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Assessment of *Lolium perenne* tetraploid clones produced from a diverse diploid breeding population

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage grasses, providing high yields with excellent forage quality. The main limiting factor for increasing the cultivation area of perennial ryegrass in the Nordic-Baltic region is insufficient winter hardiness due to unstable climatic conditions as well as insufficient persistence and drought resistance. Currently, the genetic diversity of perennial ryegrass cultivars is relatively limited; therefore, developing new, highly adaptable germplasm is of high importance in the context of changing climatic conditions.

In the framework of the Nordic-Baltic Public-Private Partnership (PPP) project in pre-breeding of perennial ryegrass, 250 tetraploid plants (hereinafter genotypes), created by chromosome doubling using colchicine at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, were evaluated in open field conditions at the Research Institute of Agronomy of Latvia University of Life Sciences and Technologies. Detailed phenological scoring of all genotypes was performed over a three-year (2016–2018) period. For data analysis, the plants were grouped according to heading time and growth habit. Significant differences among the groups were found in winterhardiness, regrowth rate, development rate of generative shoots, susceptibility to rust, etc. The seed from plants that survived well and showed some promising properties were harvested in the 2nd ley year – a total of 199 genotypes or 358 individuals (80% and 48% of all, respectively).

Results of genotyping of randomly selected genotypes with the highest and lowest winter hardiness showed that the clones were genetically differentiated from the cultivars developed in Baltic countries – unique alleles were found in the tetraploid clones that were not present in the analysed cultivars. This suggests that these developed tetraploid clones or genotypes could provide valuable breeding material to improve the suitability of perennial grass cultivars to local environmental conditions in the future.

Key words: DNA markers, genotype, perennial ryegrass, phenotyping, pre-breeding, cultivar.

Introduction

Milder winters and increase in the length of growing seasons provide opportunities for the cultivation of forage grasses, which have previously been limited in northern latitudes. One of these species is perennial ryegrass (*Lolium perenne* L.), the proportion of which is increasing in seed mixtures used for forage grass production.

Perennial ryegrass is a very important component in forage production in more maritime regions south of 60° N, because of its high dry matter yield, regrowth capacity and excellent forage quality (Kemešytė et al., 2016; Berzins et al., 2018 b). It is a widely used forage species in temperate regions of the world, particularly in Western Europe, including Denmark, Ireland and United Kingdom (Deleuran, Boelt, 2009; Grogan, Gilliland, 2011; McDonagh et al., 2016). Perennial ryegrass is also successfully grown in the southern parts of Sweden in mixtures with other grasses. Norwegian studies have concluded that the inclusion of perennial ryegrass increases yield and digestibility as well as decreases weeds in the first year, both in the warmer southern regions as well as in more northerly regions (Jørgensen et al., 2019). Climate change scenarios predict that in the

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near future, the cultivation range of perennial ryegrass will expand northwards. With its high biomass yield, regrowth capacity and superior feed quality perennial ryegrass will undoubtedly become a promising forage crop at higher latitudes experiencing prolonged growing seasons and milder winters (Helgadóttir et al., 2014).

However, large-scale cultivation of this species in Baltic and Nordic conditions still tends to be relatively risky (Wilkins, Humphreys, 2003; Østrem et al., 2013; Berzins et al., 2015). Perennial ryegrass grown inland and north of 60° N generally shows poor survival because of extensive winter damage (Helgadóttir et al., 2018). Due to unstable wintering conditions in the Baltic region and extreme fluctuations in temperature during spring, perennial ryegrass tends to disappear from swards, especially in the 2nd and subsequent ley years (Lemežienė et al., 2004; Berzins et al., 2018 b). To reduce the risks for ryegrass cultivation in these regions and to expand production into new areas, it is necessary to develop improved perennial ryegrass germplasm and first of all to select a range of traits within different germplasm types, varying in ploidy, heading time, growth habit, etc. The main challenge for breeders is to increase winter hardiness, survival, drought resistance and other traits significant for Northern Europe in the context of climate change (Rognli et al., 2018; Rancane et al., 2019). Therefore, it is important to evaluate the available genetic resources of perennial ryegrass in addition to other breeding tools, e.g., production of tetraploid germplasm.

Drought resistance is also increasing in importance, and phenotyping in combination with genomic tools could improve the assessment of perennial ryegrass breeding material. Plant recovery after drought periods is an important trait, and there is a clear effect of ploidy level on recovery after drought stress (Lee et al., 2019). One explanation for this is that at comparable drought stress levels, tetraploid plants showed signs of senescence one week later than diploid plants. Other possible reasons are differences in stomatal architecture of tetraploid plants, enhanced water use efficiency or accumulation of storage carbohydrates (Westermeier, Hartmann, 2019).

Within the Nordic-Baltic public-private partnership, new and commercial perennial ryegrass populations and genotypes were assessed in differing environmental and climatic conditions. Growing conditions in Northern Europe are unique with different day length, temperature fluctuations, frost and thaw conditions, etc. in comparison to other cultivation regions. Therefore, to increase the cultivation of perennial ryegrass and use in grass mixtures in Northern Europe, germplasm must be selected to be adapted to these specific environmental and climatic conditions (Helgadóttir et al., 2014; 2016; Østrem et al., 2015 a). It would be desirable to breed material with as wide adaptation as possible, so that the material can be used safely in different climatic conditions, especially in view of the rapidly changing wintering conditions in the Nordic-Baltic region. Such an approach would require considerable pre-breeding efforts in line with the already initiated Nordic Public-Private Partnership for pre-breeding in perennial ryegrass (Rognli et al., 2013).

Tetraploid perennial ryegrass genotypes induced from a diploid population were analysed in this study with the aim of obtaining detailed phenotypic information on tetraploid genotypes in different growing seasons. The plants were grouped according to heading time and growth habit. Several other significant phenotypic differences between these groups were identified indicating that this material is promising for further breeding efforts. A subset of individuals was analysed with genetic markers to compare these novel tetraploid genotypes with previously developed Baltic perennial ryegrass cultivars.

Materials and methods

Perennial ryegrass (*Lolium perenne* L.) tetraploids were induced at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry by chromosome doubling of the broad diploid breeding population using colchicine. Previously results indicated that there is high genetic variability in the diploid population, therefore, seeds from four 2nd generation seed lots were randomly selected for doubling. Seed-excised embryos were treated with colchicine with subsequent culture *in vitro* (Dabkevičienė, 2009).

Field trials. Induced tetraploids were evaluated in field trials at the Research Institute of Agronomy (56°37 N, 25°07 E) of Latvia University of Life Sciences and Technologies. In June 2016, 250 tetraploid plantlets (hereinafter genotypes) were placed in pots with peat substrate and grown before cloning for approximately two months. At the end of August, the genotypes were cloned and planted in a completely randomised design in three replicates in sod-podzolic loamy sand soil *Eutric Retisol* (WRB, 2014) with pH_{KCl} 5.7, organic matter 18 g kg^{-1}, plant available phosphorus (P_2O_5) 66 mg kg^{-1} and potassium (K_2O) 69 mg kg^{-1}. Individuals were planted in a 60 × 60 cm grid.

Phenological and phenotypic evaluations were carried out repeatedly over a three-year (2016–2018) period. This study evaluated a number of features: growth habit, leaf width, rust susceptibility (all these features were re-evaluated over the experimental period), winter hardiness, regrowth in spring and after harvesting, tillering density, plant height (in 2017 and 2018), fresh matter per plant (in 2017), dry matter yield and seed weight per plant (in 2018). Phenotypic properties were scored on a 9-point scale with lower scores indicating weaker expression and higher scores showing a more pronounced expression of the trait. For example, a score of 1 for winter hardiness indicated that the individual was very weak and barely survived overwintering, while a score of 9 indicated that the individual did not show any signs of damage during overwintering. Growth habit was assessed according to UPOV (2006) guidelines: 1 – erect (~80–90°), 3 – semi-erect (70–75°), 5 – medium (~45°), 7 – semi prostrate (20–25°), 9 – prostrate (<15°).

Rust susceptibility was assessed according to the EUCARPIA Multisite Rust evaluation methodology (Schubiger et al., 2010). A scale from 1 to 9 was used: 1 – no rust disease, 2 – traces of rust, 3 – 5% of the leaf surface is covered with rust pustules, 4 – 10%, 5 – 25%, 6 – 40%, 7 – 60%, 8 – 75%, 9 – more than 75% of the foliage covered with rust, dominated by necrotic leaves. The rating values represented a relative estimate of leaf area occupied by rust pustules. Rust susceptibility was scored for the first time in 2016, when due to atypically warm and humid weather conditions in mid-September, part of the genotypes were heavily infected shortly after planting in the field. In the following years, the rust susceptibility was scored several times a season, if necessary. During the interpretation of the obtained data, the rust susceptibility was recalculated to the rust resistance, because the task is to select a material with a higher rust resistance.

Regrowth rate was assessed visually by comparing the growth rate of genotypes in spring after vegetation resumption and after mowing. To find out differences between genotypes in terms of plant height increase over a certain period, the tallest stem was measured at the heading–flowering stage (on 21st and 27th of June, 2017), and the differences were calculated. Leaf width and intensity of ear formation at the heading–flowering stage were also assessed visually using a 9-point scale. Heading time i.e. the date, when the first three tillers emerge was fixed. Twice (at 1st and 2nd cut) during the vegetation period in the 1st ley year (2017), an assessment of above ground plant mass was done by cutting each plant individually during full heading. In the 2nd ley year (2018), all surviving plants were cut, and the weight of dried straw and seeds of each plant was determined.

Genetic analysis. Agronomic properties and genetic diversity sub-set of six tetraploid genotypes were compared to five perennial ryegrass cultivars developed in the Baltic countries. Two of the cultivars are tetraploid intermediate '(Elena DS' and 'Raite'), two are tetraploid late type ('Spidola' and 'Raminta'), and one cultivar is diploid intermediate type ('Gunta'). In the spring of the 2nd ley year, three tetraploid genotypes (Vg169, Vg26 and Vg213) were randomly chosen from among the best wintering clones, and three (As204, Vg144 and Vg149) – from the poorest overwintering clones. Dry matter yield, winter hardiness, heading date, ear formation and rust resistance were assessed over four years in continuous sowing for the cultivars and over two years for the tetraploid clones.

Sward cover was assessed in the 4th ley year for the cultivars; an assessment of the overall condition of clones was performed in the 2nd ley year. Genetic analyses were done on 12 individuals from each of five cultivars, and one individual of six selected tetraploid clones (Table 4). DNA was extracted from a leaf sample from a single individual using a CTAB-based method (Porebski et al., 1997). Genotyping was done using eight simple sequence repeat (SSR) markers: G03_020, G05_033, G07_037, G01_053, G07_065, G05_071, G05_088 and G05_099 (Studer et al., 2008). Each forward primer was labelled with a different fluorophore (6-FAM, HEX or TMR) to facilitate visualization using capillary electrophoresis. The polymerase chain reactions (PCRs) were carried out in a 10 µl solution, containing 2 µL Hot FirePol Blend MasterMix (Soltis BioDyne, Estonia) with 10 mM $MgCl_2$, 0.5 µM of each primer, 2 µL (approximately 50 ng) DNA solution. The PCR cycling conditions were: 95°C for 15 min, 40 cycles of 95°C for 20 s, 58°C for 30 s and 72°C for 45 s, followed by 72°C for 5 min. The PCR products were size separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) and genotyped using software *GeneMapper*, version 4.0 (Applied Biosystems). As the analysed tetraploid clones and cultivars were derived from diploid germplasm, the utilised SSR loci were scored and analysed as diploid. Genetic diversity parameters were calculated using software *GenAlEx*, version 6.0 (Peakall, Smouse, 2006). Neighbour-joining trees and consensus trees were constructed using the programs *Neighbour* and *Consense* in PHYLIP (Felsenstein, 1989). Phylogenetic trees were visualized using software *FigTree*, version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Meteorological conditions. During the winter 2017–2018 in general, the temperature was higher than the long-term average, but there were sharp fluctuations: unusually warm conditions in December and January were followed by sharp cold periods in February. As a result of it, the average air temperature in February and March was below long-term average: −3.5°C and −2.1°C, respectively. In the second half of the year 2017, heavy rains followed one another; the monthly precipitation rate was significantly exceeded. The atypically wet season in 2017 was followed by an intensified drought in 2018, when precipitation during the whole vegetation period from May to September was well below the norm – in some months the precipitation was 2–3 times less compared to the long-term average. Along with the limited amount of humidity, long periods of heat prevailed: from April to October the average monthly temperature was +2…+4°C above long-term average (Fig. 1).

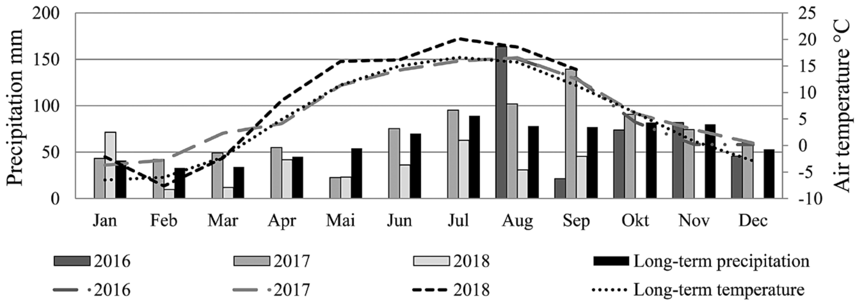

Figure 1. Air temperature and precipitation by months in 2016–2018 and long-term averages

Statistical analysis. The perennial ryegrass genotypes were divided into several groups according to phenological and phenotypic properties. Genotypes were divided into two groups according to heading time calculating from 1st May: intermediate (IM) (flowering prior to day 33, including) and late (L) (flowering after day 34, including). Two groups were defined for growth habit: erect (>45°) (E) and prostrate (<45°) (P). Based on these initial groupings, individuals were further divided into four sub-groups combining heading time and growth habit: intermediate-erect (IM-E), intermediate-prostrate (IM-P), late-erect (L-E) and late-prostrate (L-P). The number of genotypes in each group is not completely identical, as the mean scores were taken into account and genotypes with the same scores were not divided into different groups. All genotypes survived during three years were grouped: a) non-persistent (G0) – genotypes with no seed collected, b) weak persistent (G1) – genotypes with one plant survived, c) well persistent (G2) – genotypes with two plants survived, and d) excellent persistent (G3) – genotypes with all three plants survived. Two groups were formed for further analysis: weak persistent group (G0/G1), which combined non-persistent and weak persistent, and persistent group (G2/G3), which combined well and excellent persistent genotypes. Results were analysed by ANOVA with a significance level of 0.05.

For the analyses of data obtained, the test of statistically significant differences ($LSD_{0.05}$) and the Fisher criterion (*F*-test) were used.

Results and discussion

The tetraploid genotypes were phenologically diverse with flowering time ranging from 28 to 48 days (calculated from the 1st of May). Due to increased rainfall in July and August in trial establishment year (2016), cloning and planting in the field was delayed for almost a month. The winter of 2017 was favourable; therefore, no winter damage in the 1st ley year was found for most of the plants. Environmental conditions in the 2nd ley year were favourable for the assessment of winter hardiness and drought resistance. There were sharp temperature fluctuations during winter and spring and reduced precipitation during vegetation, which was combined with prolonged heat periods (Fig. 1).

To obtain a diverse range of genotypes for breeding, seeds were collected from all individuals that had good or satisfactory winter hardiness. In total, seeds were collected from 196 genotypes (80% of all genotypes), represented by 358 individual plants (48% of all individual plants). Of the 250 assessed genotypes, seeds were collected from 2 or 3 individuals for 121 genotypes (48.5%). Seeds from 76 genotypes (30.5%) were collected from only one individual, as the other two replicates were severely damaged over winter or did not survive due to various reasons. Seeds were not collected from 53 genotypes (21.2%), as they did not survive in any of the replicates (Table 1).

Table 1. Number of perennial ryegrass genotypes by group and averages within groups

Group, number of genotype	Number of genotypes survived for 3 years	Height increase cm	Fresh matter per plant (2017) kg		Average yield per plant survived for 3 years g	
			1st cut	2nd cut	dry matter	seed
IM, 126	103	0.6	0.66	0.39	54.6	11.3
L, 124	93	3.6*	0.77*	0.45*	51.1	10.0
$LSD_{0.05}$		0.81	0.05	0.03	10.13	2.10
E, 126	91	2.0	0.72	0.43	46.2	9.6
P, 124	105	2.2	0.70	0.42	59.4*	11.7*
$LSD_{0.05}$		0.81	0.05	0.03	10.13	2.10
IM-E, 61	46	0.7	0.67	0.4	46.4	10.1
IM-P, 65	57	0.5	0.65	0.38	62.3*	12.4
$LSD_{0.05}$		1.15	0.07	0.05	14.27	2.96
L-E, 65	45	3.4	0.78	0.45	46.1	9.0
L-P, 59	48	3.9	0.76	0.45	56.6	11.0
$LSD_{0.05}$		1.16	0.07	0.05	14.39	2.99
G0, 53	0	3.3	0.69	0.42		
G1, 76	76	1.9	0.69	0.41	64.3	11.6
G2/3, 121	121	1.6	0.74	0.43	68.7*	14.7*
$LSD_{0.05}^{1}$		1.11	0.07	0.05	10.34	2.16
$LSD_{0.05}^{2}$		1.02	0.06	0.04	9.51	1.99
Min		0.5	0.13	0.08	9.1	2.0
Max		14.5	1.54	0.82	333.0	53.5

Note. IM – intermediate, L – late, E – erect, P – prostrate; IM-E – intermediate-erect, IM-P – intermediate-prostrate, L-E – late-erect, L-P – late-prostrate; G0 – non-persistent, G1 – weak persistent, G2 – well persistent, G3 – excellent persistent; least significant difference: $LSD_{0.05}^{1}$ – between groups G0 and G1, $LSD_{0.05}^{2}$ – between groups G0 and G2/3; * – indicate to the significant differences between groups.

Regrowth rate between genotypes varied widely with scores ranging from 1.6 to 8.0 in spring and from 1.8 to 8.7 after mowing. Early genotypes had significantly higher ($p < 0.05$) regrowth intensity in spring and after mowing (Table 2). However, plant growth (height increase) at the end of June (heading of late genotypes, flowering of early genotypes) was significantly higher ($p < 0.05$) in late genotypes due to their longer vegetation period. In addition, late genotypes formed denser shoots and had higher green mass yield at both cuts. Average green mass yield per genotype in the 1st ley year ranged between 0.13–1.54 kg at the 1st cut and 0.08–0.82 kg at the 2nd cut.

High diversity was observed in growth habit from almost erect growing genotypes (scored 2–3) to prostrate types (score 8). The majority of individuals (62%) with shoots growing from the base of tillers at an angle of 45–60° had an intermediate growth habit. One-third (32%) of genotypes had a tendency to form more prostrate sward, and only about 6% of genotypes were rated as erect type with a shoot angle of 70–90°. This range of phenotypic variation enables selection of genotypes for different purposes. For example, more erect types are preferable for mowing, while more prostrate types are useful for grazing and ornamental lawns. In general, earlier genotypes had a more vertical growth habit; however, significant differences in growth habit were not found between the intermediate and late genotype groups (Table 2).

Usually, survival of perennial ryegrass in the first winter is good, and differences between genotypes and cultivars appear in subsequent years (Berzins et al., 2018 a). This was confirmed by previous studies within the Baltic region (Lemežienė et al., 2004; Aavola, 2005) as well as in our experiment. Weak winter hardiness (with a score of <5) was observed in only four genotypes in the 1st year but in 98 genotypes in the 2nd year. The number of genotypes with a winter hardiness score of >7 was 182 and 47 in the 1st and 2nd year, respectively. Prostrate genotypes had significantly better ($p < 0.05$) winter hardiness in the 2nd year (Table 2), and within the prostrate growth habit group late genotypes were more winter hardy. Prostrate genotypes had higher seed yield (Table 1) and higher growth intensity in spring and wider leaves (Table 2).

Of all the genotypes, from which seeds were collected, the largest proportion was from the intermediate-prostrate (IM-P) sub-group. Apparently, genotypes from this group cease growth earlier in the autumn, are better prepared for wintering and, therefore, are more tolerant of temperature fluctuations and other stresses in winter and early spring. It has been observed that non-native species

Table 2. Phenotypic grouping and scores (1–9) of perennial ryegrass genotypes

Group	Growth habit	Density of ears	Leaf width	Winter hardiness		Rust resistance		Regrowth	
				1st year	2nd year	1st year	2nd year	in spring	aftermath
IM	5.6	6.8*	6.8	7.3	5.4	6.5	6.3	5.0*	5.4*
L	5.2	4.9	6.9	7.3	5.1	6.8	6.7	4.6	4.9
$LSD_{0.05}$	0.14	0.36	0.27	0.21	0.40	0.41	0.36	0.37	0.38
E	4.6	5.9	6.7	7.5*	4.9	6.7	6.4	4.6	5.4*
P	6.2*	5.8	7.0*	7.1	5.6*	6.6	6.6	5.1*	5.0
$LSD_{0.05}$	0.14	0.36	0.27	0.21	0.40	0.41	0.36	0.37	0.38
IM-E	4.8	6.9	6.6	7.5*	5.1	6.3	6.1	4.8	5.6
IM-P	6.3*	6.8	6.9	7.2	5.6	6.7	6.5	5.2	5.2
$LSD_{0.05}$	0.20	0.51	0.38	0.29	0.57	0.58	0.51	0.52	0.54
L-E	4.5	4.9	6.7	7.4	4.7	7.0	6.7	4.3	5.1
L-P	6.1*	4.9	7.1*	7.1	5.6*	6.6	6.7	4.9*	4.7
$LSD_{0.05}$	0.20	0.52	0.38	0.29	0.57	0.58	0.52	0.52	0.54
G0	5.1	5.2	6.4	7.2	3.3	7.0	6.7	3.1	5.6
G1	5.4	6.0*	6.6	7.3	5.0	6.5	6.5	4.7*	5.2
G2/3	5.6	6.1*	7.2	7.4	6.2	6.6	6.5	5.6*	4.9
$LSD_{0.05}$[1]	0.34	0.49	0.35	0.28	0.40	0.57	0.50	0.38	0.52
$LSD_{0.05}$[2]	0.31	0.45	0.33	0.26	0.37	0.52	0.46	0.35	0.48
Min	2.0	2.0	3.0	3.2	1.3	2.0	3.2	1.6	1.8
Max	8.0	9.0	9.0	8.7	8.3	9.0	7.8	8.0	8.7

Explanation under Table 1

in the Nordic region do not cease growth in the autumn early enough for successful acclimation to the wintering condition. Studies in Norway (Østrem et al., 2015 b) have shown that in the north, the amount of light could be insufficient to trigger the changes in photosynthetic apparatus that are responsible for growth cessation. It is important to pay more attention to selecting genotypes with photoperiodically-controlled growth cessation in the autumn and a less rapid resumption of development in the spring, especially in the context of expected climate changes in the Nordic-Baltic region.

Genotypes with a more erect growth habit had significantly higher ($p < 0.05$) aftermath regrowth intensity and had denser tillering compared to prostrate forms (Table 2). Tetraploid perennial ryegrass individuals usually have wide, dark green leaves. Approximately 40% of genotypes were characterised as having wide leaves (score >7), and 38 genotypes (15%) had very wide leaves (score >8).

Lengthy dry periods, which influence yield, have become more frequent in recent years. The effect of drought on the growth and development of perennial ryegrass has been previously observed in studies throughout Europe. The general conclusion is that tetraploid cultivars are more drought resistant than diploid cultivars, but that drought resistance in general is low due to the shallow root system of perennial ryegrass (Westermeier et al., 2019). The vegetation period in 2018 was exceptionally hot and dry, which influenced the overall vitality of perennial ryegrass genotypes and fresh yield. Nevertheless, at seed harvest, differences were observed between genotypes for plant height and dry weight, ranging from 9.1 to 333.0 g (Table 1). Weight of dried plant for 47 genotypes (23%) ranged from 9 to 49 g, 129 genotypes (65%) 50–99 g, 19 genotypes (10%) 100–146 g, and for 4 genotypes was over 150 g. Average plant dry weight at seed harvest and seed yield were not significantly different between intermediate and late genotype groups. However, prostrate genotypes had significantly higher ($p < 0.05$) yield of both dry matter and seed compared to erect genotypes. Seed weight ranged from 2 to 53.5 g with 71 genotypes (36%) having low seed yield (2–10 g), 106 genotypes average seed yield (11–20 g) and 22 genotypes (11%) high yield (21–54 g).

Rust infection is a significant problem for perennial ryegrass. Crown rust, caused by *Puccinia coronata*, has a significant impact on yield, feed quality and digestibility; therefore, breeding for resistance is important.

The use of rust resistant parental genotypes in pre-breeding was one of the objectives of this study. The autumn of the year, when clones were established, was atypically warm and moist, and, as a result, some of the clones were heavily infected by rust, which usually does not occur in the first year. However, some of the clones did not show signs of rust infection, and overall rust resistance scores ranged from 2 to 9 (Table 2). No significant differences between groups were identified. The following year, rust resistance of genotypes ranged from 3.2 to 7.8. However, no correlations were identified between the groups or between the two experimental years. This indicates that different rust races are present in different years, against which the resistance of individual genotypes varies greatly, and, therefore, observations for several years and at several locations appear to be the best way to improve rust resistance in perennial ryegrass (Reheul, Ghesquiere, 2006). Studies in Lithuania have concluded that the prevalence and degree of infection of crown rust were strongly influenced by weather conditions during the year of study, but the resistance of locally grown perennial ryegrass cultivars also played an important role (Kemešytė et al., 2019).

Comparison of agronomic properties and genetic diversity of six tetraploid genotypes to five perennial ryegrass cultivars (including four tetraploid and one diploid) developed in the Baltic countries showed some essential differences (Table 3).

Overall, winter hardiness of the cultivars was good – average scores over the four years ranged from 4.5 to 6.9. The 'Elena DS' had a significantly lower ($p < 0.05$) winter hardiness score (4.5) compared to the other cultivars. Nevertheless, this cultivar shows excellent renewal in sward, which is usually characteristic of *Festulolium* hybrids (Berzins et al., 2019). Of the assessed cultivars, 'Elena DS' was the earliest, and it is possible that rapid development in spring enables effective use of moisture collected in soil over winter and efficient renewal of this cultivar. In addition, intensive regrowth of this cultivar after mowing and very intensive culm formation in aftermath compensated for losses caused by weak winter hardiness. Significant differences ($p < 0.05$) between the tetraploid clones were found for winter hardiness (scores ranged from 2.8 to 7.8) as well as heading date (ranging from day 30 to 37). Long-term observations indicate

Table 3. Agronomic properties of perennial ryegrass cultivars and tetraploid clones

Cultivar	Winter hardiness (1–9)[1]	Dry matter yield t ha^{-1}	Heading date[2]	Culms in aftermath (1–9)[3]	Rust resistance (1–9)[4]	Cover (1–9)[5]
Elena DS	4.5	7.65 a	32 b	7.0 a	6.6 a	2.5 c
Gunta	6.2a*	6.12 b	33 b	5.3 b	6.0 a	3.2 c
Raite	6.5 a	6.76 b	35 ab	4.6 b	6.4 a	4.5 ab
Raminta	6.9 a	6.88 ab	36 a	5.9 ab	5.9 a	5.5 a
Spidola	6.3 a	6.26 b	37 a	5.2 b	6.3 a	5.8 a
$LSD_{0.05}$	1.98	1.09	2.7	1.43	1.02	1.07
Clone	**Winter hardiness (1–9)[1]**	**Fresh matter kg plant^{-1}**	**Heading date[2]**	**Culms in aftermath (1–9)[3]**	**Rust resistance (1–9)[4]**	**Overall assessment (1–9)[6]**
As204s0	2.8 c	1.22 b	30 b	5.0 b	6.9 a	1.7 b
Vg169	7.8 a	1.16 b	33 b	8.0 a	5.7 a	8.3 a
Vg144s0	2.8 c	1.28 a	34 ab	3.3 b	6.7 a	1.3 b
Vg26	7.7 a	1.08 b	36 ab	5.7 b	4.5 a	6.3 a
Vg149	4.3 bc	1.76 a	36 ab	8.0 a	5.8 a	4.3 b
Vg213	6.2 ab	1.18 b	37 a	5.0 b	6.2 a	5.3 a
$LSD_{0.05}$	2.64	0.48	3.17	1.94	2.72	3.93

Note. [1] – 1 – very weak, almost all plant/sward is damaged, 9 – excellent, without damage; [2] – when the first three ears have appeared, day from the 1st May; [3] – 1 – absent, 2 – few, 9 – numerous; [4] – 1 – more than 75% of the foliage covered with rust, dominated by necrotic leaves, 9 – no rust disease; [5] – sward cover in the 5th ley year: 1 – about 10%, 9 – about 90%; [6] – 1 – very weak, 1 – excellent; * – different letters indicate significant differences between them; identical letters indicate that there are no significant differences between them; the letter is not placed if the number differs significantly from all of other.

that perennial ryegrass has good overwintering capacity in the 1st year, but in further years winter hardiness and sward cover are dependent on environmental conditions as well as the cultivar (Berzins et al., 2018 b). Average dry matter yield of the cultivars over four years varied from 6.12 to 7.65 t ha^{-1} with the 'Elena DS' producing a significantly higher yield. Average fresh weight of the tetraploid clones in two cuts varied from 1.08 to 1.76 kg per plant with the clone Vg149 producing significantly higher ($p < 0.05$) yield.

Intensive culm formation is characteristic of perennial ryegrass, and the cultivars were assessed to have medium to pronounced culm formation (scores ranged from 4.6 to 7.0). Variation between the tetraploid clones was higher with scores ranging from 3.3 to 8.0, which could be a result of them being assessed for only two seasons or possibly due to higher variation for this trait between the clones. It must be noted that results for the cultivars and clones cannot be compared directly, as the cultivars were assessed in sown sward, while the clones were assessed as individual plants. Therefore, an overall assessment was done of the clones, based on a range of indicators. Scoring was done in a range of 1 to 9 with 1 indicating that the clone was very weak and damaged, and 9 indicating that the clone was exceptional. Clone Vg169 received the best rating.

Significant differences in rust resistance were found between the cultivars and even more pronounced differences between the clones. However, average rust resistance scores over the years did not show such pronounced differences with scores ranging from 5.9 to 6.6 for cultivars and from 4.5 to 6.9 for the tetraploid clones. As mentioned previously, rust races can differ between years, and different genotypes can have differing resistance to various rust races. The identification and selection of rust-resistant individuals as well as the development of rust-resistant populations is one of the important tasks of perennial ryegrass pre-breeding activities.

One of the main disadvantages of perennial ryegrass is low persistence. Therefore, it is important to assess and select germplasm in varying environmental conditions with individuals being subjected to various stresses. In the autumn of the 4th ley year, persistence of the cultivars ranged from 25% to 60% with the Latvian cultivar 'Spidola' showing significantly higher ($p < 0.05$) persistence (a score of 5.8), which is probably a reflection of this cultivar being well adapted to specific local growing conditions in Latvia.

Similarly to the assessment of agronomic properties, genetic analyses were done on 12 individuals from each of five cultivars, but only one individual of each selected tetraploid clone (Table 4). The mean number of alleles (over all loci) in each cultivar was approximately 4 and in most cases was over 1 in the clones. As perennial ryegrass is an outcrossing species, some level of genetic variation is expected even in cultivars. The mean number of effective alleles in the cultivars was lower than that indicating that some of the alleles were of low frequency. The presence of low frequency alleles within cultivars is also reflected in the mean frequency of the unique alleles found in the cultivars. All cultivars had unique alleles ranging from 1 to 5 in each cultivar, but they were of

Table 4. Genetic parameters of perennial ryegrass cultivars and tetraploid clones

Cultivar and clone	Number of analysed individuals	Mean number of alleles over all loci (SE)	Mean effective number of alleles (SE)	Observed heterozygosity (SE)	Total number of unique alleles (mean freq.)
Elena DS	12	4.29 (0.84)	2.50 (0.26)	0.53 (0.09)	5 (0.07)
Gunta	12	4.14 (0.51)	2.68 (0.44)	0.45 (0.08)	4 (0.10)
Raite	12	3.57 (0.57)	2.41 (0.39)	0.49 (0.13)	1 (0.04)
Raminta	12	4.00 (0.69)	2.54 (0.30)	0.60 (0.13)	3 (0.04)
Spidola	12	4.00 (0.53)	2.61 (0.36)	0.56 (0.11)	4 (0.07)
as204s0	1	1.00 (0.22)	–	0.14 (0.14)	2 (0.50)
vg169	1	1.57 (0.30)	–	0.71 (0.18)	0
vs144s0	1	1.29 (0.29)	–	0.43 (0.20)	0
vs26	1	1.29 (0.29)	–	0.43 (0.20)	1 (0.50)
vs149	1	1.43 (0.20)	–	0.43 (0.20)	0
vs213	1	1.43 (0.20)	–	0.43 (0.20)	0

low frequency (mean frequency of unique alleles per cultivar was <0.1). This is probably again a result of the outcrossing nature of perennial ryegrass with a low level of gene flow from other perennial ryegrass germplasm. Unique alleles were also found in the tetraploid clones – 1 in vs26 and 2 in as204s0. All of the unique alleles in the tetraploid clones were heterozygous, i.e. not fixed.

The majority of analysed perennial tetraploid cultivars were genetically similar with only the 'Spidola' clustering separately. The analysed tetraploid clones clustered separately and had larger pairwise genetic distances (Fig. 2).

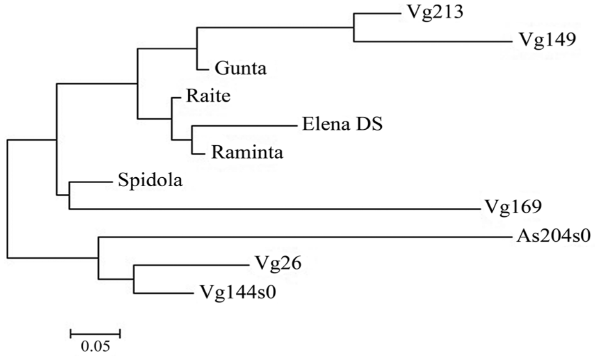

Figure 2. UPGMA dendrogram of Nei genetic distances between analysed perennial ryegrass cultivars and clones

This could partly be due to the fact that only one individual was analysed per clone; however, the clustering indicated that these clones were genetically distinct from the analysed cultivars.

Conclusions

1. The phenotypic assessment of tetraploid perennial ryegrass clones provided valuable information about novel genotypes with very varying properties. Preliminary evaluations show that tetraploid clones could be a valuable source for improving perennial ryegrass winter hardiness as well as rust resistance, which are important indicators for the Northern European region.

2. The genetic analyses indicated that the new tetraploid perennial ryegrass clones are distinct from cultivars developed in the Baltic countries, suggesting that there is sufficient diversity in the artificially generated tetraploid germplasm to provide valuable material for further breeding efforts.

3. The evaluation of tetraploid clones should be continued, because very important step is progeny assessment to determine the heritability of traits. It could help in the future to develop diverse populations with different earliness, growing type, etc. for various uses and conditions.

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Lolium perenne tetraploidinių klonų, sukurtų iš skirtingos diploidinės selekcinės populiacijos, įvertinimas

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Santrauka

Daugiametė svidrė (*Lolium perenne* L.) yra viena svarbiausių pašarinių žolių, duodanti didelį derlių ir puikios kokybės pašarą. Pagrindinis daugiametės svidrės auginimo ploto Šiaurės ir Baltijos regione ribojimo veiksnys yra nepakankamas atsparumas žiemojimui dėl nestabilaus klimato, taip pat nepakankamas išsilaikymas žolyne ir atsparumas sausrai. Šiuo metu daugiametės svidrės veislių genetinė įvairovė yra palyginti ribota, todėl kintančio klimato kontekste labai svarbu sukurti naują, lengvai pritaikomą genetinę medžiagą.

Vykdant Šiaurės ir Baltijos šalių viešojo bei privataus sektoriaus partnerystės projektą, daugiametės svidrės selekcijos pradiniame etape 250 tetraploidinių augalų (toliau – genotipų), sukurtų Lietuvos agrarinių ir miškų mokslų centro Žemdirbystės institute chromosomoms padvigubinti panaudojus kolchiciną, buvo įvertinti lauko sąlygomis Latvijos gyvybės mokslų ir technologijų universiteto Žemės ūkio tyrimų institute. Išsamus fenologinis visų genotipų įvertinimas buvo atliktas per trejus (2016–2018) metus. Duomenims analizuoti augalai buvo sugrupuoti pagal plaukėjimo laiką ir augimo pobūdį. Grupės reikšmingai skyrėsi pagal atsparumą žiemojimui, ataugimo greitį, generatyvinių ūglių vystymosi greitį, jautrumą rūdims ir kt. Antraisiais tyrimo metais buvo surinkta gerai išsilaikiusių augalų, pasižyminčių perspektyviomis savybėmis, sėkla – iš viso 199 genotipų, arba 358 augalų (atitinkamai 80 ir 48 % visų tirtų augalų). Atsitiktinai parinktų genotipų, pasižyminčių didžiausiu ir mažiausiu atsparumu žiemojimui, genotipavimo rezultatai parodė, kad klonai genetiškai skyrėsi nuo Baltijos šalyse sukurtų veislių – tetraploidiniuose klonuose buvo rasti unikalūs aleliai, kurių neturėjo analizuotos veislės.

Tyrimo rezultatai rodo, kad sukurti tetraploidiniai klonai arba genotipai galėtų būti vertinga selekcinė medžiaga, siekiant ateityje pagerinti daugiamečių žolių veislių tinkamumą vietinėms aplinkos sąlygoms.

Reikšminių žodžiai: DNR žymekliai, genotipas, daugiametė svidrė, fenotipavimas, pradinė selekcija, veislė.