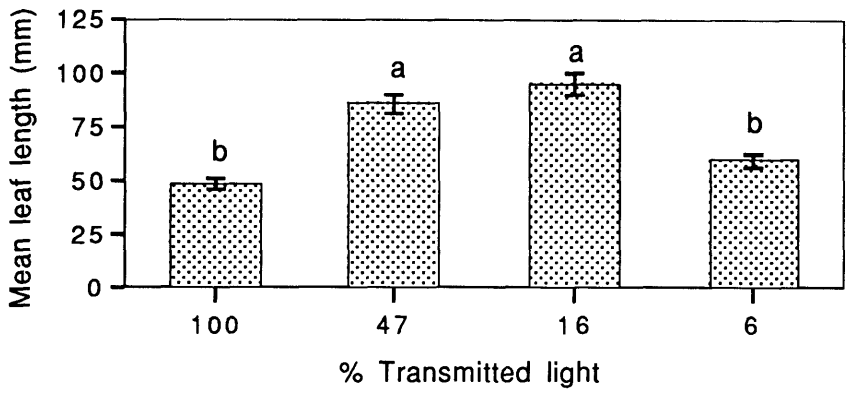


Fig. 5.3.5. Effects of light intensity treatments on mean leaf lengths of five *M. stipoides* populations. Columns which share the same letter are not significantly different at P > 0.05. Vertical bars indicate ± SE.

5.3.3.7 Leaf width

Shading had no significant effect on the average width (mm) of the leaves of the five *M. stipoides* populations. However, significant differences resulted when average leaf width of each population was computed over all light intensity levels. M (Lpe), M (Dgl) and M (Paq) had significantly (P < 0.05) wider leaves than M (Ppr) and Population 1 (Fig. 5.3.6).

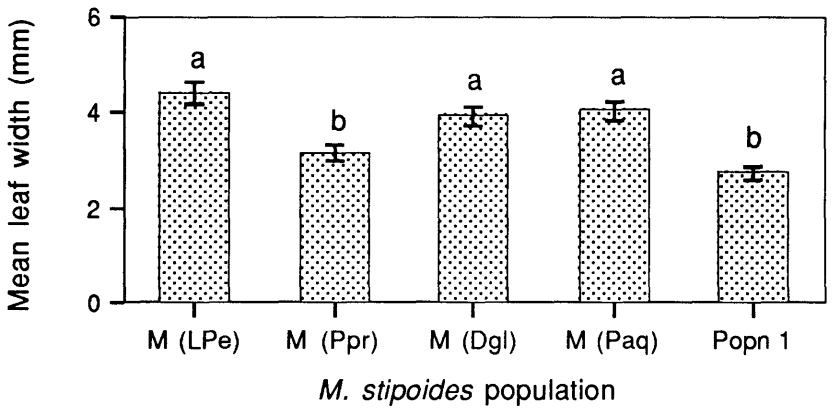


Fig. 5.3.6. Mean leaf width of five *M. stipoides* populations. Columns which share the same letter are not significantly different at P > 0.05. Vertical bars indicate ± SE.

5.3.3.8 Plant height

Significant ($P < 0.05$) differences in plant height among the two main factors analysed, i.e. population and light treatment, were found at the first measurement, November 1990, four weeks after transplanting (Fig. 5.3.7). On the three succeeding plant height measurements, significant differences were found only in the light treatment factor (Table 5.3.2). There were no significant differences among the light treatment x population interaction factor in all four measurements.

One month after setting up the field experiment, average plant heights of the five populations were significantly higher when the plants were grown under 47% and 16% transmitted light (Table 5.3.2). As the experiment progressed, average plant heights of plants grown under 16% and 6% transmitted light were significantly higher than that of plants grown under full light and 47% transmitted light, although plant height was significantly higher under 47% transmitted light than under full light (Table 5.3.2). Severe shading (6%) did not cause a significant difference in plant height compared with the height of the plants grown under 16% transmitted light (Table 5.3.1).

Table 5.3.2. Effects of light intensity on average plant height (mm) of five *M. stipoides* populations at four separate measurements. Values within rows which share the same letter(s) are not significantly different at $P > 0.05$.

Dates of plant height measurement	Light intensity (% transmitted light)			
	100	47	16	6
	Plant height (mm)			
November 1990	73.7^{b}	105.3^{a}	117.1^{a}	80.6^{b}
January 1991	35.4^{c}	91.5^{b}	136.6^{a}	128.1^{ab}
July 1991	64.3^{c}	125.0^{b}	184.8^{a}	185.3^{a}
November 1991	122.9^{c}	250.2^{b}	333.8^{a}	302.3^{a}

When averaged over all light intensity levels, M (Ppr) was significantly taller than M (Lpe), M (Dgl) and M (Paq), while Population 1 was intermediate, on the first measurement (November 1990). There were no significant differences in the heights of the five populations during the succeeding three measurements (January, July and November 1991).

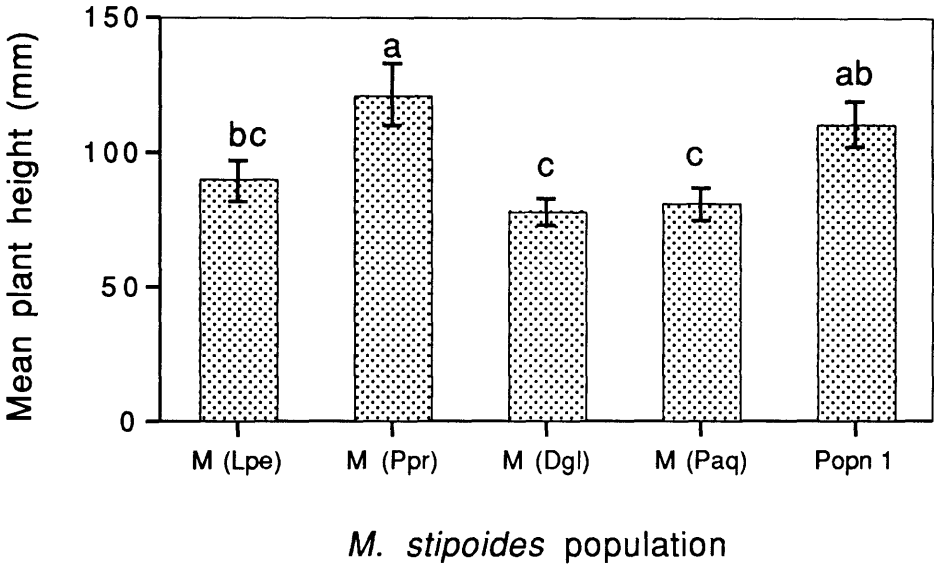


Fig. 5.3.7. Mean plant heights of five *M. stipoides* populations four weeks after transplanting. Columns which share the same letter(s) are not significantly different at P > 0.05. Vertical bars indicate ± SE.

5.3.3.9 Panicle production

Mean total number of panicles exserted for one flowering season by the ramets of the five populations was significantly (P < 0.05) higher when the ramets were grown under 47% transmitted light than when grown under 16% transmitted light (Table 5.3.3). The mean total number of panicles exserted by the ramets when grown under 16% transmitted light was, however, significantly greater than when the ramets were grown under severe shading, while those grown under full light was intermediate (Table 5.3.3). The average number of panicles exserted by M (Ppr) was significantly higher than M (Lpe) and M (Dgl) while M (Paq) and Population 1 were intermediate (Table 5.3.3). Light intensity treatment and population had no significant interacting effects on panicle production.

5.3.4 Discussion

The five populations of *M. stipoides* exhibited significant differences in their ability to tolerate full sunlight and shading. *Microlaena stipoides* (Ppr) showed the greatest shade tolerance, having higher biomass yields under 47% and 16% transmitted light and the least reduction in dry mass yield when grown under 16% transmitted light. *Microlaena stipoides* (Ppr) also produced the greatest number of tillers when grown under 16% transmitted light.

The other four populations produced the greatest numbers of tillers when grown under 47% transmitted light. It has been reported that both intensity and quality of transmitted radiation by leaf canopies influence tillering of grasses (Casal *et al.* 1987) and that the radiation environment at the base of the canopy influences the subsequent growth of the plant (Thompson 1993). Results show that with M (Ppr) low light intensity (16% transmitted light) induces maximum tillering, while for the other populations, a higher light intensity (47% transmitted light) induces maximum tillering.

Table 5.3.3. Mean total number of panicles/pot produced by five *M. stipoides* populations grown under varying light intensity levels. Within each group, means which share the same letter(s) are not significantly different at P > 0.05.

	M. stipoides population					
% Transmitted light	M (Lpe)	M (Ppr)	M (Dgl)	M (Paq)	Population 1	Mean
	Panicles/pot					
100	9.33	38	13.5	19.25	27	21.42^{bc}
47	30.25	108	59	57.25	64.5	63.80^{a}
16	19.75	49.67	24	41.75	47.25	36.48^{b}
6	4.75	20.67	3.75	15.5	10	10.93^{c}
Mean	16.02^{b}	54.08^{a}	25.06^{b}	33.44^{ab}	37.19^{ab}	

Microlaena stipoides (Ppr) had more leaves and higher chlorophyll content than the other populations when grown under 47% transmitted light. The higher chlorophyll content and total number of leaves of M (Ppr) mean that it has more photosynthetic centres to capture photosynthetically active radiation (Björkman 1981) as manifested by the high dry mass yield. *Microlaena stipoides* (Ppr) also showed a significant reduction in leaf production when grown under full light compared with its leaf production under 47% transmitted light. This could mean that M (Ppr) experienced inhibition of photosynthetic activity under strong light, producing less leaves and tillers and hence dry mass (Björkman and Holmgren 1963, Kuiper and Smid 1985). These results indicate that among the five populations, the M (Ppr) genotype was more adapted to lower light intensity.

Microlaena stipoides (Ppr) was collected from the lower south-eastern part of the paddock shaded by a big tree, where it grows in association with *P. pratensis*. *Poa pratensis* has an aggressive rhizomatous growth habit (Hunt and Dunn 1993) and occurs naturally more often in hollows than in ridges (Reader and Bonser 1993). The higher soil moisture

content and fertility levels in hollows than in ridges favour the increase in tillering of *P. pratensis* in hollows (Reader and Bonser 1993). Growth of *P. pratensis* may be reduced both by low soil moisture content (Bennett *et al.* 1972) and by low soil fertility (Skinner and Noll 1919, Hartwig 1938). Thompson and Clark (1993) observed a positive response of *P. pratensis* to added nitrogen fertiliser, resulting in an increase in the number of large tillers, initiated tillers and flowering shoots. Thompson (1993) reported that not only the alteration of light quality and quantity by neighbours, but some other aspects of interaction such as competition for nutrients, water or other environmental factors are also important in the growth of plants. Most crop species exhibit significant genotype-environment interactions such that species are best adapted to the ecological situations from which the populations were derived (Charmet *et al.* 1993).

Microlaena stipoides (Ppr) produced a larger number of panicles compared with the other four populations at all light intensity levels. Because M (Ppr) produced more panicles, it can be inferred that it has higher seed production than the other populations, assuming the same number of seeds per panicle. Seeds produced by M (Ppr) generally weigh significantly less (3.55 mg/seed) than the weight of the seeds of the other populations (Chp. 5.2). Production of larger quantities of smaller or lighter seeds could be an adaptive strategy of M (Ppr) whereby less energy resources are allocated per seed but massive quantities of seed are produced. A larger number of panicles were produced when the plants were grown at 47% transmitted light.

Microlaena stipoides (Dgl) showed a significant tolerance to high light intensity, producing larger dry mass and tiller numbers when grown under full light compared with the other *M. stipoides* populations. It was collected from the upper slope on the north-eastern part of the paddock where it was growing in association with *D. glomerata*, a tufted perennial suitable for moderate fertility and dry regions (Levy 1970). *Dactylis glomerata* was more common on the ridges where soil fertility levels and soil moisture content was lower than in the hollows (Reader and Bonser 1993).

Microlaena stipoides (Lpe) had the highest dry mass, tiller number and leaf number when grown under 47% transmitted light and exhibited reductions in all these growth parameters when grown under full light and 16% transmitted light. Like the other four populations, growth of M (Lpe) was significantly reduced under severe shading. It was collected from an exposed flat site on the south-western part of the paddock where the soil was clayey and growing in association with *L. perenne*. *Lolium perenne* has a strong clonal growth pattern with a good balance between growth at the apex and death of the old basal stem (Brock and Fletcher 1993). It is most suitable for high fertility conditions (Levy 1970) and showed a considerable increase in yield with added nitrogen (Wedderburn *et al.* 1993).

The yield of *L. perenne* is limited by its poor adaptation to summer heat and drought (Charmet *et al.* 1993).

Microlaena stipoides (Paq) was collected from an open north-facing site with a stony soil, where it was growing in association with *P. aquatica*. *Phalaris aquatica* has a rhizomatous growth habit (Rumball 1980) and survives under hard grazing (Hutchinson 1970) and exhibits pest tolerance (Stevens *et al.* 1993).

Population 1 showed sensitivity to full light and severe shading, having significant reductions in dry mass when grown under the two extreme conditions. Population 1, despite a large reduction in dry mass when grown under severe shading, however, produced the highest number of tillers among the five populations grown under severe shading. Population 1 was collected from heavily grazed, highly improved pasture on a basaltic soil.

Generally, all the populations of *M. stipoides* tested were best adapted to growth under shading, having higher dry mass, larger numbers of tillers, leaves and panicles when grown under shade (47% and 16% transmitted light). In natural pastures, *M. stipoides* is commonly found under shade trees frequented by resting stock (Taylor and Hedges 1984), around tree stumps, along margins of sheep camps, and in more fertile or protected areas of natural pastures, particularly where there is some timber cover (Whalley *et al.* 1978). The survey described in Chp. 3 revealed that high tree density in a paddock was highly associated with abundant *M. stipoides* on the Northern Tablelands.

The results of this experiment showed that generally all the five populations gave significantly higher biomass when grown under 47% transmitted light, which contrasts with the earlier findings of Rose (1986) that total plant weight decreased with a decrease in light intensity. The trends in mean tiller and leaf production were generally similar to the trend in biomass production under the varying light intensity levels. Significantly higher mean dry mass, tiller number and leaf number were produced at 47% transmitted light, followed by 16% transmitted light.

Rose (1986) found that 25% transmitted light caused significant decreases in leaf and tiller number. This was not the case with the *M. stipoides* populations that were used in the present study. Reducing the amount of transmitted light inside the cages to 16% did not cause a severe reduction in tiller production. The number of tillers produced at this light intensity was in fact significantly higher than tiller production under full light and 6% transmitted light. Reducing the transmitted light to 6% was the treatment that caused a drastic reduction in tiller production.

All the populations exhibited a significant increase in chlorophyll concentration when grown in the shade. An increase in chlorophyll content when grown in shade was also observed in *M. stipoides* (Rose 1986), *Solidago virgauera* (Björkman and Holmgren 1963) and *Plantago major* ssp. *major* (Kuiper and Smid 1985). The chlorophyll content of species adapted to shaded habitats such as *Aaenocaulon bicolor*, *Aralia california* and *Trillium ovatum* (Björkman 1968), a number of rainforest species (Boardman 1977), and *Solanum dulcamara* (Clough *et al.* 1979) was higher than species adapted to sunny habitats. Leaves of shade tolerant plants have fewer but larger chloroplasts and were richer in chlorophyll b (Rabinowitch 1945, Egle 1960, Kirk and Tilney-Bassett 1967, Boardman 1977). Grana stacks in chloroplasts of shade tolerant plants such as *Alocasia macrorrhiza*, *Cordyline rubra* and *Lomandra longifolia* were larger and contained as many as 100 thylakoids per granum (Goodchild *et al.* 1972, Anderson *et al.* 1973, Boardman *et al.* 1975). The grana are irregularly arranged within a shade tolerant plant chloroplast, possibly to increase their light absorption efficiency (Boardman 1977).

Gui-Chao 2, a variety of rice, *Oryza sativa*, adapted to shaded habitats, exhibited a larger reduction in chlorophyll content than Bellemont, a rice variety adapted to sunny habitats, when grown under strong light (Tu *et al.* 1988). This could be related to chloroplast disturbances such as the bleaching and deformation observed by Björkman and Holmgren (1963) when shade-adapted plants of *Solidago virgauera* were grown under strong light.

Leaf lengths of *M. stipoides* were significantly longer when the plants were grown under 47% and 16% transmitted light compared with full light and severe shading. Growing the plants under varying light intensity levels did not significantly alter the leaf widths of the five populations. However, significant variation in leaf width among the five populations, averaged over all light intensity levels, was recorded. Population 1 and M (Ppr) had significantly narrower leaves than M (Lpe), M (Dgl) and M (Paq).

Plant height was generally lowest when the plants were grown under full light. The increase in plant height with shading has been observed in *M. stipoides* (Rose 1986) and in *Festuca rubra* (Skálová and Krahulec 1992). This could be due to: i) a reduction in the inhibition of cell extension by light (Rose 1986); ii) the allocation of energy resources to internode elongation and leaf elongation to enable the plant to grow towards light (Smith 1982); iii) an adaptation directed at spreading the area of influence of the grass so as to catch more of the incident light; iv) a method of obtaining an open sward with the best angle for maximum light penetration (Rose 1986); or v) to enable young leaves to reach more favourable light within the canopy (Grime 1979).

The severe reduction of transmitted light down to 6%, however, caused a significant reduction in plant growth, as manifested by the significantly lower dry mass, tiller and leaf numbers and the shorter leaf lengths. The plants could have been drastically deprived of light energy for photosynthesis under these conditions.

The results presented in this chapter clearly suggest that *M. stipoides* is a shade-adapted species and that the five *M. stipoides* populations studied possess significant differences in their sensitivity or tolerance to light intensity, indicating that they are clearly different genotypes. The five populations differ in their strategies for energy allocation under varying light intensity levels. *Microlaena stipoides* (Ppr) produced a larger number of tillers and leaves when grown under shadecloth. During the initial stages of the experiment, it showed the least inhibition in leaf and internode elongation under full light, having the tallest plants among the five populations grown under full light. Tillering and leaf elongation can modify canopy structure, leaf elongation affecting vertical structure and tillering affecting horizontal structure (Skálová and Krahulec 1992). Leaf elongation and tillering influences absorption of incident light and gap colonisation, thereby affecting the survival of grasses in grassland. *Microlaena stipoides* (Ppr) also showed higher panicle production and consequently, possible higher seed production than the other four populations. *Microlaena stipoides* (Dgl), on the other hand, exhibited a different strategy, producing a larger number of tillers compared with the other populations under full light, thus yielding higher dry mass. The different behaviour of the five populations under varying light intensities infers differences in their competitive abilities under varying canopy shades and suggests that the pasture species with which they were originally associated exert differential effects on the quality and quantity of transmitted light, to which the five populations have adapted.

5.4 RESPONSES OF FIVE *Microlaena stipoides* POPULATIONS TO WATER STRESS

5.4.1 Introduction

The three basic strategies of responses to water stress are escape, avoidance and tolerance (Turner 1986, Ludlow 1989). The escape strategy is quite common among annual plants and is characterised by early flowering and fruiting and developmental plasticity in response to variations in water availability. Avoidance strategy is characterised by a deep root system to maximise access to water and adaptations to minimise water loss while tolerance strategy is characterised by dehydration tolerance of plant tissues and high osmotic adjustment (Ludlow 1989).

Features of the escape strategy include rapid germination, early root initiation, and rapid root extension, which enable the seedlings to compete with other species and avoid drought caused by evaporative water losses in the upper soil layers (Johnson and Asay 1993).

Avoidance strategy includes divergence of energy into the development of extensive root systems to extract water deep in the soil profile (Johnson and Asay 1993). O'Toole and Bland (1987) reported that genotypic variation exists for many root characteristics of crop plants. Reduction of water loss through leaf area reduction, either by leaf senescence or shedding, thickening of the epidermal cell wall and the cuticle (Schulze 1988), development of leaf hairs, leaf orientation and movement away from the sun, leaf rolling, stomatal closure and development of epicuticular wax (Johnson and Asay 1993) have been reported.

Another mechanism of adaptation to drought is osmotic adjustment, which involves the accumulation of solutes, resulting in a more negative osmotic potential of the cell sap. Turner (1986) proposed that osmotic adjustment plays an important role in survival of the developing apex and leaves. Genetic variation for osmotic adjustment has been found in many crop species including *Triticum aestivum*, *Sorghum bicolor*, *Pennisetum glaucum*, *Gossypium hirsutum*, *Oryza sativa* and *Cajanus cajan* (Ludlow and Muchow 1990).

Differences in stomatal characteristics may influence plant adaptation to water stress. Wilson (1971) found variation in stomatal length and frequency among populations of *Lolium perenne*. Walton (1974) studied stomatal length and frequency of a 7 × 7 diallel

cross of *Bromus inermis*. He found significant differences in the stomatal frequency among the seven clones using rubber silicone impressions.

Mack and Finn (1970) tested the growth of six clonal stocks of *Phleum pratense* under four nutritional (N and P) levels, two moisture levels (25% and 75% of field capacity) and three temperature levels. They found diversity in growth among the six clones. Two of the clones were more productive under lower soil temperatures (10° to 20° C), while another clone gave a higher yield under high soil temperatures (27/20° C, day/night), good nutrition (N+P) and high moisture (75% to 100% available water). The rate of regrowth of the six clones varied significantly under more favourable growing conditions of high soil moisture (75% to 100%) and nutrition. The diversity of growth of the six clones in response to changes in soil environmental conditions demonstrated genotypic diversity among the six clones of *Phleum pratense*.

Harradine and Whalley (1979) studied the relative tolerance of *Aristida ramosa*, *Danthonia linkii* and *Dichanthium sericeum* to repeated, increasing periods of water stress in a limited volume of soil. *D. linkii* depleted water more rapidly, while *A. ramosa* depleted the available water more slowly when grown in a limited volume of soil. *Danthonia linkii* showed a greater degree of drought tolerance, being able to endure a longer period and greater degree of water stress than the other two species.

Walton (1974) stated that structures associated with transpiration, respiration and photosynthesis which are important to plant growth, must have evolved under the influences of natural selection and be under genetic control. Guobin and Kemp (1992) found that the *T. repens* cultivar used in their study had poor performance under water stress, which was contrary to previous reports and hinted that genetic variation in response to water stress may exist within the *T. repens* species.

It has been shown in the previous section (Chp. 5.3) that the five populations of *M. stipoides* used in this study exhibited different responses to light intensity and morphological differences including leaf length, leaf width and tiller number. This study was designed to examine differences in the response of the five *M. stipoides* populations to water stress.

5.4.2 Materials and methods

The plants were grown in pots in a 4:1 mixture of sandy loam and river sand. The air

dry water content of the mixture was determined gravimetrically by weighing and then drying for 24 and 48 hours at 110° C. No further loss of water was recorded after 24 hours.

The field capacity of the soil mixture was determined by covering the lower end of a 1L plastic cylinder with cheese cloth tied in place with a rubber band. Six cylinders were filled with the soil mixture to 2-3 cm from the upper end and the fresh weight of the soil determined. The six cylinders were positioned vertically using an iron clamp and stand. A volume of water equivalent to 7.5% of the fresh weight of the soil was carefully added on top of the soil mixture inside the cylinders. The water was allowed to drain and the length of the wet soil column was measured after 48 and 72 hours. Since there was no change in the length of the wet column in the two consecutive daily measurements, a sample was collected from the middle of the wet portion of the soil mixture and the moisture content determined as described above.

Heavy duty plastic bags were used to line the inside of 100 (small), 125 (medium) and 150 (large) mm diameter plastic pots. Small pots were filled with 500 g, medium pots with 1,000 g and large pots with 1,800 g of the soil mixture.

Cleistogamous seeds of the five *M. stipoides* populations used in the light intensity experiment (Chp. 5.3) and seeds of *Lolium perenne* were germinated on moist germination pads in petri dishes inside a 25° C incubator. The germinated seeds were transplanted into pots when the radicle length was about 1 cm long. There were ten replicates for each population at each pot size, making 180 pots altogether. The soil mixture in the pots was brought to field capacity prior to transplanting with a 0.05% Aquasol® solution (23% N, 4% P, 18% K, 0.05% Zn, 0.06% Cu, 0.0013% Mo, 0.04% S, 0.15% Mn). The soil surface of each pot was covered with a known mass of white polythene granules to minimise water loss from the soil surface. Transplanted seedlings were maintained on a shaded work bench for two days before they were transferred to the glasshouse. Pots were arranged randomly in the glasshouse. The water content of the soil mixtures was brought up to field capacity daily with adequate 0.05% Aquasol® solution for 40 days before the first stress cycle commenced.

The first stress cycle consisted of leaving each pot unwatered for one day. Aquasol® solution (0.05%) was then added to bring each pot back up to field capacity. The next stress cycle consisted of leaving the pot unwatered for 2 days before bringing back up to field capacity. Increasing stress periods of 1, 2, 4, 5, 7, 9, 11, 11, 13, 13, 16, 20 and 25 days were used. The number of green leaves produced and dead leaves was counted regularly until each plant died. A plant was considered dead when all the leaves had totally lost green

colour or no new shoots emerged after watering. Surviving plants were subjected to the next stress cycle until all plants were dead.

At the death of each plant, the total number of leaves produced was tallied, the proportion of dead leaves at each counting date was calculated, the cumulative number of dead leaves was computed and the number of drying days at which 50% and 100% of the leaves had died was computed. Analysis of variance of the data on 50% and 100% leaf mortality was done using the STATVIEW statistical package and significant differences among means were determined using Scheffé's test at the 5% level of significance.

5.4.3 Results

5.4.3.1 50% Leaf mortality

The total number of drying days to 50% leaf mortality differed significantly ($P < 0.05$) with pot size (Table 5.4.1). Plants grown in large pots had a significantly greater number of total drying days, while those grown in small pots had a significantly lower total number of drying days to 50% leaf mortality. All the five *M. stipoides* populations had a significantly greater total number of drying days to 50% leaf mortality than *L. perenne* averaged over all pot sizes. There were no significant differences in the total number of days to 50% leaf mortality among the five *M. stipoides* populations averaged over all pot sizes. Pot size x *M. stipoides* population interaction had no significant effect on 50% leaf mortality.

Table 5.4.1. Cumulative number of drying days required to 50% leaf mortality of plants of five *M. stipoides* populations and *L. perenne* grown in three pot sizes. Within each group, means which share the same letter are not significantly different at $P > 0.05$.

Pot size	Population						
	M(Lpe)	M(Ppr)	M(Dgl)	M(Paq)	Popn 1	*L. perenne*	Mean
Small (100 mm D)	22.1	24.4	30.4	26.2	25.4	10.1	23.1[c]
Medium (125 mm D)	32.3	34.2	27.1	35.3	33.7	23.3	31.0[b]
Large (150 mm D)	41.4	40.4	49.5	47.6	42.1	26.9	41.3[a]
Mean	31.9[a]	33.0[a]	35.6[a]	36.4[a]	33.7[a]	20.1[b]	

5.4.3.2 100% Leaf mortality

Plants grown in large pots had a significantly ($P < 0.05$) greater total number of drying days to 100% mortality than when grown in medium and small pots averaged over all *M. stipoides* populations and *L. perenne* (Table 5.4.2). At each pot size, there were no significant differences in the days to 100% leaf mortality among any of the populations (Table 5.4.2). However, when comparisons of the total number of drying days to 100% leaf mortality were done for each population across all three pot sizes, significant increases were exhibited by M (Dgl) and M (Paq) when grown in the large pots compared with when grown in the small and medium pots (Table 5.4.2). Significant differences in the total number of drying days to 100% leaf mortality were observed in M (Lpe) and *L. perenne* when grown in large pots compared with when grown in small pots (Table 5.4.2). There were no significant differences in the total number of drying days to 100% leaf mortality of M (Ppr) and Population 1 when grown in all three pot sizes (Table 5.4.2). Such pot size x population interaction differences were not detected in the total number of drying days to 50% leaf mortality (Table 5.4.1).

Table 5.4.2. Cumulative number of drying days required to 100% leaf mortality of plants of five *M. stipoides* populations and *L. perenne* grown in three pot sizes. Means which share the same letter(s) are not significantly different at $P > 0.05$. Comparisons are made in both directions except for the means.

Pot size	Population						
	M(Lpe)	M(Ppr)	M(Dgl)	M(Paq)	Popn 1	*L. perenne*	Mean
Small (100 mm D)	30.2^{d}	37.9^{cd}	43.5^{cd}	42.1^{cd}	41.8^{cd}	25.5^{d}	36.8^{b}
Medium (125 mm D)	40.2^{cd}	52.6^{abc}	42.4^{cd}	46.6^{cd}	48.2^{bcd}	34.2^{cd}	44.0^{b}
Large (150 mm D)	78.4^{abc}	64.8^{abc}	97.8^{a}	94.5^{ab}	74.0^{abc}	53.3^{abc}	77.1^{a}
Mean	49.6^{ab}	51.8^{ab}	61.2^{a}	61.1^{a}	54.7^{a}	37.6^{b}	

5.4.4 Discussion

The significantly lower total number of drying days to 50% leaf mortality of *L.*

perenne compared with all five *M. stipoides* populations and to 100% leaf mortality compared with M (Dgl), M (Paq) and Population 1 could be attributed to the rapid foliar production of *L. perenne* during the initial stages of growth. *Lolium perenne* plants had a larger number of leaves during the initial stages of water stress development, thereby losing more water and reaching its lethal leaf water potential much earlier than *M. stipoides* plants.

The total number of drying days to 50% and 100% leaf mortality increased significantly with an increase in pot size averaged over all populations. Harradine and Whalley (1979) found that plants of *Aristida ramosa, Danthonia linkii* and *Dichanthium sericeum* grown in larger pots had the longest drying times. They observed slower development of initial water stress in the larger pots, giving the plants ample time to adjust physiologically as the water stress developed. There were significant variations in the increase in total number of drying days to 100% leaf mortality among three of the five *M. stipoides* populations when grown in the three pot sizes. This indicates that there were significant variations in physiological adjustment to water stress among the five populations in that M (Dgl), M (Paq) and M (Lpe) were able to adjust whereas M (Ppr) was not because the drying days to 100% leaf mortality were not significantly different for any of the pot sizes.

Physiological adjustment to developing water stress may involve several characteristic plant adaptations. Ludlow and Muchow (1990) stated that improved response to water stress cannot be attributed to a single plant characteristic. Responses to water stress of the five *M. stipoides* populations studied when growing in the field could be a combined result of any of the following avoidance or tolerance strategies, which include extensive root system development (O'Toole and Bland 1987, Johnson and Asay 1993), leaf area reduction (Schulze 1988), leaf rolling, leaf orientation, stomatal closure, development of epicuticular wax, thickening of the epidermal cell wall (Johnson and Asay 1993), adjustment of the osmotic pressure in the cell sap (Turner 1986, Ludlow and Muchow 1990), and dehydration tolerance of the leaf tissues (Ludlow 1989).

Microlaena stipoides (Dgl) and M (Paq) showed a significant increase in the total number of days to 100% leaf mortality when grown in small or medium sized pots compared with large pots. *Microlaena stipoides* (Lpe) showed a significant increase between small and large pots, whereas M (Ppr) showed no significant increase at all. *Microlaena stipoides* (Dgl) and M (Paq) were both collected from the upper slope of the paddock, which perhaps dries out faster than the areas on the lower slope such as the *P. pratensis* and *L. perenne* sites, where M (Ppr) and M (Lpe) were collected, respectively. The *D. glomerata* and *P. aquatica* sites where M (Dgl) and M (Paq) were collected were stony, while the *P. pratensis*

site was loamy and the *L. perenne* site was clayey. *Dactylis glomerata* and *P. aquatica* sites perhaps retain less moisture than the other two sites.

Poa pratensis has been reported to be sensitive to low soil moisture content (Bennett *et al.* 1972), producing a significantly lower number of tillers on the top of hills, where soil moisture levels are lower than in hollows. *Dactylis glomerata* has been reported to be suitable for dry regions (Levy 1970), with more successful establishment than *L. perenne* on dry hills (White *et al.* 1972, Barker *et al.* 1993), and had greater yields than *L. perenne* after moisture stress (Wedderburn *et al.* 1993). *Phalaris aquatica* has high persistence during drought (Robinson 1952) and can also avoid drought by becoming dormant (McWilliam and Kramer 1968).

In summary, among the four associated grasses, *D. glomerata* and *P. aquatica* were reported to persist under water stress, while *L. perenne* and *P. pratensis* were reported to be sensitive to water stress. The two *M. stipoides* populations growing in association with *D. glomerata* and *P. aquatica*, M (Dgl) and M (Paq) respectively, showed a significant increase in total number of days to 100% mortality when grown in larger pots compared with their total number of days when grown under medium or small pots. In addition, they had the greatest number of days to 100% leaf mortality although these differences were not significant ($P > 0.05$). *Microlaena stipoides* (Lpe) also exhibited some adjustment to the slower rate of water stress development in the larger pots, whereas with M (Ppr) the increase in days to 100% leaf mortality was not significant and it also had the lowest number of days to 100% leaf mortality in the largest pots, although the differences were not significant ($P > 0.05$). These findings indicate some genetic differences among the four populations which could have evolved through natural selection resulting from competitive interactions with their associated perennial grasses or from selection pressures associated with differences in water availability at the locations from which they were collected.

5.5 GENOTYPIC VARIATION AMONG *Microlaena stipoides* POPULATIONS. I. DNA AMPLIFICATION FINGERPRINTING

5.5.1 Introduction

Genetic variation within populations is a prerequisite for adaptation and evolutionary change (Ennos 1983). Measures of genetic variation include differences in the genetic composition such as altered DNA sequences, altered protein composition, altered chromosome constitution, and differences in phenotypes. Differences in phenotypes can result from changes in one or more genes or modifier loci (Gottlieb 1981, Hilu 1983, Doebley and Wendell 1989). Britten (1986) reported that alterations in the DNA sequence arising from substitutions, insertions, deletions, and rearrangements result in phenotypic variation since such changes affect genes or their regulation and influence biochemistry, development, morphology and behaviour.

Ennos (1983) reviewed several factors that maintain genetic variation in a population. Among these are disassortative mating, meiotic drive, alternate selection of an allele in the gametophyte and sporophyte generations, temporal and spatial heterogeneity, multiple niche selection, intergenotypic competition arising from annidation (two components occupying slightly different niches), apostatic selection by herbivores and plant-pest interactions.

Evidence for multiple niche selection has been generated from studies of perennial plant communities that partitioned the habitat into different niches characterised by significantly different selection pressures. For example, Snaydon (1970) found significant genotypic differences among populations of *Anthoxanthum odoratum* subjected to varying nutrient regimes and cutting treatments. Similarly, differences in stolon length of *Agrostis stolonifera* were highly correlated to wind exposure (Aston and Bradshaw 1966).

Turkington and Harper (1979c) found strong evidence of genotypic variation among *Trifolium repens* populations that were growing in association with four different perennial grasses in a permanent pasture. This is a case in which associated grass species provided a different niche within which there was selection of *T. repens* clones (Ennos 1983).

The four populations of *M. stipoides* studied here, growing in association with four different perennial grasses in permanent pasture, differed in phenotypic characters such as leaf length, leaf width, tiller and panicle production. Differences in behavioural characteristics were also observed among some of the populations, including growth under

varying light intensities and drought tolerance. This study aimed to investigate the amount of genotypic differentiation among these four populations of *M. stipoides*.

DNA amplification procedures based on polymerase chain reaction (PCR) such as random amplified polymorphic DNA (RAPD) markers (Kazan *et al* .1993a), DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al.* 1991), and arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh *et al.* 1991) are recent advances in DNA fingerprinting. The techniques do not require prior knowledge of DNA sequences for designing appropriate oligonucleotide primers (Halward *et al.* 1992), are claimed to be independent of the amount of DNA template, and do not require labelling of probes (Caetano-Anolles *et al.* 1991) and provide quick, efficient and economical methods for detection of differences in genotypes. DNA polymorphism is identified as the presence or absence of an amplification product on an ethidium bromide-stained gel (Kazan *et al.* 1993b). DNA amplification fingerprinting is simple and rapid as it utilises only one oligonucleotide primer, of random sequence, in the amplification of DNA by polymerase chain reaction (Williams *et al.* 1990, Caetano-Anolles *et al.* 1991, Kazan *et al.* 1993a).

The original polymerase chain reaction (PCR) process is an *in vitro* process of enzymatically amplifying specific genomic DNA directed by a pair of highly specific oligonucleotide primers (Caetano-Anolles *et al.* 1991, Taylor 1991). Components of the polymerase chain reaction mixtures include deoxynucleotide triphosphates to provide energy and nucleosides for the synthesis of new strands of DNA, DNA polymerase for DNA synthesis, usually two, sometimes one oligonucleotide primer to direct extension of complementary strands of DNA, DNA template and a buffer containing magnesium (Taylor 1991). Conventional PCR requires prior knowledge of the DNA sequence, DNA hybridisation and enzymatic conditions for the specific amplification of a known target sequence with a pair of tailored primers (Caetano-Anolles *et al.* 1991). Random amplified polymorphic DNA, DNA amplified fingerprinting (DAF) and arbitrarily primed-polymerase chain reaction (AP-PCR) are similar to PCR in that they involve enzymatic amplification of DNA segments, but they require only one oligonucleotide primer and the primers flank multiple unspecified priming sites on the DNA strand (Caetano-Anolles *et al.* 1991, Halward *et al.* 1992, Kazan *et al.* 1993a).

Caetano-Anolles *et al.* (1991) used a DNA amplification fingerprinting procedure to successfully generate DNA fingerprints of a wide range of organisms including *Bradyrhizobium* sp., *Azolla amma*, *Candida albicans*, *Cynodon dactylon*, *Eremochloa ophiuroides*, *Cornus florida*, *Oryctolagus cuniculus*, *Macaca mulatta* and *Homo sapiens*. Kazan *et al.* (1993a, b) employed the RAPD technique in determining genetic relationships of taxa within the *Stylosanthes guianensis* complex. They found good agreement between

the genetic similarities determined by RAPDs and morphological and agronomical properties of *Stylosanthes guianensis* (Kazan *et al.* 1993a). Fritsch and Rieseberg (1992) found that RAPD markers are useful in the estimation of outcrossing in *Datisca glomerata,* particularly when isozymes fail to provide sufficient polymorphism. Results of experiments using single primer DNA amplification conducted by Halward *et al.* (1991, 1992) showed that segments of peanut genomic DNA can be successfully amplified using short primers of arbitrary sequence. The results were useful for detecting polymorphism among wild peanut species and can be used in band sharing analyses to generate dendrograms of species relationships. However, they found that the markers produced from arbitrarily primed PCR for genetic mapping studies in peanut were inadequate in separating homozygotes from heterozygotes (Halward *et al.* 1992). Welsh *et al.* (1991) used AP-PCR to identify inbred parents of hybrid maize plants but also found difficulty in distinguishing heterozygotes acurately.

This study was designed to detect polymorphic differences in the amplified DNA segments of the four *M. stipoides* populations and another species, *Lolium perenne* using the DNA amplification fingerprinting technique, so that the amount of genotypic differentiation among these populations could be determined. The study was also conducted to develop an appropriate DNA amplification methodology based on PCR for *M. stipoides* and *L. perenne.*

5.5.2 Materials and methods

5.5.2.1 DNA extraction

Leaves of four *M. stipoides* populations and of *L. perenne* were harvested, washed with distilled water and stored in a freezer at -70° C. Plant genomic DNA was extracted following a procedure modified from Guidet *et al.* (1991). Leaves were cut into small pieces and ground in liquid nitrogen using a mortar and pestle. The powdered leaves were transferred to 10 ml plastic centrifuge tubes and suspended in 4 volumes (e.g. 4 ml/g powdered leaf) of extraction buffer (0.1 M Tris-HCl, pH 8, 10 mol m^{-3} EDTA, 4% sarkosyl), then an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was shaken end over end (20 rpm) for 1 h at 4° C. The tubes were centrifuged for 5 min at 7,000 rpm and 4° C, then the organic layer was discarded and the aqueous layer and interphase containing DNA were re-extracted as described above. Following centrifugation for 5 min at 7,000 rpm and 4° C, the aqueous layer was transferred to another tube and extracted by shaking end to end with an equal volume of chloroform-isoamyl alcohol (24:1) for 1 h at 4° C. After centrifigation (7,000 rpm, 4° C for 5 min), the

aqueous layer was transferred into another centrifuge tube and genomic DNA was precipitated with 0.1 volume of 3M Na acetate (pH 4.8), and then 2.5 volumes of 99% ethanol was layered on top of the solution. The mixture was carefully hand-shaken end over end for 1 min and then centrifuged for 15 min at 15,000 rpm and 4° C. Ethanol was carefully removed using a pasteur pipette and the precipitated DNA was washed three times with 2 ml of 70% ethanol and dried under vacuum for 5 min. DNA was redissolved in 300 µl of TE buffer (10 mol m^{-3} Tris-HCl, 1 mol m^{-3} EDTA, pH 8.0).

DNA concentrations and purity were determined using a UV spectrophotometer and electrophoresis through 1.5% agarose gel. A 1 µl aliquot of DNA solution was diluted to 100 µl with sterile water and UV absorbance was determined using a UV spectrophotometer. One µl of DNA solution was diluted to 10 µl with 8 µl sterile water and 1 µl loading buffer and was electrophoresed through 1.5% agarose gel using TBE (Tris-borate-EDTA) buffer. The DNA was further purified of RNA impurities by RNase digestion. Ten µl of RNase (10ng/µl) was added to the DNA solution and incubated at 35° C for at least 1 h. The DNA was re-precipitated by adding 0.1 volume of 3M Na acetate, mixing well and adding 2.5 volumes of 99% ethanol. After centrifugation for 10 min at 13,000 rpm in a microfuge, ethanol and Na acetate were removed using a pasteur pipette. DNA was rinsed three times with 70% ethanol and dried under vacuum for 5 min. DNA was redissolved in 50 µl of TE buffer and 100 µl of sterile water was added.

5.5.2.2 DNA amplification fingerprinting (Random amplified polymorphic DNA)

Amplification of DNA fragments was performed in a polymerase chain reaction (PCR) mixture consisting of 2 µl 10 X PCR reaction buffer, 2 µl 25 mol m^{-3} $MgCl_2$, 0.25 µl 16 mol m^{-3} each of dATP, dCTP, dTTP, and dGTP (or 1 µl 16 mol m^{-3} dNTPs), 2 µl 20 mol m^{-3} primer, 1 µl *Thermus aquaticus* DNA polymerase (1 unit/µl), 2 µl template DNA and 10 µl sterile water. The reaction was overlaid with a drop of mineral oil to prevent evaporation of the reaction mixture when heated at 94° C and the mixture was incubated in a Gene Machine® thermocycler (Bartelt Instruments, Melbourne, Australia).

The Bresatec® 10 X PCR reaction buffer contained 0.67 M Tris, 0.166 M $(NH_4)_2SO_4$, 4.5% Triton X and 2 mg/ml gelatin. The non-ionic detergent (Triton X) and gelatin are essential to obtain maximum processivity of the enzyme (Taylor 1991).

Several trials were performed to develop a reproducible DNA amplification procedure. The combination of oligoprimers, number, temperatures and lengths of time for denaturing,

annealing and extension in each amplification cycle were identified. In the first three trials (Table 5.5.1), oligoprimer I was used as a primer and two concentrations (2 and 0.2 ng/µl) of genomic DNA from one of the *M. stipoides* populations, M(Lpe), was added in the PCR reactions. PCR products were separated by electrophoresis through gels, using TBE buffer, followed by staining with ethidium bromide and viewing under UV light.

Table 5.5.1. Combination of number, temperatures and lengths of time for denaturing, annealing and extension in each amplification cycle in the first three trials.

Trial	No. of cycles	Denaturing		Annealing		Extension		Separation gel
		Temp (° C)	Time	Temp (° C)	Time (sec)	Temp (° C)	Time (sec)	
1	35	94	2 min	29	60	72	60	1.5% agarose
2	10	94	5 min	29	60	72	90	
then	25	94	2 min	29	60	72	60	4% acrylamide
3	35	94	20 sec	29	30	72	60	1.5% agarose

The fourth trial was done to determine the best primers for amplification of *M. stipoides* genomic DNA. Five different nucleotides including oligoprimer I, 12MER1 (5'-TGATAGTGTG GG-3'), 12MER2 (5'-GGCCTCGCCT AC-3'), 12MER10 (5'-GTTGCCGCCG GT-3'), and 12MER11 (5'-GTTGCCACCG GT-3') and three concentrations (2, 0.2 and 0.02 ng DNA/µl) of template DNA (M (Lpe))were used in the PCR reactions. Genomic DNA was denatured at 94° C for 5 min, annealed at 29° C and polymerased at 72° C for 60 sec in the first cycle. This was followed by amplification of the DNA fragments consisting of 45 cycles of denaturation at 94° C for 20 sec, annealing at 29° C for 30 sec and polymerisation at 72° C for 60 sec. The PCR products were separated by electrophoresis through 1.5% agarose gel using TBE buffer at 80 V for 1.5 h.

The fifth trial was to determine the ideal concentration of template DNA (0.5, 1, 2 and 4 ng DNA/µl). The same combination of amplification steps used for testing 12MER1 and 12MER10 oligoprimers was used in the PCR reactions. PCR products were separated as above.

The sixth trial examined the effect of $MgCl_2$ concentration in the PCR reactions. The $MgCl_2$ concentrations used were 1, 1.25, 1.5, 1.75, 2 and 2.25 µl of 25 mol m^{-3} stock solution of $MgCl_2$. Each PCR reaction consisted of 2 µl buffer, 2 µl oligoprimer 12MER10,

1 µl 16 mol m^{-3} dNTPs, 1 µl Taq polymerase enzyme, varying concentrations of $MgCl_2$, template DNA (1, 2 and 4 ng/µl) and sterile water to make up to 20 µl final volume. The first cycle involved denaturation at 94° C for 5 min, annealing at 31° C for 30 sec and polymerisation at 72° C for 60 sec. This was followed by 44 cycles of denaturation at 94° C for 20 sec, annealing at 31° C for 30 sec and polymerisation at 72° C for 60 sec. PCR products were separated as above.

The seventh trial involved PCR reactions consisting of 2 µl $MgCl_2$, 2 µl buffer, 2 µl oligoprimer 12MER10, 1 µl 16 mol m^{-3} dNTPs, 1 µl Taq polymerase, 5 µl DNA template (0.4 and 0.2 ng/µl) and 7 µl sterile water. The first cycle of DNA amplification consisted of denaturation at 94° C for 5 min, annealing at 35° C for 30 sec, polymerisation at 72° C for 30 sec followed by 44 cycles of denaturation at 94° C for 20 sec, annealing at 35° C for 30 sec and polymerisation at 72° C for 30 sec. PCR products were separated on a 1.5% agarose gel electrophoresis at 80 V for 1.5 h as above.

The technique was further refined by increasing the annealing temperature to 37° C. Basically, the same composition of the PCR reactions was prepared using 1 and 2 ng/µl of template DNA. The DNA amplification cycle was: denaturation at 94° C for 5 min, annealing at 37° C for 30 sec and polymerisation at 72° C for 60 sec in the first cycle followed by 44 cycles of denaturation at 94° C for 20 sec, annealing at 37° C for 30 sec and polymerisation at 72° C for 30 sec. Fragments of genomic DNA of all the populations of *M. stipoides* and *L. perenne* were amplified following this protocol.

5.5.3 Results

In the first three trials where oligoprimer I was used as a primer and a maximum of 35 cycles was employed in the amplification of DNA fragments from genomic DNA of *M. stipoides* population M(LPe), no amplified products were visible in the ethidium bromide-stained agarose gel. When the number of amplification cycles was increased to 45, and with DNA template concentrations of 0.02 and 0.2 ng/µl, a few DNA bands were observed on the agarose gel using oligoprimer I. Four other oligonucleotides were used as primers in the fourth trial and three of them gave visible bands on the gel after amplification for 45 cycles (Fig. 5.5.1). The number of bands of amplified fragments increased with increasing concentrations of template DNA when oligonucleotide 12MER1 (5'-TGATAGTGTG GG-3') was used as the primer in the PCR reactions (Fig. 5.5.1). With 12MER2 (5'-GGCCTCGCCT AC-3'), more bands of amplified DNA fragments were observed at 2.0 ng/µl template DNA concentration, while there was only one band at 0.02 ng/µl and three bands at 0.2 ng/µl template DNA concentration. PCR reactions containing the primer

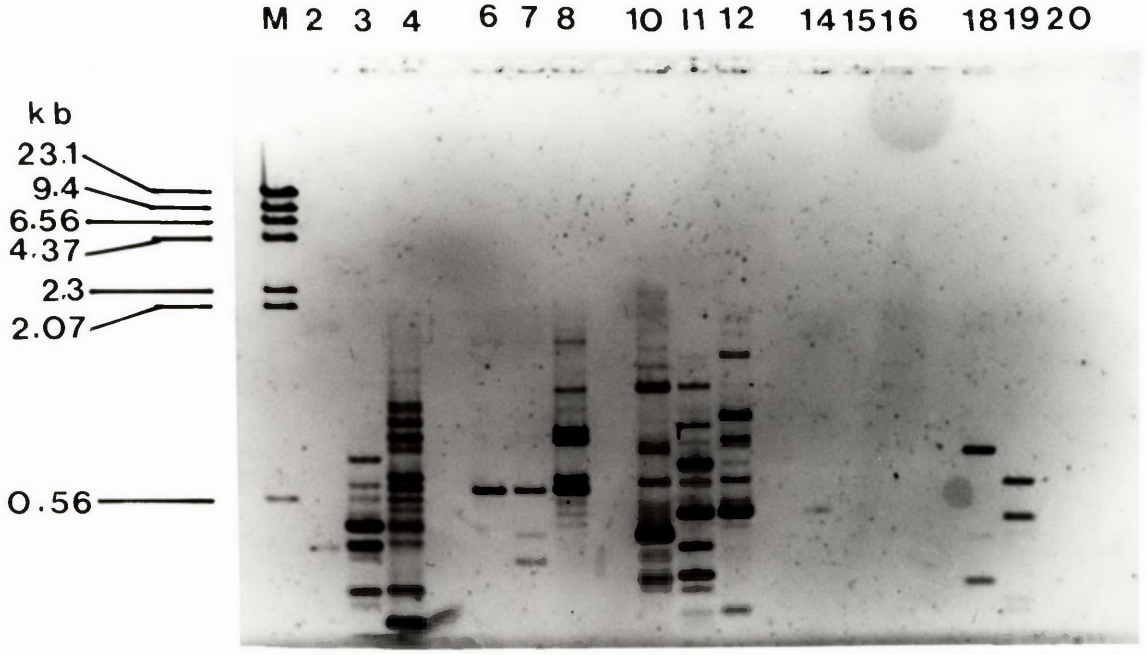
oligonucleotide 12MER10 (5'-GTTGCCGCCG GT-3') gave a consistently high number of visible bands of amplified DNA fragments at all three concentrations of template DNA, while those containing oligonucletide 12MER11 (5'-GTTGCCACCG GT-3') gave no visible band of amplified DNA fragments at any concentration of template DNA (Fig. 5.5.1).

The two promising oligonucleotides, 12MER1 and 12MER10, were selected and used in the fifth trial in which the concentration of template DNA was varied (0.5, 1, 2 and 4 ng/µl). The oligonucleotide 12MER10 gave a consistently high number of bands of amplified DNA fragments at all four concentrations of template DNA, while 12MER1 gave an increasing number of DNA bands with increasing concentration of template DNA (Fig. 5.5.2). The location and intensity of DNA bands in the lanes where 12MER10 was used as the primer, however, were not reproducible at all concentrations of template DNA.

The concentration of $MgCl_2$ in the PCR mixtures was very crucial in the amplification of DNA fragments and the sixth trial involved variation of the $MgCl_2$ concentration. No DNA fragments were amplified when the concentration of $MgCl_2$ added in the PCR mixture was reduced to 1.25 mol m^{-3}. There was poor reproducibility of amplified DNA bands in all three template DNA concentrations at concentrations of $MgCl_2$ less than 2.5 mol m^{-3} (Fig. 5.5.3). At 2.81 mol m^{-3} $MgCl_2$, the number of amplified bands increased with an increase in concentration of template DNA.

The concentration of $MgCl_2$ in the PCR reactions in the next trial was 2.5 mol m^{-3} and the annealing temperature in the amplification cycles was increased to 35° C. There was marked improvement in the reproducibility of bands of the amplified DNA fragments for three replicates at 1 and 2 ng template DNA (Fig. 5.5.4).

The technique was further refined by increasing the annealing temperature 2° C higher to 37° C. Very good, reproducible results were obtained from the bands of the amplified DNA fragments at two concentrations of template DNA (1 and 2 ng/µl; Fig. 5.5.5). The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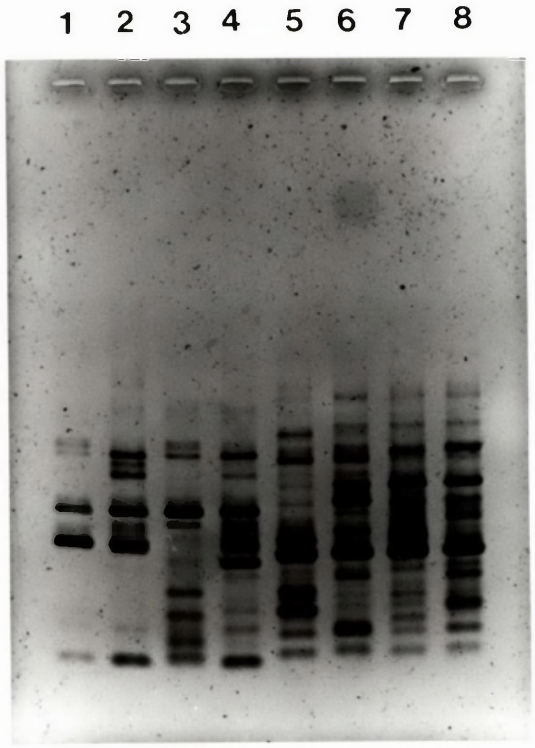
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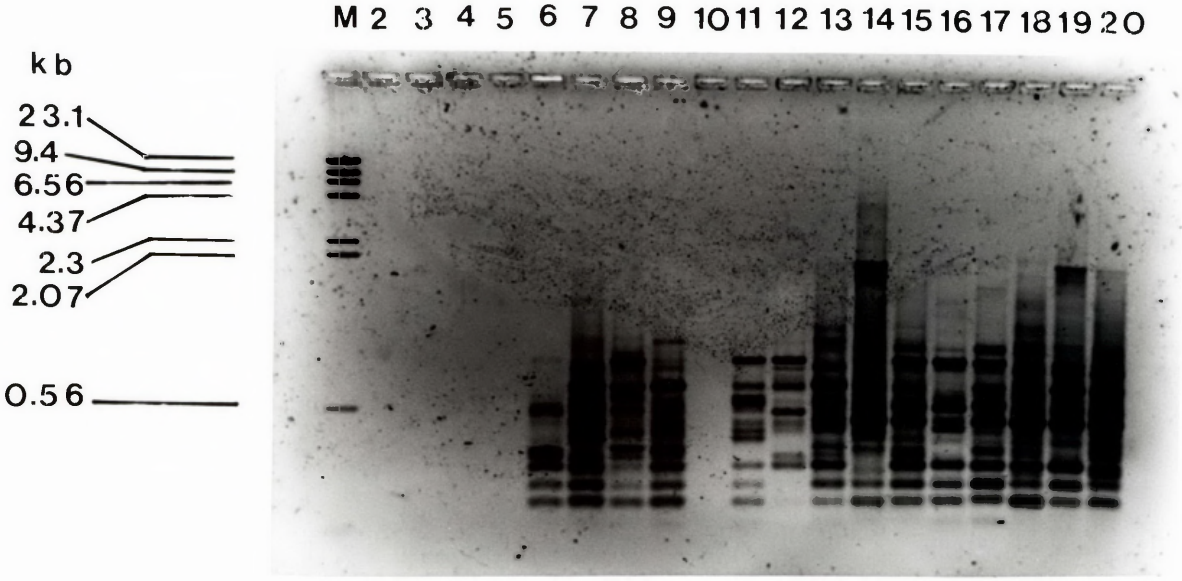
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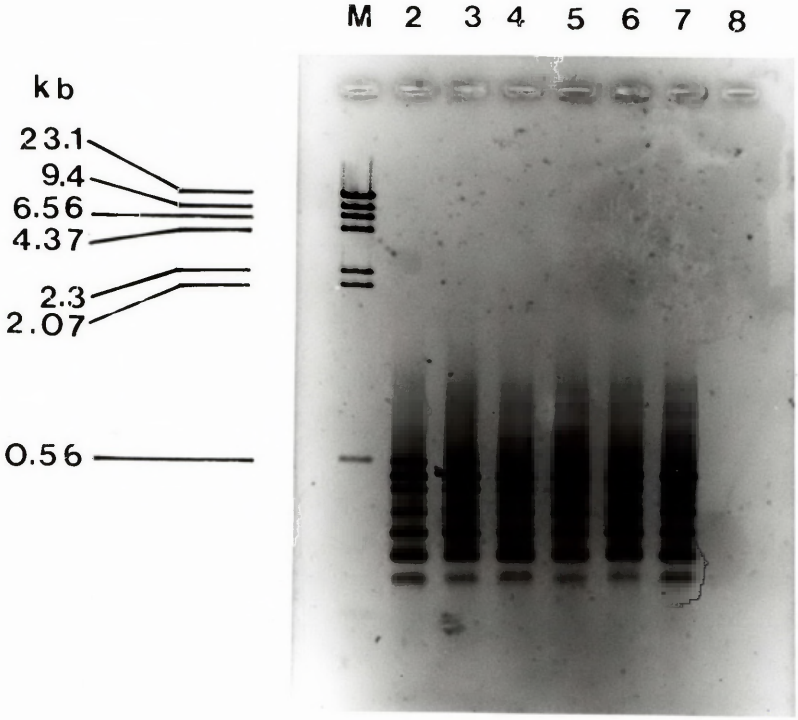
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Fig. 5.5.3. Effect of $MgCl_2$ concentration on the polymorphic patterns detected in *M. stipoides* (Lpe) genome amplified with primer 12MER10 using 3 levels of genomic DNA concentration: 1, 2 and 4 ng/µl. Lanes 3-5: 1.25 mol m^{-3} $MgCl_2$ lanes 6-8: 1.56 mol m^{-3} $MgCl_2$, lanes 9-11: 1.92 mol m^{-3} $MgCl_2$, lanes 12-14: 2.19 mol m^{-3} $MgCl_2$, lanes 14-16: 2.5 mol m^{-3} $MgCl_2$, lanes 18-20: 2.81 mol m^{-3} $MgCl_2$.

Fig. 5.5.4. Effect of increasing the annealing temperature to 35° C on the polymorphisms detected in genomic DNA from *M. stipoides* (Lpe) amplified with 12MER10. Lanes 2-4, triplicate reaction: 1 ng DNA/reaction, lane 5-7, triplicate reaction: 2 ng DNA/reaction.

technique used PCR reactions composed of 2 µl 25 mol m^{-3} $MgCl_2$, 2 µl buffer, 2 µl oligoprimer 12MER10, 1 µl 16 mol m^{-3} dNTPs, 1 µl Taq polymerase, 5 µl DNA template and 7 µl sterile water and DNA amplification cycles consisting of denaturation at 94° C for 5 min, annealing at 37° C for 30 sec and polymerisation at 72° C for 60 sec in the first cycle followed by 44 cycles of denaturation at 94° C for 20 sec, annealing at 37° C for 30 sec and polymerisation at 72° C for 30 sec. This was the standard protocol used in the subsequent assessment of random amplified polymorphic DNA markers among the four *M. stipoides* populations and *L. perenne*.

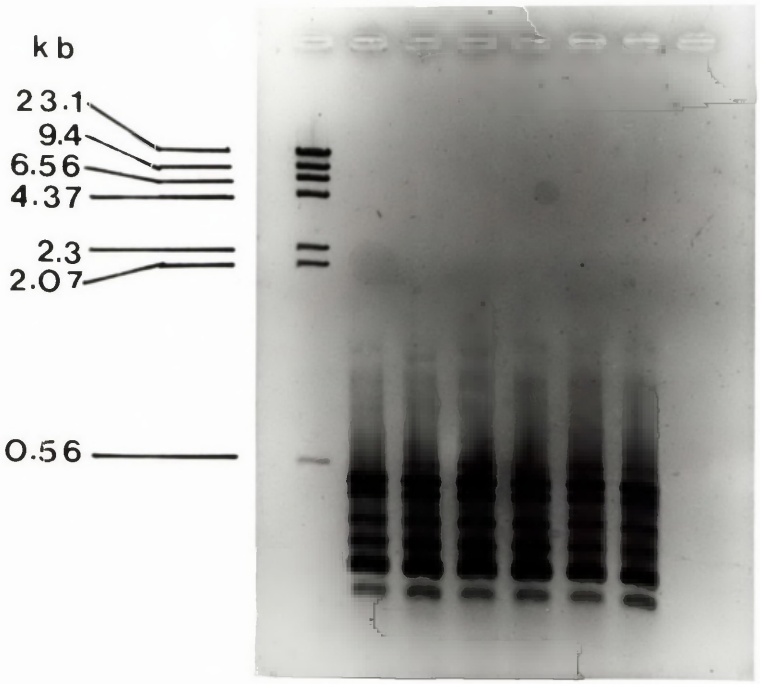
The DNA fingerprints of the four populations showed polymorphism although there were similarities in the location of three major conserved bands (labelled a, b and c; Fig. 5.5.6) and a minor conserved band (labelled ';'; Fig. 5.5.6) among the four populations. Among the four *M. stipoides* populations, M (Ppr) showed the greatest variation in the banding pattern of major and minor bands from the other three populations (Fig. 5.5.6). Two major variable bands (labelled f and g; Fig. 5.5.6) and a minor variable band (labelled h; Fig. 5.5.6) were present in M (Ppr) fingerprints, but not in the other three populations. Two minor bands (labelled d and e; Fig. 5.5.6) were present in the three *M. stipoides* populations, (M (Lpe), M (Dgl) and M (Paq)), but absent or very faint in the M (Ppr) banding pattern. A minor band (labelled i; Fig. 5.5.6) was distinctly present in the M (Dgl) and M (Paq) banding patterns and faintly present in the M (Lpe) banding pattern, but not in the M (Ppr) banding pattern. The other species, *L. perenne*, gave distinctly different banding patterns compared with those of the four *M. stipoides* populations both in the major and minor bands.

5.5.4 Discussion

Amplification of genomic DNA based on PCR using single primers of arbitrary sequence is currently used in detection of genetic polymorphism (Caetano-Anolles *et al.* 1991, Halward *et al.* 1992, Kazan *et al.* 1993 a, b). Initial trials in this study revealed that the number of amplification cycles was crucial in the amplification of DNA segments. With the *M. stipoides* genome, about 45 amplification cycles were sufficient to generate amplified bands in the agarose gel. After several cycles, the reaction products included copies of the segments of DNA flanked by the primers and the primers themselves (Taylor 1991). DNA amplification with arbitrary primers detects changes in the DNA sequence at sites in the genome which are defined by the primer used (Halward *et al.* 1992).

Fig. 5.5.5. Effect of increasing the annealing temperature to 37° C on the reproducibility of polymorphic banding patterns of genomic DNA from *M. stipoides* (Lpe) amplified with 12MER10. Lanes 2-4, triplicate reactions: 1 ng DNA/reaction, lanes 5-7, triplicate reactions: 2 ng DNA/reaction.

Fig. 5.5.6. Intraspecific polymorphisms among four *M. stipoides* populations and interspecific polymorphism between *M. stipoides* and *L. perenne* revealed by amplification with primer 12MER10. Each population is represented with four lanes: two replicates at each concentration: 1 and 2 ng/µl. Lanes 2-5: M (Lpe), lanes 6-9: M (Ppr), lanes 10-13: M (Dgl), lanes 14-17: M (Paq) and lanes 18-21: *L. perenne*.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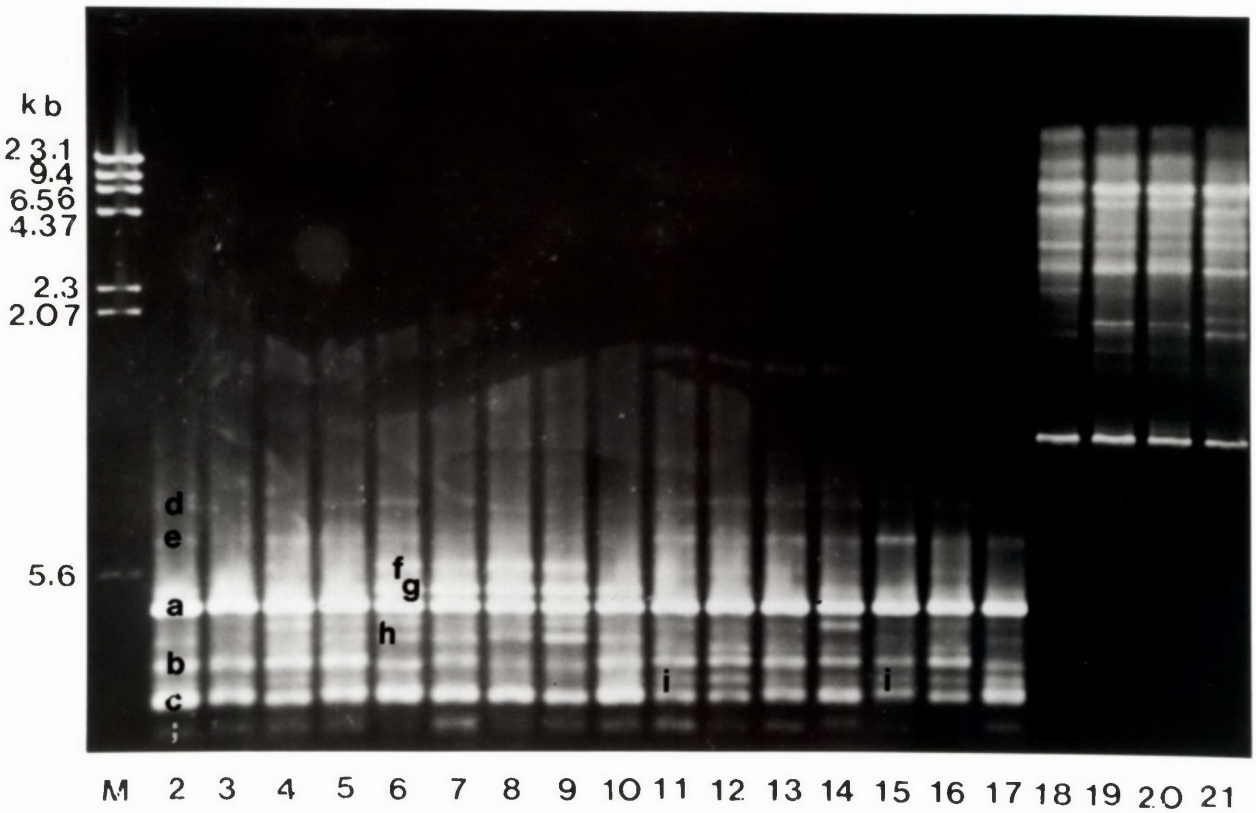
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Different primers resulted in different or variable bands of amplified DNA segments. Caetano-Anolles *et al.* (1991) found in their DNA fingerprinting study of a wide range of organisms that the spectrum of products changed with each primer and template combination. The use of a high G+C content (12MER10) primer with *M. stipoides* resulted in a consistently high number of amplified bands similar to the findings of Caetano-Anolles *et al.* (1991). Caetano-Anolles *et al.* (1991) reported that the number of amplification products obtained was not strictly related to the length of the primer used but rather to the particular sequence chosen. This is supported by the marked difference in amplified products between 12MER10 and 12MER11. The two primers differed only in the seventh base, where 12MER11 had Adenine instead of Guanine. There were no amplified products in the reactions in which 12MER11 was used as the primer.

The concentration of template DNA was critical in the amplification of DNA. The number of amplified bands was fewer at a low concentration of template DNA (0.5 ng/µl). Results also show variation of amplified minor bands at different concentrations of template DNA. These results are contrary to the findings of Caetano-Anolles *et al.* (1991) who found that the technique was independent of template DNA concentration. These discrepancies could arise from the fact that the primers used by Caetano-Anolles *et al.* (1991) were less prone to production of variable patterns. The features of each primer clearly need to be established before such assumptions can be made.

The results of the sixth trial demonstrated the crucial importance of the concentration of $MgCl_2$ in the amplification of DNA. At very low concentrations (1 µl of 25 mol m^{-3} $MgCl_2$), no amplification products were visible in the gel. Increasing concentrations of $MgCl_2$ resulted in an increased number of amplified bands. Kazan *et al.* (1993b) found that Mg^{2+} ion concentration was vital for successful amplifications. They reported that at lower Mg^{2+} concentrations (i.e. 2 mol m^{-3}), the number of fragments produced from a given primer was either drastically reduced or no fragments at all were produced and the number of bands resolved increased with higher concentrations (4 mol m^{-3}). High Mg^{2+} concentrations enhanced the stability of primer hybridisation to template DNA (Simon *et al.* 1991, Welsh *et al.* 1991, Kazan *et al.*1993b) and affected enzyme activity (Simon *et al.* 1991). Taylor (1991) noted a stoichiometric interaction between the dNTPs and magnesium. Higher concentrations of dNTPs bind to magnesium, thereby reducing the availability of magnesium.

Raising the annealing temperature to 37° C resulted in reproducible bands of amplified DNA. The patterns were not always identical though, and this raises questions about the significance of minor band variations in AP-PCR results. Taylor (1991) remarked that the selection of times, temperatures, and number of cycles depends on the DNA being amplified

and the primers chosen. He also added that in PCR reactions it is vital to ensure full denaturation of the template DNA before commencing amplification. It was also observed that agarose gel electrophoresis and ethidium bromide staining of amplification products detect fewer fragments than polyacrylamide gel electrophoresis (Caetano-Anolles *et al.* 1991).

The molecular basis for RAPD, DAF and AP-PCR may be the detection of changes in DNA sequence at arbitrary sites in the genome which are defined by the primer (Caetano-Anolles *et al.* 1991). These alterations could refer to either single base changes in the primer binding sites or insertions and deletions in the region between priming sites (Kazan *et al.* 1993a). These changes result in different numbers and lengths of amplified products and are not linked to a particular locus. Primer sites are randomly distributed along the target genome and primers flank both conserved and highly variable regions, thus both phylogenetically conserved and individual-specific bands are amplified (Caetano-Anolles *et al.* 1991). Each amplified fragment represents an allele (Kazan *et al.* 1993a).

DNA fingerprints of the four *M. stipoides* populations exhibited amplified fragments common to all populations which may indicate the phylogenetically conserved regions as well as individual-specific bands. *M. stipoides* (Ppr) showed greater variation in the banding patterns compared with the other three populations, which may indicate a more advanced genetic change or microevolution. Distinct DNA polymorphism between *M. stipoides* populations and *L. perenne* clearly distinguishes that the latter is a different species. These genetic diferences among populations of *M. stipoides* parallel their morphological and behavioural differences.

5.6 GENOTYPIC VARIATION AMONG *Microlaena stipoides* POPULATIONS. II. ASSESSMENT OF DNA-DNA HYBRIDISATION TECHNIQUE

5.6.1 Introduction

Comparison of the degree of DNA sequence homology by means of DNA-DNA hybridisation is a widely used and effective method of determining genetic relatedness. Nucleic acid hybridisations yield quantitative data on genotypic relationships as they enable one to compare the proportion of genes shared by different species (Entingh 1970) and to measure differences at the level of nucleotide substitutions (Bot *et al.* 1989). It has been employed in the phylogenetic studies of bacterial strains (Hudman and Gregg 1989, Adnan *et al.* 1993), *Atriplex* sp. (Belford and Thompson 1981a, b), humans (Britten 1986), *Drosophila* sp. (Laird and McCarthy 1968, Entingh 1970, Zwiebel *et al.* 1982), *Cladophora* sp. (Bot *et al.* 1989), rodents (Santiago and Rake 1973, Brownell 1983), sea urchins (Britten *et al.* 1978), primates and avians (Brownell 1983), *Gymnobelideus leadbeateri* (Edwards and Westerman 1992), and wheat (Bendich and McCarthy 1970a, b).

The molecular hybridisation technique involves annealing *in vitro* single-stranded DNA sequences from two species and the hybrid duplexes are subjected to thermal dissociation to determine divergence of the base sequences (Belford and Thompson 1981b). Several methodologies have been developed to determine DNA-DNA hybridisation, including nitrocellulose binding, renaturation techniques, microdilution plate and photobiotin labelling (Adnan *et al.* 1993). Nitrocellulose binding consists of annealing a labelled hybridisation probe with another unlabelled nucleic acid that had been immobilised onto nitrocellulose filter. Nitrocellulose filter efficiently binds single-stranded nucleic acid (Ivanov and AbouHaidar 1992) and is a widely used membrane filter. The constructed hybrid is separated by thermal denaturation (Brownell 1983) and thermal stability profiles of DNA hybrids are plotted (Belford and Thompson 1981a) to determine the degree of base pair mismatch between two pools of DNA (Belford and Thompson 1981b, Ruvolo and Smith 1986).

The nitrogenous bases (adenine, cytosine, guanine and thymine) are arranged in complementary pairs and held together by hydrogen bonds in the native DNA. Under physiological salt concentrations, the double stranded structure can be denatured only at high temperature levels (95° C and above) giving off single stranded DNA. Variation from the native complementarity of the double stranded DNA lowers this melting point by an interval proportional to the base pair mismatch (Brownell 1983). Several studies have shown that on

average, 1% base pair mismatch corresponds to 1.0-1.5° C depression (Marmur and Doty 1962, Britten and Bonner 1970, Kohne *et al.* 1971, McCarthy and Farquhar 1972, Britten *et al.* 1974, Angerer *et al.* 1976, Stein *et al.* 1979). The changes in the melting temperature of the hybridising fraction of the genomes can be used as indices of heteroduplex similarity, and of genetic relatedness (Marmur and Doty 1959), and is an accurate linear measure of the degree of base pair mismatch between the pairs of homologous DNAs being compared (Powell *et al.* 1993).

DNA-DNA hybridisation experiments entail all of a genome's single copy DNA with at least 10^8 base pairs (Ruvolo and Smith 1986) as well as repeat sequences if unfractionated genomic DNA is used. Hybrids formed from single-copy DNA are composed only of sequences from the same ancestral sequence and thus should provide a convincing gauge of lineage (Belford and Thompson 1981b). Single-copy DNA is the fragment containing one copy of all genomic sequences, including at least one copy of each repeated DNA sequence. It may contain 95-98% of the total genomic sequence complexity, although it may only be 60% of the total DNA (Britten 1971). Belford and Thompson (1981a) observed that 80-100% of all single copy DNA within a genus is represented in every species with 0-3% modification in the base sequence.

Bot *et al.* (1989) found remarkably high interspecific divergence in the genus *Cladophora* when he evaluated DNA sequence divergence between *Cladophora sericea* isolates from France, Norway, Iceland and East Canada by means of DNA-DNA hybridisations. Bendich and McCarthy (1970b) found minimal differences in the thermal stabilities of DNA-DNA heteroduplexes when only the fast annealing 11% of homologous DNA was measured. However, large differences in thermal stabilities were observed when 95% of homologous DNA strands were reassociated, indicating base sequence divergence in the major fractions of the various genomes of hexaploid, tetraploid and diploid wheat and *Aegilops squarrosa.*

Separation of populations of organisms into genetically isolated subgroups leads to accumulation of differences in the DNA of the subgroups. These differences could arise from unpredictable events including deletions or amplifications of large DNA fragments and from a clocklike process of point mutations altering single base pairs (Wilson *et al.* 1977). Brownell (1983) postulated that large variation in interspecific hybridisation values may have resulted from a series of genomic deletion events during the evolution of different lineages.

DNA-DNA hybridisations also yield data that could determine the nature of genetic change between species. Zwiebel *et al.* (1982) and Ruvolo and Smith (1986) found that DNA-DNA hybridisation could demonstrate the presence of quickly-evolving single-copy

DNA and the regular, slow accumulation of mutation in *Drosophila* species. Entingh (1970) found it interesting that a fraction of the eukaryotic DNA reanneals rapidly with the rest of the DNA in interspecific hybridisation.

Entingh (1970) investigated intraspecific and interspecific DNA/DNA duplex formation in the genus *Drosophila* using hybridisation experiments. Entingh (1970) determined the fast annealing fragment of various *Drosophila* spp. DNA by incubating filterbound DNA with labelled DNA at 65° C for 16 h. After washing the filter with 3 X SSC and drying overnight, the radioactivity in the filter was counted in a liquid scintillation counter. The slow annealing fraction was determined by incubating the remaining DNA with fresh filterbound DNA for a specified number of days after dialysing, lyophilisation, adjusting the concentration to 3 X SSC and denaturation of the remaining labelled DNA. After incubation, the fresh filters were washed and counted as described above. The proportion of fast annealing DNA fragments bound to homologous filterbound DNA reached a maximum of 20% after a 12 h incubation. Fast annealing DNA sequences consist of repeated nucleotide sequences (Entingh 1970).

Higher variation was detected in many mammalian species when the slow annealing DNA fractions were compared instead of the fast annealing fragments. Entingh (1970) postulated that changes in slow annealing sequences occur more rapidly than in the fast annealing sequences and that mispairing is likely to happen when a number of similar sequences are present, a phenomenon inherent in the technique.

Entingh (1970) reported that the degree of similarity among different species derived from DNA comparisons was reflected in the degree of morphological similarities. Therefore, greater nucleotide sequence similarity would be expected between siblings than between morphologically distinct species.

The objective of this study was to assess the applicability of using the DNA-DNA hybridisation technique in determining the degree of divergence of the base sequences of genomic DNA of four *M. stipoides* populations and *L. perenne*.

5.6.2 Materials and methods

5.6.2.1 Preparation of nylon membrane filters

Genomic DNA from the four *M. stipoides* populations and *L. perenne* was spotted

onto individual 12 mm squares of neutral nylon filters (Hybond N®, Amersham) to be used for cross-hybridisation studies using the following procedure:

Genomic DNA concentration was determined by running electrophoresis of a series of two-fold dilutions of genomic DNA solutions on 1.5% agarose gel using TBE buffer, stained with ethidium bromide, exposed to UV light and photographed. Photographic images of DNA bands were scanned using a laser densitometer and the areas of peaks of DNA bands were determined. Concentration of DNA stock solutions was adjusted by determining the relative ratio of the area of peaks of DNA bands to one another and diluting the more concentrated solutions with milli Q distilled water.

Two-microgram aliquots (10 µl) of genomic DNA (200 µg/ml) from each *M. stipoides* population were taken and diluted with 6 µl of Tris·HCl- EDTA (TE) buffer pH 8.0 in Eppendorf tubes. After spinning down the mixture, DNA was denatured by heating at 98° C in boiling water for 10 minutes. The tubes were immediately transferred to ice-water to slow down re-hybridisation of the single stranded DNA and 4 µl of 20 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M Na_3 citrate) was added to each tube. The tubes were placed back in ice-water after spinning down the mixture.

While heating the DNA, 12 mm squares of nylon membrane were cut, labelled with pencil and soaked in 5 X SSC. Denatured DNA was spotted onto the middle of the presoaked filters, placed on top of parafilm, and allowed to dry. Each filter was placed inside an individual paper envelope and placed on top of a 35° C thermoblock for 30 min, then incubated at 80° C for 2 h. Filters were transferred to individual plastic hybridisation bags, a pre-hybridisation solution was added (50% formamide, 5 X SSC, 5 X Denhardt's (2% polyvinylpyrrollydone, 2% Ficoll, 2% BSA), 1% glycine, 50 mM KPO_4, 200 µg/ml denatured stock DNA) and the bag was thermally sealed before pre-hybridising overnight at 42° C.

The pre-hybridisation solution was squeezed out through a slit in one corner of the hybridisation bag, replaced with 400 µl of hybridisation probe solution and the bags resealed. Filters were incubated for a total of three days at 42° C, 37° C and 30° C (24 h at each temperature).

5.6.2.2 Preparation of hybridisation probe

For each DNA type used as a hybridisation probe, a reaction mixture was prepared by mixing 0.5 µg of genomic DNA (2.5 µl of DNA stock solution 200 ng/µl), 2.5 µl of 10 X

restriction enzyme buffer (REB), 2.5 µl of 10 mol m^{-3} dCTP, 2.5 µl of 10 mol m^{-3} dTTP, 2.5 µl of 10 mol m^{-3} dGTP, 1.0 µl of DNAse I (1.0 µg/µl), 1.0 µl of Polymerase I (10 units/µl), 5.0 µl of [α-^{32}P] dATP and 5.5 µl sterile water in an Eppendorf tube suspended in iced-water. Genomic DNA was radio-labelled (nick-translated) by incubating the reaction mixture at 15° C for 90 min.

Radioactive products (*DNA) were separated from the remaining ^{32}P-nucleotide triphosphates by running the reaction mixture through a Sephadex-G100 desalting column in TE buffer. Collected *DNA was combined and precipitated with 0.5 X volume 3M NH_4Ac and 3 X volume 99% EtOH. The tube was chilled at -20° C for 30 min after mixing. *DNA was precipitated by centrifuging the mixture at 13,000 g for 10 min and the supernatant solution was removed. *DNA was resuspended in TE buffer at 10% final hybridisation volume and denatured at 98° C for 10 min in a boiling water bath. After denaturation, *DNA was added to 9 volumes of hybridisation solution (50% formamide, 5 X SSC, 1 X Denhardt's, 20 mol m^{-3} KPO_4, 10% Dextran sulphate, 100 µg/µl *E. coli* DNA).

5.6.2.3 Heteroduplex melting experiment

After incubation with the hybridisation probe for three days, the filters were washed free of non-specifically bound *DNA with 5 X SSC, 0.1% SDS 5 times for 5 min each time and finally with elution buffer (2 X SSC, 30% (v/v) dimethylsulfoxide (DMSO) for 5 min.

*DNA bound to the filters was eluted by sequential incubation of each filter in a series of 2 ml elution buffer vials for five min each at 5° C temperature increments ranging from 40° C to 95° C in a hot water bath. The amount of *DNA released at each step was measured using the [^{3}H] channel of a Tri-Carb Liquid Scintillation counter (Packard). The amount of radioactive DNA released at each step was sequentially summed, and percent *DNA released at each step was calculated and plotted against temperature. This yielded a thermal denaturation curve of DNA complexes for each population genome pair. The temperature at which 50% of bound *DNA was released was read directly from the thermal denaturation curve. The relative percentage bound *DNA was computed by obtaining the ratio of the total amount of radioactive DNA released by each heterologous duplex DNA and homologous duplex DNA.

5.6.3 Results

5.6.3.1 Relative percentage bound DNA

When M (Lpe) DNA was used as the hybridisation probe, the relative percentage bound DNA during hybridisation between M (Lpe) and the other three *M. stipoides* populations ranged from 81 to 86% (Table 5.6.1). The relative percentage bound DNA values during hybridisation in heteroduplexes when M (Ppr) was used as the hybridisation probe ranged from 62.4 to 76.1, lower compared with when M (Lpe) was used as the hybridisation probe (Table 5.6.1). Likewise, when M (Dgl) was used as the hybridisation probe, the relative percentage bound to filter-bound M (Lpe) (78.4%) and M (Paq) (60.8%) were lower compared with when M (Lpe) was used as the probe (Table 5.6.1). The high relative percentage bound of labelled M (Dgl) to filter-bound M (Ppr) is anomalous and this could be explained by a discrepancy in the DNA concentration. It could be possible that the filter-bound M (Ppr) DNA concentration was lower than expected, thus higher binding occurred compared with the homologous duplex which had the correct DNA concentration. There were more serious anomalies in the results when M (Paq) was used as the hybridisation probe, all the heteroduplexes had relative percentage bound values greater than 100% which is not theoretically possible. In this case, the problem could have resulted from failure to clean *DNA effectively on the desalting column, resulting in the presence of [^{32}P] dATP which could artificially raise the level of ^{32}P binding to the filters. Due to the shortage of time, the experiment was not repeated. To minimise errors due to slight variations in DNA concentration, mean relative percentage binding was calculated for the reciprocal duplexes (Table 5.6.2). Because they probably represented artefactual retention of radioactivity, relative percentage binding values greater than 100% were eliminated in the calculations.

Table 5.6.1. Relative percentage bound DNA during hybridisation.

^{32}P-labelled DNA (probe)	Filter-bound DNA				
	M (Lpe)	M (Ppr)	M (Dgl)	M (Paq)	*L. perenne*
M (Lpe)	100	81	82	86.2	89.6
M (Ppr)	76.1	100	71.8	62.4	85.3
M (Dgl)	78.4	125	100	60.8	65.6
M (Paq)	131.3	162	154.8	100	131.6
L. perenne	40.3	66.9	67	65.5	100

Despite the preliminary nature of the results of this experiment, it is possible to propose degrees of relatedness among the four *M. stipoides* populations and *L. perenne* from the mean relative percentage bound data (Table 5.6.2). The three *M. stipoides* populations, M (Lpe), M (Ppr) and M (Dgl), appear to be more closely related to each other than M (Paq). The relationship of these three populations to *L. perenne* is not particularly different from their relationship to M (Paq). The data also illustrate more remote relatedness of *L. perenne* to *M. stipoides* populations.

Table 5.6.2. Mean relative percentage bound DNA during hybridisation of the reciprocal DNA duplexes. Relative percentage bound values greater than 100% were eliminated in the calculation of the mean.

	M (Lpe)	M (Ppr)	M (Dgl)	M (Paq)	*L. perenne*
M (Lpe)	100				
M (Ppr)	78.6	100			
M (Dgl)	80.2	71.8	100		
M (Paq)	86.2	62.4	60.8	100	
L. perenne	65	76.1	66.3	65.5	100

5.6.3.2 Thermal denaturation curves

Thermal denaturation curves of the four populations generally show that the three *M. stipoides* populations originating from the same paddock at 'Karuah' gave similar levels of cross hybridisation, irrespective of which *M. stipoides* population was used as the hybridisation probe (Fig. 5.6.1). The slope of the thermal denaturation curves when M (Lpe) (Fig. 5.6.1a) was used as the hybridisation probe was steep compared with the slopes of the thermal denaturation curves when the other three *M. stipoides* populations were used as hybridisation probes. On the other hand, the slope of the thermal denaturation curves when *L. perenne* (Fig. 5.6.1e) was used as the probe was slower and longer compared with the curves when the *M. stipoides* populations were used as probes. A steep curve is characteristic of a population of nucleotide sequences with similar thermal stabilities, while a shallower slope indicates greater diversity of thermal stabilities among the component nucleotide sequences.

The thermal denaturation curves of the heteroduplexes formed between *L. perenne* and the four *M. stipoides* populations diverged clearly from the thermal denaturation curve of the homologous duplex of another species, *L. perenne* (Fig. 5.6.1e). Even the three *M.*

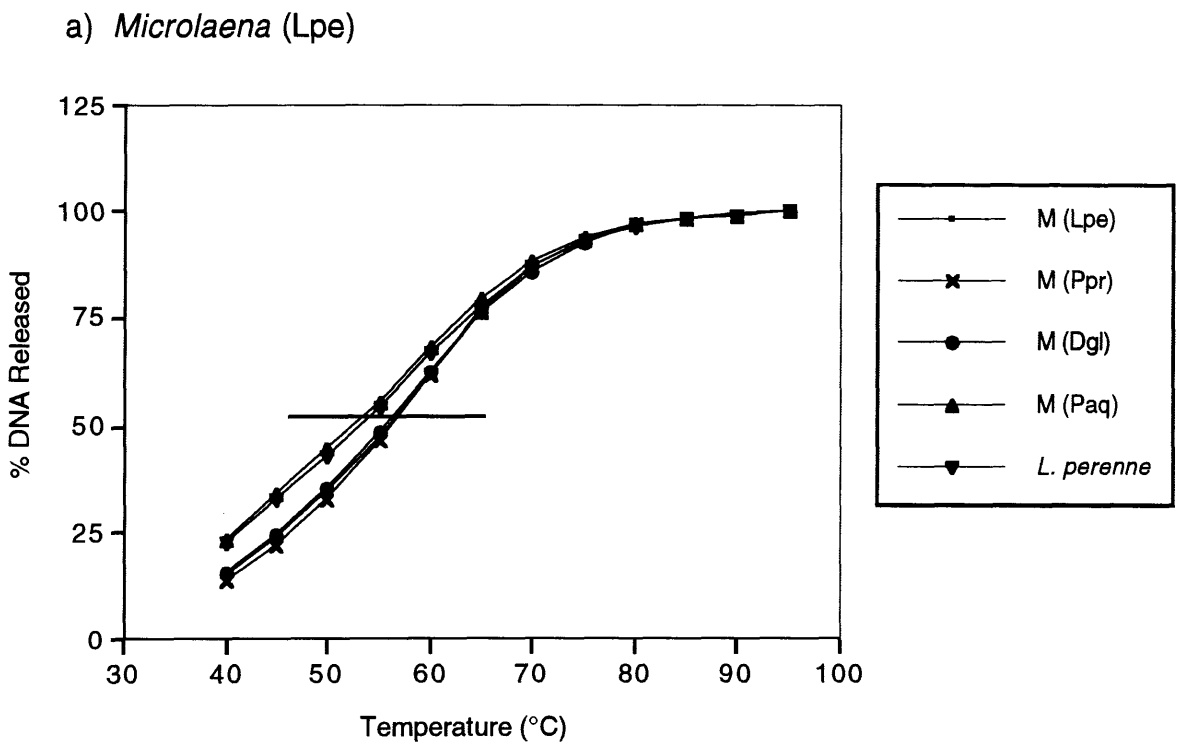
stipoides populations collected from the same paddock showed divergence in the thermal denaturation curves of their heteroduplexes formed with *L. perenne*.

5.6.3.3 ΔTm values

Theoretically, the temperature at which 50% of bound radioactive DNA was released from heteroduplex hybrid DNA should be lower than from homoduplex hybrid DNA. Therefore, the ΔTm values for all heteroduplexes should be negative. When M (Lpe) was used as the probe, all the heteroduplexes, except for the M (Lpe)/M (Ppr), had negative ΔTm values. It can be considered that the +1° C ΔTm value for the M (Lpe)/M (Ppr) duplex is within the error range. When the other *M. stipoides* populations were used as hybridisation probes, difficulties were encountered. However, considering the range of ΔTm values for the homoduplex and the heteroduplexes hybrids, narrower ranges were observed when M (Ppr) (-0.2 to +0.8° C) and M (Dgl) (0 to +0.5° C) were used as the hybridisation probes (Table 5.6.3). On the other hand, the range of the ΔTm values was wider when M (Paq) and *L. perenne* were used as the hybridisation probes (Table 5.6.3). The range of errors observed in this experiment clearly demonstrate a need for extensive repetition.

5.6.4 Discussion

The main aim of this experiment was to assess the applicability of the DNA-DNA hybridisation technique in determining genetic relatedness among four *M. stipoides* populations and *L. perenne*. The sensitivity of the DNA/DNA hybridisation technique varies with stringency of experimental conditions (Walker and McClaren 1965, Martin and Hoyer 1966). The series of experiments was performed twice as serious technical problems were encountered during the first series regarding discrepancies in the concentrations of genomic DNA. Adjustment of genomic DNA concentrations after scanning of the DNA bands using a laser densitometer yielded more consistent hybridisation results. Other technical problems were also encountered in the experiments such as the accuracy of genomic DNA concentrations applied on hybridisation filters and added into hybridisation probes both for heteroduplexes and homoduplex hybrids, efficient cleaning up of the radioactive probe so that the radioactive binding material is purely DNA, and constancy of incubation conditions. Nevertheless, with further refinement of the technique and replication of experiments, the technique has potential as a method in determining the degree of divergence of base sequences. Entingh (1970) has reported that although DNA-DNA hybridisation data do not yield absolute measures of sequence similarity, under constant incubation conditions the technique will yield reliable indications of relative nucleotide sequence similarity.



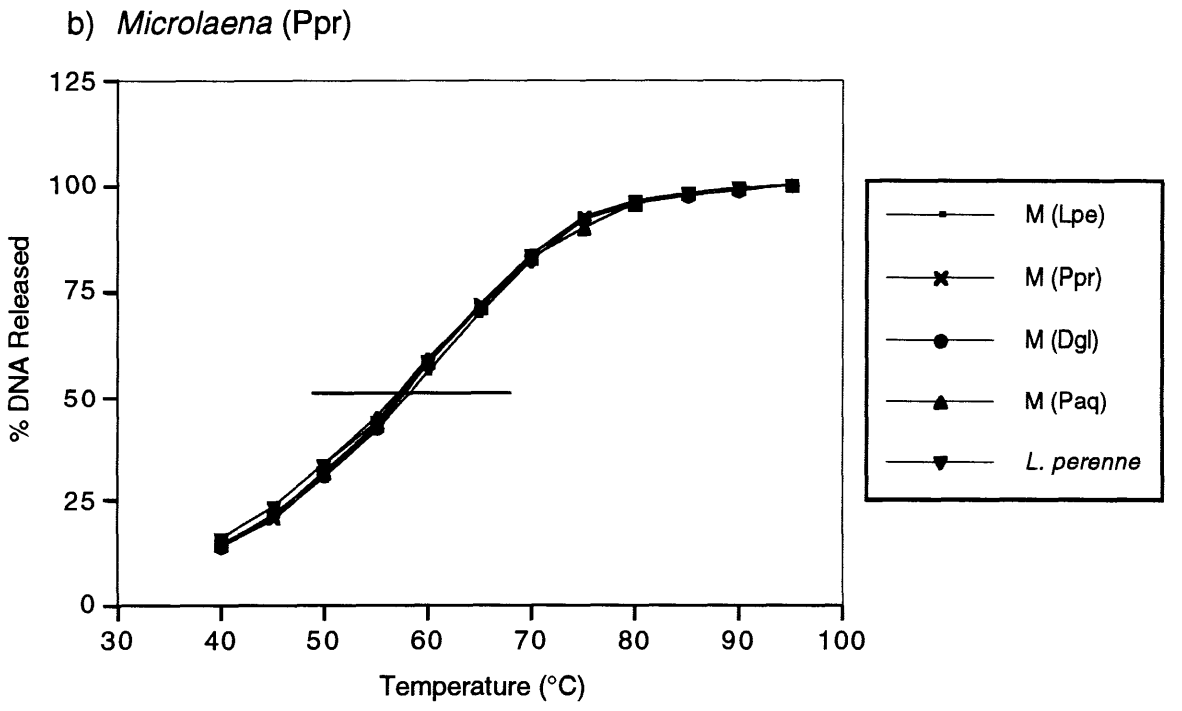
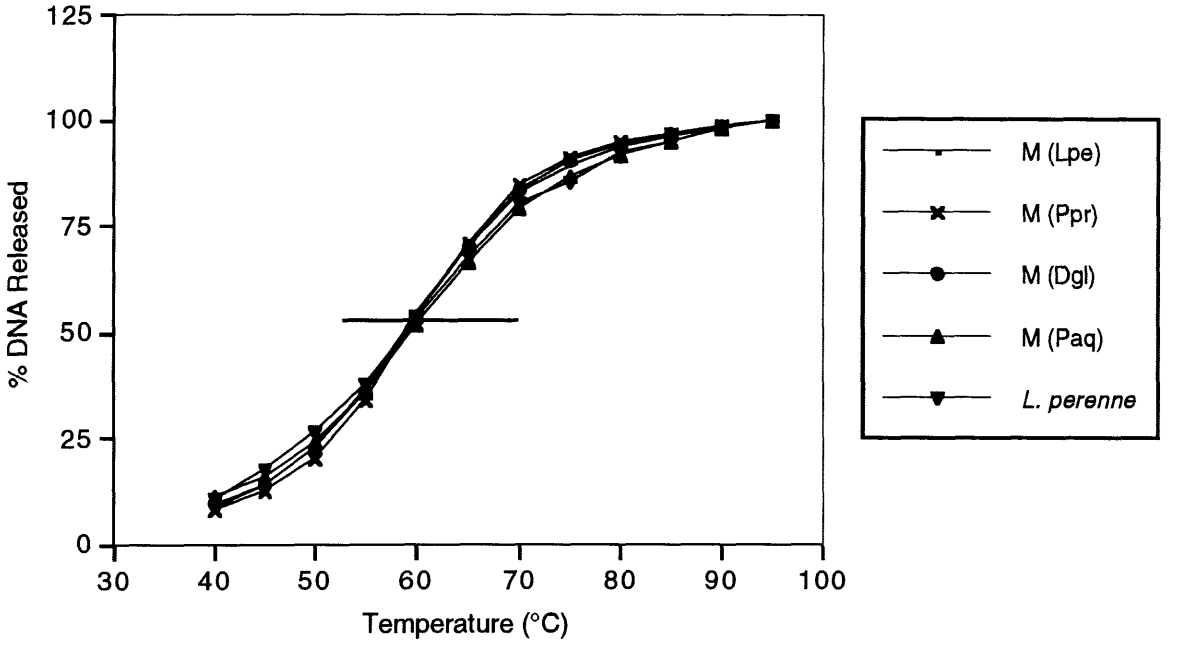
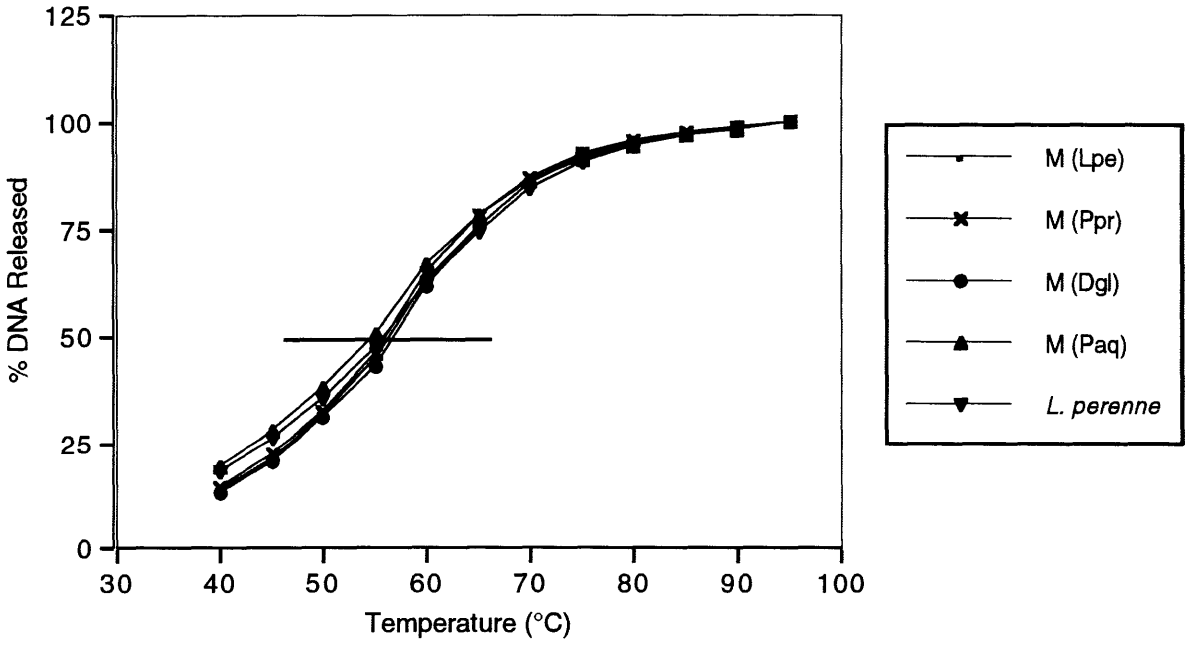


Fig. 5.6.1. Thermal denaturation curves of homologous and heterologous duplexes DNA using a) *Microlaena* (Lpe), b) *Microlaena* (Ppr), c) *Microlaena* (Dgl), d) *Microlaena* (Paq) and e) *Lolium perenne* as the hybridisation probe.

c) *Microlaena* (Dgl)



d) *Microlaena* (Paq)



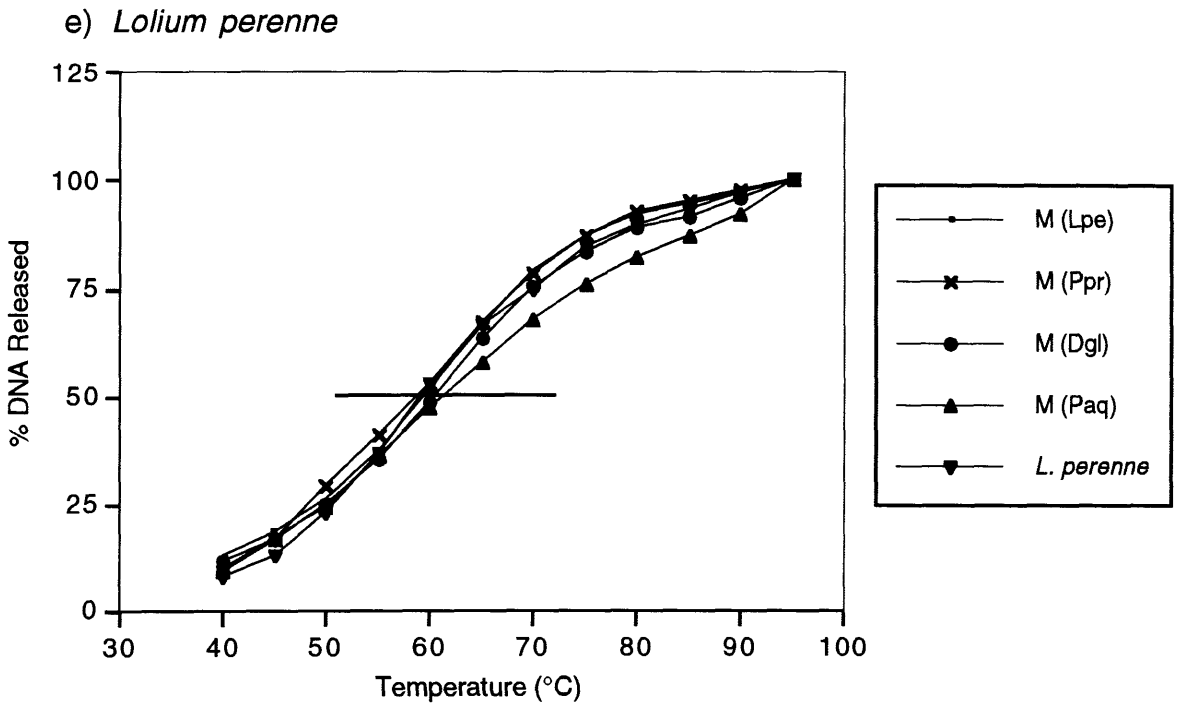


Table 5.6.3. Changes in temperature (° C) at which 50% bound radioactive DNA was released (ΔTm) from homoduplex to heteroduplex hybrid DNA.

P^{32}-labelled DNA (probe)	Filter-bound DNA				
	M (Lpe)	M (Ppr)	M(Dgl)	M (Paq)	*L. perenne*
M (Lpe)	--	+0.1	-0.7	-3.7	-2.9
M (Ppr)	+0.8	--	+0.4	-0.2	--
M (Dgl)	--	--	--	+0.5	--
M (Paq)	+1.7	+1.5	+2.3	--	+1.5
L. perenne	+0.3	-0.3	+1.2	+2.4	--

The nature of DNA/DNA duplexes formed *in vitro* may vary depending on hybridisation conditions, including incubation temperature, salt concentration and formamide concentration (Bendich and McCarthy 1970a). Hybridisation solutions are formulated to prevent non-specific binding of labelled probes to the filter and to ensure maximal efficiency of specific hybridisation (Ivanov and AbouHaidar 1992). In these experiments, hybridisation was extended over three days of three progressively lowered temperatures, to ensure maximum binding of both well matched and poorly matched hybrids. Ivanov and AbouHaidar (1992) found that pre-treatment of DNA with formaldehyde prior to loading on

nitrocellulose membrane filters increased the intensity of hybridisation tenfold. The ratio of filter-bound DNA to labelled DNA affects the Tm and ΔTm values. Bendich and McCarthy (1970a) reported that filter-bound DNA sites form stable base pairs with labelled DNA, however, less stable base pairs are formed when there is an excessive number of available sites.

The DNA-DNA hybridisation technique is based on the assumption that the accumulation of heritable changes in the genes of organisms is reflected in alterations of the sequential order of bases in the DNA structure (Bendich and McCarthy 1970a). The extent of these single base changes in the DNA structure can be calculated using thermal stability profiles or thermal denaturation curves (Belford and Thompson 1981a). The fraction of base pair substitution between two DNAs can be estimated using the ΔTm values generated from the thermal stability profiles (Laird *et al.* 1969). Thermal stability of a DNA/DNA hybrid is a function of the complementarity in the base sequence of the component strands (Bendich and McCarthy 1970a) and provides an estimate of the average or median DNA sequence differences (Britten 1986).

The difference in mean thermal temperature at which 50% of bound DNA was released (ΔTm) of homologous and heterologous duplexes is a gauge of base sequence divergence between these DNAs (Laird *et al.* 1969). The narrow range of ΔTm values of the thermal stability profiles of hybrid duplexes using labelled M(Dgl) DNA and M(Ppr) DNA show that their base sequences were similar to the base sequences of the other three *M. stipoides* populations and *L. perenne*. The base sequence of M(Lpe) DNA appeared closer to M(Ppr) DNA and M(Dgl) DNA and more divergent from the base sequences of M(Paq) and *L. perenne* DNA. The greatest divergence was observed between M(Paq) DNA and all the other three *M. stipoides* populations and *L. perenne*, implying that M(Paq) is distantly related to the other three *M. stipoides* populations and *L. perenne*. The base sequence of *L. perenne* DNA is closer to M(Lpe) DNA and M(Ppr) DNA and more divergent from M(Paq) DNA, while proximity to the base sequence of M(Dgl) is intermediate. All these data seem to indicate the wide variability in the base sequences of the four *M. stipoides* populations. Comparing the degree of divergence among the four *M. stipoides* populations, it appears that M (Paq) has diverged significantly from the other three *M. stipoides* populations.

The good agreement in binding reciprocity between M(Lpe) and the DNAs of the other two populations, M(Ppr) and M(Dgl), shows a close similarity of their polynucleotide base sequences in their DNA structures and that approximately 80% of their genomic DNA complements each other. The large discrepancies in the other reciprocal comparisons can be attributed to the variation in the size and number of families of related DNA sequences forming the hybrid duplex DNA (Bendich and McCarthy 1970a). The M(Lpe) filter

contained a sufficient concentration of base sequences complementing the labelled M(Paq) DNA, allowing duplex formation similar to the homologous binding, whereas the M(Paq) filter contained base sequences that complement only 86% of labelled M(Lpe) DNA. Likewise, M(Ppr) and M(Dgl) filters contained sufficient concentrations of base sequences complementing the labelled M(Paq) DNA to allow duplex formation similar to homologous binding, while the M(Paq) filter contained base sequences that complemented 62.4% of labelled M(Ppr) DNA and 60.8% of labelled M(Dgl) DNA. On balance, these results appear to imply that M(Paq) had a more divergent base sequence compared with the base sequences of the other three *M. stipoides* populations collected from the same paddock at 'Karuah'. It could be possible that geographical isolation of M (Paq) led to its divergent nucleotide base sequences. Among the three *M. stipoides* populations situated in one paddock but growing in association with different perennial pasture grasses, percentage binding varied from 72% to over 100%.

It was unfortunate that further pursuit of these methods was not feasible, because more certain results are required to make a useful analysis of population divergence. Nevertheless, the results of the DNA-DNA hybridisation experiments seem to show that there is a wide base sequence divergence among the four *M. stipoides* populations. The *M. stipoides* population (M(Paq)) collected from 'Powalgarh' exhibited a distant relationship to the other three *M. stipoides* populations collected from the same paddock at 'Karuah'. Among the three populations collected from the same paddock, differences in thermal stability profiles, ΔTm and percentage binding also appear to indicate base sequence divergence. It is shown in the results that *L. perenne* is genetically different from the four *M. stipoides* populations, as indicated by the varying thermal stability profiles and low percentage binding. The difference here between *M. stipoides* and *L. perenne* was predicted and the results confirmed it. Therefore, despite the numerous difficulties encountered in this experiment, the DNA-DNA hybridisation technique has a promising value in phylogenetic studies. Analysis of DNA sequences within plant genomes is becoming a major taxonomic feature.

5.7 DISCUSSION

The five populations of *M. stipoides* exhibited differences in their growth performance under varying light intensity levels. Generally, all the populations produced the highest total dry mass when grown under 47% transmitted light. *Microlaena stipoides* (Ppr) showed greater shade tolerance than the other populations. When grown under 16% transmitted light, M (Ppr) gave the smallest reduction in total dry mass and produced the largest number of tillers compared with the other three populations. The other four populations, on the other hand, produced a larger number of tillers when grown under 47% transmitted light. This indicates that maximum tillering is induced in M (Ppr) at lower light intensity (16% transmitted light), while the other three populations had their maximum tillering at a higher light intensity (47% transmitted light). *Microlaena stipoides* (Ppr) had more leaves and a higher chlorophyll content than the other populations when grown under 47% transmitted light, perhaps explaining its higher dry mass. It was also M (Ppr) that showed a significant reduction in leaf production when grown under full light compared with its leaf production under 47% transmitted light. This could mean that M (Ppr) experienced inhibition of photosynthetic activity under strong light, thus producing less leaves and tillers, and hence dry mass (Björkman and Holmgren 1963, Kuiper and Smid 1985). All these results indicate that among the five populations, the M (Ppr) genotype was more adapted to lower light intensity than the other four populations. Population 1 showed sensitivity to full light and severe shading, having significant reductions in dry mass when grown under the two extreme conditions.

Microlaena stipoides (Ppr) produced a larger number of panicles at all light intensity levels compared with the other four populations. Because M (Ppr) produced more panicles, perhaps it has higher seed production than the other populations, assuming the same number of seeds per panicle. Seeds produced by M (Ppr) generally weighed significantly less (3.55 mg/seed) and had a faster rate of germination than seeds of the other populations. Production of larger quantities of smaller or lighter seeds could be an adaptive strategy of M (Ppr) by allocating less energy resources per seed but producing larger quantities of seed.

Generally, the number of drying days to 100% leaf mortality increased with an increase in pot size for all four *M. stipoides* populations. *Microlaena stipoides* (Ppr) did not show a significant increase in the total number of drying days to 100% leaf mortality with an increase in pot size, indicating an inability to adjust physiologically to developing water stress. Among the five *M. stipoides* populations, M (Ppr) had the lowest number of days to 100% leaf mortality in the large pots compared with the other *M. stipoides* populations, though it was not statistically significant. *Lolium perenne* had the lowest tolerance to water

stress, as exhibited by a significantly lower total number of drying days to 50% and 100% leaf mortality, compared with all *M. stipoides* populations.

DNA fingerprints of the four *M. stipoides* populations exhibited amplified fragments common to all populations, which may indicate the phylogenetically conserved regions as well as individual-specific bands. *M. stipoides* (Ppr) showed the greatest divergence in banding patterns compared with the other three populations, which may indicate a more advanced genetic change or microevolution. This result parallels the divergent growth performance of M (Ppr) compared with the other three *M. stipoides* populations when grown under different light treatments. DNA polymorphisms clearly distinguished *L. perenne* as a different species compared with all *M. stipoides* populations.

Microlaena stipoides (Ppr) was collected from the lower south-eastern part of the paddock at 'Karuah', where it was shaded by a big tree and growing in association with *P. pratensis*. *Poa pratensis* naturally occurs more often in hollows than on ridges because the higher soil moisture content and fertility levels in the hollows favour its increased tillering (Skinner and Noll 1919, Hartwig 1938, Bennett *et al.* 1972, Reader and Bonser 1993).

Microlaena stipoides (Dgl) showed a significant tolerance to high light intensity, producing greater dry mass and tiller numbers when grown under full light compared with the other *M. stipoides* populations. *Microlaena stipoides* (Dgl) and M (Paq) showed a relatively longer period to 100% leaf mortality when grown in large pots compared with when grown in small and medium pots. The other two populations, M (Ppr) and M (Lpe) did not exhibit a significant increase in the the total number of drying days to 100% leaf mortality when pot size was increased from medium to large. Averaged over all pot sizes, M (Dgl) and M (Paq) took a longer time to reach 100% leaf mortality compared with M (Ppr) and M (Lpe), although not statistically significant.

Microlaena stipoides (Dgl) was collected from the upper slope on the north-eastern part of the paddock at 'Karuah', where it was growing in association with *D. glomerata*, a tufted perennial suitable for moderate fertility and dry regions (Levy 1970). *Dactylis glomerata* was more common on the ridges where soil fertility levels and soil moisture content were probably lower than in the hollows (Reader and Bonser 1993). *Dactylis glomerata* has been reported to be suitable for dry regions (Levy 1970), with more successful establishment than *L. perenne* on dry hills (White *et al.* 1972, Barker *et al.* 1993), and had greater yields than *L. perenne* after moisture stress (Wedderburn *et al.* 1993).

Microlaena stipoides (Paq) was collected from an open north-facing site with a stony soil at 'Powalgarh', where it was growing in association with *P. aquatica*. *Phalaris aquatica*

has a rhizomatous growth habit (Rumball 1980) and survives under hard grazing (Hutchinson 1970) and pest tolerance (Stevens *et al.* 1993). In addition, *P. aquatica* has high persistence during drought (Robinson 1952) and also can avoid drought by becoming dormant (McWilliam and Kramer 1968).

Microlaena stipoides (Lpe) had the highest dry mass, tiller number and leaf number when grown under 47% transmitted light and exhibited reductions in all these growth parameters when grown under full light and 16% transmitted light. Like the other four populations, growth of M (Lpe) was significantly reduced under severe shading. *Microlaena stipoides* (Lpe) showed a significant increase in the length of time to 100% leaf mortality between small and large pots, whereas M (Ppr) showed no significant increase. It therefore exhibited some adjustment to the slower rate of water stress development in the larger pots whereas M (Ppr) did not.

Microlaena stipoides (Lpe) was collected from an exposed flat site on the south-western part of the paddock where the soil was clayey and growing in association with *L. perenne*. *Lolium perenne* has a strong clonal growth pattern with a better balance between growth at the apex and death of the old basal stem (Brock and Fletcher 1993). It is most suitable for high fertility conditions (Levy 1970) and showed considerable increase in yield with added nitrogen (Wedderburn *et al.* 1993). However, the yield of *L. perenne* is limited by its poor adaptation to summer heat and drought (Charmet *et al.* 1993).

The significant variations in the increase of total number of drying days to 100% leaf mortality among three of the four *M. stipoides* populations indicates variations in physiological adjustment to water stress. Among the four populations, M (Dgl), and M (Paq) exhibited a greater tolerance to water stress compared with M (Lpe) and M (Ppr). These two *M. stipoides* populations which exhibited greater tolerance to water stress were growing in association with neighbouring grass species that have been noted for water stress tolerance and were situated in the upper slopes of the paddock where soil moisture is likely to be more limiting compared with the lower slopes or flat areas where the other two populations were situated. It is possible that the neighbouring grass species as well as the abiotic environmental conditions such as soil moisture availability and soil physical properties exerted natural selection forces on the *M. stipoides* populations for water stress tolerance.

Microlaena stipoides (Lpe) also exhibited some adjustment to the slower rate of water stress development in the larger pots, whereas with M (Ppr) the increase in days to 100% leaf mortality was not significant. It also had the shortest time to 100% leaf mortality in the largest pots, although the differences were not significant ($P > 0.05$). These findings

indicate that there may be some genetic differences among the four populations which could have evolved through natural selection resulting from competitive interactions with their associated perennial grasses or from selection pressures associated with differences in water availability at the locations from which they were collected.

Results of the DNA hybridisation experiments seemed to show that the base sequence of M (Paq) was different from the base sequences of the other three *M. stipoides* populations. This implies that the *M. stipoides* population (M(Paq)) collected from 'Powalgarh' was distantly related to the other three *M. stipoides* populations collected from within the same paddock at 'Karuah'. These findings may indicate that geographical isolation results in divergence in the base sequences. Among the three populations collected from the same paddock, differences in thermal stability profiles, ΔTm and percentage binding also appear to indicate some base sequence divergence.

The results presented in this chapter clearly suggest that *M. stipoides* is a shade-adapted species and that the four *M. stipoides* populations studied possess significant differences in their sensitivity to or tolerance of light intensity and responses to water stress. The four populations differ in their strategies for energy allocation under varying light intensity levels. *Microlaena stipoides* (Ppr) produced a larger number of tillers and leaves when grown under shade and also produced more panicles and consequently, possibly higher seed production than the other three populations. *Microlaena stipoides* (Dgl), on the other hand, exhibited a different strategy, producing a larger number of tillers compared with the other populations under full light, thus yielding a higher dry mass. *Microlaena stipoides* (Dgl) and M (Paq) exhibited greater tolerance to water stress. *Microlaena stipoides* (Lpe) showed physiological adjustment to water stress with an increase in pot size, while M (Ppr) did not. Genetic analyses also showed that genetic divergence occurred among the four *M. stipoides* populations. Amplified polymorphic DNA segments of M (Ppr) were different from the other three populations, while the thermal denaturation curve of geographically isolated M (Paq) was distinct from the other three populations which were collected from within the same paddock.

These findings show that local population differences occur among *M. stipoides* populations growing in association with different neighbouring perennial grasses in a permanent pasture. The four populations exhibited behavioural and genetic differences which may have evolved through natural selection resulting from competitive interactions with their associated perennial grasses or from selection pressures associated with differences in water availability and perhaps, other abiotic microenvironmental factors, at the locations from which they were collected. The next chapter will test the hypothesis that

competitive interactions with neighbouring grass species was the driving force that caused this divergence among *M. stipoides* populations.