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AGRONOMIC TRAIT AND GENETIC ANALYSIS OF LATVIAN FLAX GERMPLASM

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Abstract. *There is a long history of flax cultivation in Latvia, and breeding programs were active until 1970's, when flax breeding in Latvia was halted. Since 1992, the Agriculture Science Centre of Latgale (ASCL) has repatriated Latvian flax germplasm from various genebanks, as well as renewed limited breeding activities in flax. Currently, the ASCL holds a collection of 497 flax accessions, as well as 9865 accessions of various lines and hybrids developed at the LLZC since 1993. To assist in the characterization of this Latvian flax germplasm, we have utilised DNA markers to assess genetic diversity and relatedness, as well as surveying functional polymorphism. We have utilised Simple sequence repeat (SSR) markers developed from both genomic libraries as well as expressed sequences. The results of the DNA marker survey were utilised to determine the genetic polymorphism and relatedness within Latvian flax germplasm, and these results were compared with the analysis of agronomic traits carried out in field trials at the ASCL. The development of DNA markers linked to traits of agronomic importance will assist in the development of a Latvian flax breeding program. The use of DNA marker technology will allow more efficient assessment and rational utilization of Latvian flax germplasm*.

Keywords: *flax breeding, DNA markers, characterisation and evaluation*.

Introduction

During the 1920's and 1930's, flax (*Linum usitatissinum* L.) was grown throughout the territory of the Republic of Latvia. Flax breeding was undertaken at Priekuļu, Stende and Ošupe breeding stations, and many flax varieties were developed, including 'Balva', 'Rota', 'Rota 2', 'Priekuļu 665', 'Priekuļu uzlabotie', 'Ošupe 30', 'Ošupe 31', etc. In the 1970's, flax breeding activities were discontinued in Latvia [1].

Currently, the flax varieties grown in Latvia are foreign varieties, which are not suited to Latvian soil and climatic conditions, and do not ensure stable flax fibre and seed yield. Successful development of the Latvian flax industry requires the creation of new, locally adapted varieties of flax.

Since 1992, the ASCL has actively engaged in research on flax fibre, oil and genetic resources, as well as repatriation, propagation, research, preservation and maintenance of a Latvian flax collection. A large part of the collection comprises of varieties and lines of Latvian origin repatriated from the N. Vavilov All-Russian Plant Institute, the Russian Flax Research Institute, and German gene banks. The ASCL flax collection also contains 497 flax accessions from other countries, including 427 fibre accessions and 70 oil accessions. Flax breeding activities were started at the ASCL in 1993, and accessions with valuable qualitative and quantitative characteristics are used for breeding and hybridisation. The flax collection also contains 9865 lines and hybrids of Latvian origin developed at the ASCL in recent years. Only a small proportion of the resulting hybrids and lines have been evaluated, the other varieties and lines are maintained in the ASCL gene bank.

The use of DNA markers has become widespread in the study of a wide range of crop species. In contrast to phenotypic traits and markers, they have the advantage of not being influenced by environmental conditions; they reveal a higher level of polymorphism; and markers linked to specific genes or quantitative trait loci (QTLs) can be characterized. DNA markers are utilised in characterization of genetic resource collections and have been integrated into

breeding programs for various crop species. While not as widely studied as other crop species, DNA markers have been extensively studied in flax, and a range of molecular tools have been developed and utilised, including SSR markers, genetic linkage maps, QTL studies, microarray and proteomics studies [2; 3, 4, 5].

This study utilised DNA markers to characterize a subset of the best established Latvian oil and fibre flax varieties and lines, and these genotyping results were compared to agronomically important traits measured in field trials of these varieties.

Materials and Methods

The flax material analysed included older Latvian cultivars, which were developed before the 1950's, as well as newly developed breeding lines, derived from crosses between these old Latvian varieties and more modern, foreign cultivars, as well as crosses exclusively between modern, foreign cultivars.

The flax material was planted in the field at the ASCL, and the following agronomic properties assessed for: plant height (total and technical), yield (straw and seed), boll number, seed count, 1000 seed weight, bast content, oil content, unretted bast and shive yield. Agronomic trait values reported in this study were measured in 2010.

Seed material was obtained from the ASCL collection. Seeds were germinated and genomic DNA was extracted from 1-2 week old seedlings using a modified CTAB protocol [6]. Samples were ground in a mortar with 500-800µl 2x CTAB buffer (heated to 65° C), and transfered to 1.5ml centrifuge tubes. After incubation at 65° C for 15-20 min., chloroform (1:1) was added and mixed for 3-5 min. The tubes were centrifuged for 10 min at 13000 rpm., the supernatant tranfered to new tubes and 0.2 volumes 5x CTAB buffer (65° C) added. Samples were gently mixed for 3-5 min and then incubated for 10 min at 65° C. Chloroform (1:1) was added and mixed for 3-5 min., then samples were centrifuged for 10 min at 13000 rpm. Supernatant was transfered to a new tube, 0.7 volumes of isopropanol added, gently mixed and incubated for 20-30 min at room temperature. Tubes were centrifuged for 10 min, the supernatant removed, and the remaning pellet washed twice with 70% ethanol (-20° C), centrifuging for 2-3 min between washes. The pellet was air-dried, and resuspended in 75µl TE buffer. DNA from 6 individuals of each cultivar was extracted and analysed separately.

Nine SSR markers were used to genotype these cultivars (Lu 002, Lu 013, Lu 021, Lu 023, Lu 031, Lu 032, Lu44E4, Lu178, Lu765B) [2, 5, 7]. The forward primer was synthesised with a 6-FAM, HEX or NED fluorescent label to allow visualisation of amplification products on a fluorescent sequencer (Table 1).

SSR locus amplification was carried out using the following PCR conditions: 95ºC for 3 min, 38 cycles of 95 ºC for 30 sec, 55ºC – 30 sec, 72ºC – 30 sec; 72ºC - 10 min; in a total volume of reaction 20µl containing 50 ng template DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U *Taq* polymerase (*Fermentas*), 0.5 mM of forward (labelled) and reverse primers. Amplification fragments were separated on an ABI Prism 3130xl Avant Genetic Analyzer (*Applied Biosystems*) and analyzed with GeneMapper 3.5. Population analyses were performed with GenAlEx version 6.4 [8], and dendrograms constructed using MEGA version 4 [9].

Table 1.

SSR markers utilised in this study

Locus	Primer name	Primer sequence	Repeat motif
LU002[a]	LU002F	AACCGGAACTTTCGGCTGAG	$(TC)_{18}$
	LU002R	GGTTGGAGTAATCGCCGGAG	
LU013[a]	LU013F	TGTGCCAATAGCCATGTGAG	$(AC)_4(AG)_{18}$
	LU013R	GTATGGCTTCCTATGGGCTAAC	
LU021[a]	LU021F	CCGAGTCCGAAAGAATCTGG	$(GA)_{15}(A)_4$
	LU021R	CAGCTCCCATTGTTGTTCCC	
LU023[a]	LU023F	CATGACCATGTGATTAGCATCG	$(CA)_8(GA)_{22}$
	LU023R	CATAGGAGGTGGGTTGCTGC	
LU031[a]	LU031F	CACGAATCTCTCCCAGACA	$(TC)_8$
	LU031R	GAGAGTTTAGGGTATGCACTGA	
LU032[a]	LU032F	GAAAAGGAAGGCTTAGAAGAAGAAG	$(AG)_{10}$
	LU032R	AGTTTCTCAATACACAGATCGAAGG	
Lu44E4[b]	Lu44E4F	TCCTCAACCCACCACCTAAA	Not reported
	Lu44E4R	ATCGTGCAGTCTTGCAACC	
Lu765B[c]	Lu765BF	CCTCATTCCGCTCAGCAA	$(TTC)_8$
	Lu765BR	CGAAAATGGGGAAGATGATG	
Lu178[c]	Lu178F	CAAGGGAGTGCTGCTCTGAT	$(TC)_9$
	Lu178R	ATTGCGAGCAACAACAACAG	

Cited in: a – [2], b – [7], c – [5].

Results and discussion

SSR marker analysis

The number of alleles detected by the 9 analysed markers ranged from 4-16 (average 8.56); however, the distribution of these alleles was uneven, as seen by the low figures for the effective number of alleles, which ranged from 1.13-4.58 (average 2.68). The observed heterozygosity ranged from 0.00-0.95 (average 0.42), while the expected heterozygosity ranged from 0.12-0.78 (average 0.55). The inbreeding coefficient *F*, ranged from -0.58-1.00 (average 0.23), indicating an overall excess of homozygotes, which is to be expected from a mainly inbreeding species, that has been homogenised by line selection over multiple generations. One interesting observation was that the SSR markers developed from expressed portions of the genome (Lu178 and Lu765B) were the most highly polymorphic markers (allele numbers were 10 and 16, respectively). However, most of these alleles were of low frequency, as reflected in the number of effective alleles (1.93 and 3.44, respectively) (Table 2).

The level of genetic polymorphism was compared between the old cultivars and the new lines. The mean number of alleles in the cultivars was 4.56, while in the lines then mean number of alleles was 7.44. This difference was a function of the number of individuals analysed, with 121 individuals from the lines analysed, and only 33 from the cultivars. When 33 individuals were randomly chosen from the lines, the mean number of alleles was similar (4.67 in the lines, 4.56 in the cultivars). Analysing the full set of data, the other measures of genetic diversity were similar between the cultivars and lines. The number of alleles with a frequency over 5% was 2.67 and 3.00 respectively, the number of effective alleles was 2.44 and 2.46, and the expected heterozygosity was 0.52 in both groups. The Nei genetic distance between

all analysed individuals was calculated, and a principal co-ordinates analysis plot calculated (Fig. 1). It can be seen that there is no clear differentiation between the cultivars and lines, and that the genetic diversity of the old cultivars has been retained in the newly developed lines. This low differentiation between the two groups was confirmed by analysis of molecular variance (AMOVA), where 95% of the genetic variance was within the groups, and only 5% between ($p \leq 0.001$).

Table 2.

Genetic parameters of analysed SSR markers

Locus	LU002	LU013	LU032	Lu21	Lu23	Lu31	Lu44E4	Lu178	Lu765B	Average over loci
Number of alleles	5	9	7	5	7	4	14	10	16	8.556
Effective number of alleles	1.539	4.581	2.360	1.130	4.426	2.247	2.502	1.929	3.441	2.684
Observed heterozygosity	0.333	0.632	0.730	0.103	0.769	0.000	0.946	0.149	0.113	0.419
Expected heterozygosity	0.350	0.782	0.576	0.115	0.774	0.555	0.600	0.481	0.709	0.549
Inbreeding coefficient	0.049	0.191	-0.266	0.107	0.006	1.000	-0.576	0.691	0.840	0.227

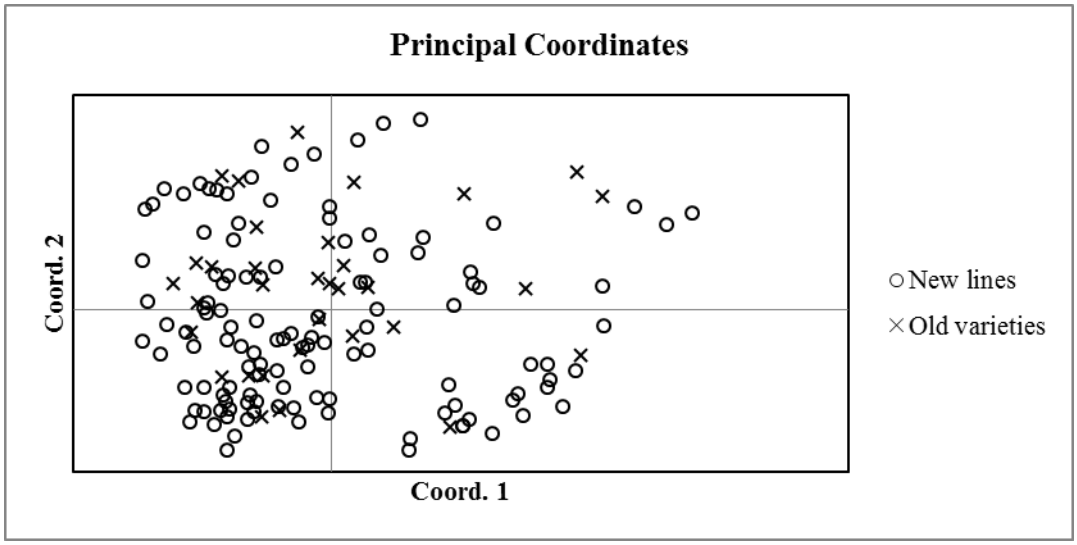

Fig. 1. **Principal coordinate analysis of analysed flax accessions**

The pairwise Nei genetic distance between the cultivars and lines was calculated, and a dendrogram constructed using the Neighbour-Joining algorithm (Fig. 2). No distinct clusters were identified within the dendrogram, however, lines derived from the same cross tended to cluster closely to each other (but not always), and the old varieties were found to cluster together. This lack of defined clusters within the dendrogram is probably a reflection of the fairly low level of genetic polymorphism, and the low differentiation between the lines and cultivars

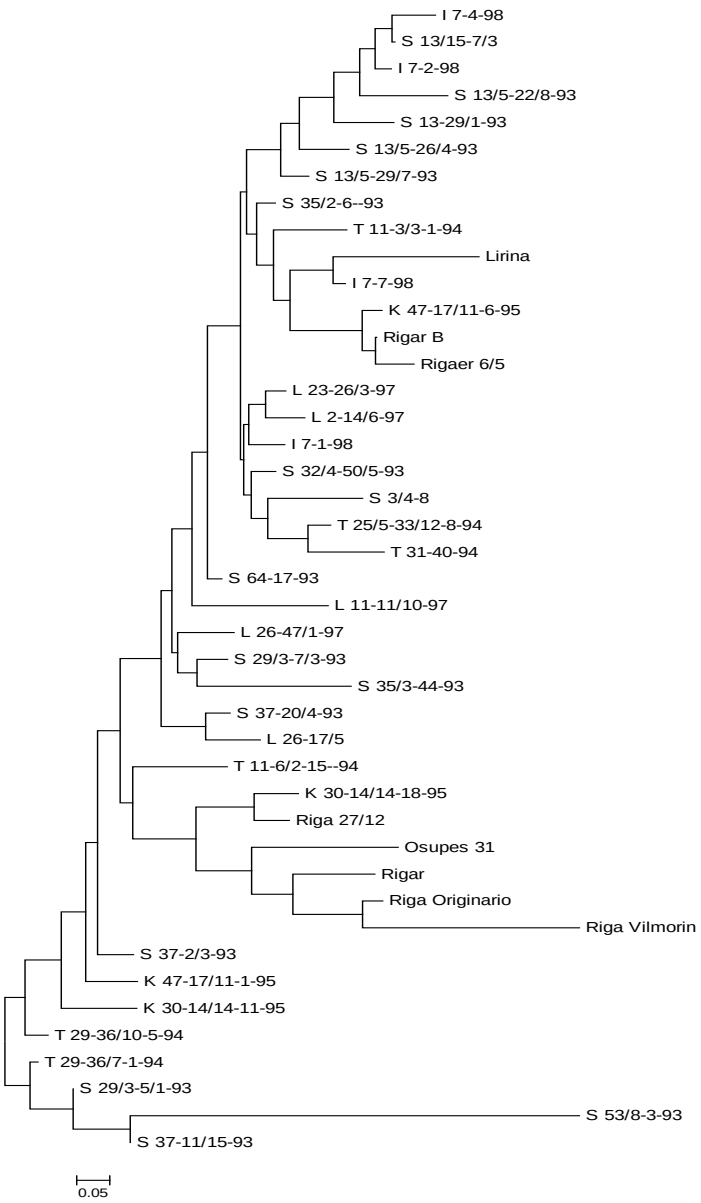

Fig. 2. **Neighbour-joining dendrogram of analysed flax accessions**

Correlation of genetic analysis with agronomic traits

The lines included in this study were selected for having agronomic properties that are desirable, and that could be potentially utilised in the development of new, locally adapted varieties. Therefore, the agronomic trait values in the lines usually exceeded those of the control varieties (Vega2 and Lirina), particularly for fibre-related traits. Examination and ordering of the agronomic trait data showed that in some cases, lines derived from the same cross yielded the best agronomic trait scores. In particular, lines derived from the cross Blakitnij x Ošupes 31 (K 30) and M-12 x Eva (S 37), were consistently ranked in the top five best lines, especially with regard to fibre traits. The agronomically best performing lines do not show any particular clustering within the dendrogram, and they are not distinct when considering the measures of genetic diversity such as the number of alleles, the number of effective alleles and heterozygosity. The old varieties tended to show better results for such parameters as seed yield, boll number and 1000 seed weight (Table 3).

Table 3.

Agronomic trait data from 2010 field trials

Variety or line	Plant height, cm		Yield, g/m²		Boll number	Seed count	1000 seed weight	Bast content	Oil content	Unretted bast	Unretted shives
	total	technical	straw	seed	per plant	per boll	g	%	% dry weight	g/m²	g/m²
I7-1-98	84.2	71.9	597	128.7	5.9	8.8	4.4	27.7	43.2	260	260
I7-2-98	85.1	74	617	142.1	7.1	8.8	4.41	32.3	42	260	270
I7-4-98	89.2	78.3	540	126.1	6.9	8.5	4.28	32.7	42.2	210	250
I7-7-98	83.9	72.2	623	117.6	5.8	9	4.15	31.5	40.2	220	240
K30-14/14/-11-95	88.9	78.2	637	96.8	6.1	8.9	3.95	35.3	46.9	290	270
K30-14/14-18-95	91.6	81.7	630	72.4	6.4	8.8	3.95	32.6	47.1	260	300
K47-17/11-1-95	80.6	68.8	553	115.9	7.2	8.6	4.08	34.8	46.6	210	200
K47-17/11-6-95	80.9	68.7	537	112.1	8.6	8.3	4.1	32.7	44.7	200	200
L11-11/10-97	74.7	64.3	510	126.9	8	9.1	4.72	30.9	45.6	159.8	190
L2-14/6-97	72.7	61.6	377	99.3	6.5	8.2	3.95	30.6	42.9	170	170
L23-26/3-97	78.6	65.4	410	82	7.9	8.3	3.73	32.5	41.6	116.3	160
L26-47/1-97	79.1	68.4	407	80	6.7	8.2	3.33	31.8	45.6	190	180
S 13/15-7/3-93	79.4	69.7	5.67	81.2	6.6	9.3	4.06	29.5	43.3	26.0	32.0
S13/5-22/8-93	79.2	67.8	497	62.1	8	9	3.72	25.3	42.3	127.8	190
S13/5-26/4-93	78.3	68.4	480	97.3	6.3	8.9	3.75	32.6	42.8	173.1	190
S13/5-29/7-93	79.3	70.1	453	98.7	5.5	8.9	3.8	28.7	42.5	163.4	170
S13-29/1-93	79.2	70.3	483	77.1	5.6	8.3	3.85	29.1	42.5	190	230
S29/3-5/1-93	72.4	62.4	507	82.9	6.1	8.2	3.9	36.8	43.8	172.8	210
S29/3-7/3-93	80.5	69.8	607	79.7	6.7	8.9	3.86	35.6	43.7	220	250
S32/4-8-93	76.6	63.9	420	75.7	8.2	8.6	3.96	32.5	42.6	210	200
S32/4-50-93	72.1	60.2	400	71.8	8.1	8.7	3.97	35.8	43.3	127.9	130
S35/2-6-93	72.5	61.4	417	102.9	5.9	8.5	3.41	34.1	44.6	210	160
S35/3-44-93	76.8	65.7	410	76.8	7.6	8.9	2.63	29.5	43.7	136.1	200
S37-11/15-93	86.2	73.8	543	116.6	7.1	9	4.56	38.7	44.8	175.1	190
S37-2/3-93	78.3	66.2	507	77.9	7	8.8	4.18	38.4	43	190	220
S37-20/4-93	82.7	71.4	557	72.9	6	8.5	4.56	36.1	44.6	210	220
S53/8-3-93	80.7	71.2	507	91	7.1	8.3	3.92	33.4	44.8	200	150
S64-17-93	81.5	70.7	587	135.2	7.4	8.9	4.56	30.2	44.5	250	290
T11-13/3-1-94	77.9	68	520	98.9	5.5	8.5	4.23	32.1	43.2	210	190
T11-6/2-15-94	77.5	66.4	417	110.6	7.5	8.6	3.84	35.3	41.2	190	170
T25/5-33/12-8-94	85.4	73.1	430	65.5	5.8	8.6	3.61	31.1	44.9	240	140
T29-36/10-5-94	79.2	69.6	573	83.4	4.8	8.8	4.17	34	42.9	230	170
T29-36/7-1-94	82.4	70.7	577	97.1	6.7	9.1	4.47	33.1	44.1	250	150
T31-40-94	83	69.8	480	102.7	8	9.1	3.71	33.1	45.2	210	150
Vega 2 (standard)	71.8	61.6	500	108.6	5.6	8.1	4.59	30.1	45.4	151.4	170
Ošupes 31	70.9	59.5	537	113.4	8.5	8.2	3.77	28.6	42	190	210
Riga 27/12	57.7	45.6	373	114.3	7.7	7.9	4.1	32.3	42.3	137.7	170
Riga Originario	59.9	47.5	460	161.3	5.7	8.2	5.25	31.1	44.1	165.9	200
Riga Vilmorin	62	51.4	397	84.8	6.9	8.6	4.73	31.2	42.7	159.9	200
Rigaer 6/5	65.6	51.3	390	139.5	5.7	8.2	4.94	31.9	44.4	161.9	190
Rigar	61.2	48.9	360	122.4	6.8	7.7	5.86	29.2	45	110.3	130
Rigar B	66	53.5	410	93.7	7.1	8.2	4.49	31.5	44.3	148.2	130

Trait values in shaded cells exceed that of standard varieties (Vega 2 and Lirina).
Lines with identical initial letter/number combinations are derived from a single cross

The level of genetic polymorphism identified with the utilised SSR markers was modest. While the number of alleles detected with the analysed SSR markers is not extremely low, the distribution of the alleles is very uneven, with only 2 or 3 alleles with a frequency above 5% found at most loci. A fairly low level of polymorphism is found in many crop species, which have been subjected to intensive selection pressures in breeding programs. There was very little genetic differentiation found between the old cultivars and the newly developed lines. This is a reflection of the fact that some of the lines are derived from crosses between these old Latvian cultivars, and more modern foreign cultivars. However, some of the new lines are derived entirely from crosses between foreign cultivars, and these lines are also not genetically differentiated from the old Latvian cultivars. This indicates the narrow genetic base of cultivated flax in general, which has been reported previously [2, 5].

An interesting observation was that the two SSR markers derived from expressed gene sequences (EST-SSRs) (Lu178 and Lu765B) were the most highly polymorphic markers in term of the number of alleles detected. This is surprising, as SSR markers derived from expressed sequences have often been reported to detect a lower level of genetic polymorphism in comparison to SSR markers derived from genomic sequences [10]. This is presumed to be a result of evolutionary constraints upon these repeat sequences due to their proximity to coding sequences. It would be interesting to survey larger amount of flax SSRs derived from expressed sequence to determine if this tendency is maintained.

There was genetic polymorphism detected within all lines and cultivars surveyed. This may be a consequence of the type of flax material included in this study – old cultivars dating from the first half of the 20^{th} century, and breeding lines. It is interesting that most of the alleles detected were of a low frequency, however, they were often found in multiple individuals from a particular line or cultivar, indicating that these rare alleles are probably due to remaining heterozygosity within these lines and cultivars, and not to pollen contamination from other flax lines and cultivars. It is not surprising to find such intra-varietal variation within older cultivars, which were not bred to such strict uniformity as required by modern cultivar registration regulations. Furthermore, this genetic heterogeneity is to be expected in breeding lines, which have not been fully homogenised through many generations of self-pollination.

That fact that the agronomically best performing lines did not show an increased degree of genetic similarity (except for those lines derived from a single cross), can be explained by the fact that the agronomic traits measured are complex, and are probably influenced by a large number of genes. In addition, the dendrogram was not particularly well-resolved, probably due to the low level of genetic polymorphism. Lines derived from the same cross did not always cluster together (e.g. lines derived from the cross I7, S37 and K47). The SSR markers utilised in this study will be useful for monitoring the overall genetic diversity within Latvian flax germplasm and breeding programs. In order to find markers that are linked to traits of agronomic interest, it will be necessary to develop markers based on functional regions of the genome, such as polymorphisms within expressed genes and gene copy number variations. Further work in this area will be the assessment of polymorphism in candidate genes that have been identified from previously published microarray and proteomic studies of fibre development in flax.

Conclusions

This study was an initial survey of the genetic polymorphism found within the Latvian flax collection using SSR markers. The data obtained will provide a baseline for further characterization and assessment of Latvian flax germplasm. Use of these SSR markers to further investigate the accessions in the flax collection held at the ASCL which have yet to be

characterised will enable genetically unique and differentiated germplasm to be identified, thus making process of screening for agronomically important traits more efficient.

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Anotācija. *Latvijā jau izsenis ir kultivēti lini, un linu selekcija Latvijā tika veikta līdz 20. gadsimta septiņdesmitajiem gadiem, līdz tā tika apturēta. Kopš 1992. gada linu selekciju Latvijā ir atjaunojis Latgales lauksaimniecības zinātnes centrs (LLZC) un veicis Latvijas linu šķirņu sēklu repatriāciju no vairākām pasaules gēnu bankām. LLZC šobrīd atrodas kolekcija, kurā ir 497 šķirnes un 9865 līnijas un hibrīdi, kas izveidoti kopš 1993. gada. Latvijas linu raksturošanai mēs izmantojām DNS marķierus, ģenētiskās daudzveidības un līniju savstarpējās radniecības izvērtēšanai, kā arī funkcionālā polimorfisma izpētei. Mēs esam izmantojuši mikrosatelītu (SSR) marķierus, kuri izveidoti no genomiskām un ekspresētām DNS sekvencēm. Ar DNS marķieriem iegūtie rezultāti tika izmantoti, lai noteiktu Latvijas linu ģenētisko polimorfismu un ģenētisko radniecību. Iegūtie dati tika salīdzināti ar LLZC veiktajiem lauka izmēģinājumu rezultātiem. Ar agronomiski nozīmīgām īpašībām saistītu DNS marķieru lietošana palīdzēs linu selekcijai Latvijā. DNS marķieru izmantošana palīdzēs racionālāk un efektīvāk izvērtēt linu selekcijas materiālu, un varēs atbalstīt un attīstīt linu selekciju Latvijā.*

Atslēgas vārdi: *linu selekcija, DNS marķieri, aprakstīšana un izvērtēšana*.