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Tetraploid Lolium Perenne genotypes Identified in Danish Semi-Natural Habitats

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Abstract

The ploidy of perennial ryegrass, *Lolium perenne,* was studied in order to assess the potential of tetraploidity as a means of preventing transgenes from spreading to natural populations.In contradiction to earlier observations, we found that tetraploid *L. perenne* genotypes was present in a semi-natural habitat in non-trivial proportions. Consequently, we conclude that tetraploidy will not prevent GM traits from spreading to natural rye grass populations by either an invasion or an introgression pathway.

Keywords: Ecological risk assessment of GMO; invasion; introgression; perennial ryegrass

Introduction

Early information suggests that only diploid *Lolium perenne* (L.) (perennial ryegrass)is found in natural habitats (Jenkin, 1959), and thus, even though diploid and tetraploid genotypes may hybridize and produce viable offspring(e.g. Griffiths, Pegler and Tonguthaisri 1971), it could be argued that the use of GM tetraploid varieties may form a hybridization barrier between natural diploid and GM tetraploid varieties of *L.perenne*. All the naturally occurring Lolium species that have been examined up to the publication of Jenkin (1959) were found to be diploids, with 7 as the basic chromosome number(references in Jenkin 1959), but since then, tetraploids, triploids and anaploids of *L. perenne* have been artificially produced in breeding efforts and constitute nearly as big a share in the modern forage varieties as the diploid varieties do.

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It is very likely that such non-diploids have also been spontaneously produced from time to time in nature, but, if so, they have probably not succeeded in establishing distinct polyploid Lolium races (Jenkin, 1959); at least,such populations were not observed by Jenkinor described in the literature at that time. However, with the increasing use of tetraploid Lolium varieties in the past 50 years, an establishment of semi-natural tetraploid populations cannot be excluded. Consequently, today's frequency of tetraploid individuals in naturally occurring *L. perenne* populations is an open question and needs to be clarified in order to be incorporated in an environmental risk assessment on the use of tetraploidy as a possible way to prevent the spread of transgenes.

L.perenne is a wind-pollinated and self-incompatible grass (Frakes 1973), which was intentionally introduced in Denmark late in the 18th century and is the most important fodder grass in temperate silage production areas. Lolium varieties are well-adapted, productive grasses widely distributed in temperate and cool climates. In addition to the use as forage, Lolium varieties are also widely used in lawns and sport fields (Jauhar 1993).

*L. perenne*is genetically compatible with varieties and wild ecotypes of other Lolium species, including *L. multiflorum*. In addition, Lolium species also show interbreeding affinities with the closely related grass genera Festuca(Thomasa and Humphreysa 1991). *L. perenne* is commonly found naturalized in semi-natural grasslands and along roadsides in Denmark (Frederiksen 1981), although it has been suggested that *L. perenne* may not be able to maintain viable populations in undisturbed natural habitats for a longer time period (Erneberg, Strandberg, Strandberg, Jensen and Weiner 2008). Seed banks of perennial ryegrass are limited and transient, even where perennial ryegrass is a major component of pastures in grasslands(Grime 1979, van Altena and Minderhord 1972). However, a systematic study of the abundance and spatial distribution of *L. perenne* in terrestrial ecosystems has not been performed.

In this study, we investigated the ploidy of Danish *L. perenne* populations in different light-open natural habitats in order to assess the possibility of the spread of transgenes or tetraploid cultivar genotypes into natural populations.

Materials and Methods

Natural Populations of L. Perenne

The average plant cover of *L. perenne* in different Danish terrestrial habitats was estimated bycover data from the database "Danmarks Naturdata". A total of 32711 plots from579 sites encompassing 17 different light-open habitat types(Table 1) in an unbalanced design with between 20 and 60 plots per site in the period from 2004 to 2009 were used in the analysis(Nielsen, Bak, Bruus Pedersen, Damgaard, Ejrnæs, Fredshavn, Nygaard, Skov, Strandberg and Strandberg 2012). The cover of the higher plant species at each plot was determined by the pin-point method, using a square frame of 16 grid points that were equally spaced by 10 cm; at each grid point, a thin pin was inserted vertically into the vegetation, and the plant species that touched the pin were recorded (Kent and Coker 1992). The mean cover was estimated by fitting the data to a generalised binomial distribution (Damgaard 2009, Damgaard 2012).

Frequency of Tetraploid L. Perenne in a Study Area

Based on a map of the sites where *L. perenne* was known to be present according to "Danmarks Naturdata" (Nielsen, Bak, Bruus Pedersen, Damgaard, Ejrnæs, Fredshavn, Nygaard, Skov, Strandberg and Strandberg 2012)and the fields where tetraploid *L. perenne* varieties had been grown for seed production, a sampling area was selected. From nine possible candidate sampling areas, a sampling area close to a farm situated at Hvilsom was selected, because the farmer had been growing the tetraploid *L. perenne* variety "Tivoli" for the past thirteen years (Fig.1).

During the summer of 2010, leaves from 487 *L. perenne* plants were sampled in uncultivated grass lands along the stream Simested Åat variable distances from the farm at Hvilsom (Fig. 1). Part of the sampled area was within a NATURA 2000 site (Hannerup Bro, Simested Å –site number: 1082), which is classified as belonging to the habitat type "species-rich *Nardus* grasslands" or acid grasslands(habitat type 6230 EU 2003). The cover of *L. perenne* at the NATURA 2000 site was estimated at 19%in 2007.

Immediately uponsampling, the leaves were placed in a cooled box (approximate 5 to 10 °C), and after returning to the laboratory in the evening they were frozen at -18 °C. The ploidy level of the sampled *L. perenne* leaves was later determined by flow-cytometry, using the Partec CyStain UV precise P kit. Ploidy determinations with the Partec Ploidy Analyser (http://www.partec.de) were performed by measuring the total DNA content of the individual nuclei. Up to 1 cm^2 of leaf material from each individual plant (two to four leaf pieces of 2 cm length, depending on the width of the leaf blades) were chopped for 30 seconds with a sharp razorblade in 0.5 ml of Partec HR-A solution in a small plastic petri dish.After 2 minutes of incubation at room temperature, the sample was filtered through a 30 μm mesh in order to remove cell debris (Partec CellTrics disposable filter). Two ml of HR-B solution was added, and after an additional two minutes at room temperature the sample tube was connected to the analyser. After around 30 to 120 seconds, an optimal DNA-histogram was produced and could be analysed.

The output of a typical ploidy analysis depicts the number of cells (nuclei) with a specific DNA content in a histogram. All nuclei within a peak contain the same quantity of DNA and represent one ploidy level. In a non-synchronized mixture of cells, found when extracting cells from grass leaves, two peaks with DNA contents in the relative proportions 1:2 are expected. These peaks represent cells in different stages of the mitotic cell cycle. The peak of lowest DNA content represents cells before DNA replication (stages G0/G1), while the peak with a DNA content of double that of the first peak represents cells having duplicated the DNA content in preparation for the mitotic cell division (stages G2/M). Five 2N and five 4N control plants were analysed individually at different points in time during the analysis to calibrate the histogram for the 2N and 4N peaks and to make sure that the measurements did not show a drift throughout the use of the instrument. Three outlier plants, with DNA content at the tails of the distributions, were omitted from further analysis, as the assignment to ploidy was uncertain.

In order to validate the results of the reading of ploidy level of the individual plant, the bimodal distribution of the measured DNA content (Fig. 2) was analysed using a mixture distribution of two Poisson distributions, $p(n, n_4, \lambda) = \frac{n - n_4}{n} Pois(\lambda) + \frac{n_4}{n} Pois(2\lambda)$, where n is the sample size, and n_4 is the estimated number of tetraploid plants in the sample.

The scale parameters of diploid and tetraploid plants were assumed to be □□ and 2□, respectively, i.e. it was assumed that the average measured DNA-content of sampled tetraploid leaves was twice as high as the average DNA content of the sampled diploid leaves. The parameters were estimated using a Bayesian MCMC approach (Carlin and Louis 1996),

Results and Discussion

The estimated average plant cover of the different habitat types (Table 1) shows that *L. perenne* is mainly present in relatively dry habitat types dominated by grasses. Among the monitored light-open habitat types the highest average cover of *L. perenne*(12 %) was observed in semi-natural dry grasslands.

Based on the measured DNA content of the sampled *L. perenne* leaves, the proportions of diploid and tetraploid plants were estimated. Since the DNA content was measured on an arbitrary scale, measures of DNA content can only be compared relatively. The average DNA content of fresh leaves of known diploid and tetraploid *L. perenne* plants yielded a signal intensity of 36 and 72, respectively, and plants with signal intensity above 40 were considered having a DNA content above 2N.Sixteen samples out of 437analysed leaves had a DNA content indicating a ploidy level higher than 2N (Fig. 2). The sixteen plants came from different sampling areas and did not display any clear geographic pattern (results not shown).

In order to validate the above result, the bimodal distribution of the measured DNA content (Fig. 2) was analysed using a mixture distribution of two Poisson distributions, where the median number of tetraploids, n_4, was estimated to be 19.2, with a 95% credibility interval between 11.6 and 29.1, out of a sample size 439, i.e. a median frequency of tetraploids of 4%.

Generally, *L. perenne* was commonly found in semi-natural dry grasslands, and the analyses of the DNA content of naturally occurring *L. Perenne* showed that a proportion of these were tetraploid and it could not be ruled out that the sampled tetraploid plants originated from the nearby field. Consequently, in contradiction to the earlier observation of Jenkin (1959), we conclude that tetraploid *L. perenne* genotypes are present in semi-natural habitats in non-trivial proportions and that tetraploidy alone will not prevent GM traits from spreading to natural rye grass populations by either invasion or introgression.

It is not possible to make a quantitative risk assessment of the likelihood of the invasion and introgression pathways due to the limited sampling intensity and the lack of land use history data. Furthermore, other farmers in the area have been growing *L. perenne*, and some of these farmers most likely also used tetraploid varieties, which renders the origin of the tetraploid plants uncertain.

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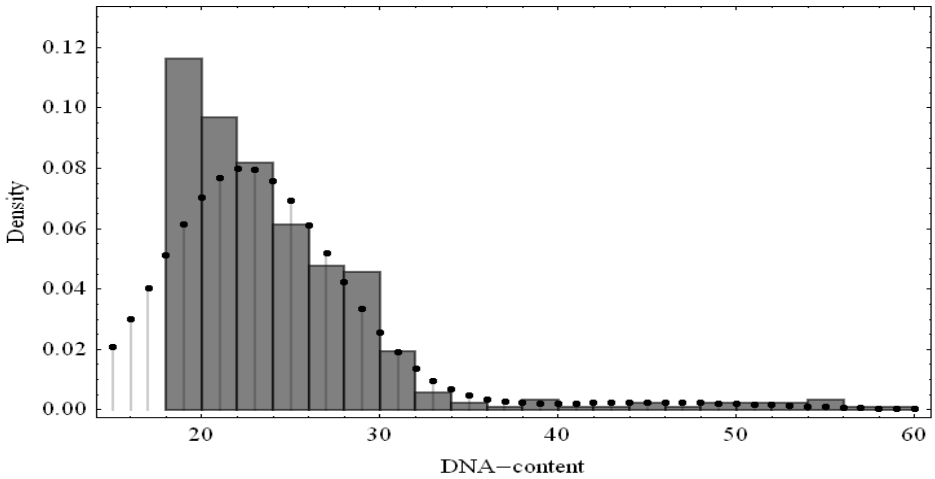
Table 1: Estimated Average Plant Cover of *L. Perenne* in Different Danish Light-Open Natural and Semi-Natural Habitat Types

Habitat code	Habitat type	Cover of *L. perenne* (%)
1330	Atlantic salt meadows	3
2130	Fixed coastal dunes with herbaceous vegetation	0.2
2140	Decalcified fixed dunes with *Empetrum nigrum*	0
2190	Humid dune slacks	0
2250	Coastal dunes with *Juniperus* spp.	0
4010	Northern Atlantic wet heaths with *Erica tetralix*	0
4030	European dry heaths	0.05
6120	Xeric sand calcareous grasslands	4
6210	Semi-natural dry grasslands and scrubland facies on calcareous substrates	12
6230	Species-rich*Nardus* grasslands	7
6410	*Molinia* meadows on calcareous, peaty or clayey-siltladen soils	0.4

7110	Active raised bogs	0
7140	Transition mires and quaking bogs	0
7150	Depressions on peat substrates of the *Rhynchosporion*	0
7210	Calcareous fens with *Cladiummariscus* and species of the *Caricion davallianae*	0
7220	Petrifying springs with tufa formation	0.6
7230	Alkaline fens	0.7

Fig.1. Map of the sampling area along the stream Simested Å (marked with a white line). Sampling was performed along the stream from the field wherethe tetraploid *L. perenne* variety "Tivoli" had been grown for the last thirteen years (marked with blue) down to the NATURA 2000 site (marked with red).

Fig.2. Bimodal distribution of the measured DNA-content of the sampled *L. perenne* leaves; observations are shown as columns, and the points represent the distribution of the expected DNA-content, assuming a mixture distribution of two Poisson distributions.



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