The impact of differences in land use on the clonal structure and genotypic diversity of the salt marsh species *Elymus athericus*

- A study based on Microsatellite markers-

by Roos Veeneklaas

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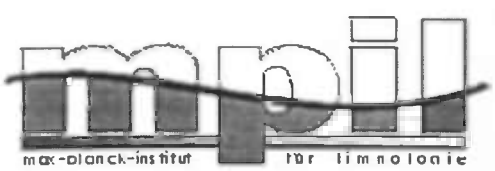

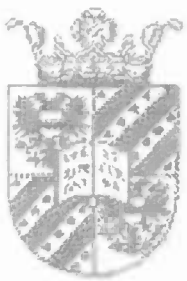

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Abstract

In recent years evidence has accumulated that certain plant species can rapidly adapt to drastic environmental changes (e.g. develop heavy metal tolerance). The ability for adaptation might enable some species to expand their distribution in a situation of rapid change, whereas evolutionary more conservative species might disappear. In 1972 different management forms (mowing, grazing) have been applied to certain abandoned areas of a salt marsh section on the Dutch Wadden Sea Island Schiermonnikoog. Mowing and grazing can be considered as drastic environmental change in the salt marsh ecosystem. We observed that *Elymus athericus*, an invasive clonal grass, exhibited large phenotypic differences between the different treatments. We hypothesised that different management regimes can change selection pressures on *Elymus athericus* when compared with the natural situation. As a consequence populations could have developed not only phenotypic differences between treatments but also differences in clonal and genetic structure. In this study we analysed clonal structure and genetic diversity between treatments with the help of five polymorphic Microsatellite markers.

Differences in genotypic diversity were found between the management regimes. In the dense vegetation of the unmanaged areas clonal propagation was favoured. The highest genotypic diversity was found in the mown plots, where seedlings have the highest likelihood to recruit.

Introduction

In recent years evidence has accumulated that certain plant species can rapidly adapt to drastic environmental changes, e.g. the evolution of the heavy-metal tolerant plant populations (Bradshaw 1991, Vekemans & Lefèbvre 1997). Studies on rapid adaptation have highlighted the existence of close associations between local pattern of environmental changes and population genetic differentiation, as a consequence of low plant mobility (Vekemans & Lefèbvre 1997). The ability for rapid adaptation might enable some species to expand their distribution in a situation of global change, whereas evolutionary more conservative species might disappear. Evolutionary models on bacteria as well as experimental results on plants have suggested that more rapid evolutionary rates would be found in stressful than in non-stressful environments (Lenski & Bennet 1993, Potvin & Tousignant 1996). Changes in environment could lead to high phenotypic plasticity or genetic adaptation to specific abiotic conditions in plant populations, being two alternative strategies. These adaptation processes can change the genetic structure through fitness correlated phenotypic traits (Bockelmann 2000). Phenotypic and genetic adaptability to new environmental conditions often seem to be involved in plant invasions (Crawley 1986, Dietz 1999), e.g. during establishment of a population in a new or heterogeneous environment.

After cessation of grazing in the salt marsh succession results in a dominance of a few species and hence a decrease in the number of plant communities and species (van Wijnen et al. 1997). In the salt marshes of the Wadden Sea area change of management regime have led to the spread of the native clonal grasses of the genus *Elymus*. *Elymus athericus* is extending in the nutrient rich upper salt marsh (Fig. 1), now frequently dominating large areas (van Wijnen et al. 1997). This species recently also invades lower parts of the salt marsh gradient (Bockelmann & Neuhaus 1999). Phenotypic and genetic adaptability could be involved in the rapid invasion of *Elymus athericus* on the salt marsh in the past three decades.

In order to detect differences in phenotypic or genotypic induced by environmental changes, long-term observation of clones under different conditions are needed. Alternatively, direct experimental manipulation of environmental conditions over a long time can be performed. Schläpfer & Fisher (1998) studied the clone structure of the grass *Brachypodium pinnatum* after 16 years of difference in management, and concluded that the clone diversity remained

unaffected by the treatments. They suggested that management-induced changes in clone structure can only be expected after much longer periods of time. In 1971 different management forms (mowing, grazing) have been applied to certain areas of the salt marsh on the Dutch Barrier Island Schiermonnikoog. The experimental set up on this island is ideal to study the impact of the management practices on phenotype, genotype and clonal structure of plant species. Different management regimes have been applied for a period of 30 years. The vegetation has been monitored throughout the whole period to assess the effect of these management regimes on the vegetation succession.

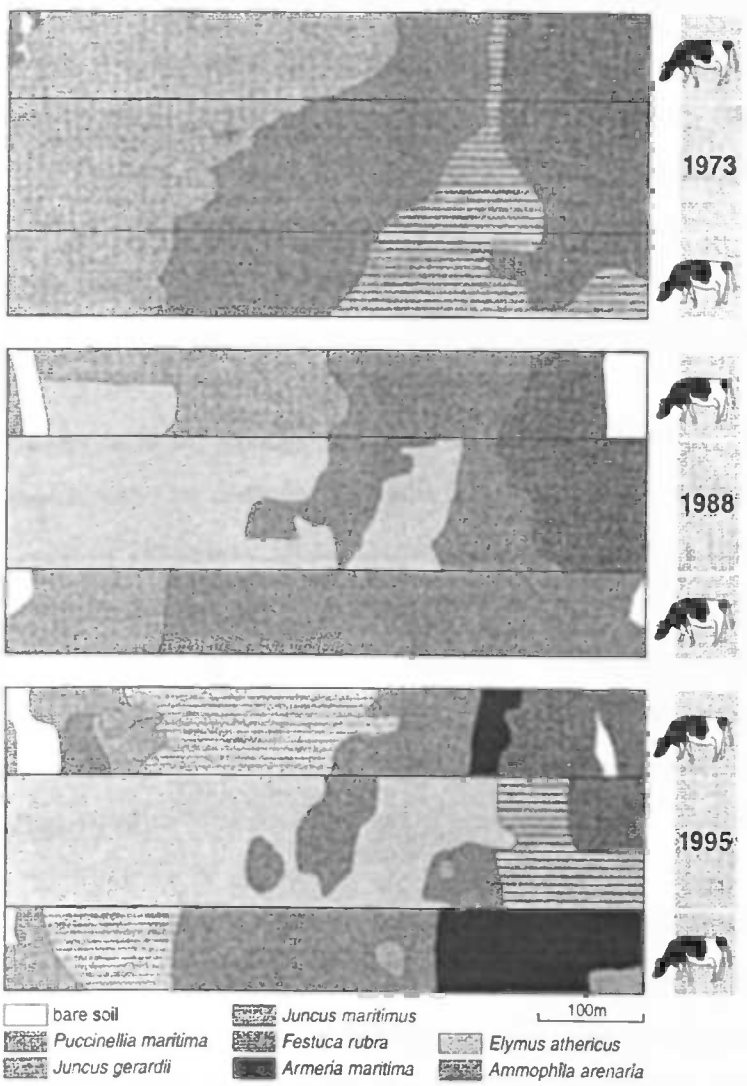


Figure 1. Vegetation development during 22 years in the higher parts of the salt marsh of Schiermonnikoog. The influence of cattle grazing can be judged from the exclosure in the centre of the study area established in 1973 (Van Wijnen *et al.* 1997.

Mowing and grazing can be considered as drastic environmental change in the salt marsh ecosystem. The selection present through the different management regimes applied could be strong enough to lead to phenotypic and genetic population differentiation between treatments. Selective grazing by large herbivores can favour unpalatable plant species at the

expense of palatable species (Steigner & Klein 2000). Under grazing pressure clonal propagation may be favoured because new ramets can quickly attain large sizes early in the growing season thereby escaping herbivory. Steigner & Klein (2000) found in their study that clonal reproduction was of greater importance in grazed populations, whereas mown populations reproduced mainly by seeds. Souilljee (2001) found a higher ramet reproduction in the grazed population of Schiermonnikoog. This means that the relative importance of sexual reproduction versus vegetative propagation can be influenced by ecological factors as has also been shown by Harada et al. (1997) and Lehmann (1997). In the following, these ecological factors may determine the clonal diversity and the spatial distribution of clones. On Schiermonnikoog, large differences in growth and vegetative reproduction have been found between the managed areas and the control. The grazing and mowing regimes reduced flowering of *E. athericus*, seed production was mainly found in the control plots (Souilljee 2001). These differences persisted in a common environment experiment (Souilljee 2001). Present differences in the height of canopy and percentage of bare soil may additionally affect the probability of recruitment of seedlings. Our hypothesis is that differences in the level of clonal diversity have been induced by the management regimes.

To understand the change in clonal diversity, individual clones need to be identified, which cannot always be done in the field. The clayey soil on the salt marsh prohibits digging and tracking of rhizomes. Quantitative characters, such as leaf morphology, cannot be used to identify individual genets (Widén et al 1994, Ellstrand & Roose 1987), because these are often influenced by phenotypic plasticity. In this respect the inclusion of genetic analysis is particularly useful for reconstruction of demographic processes, e.g. by the comparison of patterns of spatial distribution of plants in different environments (Dietz 1999, Schläpfer & Fischer 1998).

The genetic study of natural populations is dependent on the availability of polymorphic neutral markers (Schlötterer & Pemberton 1994). Until recently isozyme electrophoresis has been successfully used to identify clones and to examine the clonal structure of plant populations (Lehmann 1997). Because their number and polymorphism is limited, and the ongoing discussion whether they are neutrally selective, the preference is shifting to the use of DNA based markers (Reusch 2001, Jarne & Lagoda 1996). In a previous study the clone structure of *E. athericus* was assessed with allozyme electrophoresis (Bockelmann et al 2001, in prep.). In this study it appeared that variation within individuals was found, but that the

results were difficult to reproduce. Therefore we decided to use Mircosatellite markers for this study.

Microsatellites are short, tandemly repeated simple sequences whose unit of repetition is between one and six base pairs (Ashley & Dow 1994, Jarne & Lagoda 1996). They are highly abundant in the eukaryotic genome and can normally reach a length of up to 150 bp. Such length variant alleles are inherited in simple Mendelian fashion and are likely selectively neutral. Polymorphisms at the Microsatellite level are caused by DNA slippage (Levinson and Gutman 1987). During replication the repeat units on the two DNA strands may anneal out of register. The consequence of this out of register annealing, followed by DNA repair, is either an expansion or a contraction of the Microsatellite. The most common alternation comprises gain or loss of a single repeat unit, but larger changes are observed as well (Schlötterer & Pemberton 1994).

Co-dominant alleles at single Microsatellite loci can unambiguously scored by size, making gel to gel comparisons straightforward. Microsatellites rely on polymerase chain reaction amplification of DNA. It is therefore fast and needs little DNA of low quality or quantity (Ashley & Dow 1994). Microsatellites are superior to RAPD or RFLP because they are inherited in co-dominant fashion, allowing an assessment of within-population structure, for example of inbreeding (Reusch *et al* 1999).

There are no specific Microsatellite markers developed for *Elymus athericus*. Therefore, we used Microsatellite markers that were developed for *Elymus caninus* and Wheat (*Triticum aestivum*). The advantage of using different markers is that no time and expenses need to be invested in developing new Mircosatellite markers. Though the adjustment of protocols can be time consuming.

There are disadvantages in using non-specific Microsatellites. Cross-species use of primers appears not to work in a lot of species, e.g. *Zostera maritima* primers do not amplify *Zostera nana* DNA (T.B.H. Reusch, personal communication). The main disadvantage is that one cannot be sure whether real Microsatellites (repetitive DNA motive) or just any randomised fragments of DNA are analysed. To solve this problem the Mircosatellite fragments can be sequenced. Though even if one has degenerated Microsatellites, they can be analysed if they

are polymorph and results are reproducible. The Microsatellites can be seen as oligo fingerprints, which are still more specific than RAPD's for example.

In this study we analysed the clonal structure and diversity between the treatments grazing, mowing and abandoning with Microsatellites to determine the ability of *Elymus athericus* to adapt rapidly to environmental changes. The hypothesis is that the clonal structure will significantly differ between treatments. We expect a higher clonal diversity in the mown and grazed plots than in the control plots.

Method

Study site

The study site is part of the Oosterkwelder salt marsh of the Dutch Friesian Island of Schiermonnikoog (53° 30'N, 6° 10'E, Fig. 2). Until 1958 the Oosterkwelder has been grazed by young cattle, after which large parts of the salt marsh were abandoned, area A remained grazed (fig. 2). In 1972, grazing was resumed (from the end of May until September/October) in a fenced part with a stocking rate of 1.3 to 1.7 cattle/ha (area B). In the same year a mowing experiment was carried out in five different plant communities on an area of 40 m^2 outside the fence. The swath was removed immediately after cutting (Bakker & de Vries 1992). Since 1972, the mowing was done annually in August and changes in the vegetation have been examined in permanent plots of 2m x 2m.

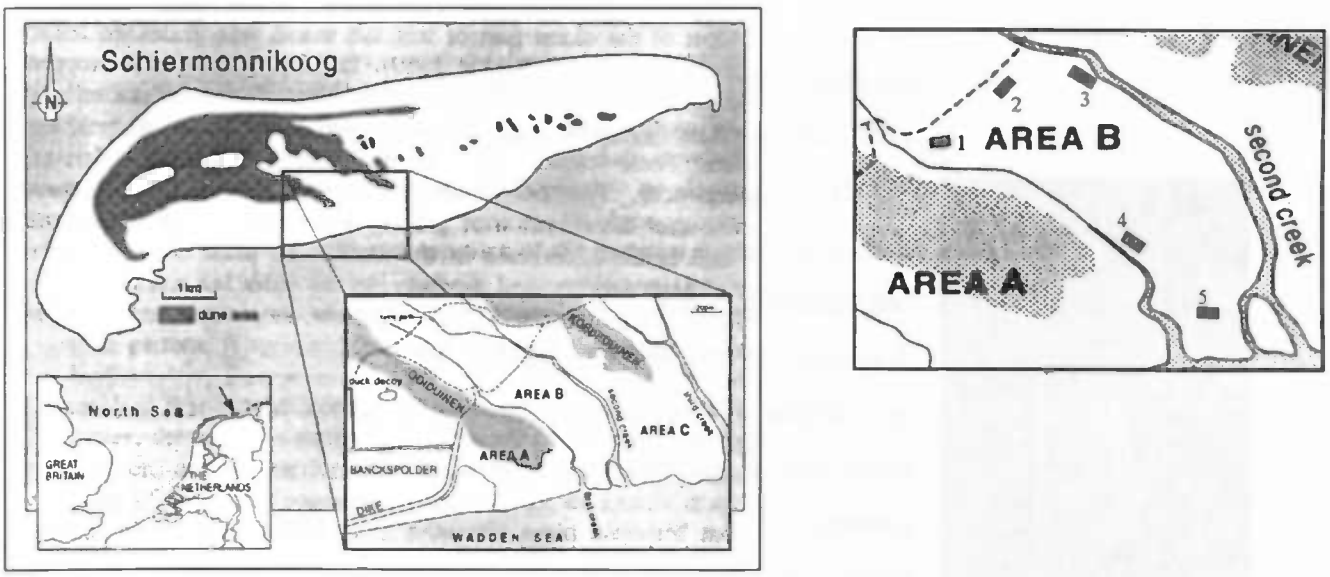


Figure 2. Map of the study site on the Barrier Island of Schiermonnikoog. In 1972 the experiment was set up in five blocks in area B.

In this study samples were obtained from five blocks on the Oosterkwelder. Each block contained three treatments, e.g. grazed, mown and control (Fig. 3).

Vegetation parameters

Since 1971, changes in the vegetation have been examined by the annual recording op %cover of all species occurring in permanent plots of 2m x 2m. The vegetation was described by estimating the cover percentage of each species (Bakker & de Vries 1992). Nomenclature of species was according to van der Meijden (1990).

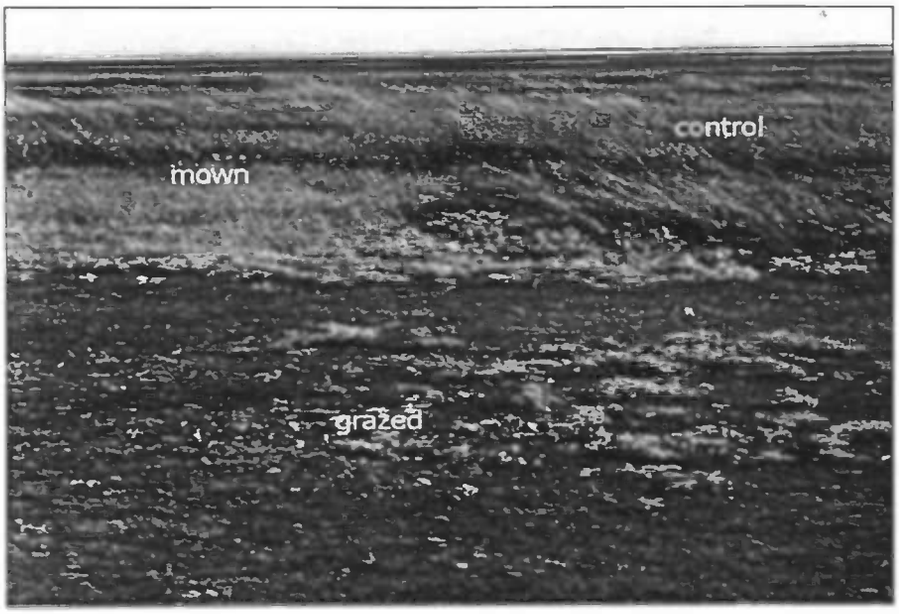


Figure 3. Overview of the management regimes in block 5.

Sampling design

We sampled in five blocks. Each block consisted of three treatments, mown, grazed and control. In each treatment we sampled randomly 20 ramets. Each ramet was marked with cable ties and a 40 cm flexible stick (Fig. 4). The sampled ramets were mapped in a grid. Fresh leaf material (approximately 2 cm^2) was collected and preserved in a tube with silica gel. In total 450 ramets were collected, each block 90 samples. Due to low densities of *Elymus athericus* in the grazed treatments, we had to extend the sampling area to attain similar sampling sizes (N=30). In 1999 during the Vegetation Dynamic course morphological characters of *E. athericus*, e.g. shoot length, ramet density, leaf length and width were measured for the three treatments per block.

Figure 4. An *Elymus athericus* ramet marked in the grazed plot.

Microsatellite analysis

The leaf tissue of each sampled ramet was preserved through drying in silica gel for later DNA extraction. In the laboratory, approximately 0.5 cm^2 dry tissue was crushed in liquid nitrogen. The total DNA was extracted for 20 minutes at 60°C using the CTAB method (Doyle & Doyle 1987, Appendix 1). The extracts were purified by two chloroformisoamyl extractions (24:1 vol : vol) and an isopropanol precipitation The crude DNA extract was stored at -20°C.

One microlitre of the DNA- was used in a 10 µl polymerase chain reaction (PCR) solution for 5 fluorescent Microsatellite markers (Appendix 2). The PCR products were electrophorised and visualised by using an ABI-Prism 377 fragment analyser (Perkin-Elmer, Appendix 3), together with a base-ladder (Rox 350). Microsatellite markers developed for other plant species were used (Table 1).

Table 1. The cross-species Microsatellite markers used in this study

Locus	Primers (5'→ 3')	Species	Assessment
ECGA22	GAA GGT GAC TAG GTC CAA C ATA GTC TCG GTC AGG CTC	*Elymus caninus*	Sun *et al*, 1998
ECGA89	TTA GCT CTT TAC TTA TTC AAA C TCC TAT GAT CAA GCA CAA G	*Elymus caninus*	Sun *et al*, 1998
WMS2	CTG CAA GCC TGT GAT CAA CT CAT TCT CAA ATG ATC GAA CA	*Triticum aestivum*	Röder *et al*, 1998
WMS6	CGT ATC ACC TCC TAG CTA AAC TAG AGC CTT ATC ATG ACC CTA CCT T	*Triticum aestivum*	Röder *et al*, 1998
WMS44	GTT GAG CTT TTC AGT TCG GC ACT GGC ATC CAC TGA GCT G	*Triticum aestivum*	Röder *et al*, 1998

Per gel-scan three primers were per sample were loaded and a base-ladder. Figure 5 shows a gel in which each lane represent a sample and the three colours represent the three different primers. The electrophoresis gels were analysed in the programme Genescan (ABI-Prism, City), in which the fragments were given a specific size using the base-ladder. The programme Genotyper (ABI-Prism, City) was used to score the fragments on presents and assign each sample to a specific genotype.

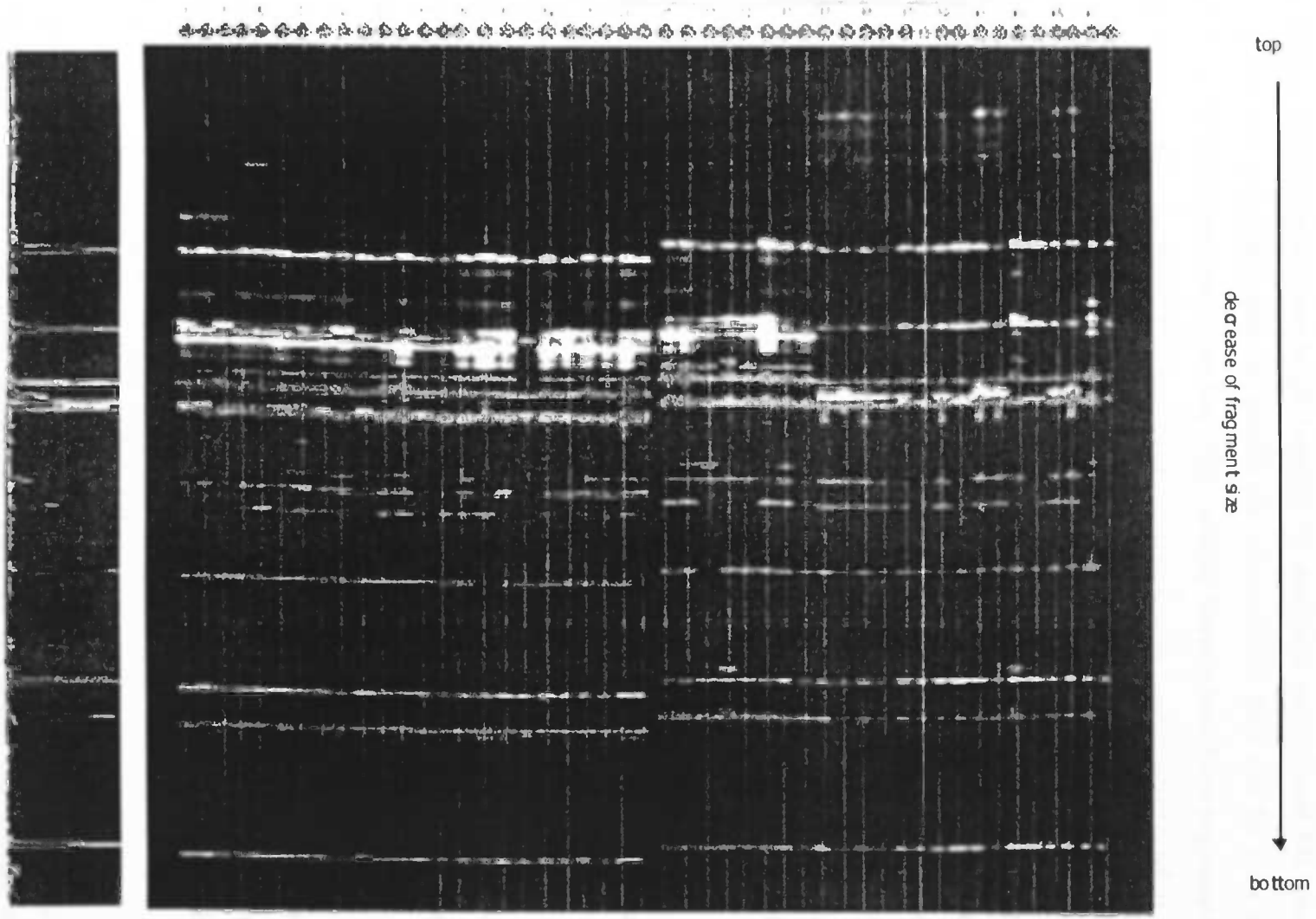


Figure 5. A gel image of the fragment analyser, with the primers ECGA22 (green), WMS6 (blue) and WMS44 (yellow). Each lane is represents on sample.

Data analysis

In this study we used Microsatellite markers developed for different species (Table 1). With cross-species use of primers one might possibly be analysing degenerated Microsatellites. Thus, if they are polymorph and the results are reproducible, they can be seen as oligo fingerprints. The PCR-products of the five Microsatellite markers have been sequenced. These products contain Microsatellites with inserts of different lengths (unpublished results). *Elymus athericus* is a hexaploid plant, this leads to difficulties in scoring alleles. When DNA concentrations are consistent in the samples, the proportion in size between peaks could be used to determine the number of alleles amplified in the analysis. As a result of the extraction method used in this study the DNA concentration varied between the samples. The concentration differences result in variation in amplification of the peaks. Samples with high DNA concentration will have higher amplified peaks, whereas samples with low concentration will give very weak banding patterns. To correct for these concentration differences a priori selection criteria were made to score the presence or absence of bands. Appendix 4 shows the criteria that have been used to score the peaks.

The five Microsatellite markers used in this study were highly polymorphic. We repeated the PCR and fragment analysis of several samples. The repetition lead to identical banding pattern. Therefore we may assume that the Microsatellite pattern is consistent and can be used to determine whether plants are identical to each other or not. For each ramet five single primer genotypes were scored, those were added up to one multilocus genotype, an oligo fingerprint.

To assess the likelihood with which ramets with the same multilocus genotype really belonged to one genet we calculated the expected frequency (P_{gen}) of the 5 primers (Reusch *et al* 1999, Parks & Werth 1993). These P_{gen} values represent the probability that two individuals display the same genotype by chance as a result of recombination.

For each treatment per block we plotted the samples in a grid to visualise the spatial structure and the clone diversity per plot. Descriptive statistics developed for species diversity measurements can be used to characterise clone diversity within populations (Ellstrand & Roose 1987). For each plot we determined two measures of clone diversity, the Simpson index of diversity and the Shannon index of diversity.

Simpson: $1-D_s$ where $D_s = \sum (n_i (n_i - 1)) / (N (N - 1)$
n_i is the number of individuals in the *i* th genotype, N is sample size (Pielou 1969, Ellstrand & Roose1987).

Shannon: $H' = - \sum p_i \ln p_i$ p_i is the proportion of individuals found in the *i* th genotype, ln is the natural logarithm (Ellstrand & Roose 1987, Stiling 1999).

Statistical Analysis

Homogeneity of variances was estimated by the Levene's test. To achieve homogeneity the parameters, e.g. leaf length and leaf width, were log transformed (Zar 1996). The frequency of ramets was square root transformed to achieve homogeneity. The morphological parameters were tested with a two-way analysis of variance (ANOVA) with treatment and block as independent factors. A Tukey-Kramer post-hoc test was used to distinguish between levels of one factor.

The indices of clone diversity were tested with a two-way ANOVA with treatment and block as the two independent factors. Samples areas differed between mown/control and grazed treatments. Because this could lead to errors in the statistical analysis, a contrast comparison was performed between the treatments control and mown only.

All statistical analysis were performed in the programmes SPSS 10.0 (SPSS Inc.) and SuperANOVA (1.1 (Abacus Concepts Inc.).

Results

Vegetation parameters

Since 1971 permanent quadrates have been monitored in each plot. The vegetation present in the different blocks belonged to 5 different plant communities. These were *Juncus maritima/Juncus gerardi* (block 1), *Festuca rubra/ Armeria maritima* (Block 2), *Elymus athericus* (Block 3), *Festuca rubra/ Limonium vulgare* (Block 4), *Festuca rubra/ Artemesia maritima* (Block 5). In this thesis I will concentrate on the change in *Elymus athericus*-cover. After 15 years *Elymus athericus* started to dominate the abandoned areas (Fig. 6). Thus, grazing and mowing suppressed the invasion of *E. athericus*. In contrast the cover of *E. athericus* decreased in comparison to 1971 in the mown and grazed treatments of block 3 which started out with an *E. athericus* stand in the beginning (Appendix 6).

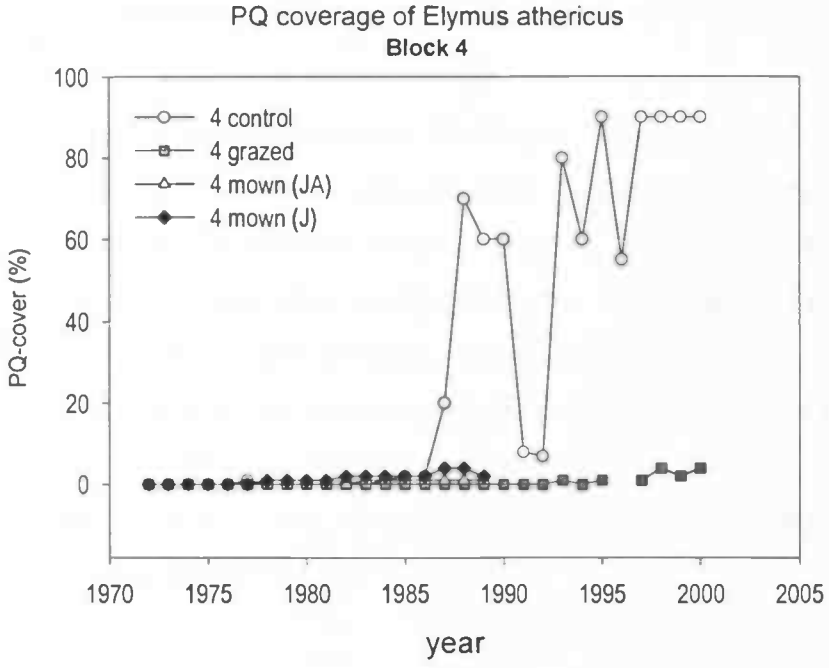


Figure 6. Change in the cover of *Elymus athericus* in permanent quadrates (PQ) with different management regimes in the *Festuca rubra/ Limonium vulgare* community since 1971.

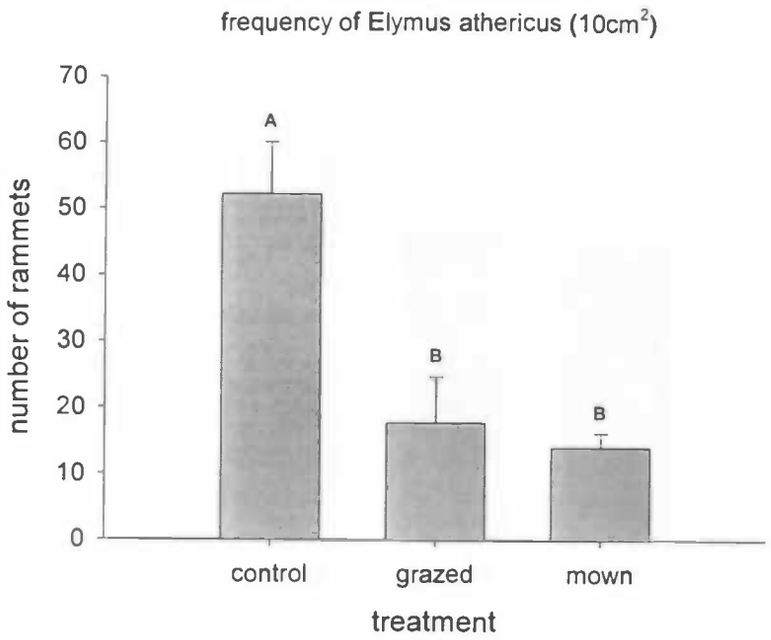


Figure 7. The density (frequency of ramets/10cm^2) of *Elymus athericus* under different treatments in June 1999, significant differences are indicated by different letters (ANOVA, post Hoc Tukey, p=0.01), with S.E. bars.

During the Vegetation Dynamics course of Groningen University on Schiermonnikoog in 1999 the density of *E. athericus* in all plots has been measured. *E. athericus* was present in all permanent plots though in different densities (Fig. 7). A significant lower density was found in the mown and grazed plots compared to the control plot (Two-way ANOVA, 'treatment' : MS= 2.144, F= 13.686, P<0.01). The management regimes did not only affect the density of *Elymus athericus* but also morphological characters, e.g. leaf length and width. The grazed and mown plants have significant wider leafs (Two-way ANOVA, 'treatment' : MS= 1.995, F= 43,322, p<0.01, Fig. 8) and longer leafs (Two-way ANOVA, 'treatment' : MS= 20.703, F= 38,564, p<0.01, Fig. 9).

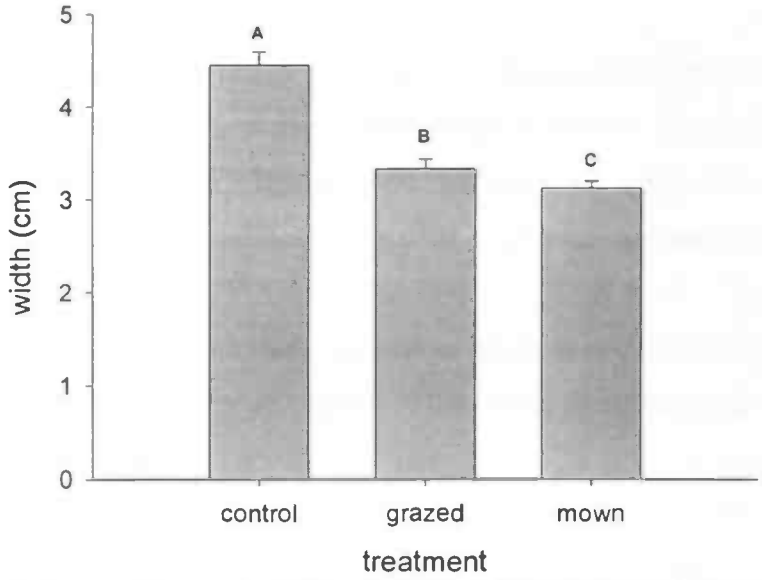


Figure 8. Significant differences in width of the 2nd and 3rd leaf of *Elymus athericus*-ramets between different treatments in June 1999, indicated by different letters (ANOVA, Tukey-Kramer post-hoc test, $p < 0.01$), with S.E. bars.

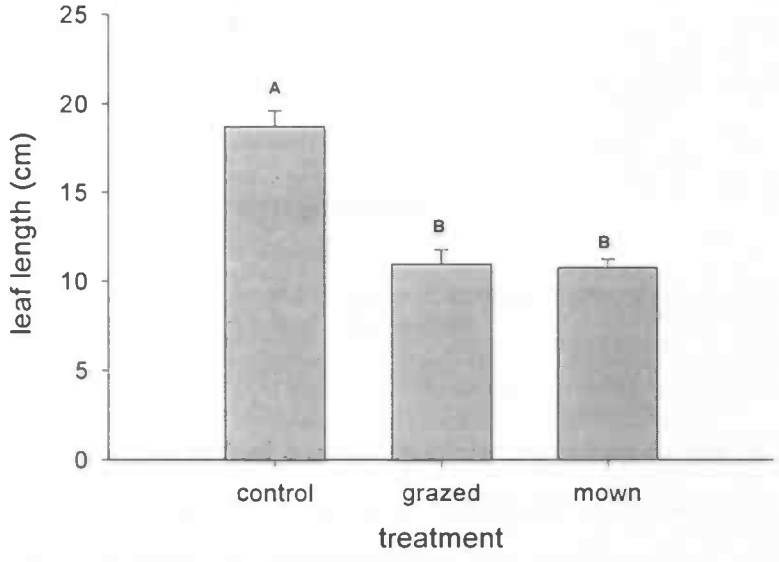


Figure 9. Significant differences in length of the 2nd and 3rd leaf of *Elymus athericus*-ramets between different treatments in June 1999, indicated by different letters (ANOVA, Tukey-Kramer post-hoc test, $p < 0.01$), with S.E. bars.

Microsatellite markers

In this study we were able to use 5 cross-species Microsatellite primers. In analyses the increase of genotypic variation with the number of primers used in the analysis (Fig. 10, for block 1). To test whether five primers were sufficient to achieve maximum genotypic variation we used a hyperbola trend line. For a 95% confidence to assess all present genotypes 4.7 primers are needed.

For the further analysis I had to exclude samples of block five. This is because many samples of block five were not scorable for all primers. Thus, these samples could not be assigned to a correct multilocus genotype.

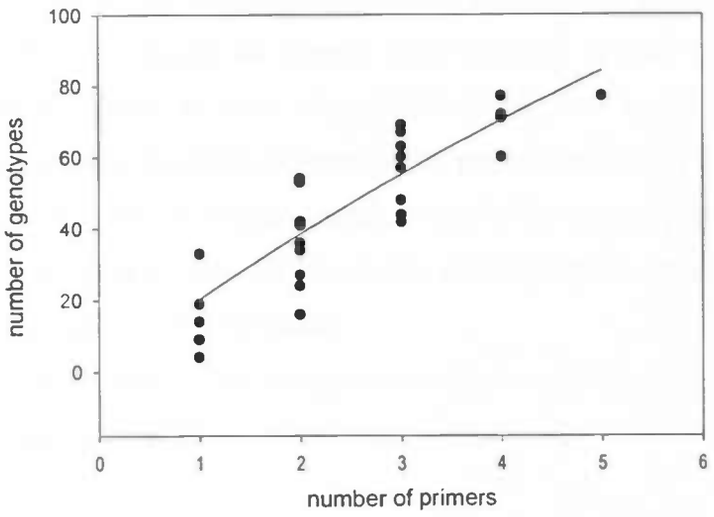


Figure 10. Increase in the number of detected multilocus genotypes of *Elymus athericus* with an increasing number of primers are added to the analysis.

I scored per ramet five single primer genotypes, which added up to one multilocus genotype. In order to test whether ramets with the same multilocus genotype really belonged to one genet we calculated the expected frequency P_{gen} (the likelihood of a 5 primer genotype occurring more than once by chance). In all cases the values of P_{gen} were <5 %, with most values <1%. Consequently all samples with identical multilocus genotype where assigned to the same genet in the present study.

Clone diversity

To visualise the spatial structure of the clones and the clone diversity, 20 sampled ramets of each plot were mapped in a grid and the ramets assigned to the same genet were given the same symbol. The most distinctive difference in clone diversity was found between the treatments in the *Juncus maritima /Juncus gerardi* (Block 1) community. In the grazed plot all ramets collected were assigned to different genets. In the mown plot two ramets were assigned to the same genet, the other 28 ramets were all different genets. In the control plot I found one large clone consisting of 9 ramets, a clone of two ramets, and a few individual genets. In the *Festuca rubra /Armeria maritima* (Block 2) and *Festuca rubra/ Limonium vulgare* (Block 4) community a less distinctive pattern was found. In these blocks a larger number of clones was found in the control and grazed plots in comparison to the mown plots. In the mown plots almost all ramets were assigned to individual genets. In the *Elymus athericus* stand (Block 3) more clones were found than in all other blocks. The different management regimes do show different clone patterns as well. The control plot contains three large clones and a few individual ramets, whereas the grazed plot consists of one large clone and many individual ramets. In the mown plot I found 5 small clones, each containing 2 ramets, and several ramet individuals.

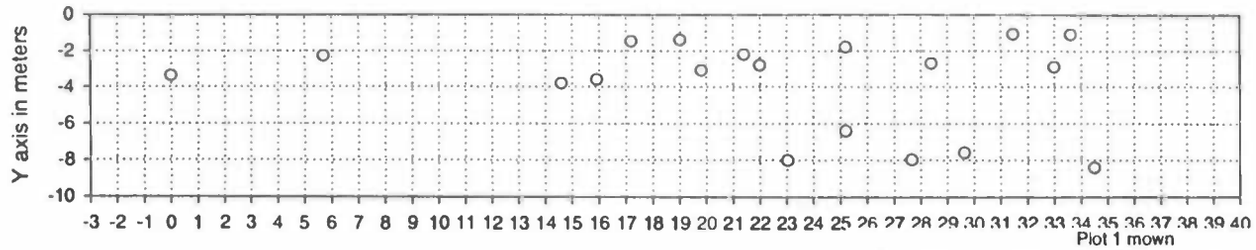
In general, large clones were mainly found in the control plots, whereas in the mown and grazed plots many individual ramets were found.

Figures 11,12,13 and 14. Clone maps of each plot. Clones are indicated with identical symbols. The open symbols represent single genets.

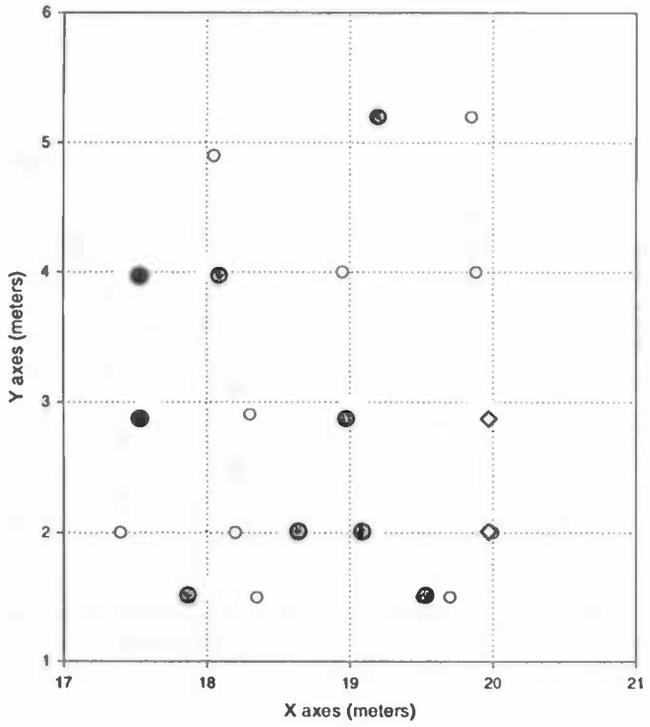
Block 1

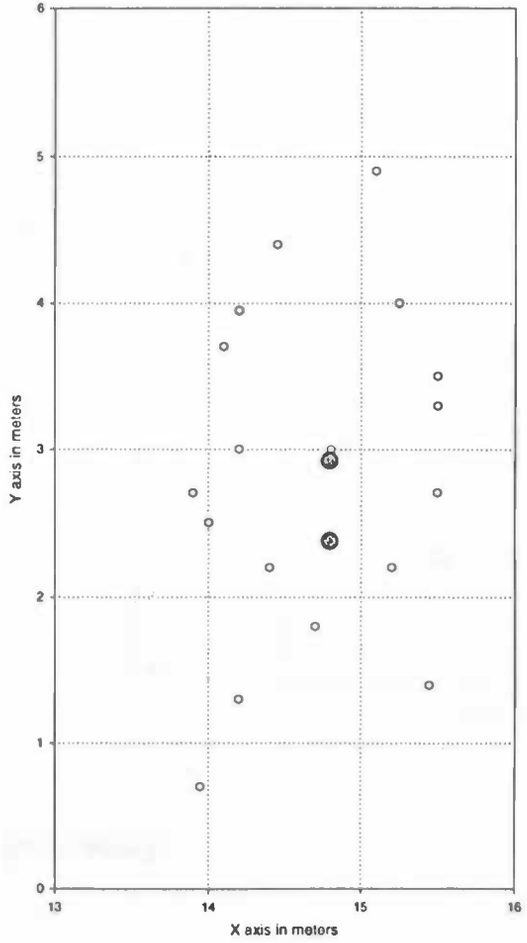
Juncus maritima / Juncus gerardi

Plot 1 grazed



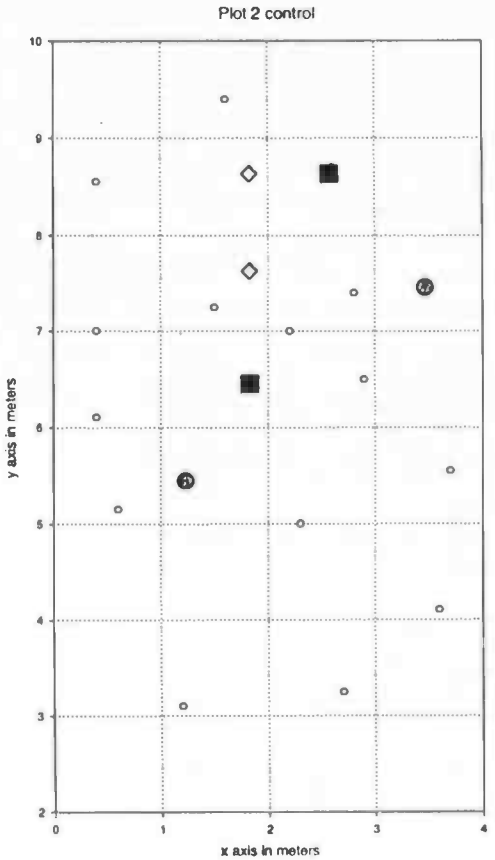
Plot 1 control

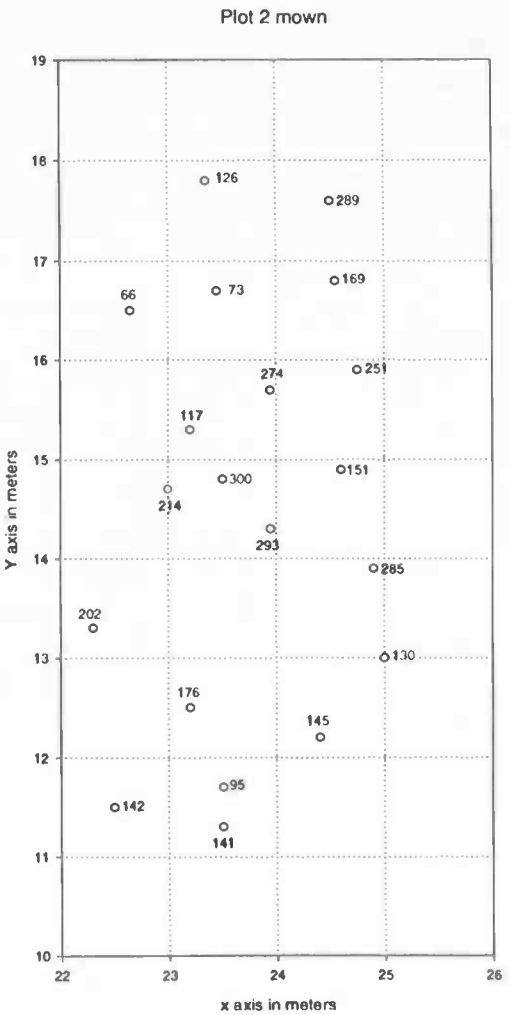


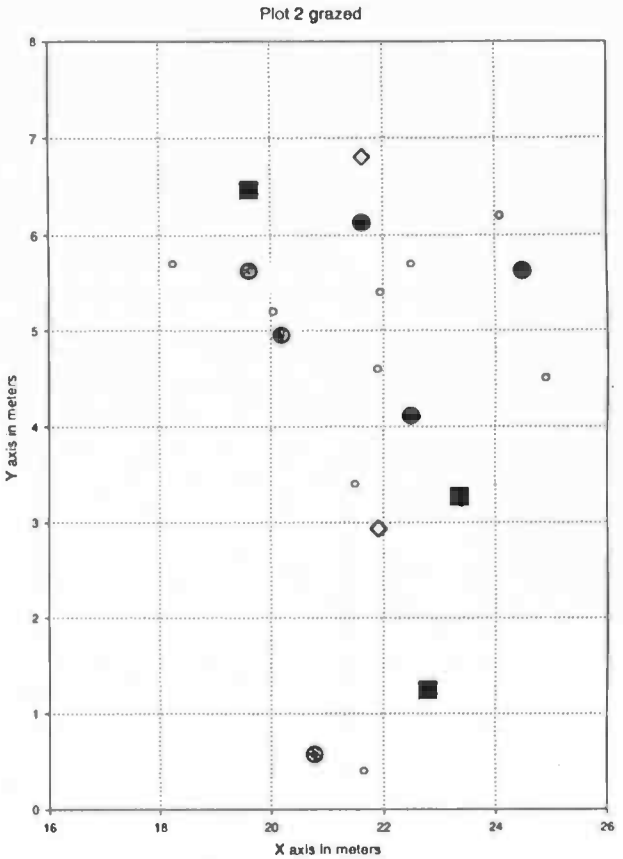


Block 2

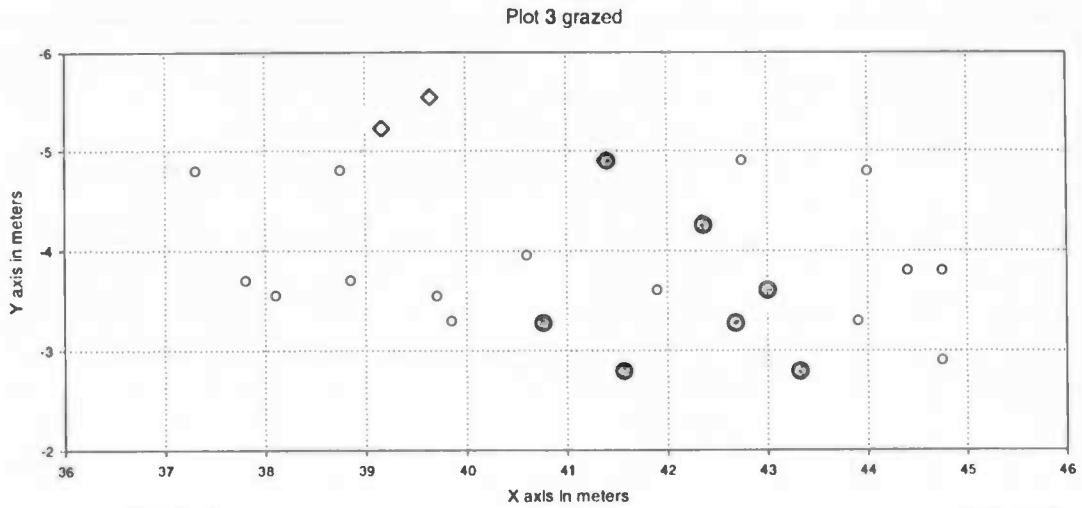
Festuca rubra / Armeria maritima

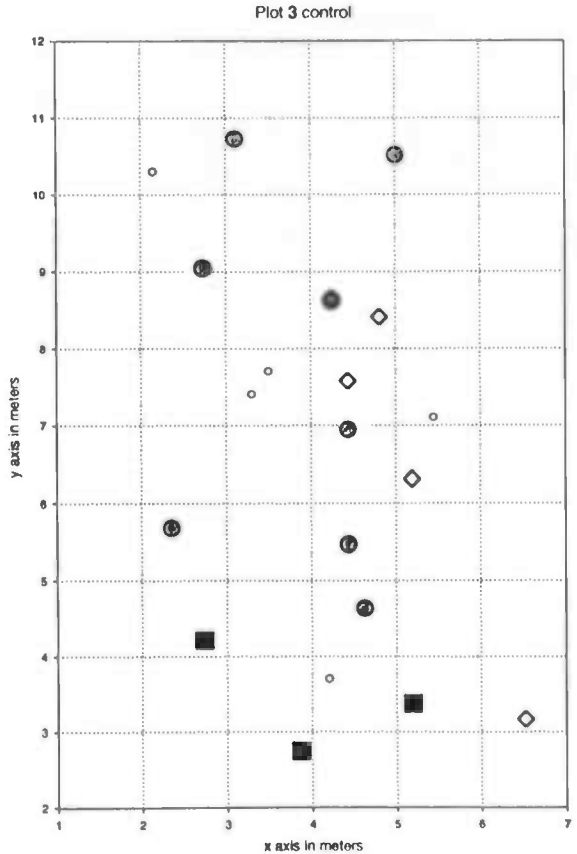


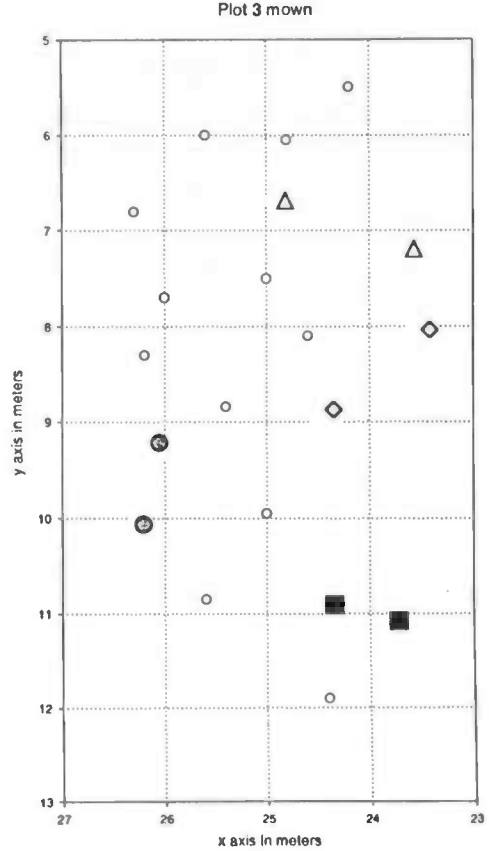


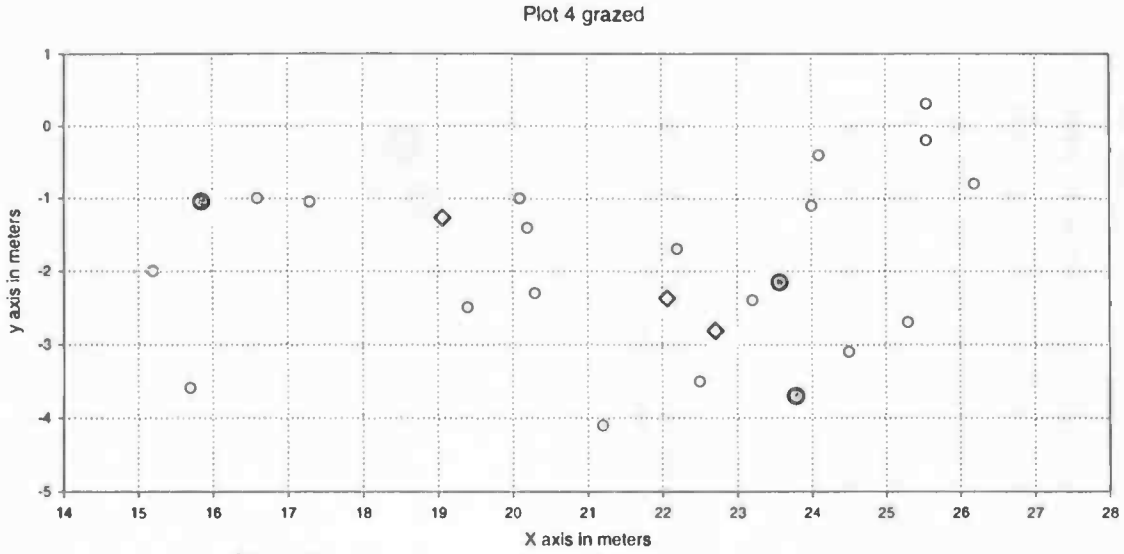


Block 3
Elymus athericus

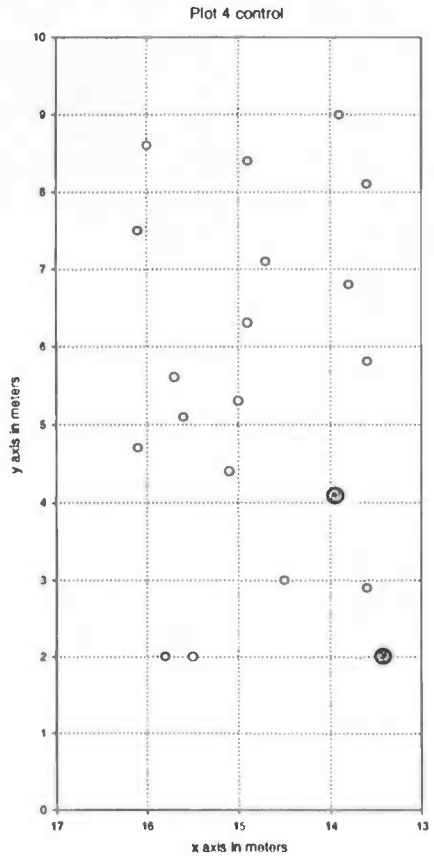


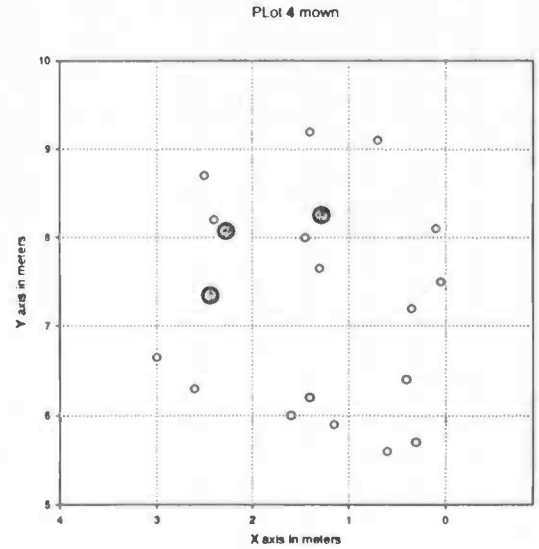






Block 4
Festuca rubra /
Limonium vulgare





The clones were not only mapped within a plot, but also mapped for the whole block. In block 3 several clones had ramets in different treatments (Fig. 15). The largest distance found between two ramets was approximately 40 meters.

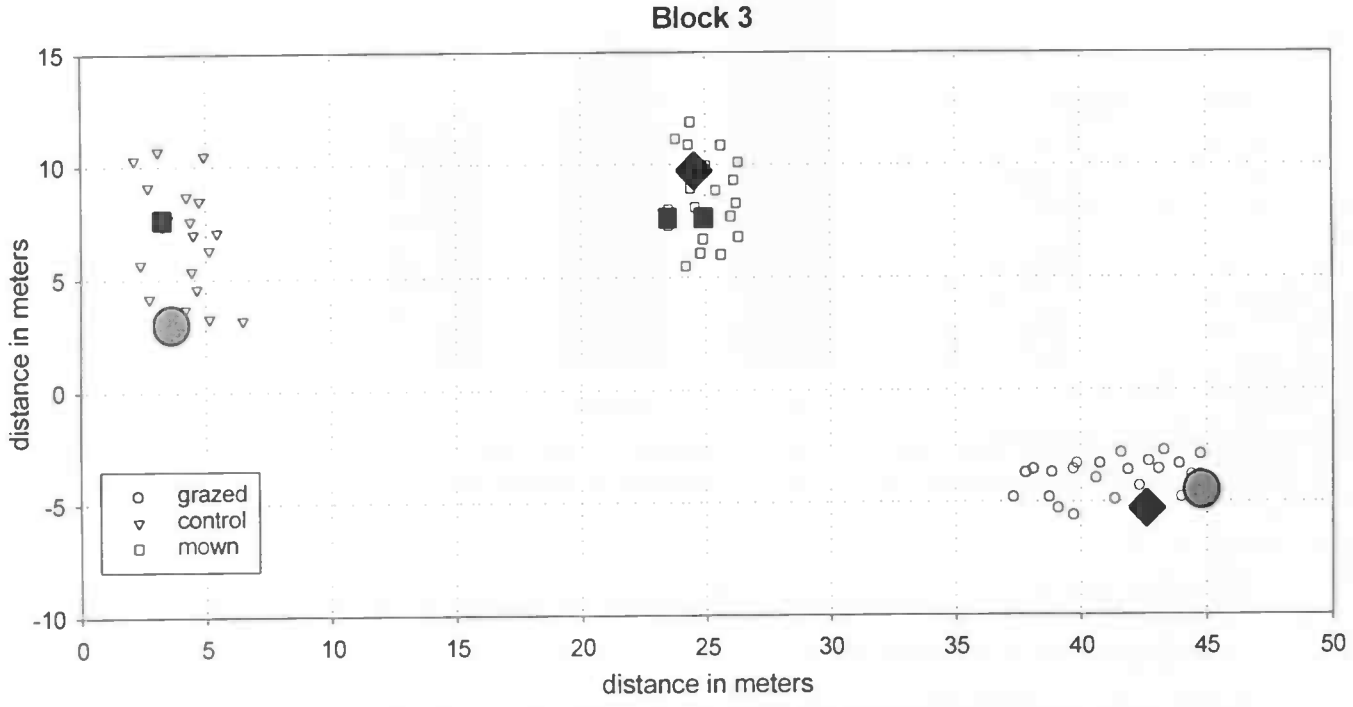


Figure 15. Large scale clonal structure of *Elymus athericus* in block 3. Open symbols represent the management regimes in which the samples were collected. The filled symbols are clones, the 3 different symbols represent 3 separate genets.

To analyse the clone diversity seen in the maps, descriptive statistics developed for species diversity measurements were used. In this study we used Simpson's index of diversity and Shannon's index of diversity. When only mapped samples were included in the analysis, no significant differences in clone diversity were found between blocks or plots. This was found using both indices. In the previous year during the Vegetation Dynamic course samples were collected in the same plots. When these samples were included in the analysis we found no significant difference between blocks for the Simpson's index. Significant results were found when all analysed samples of both years were included in the analysis, the sample size per block per plot varied between the 27 and 34 samples (Appendix 5).

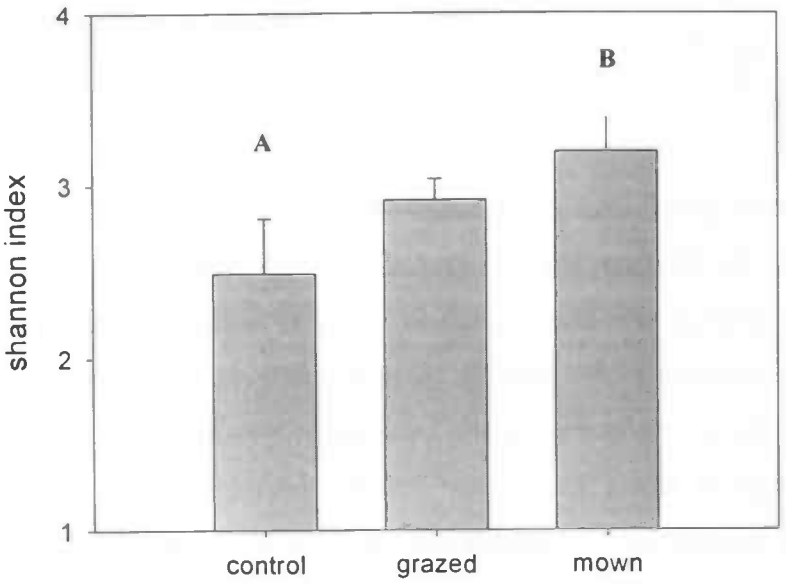


Figure 16. Mean genotypic diversity (over 4 replicates) of *Elymus athericus* under different treatments expressed with the Shannon index, significant differences (ANOVA with contrast) are indicated by different letters, with S.E. bars.

We expected the clone diversity only to increase, as the maximum clone diversity is when all ramets belong to separate genets, and therefore test one sided. No significant differences were found between plant communities (blocks). An almost significant difference was found between treatments (Table 2). A contrast comparison by means was done between the treatments control and mown. The mown plots had significantly higher clone diversity than the control plots (Table 2, Fig. 16).

Table 2. Genotypic diversity of *Elymus athericus* under different treatments expressed as Shannon's and Simpson's indices of diversity (ANOVA).

Index	Source of variation	df	Mean Squares	F	*p*
Simpson	block	3	.005	1.542	.2978
	treatment	2	.008	2.596	.1540
	error	6	.003		
Shannon	block	3	.376	2.988	.1177
	treatment	2	.505	4.014	.0783
	error	6	.126		
	contrast (C – M)	1	.995	7.913	.0306

Discussion and Conclusions

Use of Microsatellite markers

This study has shown that Microsatellite-based DNA genotyping is a valuable tool in the study of natural populations, and particularly for the analysis of clonal diversity. Until recently, in population genetics isozyme electrophoresis was mainly applied. In June 1999 students from Groningen University sampled *Elymus athericus*-ramets in the same plots on Schiermonnikoog as in my study. They used allozyme markers for the analysis. The genetic variation (number of polymorphic bands) was much lower in their study than in my investigation with Microsatellites. A.C. Bockelmann (unpublished results) analysed the number of clones found in $9m^2$ plot in different salt-marsh areas on Schiermonnikoog and found interestingly a higher genetic variation when using allozyme makers instead of Microsatellite markers. It has been discussed that genetic variation in enzyme activity is not necessarily due to variation in the structural gene encoding the enzyme, but can also vary because of differences in metabolic flux rates, efficiency or concentration (Clark & Koehn 199, Bockelmann et al 2001b, in prep). This implies that when using isozyme markers one might overestimate genetic variation by differentiating between individuals that are actually genetically the same but vary in enzyme activity.

The cross-species use of Microsatellite primers may lead to some difficulties in the analysis as discussed in the introduction. When the primers give interpretable banding patterns, which are reproducible and polymorph, the banding patterns can be seen as oligo fingerprints. Bockelmann and Reusch (personal communication) tested 25 primers, which were developed for *Elymus caninus, Elymus alaskanus and Triticum aestivum.* Five of these primers amplified and gave interpretable banding patterns. The banding patterns were consistent for repeated analysis of some samples and therefore reproducible. The primers varied in polymorphism. Per primer 8 to 25 different genotypes were scored, and 253 multilocus genotypes were scored for 330 samples. The level of polymorphism was sufficient to make detection of most unique multi-locus genotypes highly probable, as shown in Figure 10. With 95 % confidence all present genotypes could be assessed, when using five Microsatellite markers.

As expected, ramets often possessed identical genotypes. The P_{gen} (the likelihood of a 5 primer genotype occurring more than once by chance) values of the identical genets were

<0.05 and in most cases <0.01. Statistically, the likelihood that ramets were erroneously assigned to the same genet because they exhibited the same multilocus genotype by chance was small. Thus, all ramets with identical genotypes were identified as genets (clones).

Use of diversity Indices

To characterise the genetic diversity within populations descriptive statistics developed for species diversity measurements can be used (Ellstrand & Roose 1987). In the present study we used the Simpson Index of diversity and the Shannon Index of diversity. The Simpson Index did not show any significant results, unlike the Shannon Index. It can be argued that both measures are equivalent to each other, and therefore need not to use both indices. Magnussen & Boyle (1995) argued that the statistical efficiency of the Shannon index was so high compared to the efficiency of the Simpson index that it should be the preferred one. For the same statistical resolution the Simpson index requires about nine times as many samples as the Shannon index. This is in agreement with our study where we only found significant results when all samples of both years were included.

Effect of management regimes on vegetation

I hypothesised that direct experimental manipulation of environmental conditions over a long time may induce changes in clonal and genetic diversity. The management regimes applied in our study, mowing and grazing, have led to different clonal diversities of the *Elymus athericus* populations. A significant higher genetic diversity was found in the mown in comparison to the control plot. In the mown plots we found almost only ramets of individual genets. In the control plots we found large clones, consisting of 3 to 9 ramets with maximum distances of 6 meter. In the clone maps, variation in genotypic diversity between the blocks can be seen, but was not statistically significant.

Differences in clonal structure between the treatments found in the present study may be affected by the abundance of *Elymus athericus*. In the control plots the abundance of *E. athericus* was very high and it was easy to sample at random. In the grazed plots this was not the case, in most plots the sample area depended on the abundance of *Elymus athericus*. We used a sample size of 30, but it was not always possible to find 30 ramets in an area of 5 by 10

meters. We adjusted the size of sample area to obtain an equal number of samples in each plot. Consequently, in the grazed and some mown areas all *Elymus athericus* ramets, which were present, were sampled in each plot, while in the control we sampled at random. To statistically compare clonal structures between the different management regimes, samples should be collected at randomly with an equal sample size and equal sample area (Zar 1996, Stiling 1999). This is a simple mathematical criteria, but often not applicable in field situations. I tried to randomise my sampling as much as possible and tried to stay within a fixed sample size of $50m^2$ per plot.

Genotypic diversity is affected by management regimes. The genotypic diversity was lowest in the unmanaged plots, in which we found a clone consisting of 9 ramets. Clonal propagation may be favoured in control plots because new ramets get a head start by starting to grow early in growing season, and are therefore less affected by light limitation than seedlings. The high density of the vegetation and the tall canopy of the control plots may prevent new seedlings to recruit. This is supported by a sowing and seedling recruitment experiment with *Elymus athericus* (Bockelmann et al. 2001). The authors showed than that germination is limited in older, densely vegetated marshes and that recruitment is reduced under competition with older plants.

Mown plots consisted of more different genets than grazed plots. This finding would suggest that in mown plots propagation would mainly occur more by seedlings than in the grazed areas. In the grazed plots mean vegetation height was ±4 cm and in the mown ±10 cm (Bakker & de Vries 1992). The percentage of bare soil was higher in the grazed plots (Bakker & de Vries 1992). Therefore, it could be expected that seedlings have a higher recruitment chance in grazed areas, because there is more bare soil. Bakker and De Vries (1992) performed a study on germination of six salt-marsh species (Artemisia maritima, Aster tripolium, Atriplex prostrata, Cochlearia danica, Plantago maritima and Suaeda maritima) at the same study site. They showed that the number of seedlings which emerged was significantly higher in the grazed than in the mown sites in all plant communities. This contrasts with our findings. However, the destruction of seedlings by trampling might cause a lower survival in the grazed plots than in mown (Bakker and de Vries 1992). Steigner and Kleijn (2001) found in their study on alpine meadows that clonal reproduction was of greater importance in grazed populations, whereas mown populations reproduced mainly by seeds.

The management regimes have not only resulted in change of genotypic diversity but also resulted in phenotypic differences of *Elymus athericus*. The leaf length and width was significantly larger in the control plots compared to the grazed and mown plots. It appears that the mowing and grazing have the same effect on the morphological traits of the plants. In a common environment experiment, Souilljee (2001) showed that the morphological differences of *Elymus athericus* were not only plastic, but also probably genetically fixed.

We expect that the differences in clonal diversity will become more striking as time passes by. Schläpfer and Fischer (1998) found in their study that the phenotypic differences between management-regimes were not reflected in clone diversity. They concluded that management-induced changes in clone diversity can only be expected after a much longer periods of time than after only 16 yr. In this study 30 years were enough time for differences in genotypic diversity induced by management regimes to develop.

Elymus athericus may have adapted to the management regimes by changing the ways to propagate. The differences of genotypic diversity between the treatments could then be explained by the preference of sexual reproduction over vegetative propagation in the grazed and mown plots. However, in the salt marsh of Schiermonnikoog the production of seeds was heavily reduced or even prevented by grazing and mowing during flowering season (Souilljee 2001, R.M. Veeneklaas, personal observations). In a common environment experiment further studies could be done to assess whether the management regimes affect the investment in seed production.

This study has shown that human activity can affect the importance of sexual reproduction versus vegetative propagation, which leads to change of the genotypic diversity in *Elymus athericus*.

Acknowledgement

The quest to reveal the secretes of the clonal *Elymus athericus* in the salt marsh of Schiermonnikoog was very challenging and exciting. I think in particular of the question whether we were able to use the available primers, and how we should interpret the banding patterns. With the supervision and advice of Anna Bockelmann and Thorsten Reusch I managed to unravel the clonal patterns of *Elymus athericus.* I would like to thank Prof. Dr. W. Lampert and the Max-Planck-Institute for Limnology in Plön to give me the opportunity to work in this institute and use the laboratory facilities. I would like to thank Silke Carstensen for her assistance in the laboratory. I would like to thank Prof. Dr. J.P. Bakker for his support and the insights of the salt marsh. Without the foresight and work of Jan Bakker and Yzaak de Vries since 1971 on the Oosterkwelder, I would not have been able to do this study. I am very grateful that the Vereniging Natuurmonumenten has enabled me to work in the National Park of Schiermonnikoog.

Colleagues and friends in Plön made sure that I did not only enjoy the wonderful laboratory facilities, but also the beautiful surrounding. The Haring fishing, barbecues and the dives in the 'Schöhsee' were great, thanks. I would especially like to thank Meike Bulten for her support throughout the whole project. The discussions but also relaxing moments have contributed a great deal to this thesis.

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Appendix 1.

DNA-extraction from dried plant tissue with CTAB

Following chemicals necessary:

- fluid nitrogen
- CTAB-extraction-buffer
- Isopropanol p.A. (-20°C)
- Ethanol 70% (+4°C)
- Chloroform:Isoamyl-Alcohol (24:1 vol:vol)

Preparaption of 100 ml CTAB-extraction-buffer (for 100 samples) mix:

- CTAB 2 gr
- NaCl 0.14 Mol = 8.2 gr
- EDTA 4 ml of 0.5 M solution
- Tris-HCL pH 8 10 ml of 1M solution
- Polyvinyl-Pyrrolidon (PVP) 0.4 gr

(This solution does not need to be autoclauved and can be stored at room temperature for a few weeks). The EDTA-solution and the TrisHCL needed to be renewed after two months.

DNA-extraction

1. Just before extraction add 1µL Mercapto-Ethanol to 1 ml buffer.
2. About 0.5.cm^2 plant tissue, clipped in little pieces (2mm) is put in a 2 mm Eppendorf tube. Keep half of the sample in the Silica-gel as reserve. Cool the cup and the mini-mortar in the fluid nitrogen. Crush the plant tissue for 30 seconds.
3. Clean the mortar with 70% ETOH (avoid contamination), and dry with a Kimwipe-tissue.
4. Add 1 ml of buffer per cup, and mix cup briefly.
5. Place cups in 60°C shaker for 10-20 minutes.
6. Place the cups on the rocking bath for 45 minutes at room temperature.
7. Add per cup 1 ml Chloroform:Isoamyl-Alcohol (24:1) and mix 1 minute by hand.
8. Centrifuge cups at 10000 rpm for 5 minutes at 15°C.
9. Bring the waterphase (upper layer) in new 2 ml cup using a pipette.
10. Repeat step 6-8 once more (about 650-700 µl waterphase will be left).
11. Add 2/3of the waterphase volume (416-466 µl) of ice-cold Isopropanol. Keep the cups at 4°C for 30 minutes **(not longer!!!)**
12. Centrifuge cups at 10000 rpm for 12 minutes, mind pellet position.
13. Remove the Isopropanol carefully, keep pellet in sight. Dry the cup up side down on tissue.
14. Add per cup 800 µl cold 70% Etoh, keep the cups at 4°C for 10 minutes.
15. Centrifuge the pellet at 10000 rpm for 3 minutes, and remove the Etoh.
16. Dry the pellet (air or vacuum).
17. Dissolve the pellet in 50 µl HPLC-water, leave it dissolve for 1 hour at room temperature. Solution should be viscose (high molecular DNA).
18. Store DNA at –20°C.

The quality of the DNA can be determined with electrophoresis using 5-10 µl of the DNA on a 1.5% Agarose-gel.

Appendix 2

PCR Protocol

Per sample 1 µl extracted DNA and 9 µl master mix.

Master-Mix

		per reaction add uL vol
PromegaBlau	10xBuff	1.00
	10xdNTP	1.00
	MgCl2	0.80
labelled	fwd-primer	1.00
unlabelled	rev-primer	1.00
1%	BSA	1.00
PromegaBlau	Taq	0.05
	HPLC-H2O	3.15
		9.00

Primer	WMS 2		WMS 6		WMS 44		ECGA 22		ECGA 89	
	°C	time	°C	time	°C	time	°C	time	°C	time
hot start	94	3:00	94	3:00	94	3:00	94	3:00	94	3:00
Denaturation	94	1:00	94	1:00	94	1:00	94	1:00	94	1:00
Anneal 1	50	1:00	50	1:00	50	1:00	55	1:00	55	1:00
Increment					-0.5°C					
Cycle	9		9		9		9		9	
Extension	72	1:00	72	1:00	72	1:00	72	1:00	72	1:00
Denaturation	94	0:30	94	0:30	94	0:30	94	0:30	94	0:30
Anneal 2	50	1:00	50	1:00	60	1:00	50	1:00	50	1:00
Cycle	22		19		17		19		19	
ext.	72	1:00	72	1:00	72	1:00	72	1:00	72	1:00
final ext.	72	5:00	72	5:00	72	5:00	72	5:00	72	5:00
Pause	4		4		4		4		4	
slow slope	2°C/s		2°C/s		2°C/s		2°C/s		2°C/s	

Appendix 3

Electroforese ABI PRISM™ 377

Preparation of Fragment gel

using Lang-Ranger™ gel sen. 25 ml 5%

- use a 50ml beaker and place on magnetic-stirrer
- add:
  - LR50% 2.5 ml
  - 10x TBE 2.5 ml
  - Urea 9 gr
  - H_2O fill up to 25 ml

stir mixture a few minutes

10x TBE-Buffer (1l):

- Tris Base 108.0 gr
- Boric Acid 55.0 gr
- Na_2EDTA 9.3 gr

mean while

Preparation of Glass plates

- place the glass plates on two boxes, covered with tissue
- clean glass plate with nanopore water
- dry the plates with Isopropanol p.a./Water (9:1)
- place the plates, so that the text on the plate is in reverse image
- put on drop of water on the plate
- place the spacers on each side of the plate
- place the other plate on top of the plate with spacers
- tighten the plates with clips

Finishing gel off

- add:
  - TEMED 18 µl
  - APS 10% 125 µl
    - Ammoniumperoxodisulfate 1.0 gr
    - Water (for chromatography) 10.0 ml
- place the glass plates in an angle
- use a syringe with a 0.2 µm filter, and pour the squeeze the gel between the plates
- place the glass plates horizontal
- place the backside of a comb in the gel
- let the gel polymerise for about an 1 to 1½ hour

Plate Check

- place the glass plate in frame in the sequencer, no buffer needed
- activate the program GeneScan, make a new file
- press PlateCheck

PreRun

- when glass plate is clean apply the buffer buckets in the frame, pour buffer in the buckets
- 1x TBE buffer is being used
  - used the 10x buffer which is used for preparing the gel to make 1x TBE
- press PreRun and let the program run for at least 10 minutes

Preparation of samples

- make a loadingbuffersolution
  - Formamid : loadingbuffer : Rox 350
    5 1.5 1

    example: 50 samples * 3 µl loadingsolution
    - 20 x 5 = 100 µl Formamid
    - 20 x 1.5 = 30 µl loadingbuffer
    - 20 x 1.0= 20 µl Rox 350

- pool the samples
  - pipette 7 µl of one sample in the other sample
- in microtitterplate: 3 µl loadingbuffer and 3 µl sample
- just before application denaturate samples at 91°C for 2 minutes, keep on ice afterwards and while applying samples on gel.

Running gel

- stop PreRun, clean the comb area with a syringe and buffersolution, removing the urea crystals.
- Place the comb in the gel
- Apply the samples in the gel, when many samples a PreRun is done in between.
- After all samples are applied, select in the programme the comb size. Select the sample sheet and press RUN. Next window select SAVE. The gel is running now.

Gel finished running

- Switch programme off, save running file on ZIP-disk.
- Remove case with glass plate and top buffer and the bottom buffer bucket.
- Clean glass plates and comb with Alconox and rinse thoroughly with demi water.

Appendix 4

Filter criteria of peak scoring in Genotyper

Category (primer)	Peaks have to have at least a % height of highest peak in category	Peak ignored when preceeded by a higher scored peak within	Peak ignored when followed by a higher scored peak within
ECGA 22	20%	0.00 to 0.80 bp	0.00 to 0.80 bp
ECGA 89	20%	0.00 to 1.10 bp	0.00 to 1.10 bp
WMS 2	20%	0.00 to 2.00 bp	0.00 to 2.00 bp
WMS 6	15%	0.00 to 1.60 bp	0.00 to 1.60 bp
WMS 44	10%	0.00 to 1.60 bp	0.00 to 1.60 bp

Primer	Peaklabel	Minimum peak size	Maximum peak size
ECGA 22	128	127.30	128.50
	130	129.00	130.60
ECGA 89	196	195.60	196.10
	197	196.50	197.10
	199	197.80	199.20
	201	201.00	202.20
	208	207.40	208.40
WMS 2	185	183.50	186.50
	235	232.00	237.50
	266	261.50	268.00
	283	279.50	285.50
	295	293.00	296.50
WMS 6	138	138.00	138.80
	140	139.40	140.00
	142	141.20	142.60
	147	146.00	147.00
	148	147.40	148.00
	149	148.20	149.20
	151	150.00	151.20
	153	152.50	153.50
	155	154.00	155.00
	158	158.00	159.00
	160	160.00	161.00
	162	161.80	162.60
	164	163.80	164.80
WMS 44	115	113.00	116.00
	125	124.50	125.50
	128	127.50	128.50
	135	134.00	136.50
	138	137.70	138.60
	139	139.00	140.00
	142	141.30	142.30
	144	143.50	144.30
	147	146.50	147.50

Appendix 5

Sample sizes

Table showing the total number of samples of which the multilocus genotype is scored per block per plot.

	Block 1	Block 2	Block 3	Block 4
Control	29	27	27	27
Grazed	28	27	30	32
Mown	34	30	27	30

Appendix 6

Change in cover of *Elymus athericus* in permanent quadrates monitored since 1971.