Genetic variability in Festuca pratensis Huds. effect of management on natural populations and comparison of cultivars to other species

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Genetic variability in *Festuca pratensis* Huds.: effect of management on natural populations and comparison of cultivars to other species

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I SUMMARY

Drastic changes in agricultural practice during the past 100 years resulted in a loss of desired species in many swards in alpine regions of central Europe. _Festuca pratensis_ Huds. is a significant component of species-rich permanent pastures and meadows, ensuring high forage yield in areas with harsh climates, where other forage grasses of high quality are lacking. Unfortunately, the relative abundance of _F. pratensis_ has been decreasing over the years, and it is now only rarely found in intensively managed grasslands. Genetic variability may influence the performance of an individual species and, thus, its proportion in the sward. Limited genetic variability could, therefore, have contributed to the decline of _F. pratensis_. The objective of this study was (i) to assess the genetic variability of _F. pratensis_, (ii) to determine whether or not intensive management influences genetic variability within natural populations, and (iii) to compare the variability of _F. pratensis_ with the variability of more abundant grass species.

Six natural populations of _F. pratensis_ and three cultivars of each of the species _F. pratensis_, _Lolium perenne_ L., and _Dactylis glomerata_ L. were used in this study. Natural populations were sampled in two unrelated long-term experiments, where different fertilization and cutting treatments had been applied for 11 to 38 years. Genetic diversity was assessed using randomly amplified polymorphic DNA (RAPD) markers and agronomic traits.

The results of this study clearly demonstrate considerable genetic variation within and among natural populations and cultivars of _F. pratensis_. The proportion of polymorphic RAPD markers within single populations ranged from 35 – 49%, which is rather low for a species as widespread as _F. pratensis_. In general, genetic variability within cultivars was lower than in natural populations. The major part of total genetic variation in natural populations was due to variation between genotypes within populations. Differences between the two sites had only a slight effect on genetic variability. However, fertilization and frequent defoliation led to a significant reduction in genetic variability within natural populations. Comparing
two natural populations and one cultivar for mean values of agronomically important traits, the cultivar differed significantly from the natural populations. This corresponds to the RAPD results that showed close grouping of the natural populations and a clear separation of the cultivars. However, estimates of genetic variability based on agronomic traits depended strongly on the specific trait.

Genetic variability based on RAPD markers was considerably lower for *F. pratensis* cultivars than for *L. perenne* and *D. glomerata* cultivars. The proportion of variability due to variation within cultivars, determined by an analysis of molecular variance, was lower for *F. pratensis* (64.6%) than for *L. perenne* (82.4%) and *D. glomerata* (85.1%). A comparison of *F. pratensis* and *L. perenne*, based on agronomic traits, confirmed the differences in genetic variability within cultivars.

This study showed that genetic variability within natural populations of *F. pratensis* is reduced by intensive management. It is, therefore, desirable to conserve, *in situ*, unfertilized and rarely cut or grazed populations of *F. pratensis* as a gene pool. Moreover, genetic variability within *F. pratensis* cultivars was lower when compared to cultivars of other forage grasses. Although plant adaptation is induced by environmental factors, it depends on genetic resources for a specific response. Thus, the low genetic variability may contribute to the decline of *F. pratensis*. 
II ZUSAMMENFASSUNG


Die Resultate dieser Studie zeigen deutliche genetische Variabilität sowohl innerhalb als auch zwischen natürlichen *F. pratensis* Populationen und Sorten. Der Anteil polymorpher RAPD Marker innerhalb einzelner Populationen lag zwischen 35 und 49%. Dies ist relativ wenig für eine so weitverbreitete Arten wie *F. pratensis*. Die genetische Variabilität innerhalb der Sorten war deutlicher geringer als in natürlichen Populationen. Der grösste Anteil der genetischen Variabilität natürlicher

Die genetische Variabilität basierend auf RAPD Markern war deutlich tiefer in den *F. pratensis* Sorten als in den *L. perenne* und *D. glomerata* Sorten. Der Anteil der Variabilität verursacht durch die Variabilität der Genotypen innerhalb der Sorten war ebenfalls geringer für *F. pratensis* (64.6%) als für *L. perenne* (82.4%) und *D. glomerata* (85.1%). Ein Vergleich von *F. pratensis* und *L. perenne* basierend auf agronomischen Merkmalen bestätigte die Unterschiede in genetischer Variabilität innerhalb der Sorten.

Natural and managed grasslands are of great ecological and agricultural importance worldwide. In humid temperate regions, permanent pastures and meadows are multi-species plant communities, often dominated by graminaceous plant species. These grasslands evolved over thousands of years as the result of anthropogenic influence and would, for the most part, revert to forest without management. During the past 100 years, agricultural practices have changed drastically. On the one hand, large areas of permanent grasslands were plowed, resulting in a loss of habitat for many plant species. On the other hand, most of the remaining grassland was managed more intensively which lead to a reduction of species diversity in pastures and meadows (Green 1990).

Permanent grasslands are complex ecological systems; many factors (e.g. biological diversity, soil fertility and climatic conditions) influence their functioning and stability. In alpine regions of central Europe, where permanent grasslands are an important source of roughage for ruminants, many swards have lost their species richness. Limited species diversity may be partly responsible for a reduced stability of grasslands (Tilman and Downing 1994). Diversity may be important not only in terms of species diversity in a sward, but also in terms of diversity within individual species and populations. However, little is known about the importance of diversity within species for their persistence and for the stability of the entire ecosystem. Analyzing genetic variability within species is, therefore, important for understanding ecological processes within grasslands.

1 GENERAL ASPECTS OF VARIATION

The extent of variability among plants, animals, and other organisms is indicative of the immeasurable diversity of life at all levels of organization. Diversity is also
found within species and within populations. The diversity among different life-
forms allows the colonization of a broad range of ecosystems. Diversity within
species, however, enables populations to live in a number of different
environments.

1.1 Phenotypic plasticity and genetic variability are two major
components of variation
Three processes are basically responsible for variation between individuals:
environmental modification, genetic recombination, and mutation (Stebbins 1950).
The phenotype of a single individual is, therefore, always the result of the effect of
a given environment on an individual genotype. The variation observed between
two individuals is due to phenotypic plasticity and genetic variability.

Phenotypic plasticity is the ability of one genotype having different
phenotypes in contrasting environments (Bradshaw 1965). The magnitude of
plastic response in different environments varies between species, populations,
and individuals (Blackman and Bunting 1954; Kahn 1963). Such differences in
plastic response lead to the conclusion that phenotypic plasticity is under its own
specific genetic control (Bradshaw 1965). A certain phenotype is, therefore,
defined by its genotype, the particular environment, and by the ability of the
genotype to interact with a certain environment. It can be described as:

\[ P_{ijk} = G_i + E_j + GE_{ij} + e_{ijk} \]

where \( P_{ijk} \) is the phenotype of the \( k^{th} \) individual of genotype \( i \) in environment \( j \), \( G_i \)
the effect of the genotype, \( E_j \) the effect of the environment, \( GE_{ij} \) the effect of the
genotype-environment interaction, and \( e_{ijk} \) is the residual (Mitchell-Olds and
Rutledge 1986).

Genetic variability is that part of phenotypic variation which is independent
of environmental effects and can be transferred from parent to offspring. Genetic
variability ultimately arises from gene mutation (DeVries 1905) but is amplified by
sexual reproduction, which brings about novel genotypes by recombination.
Genetic variability is the raw material for evolution (Silvertown and Lovett Doust 1993).

1.2 Genetic variability is a prerequisite for adaptation and evolution
Plant populations can adapt to short-term environmental changes as well as to long-term evolutionary changes. Short-term adaptation is possible because of the phenotypic plasticity of a certain individual (genotype) and can occur within a short period of time (Snaydon 1987). Adaptation to long-term evolutionary changes on the other hand results in population differentiation that usually takes several generations (Bradshaw 1984). Although plant adaptation (short- and long-term) is induced by environmental factors, it depends on the existence of genetic resources (within a population or within a single individual) for the specific response: e.g., only some plant species can develop heavy metal tolerance (Bradshaw 1984).

A population can not evolve or adapt to environmental changes without genetic diversity (Templeton 1994). Genetically depauperate populations are, therefore, at greater risk of extinction than populations rich in genetic variation (Dolan 1994). Although it is accepted since Darwin that genetic variability is adaptive and enables the better utilization of habitats diverse in time and space, there has been much controversy about the extent to which genetic diversity influences the survival of populations or individuals (Beardmore 1983). However, polymorphic populations of fruit fly (Drosophila pseudoobscura) are able to utilize a defined level of resources more efficiently than monomorphic populations (Beardmore 1983). The performance of individuals is also influenced by genetic variability: a study of the ability to survive and the fecundity of sweet vernal grass (Anthoxanthum odoratum L.) showed that genetically variable plants are more fit (estimated by the net reproductive rate) than genetically uniform plants (Antonovics 1984). Genetic diversity also has an impact on higher levels of biodiversity. Species can be regarded to be evolving lineages, and their capacity to evolve is based on genetic diversity (Templeton 1994).
2 GENETIC VARIABILITY IN PLANTS

The level of genetic variability found in plants is roughly comparable to that of invertebrates but higher than that of vertebrates (Nevo 1978). Genetic variability varies considerably among species (Hamrick et al. 1979) and can be observed at various levels: heterozygosity occurs within single plants, polymorphism among individual plants within a population, and population differentiation among populations (Snaydon 1987).

2.1 Many variables influence genetic variability

Hamrick and Godt (1990) classified ecological and life history variables that influence genetic diversity by reviewing more than 650 allozyme studies covering more than 480 plant species. At the species level, geographic range was the most important factor influencing variability: widespread species showed up to twice the genetic diversity as endemic plants. At the population level, the breeding system and, again, the geographic range were the most important variables. Out-crossing, wind-pollinated, widespread species showed the highest variability within populations (Hamrick and Godt 1990). Gene flow between genotypes is of great evolutionary importance, since a recombination of traits allows genotypes to adapt to different growth conditions (VanDijk 1989). In addition, gene flow through the dispersal of pollen or seeds allows the introduction of new genes into a population. Other important factors influencing diversity at the species and the population levels are: life form (annual/perennial; woody/herbaceous), regional distribution (altitude, latitude), taxonomic status (gymnosperms/monocots/dicots), and mode of seed dispersal (Hamrick and Godt 1990).

A number of variables influence genetic variability, but adaptive evolutionary changes occur only when selective forces (e.g. limited resources or changes in environmental conditions) act on a number of individuals. This process is referred to as natural selection and can result in the differentiation of populations or, on a smaller scale, in differences among individuals within a population.
2.2 Populations differentiate in distinct environments

Variability within populations and population differentiation are part of a continuous phenomenon in which variability becomes manifest and is then sifted by the forces of the environment. Genetic differences between initially similar populations, which have been exposed to different conditions, may evolve over centuries or over periods as short as six years (Snaydon and Davies 1982). Differentiation of populations occur over very large distances (hundreds of kilometers) or on a local scale (over just a few meters). The extent of the differences among populations depends on the reciprocal action of natural selection (enhances differences) and migration (reduces differences) (Bradshaw 1984). Due to the sedentary nature of plants, migration can be overruled easily by selection (Bradshaw 1972), and patterns of differentiation of populations tend to follow patterns of environment very closely.

Population differentiation has been observed among geographically separated populations, but the responsible factors were not further investigated (Bahrman et al. 1994; Besse et al. 1994; Delavega et al. 1994; Holt 1994). Management may be an important factor causing population differentiation, since it represents fundamental interference in the functioning of the ecosystem. In grassland, where management basically involves fertilization and defoliation, these two processes have been shown to influence population differentiation.

2.2.1 Response to fertilization

Of the various factors influencing soil conditions, animal excreta and applications of mineral fertilizer seem to cause the greatest variation in grasslands (Snaydon 1987). Effects of urine are very short-lived and are, therefore, unlikely to cause genetic changes over short distances. Differences over larger distances (e.g. between fields) may be due to average differences in nutrient turnover (Snaydon 1987). Although the effects of dung are more prolonged (1-2 years) and large plastic responses in morphology occur (Rebuffo 1986), population differentiation over such short distances is again unlikely. However, animal excreta are likely to increase genetic polymorphism within plant species (McNeilly and Roose 1984). The effects of mineral fertilizers are generally not as localized, last longer and can,
therefore, induce population differentiation (Snaydon 1987). In the Park Grass Experiment in Rothamsted, where different fertilization and liming treatments have been applied for 50 to 100 years (Johnston 1997), populations of *A. odoratum* differed significantly in a number of morphological characteristics (Snaydon and Davies 1972). Population differentiation was closely correlated with differences in environmental conditions of the plots.

### 2.2.2 Response to defoliation

Significant differences among populations in response to defoliation have been detected over distances of only a few meters (Watson 1969) and over periods of about 10 years (Pike et al. 1979). *F. idahoensis* Elmer plants, that were never defoliated, grow taller than regularly defoliated plants when grown under similar conditions (Jaindl et al. 1994). Populations of *Poa annua* L. from closed habitats (grazed or mown) perform better under less favorable conditions imposed by cutting or competition than populations from open habitats (McNeilly 1981).

### 2.3 Genetic variability within populations

Numerous studies showed significant genetic variability within plant populations (Stebbins 1950). This variability is influenced by ecological variables (see above) and also varies considerably among species. While average heterozygosity within populations of 114 taxa was 0.16, values ranged from 0.00 for *Lycopersicon cheesmani* Riley to 4.81 in *Opuntia basilaris* Engelm. & Bigelow (Hamrick et al. 1979). The same was observed for the genus *Lolium* where heterozygosity within natural populations ranged from 0.00 (*Lolium temulentum* L.) to 0.39 (*L. multiflorum* L.) (Charmet and Balfourier 1994).

Domestication may also influence genetic variability within populations: commercial cultivars of *Phaseolus vulgaris* L. show a significantly reduced level of genetic variability than wild germplasm (Sonnante et al. 1994). Selection certainly played a significant role during domestication. Moreover, breeding may also influence genetic variability within forage grass cultivars (Huff 1997).

Only little information is available on variability within grassland species, but some studies indicate that management influences genetic variability within...
populations. *Poa annua* L. populations from open habitats (i.e. unmanaged populations) maintain greater genetic variation than populations from closed habitats (i.e. managed populations) (McNeilly 1984). Comparing grazed and non-grazed areas, Gray et al. (1979) found a larger number of genotypes of *Puccinellia maritima* Huds. in non-defoliated grasslands. However, these studies allow only a rough estimation of genetic variability, and there is no detailed information on the effect of current agricultural practices on populations within species-rich grasslands.

3 MEADOW FESCUE, A VALUABLE COMPONENT OF PERMANENT GRASSLAND IN COOLER REGIONS

Meadow fescue (*F. pratensis*) is a forage grass whose quality is comparable to *Lolium* spp.; it is quite winter-hardy and has a high yield potential (Meister and Lehmann 1990). Stebler and Schröter (1887) analyzed the floristic composition of grasslands in Switzerland at the end of the last century and found proportions of *F. pratensis* between 9% and 36% in fertilized meadows and pastures below 900 m a.s.l. Today meadow fescue has nearly disappeared from intensively managed meadows and pastures. Under mild climatic conditions, other high quality grasses (e.g. *Lolium* spp.) can mainly replace meadow fescue, but these species are not adapted to harsher climates. Consequently, intensively managed grasslands in higher and cooler regions are often dominated by undesirable species of *Umbelliferae, Ranunculaceae* or *Polygonaceae*. Decreasing the intensity of management may lower the proportion of undesirable species, but this is not possible without significant losses in forage yield and quality. It is, therefore, necessary to improve quality and floristic stability of these grasslands by introducing species such as *F. pratensis* that have high forage quality, are winter-hardy and produce high yield.
3.1 Cytogenetics of *F. pratensis*

*F. pratensis* is a diploid (2n = 14) out-breeding species that belongs to the genus *Festuca*, together with *Lolium* the most important genus of forage grasses in temperate regions. The DNA content of the unreplicated haploid genome of *F. pratensis* is 1.98 pg, corresponding to a genome size of approximately $1.98 \times 10^6$ kb (Xu and Sleper 1991). Tetraploid *F. pratensis* plants have been produced by means of colchizine treatments (Kostoff 1949), but these plants showed poor fertility. However, a tetraploid cytotype, *F. apennina* De Not. (2n = 4x = 28), is found at higher altitudes (above 1300 m a.s.l.) and is well-adapted to the harsh conditions (Jauhar 1993). *F. pratensis* is closely related to *Lolium* spp. (Jauhar 1993) and it was suggested that all outbred *Lolium* species and *F. pratensis* diverged from a common diploid ancestor (Charmet et al. 1996). However, *Festuca* and *Lolium* plants are clearly differentiated by the shape of the inflorescence (*Festuca* forms a panicle; *Lolium* forms an ear).

3.2 Reasons for the decline of meadow fescue

Intensive management can not be the main cause for the disappearance of meadow fescue, since it thrives under high fertilization and frequent defoliation when grown in monoculture (Gügler 1993). However, meadow fescue is a weak competitor when grown in mixture with *Dactylis glomerata* L. The low competitive ability of *F. pratensis* relative to *D. glomerata* is due mainly to its lower shoot competitive ability (Carlen 1994). While shoot competitive ability declined progressively during a two year field experiment, root competitive ability was high and varied only with the season. Leaf growth morphology may be a key factor influencing the competitive ability of meadow fescue. A low rate of leaf elongation during the second half of the re-growth cycle was partly responsible for the low competitive ability of meadow fescue in competition with *D. glomerata* (Messerli 1997).

As well as low competitive ability, other factors may influence the low persistence of meadow fescue in intensively managed swards. Propagation of seed, for example, is a very important mechanism in the survival strategy of meadow fescue in permanent grasslands. When reproductive tillers are cut before
anthesis, there is no possibility of reproduction by seed, thus causing a decline of the meadow fescue population (Zimmermann 1995).

According to observations of plant breeders, phenotypic variability of meadow fescue seems to be low when compared to other forage grasses (U. Feuerstein, personal communication). Furthermore, the variation for the date of ear emergence is smaller for meadow fescue cultivars (approx. 10 d) (Lehmann et al. 1996) than for other out-crossing forage grasses such as perennial ryegrass (approx. 30 d). Limited genetic variability may be an additional factor influencing the low persistence of meadow fescue.

4 DETECTING POLYMORPHISM IN PLANTS

Genetic differences between and, therefore, the diversity among individuals and groups of individuals can be estimated in many different ways. Although environmental effects can be largely excluded by using modern techniques such as enzyme and DNA markers, no method unambiguously describes the overall genetic diversity of an individual, population or species. Each method measures a different portion or expression of the genome, and the choice of method must be based on the particular question to be answered and the resources available.

4.1 Morphological traits
Morphological traits such as height, shape, size, number of leaves, date of flowering, etc. have been widely used to estimate genetic diversity (Snaydon and Davies 1972; Foster and Shaw 1988; Bachmann and Battjes 1994). Morphological characters allow the most direct approach for studying the variability of agronomically important traits, but it is often difficult to separate phenotypic plasticity from genetic variability. Therefore, most quantitative genetic studies have been conducted under controlled environments with cultivated plants and little work has been done on wild species in natural conditions (Primack and Kang 1989). Because the requirements necessary for a precise estimation of the genetic
variability in natural populations are difficult to meet, carefully designed field experiments and detailed statistical analyses are inevitable.

4.2 Isozyme markers
Differences in a specific enzyme allow the investigation of the corresponding alleles. Different molecular forms of enzymes with the same general function are usually referred to as isozymes. Since their discovery (Hunter and Markert 1957), various isozyme systems have been used to explore a large number of plant species (Hamrick and Godt 1990). Although isozymes allow a close approach to the genome, they still represent a part of the phenotype; banding patterns may depend on the type of tissue and the physiological status of the plant (Wendel and Weeden 1989). Isozymes deal with a limited number of functional enzymes and mainly concern variation in the coding sequences of the genes. Moreover, only a part of the variation on the DNA level will be visible at the isozyme level due to the redundancy of the genetic code.

4.3 DNA markers
In contrast to isozymes, DNA polymorphism can occur anywhere in the genome including coding and non-coding, single-copy or repetitive DNA (Bachmann 1994). The great advances made in molecular biology over the past 15 years have provided plant biologists with an almost unlimited array of tools for genome analysis (Jauhar 1996), and new methods continue to be developed. Techniques used to detect polymorphism at the DNA level can basically be divided in two categories: either DNA is cut at specific sites with restriction enzymes to detect restriction fragment length polymorphism (RFLP; Kochert 1994) or a stretch of target DNA is amplified between specific sites to which primers attach and serve as starting points for a polymerase chain reaction (PCR; Mullis and Faloona 1987). Of the various RFLP and PCR based techniques most frequently used for germplasm analysis (e.g. Gresshoff 1994; Phillips and Vasil 1994; Weising et al. 1995; Jauhar 1996), randomly amplified polymorphic DNA (RAPD) markers represent a technique that is cheap, quick, and easy to implement.
4.3.1 Randomly amplified polymorphic DNA (RAPD)

RAPD is a PCR-based technique that uses only one short (usually 10 base pairs) primer of arbitrarily sequence instead of the two primers of known sequence used in conventional PCR. The result of a RAPD amplification is several fragments of different size, randomly dispersed in the genome. These fragments are separated by agarose-gel-electrophoresis, stained with ethidium bromide, visualized under UV light and represent the RAPD fingerprint of the individual under study. Different primers produce different fingerprints and, because there are $4^{10}$ different potential decamers, the number of loci which may be assayed by RAPD is very large. RAPD polymorphisms are the result of insertion or deletion events within the amplified region (Williams et al. 1990) or arise due to sequence variation in the primer binding sites (Paran and Michelmore 1993).

Since its discovery in 1990 (Welsh and McClelland 1990; Williams et al. 1990), a large number of studies using RAPD for various applications, including genetic mapping, systematic and ecological studies, and identification of controlling regions for agronomic traits (Sobral and Honeycutt 1994) have been published. RAPD is quicker and more convenient than most other molecular marker systems and allows many loci to be assayed simultaneously. In addition, the cost of locus discovery is very low. For this reason, RAPD is particularly useful for large studies of genetic diversity, particularly for organisms for which there is no pre-existing molecular data. However, RAPD markers are usually dominant markers, and this limits their use in mapping projects. In addition, the method sometimes suffers from reproducibility problems which requires a very careful standardization of protocols and may limit a transfer between laboratories.

4.4 Morphological traits may complement RAPD markers

RAPD markers are very convenient for analyzing genetic variability of a large number of individuals. The analysis of morphological characters, on the other hand, represents the most direct approach to the variability of agronomically important traits. Therefore, combining morphological traits, which reflect a particular part of the genome, with RAPD markers, which detect anonymous
variation, may provide better insight into genetic variability of species, populations and individuals.

5 OBJECTIVE OF THE STUDY

The aim of this study was to investigate the genetic variability within species of permanent grassland using Festuca pratensis Huds. as a model plant. For this purpose, the randomly amplified polymorphic DNA technique was established and adapted to provide a tool for the detection of genetic variability in F. pratensis populations. Since the RAPD technique detects a rather anonymous variability, plants were grown under controlled conditions and variability was also estimated using key agronomic traits. In order to investigate the effect of intensive management on genetic variability in natural populations, plants were sampled from long-term experiments in which treatments had been applied for several decades. The genetic variability of natural populations was compared with that of commercial cultivars. Genetic variability may vary between species. Therefore, the genetic variability of F. pratensis was compared with that of Dactylis glomerata L., a companion species in permanent grasslands, and Lolium perenne L., one of the most important forage grasses in temperate regions. The investigation was based on the following hypotheses:

(I) Intensive management (i.e. fertilization and defoliation frequency) of Swiss grasslands for a number of decades caused a reduction in genetic variability within natural F. pratensis populations.

(II) Intensive breeding programs lowered genetic variability of commercial cultivars when compared to natural populations that had been established for 50 years or longer.

(III) Genetic variability within F. pratensis cultivars is lower than genetic variability of cultivars of other forage grasses.
1 ABSTRACT

Permanent pastures and meadows are species-rich vegetation systems that play an important role in the ecology and agriculture of temperate climates. Intensive management reduced species diversity and may also influence the genetic diversity within individual species and populations. The objective of this study was to assess genetic variability of meadow fescue, an important component of species-rich grasslands, and to determine whether or not fertilization and defoliation frequency influence genetic variability within natural populations. Genetic diversity of six natural populations and three cultivars of *Festuca pratensis* Huds. (meadow fescue) was investigated using randomly amplified polymorphic DNA (RAPD) markers and agronomic traits. Samples of natural populations were taken from two unrelated long-term experiments, where treatments had been applied for 11 to 38 years. RAPD analysis detected a clear genetic distinction of the cultivars from the natural populations. Genetic variability within cultivars was lower than within natural populations. Analysis of molecular variance (AMOVA) showed a significant effect of management on genetic variability. Fertilization and frequent defoliation led to a reduction in genetic variability within natural populations. Analysis of agronomic traits was only partially congruent with the results of RAPD analysis. This study shows that significant genetic variability exists within cultivars and natural populations of meadow fescue and can be reduced by intensive management.
2 INTRODUCTION

Permanent pastures and meadows of temperate climates are species-rich, complex ecological systems which evolved under thousands of years of anthropogenic influence (Green 1990). They play a key role in the agriculture of alpine regions and eastern Europe and are of great ecological value. In such species-rich communities, many factors, including environment and management, influence the performance of an individual species and, thus, its proportion in the sward. During the past 100 years, agriculture shifted towards more intensive management systems, resulting in a loss of species diversity in permanent grasslands (Green 1990). Swards with reduced species diversity are known to lose stability and to respond more rapidly to disturbances (Tilman and Downing 1994).

However, diversity may be crucial not only in terms of species diversity in a sward, but also in terms of genetic diversity within individual species. Populations can not evolve without genetic diversity and populations with low genetic variability are at greater risk of extinction (Dolan 1994; Templeton 1994). Genetic variability within and among populations is influenced by various factors, some of which (e.g. soil fertility) can be influenced by management. Fertilization and defoliation are known to cause population differentiation (Snaydon 1987), but little is known about their influence on genetic variability within populations.

*Festuca pratensis* Huds. (meadow fescue) is a significant component of species-rich permanent pastures and meadows, ensuring high forage yield in harsher climates, where other forage grasses of high quality are lacking. Unfortunately, the relative abundance of meadow fescue has been decreasing over the years. Limited genetic variability could have contributed to the decline of meadow fescue. Therefore, we chose meadow fescue as a model plant to investigate the effect of fertilization and defoliation frequency on genetic diversity. We investigated natural populations, sampled from permanent grassland, where different fertilization and defoliation treatments had been applied for a number of decades. Although significant genetic variability was found in cultivars of other forage grasses (Huff 1997; Xu et al. 1994), little is known about genetic variability of meadow fescue cultivars. Therefore, we included three unrelated synthetic
cultivars in our investigation to be able to compare the genetic diversity within cultivars in relation to the genetic diversity found in natural populations.

Of the different methods for detecting genetic diversity in plants, the analysis of morphological traits allows the most direct approach to studying variability of agronomic importance. However, the major problem in studying morphological traits is the partitioning of phenotypic plasticity and genetic variability; relying on agronomic traits may not be sufficient to differentiate between closely related germplasm (Casler 1995). Molecular markers may allow greater resolution because they enable genetic diversity to be estimated regardless of the phenotype (Bachmann 1994). Randomly amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990; Williams et al. 1990) have been used successfully to detect genetic variability in various plant species (Schierenbeck et al. 1997) including perennial ryegrass (Huff 1997) and switchgrass (Panicum virgatum L.) (Gunter et al. 1996), as well as to separate closely related genotypes of meadow fescue (Vallés et al. 1993).

In this study we used RAPD markers and agronomic traits (i) to assess genetic variability within and among cultivars and natural populations of meadow fescue, (ii) to determine whether or not fertilization and defoliation frequency influence genetic variability of natural populations and (iii) to compare genetic variability estimates derived from each of the methods.

3 MATERIALS AND METHODS

3.1 Plant material
Six natural populations and three cultivars of meadow fescue (Festuca pratensis Huds.) were used in this study. Natural populations were sampled at two sites in the Swiss Alps, near Zweisimmen (Eggenalp, 1340 m above sea level) and Martigny (Rosière, 1190 m a.s.l.). The plant community was a Trisetetum flavescentis at both sites. The experimental setup was a randomized complete block design with four replicates and a plot size of 50 m² (Eggenalp) and a split
plot design with four replicates and a plot size of 15 m² (Rosière). The experiments were established by Kali AG, Bern, Switzerland, in unchanged permanent grassland and treatments were applied for 38 (Eggenalp) and 11 years (Rosière) (Table 1). Few such experiments have been conducted but are very valuable; they represent common management systems and allow the investigation of long-term effects. Single tillers of 36 plants of each treatment were randomly sampled as single tillers from all four replicates. Plants of the three cultivars (Table 1) were grown from basic seed on sand for three weeks before 36 single plants were randomly selected. Each plant was transferred to a soil-filled pot and kept in the growth chamber. Due to the out-crossing nature of the species, in the following each individual plant will be referred to as a genotype. The term population will be used for plants from the same treatment at a particular site and for plants from the same cultivar (Table 1).

### Table 1 Meadow fescue (*Festuca pratensis* Huds.) populations used for investigation

<table>
<thead>
<tr>
<th>Population</th>
<th>Origin</th>
<th>Treatment</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>E20</td>
<td>Eggenalp</td>
<td>no</td>
<td>2</td>
</tr>
<tr>
<td>E2N</td>
<td>Eggenalp</td>
<td>yes</td>
<td>2</td>
</tr>
<tr>
<td>R20</td>
<td>Rosière</td>
<td>no</td>
<td>2</td>
</tr>
<tr>
<td>R2N</td>
<td>Rosière</td>
<td>yes</td>
<td>2</td>
</tr>
<tr>
<td>R30</td>
<td>Rosière</td>
<td>no</td>
<td>3</td>
</tr>
<tr>
<td>R3N</td>
<td>Rosière</td>
<td>yes</td>
<td>3</td>
</tr>
<tr>
<td>PRE (Préval)</td>
<td>cultivar</td>
<td>--</td>
<td>14</td>
</tr>
<tr>
<td>DAR (Darimo)</td>
<td>cultivar</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>FUR (Fure)</td>
<td>cultivar</td>
<td>--</td>
<td>84</td>
</tr>
</tbody>
</table>

- **Treaments were applied for 38 years at Eggenalp and for 11 years at Rosière**
- **Parental clones on which the cultivar is based**
- **Fertilization according to the official recommendation**
- **Swiss Federal Research Station for Plant Production, Changins, Switzerland**
- **Mommersteeg International, Vlijmen, The Netherlands**
- **The Norwegian Crop Research Institute, Ås, Norway**

### 3.2 DNA extraction

Fresh leaf material of each genotype was sampled 10 weeks after planting and stored at -80 °C. Genomic DNA was isolated by a modified hexadecyltrimethyl
ammonium bromide (CTAB) extraction procedure (Doyle and Doyle 1990). Frozen leaf tissue (approx. 100 mg) was ground in liquid nitrogen, transferred to a sterile Eppendorf tube and lyophilized overnight. Samples were incubated with 1 mL of CTAB buffer (100 g L⁻¹ CTAB, 1 M Tris pH 7.5, 5 M NaCl, 0.5 M EDTA pH 8.0) for 90 min at 60 °C, treated with 10 µg mL⁻¹ ribonuclease A (Boehringer Mannheim, Germany) at 37 °C for 30 min, extracted for 5 min with 450 µL chloroform/isoamyl alcohol (24:1 v:v) and centrifuged for 10 min at 7000 rpm. The aqueous phase was transferred to a new tube and the DNA was precipitated with cold isopropanol (900 µL). DNA pellets were recovered by centrifugation (15 min at 5000 rpm), washed in 75% ethanol / 10 mM ammonium acetate, dried under vacuum and dissolved in 150 µL sterile H₂O. DNA concentration was estimated with a LS-2B filter fluorimeter (Perkin-Elmer Ltd., Buckinghamshire, England) as well as visually after electrophoresis in a 20 g L⁻¹ agarose gel at 125 V for 1 h in TAE buffer (400 mM Tris, 20 mM EDTA, 200 mM sodium acetate) and staining with ethidium bromide.

3.3 DNA amplification and separation
Reactions were performed in 20 µL volumes containing: 1 x reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), 100 µM dATP, dGTP, dTTP, dCTP, 0.35 µM primer, 0.6 units EUROBIOTAQ® DNA polymerase (Eurobio, Les Ulis Cedex B, France) and 10 ng genomic DNA. Amplification was carried out in a Hybaid OmniGene temperature cycling system (Control Module with MicroBlock; Hybaid Ltd., Middlesex, UK) and was initiated by denaturation for 1 min at 94 °C, followed by 35 cycles of 30 sec at 35 °C, 2 min at 72 °C and 5 sec at 94 °C. The amplification was completed after 10 min at 72 °C. Reaction products were separated by electrophoresis in a 20 g L⁻¹ agarose gel at 100 V for 2.48 h in TAE buffer, stained with ethidium bromide (0.5 µg mL⁻¹) and photographed under UV light with a Polaroid type 667 film (Polaroid Corp., Cambridge, MA, USA). Amplification products were considered RAPD markers and reproducibility was confirmed by running independent duplicate samples. To estimate the size of the RAPD markers, a 100 base pair (bp) marker was used as a standard. To select suitable primers, 140 decamer primers (Operon Technologies, Inc., Alameda, CA,
USA) were initially screened using four unrelated ecotypes of meadow fescue, not associated with the nine populations used in this study. According to the quality of the banding patterns and the number of polymorphic markers detected between the ecotypes, 13 primers (B01, B08, B11, B12, B14, B15, H02, H19, Q5, R3, R11, R19, V16) were selected to analyze the 36 genotypes of the nine populations.

### 3.4 RAPD marker analysis

RAPD markers were scored for presence (1) or absence (0) and entered into a binary vector representing the RAPD phenotype of each individual genotype. Only polymorphic markers that were reproducible and could be scored unequivocally in all genotypes were included in the analysis. The pairwise distances between genotypes were estimated using the Euclidean distance of Excoffier et al. (1992),

\[
E_{ij} = \{ \beta_j^2 \} = n \left[ 1 - \frac{2n_{ij}}{2n} \right]
\]

where \( n_{ij} \) is the number of bands shared by the two genotypes \( i \) and \( j \) and \( n \) the total number of polymorphic bands. Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used to calculate the proportion of total RAPD variation between two populations (\( \Phi_{st} \)), which was used as a measure of genetic distance. A hierarchical dendrogram was constructed with \( \Phi_{st} \) values, following the unweighted pair group procedure with an arithmetic mean (UPGMA) (Gordon 1981). Reliability of fit of the clustering to the original data set was tested with a cophenetic correlation coefficient (Rohlf 1993). To investigate the treatment and site effects, a data set of the natural populations only was subjected to AMOVA analysis. For comparison within sites and treatments, subsets containing only two populations were also analyzed. Significance of variance components and \( \Phi_{st} \) values was tested by means of a nonparametric procedure that involved 1,000 permutations (Excoffier et al. 1992). AMOVA analysis was performed using the WINAMOVA 1.55 program (http://anthropologie.unige.ch/~laurent/default.htm#Software_programs), kindly provided by L. Excoffier. The NTSYS-pc package (version 1.8) (Rohlf 1993) was used for principal coordinate analysis and UPGMA clustering.
3.5 Variation in agronomic traits

Variation in agronomic traits was assessed in a growth chamber experiment. Fifteen genotypes were randomly chosen from each of the populations from Eggenalp (fertilized and unfertilized) and from the cultivar Préval. Three clonal replicates per genotype were produced using three single tillers of similar weight. Replicates were planted in individual cylindrical pots (75 mm diam.; 1.1 L volume) filled with silica sand (particle size 0.8 - 1.2 mm). After establishment (2 weeks), replicated plants were arranged in three randomized blocks. Each block with one replicate of each genotype was surrounded by border plants. The plants were cultivated in a growth chamber (PGV36, Conviron Instruments, Winnipeg, MB, Canada) at temperatures of 13/18 °C (day/night), 80% relative humidity and a photoperiod of 16 h. Light (photosynthetic photon flux density 500 μmol m⁻² s⁻¹) was provided by cool-white fluorescent lamps (Sylvania, CW/VHO, 215 W) and incandescent bulbs (100 W) at a ratio of 5:1. Plants were watered twice a day with 40 mL nutrient solution (Hammer et al. 1978). After growing for 59 days, plants were harvested and separated into roots, leaf laminae and sheaths. The area of the leaf laminae was measured using a photoelectric meter (Model LI-3000A; Li-Cor, Lincoln, NE, USA), and leaves and tillers were counted. All the fractions were dried at 65 °C for 48 h. Tiller and leaf dry weight, based on the dry weight of the shoot (leaf laminae and sheaths) and the leaf laminae respectively, were calculated. The data were subjected to an analysis of variance using the GLM procedure of the SAS statistical package (Statistical Analyses System, Version 6.12, SAS Institute, Cary, NC, USA). Means of populations were compared by Duncan’s multiple range test (P < 0.05). Variance components were estimated using the VARCOMP procedure of the SAS package. Coefficients of genetic variation were calculated according to Helgadottir and Snaydon (1986) as

\[
CV_g = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100
\]

where \( \sigma^2_g \) is the genotypic component of variance and \( \bar{x} \) is the population mean of the character measured. For factor analysis, traits were averaged by genotype and the values were transformed to standard deviates (Sokal and Rohlf 1995). Factor analysis was applied using the varimax rotation method (Kaiser 1958).
4 RESULTS

4.1 Characteristics of RAPD markers
The 13 primers yielded a total of 101 reproducible bands, ranging in size from 380 to 2000 bp. After excluding monomorphic bands, 69 polymorphic markers were left for the analysis. Each of the 324 individual plants had a unique RAPD phenotype (Fig. 1). The number of polymorphic markers within each population ranged from 34 to 49 (Table 2).

Marker frequency varied greatly across populations. None of the markers that were found in all the individuals of one population were at the same time missing in the other populations. While 33 markers (48%) were found in all populations, one marker was not found in any of the cultivars (PRE, DAR, FUR), two were found only in the populations at Rosière and one marker was found exclusively in the populations at Eggenalp.

![Fig. 1 RAPD pattern of meadow fescue genotypes from an unfertilized (Eggenalp-2-0) and a fertilized (Eggenalp-2-N) population generated by primer OPB-11. Numbers indicate individual genotypes (two independent replicates). Arrows point to scorable polymorphic markers. M = 100bp molecular marker](image)

4.2 Comparison of cultivars and natural populations
The genetic distances, expressed as the proportion of total RAPD variation between two populations ($\Phi_{ST}$) (Table 3), were significant for all pairs of populations and ranged from 0.079 (E20 / E2N) to 0.430 (PRE, DAR / R30). The
average $\Phi_{st}$ value, when one population was compared to the rest, was highest for PRE (0.351) and lowest for E20 (0.263). UPGMA clustering of $\Phi_{st}$ values resulted in two main clusters, the cultivars separated from the natural populations (Fig. 2).

**Table 2** Genetic diversity within populations, origins and treatments for three cultivars and six natural populations of *Festuca pratensis* Huds.

<table>
<thead>
<tr>
<th></th>
<th>Number of polymorphic markers</th>
<th>Euclidean distance (^2) (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within populations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>48</td>
<td>14.8</td>
</tr>
<tr>
<td>DAR</td>
<td>36</td>
<td>10.2</td>
</tr>
<tr>
<td>FUR</td>
<td>34</td>
<td>11.1</td>
</tr>
<tr>
<td>E20</td>
<td>49</td>
<td>18.7</td>
</tr>
<tr>
<td>E2N</td>
<td>48</td>
<td>16.5</td>
</tr>
<tr>
<td>R20</td>
<td>45</td>
<td>14.8</td>
</tr>
<tr>
<td>R2N</td>
<td>44</td>
<td>15.1</td>
</tr>
<tr>
<td>R30</td>
<td>47</td>
<td>14.5</td>
</tr>
<tr>
<td>R3N</td>
<td>42</td>
<td>12.7</td>
</tr>
<tr>
<td><strong>Average within origins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultivars</td>
<td>39.3</td>
<td>12.0</td>
</tr>
<tr>
<td>Eggenalp</td>
<td>48.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Rosière</td>
<td>44.5</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Average within fertilization treatments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not fertilized</td>
<td>47.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Fertilized</td>
<td>44.6</td>
<td>14.8</td>
</tr>
<tr>
<td><strong>Average within cutting treatments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two cuts per year</td>
<td>44.5</td>
<td>15.0</td>
</tr>
<tr>
<td>Three cuts per year</td>
<td>45.0</td>
<td>13.6</td>
</tr>
</tbody>
</table>

a Total number of markers scored: 101
b Average distances for pairwise comparisons of genotypes
c See Table 1 for a description of populations

Although populations from Eggenalp were closely grouped, populations from Rosière did not form a distinct cluster. The correlation coefficient between cophenetic values of the dendrogram and the $\Phi_{st}$ values was $r = 0.85 \ (P < 0.001)$, indicating that the clustering was congruent with the original data set (Rohlf 1993). Genetic polymorphism within populations and treatments, estimated from the number of polymorphic markers and the average Euclidean distance (E), is shown.
in Table 2. The cultivars DAR and FUR showed the least polymorphism within populations, whereas E20 revealed the greatest divergence of all the populations (Table 2). Comparing the average values of the three origins (cultivars, Eggenalp and Rosière), the cultivars (E = 12.0) were not as polymorphic as the natural populations.

![UPGMA clustering of nine Festuca pratensis Huds. populations](image)

**Fig. 2** UPGMA clustering of nine *Festuca pratensis* Huds. populations (see Table 1 for specification) based on $\Phi_{st}$, the proportion of total RAPD variation between populations. $\Phi_{st}$ values were calculated using AMOVA and an Euclidean metric distance matrix based on RAPD profiles of 36 genotypes per population.

### 4.3 Comparison of natural populations from different sites and treatments

The total amount of genetic variation found in the natural populations was mainly due to the variation among genotypes within populations (71%), whereas the variation among populations accounted for 20% and among sites for only 9% of the genetic variation (Table 4). Analysis of molecular variance for all six populations showed no significant variance component for fertilization (-0.383) and cutting (-0.181) treatments. However, variation averaged over treatments was lower within fertilized or more frequently cut than in unfertilized or less frequently cut populations, respectively (Table 2).
**Table 3** Genetic distance, expressed as $\Phi_{st}$, the proportion of total RAPD variation residing between populations, for three cultivars and six natural populations of *Festuca pratensis* Huds. Thirty-six genotypes per population were analyzed.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Natural populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE$^a$</td>
<td>DAR</td>
</tr>
<tr>
<td>DAR 0.377$^b$</td>
<td>--</td>
</tr>
<tr>
<td>FUR 0.260</td>
<td>0.276</td>
</tr>
<tr>
<td>E20 0.275</td>
<td>0.375</td>
</tr>
<tr>
<td>E2N 0.318</td>
<td>0.418</td>
</tr>
<tr>
<td>R20 0.358</td>
<td>0.391</td>
</tr>
<tr>
<td>R2N 0.366</td>
<td>0.357</td>
</tr>
<tr>
<td>R30 0.430</td>
<td>0.430</td>
</tr>
<tr>
<td>R3N 0.427</td>
<td>0.397</td>
</tr>
</tbody>
</table>

---

$^a$ See Table 1 for a description of populations

$^b$ All $\Phi_{st}$ values were significantly larger than a random $\Phi_{st}$ value ($P < 0.001$)
Comparing fertilization treatments at the same sites under the same cutting regime, AMOVA revealed significant components of variance \((P < 0.001)\) for fertilization (Table 4). The same was true for cutting treatments with the same level of fertilization. Although fertilization explained only 8\% of the variation in Eggenalp, fertilization was responsible for 28\% (2 cuts y\(^{-1}\)) and 29\% (3 cuts y\(^{-1}\)) of the variation in Rosière. Fertilization and higher cutting frequency generally reduced variability within populations, with the exception that the Euclidean distance for R2N was slightly higher than for R20 (Table 2).

**Table 4** Analysis of molecular variance (AMOVA) for six natural populations of *Festuca pratensis* Huds. from two sites (Eggenalp and Rosière) and four cultivation treatments (fertilization/no fertilization; 2/3 cuts y\(^{-1}\)) using 69 RAPD markers on 36 genotypes per population

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Mean square deviation</th>
<th>Variance component(^a)</th>
<th>% of total variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>1</td>
<td>183.03</td>
<td>0.99</td>
<td>9</td>
</tr>
<tr>
<td>Population (site)(^b)</td>
<td>4</td>
<td>88.15</td>
<td>2.24</td>
<td>20</td>
</tr>
<tr>
<td>Genotype (population)</td>
<td>210</td>
<td>7.69</td>
<td>7.69</td>
<td>71</td>
</tr>
</tbody>
</table>

**Within Eggenalp**

- Fertilization: 1 35.88 0.75 8
- Genotype (fertilization): 70 8.79 8.79 92

**Within Rosière**

- Fertilization, 3 cuts y\(^{-1}\): 1 107.01 2.78 29
- Genotype (fertilization): 70 6.80 6.80 71
- Fertilization, 2 cuts y\(^{-1}\): 1 113.08 2.93 28
- Genotype (fertilization): 70 7.48 7.48 71
- Cutting, fertilized: 1 53.69 1.30 16
- Genotype (cutting): 70 6.96 6.96 84
- Cutting, unfertilized: 1 158.90 4.21 37
- Genotype (cutting): 70 7.31 7.31 63

\(^a\) All components were significant at \(P < 0.001\), giving the probability of obtaining a more extreme random value computed from non-parametric procedures (1,000 data permutations)

\(^b\) Nested analysis: Population (site) = variation among populations within site
4.4 Variation in agronomic traits

Of the agronomic traits, PRE showed the highest mean values, with the exception of specific leaf area (Table 5). Although PRE was generally significantly different from natural populations, except for tiller dry weight, natural populations differed from each other by three traits only (Table 5). E20 plants had significantly more tillers but lighter leaves and a lower shoot/root ratio than E2N plants. Ranking populations according to \( CV_g \) values depended strongly on the traits investigated (Table 5). While PRE showed the highest \( CV_g \) for leaf dry weight, tiller dry weight and shoot/root ratio, the population E2N had highest values for the other investigated parameters (Table 5). Except for shoot/root ratio, the component of genotypic variance was always lower for E20 than for E2N.

Table 5 Mean values (\( \bar{x} \)) and coefficient of genotypic variation (\( CV_g \)) for seven agronomic key traits of 45 genotypes of meadow fescue from an unfertilized natural population (E20), a fertilized natural population (E2N) and the cultivar Préval (PRE)

<table>
<thead>
<tr>
<th>Trait</th>
<th>E20</th>
<th>E2N</th>
<th>PRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight plant (^1) [g]</td>
<td>( \bar{x} ) 2.8 a(^*)</td>
<td>2.7 a</td>
<td>3.6 b</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 19.6 ***</td>
<td>39.1 ***</td>
<td>17.9 **</td>
</tr>
<tr>
<td>Leaf area plant (^1) [cm(^2)]</td>
<td>( \bar{x} ) 255.5 a</td>
<td>241.6 a</td>
<td>340.6 b</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 16.9 *</td>
<td>38.4 ***</td>
<td>16.7 **</td>
</tr>
<tr>
<td>Tillers plant (^1) [no.]</td>
<td>( \bar{x} ) 18.9 a</td>
<td>17.1 b</td>
<td>21.9 c</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 21.4 ***</td>
<td>32.7 ***</td>
<td>24.5 ***</td>
</tr>
<tr>
<td>Dry weight leaf (^1) [mg]</td>
<td>( \bar{x} ) 22.4 a</td>
<td>24.2 b</td>
<td>30.4 c</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 8.9</td>
<td>15.9 **</td>
<td>18.8 ***</td>
</tr>
<tr>
<td>Dry weight tiller (^1) [mg]</td>
<td>( \bar{x} ) 147.0 a</td>
<td>156.4 ab</td>
<td>164.1 b</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 13.4 **</td>
<td>14.9 ***</td>
<td>15.9 ***</td>
</tr>
<tr>
<td>Shoot / root ratio [no.]</td>
<td>( \bar{x} ) 3.0 a</td>
<td>3.4 b</td>
<td>3.6 c</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 14.0 ***</td>
<td>7.3</td>
<td>13.1 ***</td>
</tr>
<tr>
<td>Specific leaf area [cm(^2) g(^{-1})]</td>
<td>( \bar{x} ) 203.3 a</td>
<td>204.1 a</td>
<td>181.5 b</td>
</tr>
<tr>
<td></td>
<td>( CV_g ) 2.8</td>
<td>7.8 *</td>
<td>5.6</td>
</tr>
</tbody>
</table>

\(*\), **, *** \( CV_g \) are significant at \( P < 0.05, 0.01 \) and 0.001 respectively

a Means within rows followed by the same letter are not significantly different at the \( P < 0.05 \) level, according to Duncan's Multiple Range Test
Table 6 Mean square deviations and variance components for RAPD phenotypes and agronomic key traits of 45 *Festuca pratensis* Hud. genotypes from three populations

<table>
<thead>
<tr>
<th>Trait</th>
<th>Source of variation</th>
<th>df</th>
<th>Mean square deviation</th>
<th>Variance component</th>
<th>% of total variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD phenotypes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>population</td>
<td>2</td>
<td>47.27</td>
<td>2.58 ***</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>genotype (population)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42</td>
<td>8.46</td>
<td>8.45 ***</td>
<td>77</td>
</tr>
<tr>
<td>Shoot dry weight plant&lt;sup&gt;1c&lt;/sup&gt; [g]</td>
<td>population</td>
<td>2</td>
<td>11.89</td>
<td>0.22 **</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>2.15</td>
<td>0.60 ***</td>
<td>52</td>
</tr>
<tr>
<td>Leaf area plant&lt;sup&gt;1&lt;/sup&gt; [cm&lt;sup&gt;2&lt;/sup&gt;]</td>
<td>population</td>
<td>2</td>
<td>129443.65</td>
<td>2502.95 ***</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>16810.97</td>
<td>4565.71 ***</td>
<td>45</td>
</tr>
<tr>
<td>Tillers plant&lt;sup&gt;1&lt;/sup&gt; [no.]</td>
<td>population</td>
<td>2</td>
<td>353.27</td>
<td>5.94 *</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>85.74</td>
<td>25.78 ***</td>
<td>64</td>
</tr>
<tr>
<td>Dry weight leaf&lt;sup&gt;1&lt;/sup&gt; [mg]</td>
<td>population</td>
<td>2</td>
<td>785.32</td>
<td>15.97 ***</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>66.62</td>
<td>17.13 ***</td>
<td>35</td>
</tr>
<tr>
<td>Dry weight tiller&lt;sup&gt;1&lt;/sup&gt; [mg]</td>
<td>population</td>
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<td>3297.60</td>
<td>25.69 **</td>
<td>2</td>
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<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>2141.45</td>
<td>536.64 ***</td>
<td>49</td>
</tr>
<tr>
<td>Shoot / root ratio [no.]</td>
<td>population</td>
<td>2</td>
<td>3.56</td>
<td>0.06 **</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>0.67</td>
<td>0.15 ***</td>
<td>36</td>
</tr>
<tr>
<td>Specific leaf area [cm&lt;sup&gt;2&lt;/sup&gt; g&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>population</td>
<td>2</td>
<td>7407.51</td>
<td>145.57 ***</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>42</td>
<td>856.96</td>
<td>130.79 **</td>
<td>18</td>
</tr>
</tbody>
</table>

* * * Significant at $P < 0.05$, 0.01 and 0.001, respectively

<sup>a</sup> Analysis of molecular variance based on Euclidean distance measure using 69 RAPD markers
<sup>b</sup> Nested analysis: genotype (population) = variance among genotypes within populations
<sup>c</sup> Analysis of variance based on three clonal replicates per genotype
Variance among populations accounted for 2% (tiller dry weight) to 33% (leaf dry weight) of the total variance observed (Table 6). Comparing RAPD phenotypes for the same genotypes as investigated for agronomic traits, 23% of the variance was explained by variation among populations (Table 6). This proportion remained the same when all 108 genotypes were included in the RAPD analysis. Factor analysis with seven factors resulted in four main factors which explained 97% of the variation. Factor 1 (which accounted for 40% of the total variance) included the parameters shoot dry weight, number of tillers and leaf area, factor 2 (which accounted for 25% of the total variance) included leaf dry weight and tiller dry weight, while factor 3 (which accounted for 17% of the total variance) included specific leaf area, and factor 4 (which accounted for 15% of the total variance) included shoot/root ratio. While principal coordinate analysis of RAPD markers resulted in a clear separation of PRE from natural populations, factor analysis of agronomic traits failed to separate the cultivar PRE from the other populations (Fig. 3).

Fig. 3 Principal coordinate analysis of 69 RAPD markers (A) and factor analysis of seven agronomic traits (B) for 45 Festuca pratensis Huds. genotypes from the cultivar Préval (PRE), a fertilized (E2N) and an unfertilized natural population (E20).
5 DISCUSSION

The results of this study clearly demonstrate considerable genetic variation within and among natural populations and cultivars of meadow fescue. Moreover, the results based on Euclidean distance were confirmed by different measures of genetic diversity such as matching (Puterka et al. 1993) and similarity (Nei and Li 1979) coefficients (results not shown). The genetic distance between populations, expressed as $\Phi_{st}$, showed a high correlation with Nei's genetic distance (Nei 1972), as determined by calculating the normalized Mantel statistics ($r = 0.87; P < 0.001$). Of all RAPD markers scored, 68% were polymorphic across all populations. This proportion dropped to 49 - 34% within single populations (Table 2), which seems rather low for a widespread species such as meadow fescue, when compared to the amount of variability found in endemic species (63% polymorphic markers across three populations of *Erodium paularense* Fern. Gonz. & Izco) (Martin et al. 1997).

Cluster analysis of RAPD data resulted in a clear separation of the cultivars from the natural populations (Fig. 2), suggesting that the natural populations were not closely related to any of the three cultivars. Furthermore, $\Phi_{st}$ values for most cultivar / cultivar comparisons were lower than values for cultivar / natural population comparisons, although PRE, DAR and FUR are unrelated synthetic cultivars. Genetic variability within cultivars, expressed as mean square (MS) values of AMOVA, ranged from 5.11 (DAR) to 7.40 (PRE). Similar levels of diversity were found in perennial ryegrass (*Lolium perenne* L.) cultivars by Huff (1997). One possible reason for the low genetic variability within DAR is that this synthetic cultivar is based on only five parental clones, four of which originated from the same variety, whereas the other cultivars (PRE and FUR) originated from a larger number of clones. However, the variability within FUR was less than within PRE, although PRE had fewer parental clones. This indicates that not only the number of clones, but also factors such as the selection intensity, the pedigrees of, and the heterozygosity within parental lines are essential for variability within cultivars.

The major part of total genetic variation in natural populations was due to variation between genotypes within populations; only 9% of the variation was
between the two sites (Table 4). This high proportion of variability within a population is commonly found in outcrossing species (Hamrick 1990). Fertilization treatments had a significant effect at both sites (Table 4), but, surprisingly, this effect was stronger on populations from Rosière (approx. 30% of total variation), although treatments were applied for only 11 years as compared to 38 years in Eggenalp. Snaydon and Davies (1982) showed that genetic differentiation may occur over periods as short as six years and can be caused by increased fertilization. Because population differentiation is known to be due to a combination of various ecologically important factors (Ernst 1987), and fertilization regimes were comparable at both sites, unidentified factors, such as the degree of initial genetic variability, microhabitat variation, and competition, were probably partially involved in differentiation. Differences in gene flow can also be excluded to a great extent since the size of the experiment, the spacing between treatments, the surrounding vegetation and the mating possibilities were comparable at both sites.

Fertilized populations under different cutting regimes in Rosière, were genetically more similar than were unfertilized populations (Fig. 2). Thus, the impact of cutting regimes was stronger on unfertilized plots. This was also reflected in AMOVA where cutting accounted for 37% of the total variation in unfertilized, but for only 16% of the total variation in fertilized populations (Table 4). In a competition experiment with orchardgrass (Dactylis glomerata L.), a companion species in natural grassland, Gugler (1993) showed that meadow fescue requires high levels of fertilization for good performance. This high nitrogen demand could result in higher selection pressure in the unfertilized populations than in the fertilized populations. Such a difference may be accentuated by cutting, which could explain the greater impact of cutting frequency on genetic variation in unfertilized populations.

Genetic differentiation, as a response to changes in management regimes may occur between populations as well as within populations (Snaydon 1987). In this study it was shown that fertilization and more frequent cutting reduced mostly the variability within natural populations (Table 2). Although there is some evidence of population differentiation as a response to fertilization and defoliation
based on morphological investigations, there is little information about the influence of these factors on variability within populations (Snaydon 1987).

In general, variability within cultivars was lower than variability within natural populations (Table 2). Results of AMOVA showed significant variance components, based on the origins as group parameters (data not shown). This lower variability of cultivars contradicts the findings of Huff (1997) who showed similar levels of variability within ecotypes and cultivars of *L. perenne*. In addition to various factors, such as breeding history, that influence variability, the variability within ecotypes of *L. perenne* may have been underestimated due to the small sample size (10 individuals per population) (Marshall and Brown 1975).

Comparing mean values of agronomically important traits, PRE differed significantly from the investigated natural populations of Eggenalp, whereas four traits of the latter were found to be similar (Table 5). This is in agreement with the RAPD results that showed close grouping of Eggenalp populations and clear separation of PRE (Fig. 2). Plants from the unfertilized population performed better than did plants from the fertilized populations, as was also observed by Helgadottir and Snaydon (1986). The significantly lower shoot/root ratio of the unfertilized plants (Table 5) may be an adaptive response to limited nitrogen availability since the shoot/root ratio affects a plant's ability to capture resources (Berendse and Elberse 1990). Population responses to fertilization were reported for root and shoot systems as evidence for morphological plasticity (Snaydon 1987). Our results clearly demonstrate that such responses have a genetic background, because both populations were grown under identical conditions. To ensure that the differences in performance between the natural populations were not influenced by fungal endophytes (Schmidt 1993), we examined all plants according to the method described by Latch and Christensen (1982); all the genotypes were infected.

Most of the variation observed in agronomic traits was due to differences between genotypes within populations rather than to differences between populations (Table 6), which is in agreement with results of the RAPD analysis. The ranking of the populations according to their coefficient of genotypic variation depended on the trait investigated (Table 5). Homogeneity of variances was tested by Bartlett's test (Sokal and Rohlf 1995) and significant heteroscedasticity was
found only for shoot dry weight ($P < 0.05$) and for dry weight per leaf ($P < 0.001$). Helgadottir and Snaydon (1986) showed that variability not only varied depending on traits but also on the site and the year of the investigation. In our experiment, variability of agronomic traits was greater within the E2N than within the E20 population, which is in contradiction to the lower RAPD variability of the fertilized populations (Table 2). The clear separation between the cultivar and the natural populations determined by principal coordinate analysis of RAPD markers (Fig. 3 A) could not be achieved by factor analysis based on seven key agronomic traits, even though the same genotypes were investigated (Fig. 3 B). Low correlation between patterns of variability of morphological traits and DNA markers was also reported by Beer et al. (1993) for *Avena sterilis*; they found that agronomic traits may be strongly influenced by environmental conditions and recommended a combination of DNA and morphological markers for estimating genetic diversity. Because our experiment was carried out under controlled conditions, environmental effects were uniform for all plants. The main reason for the differences in patterns of variability of RAPD markers and agronomic traits is probably that different parts of the genome were investigated with each method: RAPD markers enable the detection of small changes in DNA, such as nucleotide sequence changes at a primer binding site (Timmerman and McCallum 1993), while agronomic traits are usually quantitative and are governed by a larger number of genetic loci.

In this investigation we sampled plants from replicated experiments at the same time to ensure that conditions were similar for all populations under investigation. No information about the genetic diversity of the initial populations is available; thus, genetic changes over time can not be assessed. However, it is not feasible to compare our experimental plants with those of unmanaged control populations; at this elevation, unmanaged grasslands would be quickly overcome by shrubs and trees, with a dramatic effect on the target species. Long-term experiments in such grasslands provide insight into processes which drive micro-evolution, but they illustrate also the difficulty of establishing true experimental controls.
In conclusion, there is significant genetic variability within and among natural populations and cultivars of meadow fescue. Fertilization and frequent cutting decreased the genetic variability within populations. Therefore it is desirable to conserve, \textit{in situ}, unfertilized and rarely cut populations of meadow fescue as a gene pool. To the best of our knowledge, this is the first report about the effect of intensive management on genetic diversity within populations determined by using RAPD markers and agronomic traits. Further investigations of other species are necessary to learn more about the way in which management systems influence the diversity of plant communities and the genetic architecture of species.

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1  ABSTRACT

Three widely used cultivars of each of the species Festuca pratensis Huds., Lolium perenne L., and Dactylis glomerata L. were investigated by means of randomly amplified polymorphic DNA (RAPD) markers and vegetative growth traits in order to investigate genetic variability within each cultivar and to compare the level of diversity among cultivars and species. RAPD markers allowed a clear separation of the three species. Genetic variability based on RAPD markers was considerably lower for F. pratensis cultivars than for L. perenne and D. glomerata cultivars which showed similar levels of variability. The proportion of variability due to variation within cultivars, determined by an analysis of molecular variance, was lower in F. pratensis (64.6%) than in L. perenne (82.4%) and D. glomerata (85.1%). A comparison of F. pratensis and L. perenne, based on vegetative growth traits, confirmed the differences in genetic variability within cultivars. F. pratensis showed lower coefficients of genetic variation for eight of ten traits when compared to L. perenne. This study demonstrates considerable differences in genetic variability which may have consequences for the adaptability and persistency of individual cultivars.
INTRODUCTION

The pattern of genetic variability within the available germplasm substantially influences the choice of breeding material and with it the success of a plant breeding program. Variability among cultivars is required for a successful forage crop species in order to provide farmers with suitable cultivars for different environments and utilization systems. However, for some purposes such as the renovation or the overseeding of degenerated permanent pastures and meadows, single cultivars that are adapted to a broad range of environments may be more desirable: permanent grasslands are specially important in the uplands of central Europe, which often represent marginal habitats where environmental conditions vary greatly over time and space.

Within a cultivar, phenotypic plasticity may enable plants to adapt rapidly to a range of environments (Bradshaw 1965). Phenotypic plasticity has been reported for many traits and species (MacDonald and Chinnappa 1989, Brock et al. 1996, Petit et al. 1996), but the genetic basis of plasticity is very complex (Scheiner 1993). Since genetic variability is crucial for adaptation (Silvertown and Lovett Doust 1997), genetic variability within cultivars may be particularly important for long-term adaptability. There is only little information on the significance of variability within populations and these results are derived from studies with wild species (Dolan 1994; Templeton 1994). However, it was shown that genetic diversity can increase disease resistance in barley (Wolfe and McDermott 1994). It was also suggested, that increasing the heterogeneity may enhance the adaptability of forage grass cultivars (Hayward 1997). Molecular markers such as randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990) allow an easy and rapid approach to genetic variability and have been used in various plants species (Schierenbeck et al. 1997). Although some of these studies focused on forage grasses (Charmet and Balfourier 1994; Gunter et al. 1996), information on genetic variability within cultivars is available for only a few forage grass species (Loos 1994; Xu et al. 1994; Huff 1997). Therefore, our objective was to examine the genetic variability within cultivars of three important forage grass species in order to provide data which may help to
better understand the genetic architecture of species and cultivars and which is important for plant breeding and for further investigations on the significance of genetic variability.

Festuca pratensis Huds. (meadow fescue) is a forage grass of high quality and yield potential, comparable in many respects to perennial ryegrass (Lolium perenne L.). Due to its winter-hardiness, it has a competitive advantage as a hay or as a silage crop in cooler regions (Aastveit and Aastveit 1989). It is also a significant component of species-rich permanent pastures and hay fields in alpine regions and in eastern Europe. However, meadow fescue is only rarely found in intensively managed grasslands and shows low persistency when sown in mixture with other forage species. L. perenne is a highly productive species with a very good nutritive value and a high palatibility. It is one of the most important forage grasses of temperate regions, but its distribution in cooler regions and at higher altitudes is limited by a low tolerance to unfavourable climatic conditions and a high susceptibility to pink snow mould (Fusarium nivale (Fr.) Ces). Dactylis glomerata L. (orchardgrass) is a widespread species of good forage quality, well adapted to moderate fertility and low soil moisture. It is a companion species and strong competitor of F. pratensis in species-rich grasslands (Gügler 1993).

We used RAPD markers as well as vegetative growth traits to assess genetic variability within three cultivars of each of the three species.

3 MATERIAL AND METHODS

3.1 Plant material
Three cultivars of each of the species Festuca pratensis Huds., Lolium perenne L., and Dactylis glomerata L., currently recommended for cultivation in Switzerland (Lehmann et al. 1996), were used (Table 7). Care was taken to avoid tetraploid L. perenne cultivars and to select cultivars as distantly related as possible. Plants were grown from basic seed on autoclaved silica sand (particle size 0.8 - 1.2mm). Two weeks after sowing, 28 single plants were randomly selected, transferred to
hydroponics and kept in the growth chamber as individual plants; they were considered to be genotypes.

Table 7 Breeding history of nine cultivars of *Festuca pratensis*, *Lolium perenne* and *Dactylis glomerata* used for investigation

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Origin and number (in parentheses) of parental clones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Festuca pratensis</strong></td>
<td></td>
</tr>
<tr>
<td>Darimo&lt;sup&gt;b&lt;/sup&gt;</td>
<td>German cultivar NFG (4) and Dutch cultivar Belimo (1)</td>
</tr>
<tr>
<td>Fure&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Norwegian ecotypes and older cultivars (84)</td>
</tr>
<tr>
<td>Préval&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Swiss cultivar Préfest (6), German cultivar Cosmos (1), and Swiss ecotypes (7)</td>
</tr>
<tr>
<td><strong>Lolium perenne</strong></td>
<td></td>
</tr>
<tr>
<td>Arione&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Swiss ecotypes (28; 26 of these clones originate from only 5 ecotypes)</td>
</tr>
<tr>
<td>Cavia&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Swiss ecotypes and older cultivars (total 12 clones)</td>
</tr>
<tr>
<td>Respect&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Cultivars Amigo (2), Melino (5), Talbot (2), and Dutch ecotypes (4)</td>
</tr>
<tr>
<td><strong>Dactylis glomerata</strong></td>
<td></td>
</tr>
<tr>
<td>Loke&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Swedish ecotypes</td>
</tr>
<tr>
<td>Prato&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Swiss variety Lara (11) and Dutch variety Baraula (2)</td>
</tr>
<tr>
<td>Reda&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Swiss ecotypes (9) and older cultivars (6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Investigated cultivars are diploid (2n = 2x = 14)
<sup>b</sup> Mommersteeg International, Vlijmen, The Netherlands
<sup>c</sup> Norwegian Crop Research Institute, Ås, Norway
<sup>d</sup> Swiss Federal Research Station for Plant Production, Changins, Switzerland
<sup>e</sup> Swiss Federal Research Station for Agroecology and Agriculture, Zurich, Switzerland
<sup>f</sup> Cebeco Zaden B.V., Vlijmen, The Netherlands
<sup>g</sup> Investigated cultivars are tetraploid (2n = 4x = 28)
<sup>h</sup> Svaldf Weibull AB, Svalov, Sweden

3.2 DNA extraction

Fresh leaf material of each genotype of *L. perenne* and *D. glomerata* cultivars was sampled seven weeks after sowing and stored at -80 °C. Genomic DNA was isolated by a modified hexadecyltrimethyl ammonium bromide (CTAB) extraction procedure (Doyle and Doyle 1990). Frozen leaf tissue (approx. 100 mg) was ground in liquid nitrogen, transferred to a sterile Eppendorf tube, and lyophilized overnight (Hetovac VR-1, Heto Lab Equipment A/S, Birkerød, Denmark). Samples were incubated with 1 mL of CTAB buffer (100 g L⁻¹ CTAB, 1 M Tris pH 7.5, 5 M...
NaCl, 0.5 M EDTA pH 8.0) for 90 min at 60 °C, treated with 10 μg mL⁻¹ ribonuclease A (Boehringer Mannheim, Germany) at 37 °C for 30 min, extracted for 5 min with 450 μL chloroform/isoamyl alcohol (24:1 v:v), and centrifuged for 10 min at 7000 rpm. The aqueous phase was transferred to a new tube, and the DNA was precipitated with cold isopropanol (900 μL). DNA pellets were recovered by centrifugation (15 min at 5000 rpm), washed in 75% ethanol / 10 mM ammonium acetate, dried under vacuum, and dissolved in 150 μL sterile H₂O. To remove compounds inhibiting PCR reactions, DNA of D. glomerata was cleaned using polyethylene glycol (13% w/v; PEG 8000). DNA concentration was estimated with a LS-2B filter fluorimeter (Perkin-Elmer Ltd., Buckinghamshire, England) as well as visually after electrophoresis in a 20 g L⁻¹ agarose gel at 125 V for 1 h in TAE buffer (400 mM Tris, 20 mM EDTA, 200 mM sodium acetate) and staining with ethidium bromide.

3.3 DNA amplification and separation
Reactions were performed in 20 μL volumes containing: 1 x reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), 100 μM dATP, dGTP, dTTP, dCTP, 0.35 μM primer, 0.6 units EUROBIOTAQ® DNA polymerase (Eurobio, Les Ulis Cedex B, France) and 10 ng genomic DNA. Amplification was carried out in a Hybaid OmniGene temperature cycling system (Control Module with MicroBlock; Hybaid Ltd., Middlesex, UK) and was initiated by denaturation for 1 min at 94 °C, followed by 35 cycles of 30 sec at 35 °C, 2 min at 72 °C and 5 sec at 94 °C. The amplification was completed after 10 min at 72 °C. Reaction products were separated by electrophoresis in a 20 g L⁻¹ agarose gel at 100 V for 2.48 h in TAE buffer, stained with ethidium bromide (0.5 μg mL⁻¹) and photographed under UV light with a Polaroid type 667 film (Polaroid Corp., Cambridge, MA, USA). Amplification products were considered RAPD markers and reproducibility was confirmed by running independent duplicate samples. To estimate the size of the RAPD markers, a 100 base pair (bp) marker was used as a standard.

For the selection of suitable primers, 140 decamer primers (Operon Technologies, Inc., Alameda, CA, USA) were initially screened using four unrelated ecotypes of F. pratensis, not associated with the three cultivars used in this study.
According to the quality of the banding patterns and the number of polymorphic markers detected between the ecotypes, 12 primers (B01, B08, B11, B12, B15, H02, H19, Q5, R3, R11, R19, V16) were selected to investigate the 28 genotypes of the nine populations. For *F. pratensis* cultivars, RAPD profiles of previous investigations (Kölliker et al. 1998) were specifically evaluated in this comparison with *L. perenne* and *D. glomerata*.

3.4 **RAPD marker analysis**

RAPD markers were scored for presence (1) or absence (0) and entered into a binary vector representing the RAPD phenotype of each individual genotype. Only polymorphic markers that were reproducible and could be scored unequivocally in all genotypes were included in the analysis. The pair-wise distances between genotypes were estimated using the Euclidean distance of Excoffier et al. (1992), defined for RAPD markers by Huff et al. (1993) as

$$
E_{ij} = \{\frac{1}{2n^2} - \frac{2n_{ij}}{2n}\}
$$

where \(n_{ij}\) is the number of bands shared by the two genotypes i and j and n the total number of polymorphic bands. Analysis of molecular variance (AMOVA) was performed using the WINAMOVA 1.55 program, kindly provided by L. Excoffier (http://anthropologie.unige.ch/~laurent/default.htm#Software programs). For principle coordinate analysis, the NTSYS-pc package (version 1.8) (Rohlf 1993) was used.

3.5 **Variation in vegetative growth traits**

Variation in vegetative growth traits was assessed in a growth chamber experiment using 28 genotypes of the *F. pratensis* cultivars Préval, Darimo, and Fure and the *L. perenne* cultivars Arion, Cavia, and Respect. The same genotypes as investigated by means of RAPD markers could be used for *L. perenne* but not for *F. pratensis*. Two clonal replicates per genotype were produced using two single tillers of similar weight. Replicated plants were cut to 5 cm tiller and root length, transferred into hydroponic containers (0.30 x 0.20 x 0.22 m; eight plants per container), arranged in two randomized blocks and grown in a complete nutrient
solution modified according to Hammer et al. (1978) containing 1 mol m\(^{-3}\) NO\(_3\)- and with a pH of 5.5. The medium was aerated continuously and replaced every seventh day. The pH was controlled daily (Sentron 1001 pH, Sentron Europe BV, Roden, The Netherlands) and adjusted if necessary (1M H\(_2\)SO\(_4\)). The plants were cultivated in two growth chambers (PGV36, Conviron Instruments, Winnipeg, MB, Canada) at temperatures of 13/18 °C (day/night), 80% relative humidity, and a photoperiod of 16 h. Light (photosynthetic photon flux density 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) was provided by cool-white fluorescent lamps (Sylvania, CW/VHO, 215 W) and incandescent bulbs (100 W) at a ratio of 5:1. Seven days after cloning, the tip of the youngest leaf of the oldest tiller was marked with nail polish. Subsequently, the growth of the following three leaves was recorded and average leaf elongation duration (number of days between the lamina and the ligule appearance of one leaf) and average phyllochron (number of days between lamina emergence of two successive leaves) were calculated. Twenty-eight days after propagation, the growth habit of the plants was determined by visual scoring of the angle formed by the imaginary line through the region of the greatest leaf density and the vertical (1 = erect; 9 = prostrate). Plants were then harvested and separated into roots, leaf laminae, and tillers. The first two undamaged leaves of the tiller on which leaf growth was recorded were separated and used for the determination of single leaf area and single leaf length. The area of the leaf laminae was measured using a photoelectric meter (Model LI-3000A; Li-Cor, Lincoln, NE, USA), and leaves and tillers were counted. All fractions were dried at 65°C for 48 h. The data were subjected to an analysis of variance using the GLM procedure of the SAS statistical package (Statistical Analyses System, Version 6.12, SAS Institute, Cary, NC, USA). Means of cultivars were compared by Duncan’s multiple range test (\(P < 0.05\)). Variance components were estimated using the VARCOMP procedure of the SAS package. Coefficients of genetic variation were calculated according to Helgadottir and Snaydon (1986) as

\[
CV_g = \frac{\sqrt{\sigma_g^2}}{\bar{x}} \times 100
\]

where \(\sigma_g^2\) is the genotypic component of variance and \(\bar{x}\) the population mean of the character measured. For factor analysis, traits were averaged by genotype,
and the values were transformed to standard deviates (Sokal and Rohlf 1995). Factor analysis was applied using the varimax rotation method suggested by Kaiser (1958). Rotated factor values of 0.50 or greater were considered to be important in interpreting factor associations (Backhaus et al. 1996).

4 RESULTS

4.1 Characteristics of RAPD markers

The 12 primers generated 104 reproducible bands which were polymorphic and could be scored unequivocally across all genotypes (Fig. 4). Fragment size ranged from 320 to 1500 bp. Each of the 252 genotypes was characterized through a unique RAPD phenotype. The percentage of polymorphic markers within each cultivar ranged from 22 to 56 (Table 8). There were four markers that occurred in all genotypes of *D. glomerata* but never in the other species. Such a fixed marker difference was also found for *F. pratensis* but not for *L. perenne*. While 41 markers were found in all three species, four were found only in *F. pratensis* and in *L. perenne* and 15 were only found in *D. glomerata*. Although marker frequency varied greatly across cultivars, no cultivar specific markers were identified.

![Fig. 4 RAPD patterns of F. pratensis, L. perenne, and D. glomerata cultivars generated by primer OPB-12. Numbers indicate individual plants (two independent replicates). Arrows point to scorable polymorphic markers. M = 100 bp molecular marker](image-url)
4.2 Genetic variation based on RAPD markers

The first three eigenvectors of Principle Coordinate Analysis (PCO) extracted 88% of the total RAPD variation observed in the whole data set. All genotypes were clearly separated into three groups according to species (Fig. 5). While *D. glomerata* genotypes were clearly separated from the other genotypes by eigenvector 1, the distance between *F. pratensis* and *L. perenne* was due mainly to eigenvector 2. PCO resulted in a clear grouping of cultivars within *F. pratensis*, but grouping was poor within *L. perenne* and was not found in *D. glomerata*. However, PCO per species greatly improved the separation of cultivars within all three species (data not shown).

![Principal coordinate analysis of 104 RAPD markers for three cultivars of *Festuca pratensis* (circles; black = Fure, grey = Préval, white = Darimo,), *Lolium perenne* (squares; black = Arion, grey = Cavia, white = Respect) and *Dactylis glomerata* (triangles; black = Prato, grey = Reda, white = Loke)](image)

**Fig. 5** Principal coordinate analysis of 104 RAPD markers for three cultivars of *Festuca pratensis* (circles; black = Fure, grey = Préval, white = Darimo,), *Lolium perenne* (squares; black = Arion, grey = Cavia, white = Respect) and *Dactylis glomerata* (triangles; black = Prato, grey = Reda, white = Loke)

The three cultivars of *F. pratensis* showed the lowest variability within cultivars based on polymorphic markers and average Euclidean distance (Table 8), while Prato (*D. glomerata*) revealed the greatest diversity of all cultivars investigated. The genetic diversity within species was much lower for *F. pratensis* than for *L. perenne* and *D. glomerata*.
The variation observed in the whole data set was due mainly to variation among species (63%), while the variation among cultivars accounted for 8% and among genotypes for 29% (Table 9). Within species the sum of squares from analysis of molecular variance were lowest for *F. pratensis* (516.2) and highest for *D. glomerata* (843.1). Pair-wise comparison of variance heterogeneity within species (Bartlett's heteroscedasticity index) was significant only for the pairs *F. pratensis* / *D. glomerata* (*P* < 0.05) and *F. pratensis* / *L. perenne* (*P* < 0.1), but not for *D. glomerata* / *L. perenne* (*P* < 0.7). More than 80% of the variation within the species *L. perenne* and *D. glomerata* was due to variation between genotypes within cultivars (Table 9). This proportion dropped to 65% in *F. pratensis*, where 35% was due to variation between cultivars.

**Table 8** Genetic diversity within cultivars and species of *Festuca pratensis*, *Lolium perenne*, and *Dactylis glomerata*. Twenty-eight individual plants of three cultivars of each species were investigated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage of polymorphic markers</th>
<th>Euclidean distance (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Festuca pratensis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darimo</td>
<td>25</td>
<td>7.4</td>
</tr>
<tr>
<td>Fure</td>
<td>22</td>
<td>8.1</td>
</tr>
<tr>
<td>Préval</td>
<td>34</td>
<td>11.7</td>
</tr>
<tr>
<td>Within species</td>
<td>45</td>
<td>12.4</td>
</tr>
<tr>
<td><strong>Lolium perenne</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arion</td>
<td>52</td>
<td>17.1</td>
</tr>
<tr>
<td>Cavia</td>
<td>50</td>
<td>15.6</td>
</tr>
<tr>
<td>Respect</td>
<td>46</td>
<td>15.5</td>
</tr>
<tr>
<td>Within species</td>
<td>64</td>
<td>18.4</td>
</tr>
<tr>
<td><strong>Dactylis glomerata</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loke</td>
<td>49</td>
<td>17.1</td>
</tr>
<tr>
<td>Prato</td>
<td>56</td>
<td>19.4</td>
</tr>
<tr>
<td>Reda</td>
<td>50</td>
<td>18.0</td>
</tr>
<tr>
<td>Within species</td>
<td>64</td>
<td>20.3</td>
</tr>
</tbody>
</table>

a Total of markers scored: 104
b Average distances for pair-wise comparisons of genotypes
4.3 Variation in vegetative growth traits

Mean values of vegetative growth traits showed significant differences between cultivars and species. The number of tillers and the leaf area per plant were significantly lower for *F. pratensis* cultivars than for *L. perenne* cultivars with the exception of the low leaf area of Respect (Table 10). However, *F. pratensis* cultivars had significantly longer leaves and a more erect growth habit than cultivars of *L. perenne* (Table 10). Differences between the two species, found by comparing average values over all three cultivars were significant ($P < 0.05$) for all parameters except for specific leaf area.

Table 9 Analysis of molecular variance (AMOVA) for *Festuca pratensis*, *Lolium perenne*, and *Dactylis glomerata*, using 104 RAPD markers on three cultivars per species and 28 genotypes per cultivar

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Variance component*</th>
<th>% of total variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>2</td>
<td>2711.6</td>
<td>15.4</td>
<td>63</td>
</tr>
<tr>
<td>Cultivar within species</td>
<td>6</td>
<td>368.0</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td>Genotype within cultivar</td>
<td>243</td>
<td>1754.9</td>
<td>7.2</td>
<td>29</td>
</tr>
</tbody>
</table>

Within *Festuca pratensis*

| Cultivar | 2  | 148.3          | 2.5                 | 35                 |
| Genotype within cultivar | 81  | 367.9          | 4.5                 | 65                 |

Within *Lolium perenne*

| Cultivar | 2  | 112.5          | 1.7                 | 18                 |
| Genotype within cultivar | 81  | 651.1          | 8.0                 | 82                 |

Within *Dactylis glomerata*

| Cultivar | 2  | 107.3          | 1.6                 | 15                 |
| Genotype within cultivar | 81  | 735.8          | 9.1                 | 85                 |

* All components were significant at $P < 0.001$, giving the probability of obtaining a more extreme random value computed from nonparametric procedures (1,000 data permutations)
Table 10 Mean values ($\bar{x}$) and coefficient of genotypic variation (CV$_g$) of five vegetative growth traits of *Festuca pratensis* and *Lolium perenne* cultivars

<table>
<thead>
<tr>
<th>Trait</th>
<th>Festuca pratensis</th>
<th>Lolium perenne</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Darimo</td>
<td>Fure</td>
</tr>
<tr>
<td>Tillers plant$^{-1}$ [no.]</td>
<td>$\bar{x}$</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>CV$_g$</td>
<td>14.1</td>
</tr>
<tr>
<td>Leaf area plant$^{-1}$ [cm$^2$]</td>
<td>$\bar{x}$</td>
<td>214.6 a,b</td>
</tr>
<tr>
<td></td>
<td>CV$_g$</td>
<td>5.9</td>
</tr>
<tr>
<td>Single leaf length$^b$ [cm]</td>
<td>$\bar{x}$</td>
<td>21.9 d</td>
</tr>
<tr>
<td></td>
<td>CV$_g$</td>
<td>7.5</td>
</tr>
<tr>
<td>Specific leaf area [cm$^2$ g$^{-1}$]</td>
<td>$\bar{x}$</td>
<td>238.0 a</td>
</tr>
<tr>
<td></td>
<td>CV$_g$</td>
<td>4.8</td>
</tr>
<tr>
<td>Growth habit$^c$ [no.]</td>
<td>$\bar{x}$</td>
<td>3.4 b</td>
</tr>
<tr>
<td></td>
<td>CV$_g$</td>
<td>26.8</td>
</tr>
</tbody>
</table>

* *, **, *** Significance of the mean square associated with the variance component at $P < 0.05$, 0.01 and 0.001

a Means within rows followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test

b Average of two fully developed leaves

c Visual scoring of the angle between the imaginary line through the region of the greatest leaf density and the vertical (1 = erect; 9 = prostrate)
Table 11 Varimax rotated scores for four factors of ten traits of Festuca pratensis and Lolium perenne from three cultivars per species and 28 genotypes per cultivar

<table>
<thead>
<tr>
<th>Traits</th>
<th>Factors 1</th>
<th>Factors 2</th>
<th>Factors 3</th>
<th>Factors 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area plant(^1)</td>
<td>0.97</td>
<td>0.04</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>Shoot dry weight plant(^1)</td>
<td>0.94</td>
<td>0.13</td>
<td>0.06</td>
<td>-0.21</td>
</tr>
<tr>
<td>Tillers plant(^1)</td>
<td>0.71</td>
<td>-0.28</td>
<td>-0.48</td>
<td>0.00</td>
</tr>
<tr>
<td>Leaf elongation duration</td>
<td>0.15</td>
<td>0.83</td>
<td>0.22</td>
<td>-0.10</td>
</tr>
<tr>
<td>Phyllochron</td>
<td>-0.30</td>
<td>0.71</td>
<td>0.24</td>
<td>0.04</td>
</tr>
<tr>
<td>Single leaf area(^a)</td>
<td>0.20</td>
<td>0.50</td>
<td>0.75</td>
<td>0.02</td>
</tr>
<tr>
<td>Single leaf length(^a)</td>
<td>0.34</td>
<td>0.59</td>
<td>0.61</td>
<td>-0.05</td>
</tr>
<tr>
<td>Growth habit(^b)</td>
<td>0.19</td>
<td>-0.14</td>
<td>-0.80</td>
<td>0.15</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>-0.03</td>
<td>-0.31</td>
<td>0.08</td>
<td>0.88</td>
</tr>
<tr>
<td>Shoot / root ratio</td>
<td>0.10</td>
<td>-0.34</td>
<td>0.32</td>
<td>-0.75</td>
</tr>
</tbody>
</table>

Variance explained by each factor 26.7% 21.3% 20.4% 14.2%

\(^a\) Average of two fully developed leaves
\(^b\) Visual scoring of the angle between the imaginary line through the region of the greatest leaf density and the vertical (1 = erect; 9 = prostrate)

Factor analysis explained 82.6% of the total variability (Table 11). Factor 1 included total leaf area, shoot dry weight, and number of tillers, factor 2 comprised leaf elongation duration and phyllochron, and factor 3 contained growth habit. Single leaf parameters (single leaf area and length) were almost equally distributed between factors 2 and 3 (Table 11). While factor 1 did not separate the two species, factors 2 and 3 (single leaf parameters and growth habit) tend to separate Festuca pratensis plants from Lolium perenne plants (Fig. 6). However, factor analysis did not enable the separation of cultivars within species. This was also true for factor analysis within each species (data not shown).

Ranking of cultivars according to their genetic variability (expressed as coefficient of genotypic variation) depended greatly on the parameter investigated (Table 10). Except for Darimo, each cultivar showed the highest coefficient of genotypic variation for at least one parameter. Furthermore, Darimo showed very low values for most parameters investigated (Table 10). Average coefficients of variation were lower for Festuca pratensis than for Lolium perenne with the exception of specific leaf area and growth habit (Fig. 7).
Fig. 6 Factor analysis of ten vegetative growth traits for three cultivars of *Festuca pratensis* (black; circles = Préval, squares = Darimo, triangles = Fure) and *Lolium perenne* (grey; circles = Arion, squares = Respect, triangles = Cavia)

Fig. 7 Average coefficient of genotypic variation of ten vegetative growth traits for *Festuca pratensis* (white) and *Lolium perenne* (black) based on three cultivars per species and 28 genotypes per cultivar
5 DISCUSSION

Genetic variability within cultivars varied considerably depending on the species. This was true for the assessment of genetic variability by means of RAPD markers as well as by means of vegetative growth traits.

RAPD markers allowed a clear separation of the three species (Fig. 5). *D. glomerata* plants were separated from *L. perenne* and *F. pratensis* plants primarily through eigenvector 1 which explained 57% of the variation, while the *L. perenne* and *F. pratensis* plants were separated by eigenvector 2, explaining only 27% of the variation. The close affinity of *L. perenne* and *F. pratensis* has also been reported in previous studies based on morphological traits (Bulinska-Radomska and Lester 1988), isozymes (Charmet and Balfourier 1994), and molecular markers (Stammers et al. 1995). Variability within *L. perenne* and *D. glomerata* cultivars was similar, while variability in *F. pratensis* cultivars was substantially lower (Table 8). The genetic variability found within *L. perenne* and *F. pratensis* cultivars is comparable to the findings of Huff (1997) and Kölliker et al. (1998); a comparable study for *D. glomerata* is not available. Genetic variability within natural populations of meadow fescue was found to be higher than that within cultivars (Kölliker et al. 1998), but is lower when compared to the variability within *L. perenne* and *D. glomerata* cultivars (Table 8).

Variability within species is due not only to variability within, but also between cultivars; thus, we used AMOVA to partition the sources of variation. The low genetic variability within *F. pratensis* was confirmed by AMOVA results per species (Table 9). While the total sum of squares was lowest for *F. pratensis*, the amount of variation due to cultivars was substantially higher when compared to *D. glomerata* and *L. perenne* (Table 9). The greater variation between cultivars could be explained in part by more diverse sources of parental genotypes (Table 7) or different breeding objectives for single cultivars. The genetic variability within individual cultivars may be influenced by the number of parental clones involved in breeding. This is a possible explanation for the low variability within Darimo which is based on only five parental clones. However, the other cultivars are all based on a comparable number of clones except for Fure which showed very low variability.
despite the 84 clones upon which it is based. Intense selection may also limit genetic variability within cultivars (Huff 1997). It is difficult to estimate selection intensity based on information about the cultivars used in this study. However, it can be assumed that breeding methods and breeding intensity are comparable for all three species. Genetic variability is also influenced by the breeding system (Charlesworth and Charlesworth 1995), and isozyme variation in the genus *Lolium* is known to decrease with a reduced level of cross-fertilization (Charmet and Balfourier 1994). All three species investigated are true out-breeders with a high degree of self-incompatibility. Troll (1931) reported a lower self-fertilization rate for *D. glomerata* than for *F. pratensis* and *L. perenne*, but this may be due to the higher level of ploidy (Lundqvist 1969). Moreover, genetic variability was lower in *F. pratensis*, although its self-fertilization rate is lower than that of *L. perenne* (Charmet and Balfourier 1994). Genetic variability is usually higher in polyploid species (Xu and Sleper 1991; Soltis and Soltis 1993). This may play an important role in the high variability within *D. glomerata* (2n = 4x = 28) as compared to *F. pratensis* and *L. perenne* (2n = 2x = 14), although the difference between *D. glomerata* and *L. perenne* was small (Table 8). We selected the two diploid and closely related species *F. pratensis* and *L. perenne* to investigate genetic variability based on vegetative growth traits.

*F. pratensis* cultivars differed significantly from *L. perenne* cultivars for most vegetative growth traits investigated, while differences among cultivars of the same species were smaller. This is in good agreement with the RAPD results that showed clear differences between these two species but a less distinct separation of the cultivars within the species (Fig. 5). *L. perenne* plants had more tillers and a more prostrate growth habit than *F. pratensis* plants (Table 10). This is an expression of the ability of *L. perenne* to withstand continuous and intensive grazing (Jung et al. 1996). The species-specific differences were also made evident by factor analysis (Fig. 6); plants were mainly separated according to growth habit (factor 3) and single leaf parameters (factor 2) (Table 11). Although the separation was not as clear as with RAPD analysis, plants were grouped according to the respective species.
The low genetic variability within *F. pratensis* cultivars detected with RAPD markers was also reflected in morphological traits, although the ranking of the cultivars according to their coefficient of genotypic variation depended on the trait investigated (Table 10). This was also observed by Helgadottir and Snaydon (1986) who showed that variability depended not only on traits, but also on the site and the year of investigation. Different authors have reported a lack of congruence between variability estimates based on DNA markers, isozymes, and morphological traits (Beer et al. 1993; Fernando et al. 1997). Estimates of genetic variability based on morphological traits may be biased by phenotypic plasticity which can evolve independent from genetic variability (MacDonald and Chinnappa 1989). In our investigation, average variability within species was lower for *F. pratensis* for most vegetative growth traits (Fig. 7), which is in good agreement with the RAPD data. Analysis of variance showed that the variation in growth habit was mainly due to variation between species (56%) and to variation between genotypes within cultivars (14%) rather than to variation between cultivars within species (4%) (data not shown). Therefore, the high variability in growth habit is unlikely to be a result of different breeding objectives for the individual cultivars (e.g. grazing types versus cutting), which would result in large differences between cultivars. Large variability in growth habit may be an advantage in competition for light because it ensures optimal positioning of leaf area in a multi-species sward. However, even if a high variability in some traits might be advantageous, the large differences in mean values between *F. pratensis* and *L. perenne* certainly remain responsible for the different adaptation of the two species.

To the best of our knowledge this is the first report to compare the genetic variability of widely used cultivars of three important forage grasses based on genetic markers and morphological traits. The genetic variability detected with RAPD markers was considerably lower for the three cultivars of *F. pratensis* when compared to the same number of cultivars of *L. perenne* and *D. glomerata*. Analysis of key vegetative growth traits confirmed the lower variability of *F. pratensis* as compared to *L. perenne*. Although plant adaptation is induced by environmental factors, it depends on genetic resources for a specific response (Bradshaw 1984). Therefore, limited genetic variability could be one factor.
contributing to the decline of meadow fescue from intensively managed grassland. Further investigations are now needed to elucidate the significance of genetic variability for the adaptability and persistence of cultivars and species.

Submitted to Euphytica
VI GENERAL DISCUSSION

1 GENETIC VARIABILITY WITHIN GRASSLAND SPECIES – RESULTS AND INSIGHTS USING *FESTUCA PRATENSIS* HUDS. AS A MODEL PLANT

Biodiversity (i.e. functional diversity and species diversity) can have a significant influence on the functioning and stability of grassland ecosystems (Tilman and Downing 1994; Hooper and Vitousek 1997; Tilman et al. 1997). Various factors that influence the species composition in a sward have already been identified (Crawley 1997). However, at a lower taxonomic level (i.e. within species), there is virtually no information on the extent of diversity in grassland and the factors influencing it. This study was conducted to investigate genetic variability within *Festuca pratensis* Huds., a key forage grass, and to provide valuable information for understanding ecological processes within grasslands.

1.1 Intensive management reduces genetic variability within natural populations of *F. pratensis*

All six natural populations showed significant genetic variability (Table 2), roughly comparable to the variability found in other grass species (Gunter et al. 1996; Huff 1997). The major part of the total genetic variation was due to variation among genotypes within populations; only 9% of the variation was between the two sites (Table 4). On the one hand, a high variability within populations is typical of outbreeding species (Hamrick 1990). On the other, low variability between sites is a clear indication that the initial populations and the environmental conditions at both sites were comparable. This was confirmed by cluster analysis where the natural populations formed a distinct cluster (Fig. 2). However, average genetic variability was lower at the site Rosière than at the site Eggenalp (Table 2). The number of genotypes in a sward, and with it the extent of variability within a population, may decline over time (McNeilly and Roose 1984). The exact date of
establishment is not known for the swards in Rosière and Eggenalp. Both experiments were established in permanent grassland and it is highly likely that both swards have not been renewed for at least 50 years. It is, therefore, probable that other factors, such as the degree of initial genetic variability or microhabitat variation and competition, were responsible for the differences between the two sites.

1.1.1 Effect of nitrogen fertilization

Fertilization, together with defoliation, is the most important interference in managed permanent grasslands. Agricultural intensification of such grasslands always involves increased fertilization. Field experiments such as the Park Grass plots at Rothamsted have shown that increased applications of fertilizer lead not only to yield increase, but also to a lower number of species in the sward (Johnston 1997). Nitrogen (N) in particular caused a reduction of species diversity in a number of grassland ecosystems (Kalmbacher and Martin 1996).

N fertilization can also influence genetic variability within individual species and populations (Table 2). Natural populations of *F. pratensis* differed genetically after 11 to 38 years of different fertilization treatments (Fig. 2). Moreover, the genetic variability within two of the three fertilized populations was clearly reduced when compared to the unfertilized populations (Table 2). Only the fertilized population R2N showed greater variability than the unfertilized population R20, but the difference was small (Table 2). At this experimental site, treatments have been applied for 11 years, a relatively short period of time for evolutionary changes; although genetic changes, as a response to N fertilization, were found after 10 years (Snaydon and Davies 1982), shorter experiments do not lead to significant changes (Harris and Lazenby 1977). However, fertilized and unfertilized populations that were cut more frequently at the same site showed marked differences in variability (Table 2). This indicates that differences can also occur after only 11 years if the effect of N is accentuated by other factors (e.g. frequent cutting).

Fertilization treatments also lead to differences in a number of agronomic traits (Table 5). Some of these differences may be regarded as adaptive responses
to N availability in the experimental plots [soil analyses are not available but unfertilized plots had significantly more species that indicate poor nutrient availability (Baumberger et al. 1996)]. Plants may adapt to nutrient-poor environments by maximizing the assimilation of nutrients (Tilman 1988) or by minimizing losses (Berendse and Elberse 1990). Plants grown under low N fertilization have a lower shoot/root ratio than plants grown under high N fertilization (Levin et al. 1989). The significantly decreased shoot/root ratio of plants from unfertilized plots, when grown under the same conditions as plants from fertilized plots (Table 5), is therefore an expression for evolutionary adaptation. In addition, shoot dry weight of unfertilized plants was higher than that of fertilized plants (Table 5), although the difference was not significant. This may indicate that these plants had a better N use efficiency than plants from fertilized plots. The following experiment was carried out to test this hypothesis.

1.1.1.1 Do plants from fertilized and unfertilized populations respond differently to nitrogen treatments?

In a growth chamber experiment, 20 genotypes of each of the populations from Eggenalp (fertilized and unfertilized; Table 1) were grown under two N treatments (low N = 0.2 mM NO$_3$; high N = 1 mM NO$_3$). Each N treatment consisted of three clonal replicates per genotype. Plants were arranged in a randomized complete block design and grown in hydroponics as described on pp. 42-43. Forty-nine days after cloning, plants were harvested and separated into roots, leaf laminae, and tillers. The area of the leaf laminae was measured using a photoelectric meter (Model LI-3000A; Li-Cor, Lincoln, NE, USA), and leaves and tillers were counted. All fractions were dried at 65°C for 48 h. The data were subjected to an analysis of variance using the GLM procedure of the SAS statistical package (Statistical Analyses System, Version 6.12, SAS Institute, Cary, NC, USA).

Nitrogen had a significant effect on shoot dry weight, number of tillers, and specific leaf area but not on leaf dry weight and shoot/root ratio. The latter is in contrast to the current opinion that increased nitrogen supply increases the shoot/root ratio. The differences between the two N treatments may have been too small to cause differences in the shoot/root ratio. The effect of genotypes
within populations was significant for all traits (Table 12) which is a clear indication of significant variation within populations.

There was no significant interaction between populations and nitrogen treatments for any of the parameters investigated (Table 12). Moreover, no significant genotype (population) x nitrogen interaction was observed. Even when each population was analyzed separately, no interaction between genotypes and nitrogen treatments was detected (data not shown). Although genotypic differences in nutrient efficiency have been reported for cultivars of different species (Marschner 1995), investigations of interactions between grass genotypes and nitrogen fertilization have given contrasting results: while significant interactions were found for bermudagrass (Cynodon dactylon L.) genotypes (McCaslin et al. 1989), no interaction was observed for L. perenne cultivars (Wilkins 1989). The relatively low N levels (0, 96, 192, 288 kg ha\(^{-1}\)) used in the bermudagrass study, may have rather pronounced genotypic differences than did the higher levels (200, 400 kg ha\(^{-1}\)) used in the L. perenne study. In the growth chamber experiment presented in Table 12, plants did not show N deficiency symptoms, but dry matter yield was significantly lower for plants grown under low N (data not shown). Even if it can not be excluded that there might be a genotype x nitrogen interaction under more limiting conditions (e.g. no N fertilization), the experiment showed that there is no such interaction under little or extensive N fertilization.

1.1.2 Effect of defoliation frequency

Increased fertilization usually goes hand in hand with increased defoliation frequency which can lead to a reduction in genetic variability (Table 2). The impact of frequent defoliation on genetic variability of F. pratensis was more distinct in unfertilized than in fertilized populations (Table 2).
Table 12 Analysis of variance for agronomic key traits of two *Festuca pratensis* Huds. populations from fertilized and unfertilized grasslands when grown in hydroponics at two nitrogen levels (low N = 0.2 mM NO₃; high N = 1 mM NO₃). All values were transformed by natural logarithms before analysis.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Source of variation</th>
<th>df</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight plant⁻¹</td>
<td>nitrogen</td>
<td>1</td>
<td>1.369 ***</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>1</td>
<td>0.028 NS</td>
</tr>
<tr>
<td></td>
<td>nitrogen x population</td>
<td>1</td>
<td>0.035 NS</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>35</td>
<td>0.340 ****</td>
</tr>
<tr>
<td></td>
<td>nitrogen x genotype (population)</td>
<td>35</td>
<td>0.112 NS</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>124</td>
<td>0.118</td>
</tr>
<tr>
<td>Tillers plant⁻¹</td>
<td>nitrogen</td>
<td>1</td>
<td>2.000 ****</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>1</td>
<td>0.650 *</td>
</tr>
<tr>
<td></td>
<td>nitrogen x population</td>
<td>1</td>
<td>0.069 NS</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>35</td>
<td>0.338 ****</td>
</tr>
<tr>
<td></td>
<td>nitrogen x genotype (population)</td>
<td>35</td>
<td>0.092 NS</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>124</td>
<td>0.130</td>
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<tr>
<td>Dry weight leaf⁻¹</td>
<td>nitrogen</td>
<td>1</td>
<td>0.115 NS</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>1</td>
<td>1.229 ****</td>
</tr>
<tr>
<td></td>
<td>nitrogen x population</td>
<td>1</td>
<td>0.002 NS</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>35</td>
<td>0.238 ****</td>
</tr>
<tr>
<td></td>
<td>nitrogen x genotype (population)</td>
<td>35</td>
<td>0.051 NS</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>124</td>
<td>0.045</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>nitrogen</td>
<td>1</td>
<td>0.324 ***</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>1</td>
<td>0.054 NS</td>
</tr>
<tr>
<td></td>
<td>nitrogen x population</td>
<td>1</td>
<td>0.002 NS</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>35</td>
<td>0.044 **</td>
</tr>
<tr>
<td></td>
<td>nitrogen x genotype (population)</td>
<td>35</td>
<td>0.021 NS</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>124</td>
<td>0.024</td>
</tr>
<tr>
<td>Shoot / root ratio</td>
<td>nitrogen</td>
<td>1</td>
<td>0.000 NS</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>1</td>
<td>0.189 NS</td>
</tr>
<tr>
<td></td>
<td>nitrogen x population</td>
<td>1</td>
<td>0.010 NS</td>
</tr>
<tr>
<td></td>
<td>genotype (population)</td>
<td>35</td>
<td>0.267 ***</td>
</tr>
<tr>
<td></td>
<td>nitrogen x genotype (population)</td>
<td>35</td>
<td>0.069 NS</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>124</td>
<td>0.123</td>
</tr>
</tbody>
</table>

NS not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001
Since *F. pratensis* requires high levels of fertilization for good performance, a combination of frequent cutting and no fertilization may result in greater selection pressure than frequent cutting and sufficient nutrient supply.

Frequent defoliation often includes a first defoliation early in the season; this can inhibit generative reproduction and, thus, cause a reduction in genetic variability.

### 1.1.3 Other effects on genetic variability within *F. pratensis*

As well as the two factors discussed above, there are many others that can also influence genetic variability within species and populations. Some of these factors can be influenced easily by management, while others are more difficult to control (e.g. seed dispersal by animals) or can not be influenced at all (e.g. climatic conditions).

To test the influence of the mode of defoliation, genetic variability within a grazed and a cut population of *F. pratensis* was investigated using RAPD markers as described on pp. 20-22. Both populations were sampled from a long-term experiment near Yverdon (La Frétaz, 1200 m a.s.l.) (Charles and Troxler 1994), where treatments have been applied for more than 30 years. Average genetic variability within both populations, estimated by Euclidean distance (E) (Huff et al. 1993), was 15.5 which is comparable to the variability found in other natural populations of *F. pratensis* (Table 2). The variability within the grazed population (E = 15.8) was higher than within the cut population (E = 15.1). Although this difference is small, it indicates that the mode of defoliation may also influence genetic variability within populations. This is supported by the findings of McNeilly and Roose (1984) who report more genotypes of *L. perenne* in ten year old pastures when compared to cut swards of the same age.

Although gene flow between individuals is of great evolutionary importance, its influence on the results presented in this thesis is certainly small. On the one hand, gene flow in plant populations is often restricted (Jain and Bradshaw 1966), and most of the pollen or seeds do not travel far (Silvertown and Lovett Doust 1993). On the other hand, the size of the experiments, the spacing between
treatments, the surrounding vegetation, and mating possibilities were comparable at both experimental sites.

1.2 Genetic variability within _F. pratensis_ cultivars is low when compared to natural populations and to other species

Improving crops through breeding involves selection for the desired traits and has, therefore, the potential for reducing variability within cultivars. Various factors related to the breeding process, such as number, pedigree and heterozygosity of parental lines, and selection intensity, can essentially affect genetic variability within cultivars. On the other hand, cultivars may be composed of very diverse germplasm and, therefore, sometimes outrange natural populations in terms of genetic diversity (ElKassaby and Ritland 1996). This does not seem to be true for _F. pratensis_: two of the three cultivars showed lower genetic variability than the natural populations (Table 2). The fact that Préval showed greater variability than the frequently cut populations at Rosière indicates that a diverse cultivar can show greater variability than a natural population that has been subjected to intense selective forces. However, average variability within cultivars was lower than within natural populations at both sites (Table 2).

Genetic variability within _F. pratensis_ cultivars was also lower when compared to cultivars of _L. perenne_ and _D. glomerata_ (Table 8). This was true for the variation within individual cultivars as well as within species (Table 8). Among the various factors influencing genetic variability within cultivars, the reproduction system can, to a great extent, be excluded, since all three species are cross-pollinators with a low degree of self-incompatibility. A greater impact is expected from differences in the ploidy level: _F. pratensis_ and _L. perenne_ are diploid species (tetraploid cultivars are available but only diploids were used in this study), while _D. glomerata_ is a tetraploid species and, therefore, is expected to show greater variability (Soltis and Soltis 1993). However, the differences between _L. perenne_ and _D. glomerata_ were very small (Table 8), and other factors must be responsible for the low genetic variability within _F. pratensis_. It is difficult to assess the extent to which the initial germplasm and selection intensity during breeding influenced the genetic variability within the cultivars, but there are some indications that the
effect of the whole breeding process on genetic variability was stronger for *F. pratensis* than for *L. perenne* or *D. glomerata* cultivars. Genetic variability within *F. pratensis* cultivars was lower than within natural populations (Table 2) which in turn showed comparable genetic variability to that of *Panicum virgatum* L. (Gunter et al. 1996) and *L. perenne* (Huff 1997). Moreover, genetic variability within cultivars of *L. perenne* is comparable to that within natural populations (Huff 1997).

Limited genetic variability within cultivars may be disadvantageous. First, it may influence the performance of the cultivar and, second, it may bring about the loss of valuable germplasm when cultivars with low genetic variability are used for further breeding. To obtain conclusive evidence about the genetic variability of *F. pratensis* in current plant breeding programs, it is necessary to investigate a greater number of cultivars.

### 2 IS GENETIC VARIABILITY IMPORTANT FOR THE STABILITY OF PERMANENT GRASSLANDS?

Ecosystem stability, i.e. the constancy of community composition, may, among others, be due to (i) a constant external environment, (ii) a rapid return to the original community composition after perturbation, or (iii) the stability of the community composition despite a perturbation (Berendse 1993). Thus, if an ecosystem is disturbed, the response of the individual species becomes important for its stability.

#### 2.1 Studying genetic variability within grassland populations: principles and prospects

At least two requirements must be met for reliable estimates of genetic variability: a method for detecting polymorphism between individual plants and natural populations representative of the grassland type under study.
2.1.1 Detecting polymorphism at the DNA level

Randomly amplified polymorphic DNA (RAPD) markers allow the rapid detection of polymorphism within a large number of individuals. These markers are certainly the method of choice for studies, in which previous genetic information is not available and a first assessment of genetic variability is intended. They have been used successfully to detect variability within and between natural populations of *F. pratensis* (Table 2). Moreover, they proved useful in separating populations that were managed with different intensity (Fig. 2) and in assessing genetic variability of cultivars of different species (Table 8). Although RAPD markers are reproducible under strictly constant reaction conditions (from DNA extraction to PCR amplification), the method suffers from certain reproducibility problems (Jones et al. 1997), making results from different laboratories difficult to compare. To obtain reliable results, only RAPD markers that can be scored unambiguously over the whole data set should be included in the analysis. This explains the different levels of variability of the *F. pratensis* cultivars when evaluated together with natural populations (Table 2) or with cultivars of other species (Table 8). However, when scored carefully, RAPD markers allow reliable assessment of genetic variability relative to other populations.

Methods that are easier to reproduce in different laboratories would allow the comparison of genetic variability of a large number of populations from very different environments. One such method are simple sequence repeat polymorphisms (SSRP), which have, only recently, been used with plants. SSRP are based on a 1-6 nucleotide core element that is repeated in tandem in different numbers in different genotypes. Differences in length are detected by PCR using two primers that complement unique sequences flanking the SSR locus (Brown et al. 1996). SSR markers are abundant and highly informative, because they are co-dominant, and many alleles are found among closely related individuals. Detection and analysis is simple and it is reproduced easily in other laboratories (Jones et al. 1997). The major drawback of this method are the high costs of SSR marker development, involving the establishment of DNA libraries and sequencing of putative SSR clones. SSR markers for *L. perenne* are currently being developed at La Trobe University, Victoria, Australia (J. Forster pers. comm.). Due to the close
relationship of *F. pratensis* and *L. perenne*, the odds are that most of these markers also be effective for *F. pratensis*. These SSR markers would provide a powerful tool for the easy screening of a large number of populations and for the comparison of many contrasting environments. Moreover, SSR markers are being developed for many different species, and suitable systems may soon be available for other grassland species.

2.1.2 Morphological traits - a valuable complement

Although the analysis of agronomic traits was not always congruent with the RAPD analysis (Table 2, 5, Fig. 3), morphological traits are a valuable complement. They allow the detection of adaptive changes, as was shown for the decreased shoot/root ratio of plants from unfertilized plots (Table 5) and can be used to assess the variability of specific traits. Since variability based on morphological traits depends strongly on the respective trait (Table 5, 10), it is necessary to investigate a large number of different traits to obtain a reliable overall estimate of genetic variability. The analysis of phenological and reproductive traits (e.g. date of inflorescence emergence, number of reproductive tillers, seed yield) in a field experiment may, therefore, be a valuable complement to the morphological data presented in this thesis (Table 5, 10).

2.1.3 Long-term experiments: a rare opportunity for studying diversity

To obtain insight into the role of genetic variability in permanent grasslands, investigations have to be carried out within these ecosystems. Long-term experiments that have been established in permanent grasslands and have not been re-sown for several decades offer a unique opportunity for studying *in situ* genetic variability and the factors influencing it (pp. 17-36). Although such experiments are rare in permanent grassland of temperate humid regions, a number of experiments in the UK [the Park Grass experiment at Rothamsted (Johnston 1997), the Bratoft meadow in Lincolnshire (Silvertown et al. 1994), and the Little Wittenham natural reserve in Oxfordshire (Bullock et al. 1994)] would provide an excellent opportunity for studying diversity in different environments and under different management systems.
Genetic changes may be caused not only by experimental treatments, but may also occur over time due to unknown factors. To control such changes, it is necessary to have information about the genetic variability of the populations at the beginning of the different treatments. However, when these long-term experiments were established, molecular techniques were virtually unknown, and such data were not collected. In the future, it is desirable to collect information on genetic variability from experiments that are likely to be continued for the next decades (e.g. the Park Grass experiment).

2.2 Genetic variability and the performance of plant populations

*F. pratensis* plants from unfertilized experimental plots differed in a number of characters from fertilized plants when grown under similar conditions (Table 5). Thus, factors that influence genetic variability within populations can also effect plant performance. Analysis of floristic composition at the experimental site showed a clear decrease in *F. pratensis* in fertilized plots when compared to unfertilized plots (Baumberger et al. 1996). Thus, genetic variability may influence the performance of individual plants and entire populations. To obtain conclusive evidence about the significance of genetic variability, the following investigations are proposed.

Populations with different levels of genetic diversity must be grown in a range of environments to test the impact of genetic variability on population performance. Selected environments should include conditions that are stressful to the plants: e.g. lack of nutrients, drought, frequent defoliation, and intensive shading by other species. The success of such experiments will depend on the availability of suitable populations. One way to obtain such populations is to screen a large number of natural populations and to select those with the greatest differences in variability. For the purpose of comparison, such populations must be sampled from sites with comparable environmental conditions; the history of the populations must be known. Such natural populations are rare, and the design of artificial populations may be a better solution. A large number of individual plants (e.g. grown from seed of a cultivar) should be investigated at as many loci as possible by means of molecular markers. After pair-wise comparison of the
genotypes, two populations will be developed, one containing very similar genotypes (low genetic variability) and one containing genotypes that are very different (high genetic variability). These populations will be an excellent basis for further studies on the significance of genetic variability.

The success of a species in a sward largely depends on its ability to co-exist with its companion species and to compete successfully for resources (Silvertown and Lovett Doust 1993). A large variability in traits essential for successful competition could allow a species to co-exist with a large number of different species and to succeed in various contrasting environments. To test this hypothesis, traits important for competition must be identified. A number of such traits have been detected (Berendse and Elberse 1990), but their importance for each species remains to be evaluated. In _F. pratensis_ for example, the rate of leaf elongation, especially after defoliation, seems to be mainly responsible for the low shoot competitive ability of this species (Messerli 1997). In a second step, the variability of such traits must be investigated for a large number of plants. This may be achieved by growing plants under controlled conditions and assessing the variability of the respective traits directly. Such experiments usually require the transplantation of plants, are very time consuming, and allow only a limited number of individuals to be screened. Molecular markers for the specific traits represent a promising alternative: they can provide a very useful tool for the easy screening of large populations and require only a small amount of plant tissue. The development of suitable markers is costly and may be difficult due to the quantitative inheritance of many traits, but their potential use in a variety of ecological studies certainly justifies the effort. The use of existing molecular linkage maps can speed up marker identification and development, but such maps are available for only a few grassland species (e.g. _L. perenne_ and _Medicago sativa_ L.) (Hayward 1994). Once the variability of certain traits has been assessed in a number of plants, the significance of this variability may be investigated by growing plants in competition with other species and in contrasting environments.
2.3 From genetic variability to ecosystem stability

The question as to whether or not genetic variability within populations influences ecosystem stability can not be answered by investigating one species within one grassland ecosystem. Too many factors and processes are involved, and only a few are as yet understood. The complexity of the issue is reflected in the debate about the relationship of biodiversity and ecosystem stability (Grime 1997). Moreover, constancy of community composition is only one of several concepts related to ecosystem stability. An ecosystem with given structural and functional characteristics (e.g. the tropical rain forest or the tussock tundra) may survive for a long time, even when the composition of the community has changed completely (Berendse 1993). However, from an agricultural and ecological (at least as far as the conservation of species is concerned) point of view, stability in terms of constancy of community composition is important. This thesis shows that genetic variability within plant populations can be reduced by intensive management (Table 2). Studies involving different plant species and ecosystems are needed to elucidate the significance of genetic variability for ecosystem stability.

3 CONCLUSION

Intensive management (i.e. fertilization and frequent cutting) decrease the genetic variability within natural populations of *F. pratensis*. Therefore, it is important to conserve unfertilized and infrequently defoliated grasslands as gene pools (*in situ* conservation). In addition, genetic variability within *F. pratensis* cultivars was lower than that of natural populations and that of cultivars of other forage grasses. Limited genetic variability can influence the performance of the cultivar and may bring about the loss of valuable germplasm. It is suggested, therefore, that plant breeders closely observe genetic variability within *F. pratensis* cultivars and attempt to increase it by introducing more diverse germplasm into the breeding process. The results of this study suggest that the decline of *F. pratensis* in intensively managed grassland is partly due to limited genetic variability that may influence the stability of the entire sward.
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Still confused...
...but on a higher level!!!