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Molecular identification and phylogenetic analysis of promising Lithuanian grapevine

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Abstract. The Lithuanian breeder Antanas Gailiūnas (1918–2004) developed a series of interspecific grapevine hybrids suited for the northern climate. With recent climate change and milder conditions, some of these hybrids have become the main varieties cultivated in Lithuanian vineyards. However, after about 50 years of vegetative propagation by amateur growers, significant plant heterogeneity has been observed. It remains unclear whether these differences are purely phenotypic or also genotypic. Additionally, the origin of many Lithuanian grapevine seedlings is undocumented. According to EU wine regulations, only *Vitis vinifera* varieties or regionally registered hybrids may be used for wine production. The aim of this study was to assess the genetic diversity and potential origin of grape varieties commonly grown in Lithuania. A total of 78 grapevine leaf samples were collected from six vineyards. Molecular identification was carried out using eight microsatellite (SSR) markers, following the guidelines of the International Organisation of Vine and Wine (OIV). In total, 133 polymorphic alleles were identified, with allele numbers per primer pair ranging from 7 to 18. The most informative loci were VVMD7 and VVMD28. The SSR profiles revealed clear genetic distinctions among the genotypes and helped identify cases where different sample names shared the same genetic code. These results confirm the effectiveness of SSR markers for grapevine genotyping and provide valuable data for distinguishing, authenticating, and potentially registering local Lithuanian grape varieties in line with EU regulations.
Key words: *Vitis* hybrids, polymorphism, SSR.

Introduction

Grapevine (*Vitis* spp.) cultivation in northern Europe has traditionally been restricted by climatic limitations, with cold winters and short vegetation periods limiting the range of viable cultivars. In Lithuania, the pioneering breeder Antanas Gailiūnas (1918–2004) developed more than 30 interspecific hybrids adapted to local conditions, particularly valued for their winter hardiness and early fruit ripening. These hybrids remain widely cultivated in private gardens and small vineyards, forming an important component of the country's viticultural heritage.

In recent decades, regional climate warming has improved viticultural suitability across the Baltic States, with longer growing seasons and milder winters (Fraga *et al*., 2016; Santos *et al*., 2020). Several hybrids developed by A. Gailiūnas are now considered promising vineyard crops. However, their long-term vegetative propagation by amateur growers - often over a period exceeding 50 years - has resulted in notable heterogeneity. It remains unclear whether this variability reflects phenotypic plasticity under different agroclimatic conditions or underlying genetic differences. The genetic origin and authenticity of Lithuanian grapevine hybrids are largely undocumented, and under European Union regulations, only *Vitis vinifera* cultivars or officially registered hybrids are permitted for wine production (European Commission, 2019). Establishing genetic origin is therefore essential not only for compliance with regulatory frameworks but also for the sustainable development of local viticulture.

Microsatellite (SSR) markers are widely recognised as an effective tool for grapevine cultivar identification, pedigree analysis, and germplasm characterization, due to their high polymorphism and reproducibility (This *et al*., 2004; Cipriani *et al*., 2010). Their application in European grapevine

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collections has frequently revealed cases of synonymy, homonymy, and misidentification (Laucou *et al*., 2011; Migliaro *et al*., 2019). Applying these molecular markers to Lithuanian grapevine hybrids enables an evaluation of genetic diversity, clarification of cultivar identity, and provides a foundation for authentication and possible registration of local varieties in line with EU legislation.

The aim of the research was to assess the genetic diversity and possible origin of grapevine varieties commonly grown in Lithuania, using molecular identification with microsatellite (SSR) markers, to clarify the extent of genetic variation, identify cases of misidentification, and support the authentication and potential registration of local varieties in accordance with EU wine production regulations.

Materials and Methods

Plant material and DNA extraction

A total of 78 grapevine (*Vitis* spp.) leaf samples were collected during the 2024 growing season from six vineyards located in different regions of Lithuania (Figure 1). The sampling aimed to include genotypes commonly cultivated by amateur and professional growers, with emphasis on varieties and hybrids developed by A. Gailiūnas. Young leaves were collected from each plant, placed in individual sterile plastic bags, and transported on ice to the laboratory. Leaf tissue was stored at −20 °C until DNA extraction. Genomic DNA was extracted from approximately 100 mg of young leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990), optimized for grapevine material. DNA quantity and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and integrity was checked by electrophoresis on 1.5% agarose gel. DNA concentrations were adjusted to 20 ng/µL for subsequent PCR reactions.

Microsatellite (SSR) markers and PCR amplification

Genetic characterization was carried out using a set of 11 nuclear SSR loci (VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, VrZAG62, and VrZAG79). These markers were selected according to the recommendations of the International Organisation of Vine and Wine (OIV, 2009) and are widely used for grapevine cultivar identification and genetic diversity studies (This *et al*., 2004). PCR amplification was performed in a total volume of 20 µL containing 20 ng of template DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1 U of Taq DNA polymerase (Thermo Fisher Scientific, USA). The forward primers were fluorescently labelled with FAM, HEX, or NED dyes for fragment detection. The thermal cycling profile consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55–60 °C (depending on primer pair) for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

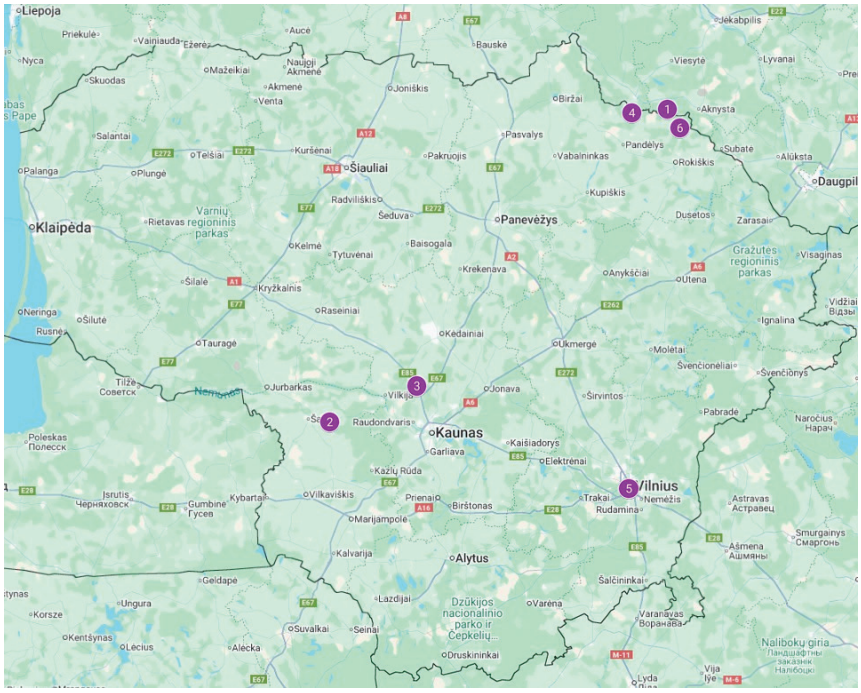

Figure 1. Map of Lithuania showing sample collection sites. Points 1–5 represent vineyard and collections; Point 6 indicates A. Gailiūnas homestead (Juodupė village, Rokiškis district).

Fragment analysis, allele scoring and data analysis

PCR products were separated using capillary electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA), with GeneScan™ 500 LIZ® as the internal size standard. Alleles were scored using GeneMapper v.4.0 software (Applied Biosystems, USA). To ensure reproducibility, a subset of samples was amplified and genotyped in duplicate. The SSR profiles were used to determine allelic polymorphism, genetic diversity and analysis SSR genotype data were imported from an Excel spreadsheet containing allele pairs for 11 loci across 78 samples. Allele calls were parsed from a/b format into numeric pairs. Missing values were excluded from frequency calculations. For each locus, allele frequencies were computed, and the following metrics were calculated using vectorized operations in NumPy (fundamental Python library for scientific computing): Na - number of observed alleles, Ne - effective number of alleles(), Ho - observed heterozygosity (proportion of heterozygotes), He - expected heterozygosity (), PIC - polymorphic information content, based on allele frequency distribution. Genetic distances among genotypes were estimated based on Nei's genetic distance, and clustering was performed using unweighted pair group method with arithmetic mean (UPGMA) in DARwin v.6.0.12 (Perrier & Jacquemoud-Collet, 2006). To visualize allelic distribution across genotypes, a heat map of alleles per locus was constructed. A binary allele matrix (presence/absence of each allele in each genotype) was generated from SSR data. Heat maps were produced in R software v.4.3.1 (Kolde, 2019; R Core Team, 2023) using the package heatmap, with rows representing grapevine genotypes and columns representing alleles. This approach facilitated the detection of genotype-specific alleles, clustering of similar individuals, and visualization of allele-sharing patterns.

Results and Discussion

SSR Polymorphism and Allelic Diversity

A total of 133 alleles were identified across the 11 SSR loci tested (Table 1). The number of alleles per locus ranged from 7 (VVMD24) to 18 (VVMD32), with an average of 12.1 alleles per loci. The highest number of alleles was observed in VVMD32 (18 alleles, 240–266 bp) and VVS2 (17 alleles, 122–161 bp), both of which are known to be highly informative for grapevine genotyping (This *et al*., 2004; Cipriani *et al*., 2010).

The allelic ranges observed in Lithuanian hybrids were generally within the expected size intervals compared to published data for *V. vinifera* cultivars. For example, with VVMD5 alleles between 226–242 bp were amplified in the current study, compared to 226–266 bp in *V. vinifera* (This *et al*., 2004; Laucou *et al*., 2011). Similarly, VVMD7 showed 15 alleles (234–256 bp) in the hybrids, close to the *V. vinifera* range of 233–265 bp.

Some loci exhibited smaller allelic number in the Lithuanian hybrids compared to *V. vinifera*. For

Table 1

SSR alleles characteristic of the studied grape varieties grown in Lithuania and A. Gailiūnas seedlings

Marker	Na	Variation of alleles in *Vitis* hybrids (current research), bp	Variation of alleles in *Vitis vinifera*, bp	Ne	MAF	Ho	He	PIC
VVMD5	9	226-242	226 – 266	3.298	0.494	0.576	0.697	0.667
VVMD7	15	234-256	233 – 265	8.753	0.198	0.919	0.886	0.875
VVMD21	10	221-268	221-268	2.048	0.686	0.430	0.512	0.495
VVMD24	7	200-220	200-220	6.085	0.203	0.953	0.836	0.814
VVMD25	11	238-273	237 – 269	4.809	0.343	0.593	0.792	0.766
VVMD27	8	176-194	176 – 198	5.513	0.291	0.895	0.819	0.795
VVMD28	16	219-263	218 – 282	7.501	0.233	0.663	0.867	0.854
VVMD32	18	240-266	232 – 292	6.931	0.250	0.381	0.856	0.841
VVS2	17	122-161	123 – 155	6.630	0.285	0.895	0.849	0.835
VrZAG62	10	180-206	180 – 206	5.702	0.285	0.733	0.825	0.803
VrZAG79	12	247-263	237 – 261	7.363	0.273	0.814	0.864	0.852

Na – number of observed alleles; Ne – effective number of alleles ($1/\Sigma p_i^2$); MAF – frequency of the most common allele; Ho – observed heterozygosity; He – expected heterozygosity; PIC – polymorphic information content.

instance, VVMD21 and VVMD24 produced 10 and 7 alleles, respectively, but published *V. vinifera* data indicate higher number (Maul *et al*., 2012; Migliaro *et al*., 2019). This may reflect a reduced genetic base in the local germplasm, consistent with its origin from a relatively small number of breeding lines. In addition, it can be assumed that the disparity in allele numbers may reflect differences in the volume and geographic origin of the analysed material rather than inherent genetic differences. Some of loci showed allelic ranges overlapping but not entirely coinciding with *V. vinifera* reference values due to the shift of 1 bp (VVMD28, Table 1). VVMD32 (18 alleles) and VVS2 (17 alleles) showed a high degree of polymorphism, indicating that these markers are especially valuable for discriminating among Lithuanian hybrids. The presence of alleles outside of the published *V. vinifera* size ranges suggests introgression from non-vinifera species in the Gailiūnas breeding material.

The high polymorphism observed confirms that SSR markers are effective for distinguishing grapevine genotypes in Lithuania. Cases where allele ranges overlapped strongly with *V. vinifera* references support the possibility of vinifera ancestry, while alleles outside published ranges point to hybrid origin. These findings are consistent with previous studies that reported the utility of SSR markers in differentiating *V. vinifera* cultivars from interspecific hybrids (This *et al*., 2004; Laucou *et al*., 2011; Migliaro *et al*., 2019; Tympakianakis *et al*. 2023).

Across the 11 SSR loci, the hybrid panel shows high polymorphism (mean Na ≈ 14–15, mean He ≈ 0.81, mean PIC ≈ 0.79), indicating strong discriminatory power for cultivar/clone differentiation and parentage testing. Most loci have MAF ≤ 0.35, which, together with large allele counts, yields informative PIC values (PIC > 0.80 for eight markers). This profile is typical of wellchosen grape SSRs and suggests the panel is suitable for finescale genetic structure analyses. VVMD7 (Na = 15, He = 0.886, PIC = 0.875) and VVMD28 (Na = 16, He = 0.867, PIC = 0.854) are the top performers, combining high allelic richness with balanced frequencies. These markers contributed strongly to identity power and exclusion probabilities. Markers VrZAG79, VVMD32, VVS2, VrZAG62 and VVMD27 (PIC 0.852, 0.841, 0.835, 0.803 and 0.795, respectively) also fall in the highly informative class. Polymorphic information content (PIC > 0.70) in loci analysis, is typically regarded as highly informative, and loci with high He and moderate MAF maximise assignment power (Foria *et al*., 2022). SSR panel in our study meets these criteria for most markers, making it robust for hybrid germplasm characterization.

Observed and expected heterozygosity with Ho ≈ He for loci VVS2 - 0.895 vs 0.849 and VrZAG62 - 0.733 vs 0.825, consistent with an interspecific breeding background typical for hybrids of north countries. A few loci show Ho > He (VVMD24, VVS2, VVMD27), which can arise from sampling of multiple hybrid groups, selection, or allele calling that favors heterozygote detection. Ho < He (0.381 vs 0.856) in loci VVMD32 shows a pronounced deficit of heterozygotes. Potential causes include null alleles (allele dropout leading to false homozygotes).

Comparing the observed allele size ranges (bp) in *Vitis* hybrids (dataset in current study) with the *V. vinifera* ranges in several loci were observed the hybrid ranges are slightly broader or shifted relative to *V. vinifera*. VVS2 (122–161 vs 123–155) and VrZAG79 (247–263 vs 237–261). The presence of alleles above *V. vinifera* range (161 at VVS2, 263 at VrZAG79) is consistent with introgression from non-V. *vinifera* species, reflecting the interspecific origin of many cold hardy hybrids. In other loci the hybrid ranges are narrower than vinifera (VVMD5 226–242 vs 226–266 and VVMD28 219–263 vs 218–282). This can indicate founder effects or targeted breeding that retain subsets of allele states while introducing adaptive traits. Loci VVMD21 align closely suggesting shared repeat architectures and mutational spectra across species.

The observed polymorphism across SSR loci provides a strong basis for varietal authentication and supports the potential registration of local varieties under EU regulations. For core identification of hybrids, we suggested markers with PIC ≥ 0.80 and He ≥ 0.80: VVMD7, VVMD28, VrZAG79, VVMD32, VVS2, VrZAG62, VVMD27.

Genetic diversity of Vitis spp. genotypes in Lithuania

A phylogenetic tree was constructed using SSR markers, which reveals the genetic relationships among 78 grapevine (*Vitis* spp.) accessions (Figure 2). The dendrogram illustrates clear clustering patterns, indicating both genetic diversity and relatedness among the studied varieties, including cultivars developed in Lithuania, international breeding lines, and local landraces.

One cluster includes accessions from the Juodupe accessions (Juodupe 7287, 7304, 7311, 7313), suggesting a close genetic relationship likely resulting from clonal propagation or breeding based on shared parental lines. Similar patterns were observed in earlier SSR-based grapevine studies, where cultivars sharing breeding histories grouped into tight clusters (This *et al*., 2004). Accessions such as Frontenac (7297), Frontenac Blanc (7302), and La Crescent (7301) formed a genetically distinct subgroup. These cultivars are known for their cold-hardy traits and are commonly used in breeding programs across North America and Northern Europe (Reisch *et al*., 2012).

Their separation from Lithuanian varieties reflects the geographic and genetic divergence between introduced hybrids and locally developed germplasm.

Lithuanian-developed varieties like Anastasija, Liepsna, and Varduva appeared in various branches of the tree, indicating that local breeding programs have incorporated a wide genetic base. This is consistent with previous research showing substantial variability among Lithuanian grapevine accessions, likely due to diverse crossing strategies. The grouping of Sirvinta, Jadvyga, and Samochvalovo genotypes in a subgroup indicates possible clonal relationships or origin from a common breeding ancestor. Such clustering patterns have been widely reported in SSR studies due to the high discriminatory power of microsatellite markers (Merdinoglu *et al*., 2005; Adam-Blondon *et al*., 2004).

Genetically similar accessions can be candidates for redundancy reduction in *ex situ* collections, while genetically unique varieties - especially those occupying distant clades - should be prioritised for conservation. This approach has been successfully employed in several national and regional grapevine repositories to balance resource use and genetic conservation (This *et al*., 2004; Reisch *et al*., 2012).

Overall, the SSR-based phylogenetic analysis confirms that Lithuanian grapevine breeding programs have managed to combine international breeding material with locally selected cultivars, contributing to both genetic diversity and climate adaptation. The tree offers a foundational framework for future breeding, selection, and germplasm management strategies in northern viticulture regions.

Genetic diversity of Lithuanian hybrids

Heatmap illustrates the clustering of grapevine accessions based on 11 SSR marker profiles, with rows representing individual samples and columns corresponding to different loci (Figure 3). The hierarchical clustering revealed several distinct genotype groups, reflecting both shared allelic composition and possible common breeding origins.

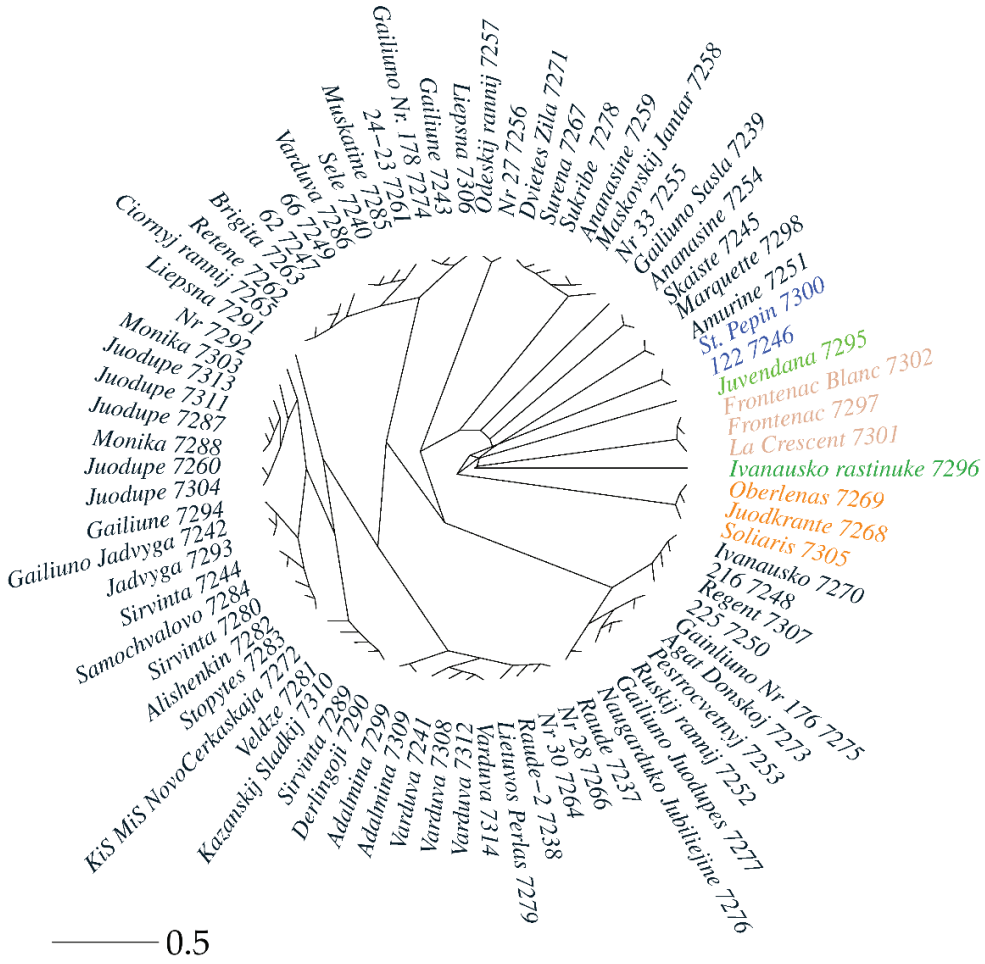

Figure 2. Radial phylogenetic tree showing genetic relationships among 78 grapevine accessions collected from Lithuanian vineyards, based on SSR marker analysis. The five most divergent clusters are highlighted in color (clustering algorithm: UPGMA; distance metric: Euclidean).

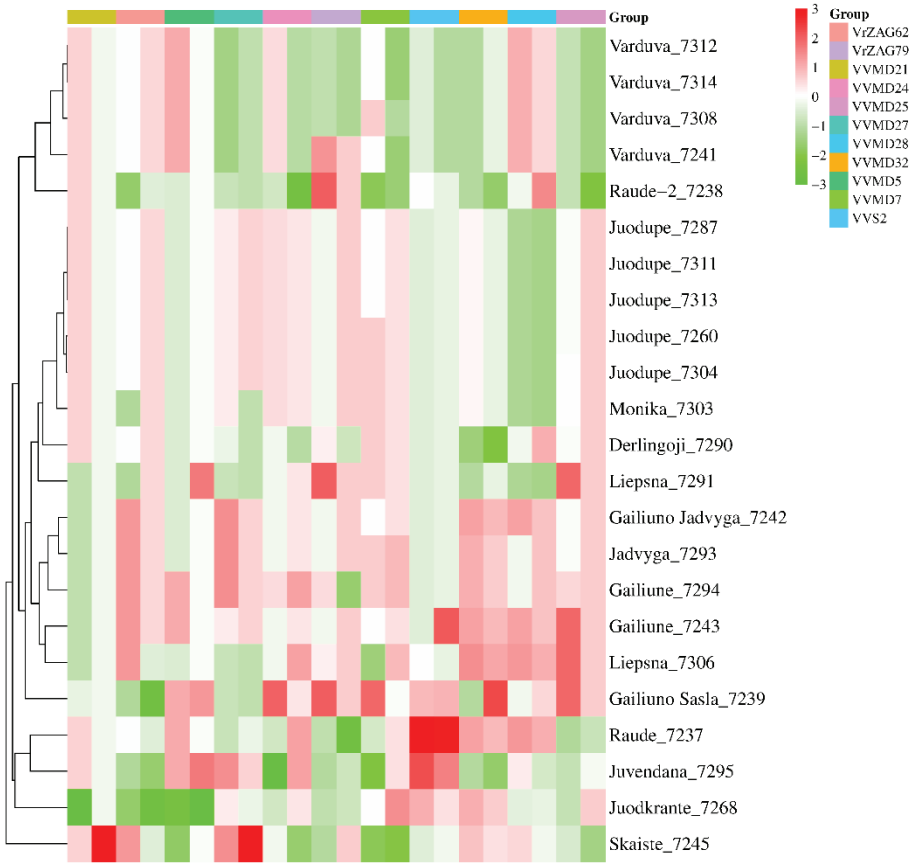

Figure 3. Clustering heatmap showing A. Gailiūnas breeding numbers grouping and SSR marker profiles.

Color intensity reflects allele variation: green indicates lower values, while red highlights higher variation or distinct allele states. The dendrogram on the left shows hierarchical grouping, revealing that certain breeding numbers (A. Gailiūnas selections) cluster closely, suggesting genetic similarity, whereas others like Varduva and Raudė form separate branches. Overall, the figure demonstrates clear genetic structuring among Lithuanian hybrid cultivars, supporting the effectiveness of SSR markers for distinguishing breeding lines.

One major cluster consisted of accessions of the cultivar group Varduva (samples 7312, 7314, 7308, and 7241), which grouped tightly together and showed relatively homogeneous allele patterns across most loci. This indicates that Varduva samples are genetically close and possibly represent clonal selections of the same hybrid genotype rather than distinct varieties. In contrast, the group of samples identified as Juodupė (7287, 7311, 7313, 7260, 7304) displayed higher allelic variability, particularly at loci VVMD32 and VVS2. This heterogeneity suggests either multiple hybridization origins under a common name or misidentification among growers. The Liepsna accessions (7291 and 7306) clustered with different branches, reflecting significant allelic differences despite their shared name. This divergence highlights potential synonymy and homonymy issues, which have also been reported in other grapevine collections (This *et al*., 2004; Laucou *et al*., 2011).

Interestingly, several unique genotypes, such as Raudė (7237, 7238), Amūrinė 7251 (*V. amurensis*), and Juodkrantė 7268, were positioned at more distant branches, emphasizing their distinct genetic profiles. The high allelic frequencies at VVMD28 and VrZAG62 in these samples suggest introgression from non-vinifera germplasm, consistent with A. Gailiūnas breeding strategy of using interspecific material for cold hardiness.

The heatmap reveals clear clustering of Lithuanian grapevine accessions, with groups such as Varduva and Juodupė forming distinct, stable clusters. These genotypes are among the most widely cultivated in Lithuania, reflecting their superior adaptation to local climatic conditions and resilience in hybrid breeding programs. Their consistent SSR profiles and strong grouping suggest genetic uniformity within each name, supporting their reliability as core cultivars for northern viticulture. This pattern underscores the importance of maintaining these well-adapted

genotypes as a foundation for future breeding and conservation efforts.

Overall, the heat map revealed a mixture of homogeneous cultivar groups and heterogeneous sets of accessions sharing the same local names. This highlights the dual challenge of genetic redundancy (clones named differently) and genetic heterogeneity (different genotypes under the same name) in Lithuanian vineyards. Such issues underline the importance of molecular authentication for the conservation, registration, and sustainable utilization of local grapevine germplasm in line with EU legislation.

Conclusions

This study confirms that SSR markers are highly effective for assessing the genetic diversity of Lithuanian grapevine germplasm. Across 11 loci, 133 alleles were identified, with VVMD7 and VVMD32 showing the highest polymorphism. Most allele ranges matched *V. vinifera* references, though unique alleles suggest introgression from non-vinifera species in local breeding.

The high allelic richness and strong informativeness across most loci reflect the composite ancestry of *Vitis* hybrids (*V. amurensis*, *V. labrusca*, *V. riparia* contributions). Expanded or shifted allele ranges relative to vinifera are expected outcomes of divergent repeat lengths and species-specific mutation dynamics. Combined with generally high heterozygosity, these results support the view that the panel captures broad genomic diversity suitable for: (i) verifying cultivar identity, (ii) detecting duplicates/near clones, (iii) reconstructing pedigrees, and (iv) assessing population structure among hybrid groups.

Phylogenetic analysis of 78 accessions revealed distinct separation between Lithuanian cultivars and introduced cold-hardy Canadian hybrids, with local varieties forming several subclades. Clustering of genotypes Juodupe, Varduva, and Širvinta indicates limited divergence, while the heat map highlighted both redundancy and heterogeneity among A. Gailiūnas hybrids. Lithuanian hybrids show significant genetic diversity, and these results support the use of SSR markers for varietal authentication, conservation, and registration under EU plant variety protection frameworks.

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