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Genetic and Phenotypic Diversity of the Sunflower Collection of the Pustovoit All-Russia Research Institute of Oil Crops (VNIIMK)

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Abstract: Publicly supported collections of cultivated germplasm are one of the key sources of new genes for crop improvement. VNIIMK is the leading organization in oil and essential oil crop breeding and seed growing in the Russian Federation with more than a century-long history. Sunflower varieties created by V.S. Pustovoit at VNIIMK became the basis for the development of the modern

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sunflower varieties worldwide. In the present study, 186 sunflower lines from the VNIIMK collection were characterized based on their genotype and general morphological and phenological economically-important traits. Additionally, for 99 sunflower lines fatty acid content, seed oil content, seed husk content, 100-seed weight, and seed number in the head were determined. Sequencing of RAD-libraries and the subsequent analysis have identified 65,553 variants including SNPs and indels. LD analysis revealed substantial variability across the genome. The longest LD blocks (>5,000 Kb) were found in the linkage groups 1, 5, and 17. The analysis revealed significant genetic and phenotypic diversity of the VNIIMK sunflower collection. Novel significant associations with linolenic acid content in the seeds were found on LGs 8, 9, and 17.

Keywords: GWAS, germplasm collections, sunflower lines, fatty acid composition, VNIIMK

Introduction

Publicly supported collections of cultivated germplasm are one of the key sources of new genes for crop improvement. Sunflower seed collections exist in Argentina, Canada, China, France, India, Russia, Serbia, Spain, United States, and some other countries, with the USDA – NPGS (Marek *et al*., 2004) and VIR (Gavrilova *et al*., 2014) being the two major sources of sunflower germplasm in the world.

Apart from VIR, sunflower seed collection is hold by the Pustovoit All-Russia Research Institute of Oil Crops (VNIIMK) (Demurin, 2003; Demurin and Borisenko, 2011; Gontcharov, 2012). VNIIMK is the leading organization in oil and essential oil crop breeding and seed growing in the Russian Federation with more than a century-long history. Growing sunflower as an oilseed crop started in the Russian Empire in the 1830s soon after D.S. Bokarev developed the technique of oil extraction from sunflower seeds (Pustovoit, 1990). Systematic sunflower breeding began in Russia in the early twentieth century. Kruglik Trial Station in Krasnodar was founded in 1912 and became one of the most important centers of sunflower breeding. Vasily S. Pustovoit was the founder and the leader of the experimental station. Later on, All-Russia Research Institute of Oil Crops was established on the basis of the Kruglik Trial Station. V.S. Pustovoit has carried out substantial work which resulted in the creation of new sunflower varieties with high yield and increased oil content. Starting with sunflower which contained only 28–32 % of oil, he obtained varieties with the oil content of more than 50 %. Among these varieties, the best-known ones are Peredovik,

VNIIMK 8931, Majak, and others. They became the basis for the development of the modern oil sunflower varieties worldwide.

Currently, VNIIMK is an important center of study and domestic breeding of oil crops in Russia. VNIIMK sunflower collection includes both the inbred lines created in the institute and the samples from the world gene banks. Notwithstanding intensive research and crop improvement work carried out in VNIIMK, a significant part of the collection has not yet been analyzed using the advanced high-throughput sequencing techniques. In this view, the objective of our study was to analyze the genotypes and phenotypes of 186 VNIIMK sunflower lines of different origin.

Materials and methods

Plant material

186 inbred *Helianthus annuus* L. lines of different origin from the VNIIMK collection were taken into the study (Supplementary Table 1). They included both the lines developed at VNIIMK and the lines from other institutions.

Genotyping

Genomic DNA was extracted from the chlorophyll-free sprouts after one week of germination without light. 100 mg of tissue for each sample was grounded using the FastPrep-96™ Automated Homogenizer (MP Biomedicals, United States). DNA was extracted using the NucleoSpin® Plant II plant DNA extraction kit (Macherey-Nagel, Germany) according to the manufacturer's recommendations and stored at −20 °C. The quality of the purified DNA samples and DNA concentration were assessed by gel electrophoresis and Qubit 3.0 Fluorometer (ThermoFisher Sscientific).

RAD-libraries were prepared using HindIII and Nlalll endonucleases as previously described (Zhigunov *et al.*, 2017) and sequenced in Illumina HiSeq4000.

Preprocessing of raw reads was performed using the Trimmomatic software (version 0.30) (Bolger *et al.*, 2014), after which 552,481,747 of good barcoded single-end reads were used as an input for the Tassel pipeline (version 5.0) (Glaubitz *et al.*, 2014) with k-mer length 65 bp. Additional filtering options were applied during variant calling: -mnMAF 0.01, -minMAPQ 10, and -mnQS 20. Phylogeny reconstruction was carried out using the Mega

software (version 7) (Kumar *et al.*, 2016) based on the SNP data matrix. For SNP marker extraction bcftools (version 1.9) was used. To identify linkage disequilibrium (LD) blocks in the genome sequences of the analyzed sunflower samples, Plink (version 1.9) (Chang *et al.*, 2015) was utilized. The variants with the minor allele frequency less than 5% were ignored in LD analysis. Maximum LD block length was limited to 246,316 Kb. Statistical analysis using the mixed linear models (MLMs) (Zhang *et al.*, 2010) implemented in the Tassel software was performed for association mapping with MDS and kinship matrixes as covariates. Multiallelic variants and the ones with high missing call rates were filtered out prior to the analysis. Sunflower samples with a large number of missing calls were excluded as well. Significant loci were identified based on Bonferroni adjusted p-values. Genome-wide association study (GWAS) results were visualized with the aid of the qqman R package (Turner, 2014).

Phenotyping

Plants were grown in field in the middle part of the Krasnodar Region, GPS coordinates 45°04′50″ N and 39°04′57″ E. Soils of the leached black earth soil type. Sunflower was sowed following the preceding crop, fall wheat, at the seeding rate of 40,000 plants per hectare in May 3–4, 2017. Sowing was carried out according to the following sowing system: 70 × 35 cm, a single plant per a planting pit. Farming techniques, as commonly used for sunflower. Each line was grown on the plot with the area of 9.1 m^2 in two replications.

The following breeding goal traits were registered in field in August:

1. Plant height (stem length from the soil level to the flower head);
2. Diameter of the flower head (distance between the flower head margins which passes through its center);
3. Stem branching (presence of lateral branches of the 1st and higher order);
4. Presence of pollen (male fertility of disk florets);
5. Presence of ray florets (pollinator attraction factor).

While the following biochemical and morphological seed traits were analyzed after harvesting:

1. Seed oil content
2. Seed husk content
3. 100-seed weight
4. Seed number in the head
5. Fatty acid composition

Phenotyping of each inbred sunflower line in field was performed for 20 typical plants. Plants with the obvious damage caused by biotic (diseases, insects, or birds) and abiotic (flooding or anthropogenic traumas) stressors were not included in the study. Weather conditions during the vegetation period were characterized as generally favorable.

Plant height was measured with a ruler as a stem length from the soil level to the flower head at the end of the flowering period, which is considered the optimal developmental stage for this kind of measurements since stem growth has already finished, but stem still retains its flexibility. Flower head diameter was also measured after the plant growth was completed as the distance between the head margins through the center. In the case of stem branching, the central head was measured.

Plants demonstrating all the multiple-head types described in sunflower, such as general, apical, and basal branching, including the "Neptune" morphotype with several lower lateral branches, were considered as branched plants. The presence of pollen was visually determined during the flowering period as male fertility of disk florets (DF) (normally androgyne). In the case of male sterility, anthers appeared to be reduced, light-colored, and lacking bright yellow pollen. In the case of fertile genotypes, pollen appeared during the primary hours (between 8 and 12 a.m.) when anthers emerge from the anther tube. The presence of ray (marginal, sterile) florets (RF), as one of the attraction factors for pollinators and a clear morphological marker character, was registered during the flowering period by the presence of clearly-detectable petals of ray flowers around the head margin.

During phenological observations, the following indices were registered: planting-budding period (DTB), i. e. the number of days from the planting date to the development of primordial flower head ("star"); planting-flowering period (DTF), the number of days from planting date to 50 % of plants in blossom; planting-physiological maturity period (DTM), i. e. the number of days from the planting date to 50 % of flower heads becoming yellow.

The data on climatic conditions during the year 2017 and previous 5 years were obtained on the basis of the climatic records of the Kruglik meteostation (Krasnodar, Russia) readily available at https://rp5.ru. HU accumulations between the planting date and physiological maturity dates were calculated according to (Kaya *et al.*, 2004).

Seed oil content, seed husk content, 100-seed weight, and seed number in the head were assesed using seeds obtained by open pollination. Three flower head were used for each line. Seed oil content was determined using the AMB-1006 M NMR analizer in the sample containing 20 g of seed in replication. Seed husk content was determined in a sample containing 10 g of seeds in replication. 100-seed weight was determined using a sampling containing 1000 seeds.

For the analysis of FA composition about 4–5 g of sunflower seeds were homogenized and mixed. 0.5 g was taken for fatty acid (FA) extraction with 4 mL of hexane. To obtain methyl esters of fatty acids, the mixture in 2–3 mL of hexane was transferred into a new tube and 0.1 mL of sodium methylate was added and mixed intensively for two minutes. The tube content was further transferred onto the paper filter with Na_2SO_4 on the bottom. The obtained filtrate was then placed into the DAG-2 M automatic dispenser tube. Gas chromatography with flame ionization detection (GC-FID) was carried out in the Chromateck-Crystal 5000 GC chromatograph with the DAG-2 M automatic dispenser. GC separation was performed in a SolGelWax column with the dimensions of 30 mm × 0.25 mm × 0.5 um, mobile gas phase – helium, speed – 25 cm/sec, and temperature range – 185–230 °C. FA detection was performed based on retention time using FA methyl ester standards (Fluke). The percentage of each FA was calculated based on the peak area with the aid of the GS chromatograph software.

Results and discussion

In the present study, 186 sunflower lines from the VNIIMK collection were characterized based on their genotype and general morphological and phenological economically-important traits such as flower head diameter, stem branching, presence of pollen, presence of ray petals, length of the planting date to budding (DTB) period, length of the planting date to flowering (DTF) period, and length of the planting date to physiological maturity period. For 99 lines, Seed oil content, seed husk content, 100-seed weight, seed number in the head, and fatty acid content in the seeds was determined.

Genotyping

Sequencing of RAD-libraries and subsequent analysis have identified 65,553 variants including SNPs and indels in 186 sunflower lines. Overall transitions to transversions ratio was 1.73.

LD analysis revealed substantial variability across the genome. Mean, median, and the 1st and 3rd quartiles of LD block length distribution were 110.517 Kb, 0.053 Kb, and 0.021 Kb and 69.132 Kb, respectively. The longest LD blocks (> 5,000 Kb) were found in the linkage groups 1, 5, and 17 with the maximum LD block length of 6156.150 Kb in the linkage group 5. This observation is in a good agreement with the previous studies (Mandel *et al.*, 2013), where the authors

accounted their observations for by the proximity of the above-mentioned LD blocks to the genes or QTLs associated with the traits that served as selection targets during sunflower domestication and/or improvement.

Phylogeny reconstruction was carried out using the Mega software (version 7) based on the SNPs data matrix.

As it can be seen in Figure 1, VNIIMK selection lines are present in all clades on the obtained tree. It can be also observed that certain samples are similar to the samples from VIR and the United States and group with them. For example, sample s54 (line L 2586) from VNIIMK fall in the same clade with s477 (RHA 298)

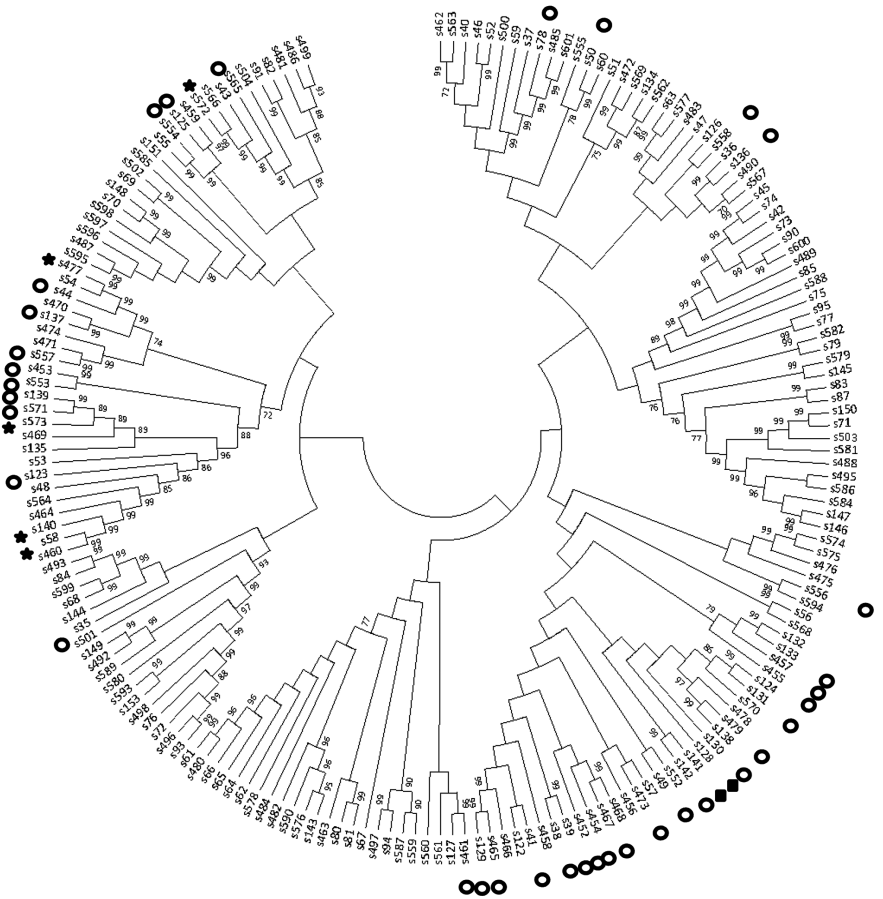

Figure 1: Tree of 186 VNIIMK sunflower lines constructed based on the SNP data. The origin of lines: circles– VIR, stars- USDA, squares–NS, unmarked – VNIIMK.

from USDA and s44 (VIR 391) from VIR. At the same time, some clades are formed solely by VNIIMK samples. Therefore, the obtained data illustrate high genetic diversity and uniqueness of the VNIIMK collection.

Phenotyping

For phenotypic data collection, plants were grown in field in the middle part of the Krasnodar Region during the year 2017.

Water scarcity is one of the critical limiting factors for sunflower growing in the Krasnodar Region. The areas on which VNIIMK experimental fields are located can be characterized as sub-humid, with the precipitation amount averaging to 738 mm in 2013–2016. During the sunflower growth period, the average rainfall is about 274 mm, and precipitation deficit can often be observed during the seed germination period. However, during the experimental period in May 2017, the rainfall was by 104 % higher, although later precipitation amount did not exceed 78 % of the average for the last 5 years, while in August it decreased to a minimum of 35 % of the average (Figure 2).

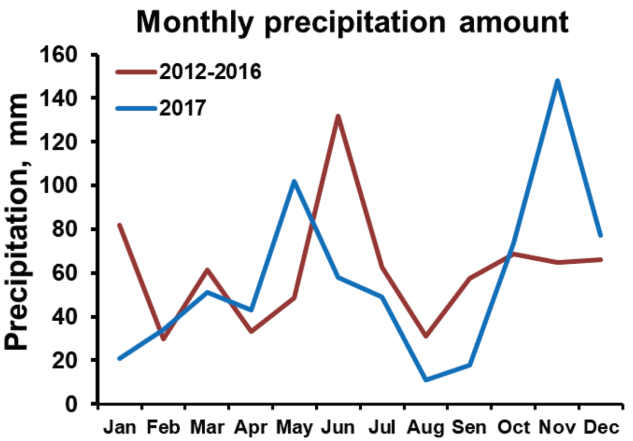

Figure 2: Monthly precipitation amount.

Temperature is another important factor affecting sunflower growth and development. The average annual temperature in 2012–2016 was + 13.6°C, with the average daily minimum of 16.1°C being observed in January, and the maximum of + 30.8 °C, in July (Figure 3).

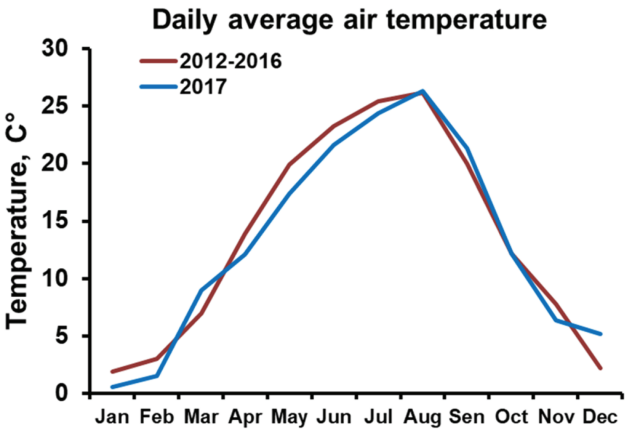

Figure 3: Daily average air temperature.

The summary positive air temperature in 2017 reached 4796 °C. The sum of the temperatures above 10 °C equaled to 4076 °C. Winters were snowless with frequent thaws. The average air temperature during the experimental period varied within the 17.4–26.3 °C range, which is 2.5–0.1 °C below the climate normal.

Plant height ranged between 50.8 and 177.4 cm (mean = 119.2 cm) in the analyzed inbred lines. Flower head diameter varied between 10.2 and 30.8 cm (mean = 18,8) (Supplementary Table 1). Among all analyzed plants, 33 plants were branched and 153 were non-branched with a single flower head. 135 sunflower lines were male fertile and 51 line, male sterile (CMS-PET1). The absence of ray flowers was observed only in 3 lines, appearing to be a very rare event. The presence of ray florets in most samples may be accounted for by strong selection pressure since ray florets are considered as a pollinator attraction factor. Seed oil content ranged from 23.4 % to 50.9 % with the mean equal to 39.9 %. Seed husk content varied from 15.9 % to 47.1 %, and the mean value was equal to 29.3 %. 100-seed weight varied between 2.2 g and 12.5 g (mean = 5.3 g). Head seed number ranged from 210 to 1796 (mean = 839.7).

Length of DTB period varied from 36 to 52 days (mean 42), DTF period ranged from 54 to 82 days (mean 68), the period from planting date to PM (DTM) ranged from 79 to 114 days (mean 100) (Supplementary Table 1).

In addition, heat units (HU) accumulation between the planting date and physiological maturity dates was calculated (Figure 4).

Significant variation in HU accumulation by the physiological maturity date was observed among the analyzed sunflower lines (from 1129 to 1828 HU).

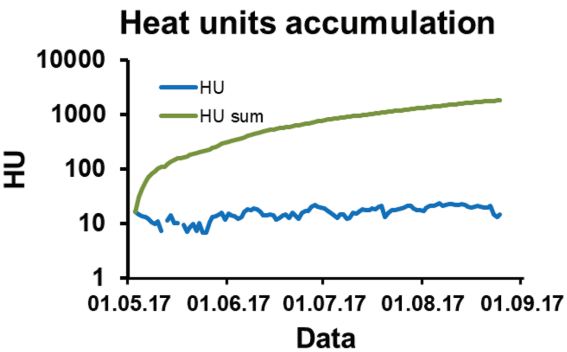

Figure 4: Heat units accumulation.

The histograms depicting quantitative morphological and phenological traits distribution among the sunflower lines from the VNIIMK collection are presented in Figure 5 (A–E).

Based on the obtained data, the major part of the analyzed lines in the collection may be described as the early-ripening or medium-ripening ones according to the existing classification (Fick and Miller, 1997). This observation may reflect the high demand for the early- and medium-ripening sunflower and its prevalence among the sunflower hybrids in the Russian Federation. The use of early-ripening varieties and hybrids allows cultivating this crop in the regions with a short growing season, for example in Siberia and Urals (Puzikov and Suvorova, 2016). In addition, the use of early-ripening varieties and hybrids makes it possible to avoid the adverse effects of drought, which is frequent in some regions of sunflower cultivation in Russia.

A significant correlation was found between the planting date to budding period and the planting date to the flowering period (Pearson coefficient = 0.851) and between the planting date to flowering and the planting date to physiological maturity periods (Pearson coefficient = 0.870). The correlation between the planting date to budding period and the planting date to physiological maturity was not as strong (Pearson coefficient = 0.695). It should be noted that the ratio between the analyzed time periods varied between different lines. For example, for the line no. 577,432, the period from the planting date to flowering was 59 days and the period from the planting date to physiological maturity was 94 days, while the line HA89 showed longer planting date to flowering period (67 days) and shorter planting date to physiological maturity period compared to the line no. 577,432 (89 days). The results obtained by us are consistent with

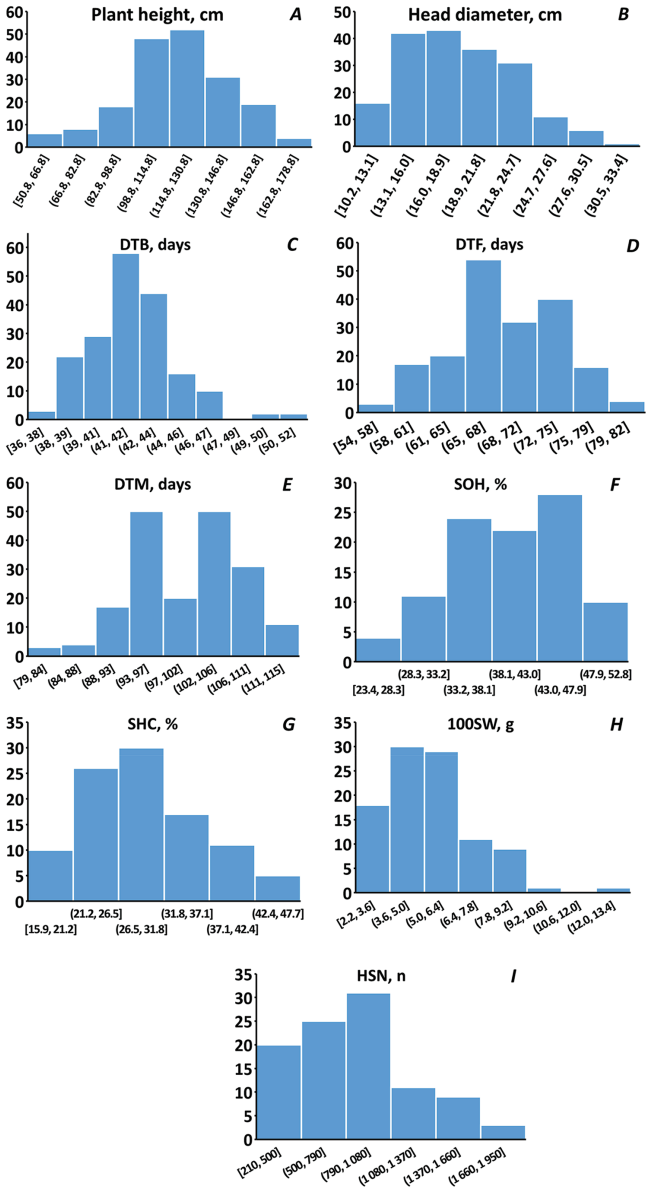

Figure 5: Quantitative agronomically important traits in sunflower. (*A*) Plant height, cm; (*B*) Head diameter, cm; (*C*) planting date to budding period, DTB, days; (*D*) length of the planting date to flowering, DTF, days; (*E*) planting-physiological maturity period, DTM, days; (*F*) seed oil content, SOH, %; (*G*) seed husk content, SHC, %; (*H*) 100-seed weight, 100 WS, g; (*I*) seed number in the head, HSN, %.

the previous data obtained in the sunflower hybrid studies (Kaya *et al.*, 2004; Thompson and Dougherty, 1998).

Fatty acid composition of seed oil from 99 sunflower line samples was analyzed using gas chromatography with flame ionization detection (GC-FID). As a result, we detected 11 FAs: C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, and C24:0. The variability in sunflower oil fatty acid content observed among 99 VNIIMK sunflower lines is presented in the Supplementary Table 2. The most abundant FAs were C16:0, C18:0, C18:1, and C18:2, which constituted a total of 97.5% of all FAs. The remaining 7 minor fatty acids constituted 2.51% of all FAs. (Table 1). The relative abundance of FAs was demonstrated to vary between samples.

Table 1: Fatty acid content variation in sunflower oil (99 VNIIMK lines).

FA		Mean	Standard deviation	Min	Max	Coefficient of variation
Myristic	C14:0	0.06	0.02	0.02	0.10	28.74
Palmitic	C16:0	6.50	0.97	3.98	9.14	14.94
Palmitoleic	C16:1	0.12	0.06	0.04	0.38	45.77
Stearic	C18:0	4.57	1.31	1.62	7.55	28.73
Oleic	C18:1	38.59	11.26	16.74	88.70	29.17
Linoleic	C18:2	47.84	11.02	3.13	70.21	23.04
Linolenic	C18:3	0.14	0.11	0.05	0.89	84.52
Arachidic	C20:0	0.36	0.09	0.11	0.60	26.18
Eicosenoic	C20:1	0.18	0.07	0.10	0.57	40.77
Behenic	C22:0	1.36	0.42	0.76	2.63	30.95
Lignoceric	C24:0	0.29	0.06	0.17	0.46	18.90

High variation coefficients calculated for each fatty acid (from 14.9% to 84.5%) point to significant differences between genotypes. Oleic acid and such essential fatty acids as linoleic and linolenic are of major interest for sunflower breeding. Among the analyzed sunflower samples, ω-9 oleic acid content varied within a broad range (between 16.34% and 88.66%). Two samples, VK 464 and LG 26, were shown to be high oleic acid lines with oleic acid content of 88.7 and 83.67%, respectively. High variability was also demonstrated for ω-6 linoleic acid (from 3.13% to 68.55%). The variability range for ω-3 linolenic acid was between 0.05% and 0.88%. This acid is present in relatively low concentrations in the oil. Detected FA abundances are in good agreement with data obtained in the previous works (Alpaslan and Gunduz, 2000; Pleite *et al.*, 2006).

GWAS

The data on the genotypes and phenotypes of inbred lines obtained in this study were used in the GWAS analysis. We have found significant associations between the branching trait and the loci on the linkage groups (LGs) 10 and 1 (Figure 6). Our results stay in good correspondence with the data on branching loci localization to the upper half of the linkage group 10 (Nambeesan *et al.*, 2015; Tang *et al.*, 2006). The data on the branching-associated loci on the chromosome 1 (for basal type branching) was also obtained previously by Nambeesan et al. (Nambeesan *et al.*, 2015). Novel significant associations with linolenic acid (18:3) content in the seeds were found on LGs 8, 9 and 17 (Figure 7).

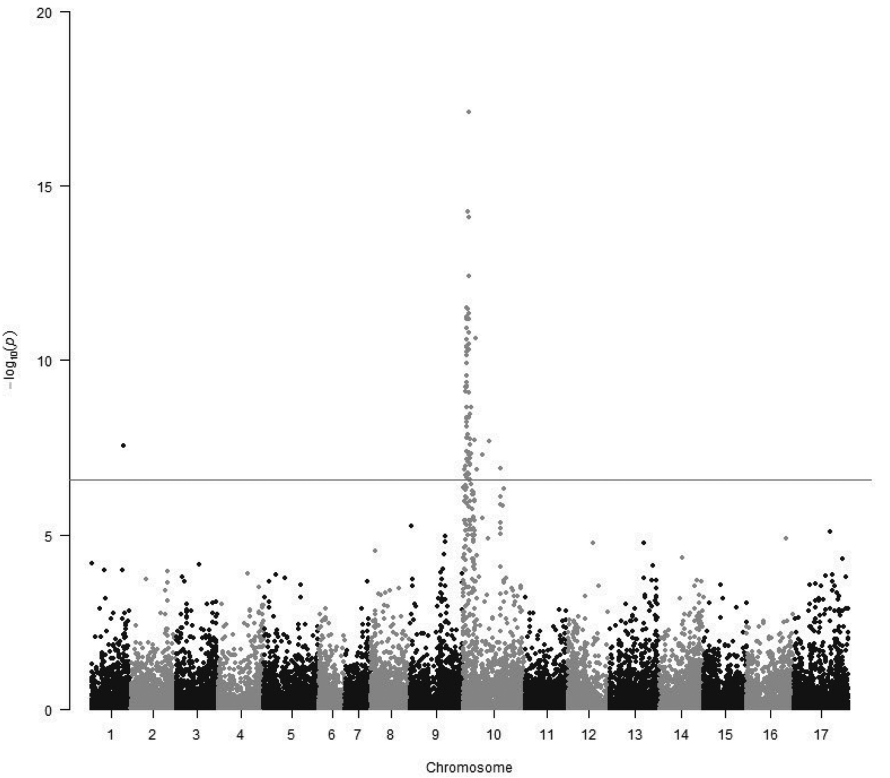

Figure 6: Manhattan plot of branching associations. The black line indicates the significance threshold based on the Bonferroni multiple testing correction (alpha = 0.01).

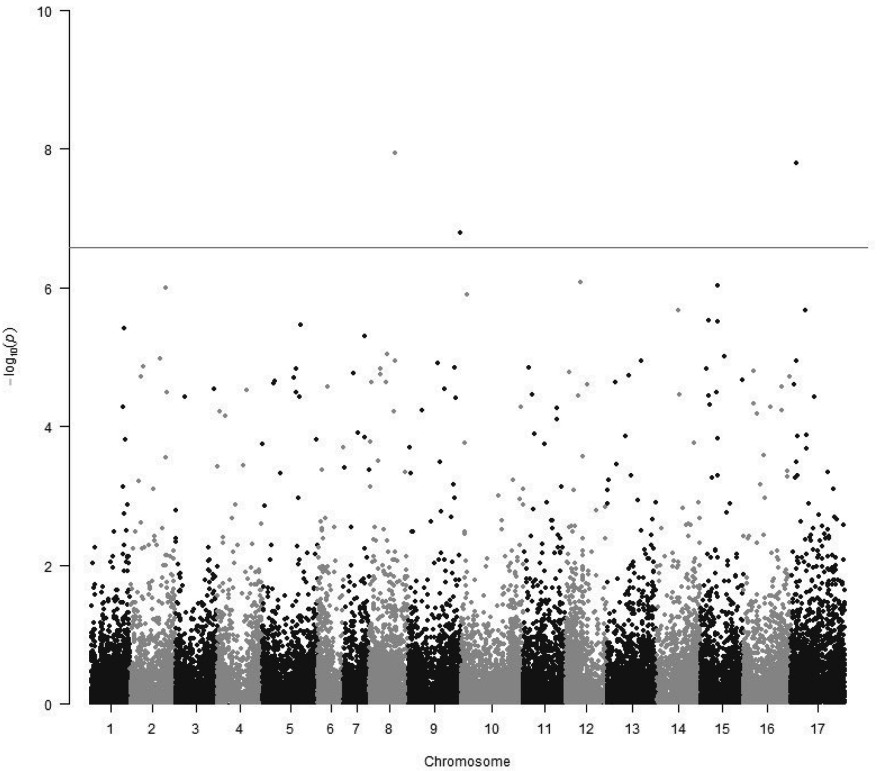

Figure 7: Manhattan plot of linolenic acid content associations. The black line indicates the significance threshold based on the Bonferroni multiple testing correction (alpha = 0.01).

Conclusions

Sunflower is the main oil crop in Russia and among the four most important oil crops in the world, with sunflower oil being used both in food industry and for technical purposes. The genetic material of the VNIIMK collection has made a significant contribution to the development of sunflower as the oilseed crop worldwide. However, notwithstanding its high value, the sunflower collection in VNIIMK remains poorly characterized in terms of using modern methods of genome-wide analysis. At the same time, a detailed study of genetic resources is essential for their effective use. In the present study, the genotypes of 186 VNIIMK lines were characterized by RAD sequencing for the first time. The data on their morphology, phenological and biochemical characteristics were obtained. The analysis revealed a significant genetic and phenotypic diversity

of the VNIIMK sunflower collection. New associations of genomic loci with linolenic acid content in seed oil have been revealed. The data obtained here will serve as the basis for further studies and may contribute to further extensive utilization of the unique VNIIMK collection in oilseed sunflower breeding.

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