MORPHOLOGICAL AND GENETIC VARIATION WITHIN PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

DISSERTATION

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By

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ABSTRACT

The relationship between plant diversity and production has been debated by grassland ecologists since it was suggested by Charles Darwin more than a century ago. Research has extensively showed that pastures with complex composition can increase production in some situations. However, there is a general lack of information on the role of within species (intra-specific) diversity and its contribution to the ecological functionality of a community. The aim of this research was to investigate the extent of intra-specific diversity, morphologically and genetically, and the mechanisms by which intra-specific mixtures influence the final production.

Perennial ryegrass (*Lolium perenne* L.) is a widely used forage species in temperate regions and BG34 is a commonly used cultivar blend composed of the cultivars Barlet, Barmaco, Barnhem, and Mara in various proportions. This research used perennial ryegrass as a model plant to (i) quantify within- and between-cultivar variation of *L. perenne* cultivar blend BG34 on the basis of several morphological characteristics and forage yield; (ii) examine the relative yield of *L. perenne* in response to genotypic diversity and how this response was affected by defoliation frequency and intensity; (iii) investigate the responses of production components i.e. tiller number, tiller weight, plant mass and survival of individual genotype, and how this was affected by the treatments of genotype and defoliation; and (iv) evaluate the suitability of SSR (simple sequence

repeats) and ISSR (inter simple sequence repeats) markers to differentiate the cultivars of *L. perenne* that comprise BG34 and investigate the genetic structure of pastures sown with BG34 ryegrass from dairy fields in Ohio.

With 100 potted plants of the four cultivars investigated, within-cultivar variation accounted for 73-97 % of the total variation for the morphological traits (i.e. height, tiller number, erectness, leaf width and yield). Measurement of cloned plants found 13.4 to 89.9% of the variation could be attributed to genetic variation depending on traits. It was concluded that although there was morphological variation between these cultivars, it was smaller than the within-cultivar variation. It was predicted that the total variation of a mixture of these cultivars (e.g. as in BG34) would result predominantly from within-cultivar variation rather than between-cultivar variation.

The relative herbage yield of monocultures and mixtures of different genotypes of *L. perenne* was evaluated using cloned plants. Eight genotypes were selected, that were a factorial combination of four yield potential levels, and two tillering potentials. Genotype treatments were composed of one, two, four and eight genotypes per plot and the defoliation treatments were frequency (cutting interval of 3 week vs. 6 week) and intensity (cutting height at 20 mm vs. 60 mm). The data showed a significant linear relationship between genetic diversity and production and a significant difference between mono-genotype and multi-genotype treatments. The highest yielding treatments had only one genotype or two genotypes, yet their yield was not significantly higher than that of the complex mixtures with eight genotypes. Defoliation frequency and height had significant effects on forage yield. Frequent (3-wk) defoliation reduced grass yield by 12.7%, while the 60 mm height clipping reduced grass yield by 14.3%. It was concluded

that genotypic diversity helped increase herbage yield and this response was modified by defoliation patterns.

Using both SSR and ISSR markers, individuals of BG34 were correctly allocated to lines and cultivars with 80.9-86.7% accuracy using discriminant analysis. There were distinct differences between cultivars, suggesting the suitability of the marker systems in identification of *L. perenne* cultivars. Divergent changes in the proportions of the respective cultivar of BG34 were found on the three 5-year old fields in Ohio. One field remained similar to the cultivar mixture in the original sowing. Change of genetic structure was found for two fields, with increases in the proportion of Mara and Barmaco, and decreases Barlet or Barnhem depending on field. Overall, SSR was proven to be highly effective for differentiating among ryegrass cultivars. It indicated that further study is required to identify the mechanisms of the genetic change and stability of genetic responses to differential management.

Dedicated to my loving and supportive wife, Duan

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AFLP	amplified fragment length polymorphism
ANOVA	analysis of variation
DA	canonical discriminant analysis
DM	dry matter
ISSRs	inter simple sequence repeats
PAC	principal component analysis
PCR	polymerase chain reaction
PROC DISCRIM	discriminant analysis procedure of SAS
PROC GLM	general linear model procedure of SAS
PROC PRINCOMP	principal component analysis procedure of SAS
PROC REG	regression procedure of SAS
PROC TREE	tree procedure of SAS
RAPD	random amplification of polymorphic DNA
RFLPs	restriction fragment length polymorphisms
SAS	SAS Institute Inc. Cary, NC
SSRs	simple sequence repeats
UPGMA	unweighted pair group method with the arithmetic averaging
UPOV	International Union for Protection of New Varieties of Plants

CHAPTER 1

INTRODUCTION

Pastures are an important land use globally. Of all the agricultural area worldwide, managed pastures occupy 69%, and these areas are estimated to be increasing at around 0.3 percent per year (FAO, 2003). Undoubtedly, forage and grazing lands form the backbone of profitable forage-livestock systems and contribute substantially to the agricultural economy (Sanderson, 2004). Conventional pasture management is mainly concerned about the optimization of the quality and quantity of herbage for animal production. Recent interest in management of pasture is moving beyond this scope to encompass a broader set of issues, such as sustainability, reduced inputs of fertilizers and pesticides, soil protection, carbon sequestration, animal biodiversity, resistance to invasion by alien plants and insects and the aesthetic value of the landscape (Spellerberg et al., 1991; Watkinson and Ormerod, 2001; Krueger et al., 2002, Sanderson, 2004). It is within this context, that increased bio-diversity in grazing systems play an important role.

In 1872 Charles Darwin was the first to state that diverse grasslands could produce more herbage than monocultures. Since then, the issue of species diversity has been a major subject of grassland ecology research. The concept of plant species diversity can be defined as the number of species and their relative abundance in a defined area. It incorporates both species richness (S, the number of plant species) and species evenness

(J, an estimate of species distribution within a community). A community is considered perfectly even when all the species in the habitat have an equal number of individuals and are of all the same size. There are many indices that combine the two factors to measure diversity in plant communities. Commonly used diversity indices may include the Shannon-Wiener index (H') and Simpson's diversity index (Peet, 1974 and Magurran, 1988).

Higher production from species-diverse communities has been widely demonstrated in grasslands, pastures and cropping systems (Hector, 1999; Tilman et al, 2001; Helland et al., 2001). The benefits of high species diversity include improved system function, greater stability and efficient nutrient cycling (McCann, 2000). Three mechanisms are hypothesized to operate in the process that plant species richness increases productivity and these hypotheses have received strong support as evidenced by substantial research: 1) niche complementarity, in which ecological differences among species result in more complete and efficient utilization of resources in diverse communities relative to less diverse ones (Tilman, 2001); 2) "sampling effect", where more diverse communities have a higher chance of containing a highly productive species (Hector, 1999); 3) positive mutualistic interactions between species in complex assemblages (Hooper, 1997).

Frequently omitted from the biodiversity (species richness) debate is the contribution of intra-species diversity to the total diversity. Some authors have suggested that similar benefits could be achieved at the intra-species scale, with natural plant populations tending to show high genetic diversity (Gustafson *et al*. 1999, Kreher *et al*. 2000). Indeed, the total biodiversity of a system is not merely the number (or

distribution) of species present, but also includes the genetic diversity of the component species (Gaston, 1996). By extrapolation from biodiversity theory, one could infer that high genetic diversity within a species might have similar potential benefits as high species diversity. However, quantitative evidence of this hypothesis is sparse.

The observation that pastures with complex composition are more productive than those with simple composition has been obtained at the intra-specific level using perennial ryegrass cultivars or genotypes (Rhodes, 1970; Surgenor, 1976). Little information is available about the detailed mechanism of this yield increase from mixtures, and how this is related to other growth parameters such as tillering character and survival. In addition, the relationship between genotypic diversity and production is often complicated by various cutting systems imposed. The effects of defoliation frequency and intensity on pasture production are controversial because of the substantial variability in the defoliation responses of different plant components and the dependence of associated conditions. Studies on the comparison of mixtures and monocultures have usually expressed yield, yield component, tillering profiles or survival in terms of unit area. More information might be obtained on the structure of the sward and individual contribution to the resultant population if the data were recorded on an individual basis.

There is a general lack of information on the genetic structure of grasslands and the extent to which this is subject to selection forces of abiotic and biotic factors. A sound knowledge of the relationships is useful in developing efficient selection strategies and to exploit plant genetic resources to human benefit. Molecular markers are powerful tools for examination of this genetic diversity. These markers are abundant compared to phenotypic markers, and they are less affected by genotype by environment interactions.

Molecular marker systems based on isozymes, RFLP, RAPD and AFLP (Sanders, 1989; Hayward et al. 1994, 1998; King et al.1998; Bert et al. 1999) have been developed for perennial ryegrass to assist identification and quantification of genetic similarity among cultivars. Among the molecular marker systems available, simple sequence repeats (SSRs) and its modification version, inter-simple sequence repeats (ISSR) are particularly suitable for genetic diversity evaluation and cultivar identification, because of a series of advantages over the above marker systems.

Perennial ryegrass (*Lolium perenne* L.) is a cool season grass native to Europe, Asia and North Africa. It is the most important forage grass species in temperate regions, and is also widely used as turf. *L. perenne* is an obligate out-crossing species with a genetically determined gametophytic self-incompatibility system (Cornish et al. 1979). Ecotypes and cultivars therefore typically have high levels of genetic variability. As one of the highest quality forage grasses, perennial ryegrass occupies about 250,000 acres in the U.S., predominantly are in the northeast and on the pacific coast. Perennial ryegrass has bunch-growing habit, low rate of horizontal spread (no or very short stolon), smooth texture in culm and leaf blade and medium size. These morphological characteristics have made perennial ryegrass an ideal model plant for the study of intra-specific biodiversity. The most commonly used perennial ryegrass in Ohio dairy pasture is BG34, which is marketed by Barenbrug Seed Company. BG34 is not a registered cultivar, but is a blend of the four cultivars Barnhem, Barmoco, Barlet and Mara, in various proportions. They are all diploid and with different breeding history.

It is hypothesized that 1) substantial morphological variability is present within and among perennial ryegrass cultivars; 2) the differences among genotypes has an

important impact on the overall production and other parameters of a stand containing multiple genotypes; 3) the morphological differences are derived ultimately from the plants genetic background, which can then be quantified. The specific objectives of this research were to (i) quantify within- and between-cultivar variation of *L. perenne* cultivar blend BG34 on the basis on several morphological characteristics and forage yield (Chapter II); (ii) examine the relative yield of *L. perenne* in response to genotypic diversity and how this response was affected by defoliation frequency and intensity (Chapter III); (iii) investigate the responses of production components i.e. tiller number, tiller size, plant size and survival of individual genotypes and how this is affected by genotype and defoliation treatments, and competition with other genotypes in mixed stand (Chapter IV); and (iv) evaluate the suitability of SSR/ISSR marker systems in differentiation of cultivars and lines of *L. perenne* that comprise BG34 and investigate the genetic structure of pastures sown with BG34 ryegrass from dairy fields in Ohio (Chapter V).

CHAPTER 2

MORPHOLOGICAL VARIATION WITHIN AND BETWEEN FOUR RYEGRASS CULTIVARS

ABSTRACT

The perennial ryegrass (*Lolium perenne* L.) blend BG34 has performed well in grazing systems across the Midwest USA and one reason for this success may be its biodiversity resulting from being a blend of four cultivars (Barlet, Barmaco, Barnhem, and Mara). The objective of this study was to quantify morphological variation (i.e. plant height, tiller number, tiller diameter, erectness, leaf width and yield) among and within these cultivars. Plants (n=100 per cultivar) were established from seed and grown in a greenhouse from February to July 2002 (Exp. 1). Random (within-genotype) variation was determined from measurement of traits on 10 clones (genotypes) from 10 plants chosen from each cultivar July 2002 to March 2003 (Exp. 2). In both Exp. 1 & 2, cultivar effects were significant ($P < 0.05$) for all morphological characters. Barnhem had the most tillers (176 tillers plant^{-1}), the smallest leaf width (3.9 mm), and was the shortest (29.6 cm) cultivar. Mara had the fewest tillers (147 tillers plant^{-1}), wide leaves (4.7 mm) and was the tallest (33.3 cm). Cultivar Barmaco had wide leaves (4.7 mm) and was prostrate, while Barlet was erect. Large within-cultivar variation (73-97 %) was found for each trait and most of the variation could be attributed to genetic variation since only

limited random variation was found in Exp. 2. It was concluded that although there was morphological variation between these cultivars, it was smaller than the within-cultivar variation. It was predicted that the total variation of a mixture of these cultivars (e.g. as in BG34) would result predominantly from within-cultivar variation rather than from between-cultivar variation.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is an important pasture species for temperate grassland agriculture, with large areas of cultivation around the world (Balfourier et al., 1997). As a diploid (2n=14), self-incompatible species, perennial ryegrass has high genetic variation between individuals (Wilkins, 1991). Cultivar development is achieved by the strategy of using small breeding populations based on limited or shared germplasm (Funk et al., 1993), so a cultivar usually consists of a heterogeneous population of genotypes. In some cases, the parent populations are closely related, thus making many ryegrass cultivars both phenotypically and genetically similar (Kubik et al., 2001). For example, Warpeha et al. (1998) showed that among 35 perennial ryegrass cultivars, which were known as genetically distinct entities, only 10 cultivars were revealed to have unique restriction fragment length polymorphism (RFLP) patterns. Similarly, Charmet and Balfourier (1994) found that the average genetic distance, measured as allelic frequencies at 13 isozyme loci, within the *L. perenne* groups was very low compared to other *Lolium* species. Both the natural breeding system and the artificial breeding strategies of perennial ryegrass make it a difficult task to determine genetic differences among many cultivars of perennial ryegrass.

Historically, ryegrass cultivar identification and property right protection have been based on morphological characteristics such as plant height, leaf width, tiller numbers, and spike length in compliance with guidelines of the Convention of the International Union for the Protection of New Varieties of Plants (UPOV). Compared with various molecular marker systems, characterization of a cultivar using morphological traits is rapid, easy to conduct, less expensive and has proven to be a

powerful means of discrimination between individuals and cultivars (Gilliland et al., 1989). For example, Loos (1993) clearly separated two inbreeding *Lolium* species *L. temullentum* and *L. persicum* from five other out-crossing species through morphological characters. Examining ryegrass morphology, Gilliland et al. (2000) correctly clustered 12 accessions into their related groups. Similarly, Roldán-Ruiz et al. (2001) found that 16 ryegrass cultivars differed from each other in one or more morphological characters, confirming they were all morphologically distinct according to the UPOV guidelines.

High genetic diversity is often associated with increased overall fitness, and enhances system productivity. This can be accomplished through efficient utilization of ecological resources, reduction of inter-plant competition and stability in face of environmental fluctuation (Tilman et al., 2001). In fact, higher yield from cultivar blends than pure-line cultivars has been observed in many crop species, such as wheat (*Triticum aestivum* L.) barley (*Hordeum vulgare* L.), sorghum (*Sorghum biocolor* L.), rice (*Oryza sativa* L.) and oat (*Avena sativa* L.) (Smithson and Lenne, 1996; Helland and Holland, 2001;). Morphological and biological characteristics of perennial ryegrass can be highly variable due to various ecological influences and forage management regimes. Perennial pasture species are subject to ever changing abiotic and biotic disturbances and stresses, and genetic variation often makes contributions to their persistence and adaptation. Rapid genetic changes in response to natural selection have been observed in perennial ryegrass (Charles, 1970; Hazard et al. 2001), tall fescue [*Lolium arundinaceum* (Schreb.) Darbys.] and orchardgrass (*Dactylis glomerata* L.) (Tsurumi et al., 1985).

BG34[1] is a winter-hardy blend of late maturing diploid cultivars of perennial ryegrass (Barnhem, Barmaco, Barlet and Mara) that were developed in Europe but now widely used in the USA. These are commonly blended in ratios of 25, 25, 40 and 10%, respectively, and marketed in the USA as BG34 by the Barenbrug Seed Company. Large phenotypic and genotypic variation is expected from the blend of these cultivars and some of its good performance might be attributable to its intra-species diversity. The specific objective of this study was to quantify within-cultivar and between-cultivar variation on the basis of several morphological characteristics and forage yield. We hypothesized that as cultivars, the four components of BG34 can be differentiated morphologically and in mixture would contribute to high genetic diversity in BG34 as a whole.

[1] Use of tradenames is for clarification and does not imply endorsement by the Ohio State University

MATERIALS AND METHODS

Exp. 1 was carried out from February to June 2002 in a greenhouse at The Ohio State University Columbus OH (40° 0' N and 82° 53' W). Seed of four cultivars (Table 2.1) was sown on 15 February 2002 in 15 cm diam., 15 cm deep plastic pots filled to identical weight with potting media (Metrox Mix 360, Scotts Co, Marysville OH). There were 100 pots of each cultivar, arranged in a completely randomized design. Seedlings were thinned to one plant per pot at 2 wks after sowing. Plants were watered daily and fertilized once a week with a solution comprising 0.5 M $Ca(NO_3)_2$ 4 H_2O, 0.3 M KH_2PO_4, 0.5 M KNO_3, 0.3 M NH_4NO_3, 0.25 M $MgSO_4$ 7 H_2O, and 5 mM Fe, pH 6.5 (J.R. Peters, Inc., Allentown PA) at the rate of 100 mg kg^{-1}. All pots were moved weekly so as to randomize location effects within the greenhouse. Measurements included plant height (H), leaf width (LW), tiller number (TN), tiller diameter (TD), and a score of erectness of the whole plant (E) (1 = upright, 5 = prostrate). The LW and TD were determined with an electronic caliper on five mature leaves or tillers randomly selected within each pot. The first measurement and harvest were conducted on 16 March and 18 June 2002, respectively. Plants were cut 10 mm above the soil surface, and dry weight (Y1, Y2, Y3) was measured three times (19 April, 21 May, and 23 June 2002). The harvested material was oven-dried at 60ºC for approximately 72 hr and weighed with an electronic scale.

Exp. 2 was conducted to partition the morphological variation into genetic and random components. For each 100 plants of a cultivar in Exp. 1, a cluster analysis was conducted with all the morphological characters measured, using Pearson correlation and 10 groups (clusters) were formed with the least within-group variation and most between-

group variation. There were various numbers of plants in each group, and one healthy plant was selected from each group, thus a total of 40 plants. Ten ramets (i.e. clones) comprising a single tiller with 2-4 leaves and some roots of each selected plant were transplanted in August 2002, in the same greenhouse and with similar conditions as in Exp. 1. Pots were arranged in randomized complete blocks with 10 replicates. One clone per genotype comprised each block of 40 plants. The same morphological measurements and methods as in Exp. 1 were used, initially during September 2002, and repeated during December 2002. Dry matter yield was measured on 28 October 2002.

Analysis of variance (ANOVA) was performed on each morphological trait of Exp. 1, assuming random between-cultivar and within-cultivar effects by the GLM procedure of SAS (The SAS System for Windows Release 8.02, SAS Inst. Inc., Cary NC) (Appendix A). The within-cultivar (or residual) variation (σ^2_{error}) was used to calculate the between-cultivar variation (σ^2_b) using the following equation:

$$\sigma^2_b = \frac{\sigma^2_{total} - \sigma^2_{error}}{n} \qquad [1]$$

where n = 100. The MANOVA statement of the GLM procedure of SAS was used to test the overall cultivar effect using all the variables measured.

The standardized data (mean = 0, and standard error = 1) were analyzed using principle component analysis (PCA) and canonical discriminant analysis (CDA). These analyses were performed with the PRINCOMP and CANDISC procedures of SAS, respectively. The scores of the populations on the first two principal components were plotted to visualize independent grouping of the populations. Mahalanobis distance (D^2)

from CDA was used to show morphological differences among cultivars and was calculated using the following equation:

$$D^2 = (\overline{X_1} - \overline{X_2})' S^{-1} (\overline{X_1} - \overline{X_2}) \quad [2]$$

where $\overline{X_1}$ and $\overline{X_2}$ were the estimated mean vectors in the respective groups, and S was the pooled within-groups variance-covariance matrix (Marcoulides, 1997).

For the 100 plants of each cultivar in Exp. 1, a combination of Pearson correlation analysis and Cluster analysis was used to obtain the similarity distance matrix, with the OUTP command within the CORR procedure, and the CLUSTER procedure of SAS. A dendrogram was then generated with the TREE procedure of SAS using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) means. Ten clusters were kept for each 100 plants of a cultivar, so that maximum morphological variation was retained with these clusters. For Exp. 2, one plant was selected from each cluster and was cloned into 10 new plants by replanting tillers. In all, there were 10 genotypes of each cultivar, and 10 clones (forming a group) of each genotype.

We calculated the genotypic variance (σ_g^2) as the difference between observed total morphological (phenotypic) variance from Exp. 1 (σ_{total}^2) and random (or pooled within-group) variance from Exp. 2 (σ_{error}^2), using the following equation:

$$\sigma_g^2 = \sigma_{total}^2 - \sigma_{error}^2 \quad [3]$$

RESULTS

There were significant differences among cultivar means for all the morphological traits and dry matter yield (Table 2.2). Multivariate Analysis of Variance (MANOVA) indicated a significant cultivar effect ($P < 0.05$) for all characters. When using $\alpha = 0.01$ (the standard for "Distantness" recommended by UPOV) all cultivar effects were significant. Nonetheless, the within-cultivar variance (σ^2_{error}) was higher than the between-cultivar variance (σ^2_b) for all traits (Table 2.2). The cultivars showed distinct differences for morphological characteristics. Barnhem had the largest tiller number, the smallest leaf width, and was the shortest. Barmaco was prostrate and Barlet was the most erect. With the lowest tiller number, Mara had the largest tiller diameter, was the tallest, and gave the highest cumulative yield. Despite the significant cultivar effect, large within-cultivar variation was observed for all morphological traits (Table 2.2). The within-cultivar contribution accounted for 93-97% for forage yield variation, and 73-96% of variation in height, tiller number, erectness, tiller diameter and leaf width.

Variation in yield over three harvests was illustrated using box-whisker-plots (Fig. 2.1). Barmaco had the most variable yield, as revealed by the length of its box, and the influence of outliers. Barnhem had the lowest yield, and showed the least variation within the box. Mara and Barlet were similar, having the greatest forage yield, but Barlet showed slightly more variation than Mara.

A principle component analysis of 12 variables found the first four principal components explained 58.9% of the observed variation (Table 2.3). The first principal component separated the populations mainly on the basis of plant size (LW and H1) and yield (Y1, Y2, and Y3). The second principal component separated populations largely

due to tillers numbers (TN1 and TN2), the first yield (Y1) and tiller diameter (TD2). A scatter plot of principle components 1 and 2 showed the degree of differentiation among cultivars (Fig. 2.2). Due to the large number of values, only the mean value and corresponding standard error for each cultivar were presented. No error bars were found overlapping among cultivars. Barnhem was the most distant from the other three cultivars, and Barlet and Mara were the most closely positioned. Similar results were found with squared Mahalanobis distance (D^2) (Fig. 2.3), which estimated the extent of morphological separation between cultivars. The largest D^2 was found between Barmaco and Barnhem ($D^2 = 4.0$), and the smallest between Barlet and Mara ($D^2 = 1.52$). Again, Barnhem was the most distinct of the four cultivars.

The residual variance of cloned plants rendered an estimate for random effects, that is, the within-group variance calculated from Exp. 2. The between-group variances (σ_b^2), were greater than within-group variances (σ_{error}^2) for all traits except for tiller diameter (Table 2.4). Genetic variance accounted for different proportions of the total variance depending on the cultivars and measurements, ranging from 13.4 to 89.9% (Table 2.4). For example, percentages of genetic variance for Barnhem were 13.4% for TD1 and 85.4% for TD2, while for Barlet they were 79.9% for TD1 and 21.3% for TD2.

Morphological characteristics were largely correlated with each other (Table 2.5). Erectness had no significant coefficient with any other traits, indicating its relative independence of other traits. Tiller number showed significantly negative correlations with plant height and tiller diameter. The largest correlation coefficient was found between tiller diameter and leaf width. Yield was strongly and positively correlated to all morphological traits except for erectness (Table 2.5).

DISCUSSION

Phenotypic variation present in a population arises due to genotypic and environmental effects. If phenotype observations are based on sufficiently large sample sizes and the traits measured show significant differences among populations, one can obtain a reasonable estimate of overall genetic performance (Humphreys, 1991). With a strong genotypic basis, quantitative characters can reasonably be used as a measure of genetic distances, even though the phenotype cannot be directly related to the genotype (Loos, 1993). For example, in a study of estimating conformity between related ryegrass cultivars, Gilliland et al. (2000) found that the magnitude of the morphological differences between 12 accessions closely reflected their known breeding history.

The within-cultivar variation for all traits was in general higher than the between-cultivar variation, though these component cultivars are of different geographic origins and breeding eras. The term "cultivar" implies a shared genetic component of all individuals within a "cultivar". However, the extent that these individuals share genes varies dramatically between species. It is very high for soybeans (*Glycine max*), wheat (*Triticum aestivum*), maize (*Zea mays*) hybrids et al, but rather low for obligate outcrosses, like perennial ryegrass and alfalfa (*Medicago sativa*). In fact, many studies have shown extensive within-cultivar variance and much less between cultivar variance for obligate outcrossing crop species. For example, Kubic et al. (2001) found the within-cultivar variance accounted for 85.35% of the total genetic variation among seven perennial ryegrass cultivars with simple sequence repeats (SSR) marker. Using AFLP markers, Guthridge et al. (2001) found 89.6% variance within populations of three ryegrass accessions with diverse breeding history. Julier et al. (2000) reported that

within-cultivar variance of alfalfa accounted for 57-100% for morphological traits and yield.

In terms of contribution to forage yield, genotypes may not perform the same under different conditions, since this would involve the traits other than yield potential *per se*, such as tolerance to environmental stresses and compatibility with other forage species. In accordance with the theory of the advantage of biodiversity, large within-cultivar variation for yield and morphological traits may be needed to survive and thrive under various environmental conditions and thus to ensure high yield. Roldán-Ruiz et al. (2001) believed that this reflected the breeders' goal to target diverse phenotypes for forage types. Apparently, when put together, these four cultivars create an even more diverse blend than each cultivar alone.

The first principle components explained less than half of the total variation, though the cultivar effects were significant for each trait. The lack of differentiation between these four cultivars was not unexpected. Speciation and domestication of *Lolium* are recent (Malk, 1967), and these species (including *Lolium perenne*) have not yet diverged sufficiently to display extensive novel genetic differentiation from their progenitors. The most common breeding strategy in perennial ryegrass is selective crossing between existing cultivars, and the progenitors of most current cultivars are a relatively small number of original wild collections (Devey et al. 1994). Breeding might have led to the reduction of genetic diversity from wild type in commercial ryegrass cultivars (Warpeha et al., 1998). It was interesting to find that Mara and Barlet, which originated from different countries, were the most closely linked based on morphological characterization, suggesting the possibility that similar phenotypes can result from

diverse plant backgrounds. This observation, however, should not be interpreted as a direct indication of close similarity of the genetic background of the two cultivars, since different gene pools can be manipulated to produce similar phenotypes. Cultivars displaying high phenotypic similarity need not be genetically similar. For instance, Roldán-Ruiz et al. (2001) found a rather poor agreement on the cultivar relationships of 16 ryegrass cultivars between morphological and molecular marker methods (AFLP and sequence tag sites, STS).

The total biodiversity of a system is not merely the number (or distribution) of species present, but also includes the genetic diversity of the component species (Gaston, 1996). In other words, the concept of biodiversity should include biological identities such as genetic diversity, as well as the commonly recognized taxonomic descriptors such as species, genus and family. In this study we showed substantial diversity within cultivars as well as among cultivars, which potentially contribute to the performance of BG34. As the ultimate measure of population diversity is not the number of species alone, but the number of genes present and being expressed, molecular work might be a complement to provide quantitative evidence of the genetic diversity found in these four cultivars.

Cultivar	Ploidy	Description	Country of origin	% in BG34
Barlet	Diploid	Bred from very diverse genetic base	Netherlands	40
Barmaco	Diploid	Late heading cultivar. Very winter-hardy and persistent	Netherlands	25
Barnhem	Diploid	Forms an extremely dense sward	Netherlands	25
Mara	Diploid	Most winter hardy	Romania	10

Table 2.1. Description of cultivars used in Exp. 1 and Exp. 2.

Morphological traits	Barnhem		Barmaco		Barlet		Mara		Cultivar effect	$LSD_{0.05}$	σ^2_{error}	σ^2_b
	mean	STD	mean	STD	mean	STD	mean	STD				
H1 (cm) †	33.4	(4.5)	31.4	(4.7)	34.6	(4.5)	35.5	(3.7)	**	1.22	19.18	3.08
H2 (cm)	29.6	(5.0)	30.5	(5.6)	30.9	(5.6)	33.3	(5.5)	**	1.5	29.14	2.18
TN1 (number)	50.2	(17.0)	41.2	(17.7)	46.4	(12.6)	45.5	(13.1)	**	4.26	234.85	10.52
TN2 (number)	176.1	(45.7)	170.8	(48.3)	155.4	(39.1)	147.4	(37.8)	**	11.97	1845.0	164.07
E1 (score)	2.7	(1.1)	3.2	(1.1)	2.3	(0.9)	3.2	(0.9)	**	0.28	1.01	0.17
E2 (score)	2.4	(0.7)	2.6	(0.8)	2.1	(0.7)	2.6	(0.8)	**	0.21	0.59	0.07
TD1 (mm)	1.7	(0.3)	1.8	(0.2)	1.8	(0.2)	1.9	(0.3)	**	0.08	0.07	0.01
TD2 (mm)	2.1	(0.3)	2.2	(0.3)	2.2	(0.3)	2.2	(0.3)	**	0.09	0.09	0.02
LW (mm)	4.0	(0.5)	4.7	(0.6)	4.6	(0.5)	4.7	(0.7)	**	0.16	0.33	0.12
Y1 (g)	2.4	(0.8)	2.3	(0.8)	2.6	(0.7)	2.6	(0.6)	*	0.21	0.25	0.018
Y2 (g)	5.7	(1.1)	5.6	(1.3)	6.1	(0.9)	5.9	(1.0)	**	0.31	1.26	0.047
Y3 (g)	12.6	(2.9)	13.5	(3.5)	13.6	(2.6)	13.8	(2.5)	*	0.81	8.36	0.22

Table 2.2. Mean, standard deviation (STD), significance of cultivar effect, LSD, within-cultivar variance (σ^2_{error}), and between-cultivar variance (σ^2_b) for each character for four ryegrass cultivars (Exp. 1).

†: H1, H2, plant height from the 1st and 2nd measurement (cm); TN1, TN2 tiller number; E1, E2, plant erectness (erect = 1, prostrate = 5); TD1, TD2 tiller diameter (mm); LW, leaf width (mm); Y1, Y2, Y3, yield (g DM).
* and ** significant at $\alpha = 0.05$ and 0.01, respectively.

Principal component	1		2		3		4	
Percentage of cumulative variance	23.11		37.61		48.29		58.89	
Principal component scores	LW	0.41	TN1	0.64	H2	-0.64	E2	0.68
	Y1	0.38	Y1	0.46	TD2	0.42	E1	0.67
	H1	0.38	TN2	0.36	TN2	0.4	LW	0.14
	Y2	0.38	TD2	-0.24	TD1	0.27	TN2	0.12
	Y3	0.35	H2	-0.24	H1	-0.27	TD2	0.11

Table 2.3. Principle component analysis results of the first four principle components, percentage of variance explained and the characters with the highest loadings on the first four principle components (Exp. 1).

	σ^2_{error}	σ^2_b	mean	STD	Barnhem	Barmaco	Barlet	Mara
					σ^2_g			
H1 (cm)	35.38	89.02	45.01	8.61	3.51 (17.2)	7.71 (34.1)	9.34 (46.6)	6.69 (49.2)
H2 (cm)	22.54	62.97	35 .28	7.67	11.09 (44.5)	16.66 (54.9)	13.89 (44.0)	14.49 (48.7)
TN1 (number)	145.75	239.66	25	15.12	184.23 (63.8)	163.7 (52.8)	95.34 (56.5)	93.93 (54.7)
TN2 (number)	680.03	754.71	61.11	29.69	1425.96 (68.2)	1748.92 (74.5)	937.89 (61.8)	1147.25 (80.5)
E1 (score)	0.66	0.75	2.96	0.93	0.75 (66.5)	0.78 (64.7)	0.23 (29.3)	0.31 (36.5)
E2 (score)	0.45	0.69	2.4	0.84	0.2 (36.5)	0.39 (55.5)	_	0.01 (16.6)
TD1 (mm)	0.11	0.08	2.56	0.34	0.01 (13.4)	0.02 (33.1)	0.04 (79.9)	0.03 (28.5)
TD2 (mm)	0.12	0.12	2.07	0.38	0.07 (85.4)	0.06 (60.9)	0.01 (21.3)	0.06 (59.7)
LW (mm)	0.40	0.86	4.63	0.88	0.09 (32.7)	_	0.1 (44.1)	0.12 (28.0)
Y1 (g)	1.26	1.51	2.63	1.3	0.1 (16.2)	0.2 (29.7)	0.45 (47.1)	_
Y2 (g)	2.32	2.68	4.49	1.8	0.27 (19.8)	0.53 (26.5)	1.63 (16.2)	1.07 (15.1)
Y3 (g)	1.96	2.35	3.63	1.67	6.73 (80.1)	10.8 (89.9)	5.12 (73.7)	4.69 (76.5)

Table 2.4. Within-genotype (σ^2_{error}) and between-genotype (σ^2_b) variance, mean, standard deviation (STD), of each character for 40 genotypes, genetic variance (σ^2_g) calculated using Eq. 3, and σ^2_g as a percentage of total variance (σ^2_t) in parenthesis (Exp. 2).

	Height	Tiller Number	Erectness	Tiller diameter	Leaf width
Tiller number	-0.31***/0.076 NS *-0.21***				
Erectness	-0.13**/0.043 NS *0.03* NS	-0.057 NS/0.054 NS *0.02* NS			
Tiller diameter	-0.002 NS/.25*** *0.15***	-0.17/-0.08 NS *-0.16****	-0.049 NS/0.027 NS *0.002* NS		
Leaf width	0.18**/0.24*** 0.26***	-0.11*/-0.12 *-0.13***	-0.015 NS/0.094 NS *0.05* NS	0.39***/0.44*** *0.51* ***	
Yield	0.059**/0.47*** *0.39****	0.033 NS/0.55*** *0.16****	-0.096 NS/0.087 NS *-0.05* NS	0.11*/0.35*** *0.32* ***	0.24***/0.32*** *0.33* ***

Table 2.5. Lowest/highest correlation coefficients between 2 measurements for each of height, tiller number, erectness, tiller diameter, leaf width and yield, and the correlation of mean values (in italic) (Exp. 1).

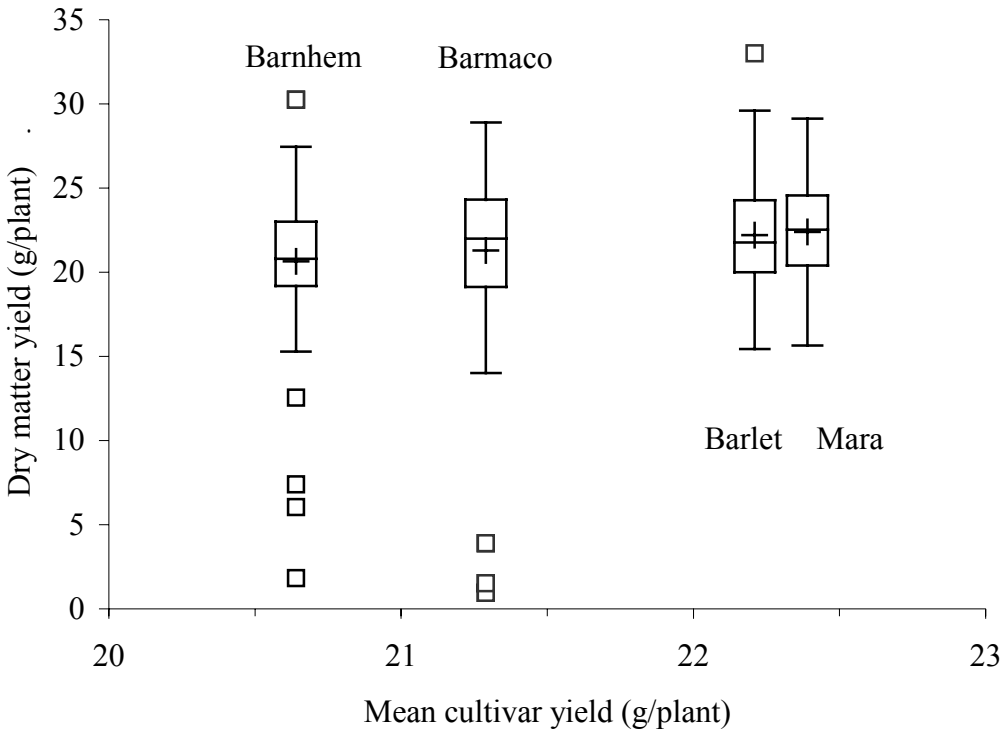


Fig. 2.1. Within- and between-cultivar variation for forage yield totaled over three harvests. In each case, the box represents the inter-quartile range, which contains 50% of the values. The whiskers are lines that extent from the box to the highest and lowest values, excluding outliers. The line across the box indicates the median, and plus sign '+' inside the box, the mean. Cases with values more than 1.5 lengths from the upper or the lower edge of the box are considered outliers (∗). The position of each box on the x-axis represents the mean yield for that cultivar (Exp. 1).

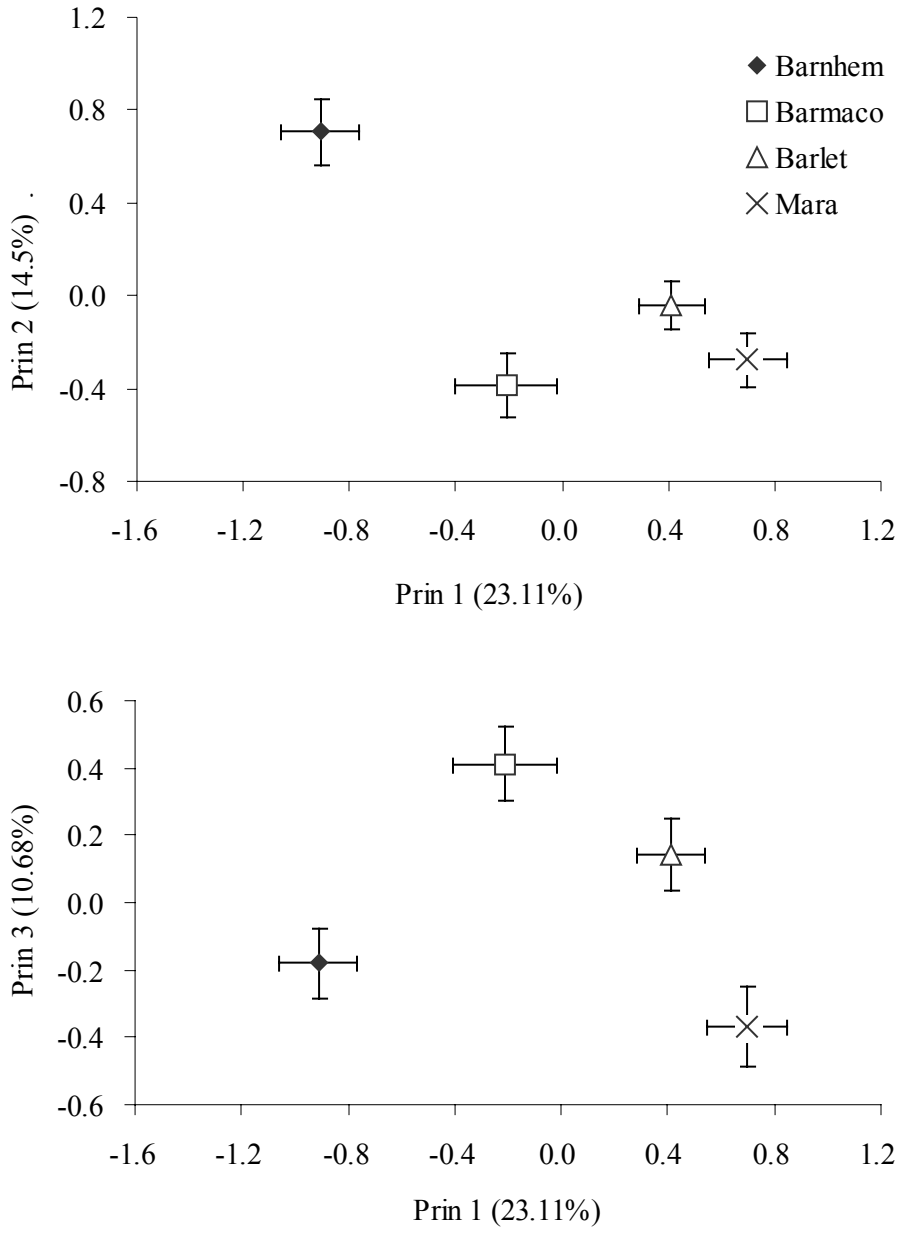


Fig. 2.2. Scatter plot of four ryegrass cultivars (n=100 per cultivar) separated on the first and second principle components (Exp. 1).

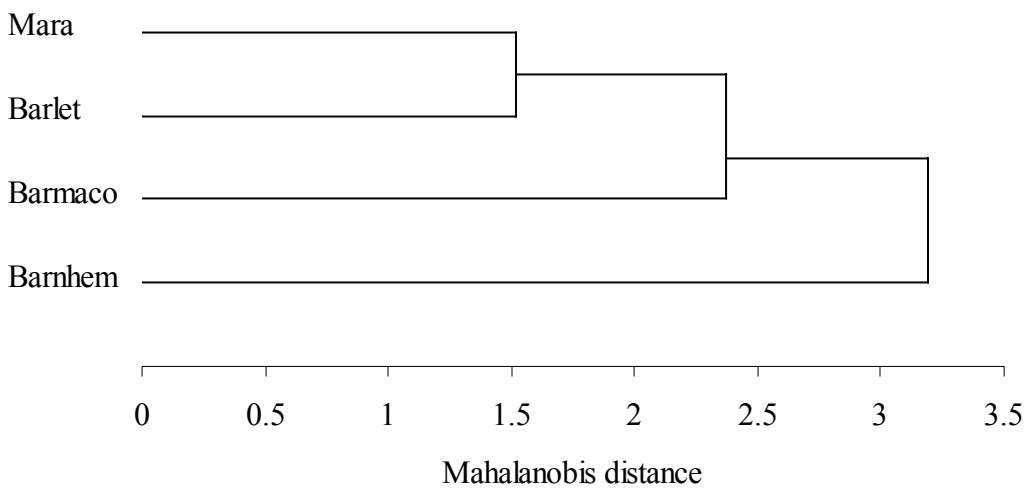


Fig. 2.3. Dendrogram based on squared Mahalanobis distances estimated from morphological data. This dendrogram was constructed using the unweighted pair-group method using arithmetic averages (UPGMA) clustering algorithm and represents the relationships between cultivars (Exp. 1).

CHAPTER 3

YIELD RESPONSES OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) TO GENOTYPE DIVERSITY AND CONTRASTING DEFOLIATION FREQUENCIES AND INTENSITIES

ABSTRACT

Perennial ryegrass (*Lolium perenne* L.) is often sown as a blend of cultivars, yet the potential advantage of blends over its pure components still remains controversial. A greenhouse experiment was conducted to evaluate the relative herbage yield of monocultures and mixtures using cloned plants. Eight genotypes of perennial ryegrass representing four yield levels, each at two tillering potentials, were grown as monocultures. In addition, 9 mixture treatments comprised of four 2-genotype, four 4-genotype and or 8-genotype were created from tillers. Defoliation treatments imposed on all genotype treatments were frequent (3 week) vs. infrequent (6 week) at 20 mm height in Exp. 1, and short (20 mm) and tall (60 mm) cutting height at a 6 week interval in Exp. 2. Four harvests were taken and dry matter yield was evaluated on the individual plant basis at each harvest. There was a significant linear relationship between genetic diversity and production per plot in Exp. 1 ($P = 0.014$) and a significant difference between mono-genotype and multi-genotype treatments in Exp. 2. Defoliation frequency and height had

significant effects on forage yield. The total forage yield was 12.7 % greater under 6 wk cutting than under 3 wk cutting, and 14.3 % more at 20 mm cutting height than at 60 mm cutting height. It was concluded that genotypic diversity increased the herbage yield and this response was modified by the defoliation regimes.

INTRODUCTION

Recent studies suggest plant species richness enhances grassland productivity (Naeem et al., 1994; Hector, et al., 1999; Loreau, 2001; Bullock et al, 2001; Tilman et al., 2001). This positive relationship between plant diversity and production has been extensively observed in both cropping and pasture systems (Simthson and Lenne, 1996; Frey and Maldonado, 1967; Benjamin, 2004). These principles also apply at a smaller scale of intra-specific genetic diversity, especially when comparisons between mixtures and pure lines of crop cultivars are made. For example, in a 3-yr survey using ryegrass six cultivars with contrasting growth habit, Jones and Roberts (1994) reported the yield from mixtures of perennial ryegrass was greater than that from monocultures by an average of 13%. In a study of the cultivar blend response of oat (*Avena sativa* L.), Helland et al. (2001) found cultivar blends had greater and more stable yield than the component pure lines in an early- maturity experiment. Allard and Adams (1969) showed the intra-specific mixtures of randomly chosen cultivars of wheat (*Triticum aestivum* L.) exceeded their component means by 2 to 5 % for grain yield. Clay and Allard (1969) reported the yield advantage of mixtures of barley (*Hordeum vulgare*) over component means ranged 2 to 5 %. In a literature review on cultivar blends in many crops, Smithson and Lenne (1996) concluded that blends generally yield more than pure lines, and the causes included stability of system production and disease control. Maintenance of heterozygosity may provide a greater chance of successful adaptation across a range of environments than a genetically homogeneous population.

The superior performance of diverse plant communities has been generally explained as “niche complementarity”, “buffering effect” and “sampling effect”. The first

explanation involves the idea that differences in resource requirements (e.g. spatial, temporal resource and habitat use) allow the community as a whole to utilize the local resources more efficiently, and thus increased productivity (Benjamin, 2001; Tilman, 2001). For the "buffering" effect, genetic diversity provides "buffering" or an insurance against environmental fluctuations, in that different species respond differently to varying conditions, leading to more stable and predictable aggregate community properties (Allard and Bradshaw, 1964; Loreau, 2000; Helland, 2001). The sampling effect is due to the greater chance of including more productive individuals in highly diverse plant populations (Huston, 1997). Presumably, these mechanisms also apply at the intra-species scale.

The practice of blending cultivars to pursue production superiority has been tested in grazing systems, where vegetative yield is the harvest target. In the 1960's, a series of studies were conducted with *Lolium* species to investigate the yield of cultivars grown in mixtures relative to their pure stand performance (Alcock & Morgan, 1966; Rhodes, 1970; Thomson, 1969, McBratney, 1978; Culleton et al. 1986). Other studies compared perennial ryegrass in monocultures or in mixtures with other forage species (England, 1965, 1968; Norrington-Davies, 1968). There were conflicting results among these studies, and no simple conclusion can be drawn on the relative merits of mixtures over monocultures.

As a dynamic system, pastures are often subject to different defoliation patterns in terms of intensity and frequency. The effects of defoliation have been generally viewed as detrimental to grasses, because defoliation removes photosynthetic tissue (from leaf and culm) which reduces carbon reserves (culm and root) and makes plants more

susceptible to pathogens. Studies dealing with the effects of defoliation on growth of pasture plants and associations have shown that as the intensity and frequency of defoliation increases, yield of shoot tissue decreases (Donald, 1963; Wagner, 1952). On the other hand, the opposite responses have also been obtained through a process known as compensatory response, or even over compensation, in which plants recover the equivalent or more yield or fitness for the removal of leaf tissue (Owen, 1980; McNaughton et al. 1983, Belsky, 1986). Plants endowed with such attributes have obvious selective advantages that result in genotype maintenance (Trumble et al., 1993).

Advantages of high versus low genotypic diversity in forages and the influence of varying defoliation patterns on these advantages have not been fully explored. Information is lacking about the actual contribution of genetic diversity to overall pasture yield at the intra-species level, and how this is associated with defoliation patterns. The objective of this study was to evaluate the relative yield of perennial ryegrass in response to the number of genotypes present, and how this response was affected by the defoliation regime, i.e. defoliation frequency and defoliation intensity. It was hypothesized that the mechanisms occurring in species-scale studies will be relevant at the intra-species scale and benefits from species diversity can be extrapolated to the intra-species scale via improvement of productivity with genotypic-diverse communities. It was further hypothesized that the favorable responses in productivity to genotypic diversity will vary under contrasting defoliation treatments (i.e. frequent vs. infrequent and low vs. high cutting height).

MATERIALS AND METHODS

Plant description

Eight genotypes used in this study were selected from a population of 400 ryegrass plants, which were established from a four-cultivar blend, BG34. The morphological traits were assessed in a previous survey when sown at one plant per pot (Chapter 2). These genotypes comprised four levels of yield potential (low, medium, high and very high), and two levels of tiller number potential (low vs. high) (Table 3.1). They were coded as A through H according to their tiller size (mg $tiller^{-1}$) and tiller number (tiller $plant^{-1}$). Eight mono-genotype and nine multi-genotype combination treatments were established (Table 3.1). In plots with multiple genotypes, the components of the mixtures were planted alternately along the row and columns.

Experiment 1

A greenhouse experiment was conducted from May to November 2003, at the Ohio State University, Columbus OH (40° 0' N and 82° 53' W). Healthy ramets (single tiller with root) from selected plants were transplanted into a 19-cm-deep tub filled with potting media (Metrox Mix 360, Scotts Co, Marysville OH). Tubs were divided with plastic sheets into 6 plots per tub (0.175 × 0.156 m). A total of 64 plants (8 rows * 8 columns) were planted in each plot with 2 cm between each row and column. For plots containing multi-genotypes, component plants of each genotype were planted alternately along the rows and columns to maximize inter-genotype interaction and were marked with colored paper clips.

Experiment 1 used a 2 x 17 factorial design, with 3 replicates in a randomized complete block design. In addition to the 17 genotype treatments (Table 3.1), there were 2 defoliation treatments that comprised cutting intervals of 3 and 6week. There were 5 harvests for the 6wk treatment and 9 harvests for the 3 wk treatment; however, the initial (pretreatment) harvest was omitted from subsequent analysis for 3 and 6 wks treatments. All treatments were randomly assigned to plots within each plot. Plants were cut individually at each harvest by drawing up the herbage and clipping to a stubble height of 2.5 to 3 cm. Dry matter (DM) yield was measured on the individual plant basis following each harvest. Herbage from each genotype was kept separate for each plot to allow calculation of the contribution of each genotype to biomass.

Experiment 2

Experiment 2 was carried out from December 2003 to July 2004 in the same greenhouse as for Experiment 1. The only difference of Exp. 2 from the Exp. 1 was the defoliation treatment, in which each plot was cut at 20 and 60 mm height each 6 weeks. The 20-mm/6 week defoliation treatment occurred in both Exp. 1 and Exp. 2.

Data analysis

Statistical analysis was conducted on the data collected at each 6 wk interval, with the yield for the 3-week defoliation treatment being the sum of the two 3-week harvests during that period. Analysis of variance was performed with PROC GLM of SAS (The SAS System for Windows Release 8.02, SAS Inst. Inc., Cary NC) to test genotypic treatment and defoliation treatment effects on total DM yield. (Appendix B) The

genotypic diversity linear relationship was tested using the CONTRAST statement with coefficient for the eight monocultures, the four 2-genotype, the four 4-genotype and the one 8-genotype treatment being (-11, -11, -11, -11, -11, -11, -11, -11, -14, -14, -14, -14, 2, 2, 2, 2, 136).

RESULTS

Significant genotype treatment effects on yield were found at each harvest as well as for cumulative yield across all harvests in both experiments (Table 3.2). On average, mixtures (treatments 9 to 17) had significantly higher yield than monocultures (treatments 1 to 8) at the Harvest 2 and 4 as well as for the total yield in Exp. 2, with the differences being . There were no differences between monocultures vs. mixtures in Exp. 1. Yield from monocultures differed significantly at all harvests in both experiments, while mixtures differed at Harvest 2, 3 and 4 and in total yield for Exp. 1, and at Harvest 2 and in total yield for Exp. 2. In addition, a significant linear regression ($P = 0.014$) of dry matter yield over genotype number was detected at the Harvest 3 and 4, and in total yield, in Exp. 1.

Defoliation frequency and intensity both significantly affected forage yield (Table 3.2). Frequent cutting (3-wk) reduced grass yield by 12.7 % compared with 6-wk cutting, while lax cutting (60 mm) reduced herbage yield by 14.9 %. There was a significant interaction between genotype treatment and cutting intensity ($P < 0.05$) at Harvests 3 and 4, as well as for total yield in Exp. 2. This was caused by genotype C and D producing higher yields under 60-mm cutting than under 20-mm cutting at those harvests.

Dry matter yield was regressed on the number of genotypes. There were a different numbers of treatments corresponding to each given number of genotypes, i.e. eight treatments for one genotype, four treatments for two and four genotypes, and one treatment for eight genotypes (Fig. 3.1). Due to the high variation in DM yield from each treatment within each group with the same number of genotypes, low R^2 values for regression were observed for all defoliation regimes. The slopes of each regression were

all positive, indicating the positive effect of genotype number on dry matter yield. The highest yielding treatments were a monoculture (genotype F) in Exp. 1 and a two-genotype treatment (genotype E and F) in Exp 2; however, their yield was not significantly different from the eight-genotype treatment 17. Two-genotype treatments had the lowest yield on average in Exp. 1, compared with other genotype treatments, but had relatively higher yield in Exp. 2.

The percentage contribution of each genotype to the final biomass (Harvest 4) was plotted against the number of genotypes in mixtures (Fig. 3.2). Large genotypes, especially genotypes E and H, tended to have high contribution to the yield. Genotype C made the least contribution to yield in all defoliation treatments. Conversely, the proportion of small genotypes was more frequently reduced in mixtures. Some obvious consistency of proportion in yield in different mixtures was detected for genotypes such as B, C, D and G. The percentage of each genotype in mixtures varied depending on defoliation regime. For example, genotype H accounted for greater proportion of yield than any other genotypes under frequent (3 week) than infrequent cutting. Genotype F was relatively more abundant in complex mixture (i.e. 8 genotype) than in simple mixtures, especially under intense (20mm) cutting (Experiment 2). It was noticeable that yield of genotype G was more like a small genotype, and that of genotype D was more like a large genotype.

DISCUSSION

This was the first study examining the effect of genetic diversity on forage yield using a number of cloned genotypes under contrasting defoliations. Our results showed there was a significant positive linear relationship between genetic diversity and forage yield in Exp. 1 and a significant higher yield from multi-genotype than from mono-genotype in Exp. 2. This finding was generally in line with the conclusion of Jones (1994) that mixtures consisting of components with contrasting growth habits had significantly positive effect on herbage yield. This reinforces the long-standing ecological paradigm that higher plant diversity enhances community productivity (Tilman 1997, 2001), even though this diversity was confined to the intra-specific level only. It has been observed that young pastures have greater yield than old ones, given the same ratio of forage species. One reason for this is the presence of higher level of genetic diversity in newly established pastures than old ones, in which many genotypes were lost through natural selection (Wedderburn et al. 2005). Studies have suggested that this gain in yield is the result of better exploitation of ecological resources and reduction of interplant competition from morphological or physiologically diverse plant populations (Knops et al., 1999). In such a diverse community, the resident plants complement each other in resource use by having different rooting depths, leaf architecture, growth rates and other characteristics.

The highest yielding treatments were a monoculture in Exp. 1, and a simple mixture in Exp. 2 (Fig. 3.1). Nonetheless, their yield was not significantly higher than the eight-genotype treatment in both experiments. McBratney (1978) and Culleton et al (1986) suggested that there was very little evidence that a mixture can have superiority

over its highest yielding component. Similarly, in a study comparing herbage yield of simple and complex mixtures with grasses and legumes, Barker et al (2003) found that the highest yielding treatment of forage in most seasons and sites were those with only one or two sown species, which varied among sites and seasons; however, there were no significant differences between these monocultures or simple mixtures and the nine-species treatment. The inconsistency of the identity of the most productive species between spring and summer in their studies is reason to suggest that the most consistent high production will be achieved from complex mixtures.

Numerous studies have investigated the plant diversity effect on yield using several cultivars, and conflicting opinions exist regarding relative advantage of mixtures over monocultures. For example, Thomson (1969) didn't find superiority of mixtures of two perennial ryegrass cultivars over monoculture in yield. Alcock & Morgan (1966) and Rhodes (1968) using Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass revealed that certain binary mixtures out-yielded their higher yielding components when grown in monoculture. These conflicting results suggest that the relationship between morphological or phenotypic diversity and mixture performance varies depending on the crop species, the sampled cultivars or the environmental conditions, as well as the methods of harvests, i.e. cutting height and frequency, initial date of cutting in a season, etc.

Forage species in pasture are under constant or systemic defoliation, which has profound impact on the overall forage yield. Defoliation is in essence detrimental to grass species by removing photosynthetic tissue and carbon reserves (Owen, 1980). Defoliation frequency and intensity have manifold effects on the final forage biomass depending on

grass species, stage of initial cutting and environmental conditions. In this study, a 20 mm defoliation height clearly increased accumulated grass yield by 14.9 %, indicating the presence of overcompensation in growth, as suggested by Hillbert et al. (1981) who stated that compensation increases exponentially with defoliation intensity. This finding was in line with that of Dovel (1996) who reported that yield from wetland meadow increased as clipping height decreased. Studies of Reid (1966) and Harrington and Binnie (1971) suggest optimal clipping height at 2.5 to 5 cm for other species. Under favorable conditions (e.g. non-limiting moisture) where rapid regrowth occurred, forage yields tended to increase as clipping height decrease (Dovel, 1996). In a 3-yr study, Bryan (2000) found the effects of cutting treatments on Kentucky bluegrass yield was affected by water availability, i.e. in wet year, biomass was increased with frequent and moderately intensive cutting, and in dry year, there were no significant differences. As expected, plants were more productive under infrequent cutting than under frequent cutting in this study. This was in agreement with the results of a number of studies (Rhodes, 1970; Surgenor and Laidlaw, 1976; Diego, 2002) that high frequency of defoliation or short time for recovery showed more negative effects on vegetative production. In addition, frequent cutting reduced ground cover of photosynthetic tissue and the interception of light after defoliation was low and much of the incident light penetrated to the soil surface.

My results revealed that the mixture superiority in yield of multi-genotype blends was associated with a certain defoliation regime, e.g. at 6 week interval and 20 mm cutting height. Some studies have indicated that yield responses from mixtures of cultivars might be achieved only when components differed in some morphological

characteristics (Rhodes, 1970; Jones, 1994). The lack of consistent relationship between genetic diversity and yield response under different defoliation treatments may be due to the insufficient genetic diversity and thus limited mixture response. Within species, the differences among individuals are restrained to the morphological level, such as leaf orientation, culm erectness, plant height or root depths etc, and there is lack of other functional diversity, such as N-fixing ability, C3 vs. C4 metabolism, phonologic variation etc., which play an important role in defining the ecological profile of a species-diverse community.

Genotypes used in this study were selected from a broad seed blend population, BG34, which is widely used in the northeast of the United States. Some of its good performance is attributable to the presence of genotypic diversity and wide adaptation across different geographical conditions. My results indicated the ability and potential to use genetic diversity to increase mixture response could be present in genotypes that are adapted to cultivation in genotypically mixed stands. This response in yield might reflect the breeder's intention that the cultivars and genotypes can maximize their capacity to interact positively with each other in the same stand, and secure a good yield across a wide range of environmental conditions.

Genotype treatment	Yield potential	Tiller size* (mg tiller^{-1})	Tiller number level	Tiller number* (tillers plant^{-1})
A	Low	63.8	High	114.5
B	Low	149.4	Low	48.2
C	Medium	112.7	High	93.2
D	Medium	208.4	Low	47.5
E	High	120.0	High	101.7
F	High	170.9	Low	70.8
G	Very high	103.2	High	149.2
H	Very high	183.4	Low	82.9
A, B	Low		High vs. low	
C, D	Medium		High vs. low	
E, F	High		High vs. low	
G, H	Very high		High vs. low	
A, C, E, G	Mixed		High	
B, D, F, H	Mixed		Low	
A, C, F, H	Mixed		Mixed	
B, D, E, G	Mixed		Mixed	
A-H	Mixed		Mixed	

Table 3.1. Genotype treatments, mean tiller size, and mean tiller number per plot of each genotype.

* Tiller size and tiller number were assessed in a previous study.

Source of variation	D.F.	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Total
Exp. 1						
Genotype (Gen)	16	27.54**	20.97**	15.05**	36.27***	338.62***
Frequency (Freq)	1	1.04	161.28**	278.61***	78.68***	1386.92***
Gen * Freq	16	3.99	9.09	3.66	8.71	63.78
Contrasts						
Mon vs. mix	1	8.24	0.93	0.55	0.19	10.45
Between mon	7	42.41**	27.06**	16.94*	55.81***	485.41***
Between mix	8	16.95	18.15*	15.21*	23.72**	251.22**
Gen diversity-linear	1	25.15	25.91	32.04*	24.72*	430.08*
Exp. 2						
Genotype (Gen)	16	1.58**	4.92***	4.93***	1.38**	37.57***
Intensity (Int)	1	5.24**	2.89	4.71*	15.56***	102.09***
Gen * Int	16	0.58	1.44	2.03*	1.12*	11.33*
Contrasts						
Mon vs. mix	1	1.41	9.93**	1.09	3.31*	51.99**
Between mon	7	2.27*	7.86***	9.09***	2.24*	63.87***
Between mix	8	1.01	1.71**	1.78	0.38	12.74**
Gen diversity-linear	1	0.01	3.85*	0.28	1.25	13.38

Table 3.2. Variance of DM yield/harvest and in total yield across all harvests for different sources of variation (Exp. 1 & 2).

*, **, ***: significant at $P < 0.05$, 0.01 and 0.001 respectively; Gen, Fre and Int, effects of the genotype, cutting frequency and cutting intensity on the 17 genotype treatments respectively; s.e., standard error.

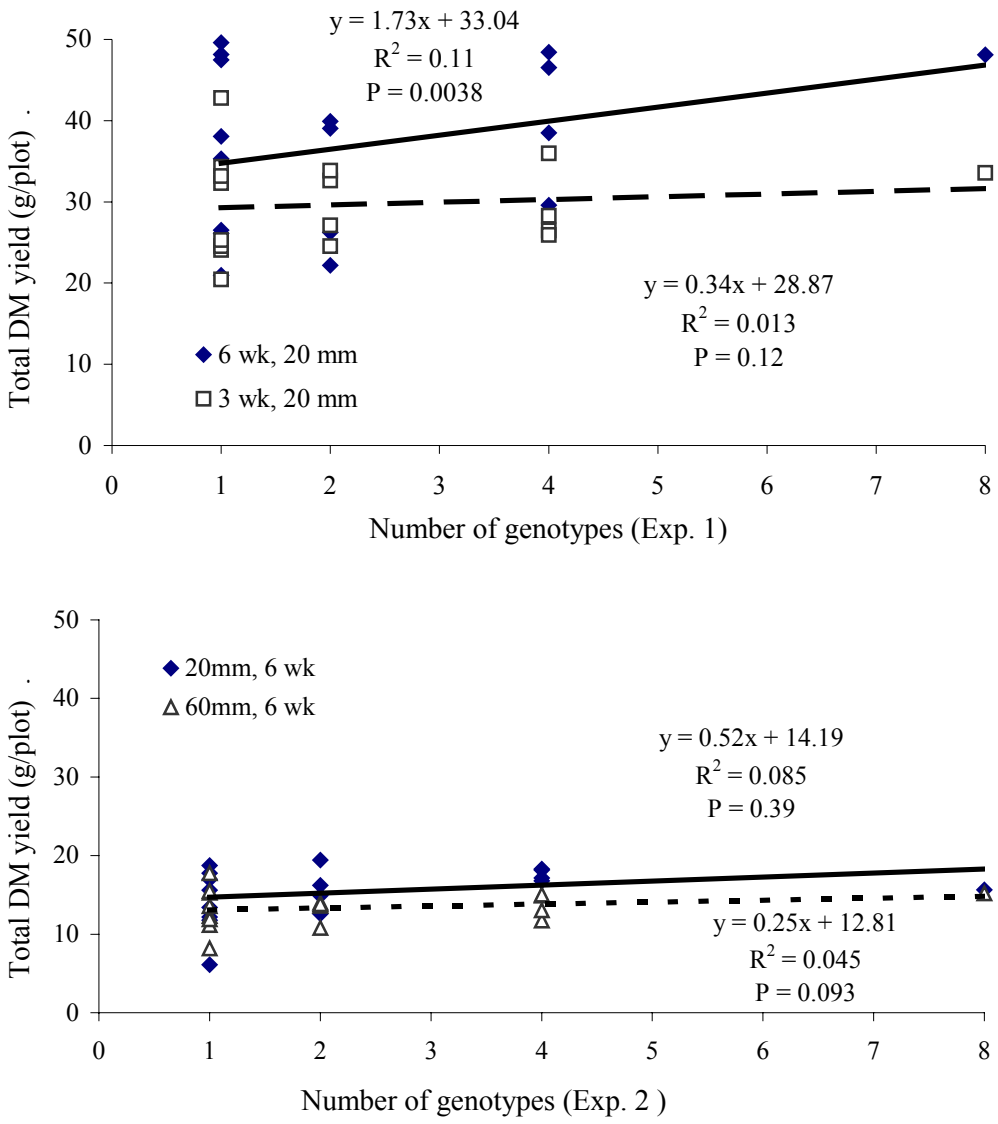


Fig. 3.1. The relationship between the total DM yield and the number of genotypes (linear least square fit shown)

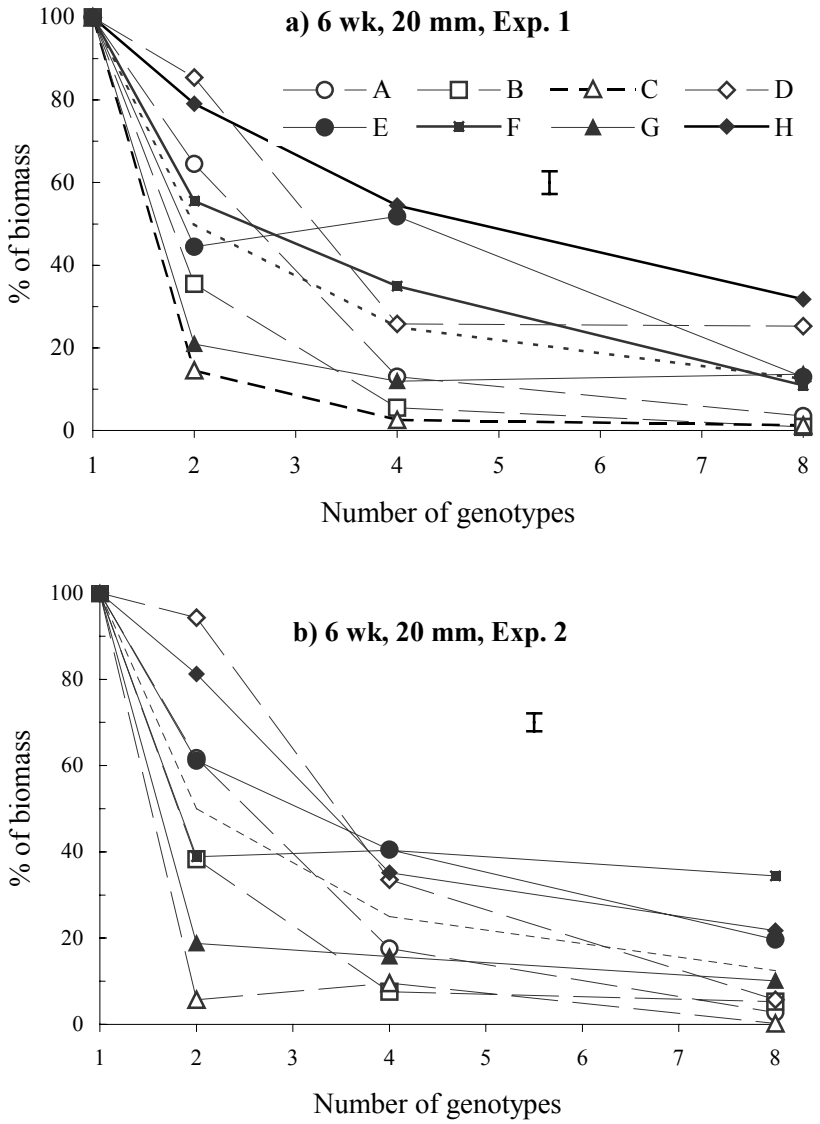
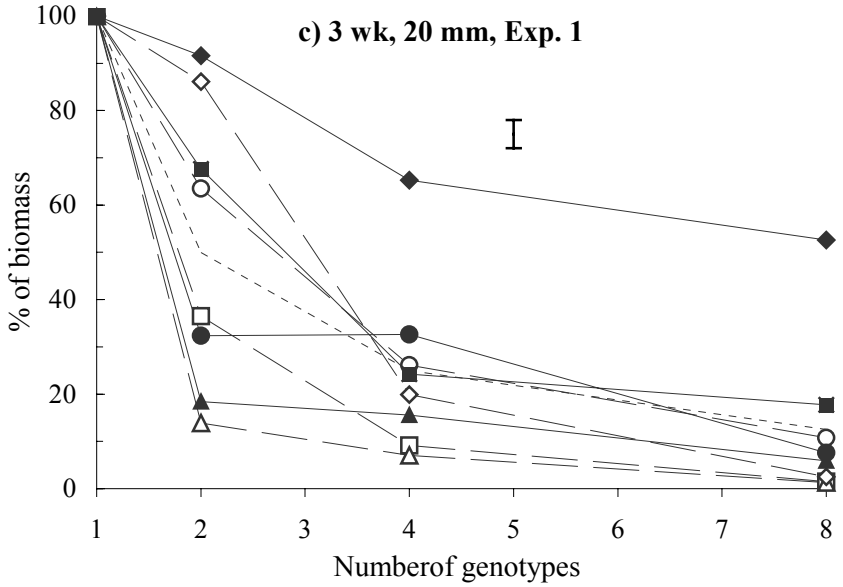
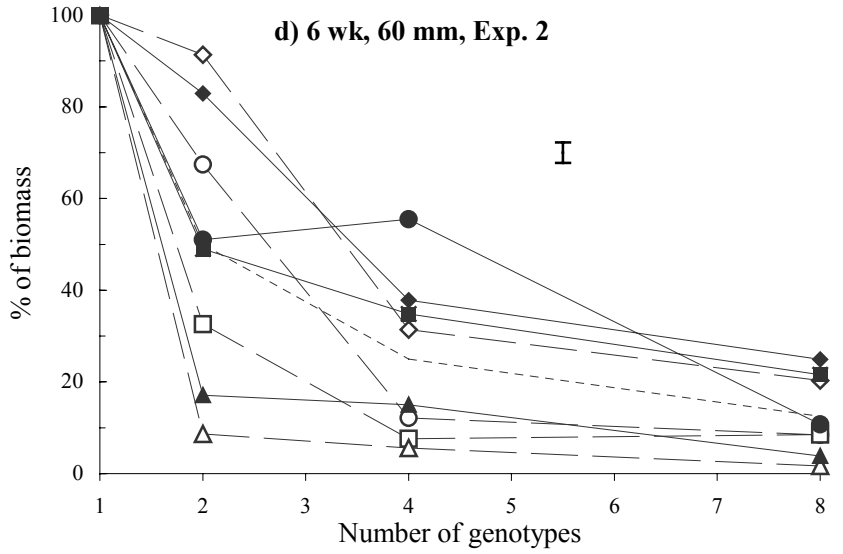


Fig. 3.2. The relationship between percentage contribution of each genotype to the final biomass (Harvest 4) and number of genotypes. (Bars represent mean standard error)

(Continued)

(Continued)


c) 3 wk, 20 mm, Exp. 1
% of biomass
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Numberof genotypes


d) 6 wk, 60 mm, Exp. 2
% of biomass
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Number of genotypes

CHAPTER 4

GENOTYPIC DIVERSITY EFFECTS ON TILLER RESPONSES AND SURVIVAL OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

ABSTRACT

The fact that greater genetic diversity generally has favorable yield responses has been well documented. Yet the there's still a lack of information on the detailed mechanism of yield increase from mixtures, and how this was related to other growth parameters such as tillering profiles and survival. The objective of this study was to investigate the effects of genotype diversity on yield components i.e. tiller number, tiller size, plant size and survival, and how these responses were affected by defoliation treatments. Eight genotypes of perennial ryegrass representing four yield levels, each at two tillering potentials was grown from tillers as monocultures and mixtures comprised of four 2-genotype, four 4-genotype and one 8-genotype plot in a greenhouse. Defoliation treatments imposed on all genotype treatments were frequent (3 wk) vs. infrequent (6 wk) at 20 mm height in Exp. 1, and short (20 mm) and tall (60 mm) cutting height at a 6-wk interval in Exp. 2. Four harvests were taken and dry matter yield was evaluated on the individual plant basis at each harvest. The results showed significant effects of genotype treatment on tiller number, tiller weight and plant mass, but not on

survival. In general, these effects became more apparent toward the late harvest (i.e. Harvest 3 and 4) and under infrequent (6 wk) and lax (60 mm) cutting. The regression of tiller number, tiller weight, plant weight and survival against number of genotypes present showed that small genotypes had negative responses to high genetic diversity and large genotypes had positive responses to genotype diversity. The survival of different genotypes didn't differ significantly when grown in monocultures under all cutting systems. However, more death of small genotypes occurred in mixtures than large genotypes, and the differences were more apparent under 6-wk cutting (infrequent), in which inter-plant competition was supposedly promoted.

INTRODUCTION

Experiments have shown that complex ecosystems, such as mixtures of grass species or cultivars, were more productive than simple ones (Foster, 1988; Tilman, 2001). Postulated benefits of pastures or grassland with diverse species or cultivars include greater primary production and more efficient environmental resource use (Sanderson, 2004). In fact, blending of cultivars or species such as perennial ryegrass (*Lolium perenne* L.) and alfalfa (*Medicago sativa* L.) has been commonly adopted in forage systems to explore this advantage. A sward of such a blend is actually composed of a broad diversity of genotypes, which differ markedly in various morphological and physiological characters. It is of considerable interest to determine how genotype mixing influences the structure of a ryegrass sward as well as the component individuals, in terms of production, tiller profiles or survival.

Perennial ryegrass is a widely used forage species in temperate regions and an ideal species for examining phenotypic characters of individual plants. The bunch-growing habit and low rate of horizontal spread make it possible to identify and observe tillers derived from or cloned from old ramets. Previous studies usually used several cultivars or genotypes of *L. perenne* to show the superiority of mixtures over their components in vegetative growth, especially when some contrasting characteristics such as growth habits or tillering ability existed among these cultivars or genotypes (Thomson, 1969; Rhodes, 1970; Surgenor, 1976). Although these studies have documented a production benefit for cultivar mixtures, they all lack information on the detailed mechanism of yield increase from mixtures, by which this response occured, and how this yield was related to other growth parameters such as tillering character and survival.

For example, Thomson (1969) reported higher yields were obtained when early and late perennial ryegrass cultivars were grown in association than when either one was grown in monoculture. Rhodes (1970) found that mixtures of perennial ryegrass genotypes with different tillering ability were more productive than their highest yielding components under infrequent cutting (34-d intervals). Surgenor and Laidlaw (1976) revealed that mixtures of late erect and early prostrate perennial ryegrass cultivars had higher yield than the individual cultivars in monoculture. Recently, Jones and Roberts (1994) demonstrated that three perennial ryegrass cultivars varying in ploidy and flowering time had an average of 13% more yield from mixtures than from monocultures.

Two aspects of defoliation management that vary readily are frequency (interval of defoliation) and intensity (height of defoliation). The effects of frequency and intensity on pasture production are of great interest in pasture management because of their influence on both production and quality. There is substantial variability of production as well as yield components in response to the defoliation patterns, which are also influenced by the associated environmental conditions (soil moisture, temperature, fertilizer management, light intensity, etc.). The complex interactions between plants diversity and different cutting systems are not well understood. In general, perennial ryegrass pasture produced more herbage when cutting less frequently (Holliday and Wilman, 1965; Leaver, 1985). In contrast, the effect of defoliation intensity on production is less significant than frequency (Ferraro, 2002).

Studies comparing mixtures have usually measured yield, yield components, tillering profiles and survival in terms of unit area. More information may be obtained on the structure of the sward and individual contribution to the resultant population if the

data were recorded on an individual plant basis. In fact, the importance of individual plant measurement has been emphasized by some authors as a means to better understand the overall sward responses (Black, 1966). In a community, plants are subject to modifications of the environment by the presence of other plants, and the effect of these modifications upon surrounding plants. The results of the interaction between individual plants may be expressed at both the population and individual level. Regarding this, increased biodiversity and well-selected species or cultivars may play an important role in affecting ecological functions (Sanderson et al, 2004).

In Chapter 3, it was found that treatments with greater genetic diversity generally had favorable yield responses. The objectives of this chapter was to: 1) investigate the production components, such as tiller number and tiller weight, along with survival of individual genotypes in monoculture and in genotype mixtures with varying components; 2) objective was to determine how these responses varied with cutting frequency and intensity; and 3) examine the effects of tiller morphology on their inter-genotype and intra-genotype performance.

MATERIALS AND METHODS

Plant material

Eight genotypes used in this study were selected from a population of 400 ryegrass plants, which were established from a four-cultivar blend, BG34. These genotypes comprised four levels of yield potential (low, medium, high and very high), and two levels of tiller number potential (low vs. high) (Table 4.1). They were coded as A through H according to their tiller weight (mg tiller^{-1}) and tiller number (tiller plant^{-1}) (Table 4.1).

Exps 1 & 2

Two greenhouse experiments were conducted at the Ohio State University, Columbus OH (40° 0' N, 82° 53' W), from April to November 2003 for Exp. 1 and from December 2003 to July 2004 for Exp. 2. Experiments used a 2 x 17 factorial design with 3 replicates as complete blocks. The 17 genotype treatments were composed of eight monocultures and nine mixtures with two, four or eight genotypes (Table 4.1), and they were identical in Exp. 1 and 2. The defoliation treatments were cutting at 3 or 6-week intervals at 20 mm height, in Exp. 1, and at 20 or 60 mm height every 6 weeks in Exp. 2. Thus, both Exps 1 & 2 had the treatment combination of 6 week cutting at 20 mm.

Plots were established by planting raments of the eight genotypes, at inter-plant spacing of 1.96 × 2.19 cm, filled 16 cm in depth with potting media (Metrox Mix 360, Scotts Co, Marysville OH). Healthy ramets of uniform size for each genotype were used. Tubs of 47 × 35 cm were divided with plastic sheets into 6 plots per tub (17.5 × 15.6 cm)

for Exp. 1. In Exp. 2, the same tubs were used, but comprised only 4 plots measuring 13.5 × 19.5 cm per tub, and also had a 4 cm planted border around each plot. A total of 64 plants (8 rows × 8 columns) were planted in each plot with approximately 2 cm between each row and column. For the multi-genotype treatments, component plants of respective genotypes were planted alternately along the rows and columns to maximize inter-genotype interaction and were marked with color paper clips to aid with subsequent genotype identification.

Measurements

The first harvest started six weeks after plant establishment, in May 2003 for Exp. 1 and January 2004 for Exp. 2. There were eight harvests for frequent cutting treatment (3 wk), and four harvests for the infrequent cutting treatment (6 wk) in Exp. 1, and four harvests each 6 wk in Exp. 2. Plants were harvested individually by drawing up the herbage and clipped to leave stubble of about 20 or 60 mm long (according to treatments) when erect. The herbage from individual genotypes within each of the nine mixture treatments was kept separate to allow calculation of average plant and tiller weight of the respective genotypes within each mixture treatment. All clipped herbage was oven dried at 72 ºC for 48 h and weighted to obtain dry-matter (DM) yield. Tiller numbers of each individual plant and number of survival plants of each genotype per plot were recorded every 6-week immediately following each harvest.

Data analysis

Analysis of variance (ANOVA) was performed with PROC GLM of SAS (SAS System for Windows Release 8.02, SAS Inst. Inc., Cary NC) to evaluate the effects of genotype treatment, cutting frequency, cutting intensity, genotype treatment by cutting frequency and genotype treatment by intensity on a set of response variables, which included: plant mass (mg plant^{-1}), tiller number (tiller plant^{-1}), tiller weight (mg tiller^{-1}) and survival (%) following each harvest in both Exp. 1 & 2 (Appendix B). The CONTRAST statement in the PROC GLM of SAS was used to make the comparison of monoculture versus mixture for the response variables. Regression models for the same response variables were conducted using the PROC REG procedure of SAS with genotype number per plot as the independent variable to examine the impact of genotype diversity.

RESULTS

Tiller number

Genotype treatment had significant effects on the number of tillers per plot, with the only exceptions occurring for the 3-week cutting at Harvest 3 in Exp. 1, and 20 mm cutting at Harvest 2 in Exp. 2 (Table 4.2). In Exp. 1, cutting frequency showed no significant effects on tiller number per plot at any harvest, while in Exp. 2, 60 mm cutting had significantly more tillers per plot than 20 mm cutting at the last two harvests (Table 4.2), with the overall mean tiller population being 161.7 and 196.7 tillers plot^{-1} at cutting height of 20 mm and 60 mm respectively at Harvest 3, and 147.7 and 181.1 7 tillers plot^{-1} at Harvest 4. A single-degree-of-freedom contrast between monoculture and mixture genotype treatments showed significant differences at Harvest 2, 3 and 4 in Exp. 1 and Harvest 4 in Exp. 2. The average tiller number at the Harvest 4 was 223.8 and 188.2 tillers plot^{-1} for monoculture and mixture, respectively, in Exp. 1, and 182.3 and 158.2 tillers plot^{-1} in Exp. 2. No significant interactions between cutting frequency or intensity and genotype treatment were observed at any harvest.

Significant differences in tiller number per plant were found between genotypes under both infrequent and frequent cuttings in Exp. 1, but none in Exp. 2. Slopes of regression of the tiller number per plant vs. number of genotypes of the smaller genotypes A-D were generally negative, with the overall mean being -0.35 tillers plant^{-1} genotype^{-1}, while the slopes of larger genotypes E-H mostly had positive values, with the overall mean being 0.34 tillers plant^{-1} genotype^{-1}. Some significant regressions were observed, though the absolute values of these regression slopes were rarely larger than 1 (Table 4.3).

Tiller weight

Significant effects of genotype treatment were found for the average tiller weight (mg tiller^{-1}) per plot, with plots with more diverse genotype composition (mixtures) having larger tillers than plots with less diverse composition (Table 4). This effect was significant at every harvest under infrequent cutting, but significant only at Harvests 1 and 2 under frequent cutting. Comparisons of monocultures and mixtures on tiller weight were significant at Harvest 4 in both Exp. 1 and 2. The average tiller weight at Harvest 4 was 41.7 and 52.5 mg tiller^{-1} for monoculture and mixtures respectively in Exp.1, and 19.9 and 24.2 mg tiller^{-1} in Exp. 2.

Both cutting frequency and intensity had significant effects on tiller weight (mg tiller^{-1}) per plot (Table 4.4). In Exp. 1, tiller weight was significantly higher under infrequent than under frequent cutting in Harvests 2 and 4; average tiller weight at Harvest 4 was 58.8 and 40.3 mg tiller^{-1} under 6 week and 3 week cutting respectively. In Exp. 2, tiller weight under 20 mm cutting was higher than under 60 mm cutting at all harvests, with the average tiller weight of Harvest 4 being 26.4 and 19.8 mg tiller^{-1} under cutting heights of 20 and 60 mm, respectively. There was a significant interaction between cutting frequency and genotype treatment at Harvests 2, 3 and 4 for Exp. 1. This was caused by the low mean tiller weight of the two-genotype treatments under infrequent cutting. No significant cutting intensity by genotype interaction was found for Exp. 2.

Significant differences were found among genotypes in monoculture for average tiller weight within each plot (mg tiller^{-1}) under all cutting systems at the Harvest 4 (Table 4.5). The regression slope of tiller weight against number of genotypes showed

small genotypes tended to decrease in tiller weight in more complex mixtures, with most slopes being negative (Table 4.5). Conversely, the tiller weight of large genotypes increased as number of genotype increased in each plot, with some regressions being significant.

Plant mass

Significant differences in average plant mass (mg plant^{-1}) were found among genotype treatments in some harvests under certain cutting treatment (Table 4.6). Averaged over all cutting treatments at Harvest 4, the plant mass for small genotypes, A-D was 109.1 mg plant^{-1}, and 174.2 mg plant^{-1} for large genotypes E-H. The maximum yield of each genotype in monoculture varied between harvests (Fig. 4.1). Large genotypes such as F and H had their highest yield at the Harvest 2, while the highest yield of small genotypes such as B and C occurred at the Harvest 4.

Cutting frequency had significant effects on plant mass of genotypes in monoculture. At the Harvest 4, plant mass of monocultures averaged 230.4 and 170.3 mg plant^{-1} under infrequent and frequent cutting respectively (Exp. 1). In contrast, cutting intensity had no significant effects on plant mass.

Significant differences were found for the average plant mass (mg plant^{-1}) among respective genotypes within each plot (Table 4.7). Regression slopes of plant mass on number of genotypes grown together were generally negative for smaller genotypes, A-D, averaging –7.2 mg plant^{-1} genotype^{-1} at Harvest 4, whereas those of larger genotypes, E-H were mostly positive with an average of 8.8 mg plant^{-1} genotype^{-1} (Table 4.7). The large values of these slopes showed the substantial change of plant size caused by

different genotypic combination and complexity. The highest regression slope for genotype H was significant ($P<0.01$) at Harvest 4 under frequent hard cutting (3 wk, 20 mm). Genotype D had two positive slopes under infrequent hard cutting (6 wk, 20 mm) from Exp. 1 and 2, indicating its growth habit resembled large genotypes.

Survival

On a plot basis, survival recorded at Harvest 4 didn't differ significantly among cutting systems (data not shown). No significant differences were observed between the genotype treatments, although their ranking for survival varied greatly with contrasting cutting regimes. The monoculture vs. mixtures comparison revealed significantly higher survival of monoculture (71.2 %) than mixture (60.7 %) under infrequent cutting, but no significant differences were found under other cuttings.

There was no significant difference between the survival of each genotype when in monoculture for any cutting treatments (Table 4.8). The regression slope of survival against number of genotypes was generally negative for small genotypes, A-D, indicating the poorer survival of small genotypes in mixtures than in monocultures. In contrast, the large genotypes (i.e. E-H) generally had higher survival in mixtures than in monoculture. This difference was most obvious in the 8-genotype treatment (Fig. 4.2) at Harvest 4.

DISCUSSION

Genotype effects on tiller dynamics

The aim of this study was to examine how yield components respond to genotypic diversity and defoliation patterns. The results showed that average tiller weight per plot was linearly related to the number of genotypes present, under 6-wk cutting in Harvests 2, 3 and 4. In contrast, average tiller weight was similar for all genotype treatments under 3-wk cutting at Harvest 3 and 4. The interaction of cutting frequency by genotype treatment was significant. This finding suggested the need for a long period (at least 6 week cutting interval) of recovery for the positive responses of tiller weight to genetic diversity to be expressed.

The genotypes selected in this study were based on their yield and tillering potential, and represented a wide range of these characters. Presumably, stands composed of genotypes with contrasting characteristics undergo less severe competition than those with more similar characteristics. Greater variability makes it possible for component plants to complement each other in resource utilization. For example, in a study on the relative yield of three perennial ryegrass cultivars, Surgenor and Laidlaw (1976) showed that more light was allowed to pass through the mixture sward consisting of erect and prostrate components than monocultures than in monocultures. On the other hand, there might be other minor physiological or morphological differences among genotypes, such as growth rhythms, which were reflected as variation of maximum yield of each genotype between harvests (Fig. 4.1). Thomson (1969) suggested that a specific balance between the growth rhythms of mixture components could be more important than diversity of growth habit *per se*. In all, such subtle physiological or morphological variations between

similar components may also result in more efficient utilization of resources (Jones and Roberts 1994).

Effects of tiller weight and tiller number

On average, there was 15.6 to 18.9 % (Exp. 1 and 2, respectively) higher tiller production in monocultures than in mixtures, especially under 60 mm cutting (Table 4.2). It seems that as a plant strategy, investment in fewer larger tillers is more successful than smaller more abundant tillers. It is reasonable to assume that competition was more severe in monocultures, and the dry matter production might only be achieved by increase in the weight of tillers, instead of tiller number since space limited any increase in tiller number (Thomson, 1969). On the other hand, in mixtures with less competition, growth rhythms of the components were different and tiller production might have been easier. Another factor that affected the total plot tiller number was the proportion surviving tillers for respective genotypes. Higer mortality of small genotypes occurred in mixtures as a result of suppression from large genotypes, and subsequently lowered the overall tiller number per plot.

In general, small genotypes were at a disadvantage in mixed communities and became smaller with fewer tillers over time. Conversely, larger genotypes were favored in mixed communities, having better survival, larger and more tillers. These relationships can be seen in the plant mass data at week 24 (Table 4.7). For example, genotype G, which when selected had relatively low tiller weight (Table 4.1), was among the highest yielding level group when grown individually, but showed drastic reduction in individual yields and tiller weight when in competition with other genotypes under infrequent

cutting. In contrast, genotype D, which had a medium yield potential and high tiller weight, increased markedly in yield under infrequent cutting. Competition studies were made by observing changes over time in artificially synthesized mixtures of genotypes. Inter-genotypic competition is an important agent of natural selection in modifying and determining the ultimate population structure. The production and tillering potential of these genotypes were assessed when they were grown as single plant per pot, an environment without competition. These parameters were modified when plants were brought into competition, and the modification was also associated with certain cutting systems.

Cutting treatment effects

Our results showed the differences were more pronounced under infrequent (6 wk) and lax (60 mm) cuttings, which was more favorable for plants to exhibit their potential without harsh stress. The primary growth unit in a pasture is individual tiller/shoot, and pasture can be regarded as a population of tillers (Korte, 1986). Increased forage production of grass can therefore be attributed to increases in tiller number or tiller weight or a combination of both (Volenec and Nelson, 1983). Experiments have suggested that tiller formation is a more important yield determinant than is tiller weight at low density (Nelson and Zarrough, 1981). On the other hand, tiller weight becomes more important when tiller population is high (Volenec and Nelson, 1983). When grown in monoculture, genotypes generally maintained their tiller production potential (Table 3), as each pair of genotype of the same yield potential was compared. However, the differences between each pair of genotypes with high and low tiller potentials were

substantially reduced, as well as the overall tiller numbers per plant (Table 1). This may be due to the limitation of space allowed for each individual plant, which was subject to competition modification. In such situation, conversion of light energy into dry matter may only be achieved by increase in tiller weight, rather than tiller number (Harris, 1970). The tillering character of each genotype was assessed when plants were grown individually with favorable conditions to reach their potential.

Genotype effects on plant mass and survival

The effects of plant mass *per se* are important in determining plant survival. This was indicated by the better survival of larger genotypes in mixtures than in monocultures. The survival of different genotypes didn't differ significantly when grown in monoculture under all cutting systems. However, more death of small genotypes occurred in mixtures than large genotypes. The differences were more apparent under infrequent cutting, in which inter-plant competition was promoted. This was in part agreement with the finding of Harris (1970) that the death of several ryegrass cultivars was greater under infrequent than frequent cutting, and the author attributed this to competition for light. In the mixtures, large genotypes had more carbon reserve and presumably recover faster from transplanting than small ones. Due to their higher growth rate, greater demand on light and possibly mineral nutrient and water supply than small ones, these large competitors began to impose stress on smaller plants as they competed for these factors. The large genotypes continued gaining these advantages and growing larger progressively. The heavily shaded plants experienced a reduction in tiller number and survival through an increased susceptibility to fungal attack (Grime, 1965).

Genotype treatment	Yield potential	Tiller weight* (mg tiller $^{-1}$)	Tiller number level	Tiller number* (tillers plant^{-1})
A	Low	63.8	High	114.5
B	Low	149.4	Low	48.2
C	Medium	112.7	High	93.2
D	Medium	208.4	Low	47.5
E	High	120.0	High	101.7
F	High	170.9	Low	70.8
G	Very high	103.2	High	149.2
H	Very high	183.4	Low	82.9
A, B	Low		High vs. low	
C, D	Medium		High vs. low	
E, F	High		High vs. low	
G, H	Very high		High vs. low	
A, C, E, G	Mixed		High	
B, D, F, H	Mixed		Low	
A, C, F, H	Mixed		Mixed	
B, D, E, G	Mixed		Mixed	
A-H	Mixed		Mixed	

Table 4.1. Genotype treatments, mean tiller weight, and mean tiller number per plant of each genotype.

*, tiller weight and tiller number were determined Chapter 2.

No. of genotypes	Harvest 1		Harvest 2		Harvest 3		Harvest 4	
Exp.1	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk
1 (8)	314.7	295.6	289.0	269.4	227.5	230.0	227.4	220.2
2 (4)	284.0	313.9	251.6	276.3	213.7	234.2	191.6	195.9
4 (4)	270.5	297.6	239.5	217.4	195.9	171.5	181.5	173.7
8 (1)	311.3	333.0	311.7	248.7	257.3	172.0	219.0	167.3
Gen	***	**	**	**	NS	*	*	*
s.e.	33.8	36.5	32.4	33.7	30.5	35.2	31.9	31.3
Fre	NS		NS		NS		NS	
s.e.	53.8		44.4		36.9		31.3	
Freq * Gen	NS		NS		NS		NS	
Mon. vs. mix	NS		*		*		**	
Exp. 2	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm
1 (8)	192.8	201.9	188.2	204.7	166.9	208.6	163.6	202.3
2 (4)	212.3	193.7	190.5	197.0	167.7	187.3	136.3	180.3
4 (4)	204.4	204.4	204.4	200.6	176.8	195.8	162.8	187.2
8 (1)	154.0	216.3	166.0	195.3	135.3	195.0	128.3	154.3
Gen	**	*	NS	**	*	*	**	*
s.e.	19.9	29.3	23.4	31.4	15.8	16.1	23.1	36.1
Int	NS		NS		*		**	
s.e.	45.4		42.2		43.3		36.4	
Int * Gen	NS		NS		NS		NS	
Mon. vs. mix	NS		NS		NS		*	

Table 4.2. Average tiller number per plot in monocultures and mixtures with different cutting frequency and intensity treatments at harvests 1-4 on weeks 6, 12, 18 and 24 of Exp. 1 and 2. Numbers in parenthesis are the number of treatments with the stated number of genotypes (geno).

*, **, *** significant at $P < 0.05$, 0.01 and 0.001 respectively; NS, not significant; Gen, Fre and Int, effects of the genotype, cutting frequency and cutting intensity on the 17 genotype treatments respectively; s.e. standard error.

	6wk		3wk		20 mm		60 mm	
Geno	Till no.	Slope	Till no.	Slope	Till no.	Slope	Till no.	Slope
A	8.39	-0.24	4.43	-0.33	5.18	-0.17	6.51	-0.39 **
B	4.42	-0.59 **	4.81	-0.18 **	3.61	-1.06	3.33	-0.74
C	4.19	-0.29 *	4.91	0.23	3.35	-0.42	5.08	-0.43
D	3.94	-0.39 ***	5.22	0.28	3.28	-0.39 *	3.19	-0.41
E	2.88	0.39	3.72	1.43	4.31	0.067	5.86	0.058
F	3.79	0.53 *	5.16	0.31	4.03	-0.27	4.04	0.18 *
G	6.81	0.11	7.91	0.5	4.93	0.41 *	7.44	0.093
H	6.73	0.82 **	3.89	0.43 *	3.71	0.11	3.91	0.31
s.e.	0.41		0.61		0.29		0.62	
F-test	NS		NS		NS		NS	

Table 4.3. Mean tiller number per plant (tiller plant^{-1}) for genotypes (Geno) in monoculture and linear regression slopes of tiller number per plant against number of genotypes (i.e. 1, 2, 4 and 8 genotypes) per plot following Harvest 4, at week 24.

*, **, *** significant level at $P < 0.05$, 0.01 and 0.001 respectively. Till no., tiller umber per plant.

No. of genotypes	Harvest 1		Harvest 2		Harvest 3		Harvest 4	
Exp.1	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk
1 (8)	37.5	39.0	29.7	45.3	18.8	36.0	36.9	46.4
2 (4)	37.2	31.7	36.0	38.8	16.0	27.9	44.2	44.4
4 (4)	39.1	41.6	32.8	54.0	18.6	52.1	43.1	68.3
8 (1)	39.0	37.3	28.4	54.9	20.1	60.4	37.1	76.0
Gen	*	*	*	***	NS	***	NS	***
s.e.	8.1	10.9	6.4	10.9	6.4	10.5	8.5	11.6
Fre	NS (11.72)		*** (11.76)		*** (10.79)		*** (14.04)	
s.e.	11.7		11.8		10.8		14.0	
Freq * Gen	NS		*		*		**	
Mono. vs. mix	NS		NS		NS		*	
Exp. 2	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm
1 (8)	13.1	11.6	25.6	22.9	25.3	22.7	23.1	16.7
2 (4)	13.0	11.4	27.1	24.0	27.1	22.7	29.5	17.1
4 (4)	16.7	12.3	28.6	23.8	30.6	22.1	25.6	17.6
8 (1)	18.2	8.6	29.2	27.7	33.2	19.9	27.5	27.7
Gen	*	*	***	***	***	***	***	**
s.e.	2.7	2.5	3.6	3.5	2.9	4.2	3.4	3.9
Int	**		**		***		***	
s.e.	3.6		5.2		6.3		4.2	
Int * Gen	NS		NS		NS		NS	
Mono. vs. mix	NS		NS		NS		*	

Table 4.4. Average tiller weight per plot (mg tiller^{-1}) in monocultures and in mixtures with different cutting frequency and intensity treatments at Harvests 1-4 on weeks 6, 12, 18 and 24 in Exp. 1 & 2. Numbers in parenthesis show the number of treatments for each of the respective number of genotypes.

*, **, *** significant at $P < 0.05$, 0.01 and 0.001 respectively; NS, not significant; Gen, Fre and Int, effects of the genotype, cutting frequency and cutting intensity on the 17 genotype treatments respectively; s.e. standard error.

Geno	6wk		3wk		20 mm		60 mm	
	Tiller weight	Slope	Tiller weight	Slope	Tiller weight	Slope	Tiller weight	Slope
A	37.1	-2.8	24.7	-0.1	14.6	-0.4	10.5	-0.9
B	34.1	-2.7	30.1	4.6	25.5	-0.1	17.7	-0.4
C	20.9	-1.0	28.4	1.3	18.0	-1.7	14.1	-0.3
D	65.3	1.8	57.4	-4.6	19.2	3.6	20.2	-0.6
E	43.4	-0.6	39.1	5.07 *	19.8	1.31 *	17.3	1.9
F	67.6	-4.2	56.4	2.5	39.9	0.5	24.3	3.12 *
G	21.2	0.5	25.8	-1.7	15.4	-1.3	10.1	1.45 *
H	74.5	2.3	45.0	0.6	32.6	0.6	20.7	1.8
F-test	**		**		**		*	
s.e.	8.83		4.03		2.57		3.19	

Table 4.5. Mean tiller weight (mg tiller^{-1}) for the genotype (Geno) monocultures and linear regression slopes of tiller weight against number of genotypes (i.e. 1, 2, 4 and 8 genotypes) per plot following Harvest 4, at week 24.

*, **, *** significant level at P < 005, 001 and 0001 respectively

No. of genotypes	Harvest 1		Harvest 2		Harvest 3		Harvest 4	
Exp.1	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk
1 (8)	177.3	171.1	144.7	192.4	88	138.4	172.8	242.9
2 (4)	170.4	155.5	149.9	174.6	70.49	115.6	158.3	186.7
4 (4)	192.5	195.1	161.4	197.4	109. 9	154.9	210.6	244.6
8 (1)	192.1	190.1	148.5	242.8	206.1	171.4	161.8	239.1
Gen	NS	NS	*	**	NS	NS	*	*
Fre	NS		***		**		**	
Fre* Gen	NS		**		NS		NS	
Mono. vs. mix	NS		NS		NS		NS	
Exp. 2	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm	20 mm	60 mm
1 (8)	53.7	46.7	95.98	96.09	96.18	96.27	91.87	73.39
2 (4)	60.4	46.7	134.5	101.8	122.3	95.6	115.5	112.5
4 (4)	69.6	53.2	124.5	98.1	120.7	91.9	97.6	67.9
8 (1)	69.4	48.9	113.8	134.7	112.4	92.4	97.6	105
Gen	NS	NS	NS	NS	NS	NS	NS	*
Int	NS		NS		*		NS	
Int * Gen	NS		NS		NS		NS	
Mon. vs. mix	NS		NS		NS		NS	

Table 4.6. Plant mass (mg plant^{-1}) in monocultures and in mixtures with different cutting frequency and intensity treatments at Harvests 1-4 on weeks 6, 12, 18 and 24 in Exp. 1 & 2. Numbers in parenthesis show the number of treatments for each of the respective number of genotypes.

*, **, *** significant at $P < 0.05$, 0.01 and 0.001 respectively; NS, not significant; Gen, Fre and Int, effects of the genotype, cutting frequency and cutting intensity on the 17 genotype treatments respectively; s.e. standard error.

	6wk		3wk		20 mm		60 mm	
Geno	Yield	Slope	Yield	Slope	Yield	Slope	Yield	Slope
A	121.9	-19.2 *	127.1	-3	75.5	-4.3 *	67.6	-7.5 *
B	158.6	-19.1 **	129.2	-9.4 *	94.6	-0.72	70.3	-2.6
C	113.8	-8.6	107.0	-7.6	58.0	-12.9 *	60.7	-4.8
D	293.3	4.1	139.5	-25.3	62.5	8.37	63.9	-22.9
E	199.4	12.1	161.0	15.3 *	84.7	2.8	79.3	12.6 *
F	324.8	-11.8	234.5	7.6	163.9	0.3	102.6	6.5 *
G	220.0	-3.4	246.3	-0.5	73.9	-4.4	66.3	5.6
H	411.1	39.8	217.7	44.1 **	121.9	6.4	76.6	7.7
F-test	***		**		**		**	
s.e.	24.1		29.4		17.9		17.4	

Table 4.7. Mean plant mass (mg plant^{-1}) for genotypes (geno) in monoculture and linear regression slopes of tiller weight against number of genotypes (i.e. 1, 2, 4 and 8 genotypes) per plot following Harvest 4, at week 24.

	6wk		3wk		20 mm		60 mm	
Geno	%	Slope	%	Slope	%	Slope	%	Slope
A	65.6	-0.4	70.3	1.5	67.7	-1.1	64.1	-0.5
B	75.5	-2.1	74.0	-5.1	68.8	-1.3	75.5	-2.86 *
C	56.8	-1.7	64.6	-5.56 *	52.6	0.3	54.2	-5.8
D	68.8	-0.1	61.6	-8.39 *	69.3	-1.0	80.7	-4.5
E	64.6	0.5	78.7	-5.1	71.9	-4.5	52.6	0.6
F	70.3	-4.9	64.1	-1.7	47.9	1.5	63.5	2.4
G	55.7	2.2	64.6	-1.4	67.2	0.0	71.4	-2.6
H	62.5	3.02 *	82.8	1.71 *	60.9	-0.3	58.9	0.84 *
F-test	NS		NS		NS		NS	
s.e.	4.7		5.5		6.2		8.5	

Table 4.8. Survival rate (% of tiller planted) of each genotype (geno) in monocultures and linear regression slopes of survival against number of genotypes (i.e. 1, 2, 4 and 8 genotypes) per plot following Harvest 4, at week 24.

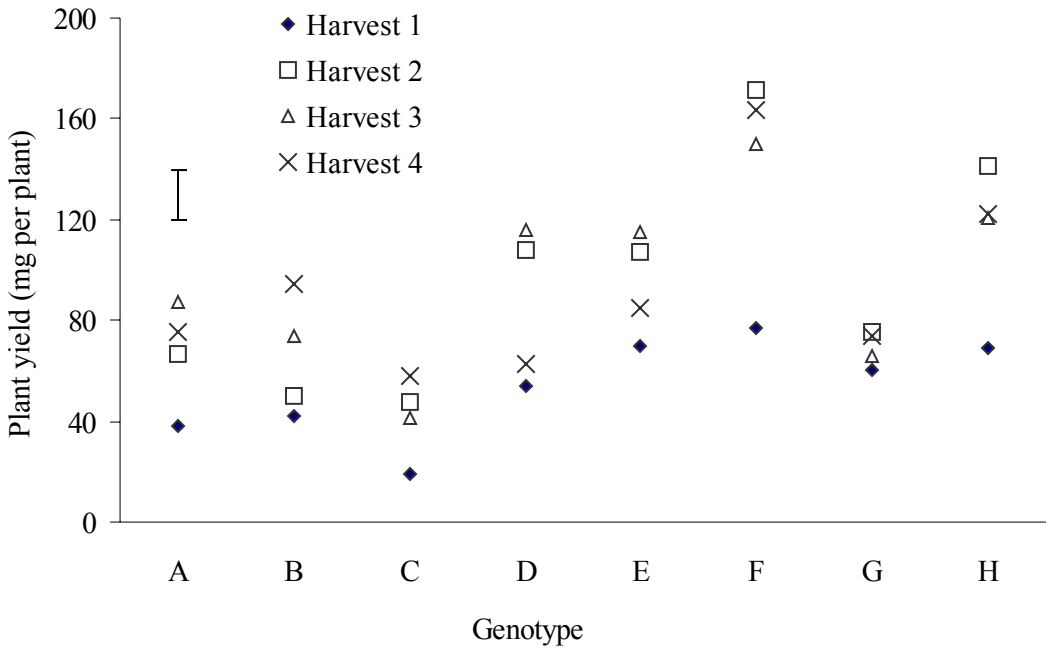


Fig. 4.1. Plant yield (mg plant^{-1}) of each genotype in monoculture for harvests 1-4 (20 mm cutting, Exp. 2). (Vertical bar represents the mean standard error)

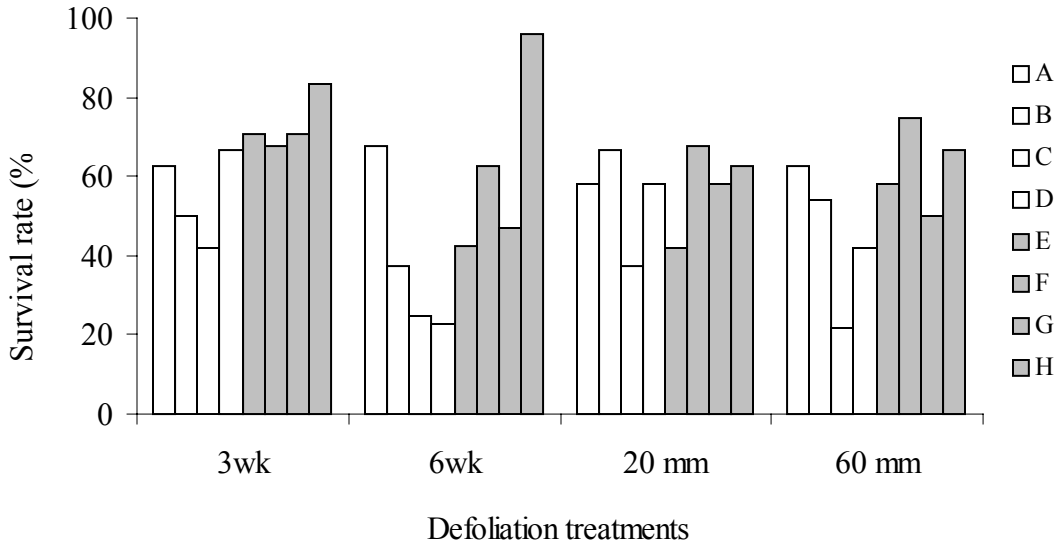


Fig. 4.2. Survival rate (% of tillers planted) of each genotype in the 8-genotype treatment under each cutting system at the Harvest 4

CHAPTER 5

IDENTIFYING CULTIVARS WITHIN PERENNIAL RYEGRASS BLENDS USING SIMPLE SEQUENCE REPEATS (SSRS)

ABSTRACT

Perennial ryegrass (*Lolium perenne*) is a widely used forage species that is often sold as a blend of several cultivars. For example BG34 is a mixture of the cultivars Barlet, Barmaco, Barnhem, and Mara in various proportions. No information is available on differentiation of these cultivars in mixtures, nor the stability of these blends after sowing. The objectives of this study were to assess the potential of SSR and ISSR markers for ryegrass cultivar differentiation, and to identify the cultivar proportions in BG34 ryegrass populations. Of each of two seed lots of the four cultivars of BG34, 50 individuals were characterized using one SSR and two ISSR markers. Individuals were correctly allocated to their respective seed lots and cultivars with 80.9-86.7% accuracy using discriminant analysis. There were distinct differences between cultivars, and in some cases, seed lots-within-cultivars. I also collected 100 ryegrass plants from three 5-year old fields of BG34 from three Ohio dairy farms, and compared their simple sequence repeats (SSRs) profiles with the reference populations using discriminant analysis. One field remained similar to the cultivar proportions in mixture blend at the

time of sowing. Change of genetic structure was found for two fields, with increases in the proportion of ‘Mara’ and ‘Barmaco’, and decreases ‘Barlet’ or ‘Barnhem’ depending on field. Overall, SSR was proven to be highly effective for differentiating among ryegrass cultivars.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is a cool season grass widely used as forage in temperate regions worldwide. This bunch-type grass is largely self-incompatible, and the cultivars are usually synthetic, i.e. developed from a limited germplasm pool (Moser, 1996). Therefore, high levels of genetic variation exist among as well as within cultivars (Jones, 2001). Determination of genetic diversity and cultivar differentiation is difficult because cultivars and populations have high morphological similarities.

One of the most commonly used ryegrass blends in Ohio dairy pasture is BG34, which is marketed by Barenbrug Seed Company. BG34 is not a registered cultivar, rather it is a blend of the four cultivars Barnhem, Barmoco, Barlet and Mara, in various proportions. They are all diploid but with different origins. Currently, no system has been used to identify the resultant proportions of these four cultivars in pastures following sowings.

Grassland populations have a distinct genetic structure that is a result of their environment and management. Large genetic diversity is usually desired for the plant materials to maintain adaptation in dynamic, non-local environments. It is of practical relevance to determine whether these proportions remain the same as sown, or how the cultivar proportions drift (more likely) in favor of the genotypes best adapted to the prevailing farm management (e.g. fertilization, grazing, rotation) and environmental conditions (e.g. climate, topography and soil characteristics). A sound knowledge of the population genetic structure might be useful in developing an efficient selection strategy and to exploit the genetic resources.

To assess the genetic structure of a pasture, one option is to profile DNA of random individuals and classify them based on a discrimination criterion developed from known "pure" or reference populations. Among the molecular marker systems available, simple sequence repeats (SSRs) are particularly suitable for genetic diversity evaluation and cultivar identification. SSR loci arise due to repetition of short nucleotide motifs in tandem arrays. SSRs are abundantly distributed throughout the eukaryotic genomes, and genetic polymorphism arises due to variation in the number of repeated units, which conventionally vary from 1 to 6 bp in length. This variation in length can be monitored with PCR primers that flank the SSRs (Weber and May, 1989). Compared with other molecular marker systems, the SSR marker system has several advantages including: uniform genome coverage, high level of polymorphism, co-dominance, and specific PCR-based assays (Pejic et al. 1998). In fact, SSRs have been successfully used in genotyping and examining the genetic diversity of many plant species. One drawback with this marker system is the requirement for sequence information from flanking regions, from which primers are designed for PCR amplification (Liu, 2001). Wide use of SSRs is thus hindered for many taxa, since the development and characterization of a large number of SSRs is time-consuming and expensive. For perennial ryegrass, several authors have reported the isolation of SSR loci (Kubik, 1999, 2001; Jones, 2001), and revealed SSRs were slightly less frequent than in some other plant taxa (Kubik, 1999).

A newly developed modification of SSR-based marker system, inter-simple sequence repeats (ISSR), has been gaining more applicability recently in many plants due to several advantages. The ISSR method uses primers that are anchored at the 5' or 3' end of a repeat region and extend into the flanking region. Since it targets the repeats *per se*,

no prior knowledge of the flanking genomic sequence is required, and thus can be potentially undertaken for any species. This technique amplifies the genomic segments between inversely oriented repeats (ISSRs), which represent multiple loci from across the whole genome. It provides highly reproducible results and generates abundant polymorphism. This approach involves the use of 5'-anchored or compound ISSR primers in which the anchor serves to fix the annealing of the primer to a single position of the target site to ensure a low level of slippage during amplification. ISSR has been proven as an ideal method for fingerprinting cultivars and can differentiate between closely related individuals (Wolfe et al. 1998). For example, Ghariani et al (2003) revealed a high degree of genetic diversity of 18 perennial ryegrass accessions with ten ISSR markers and their results concurred with those described with the application of isozymes, RFLPs and RAPDs. Other advantages of this technique include high throughput, requirement of low amounts of DNA and cost-efficiency.

In this study, I used a combination of SSRs and ISSR markers to: 1) determine the suitability of SSR/ISSR system for differentiating between cultivars of perennial ryegrass that comprise BG34, and whether cultivars can be differentiated based on SSR polymorphism; 2) use SSR to investigate the genetic structure (e.g. dynamic patterns of the 4 component cultivars) of BG34 ryegrass from dairy fields in Ohio.

MATERIALS AND METHODS

Plant material

The reference populations used in this study consisted of four cultivars of perennial ryegrass, using two seed lots from each cultivar (Table 5.1). Plants were grown from seed for four weeks in a greenhouse before sampled for DNA extraction. Fifty plants from each seed lot were profiled with SSR and ISSR markers.

One hundred ramets (tiller with roots), each from a different plant, were collected randomly from each of the three fields in northeastern of Ohio (Table 5.2) September, 2004. Sampled ramets were then transplanted into potting media (Metrox Mix 360, Scotts Co, Marysville OH) in a greenhouse, and kept for one week before DNA extraction.

DNA preparation

About 0.2 g leaf blades were ground to a fine powder in liquid nitrogen with a mortar and pestle followed by incubation with 1000 µl Shorty buffer [200mM Tris-HCl, pH 9.0; 400mM LiCL; 25mM EDTA; 1% SDS] for a short time and spun for 5 minutes at 12,000 rpm in a micro-centrifuge. The DNA was precipitated with isopropanol and then centrifuged at 12,000 rpm in a micro-centrifuge for 5 min. The pellet was air-dried and then 400 µl dd water added for storage at -20ºC as prep.

In the test of reproducibility of SSRs fingerprinting, two blades from one plant were sampled and all the above procedures were followed. The DNA prep was then used for PCR reaction as follows.

Polymerase chain reaction (PCR) SSR procedure

The reactions were performed with a SSR primer pair and two ISSR single primers. The use of these primers in their original forms has been reported in several sources (Kubik, 1999, Ghariani, 2003). Each PCR reaction contained 0.1 units 10× Buffer, 0.25 mM of each dNTP, 0.5 units of Taq polymerase, 10 pmol of each primer and about 20 ng of template DNA in a total volume of 25 µl PCR consisted of 30 cycles, each cycles with 1 min denaturation step at 94 ºC; a 1 min annealing step at temperature of 57 or 60 ºC, depending on the optimum annealing temperature for given primer pairs (Table 5.3); and 2 min at 72 ºC for elongation. The PCR concluded with a 15 min elongation step at 72 ºC. According to the sizes PCR fragments (determined by SSR primers), the amplified products were visualized on 1.2 or 3% 3:1 agarose gel at 72 V for 2 hours in 1 x TBE.

Data analysis

The amplified PCR bands from each DNA were scored within a 100 bp range as 0 for absence, and 1 for the presence. The DNA profile of one population was then transformed into a binary character matrix, which was then computed with discriminant analysis.

The discriminant analysis was performed with the PROC DISCRIM of SAS (SAS Institute, 2000) for the reference populations, i.e. seed lots of each cultivar of BG34 and field populations. The data set from the reference population was used in the DISCRIM procedure as calibration to develop the discriminant criterion, and this derived

discriminant criterion was then applied to the field data set to classify each observation into one of the groups (seed lots or cultivars).

The genetic distances were then submitted to the PROC TREE of SAS (SAS Institute, 2000) to map phylogenetic diagrams (unrooted tree and cladograms) using the Unweighted Pair Group Method with the Arithmetic Averaging (UPGMA) method.

RESULTS

We tested sixteen SSR/ISSR primers for their ability to detect genetic polymorphism in perennial ryegrass (Table 5.3). One SSR and two ISSR primers generated interpretable polymorphic amplifications, while the other 13 produced monomorphic or ambiguous band patterns and were discarded. The three primers generated a total of 30 interpretable polymorphic fragments among the 200 individuals, with fragment size ranging from 150 to 1200 bp. $(AG)_{10}C$ and $(AG)_{10}T$ generated 11 and 12 useful ISSR markers respectively, while there were only seven interpretable bands generated with primer M15-185. A typical example of ISSR amplification profiles using $(AG)_{10}T$ showed high levels of polymorphism (Fig. 5.1), which were produced at multiple loci. Bands that were common to all individuals tested were excluded from analysis due to lack of useful information in genotyping. Reproducibility of SSR markers was evaluated by repetition in DNA extraction from the same plant and parallel PCR procedure with the same DNA samples. The PCR results were identical when comparing the PCR amplified products from two leave blades, and two separate PCR (Fig. 5.2). Only slight differences in band intensity were found between two samples.

The genetic distance was calculated to demonstrate the relationship among ryegrass seed lots as well as cultivars (Fig. 5.3). The population pairwise distances ranged from 0.39 to 1.24 and suggested a high genetic diversity between populations at the DNA level. The smallest genetic distance value (0.39) was found between seed lot Mara-1 and Mara-2, indicating the high similarities between with cultivar seed lots. The maximum genetic distance (1.24) was observed between Barnhem-2 and Barlet-1. Seed lots of

Balet-1 and Barlet-2 were clustered closely together with a value next to the smallest. Barnhem-1 was found to be the most distinct seed lot from all others. Barmaco-1 was a little further away from Barmaco-2 than cultivar Mara.

The accuracy of the assignment was improved when more loci were included. Sampling 100 plants from each cultivar, there were less than 50% of individuals correctly assigned on average (Table 5.4), when only one locus was considered. The rate of correct assignment was increased to 83.52% when all three primers were used.

Percentages of each cultivar in each of the three fields were computed by classifying each observation into a cultivar according to the classification criterion generated with reference populations (Table 5.5). The Gessell field remained similar to the cultivar mixture in the original sowing, only with small increase in Barnhem and a very slight decrease in Mara. Substantial changes of genetic structure occurred for the other two fields. The proportion of Barlet dropped dramatically in the Noyes field, while Barnhem and Barmaco increased from 25% to 37 and 39 %, respectively. In the Kozak field, Barnhem and Barlet decreased from 25 and 40 % to 13 and 31%, respectively, while Barmaco and Mara increased to various degrees. These results were obtained with an accuracy rate averaged at 73.7%.

DISCUSSION

ISSR markers can be used in population genetic studies of plant species because they can effectively detect very low levels of genetic variation. They also may have potential for analyzing bio-geographic patterns among populations of a single plant species. In this study, we have shown that these markers revealed genetic variation among geographically separated samples of *Lolium perenne*, and also revealed diversity within each sub-population.

The ISSR primers that were utilized in this study contained AG-repeat motifs in their sequence (Table 5.3). Recently, in a genetic study of perennial ryegrass, Ghariani (2003) demonstrated high number of polymorphic markers with various ISSR primers containing AG-repeat motifs in their sequences. Studies on the development of SSR markers of perennial ryegrass (Jones, 2001; Kubik, 2001) indicated that AG di-nucleotide repeat was present in significant numbers in perennial ryegrass, in all regions of the genome, which include intergenic regions, introns, and exons. The number of polymorphisms produced in the present study confirmed the utility of using ISSR primers containing this repeat motif. The ISSR–PCR method has been utilized successfully for genetic characterization of many crop species such as wheat (*Triticum aestivum*), pea (*Pisum sativum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*), as well as perennial ryegrass (Plaschke et al. 1995; Lu et al. 1996; Sanchez de la Hoz et al. 1996; Nagaoka and Ogihara 1997; Parsons et al. 1997; Smith et al. 1997; Blair et al. 1999; Ghariani, 2003).

The SSR distribution was a function of the dynamics and history of genome evolution and of selective constraints (Morgante et al. 2002), and thus SSR markers reflect genetic relationship among breeding seed lots or cultivars. My results demonstrated a large genetic diversity among cultivars of BG34, as well as within each cultivar. The dendrogram clusters elucidated some of the close relationship of seed lots within cultivars. For instance, each two seed lots from Mara and Barlet were closely clustered together, indicating their genetic closeness. This reaffirmed the power of SSR/ISSR in fingerprinting genotypes and grouping closely related populations. However, it was unexpected that the seed lot Barhem-1 was more similar to other cultivars than to Barhem-2. Similarly, Ghariani (2003) reported that the ISSR produced a close clustering of perennial ryegrass cultivars with several spontaneous populations collected from different habitats. The authors hypothesized that this may be because the differences between these forms concern only a small region of the genome involved in the cultivar selection; and genetic exchange may have occurred. It is not clear if this was due to the real divergence in the process of breeding or whether this reflected the complex domestication process in this forage crop.

In discriminant analysis, the genotype was assigned to the population where its expected frequency is highest, i.e. where it has the greatest probability of occurrence (Waser and Strobeck, 1998). The test determined whether a query individual had a genotype that is typical of one cultivar or whether it better represented the genetic characteristics of a different cultivar (Kubik, 2001). This test is useful for property right protection and cultivar identification. Large sample size is required to establish reliable DNA profiles of a reference population, to which an unknown plant is compared. This is

especially the case for out-crossing species, which usually show high level of genotypic variability within cultivars or accessions. Kubik et al. (2001) suggested at least 20 individuals per cultivar for *L. perenne* would be needed for a quality genotype assignment test. In this study, we examined 50 individuals per seed lot (100 per cultivar) because of the relatively few primers used.

This is a pilot survey of identifying unknown plants using a combination of SSR and ISSR markers. Cutivars in pasture are dynamic entities that change over time. The seeded cultivars under grazing are usually different from the original seed lot due to various reasons (Vaylay, 1999). For perennial ryegrass, high mortality of sown seeds occurs during the establishment phase, with about only 10 % survival (Charles, 1961). Profiling DNA from is the only means available so far to recognize unknown individuals in a pasture. Reliable profiling of the reference population is required for correct and accurate assignment identification of unknown plants. In this study, we classified the four cultivars correctly with an average accuracy of 83.2%, which laid solid foundation for precise assignment of plants to be tested. Previous studies adopted either SSR or ISSR primers in examining genetic diversity of perennial ryegrass populations. The combination of conventional SSR and ISSR in this study apparently increased the differentiating power (Table 5.3). Our results indicated that as few as three SSR & ISSR primers can identify unknown plants with acceptable level of accuracy. Similarly, with three ISSR primers, Jesse and Jones (2005) were able to estimate the population genetic structure of a primary egg parasitoid (*Gonatocerus ashmeadi*) of sharpshooter. For a large size sample, however, a simple, rapid and cost-efficient methodology is highly desired. The SSR/ISSR method could provide efficient and fast screening for both

germplasm conservation and crop improvement. Examining field plants revealed a varying degree of genetic change in different fields since the original sowing. This paralleled the finding of Vaylar (1999) that tall fescue (*Festuca arudinaceae*) population differentiation took place in a short period of time after the pastures had been established. Only the genotypes that could adapt to the environment (e.g. infected with endophytic fungus) survived.

In this study we did not attempt to identify what factor or factors caused these divergent changes in the proportions of the respective cultivars of BG34. The fields sampled were all on the same soil type with high fertility and similar grazing management with dairy cows. We did not have access to the original seed lots sown. The genetic composition of BG34 is not necessarily constant and we had to assume the three fields were sown with the same proportions of the cultivars. Since the seed was obtained from similar sources at similar times, this assumption is not unreasonable. More detailed study is required to identify the mechanisms of genetic change and the stability of genetic responses to differential management, and we have demonstrated that the SSR/ISSR method would be a powerful tool toward that end.

Cultivar	Seed lot	Abbreviation	Ploidy	Description	Origin	% in BG34
Barlet	100978-4	Barlet-1	Diploid	Bred from very diverse genetic base	Netherlands	40
	H4-0-83416	Barlet-2				
Barmaco	NL 961369-1	Barmaco-1	Diploid	Late heading cultivar. Very winter-hardy, and persistent	Netherlands	25
	100309-3	Barmaco-2				
Barnhem	NL 120037	Barnhem-1	Diploid	Forms an extremely dense sward	Netherlands	25
	H4-1-11312	Barnhem-2				
Mara	H4-1-93611	Mara-1	Diploid	Most winter hardy	Romania	10
	L177-1-mara	Mara-2				

Table 5.1. Description of perennial ryegrass cultivars, seed lots examined using SSR markers.

Field	Location	Time established	Botanical composition	Soil	Management
Kozak	W 81° 59' N 40° 33'	Spring 1998	80 % *L. perenne* 10 % *Trifolium repens* 10 % others	Canfield, silt loam	Rotational grazing with Jerseys cows
Gessel	W 81° 58' N 40° 42'	Autumn 1998	40 % *L. perenne* 30 % *Dactylis glomerata* 10 % *Trifolium repens* 20 % *Poa pratensis*	Canfield, silt loam	Rotational grazing with Jerseys cows
Noyes	W 81° 57' N 40° 54'	Autumn 1999	25 % *L. perenne* 30 % *Poa pratensis* 10 % *Agropyron repens* 10 % *Trifolium repens* 5 % *Phleum pratense* 20 % others	Canfield, silt loam	Rotational grazing with Jerseys cows, Cut each May for silage

Table 5.2. Description of agriculture and ecological parameters at the sample sites

Locus	Primers Sequence (5'-3')	Repeat motif	No. of alleles
M4-213	F CAC CTC CCG CTG CAT GGC ATG T R TAC AAC GAC ATG TCA AGG	$(GT)_8AGGT$	2
M15-185*	F GGT CTG GTA GAC ATG CCT AC R TAC CAG CAC AGG CAG GTT C	$(GA)_5TTAGAGG(GA)_{17}$	7
M16-B	F TGC TGT GGC TCT TGT GAC R AGC CGA GGC TCA GCT CGA	$(GA)^3G(GA)_{18}GG(GA)_7$	3
M4-136	F AGA GAC CAT CAC CAA GCC R TCT GGA AGA AGA TTT CCT TG	$GATT(GA)_{12}GT(GA)_{15}$	2
M2-148	F GCA ACT TCT ATC GAG TTG R GAG GCT CGA TCT TCA CGG A	$(GT)_9(GA)_9$	3
M12-52	F CTA CAA TGC ATT CGT GCA R TAG AGG CAC CCG CGC CCT	$(GA)_9$	2
LPSSRH01A07	F TGG AGG GCT CGT GGA GAA GT R CGG TTC CCA CGC CTT GC	$(CT)_9$	2
LPSSRH01A10	F CGC AGC TTA ATT TAG TC R GCT TTG AGT ATG TAA AGT T	$(CA)_{10}$	4
LPSSRH01H06	F ATTGACTGGCTTCCGTGTT R CGCGATTGCAGATTCTTG	$(CA)_9$	3
LPSSRH02C11	F CGGCCACCCTTGATAGAG R TCGTCAAGGATCCGGAGA	$(CA)_4TA(CA)_4$	4
LP20	F TGA CTT CTC TCG ATC CT R ATG TGA CTA CAA AAC CA	$(GA)_{16}(A)_5GCA(GA)_4$	3
LP204	F GAG CTT CTC TCG ATC CT R AGT GGA TGT GAC TAC A	$(CT)_{20}$	2
PR3	F GTA TAG TAC CCA TTC CGT R GCC GCC CTG CCA TGC TG	$(CA)_{22}$	1
PR14	F CCT TTT CGC CTT CGT A R CAC CAA CAT TGC CGA GTG	$(GT)_2GG(GT)_{10}$	2
PR37	F TCT GCA TTC GTT GTC TCA CTG R GAG CCG TCG CAC CCC TG	$(GT)_{18}$	4
PR39	F CAT TCA TCC ACG TTA GAC R CTT CCA CGA CTG CTT C	$(CA)_{17}$	4
ISSR*	$(AG)_{10}C$	AG	9
ISSR*	$(AG)_{10}T$	AG	10

Table 5.3. Sequences of three primer pairs used for amplification, optimal anneal temperature, and interpretable polymorphic number.
M4-213 to M12-52, SSR loci from Kubik et al., 1999
LPSSRH01A07- LPSSRH02C11, SSR loci from Jones et al., 2001
LP20-PR39, SSR loci from Kubik et al., 2001
$(AG)_{10}C$ and $(AG)_{10}T$, primers according to Ghariani, 2003
* primers actually used in this study

Primers used	Barnhem	Barmaco	Barlet	Mara
$(AG)_{10}C$	66.7	40.1	61.5	56.4
$(AG)_{10}T$	49.4	63.2	60.6	78.3
M15-185	50.5	46.4	67.1	28.9
$(AG)_{10}C + (AG)_{10}T$	72.5	71.4	75.6	78.3
All primers	84.8	81.3	86.7	80.9

Table 5.4. % of individuals of each cultivar that were correctly classified using either one, two, or all three SSR and ISSR markers

Fields	Barnhem	Barmaco	Barlet	Mara	% of confidence
Noyes	37	39	10	14	72.2
Gessell	32	22	39	7	70.3
Kozak	13	37	31	19	78.7
Assumed	25	25	40	10	

Table 5.5. Estimated percentage of each cultivar in the four sampled fields and in the original seed lot sown (assumed) and percentage of correct assignment.

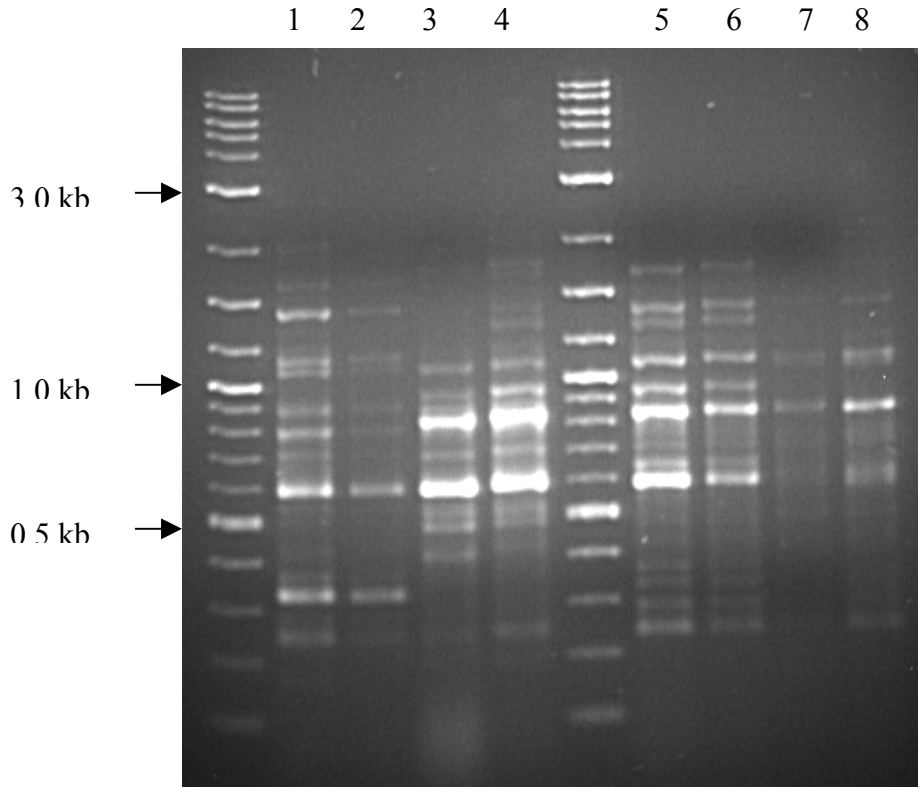


Fig. 5.1. Test of reproductivity of SSR with two leaf blades of one plant sampled (lane 1-2, 3-4, 5-6 and 7-8) for DNA extraction and PCR amplification with SSR primers $(AG)_{10}T$ (lane 1-4) and $(AG)_{10}C$ (lane 5-8). (1^{st} and 6^{th} lanes: 2-Log DNA ladder)

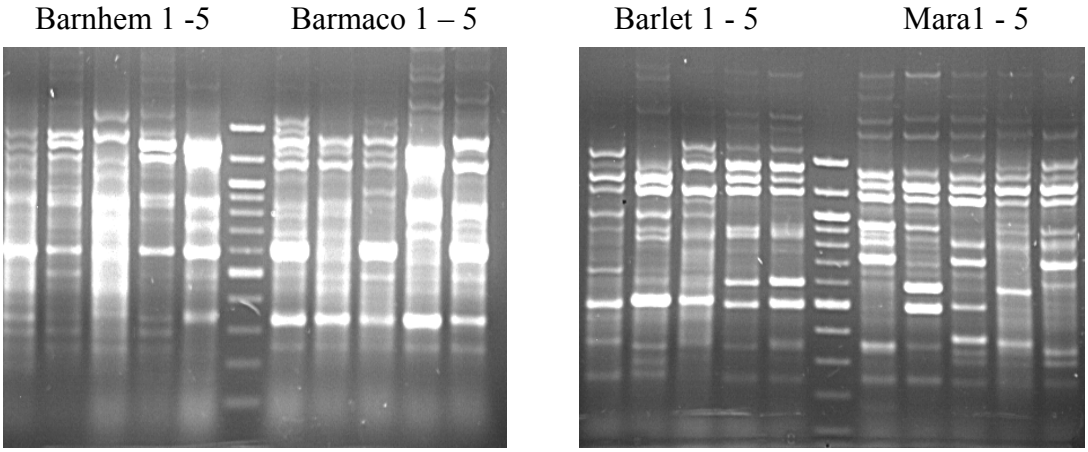


Fig. 5.2. Sample of SSR amplification products amplified by primer $(AG)_{10}C$ for five individuals from each of the four cultivars. (The middle lane was a 100 bp ladder with sizes from top to bottom: 1500, 1200 and 1000 to 100 with 100 bp intervals)

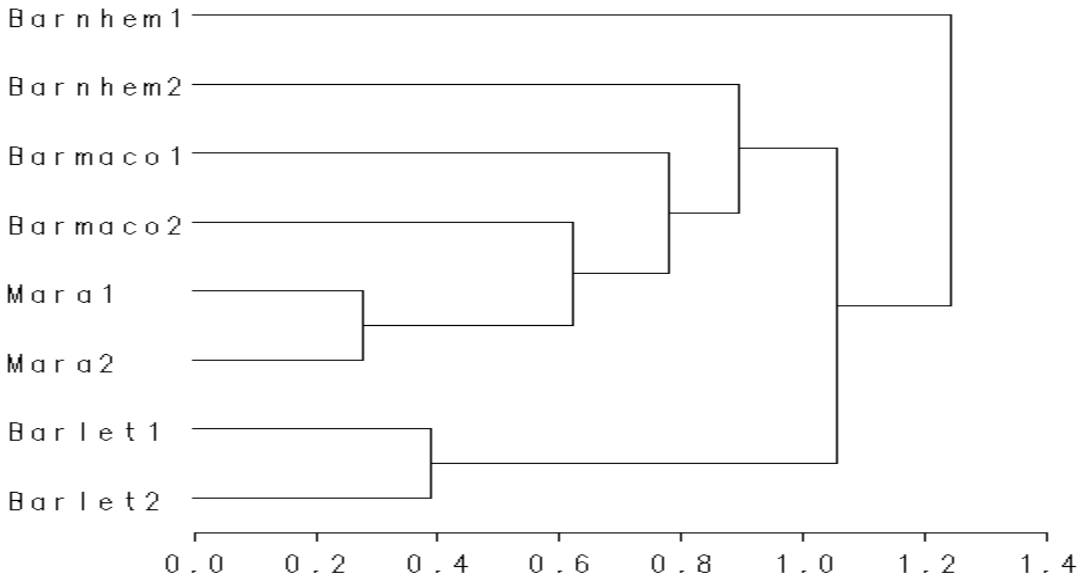


Fig. 5.3. Dendrogram showing relationship among the two seed lots from each of 4 ryegrass cultivars generated from SSR and ISSR data (Calculated with PROC CLUSTER of SAS).

CHAPTER 6

GENERAL CONCLUSION AND RESEARCH SUMMARY

As found by many ecologists on natural and experimental grasslands, the evidence for diversity effects on biomass and its components is equivocal for pastures. Positive effects of plants diversity have been reported in forage and grazing land experiments, the benefits have been attributed to the 1) niche complementarity, (Tilman, 2001); 2) "sampling effect", (Hector, 1999); 3) positive mutualistic interactions (Hooper, 1997). Previous studies have mainly dealt with α-diversity (species richness, at a small, ~ 1 m^2 scale) and primary production per area. More information may be obtained on the structure of the sward and individual dynamics in response to diversity if data is recorded on an individual plant basis. It is in these situations I conducted this research to address relevant questions as to the extent of within species diversity and the mechanisms of increased production from diverse community.

Evaluation of the morphological and genetic diversity is important to understand the relationship between plant diversity and productivity in a community. It is a complex problem for perennial ryegrass, because of its out-crossing nature and breeding practice to develop cultivars. Both morphological (Chapter 2-4) and molecular (Chapter 5) methods were used in this research to investigate the extent of genetic diversity within

and among perennial regress cultivars. The relationship between plant diversity and community productivity was discussed along with the mechanisms of this yield increase from mixtures, and how this was related to other growth parameters such as tillering character and survival rate. For the understanding of community response to genetic diversity, the measurement of individual plant is important because single plant is the unit that responds to the surrounding environment.

The first objective to quantify within- and between-cultivar variation of *L. perenne* cultivar blend BG34 on the basis of several morphological characteristics and forage yield was addressed in Chapter 2. I found substantial morphological variation between cultivars as well as within cultivars. The within-cultivar variance accounted for 93-97% of total variance for forage yield, and 73-96% of for height, tiller number, erectness, tiller diameter and leaf width. In a principle component analysis of 12 variables, the first four principal components explained 58.9% of the observed variation. The first principal component separated the populations mainly on the basis of plant mass (LW and H1) and yield (Y1, Y2, and Y3). As the ultimate measure of population diversity was not the number of species alone, but the number of genes present and being expressed, molecular work might be a complement to provide quantitative evidence of the genetic diversity found in these four cultivars. It was predicted that the total variation of a mixture of these cultivars (e.g. as in BG34) would result predominantly from within-cultivar variation rather than between-cultivar variation.

The second objective to examine the relative yield of *L. perenne* in response to genotypic diversity and how this response was affected by the defoliation frequency and intensity was addressed in Chapter 3,. There was a significant seed lotar relationship

between genetic diversity and production in Exp. 1 and a significant difference between mono-genotype and multi-genotype treatments in Exp. 2. The highest yielding treatments had only one genotype in Exp. 1 and two genotypes in Exp. 2, yet their yield was not significantly higher than that of the complex mixtures. Defoliation frequency and intensity had significant effects on forage yield. Frequent (3 wk vs. 6 wk) defoliation reduced grass yield by 12.7%, while the lax clipping (60 mm vs. 20 mm) reduced grass yield by 14.3%. It was concluded that genotypic diversity helped increase the herbage yield and this response was modified by defoliation treatments. Genotypes used in this study were selected from a broad seed blend population, BG34, which is widely used in the northeast, United States. Some of its good performance may be attributable to its presence of genotypic diversity and thus wide adaptation across different geographical conditions. The results indicated the ability and potential to use genetic diversity to increase mixture response could be present in genotypes that are adapted to cultivation in genotypically mixed stands. This response in yield might reflect breeder's intention that the cultivars and genotypes can maximize their capacity to interact positively with each other in the same stand, and adapt to a wide range of environmental conditions.

The third objective to investigate the responses of the yield components such as tiller number, tiller weight, plant mass and survival rate to genotype diversity, and how these responses were influenced by defoliation treatments was addressed in Chapter 4. My results showed significant effects of genotype treatment on tiller number and tiller mass, but not on survival rate. In general, these effects became more apparent toward the late harvest (i.e. Harvest 3 or 4) and under infrequent and lax cutting. The regression of tiller number, tiller mass, plant mass and survival rate against number of genotypes

present showed that small genotypes had negative responses to high genetic diversity and large genotypes had positive responses to genetic diversity. The survival of different genotypes didn't differ significantly when grown in monoculture under all cutting systems. However, more death of small genotypes occurred in mixtures than large genotypes, and the differences were more apparent under 6-wk cutting (infrequent), in which inter-plant competition was supposedly promoted.

In the Chapter 5, I evaluated the suitability of SSR and ISSR markers to differentiate cultivars of *L. perenne* that comprise BG34 and investigate the genetic structure of pastures sown with BG34 from dairy fields in Ohio. Of all the 16 primers tested, only three proved to be useful by generating interpretable polymorphic amplifications. Reproductivity of SSR and ISSR markers was tested by duplication of DNA extraction and PCR reaction. With these three primers, we correctly allocated individuals to cultivars and seed lots with 80.9-86.7% accuracy using discriminant analysis. The accuracy of the assignment was improved when more loci were included. Rate of correct assignment was increased from less than 50% with one locus considered up to 83.52% when all three primers were used. There were distinct differences between cultivars, and in some cases, seed lots within-cultivars. Divergent changes in the proportions of the respective cultivar of BG34 were found on the three 5-year old dairy fields of Ohio. One field remained similar to the cultivar mixture in the original sowing. Change of genetic structure was found for two fields, with increases in the proportion of Mara and Barmaco, and decreases Barlet or Barnhem depending on field. Overall, SSR was proven to be highly effective for differentiating among ryegrass cultivars. More

detailed study is required to identify the mechanisms of genetic change and the stability of genetic responses to differential management.

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APPENDIX A

SAS PROGRAMS (CHAPTER 2)

Phenotypic variation is the observable morphological present in a population and includes both genotypic and environmental components. Genetic variation is due to the genotypic differences among individual

SAS code used for the MANOVA using PROC GLM procedure and MANOVA statement.

```
data MorphDataExp1;
        input  plant $ H1 H2 TN1 TN2 E1 E2 TD1 TD2 LW Y1 Y2 Y3;
        cards;
        <400 lines of data>
        /*containing measures of 100 plants of each of the four cultivars*/
proc glm;
        class plant;
        model H1 H2 TN1 TN2 E1 E2 TD1 TD2 LW Y1 Y2 Y3 = plant;
        manova h = plant;
        lsmeans plant;
run;
```

SAS code used for the correlation analysis and clustering with PROC CORR, PROC CLUSTER and PROC TREE procedures.

```
data cultbarnhem; (cultbarmaco, cultbarlet or cult mara)
        input  plant $ H1        H2 TN1 TN2 E1 E2 TD1 TD2 LW Y1 Y2 Y3;
        cards;
        <100 lines of data>
proc transpose data=one out=two;
    id plant;
proc corr noprint outp=twop;
    var _1--_100 ;
data three (drop=_type_ _name_ type=distance);
    set twop;
    plant =_name_;
        if _type_ eq 'CORR';
        array numbs _1--_100 ;
        do over numbs;
        numbs = 1 - numbs;
        end;

proc cluster data=three(type=distance) method=ave out=four;
        var _1--_100 ;
        id plant;
proc Tree data = four horizontal vpages=10 hpages=6 maxh=2.5;
        goptions htext= .05 fontres=presentation htitle= .5;
        id plant;
        title 'Tree graph of pearson correlation';
quit;
```

Principal component analysis (PCA)

PCA transforms the original set of variables, which may or may not be correlated to each other, into a set of uncorrelated variables, called principal components. These variables are arranged in order of decreasing importance. The first principal component accounts for as much of the total variability as possible, and is thus the most meaningful. Each succeeding component accounts for as much of the remaining variability as possible. The analysis is variable-dependent, so character redundancies are weighted out.

SAS code used for the PCA using PROC PRINCOMP procedure.

```
data MorphDataExp1;
      input  plant $ H1 H2 TN1 TN2 E1 E2 TD1 TD2 LW Y1 Y2 Y3;
      cards;
      <400 lines of data> /*data were standardized*/
proc princomp out=prin;
      var H1 H2 TN1 TN2 E1 E2 TD1 TD2 LW Y1 Y2 Y3;
proc plot ;
      plot prin1*(prin2 prin3)=plant / vpos=25;
run;
```

Canonical discriminant analysis (CDA)

CDA is a dimension-reduction technique related to PCA and canonical correlation. Given a classification variable and several interval variables, PROC DISCRIM derives *canonical variables* (linear combinations of the interval variables) that summarize between-class variation in much the same way that principal components summarize total variation.

SAS code used for the canonical analysis using PROC CANDISC procedure.

```
data MorphDataExp1;
input plant$      H1    H2    TN1   TN2   E1    E2    TD1   TD2   LW
      Y1    Y2    Y3;
cards;
<400 lines of data> /*data were standardized*/

proc candisc all out=disc;
class plant;
var H1 H2   TN1   TN2   E1    E2    TD1   TD2   LW    Y1    Y2    Y3;
proc print data=disc /*to obtain can1, can2... value for Excel
plotting*/;
run;
```

APPENDIX B

SAS PROGRAMS (CHAPTER 3 and 4)

The CONTRAST statement (preceded by the MODEL statement) constructs and tests linear functions of the parameters in the MODEL statement.

SAS code used for the contrast analysis using PROC GLM procedure and CONTRAST statement.

```
Data1 DataEx3;
        input wk $ trt cut $ rep $ y1 y2 y3 y4 y5;
        cards;
        <102 lines of data>
        /***17(genotype treatment) × 2(cutting treatment) × 3(rep)***/


proc glm;
        class wk trt cut rep;
        model y1 y2 y3 y4 y5 =wk trt|cut (wk);
        repeated y;
        means trt/lsd;
*Treatment order-------------------------A---B---C---D---E---F---G---H;
*contrast 'label' variable effect values;

        contrast 'genetic diversity-linear' trt  -11 -11 -11 -11 -11 -11 -11 -11 -14 -14 -14 -
        14 2 2 2 2 136;
        contrast 'genetic diversity-quatratic' trt 20 20 20 20 20 20 20 20 -8 -8 -8 -8 -58 -58
        -58 -58 104;
        contrast 'yield potential-quadratic' trt 1 1 -1 -1 -1 -1 1 1 1 -1 -1 1 0 0 0 0 0;
        contrast 'yield potential-linear' trt -3 -3 -1 -1 1 1 3 3 -3 -1 1 3 0 0 0 0 0;
        contrast 'hi vs lo tiller potential' trt 1 -1 1 -1 1 -1 1 -1 0 0 0 0 0 0 0 0 0;
        contrast 'yield*tiller potential interaction' trt -3 3 -1 1 1 -1 3 -3 0 0 0 0 0 0 0 0 0;
        contrast 'momo vs mixture' trt 9 9 9 9 9 9 9 9 -8 -8 -8 -8 -8 -8 -8 -8 -8;
        means trt/lsd;
        means cut/lsd;
run;
```

SAS code used for the regression analysis of plot yield against number of genotypes using PROC REG procedure (Chapter 3):

Data Plotyield;

input trt cut$ y;

cards;

1	3wk	28.07	or	1	2cm	14.03
2	3wk	28.8		2	2cm	16.65
4	3wk	26.29		4	2cm	18.76
8	3wk	30.3		8	2cm	14.44
1	3wk	27.72		1	2cm	14.66
2	3wk	26.79		2	2cm	14.15
4	3wk	29.96		4	2cm	16.59
8	3wk	31.34		8	2cm	15.73
1	3wk	33.07		1	2cm	13.54
2	3wk	33.02		2	2cm	16.57
4	3wk	31.27		4	2cm	17.49
8	3wk	39.05		8	2cm	16.72
1	6wk	35.72		1	6cm	14.53
2	6wk	25.26		2	6cm	14.36
4	6wk	45.61		4	6cm	14.43
8	6wk	48.06		8	6cm	14.12
1	6wk	35.57		1	6cm	13.42
2	6wk	29.8		2	6cm	11.85
4	6wk	35.3		4	6cm	13.77
8	6wk	49.18		8	6cm	13.19
1	6wk	38.29		1	6cm	11.69
2	6wk	40.46		2	6cm	12.95
4	6wk	41.38		4	6cm	12.85
8	6wk	47.12		8	6cm	18.26

;
proc reg;

model y=trt;

by cut;

run;

APPENDIX C

SAS PROGRAMS (CHAPTER 5)

For a set of observations containing one or more quantitative variables and a classification variable defining groups of observations, the DISCRIM procedure develops a discriminant criterion to classify each observation into one of the groups. The derived discriminant criterion from this data set can be applied to a second data set during the same execution of PROC DISCRIM. The data set that PROC DISCRIM uses to derive the discriminant criterion is called the *training* or *calibration* data set.

SAS code used for the discriminant analysis using PROC DISCRIM procedure.

```
data refpop;
      input seedlot$    cult$ C1 C2 C3 C4 C5 C6 C7 C8 C9 T1 T2 T3 T4 T5
      T6 T7 T8 T9 T10 M1 M2 M3 M4 M5 M6 M7;
      cards;

      <400 lines of data>

data fieldges; /*fieldkoz and fieldnoy*/

      input plants$ C1 C2 C3 C4 C5 C6 C7 C8 C9 T1 T2 T3 T4 T5 T6 T7 T8
T9 T10 M1 M2 M3 M4 M5 M6 M7;
      cards;

      <100 lines of data>

proc discrim data=refpop testdata=fieldges testout=outges;
      class cult;
      var C1      C2    C3    C4    C5    C6    C7    C8    C9    T1
      T2    T3    T4    T5    T6    T7    T8    T9    T10 ;
proc print data =outges;
run;
```

SAS code used to develop dendragram of seed lots using PROC CLUSTER and PROC TREE procedures.

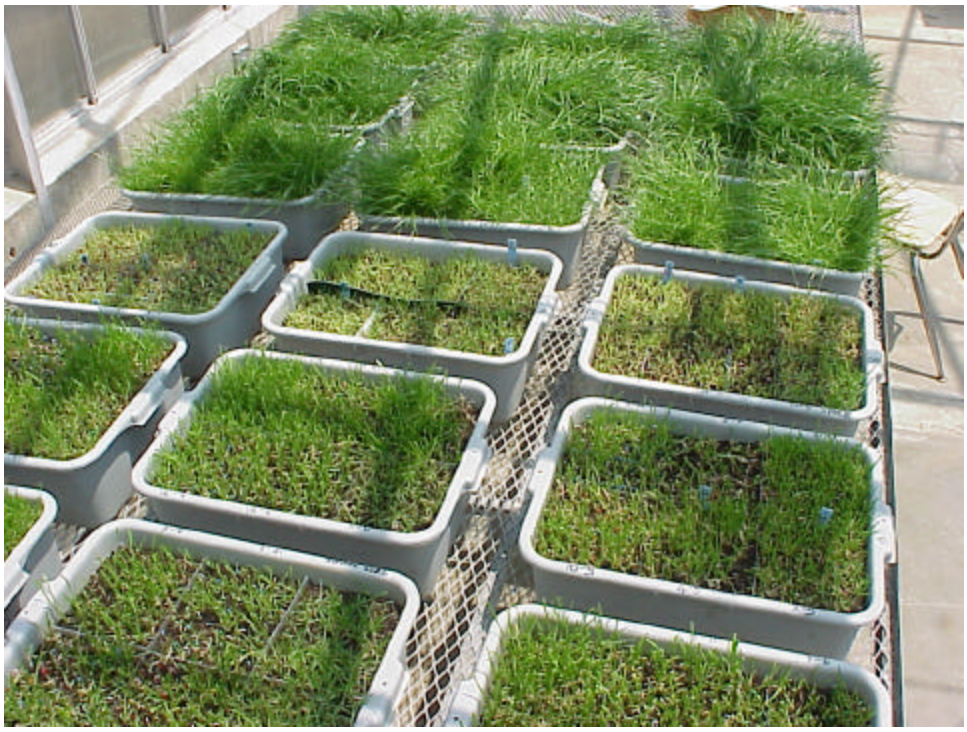
```
data one;
input   seedlot$        Barnhem1        Barnhem2        Barmaco1        Barmaco2        Barlet1
        Barlet2Mara1  Mara2;
cards;

Barnhem1          0   22.8133   16.0387   14.0165   22.7667   17.7901   19.5133   22.1864
Barnhem2    22.8133         0   15.2373   12.4942   23.2829   17.8946   16.9497   10.9643
Barmaco1    16.0387   15.2373         0   10.4121   21.3513   20.3571   13.2916   12.9164
Barmaco2    14.0165   12.4942   10.4121         0    15.232   15.9681   10.4432   9.09951
Barlet1     22.7667   23.2829   21.3513    15.232         0   6.10944   12.8989   8.37894
Barlet2     17.7901   17.8946   20.3571   15.9681   6.10944         0   13.7774   10.9174
Mara1       19.5133   16.9497   13.2916   10.4432   12.8989   13.7774         0   4.34546
Mara2       22.1864   10.9643   12.9164   9.09951   8.37894   10.9174   4.34546         0
;
proc cluster method=ave data=one(type=distance) out=outone;
id seedlot;
proc print data=outone;
data two;
set outone;
keep _NAME_ _parent_ _height_;
proc tree graphics HORIZONTAL vpages=1 hpages=.5  maxh=1.4 ntick=8;
  goptions htext=1.4 fontres=presentation htitle=2 ;
run;
```

APPENDIX D

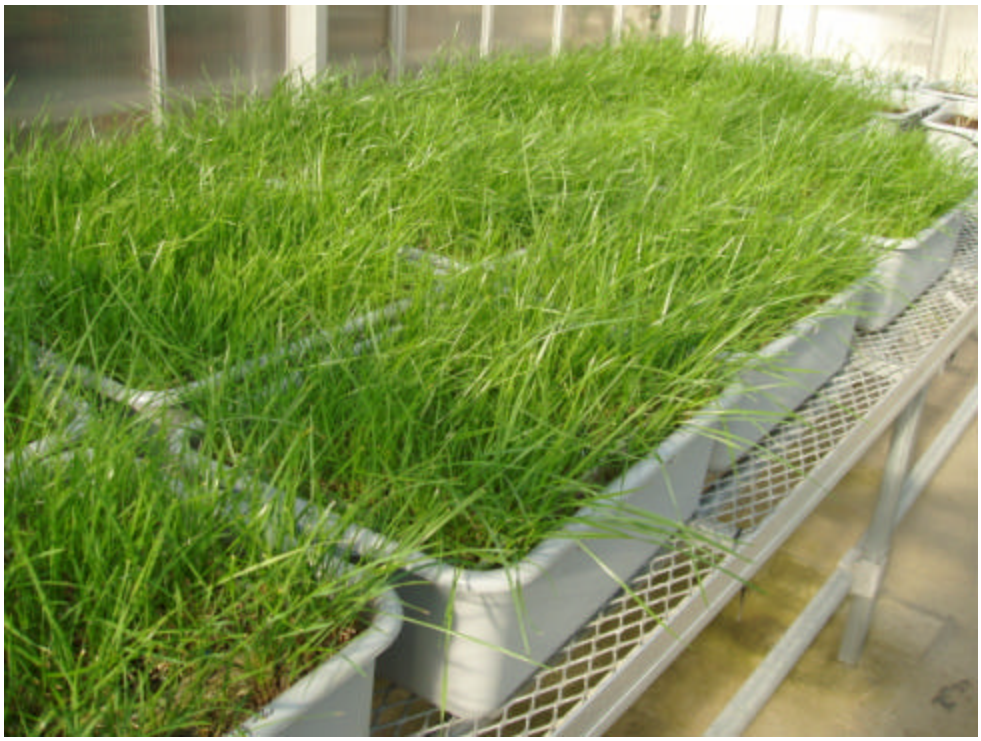
Photographs of Experiments

Chapter 2 Natural variation among 100 plants each of Mara, Barnhem, Barlet, Barmaco, 3 June 2002, Exp 1



Chapters 3 & 4. Plots of genotype mixture treatments, 3 week vs 6 week defoliation treatment, 30 May 2003, Exp. 1.

Chapters 3 & 4. Plots of genotype mixture treatments (6 per tub), 30 May 2003, Exp. 1.



Chapters 3 & 4. Plots of genotype mixture treatments during establishment, 4 August 2003, Exp. 2.

Chapters 3 & 4. Plots of genotype mixture treatments (4 per tub), 4 August 2003, Exp. 2.

Chapter 5. Transplanting BG34 tillers extracted from Ohio dairy pasture, Sep. 2004.

Chapter 5. Transplanted BG34 tillers extracted from Ohio dairy pasture, Sep. 2004.