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Research Paper

Unveiling clonal and inter-varietal diversity in Apulia's representative grape varieties

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A B S T R A C T

The Apulian wine industry, a cornerstone of Italian viticulture, benefits from the great genetic diversity of local grape varieties that are closely linked to the traditional and cultural heritage of Apulian landscape. The aim of this study is to investigate the genomic diversity of five representative Apulian grapevine varieties, 'Negramaro', 'Malvasia Nera', 'Primitivo', 'Minutolo Bianco' and 'Uva di Troia' using genotyping-by-sequencing. By analysing 59 183 high-quality single nucleotide polymorphism (SNP) markers across 75 clones, we were able to detect significant genetic variation within and between varieties. The fixation index was calculated for each individual SNP, identifying 5 363 non-redundant divergent SNPs. Of these, 1 785 were located within genes, including 887 in untranslated regions, 518 in introns, and 380 in coding sequences. The results highlighted divergent loci associated with genes crucial for secondary metabolism, stress resilience, and berry quality traits. Key genes identified in this study include *WRKY* transcription factors, *MYB* regulators, and enzymes such as glycosyltransferases and O-methyltransferases, all of which play an important role in the biosynthesis of secondary metabolites that influence wine flavour, aroma, and pigmentation. Additionally, six SNP loci were validated for varietal discrimination by PCR amplification and Sanger sequencing, confirming their potential for traceability. These results provide a solid genomic foundation for the preservation and valorisation of Apulia's rich wine heritage and for the development of targeted breeding programs to enhance the quality and resilience of Apulian wines in the face of changing consumer's preferences, market and climatic conditions.

Keywords: *Vitis vinifera* L.; Climate change; Single nucleotide polymorphism; Outlier SNP; Varietal discrimination; Traceability

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important tree crops worldwide, characterized by extensive genetic diversity resulting from its long history of cultivation and adaptation to diverse environmental conditions (Marrano et al., 2018; Dong et al., 2023). In Italy, historical migrations have introduced a vast array of allochthonous plant material, further enriching the genetic landscape of native varieties. As a result, the country boasts remarkable diversity, with over 650 wine grape varieties

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registered in the National Register of Vine Varieties and 1 774 officially recognized clones (http://catalogoviti.politicheagricole.it/catalogo.php).

Apulia is a prominent Italian wine region, encompassing 95 025 ha of vineyards and yielding 13 578.500 tonnes of grapes in 2023 (ISTAT, 2024). Its diverse landscapes and climate conditions allowed the adaptation of grape varieties to distinct local conditions (Seccia and Santeramo, 2018). The interplay between the region's unique physical and biological environment and traditional agricultural practices imparts distinctive qualities to its wines, contributing to the success of the Apulian wines (Gentilesco et al., 2023; Alba et al., 2024). In recent years, Apulia has experienced significant valorisation and enhancement of its wine production, leading to greater recognition and appreciation in both national and international markets. Apulian wines produced from the 'Negramaro', 'Primitivo', and 'Uva di Troia' grape varieties rank among the most widely produced and exported Italian wines. These varieties originate from different areas of the region: 'Negramaro' from the South, 'Primitivo' from the central zones, and 'Uva di Troia' from the North. They play a key role in the production of several QWPSR (Quality wines produced in specified regions), both CDO (Controlled Designation of Origin) or PDO (Protected Designation of Origin), and TGI (Typical Geographical Indication) or PGI (Protected Geographical Indication) wines (Tarricone et al., 2014; Gentilesco et al., 2023).

Originally recognized and cultivated in Gioia del Colle (BA), Apulia, as an early-ripening valuable variety in the late 18th century, the 'Primitivo' grape variety has undergone significant evolution, transitioning from a blending wine used to enhance weaker product of other countries or regions to a distinguished variety for monovarietal regional PDO. It is also known as 'Zinfandel' in the US, 'Pribidrag'/'Tribidrag'/'Crljenak'/'Kaštelanski' in Croatia and 'Kratosjia' in Montenegro (Marinov et al., 2024). Genetic studies revealed a strong connection between 'Primitivo' and other varieties like 'Plavac Mali' and 'Dobričić' (Maras et al., 2004; Calò et al., 2008), although the recent study of Marinov et al. (2024) excludes that 'Plavac Mali' is the offspring of the cross between 'Primitivo' and 'Dobričić'.

'Negramaro' is a red grape variety cultivated in the south-eastern Apulia and produces wines with a deep red color and a well-balanced aromatic profile. Traditionally, it is blended with other grape varieties to produce 14 Apulian PDO wines (Marzano et al., 2016).

'Uva di Troia' is a grape variety cultivated in northern Apulia, derived from a natural cross between 'Bombino Bianco' and 'Quagliano' (Lacombe et al., 2013). It produces high-quality wines with a high polyphenol content and a deep ruby red color and is used in several PDO and PGI wines in Apulia (Tavoliere delle Puglie DOP, 2025). The black grape variety 'Malvasia nera' is widely cultivated in the southern part of Apulia, where it is known with the two local names 'Malvasia nera di Brindisi' and 'Malvasia nera di Lecce'; according to Crespan et al. (2008), these are synonymous and have emerged from the crossbreeding of the aromatic 'Malvasia bianca lunga' (also known as 'Malvasia del Chianti') with 'Negroamaro'. Finally, the 'Minutolo Bianco' is an aromatic grape with yellow pulp, mainly found in the Itria Valley. Genetic studies have confirmed its relationship with 'Moscato Bianco' and 'Moscato di Alessandria' (D'Agata, 2014; Ruffa et al., 2016).

The use of single nucleotide polymorphism (SNP) markers generated by next generation sequencing (NGS) technologies has provided high-throughput and cost-effective approach for characterizing genetic variation. This data uncovers patterns of allele variability and identifies key genomic regions associated with complex traits, enhancing breeding accuracy and efficiency (Kumar et al., 2024; Prasad et al., 2024). Fully annotated, complete reference genomes (Zhou et al., 2019; Massonnet et al., 2020; Vondras et al., 2021; Minio et al., 2022; Navarro-Payá et al., 2022; Shi et al., 2023) enable the identification and mapping of allelic variants in understudied varieties, which represent an untapped reservoir of alleles potentially valuable for breeding. These rare alleles could be useful for traceability, maintaining typicality, and preserving varietal identity (Bianchi et al., 2020; Miazzi et al., 2020; Di Rienzo et al., 2017; Kaya et al., 2023). However, few studies have investigated genetic variability within Apulian varieties (Meneghetti et al., 2012; Miazzi et al., 2020; Villano et al., 2023; Montemurro et al., 2023; Procino et al., 2025a). Therefore, studying both intra- and inter-varietal genetic diversity is a crucial first step in developing a panel of SNP markers that can be used for both variety discrimination and wine traceability (Sion et al., 2021).

Preserving local viticultural biodiversity characterized by unique quality traits and a strong territorial identity has become an asset for wineries seeking to expand their market opportunities. The quality of grape berries for winemaking is determined by complex and dynamic interactions between the plant and its environment. Climate change and in particular global warming is increasingly impacting the traditional wine-producing regions, altering grapevine physiology and biochemistry and, in turn, affecting berry quality. Identifying the genomic regions and key genes that regulate these traits is crucial for developing sustainable strategies to help growers to navigate the challenges posed by the unpredictable climatic conditions (Droulia and Charalampopoulos, 2021).

With this in mind, we assessed the genomic variability both within and among five Apulian grapevine varieties ('Negramaro', 'Malvasia Nera', 'Primitivo', 'Minutolo Bianco', and 'Uva di Troia') using the genotyping-by-sequencing (GBS) approach. The specific objectives of the study were: i) to explore clonal variability within each variety, ii) to explore inter-varietal genetic variation, iii) to identify putative divergent loci/genes between varieties that could be responsible for morpho-qualitative traits relevant to breeding purposes, and iv) to validate unique allelic variants for each variety, enabling improved varietal discrimination, identification, and traceability.

2. Materials and methods

2.1. *Plant materials*

The genetic material consists of five grapevine varieties cultivated in different areas of Apulia: 'Malvasia Nera' (MN, 8 clones), 'Negramaro' (NE, 16 clones), 'Uva di Troia' (UT, 13 clones), 'Minutolo Bianco' (MI, 5 clones), and 'Primitivo' (PR, 33 clones) (Fig. 1; Table S1). The clones, identified during a long-lasting clonal and sanitary selection program started in the 1970s, were grown in the regional *ex situ* conservation center at the CRSFA - Centro Ricerca, Sperimentazione e Formazione in Agricoltura 'Basile Caramia' in

Fig. 1 The clusters of the five Apulian grapevine cultivars investigated in this study

Locorotondo (Bari), Italy, with a planting density of 1.0 m × 2.5 m, following standard agronomic practices. All samples were confirmed as clones of the five varieties using a set of simple sequence repeat (SSR) markers, as described by Montemurro et al. (2023), and their sanitary status was assessed, according to the procedure outlined in Procino et al. (2025a).

2.2. DNA extraction, SNP calling, filtering, and statistics

DNA extraction was performed according to Spadoni et al. (2019). GBS and SNP calling were performed by Elshire Group Ltd. (https://www.elshiregroup.co.nz/) using the 12X.v2 version of the *Vitis vinifera* PN40024 reference genome sequence (NCBI genome GCA_000003745.2), as detailed by Miazzi et al. (2020). GBS analysis generated a raw VCF file of 184 895 unfiltered SNPs called on 188 individuals, including Apulian, national, and international grapevine varieties, the majority of which have been previously described in the literature (Miazzi et al., 2020; Villano et al., 2023; Procino et al., 2025a). For this study, we selected a subset of 75 clones from the raw VCF file: 8 clones of 'Malvasia Nera', 16 of 'Negramaro', 13 of 'Uva di Troia', 5 of 'Minutolo Bianco', and 33 of 'Primitivo'. These were then subjected to a filtering procedure using the Golden Helix SNP and Variation Suite (SVS) v.8.8.4 (Golden Helix Inc., Bozeman, MT, USA) with a minor allele frequency (MAF) threshold of < 5%, and call rate > 20%. Various statistics were generated using VCFtools v.0.1.17 (Danecek et al., 2011), which also added gene annotations to the VCF file.

2.3. Identity-by-state, linkage disequilibrium and population structure analysis

The filtering process resulted in high-quality SNP marker datasets, which were used to calculate the pairwise identity-by-state (IBS) distance matrix between all clones using SVS. Duplicated individuals were identified based on a percentage of shared alleles ≥ 0.95. The average decay of linkage disequilibrium (LD) across the entire collection was calculated using TASSEL v. 5.2.94 (Bradbury et al., 2007) with a sliding window of 50 SNPs and a MAF threshold of 0.05, at the critical r^2 level of 0.20, as described by Remington et al. (2001).

Genetic structure was assessed via Principal Components Analysis (PCA) using TASSEL. The genetic relationships among individuals were determined using a Neighbour-Joining tree with 1 000 bootstrap replicates using MEGA XI (Tamura et al., 2021). The resulting trees were visualized using FigTree v.1.4 (Rambaut, 2014). The average observed heterozygosity (Ho) for each variety was calculated as follow:

$$Ho = nH_i/m$$

where nH_i is the number of heterozygous genotypes for each individual i, and m is the number of total markers. Pairwise genetic distances between sub-populations were estimated using Weir and Cockerham's average fixation index (F_{ST}), implemented in SVS.

2.4. Identifying candidate genes in divergent genomic regions

To assess the contribution of genomic divergence at individual loci in distinguishing the five grapevine varieties, we applied the population-based pairwise F_{ST} (Weir and Cockerham, 1984), following the approach outlined by Taranto et al. (2022). Only loci with an F_{ST} value of 1, indicating complete divergence, were considered for identifying candidate genes. Gene annotations were obtained from the URGI platform (https://urgi.versailles.inra.fr/Platform). The LD decay distance was used to define the size of regions containing each divergent marker, thus encompassing potential quantitative trait loci (QTLs) or genes influencing agronomically important traits. Functional information for these genes was retrieved through a BLAST search against the *Vitis vinifera* genome (GCA_000003745.2). SNP impacts on gene sequences were evaluated using SnpEff (Cingolani et al., 2012), with default settings applied.

The *in-silico* validation of the SNP effect was performed using BLASTx alignments (Altschul et al., 1997).

2.5. SNP validation

Based on divergent loci with $F_{ST} = 1$, six SNP markers capable of discriminating among the five grapevine varieties were selected for validation thorough PCR amplification and Sanger sequencing (Eurofins Sanger Sequencing Services, Cologne, Germany). A genomic region of approximately 500 nucleotides flanking each SNP locus was extracted, and primer design was guided using Primer3Plus (2025).

3. Results

3.1. GBS-derived SNP markers

The analyzed dataset comprised 75 clones, along with 184 895 unfiltered SNPs. After filtering, 59 183 high-quality SNP markers, distributed across the 19 chromosomes were retained (Table S2)

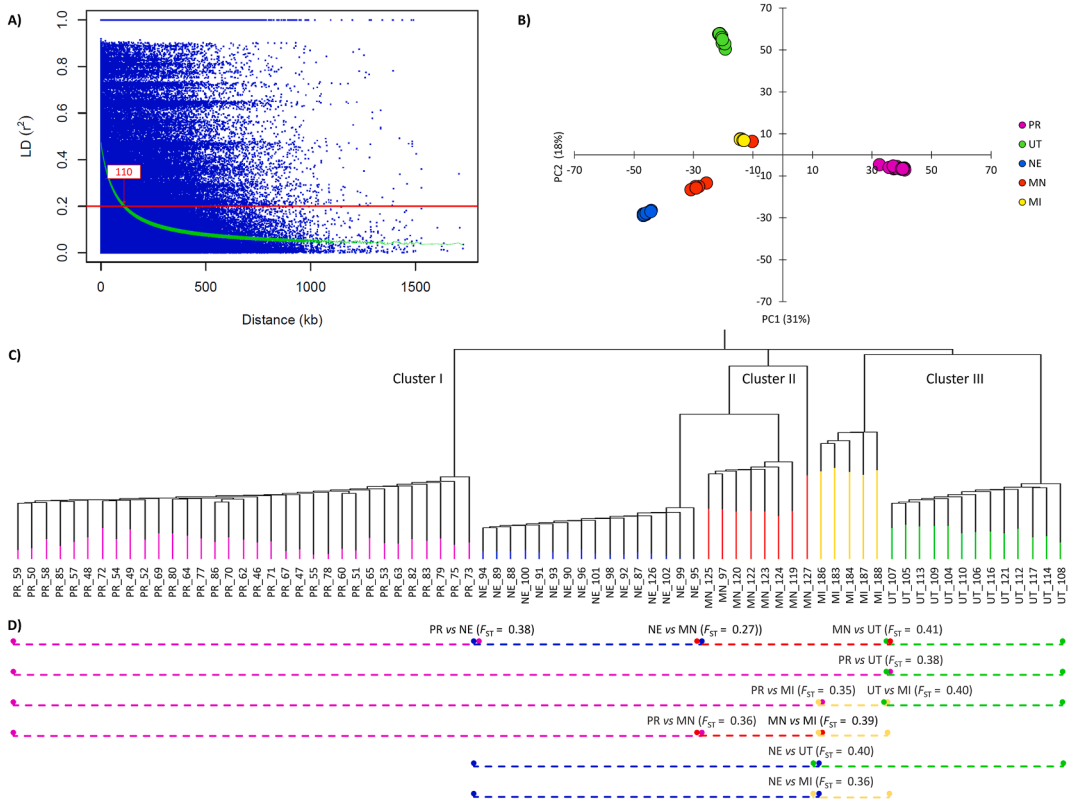

Fig. 2 Genetic diversity analysis assessed on 75 clones and 59 183 SNPs

(A) LD decay calculated at the critical r^2 level of 0.20. (B) PCA plot. (C) Neighbour-Joining tree. (D) Genetic distance between the five varieties assessed using the fixation index (F_{ST}). PR: 'Primitivo'; MN: 'Malvasia Nera'; MI: 'Minutolo'; UT: 'Uva di Troia'; NE: 'Negramaro'.

and used for downstream analysis. The filtered VCF file has been uploaded to the Mendeley data repository (Procino et al., 2025b).

Chromosomes 5 (4 146 SNPs) and 14 (3 785 SNPs) had the largest number of SNPs, while chromosomes 2 (2 224 SNPs) and 17 (1 862 SNPs) had the fewest.

The average SNP density across all chromosomes was 2 900. Of the total SNPs, 63.3% were transitions (Ts) and 36.7% were transversions (Tv), yielding a Ts/Tv ratio of 1.7 (Table S3). The most common substitution was A→G (31.7%), while the least frequent was C→G (7.29%). Based on the genomic coordinates of grapevine gene models, 28 475 (48.2%) SNPs were in genic regions, and 30 713 (51.8%) were in intergenic regions. Of the genic SNPs, 13 265 (22.4%) were found within annotated exons, affecting a total of 13 175 genes.

3.2. *Dissecting the genetic structure of the population*

No duplicate clones were detected based on the pairwise IBS distance within and between varieties. The intra-chromosomal LD decay showed a mean value of 110 kb (Fig. 2, A). The average of heterozygosity calculated for each variety was higher in 'Negramaro' (0.37), 'Primitivo' (0.33) and 'Uva di Troia' (0.30) than 'Malvasia Nera' (0.26) and 'Minutolo Bianco' (0.23).

PCA was used to assess the population structure (Fig. 2, B). The first two principal components explained approximately 49% of the variance, with 31% for PC1 and 18% for PC2. PCA revealed high variability among the five varieties and relatively low variability within clones of each variety. Clones of 'Uva di Troia' and 'Minutolo Bianco' were clustered in the II quadrant, while clones of 'Malvasia Nera' and 'Negramaro' were grouped in the III quadrant. All 'Primitivo' clones were positioned in the IV quadrant. Notably, one clone of 'Malvasia Nera' (namely MN_127) clustered closed to the 'Minutolo Bianco' clones.

The Neighbour-Joining tree corroborated the PCA results, revealing the presence of three main clusters (Fig. 2, C). Cluster I consisted of 'Primitivo' clones, cluster II grouped 'Negramaro' and 'Malvasia Nera', while the third cluster includes 'Minutolo Bianco' and 'Uva di Troia'. The MN_127 clone formed a distinct clade, closely related to the node grouping 'Negramaro' and 'Malvasia Nera'. To assess sub-population differentiation, we calculated F_{ST} values between the five varieties. Strong differentiation was observed in all comparisons, with the highest F_{ST} value of 0.41 between 'Malvasia Nera' and 'Uva di Troia' and the lowest F_{ST} value of 0.27 between 'Negramaro' and 'Malvasia Nera' (Fig. 2, D).

3.3. *Molecular signatures of divergence*

A total of 59 183 SNPs were analyzed to identify molecular signatures of divergence. The F_{ST} was calculated for each individual SNPs, with a significance threshold set at 1. The analysis identified 8 373 divergent SNPs across ten comparisons (Fig. 3), distributed throughout all 19 chromosomes (including the unknown chromosome) of the *Vitis vinifera* genome. Of these, 5 363

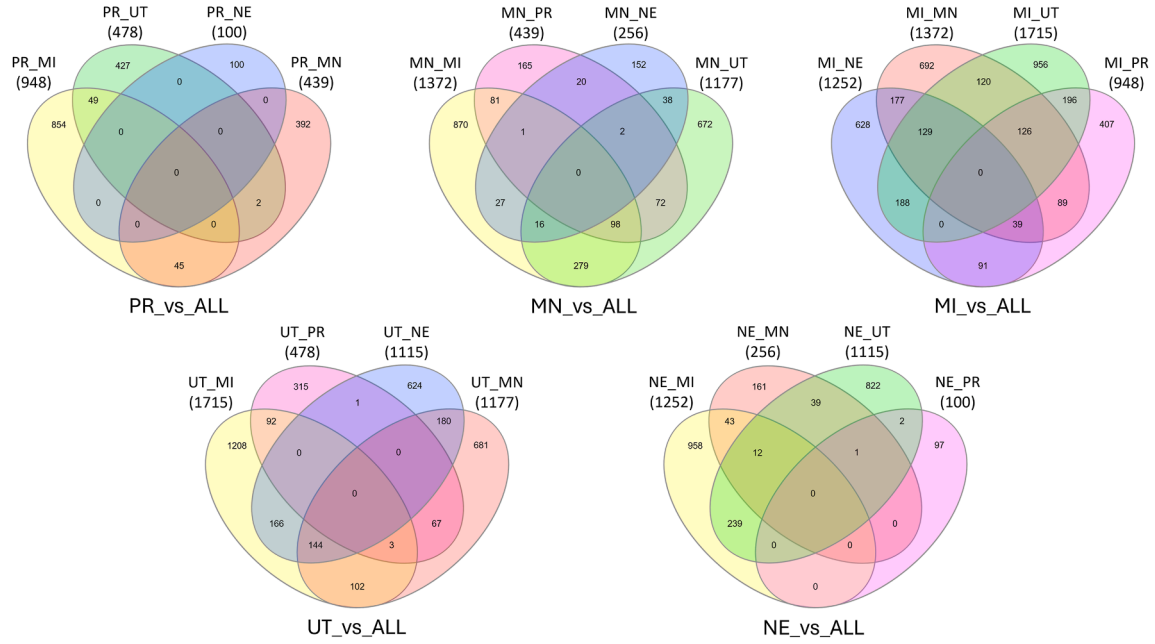

Fig. 3 Venn diagrams illustrating the number of divergent loci ($F_{ST} = 1$) among the five grapevine varieties
PR: 'Primitivo'; MN: 'Malvasia Nera'; MI: 'Minutolo'; UT: 'Uva di Troia'; NE: 'Negramaro'.

were non-redundant between comparisons (Table S4). Among the divergent SNPs, 1 785 were in genes, with 887 found in untranslated regions (UTRs), 518 in introns, and 380 in the coding sequences (CDS).

Overall, this analysis revealed that 'Primitivo' exhibited the lowest number of molecular signatures of divergence (1 979) compared to the other four grapevine varieties, while 'Minutolo Bianco' and 'Uva di Troia' showed the largest number (5 306 and 4 485). The fewest divergent loci were observed in the comparisons between 'Negramaro' with 'Primitivo' (105) and between 'Negramaro' and 'Malvasia Nera' (260).

Six divergent loci were selected to identify unique SNP markers for each of the five varieties and were validated through Sanger sequencing. All polymorphisms were confirmed, indicating that the combination of these 6 SNP markers is effective for discriminating among the five varieties (Table S5; Fig. S1).

The panel of 5 363 SNPs was analyzed using SnpEff, which identified 1 785 variants affecting the genes. Of these, 51.65% (922) were in exons, with 76.7% (707) of these being synonymous variants and 23.32% (215) non-synonymous variants. Annotation analysis revealed that thirteen divergent loci with $F_{ST} = 1$ had variants classified as "HIGH" impact, suggesting a significant effect on gene function or protein structure (Table 1; Fig. 4). Among these SNPs, seven had a stop gained effect, four were splice acceptor variants, and two were stop lost variants. The variants with a stop gained effect were found within the *MEDIATOR OF RNA POLYMERASE II TRANSCRIPTION SUBUNIT* on chromosome15, *AT5G42310-LIKE* on chromosome18, *ARMADILLO BETA-CATENIN-LIKE REPEAT-CONTAINING PROTEIN*, on chromosome19, *HYDROXYPHENYLPYRUVATE DIOXYGENASE* (HPPD) gene on chromosome 2, and the *CALCIUM ION BINDING* and *CALCIUM-TRANSPORTING ATPASE CHLOROPLASTIC* genes on chromosomes 6 and 19, respectively.

Fig. S2 presents BLASTx alignments comparing the nucleotide sequences containing the SNP to the original protein sequences of three genes: VIT_202s0012g01990, VIT_206s0004g00600, and VIT_219s0002g00042. The pairwise alignments confirm the predicted changes in the protein sequences caused by the nucleotide modification. Additional candidate genes were identified based on divergent loci with $F_{ST} = 1$, located within the genes or their proximal regions, as inferred from LD decay analysis (Table S6). Six outlier SNPs, identified in comparisons between 'Minutolo Bianco' vs the other varieties, fall within genes involved in regulating plant growth and development, including gibberellin and mycorrhizal signalling, as well as responses to abiotic and biotic stresses. These genes include *GROWTH-REGULATING FACTOR* (VIT_215s0048g01740, GRF), *APETALA2/ETHYLENE RESPONSE FACTORS* (VIT_216s0013g00890, AP2/ERFs), *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 5* (VIT_216s0013g01050), and *GRAS FAMILY TRANSCRIPTION FACTOR* (VIT_208s0007g00510, VIT_213s0019g01780). Six loci, divergent between UVA DI TROIA and the other four varieties, were all in the *VvGAI1–1* (VIT_214s0068g01610) gene. In addition, a *GIBBERELLIN (GA)-REGULATED PROTEIN 2 PRECURSOR* (VIT_218s0072g01120) was divergent between 'Uva di Troia' and 'Malvasia Nera' and 'Negramaro'. Numerous divergent loci identified WRKY transcription factors and *SAUR* (small auxin upregulated RNA) genes, which are distributed across several chromosomes.

Several genes associated with secondary metabolism were also found. Among these, *GLYCOSYLTRANSFERASES* (GTSs) genes (VIT_202s0033g00240, VIT_204s0023g02510, VIT_205s0062g00270, etc.) are involved in the glycosylation of secondary metabolites, while the *O-METHYLTRANSFERASE* (OMT; VIT_210s0003g00440) gene is involved in flavonoid decoration.

Other relevant divergent genes were involved in the production of secondary metabolites such as the family of the *GLUTATHIONE S-TRANSFERASE* (GST, VIT_201s0026g01330; VIT_201s0026g01340; VIT_201s0026g01370) which play a critical role in fruit pigmentation. Additionally, *GLUTATHIONE PEROXIDASES* (GPXs,

Table 1 List of genes containing divergent SNPs ($F_{ST} = 1$) that are predicted to have a significant effect (HIGH impact) on gene function or protein structure

Chr	SNP position	Gene ID	Gene start	Gene end	Gene function	Comparison	Variant effect
2	8634201	VIT_202s0012g01990	8633024	8634459	4-Hydroxyphenylpyruvate dioxygenase	PR vs NE	stop_gained|c.235C > T|p.Gln79*
3	9286731	VIT_203s0088g01050	9270890	9287382	Ll-diaminopimelate aminotransferase	MI vs UT	stop_gained|c.461G > A|p.Trp154*
4	183565	VIT_204s0008g00260	183115	183936	Amino acid	MN vs MI	splice_donor_variant&intron_variant |c.273+1A > G
4	22943741	VIT_204s0044g01395	22934239	22945853	Swim zinc finger-like protein	MI vs UT	splice_donor_variant&intron_variant| c.1208+2C > T
5	21880163	VIT_205s0102g00060	21880043	21882648	Ankyrin repeat-containing protein at3g12360-like	PR vs MI	splice_donor_variant&intron_variant|c.619+1G > A
6	766380	VIT_206s0004g00600	765088	767737	Calcium ion binding protein	UT vs MI	stop_gained| c.1122T > G|p.Tyr374*
8	14728017	VIT_208s0007g00420	14724285	14728537	Protein kinase	NE vs MI	stop_lost|c.1216T > A| p.Ter406Lysext*
13	3740818	VIT_213s0019g02490	3740333	3756703	Uncharacterized protein loc100259879	NE vs UT	stop_lost|c.2961A > T| p.Ter987Cysext*
15	20140123	VIT_215s0046g03620	20139683	20144426	Mediator of RNA polymerase II transcription subunit	PR vs MI	stop_gained|c.2116G > T| p.Gly706*
17	2884982	VIT_217s0000g03060	2882518	2889082	Pentatricopeptide repeat-containing protein	MN vs MI	splice_acceptor_variant&intron_variant| c.*529-1A > G
18	15161094	VIT_218s0076g00100	15159481	15161375	at5g42310-like partial	UT vs MI	stop_gained|c.601C > T| p.Gln201*
19	7844035	VIT_219s0090g01800	7842956	7844371	Armadillo beta-catenin-like repeat-containing protein	NE vs MI	stop_gained|c.1116T > A| p.Tyr372*
19	18219189	VIT_219s0027g00042	18219094	18221860	Calcium-transporting atpase chloroplastic-like	UT vs MI	stop_gained|c.1228C > T| p.Gln410*

Note: * indicates stop codon.

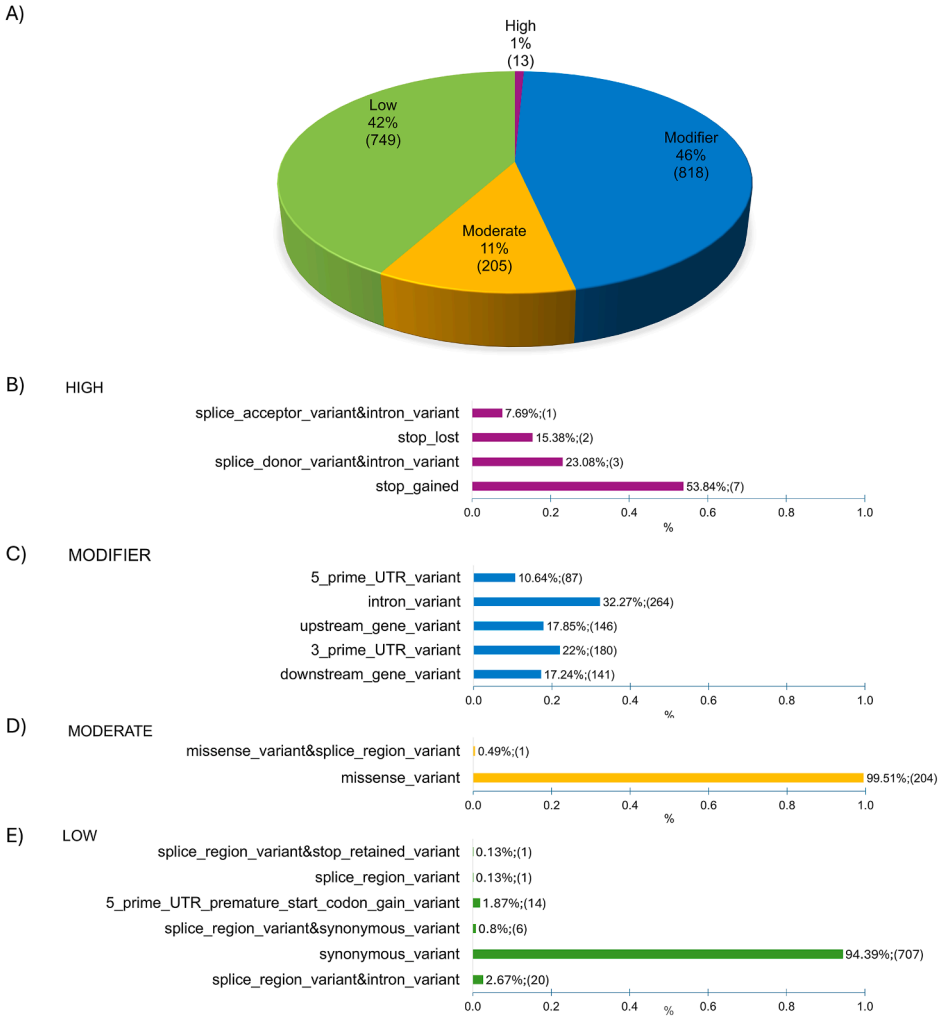

Fig. 4 The overall impact of the SnpEff tool

(A) Percentage contribution of single-nucleotide polymorphisms (SNPs) in high, low, moderate and modifier types of effects. (B–E) Detailed percentage sub-classification of high (B), modifier (C), moderate (D) and low (E) groups. Data in brackets indicate the number of SNPs identified in each effect.

VIT_202s0025g03610; VIT_202s0025g03620) may influence the accumulation and transport of anthocyanins and flavonoids through their role in maintaining cellular redox homeostasis. Several *MYB TRANSCRIPTION FACTOR* genes (e.g., VIT_201s0011g03110, VIT_201s0011g03730, VIT_201s0026g01050) were identified as divergent among the analyzed varieties.

4. Discussion

The International Organization of Vine and Wine (OIV) recognizes the importance of preserving world's grapevine heritage through intra-varietal diversity assessment and polyclonal selection, as outlined in the "RESOLUTION OIV/VITI 424/2010; RESOLUTION OIV-VITI 564B-2019". The protection of local grape biodiversity is increasingly seen as a strategic advantage for producers, especially given the impact of climate change on grapevine physiology and berry quality (Bacilieri et al., 2013; Lózsa et al., 2015). The identification of key genomic regions and genes that regulate these traits can support the development of sustainable viticultural practices (Piarulli et al., 2024). This is particularly relevant for Apulian grape varieties such as 'Primitivo', 'Negramaro', and 'Uva di Troia', which play an important role in wine production in the region (Riaz et al., 2018; Fanelli et al., 2021). Additionally, local policies promote the cultivation of other varieties such as 'Minutolo' and 'Malvasia Nera', which are valued for their contribution to fruity and aromatic wines, and have recently also been considered for the production of sparkling wines (Fanelli et al., 2019; Capozzi et al., 2022).

Building on previous studies that focused on the analysis of intra-clonal genomic diversity in the 'Primitivo', 'Uva di Troia', 'Minutolo Bianco', and 'Malvasia Nera' varieties (Villano et al., 2023; Procino et al., 2025a), this study aimed to: i) comprehensively investigate inter-varietal genetic differences, ii) identify key genes responsible for variations in major morpho-agronomic and qualitative traits, and iii) develop a reliable set of SNP markers for accurate varietal discrimination.

To better understand the genetic diversity of the studied varieties, it was essential to examine their origin and ancestry. IBS and genetic diversity analyses confirmed a high degree of clonal

variability across all five varieties. Inter-varietal comparisons revealed significant genetic divergence, with 'Malvasia Nera' displaying the least differentiation from 'Negramaro' and the greatest from 'Uva di Troia'. The genetic proximity between 'Malvasia Nera' and 'Negramaro' likely reflects the presence of 'Negramaro' in the pedigree of 'Malvasia Nera' (De Lorenzis et al., 2019).

'Primitivo' and 'Negramaro' were found to have high heterozygosity and a lower number of divergent loci compared to 'Minutolo Bianco' and 'Malvasia Nera'. These results could be due to greater intra-clonal variability as previously reported by Villano et al. (2023) and Procino et al. (2025a) for the same varieties. As two of the most important grape varieties in Apulia, 'Primitivo' and 'Negramaro' have undergone extensive selection programs targeting genomic regions associated with wine-quality traits. This has resulted in a great number of accessions/clones and increased intra-varietal variability following the accumulation and fixation of somatic mutations in a heterozygous state through clonal propagation, as shown by Vondras et al. (2019) in 'Zinfandel'. Indeed, 'Primitivo' and 'Negramaro' have a large many clones widely distributed over the territory where they show phenotypic plasticity reflected in the genome by an increased variability. In contrast, 'Minutolo Bianco' and 'Malvasia Nera' exhibited lower overall heterozygosity and fewer differences between clones. The intra-varietal variability affects the vine's physiology and phenology and is crucial for adaptability and resilience to different environmental condition. Studies carried out on 'Malbec' confirmed the effects of somatic mutations on shaping phenotypic diversity and adaptation capacity (Calderón et al., 2021), and in 'Nebbiolo clones, single-nucleotide variants (SNVs) were found to be associated with genes involved in stress response and geographical origin of the accessions (Boccacci et al., 2020). The genetic variation underlying clonal differences in the old variety 'Chardonnay' has also been proposed as a genetic marker for identifying specific clones for authenticity testing, or as breeding markers for new clonal selections (Roach et al., 2018).

The variety 'Uva di Troia' showed a significant genetic distance from the other Apulian varieties, and a high number of divergent loci, confirming the results of previous studies (Miazzi et al., 2020; Villano et al., 2023). This differentiation could be due to the French origin of this variety (D'Onofrio et al., 2021) and was referable in particular to two candidate genes (*GA-REGULATED PROTEIN 2 PRECURSOR* and *VvGAI1–1*) belonging to the *DELLA* subfamily within the *GRAS* family of plant regulatory proteins (Tyler et al., 2004; Jaiswal et al., 2022). These genes are involved into gibberellin (GA)-mediated regulatory networks during seed and berry development (Vargas et al., 2013; Wu et al., 2015; Wang et al., 2020). The *VvGAI1–1* gene (VIT_214s0068g01610) includes six SNPs that were divergent between 'Uva di Troia' and the other four varieties. Both *VvGAI1* and GA-REGULATED *PROTEIN 2 PRECURSOR* are associated with flowering time regulation, a crucial factor in plant adaptation. This regulatory mechanism enables vines to adjust to early or late frosts and seasonal temperature fluctuations. Such adaptability ensures optimal flowering and fruiting periods, ultimately improving ripening conditions (Van Leeuwen et al., 2019).

A large number of divergent loci were also identified in the comparison between 'Minutolo' and the other varieties, including genes related to *APETALA2/ETHYLENE-RESPONSIVE FACTOR* (AP2/ERF), ERF, Grass Family Transcription Factors, and growth-regulating factors (GRFs) families. These genes play key roles in regulating plant growth and development, particularly in hormone responses, root growth, and resistance to environmental stress. 'Minutolo' is particularly known for the volatile compounds that define the unique aromatic profile of its wine. 'Minutolo' is a terpenic variety closely related to 'Moscato Bianco' and 'Moscato di Alessandria', which belong to the "Muscat types" known for their high levels of free monoterpenes (> 6 mg $\cdot$ L^{-1}) (Mateo and Jiménez, 2000). In contrast, 'Primitivo' and 'Negramaro' are non-aromatic varieties (Capone et al., 2013). The aroma profile plays a fundamental role in the sensory characteristics and consumer preferences of wine, and results from significant biochemical modifications, including esterification, hydrolysis, and redox reactions, all facilitated by enzymes (Baiano et al., 2015). The aromatic composition of grapes can change during ripening (Fenoll et al., 2009), with key aromatic compounds, such as terpenes and esters, largely derived from secondary metabolites, synthesized during flowering and fruit development. Studies have shown that the expression of specific genes involved in flowering is also associated with the production of these aromatic compounds (Carmona et al., 2008; Fenoll et al., 2012). The timing of flowering and the environmental conditions during this period can greatly influence the aromatic profile of the grapes, as the accumulation of aroma precursors is closely linked to fruit maturity (Sun et al., 2011; Ubeda et al., 2017).

'Minutolo' showed divergent SNP loci also in other genes involved in the production of secondary metabolites that affect wine aroma, such as *UGTs* and *OMTs*. This was in agreement with the findings of Li et al. (2017), who found that monoterpenyl glycosyltransferases differentially contribute to the production of monoterpenyl glycosides typical of aromatic *V. vinifera* varieties.

Despite their importance, there is limited data available in the literature on these Apulian grape varieties, and the focus is mainly on 'Uva di Troia', 'Negramaro' and 'Primitivo'. These varieties were studied in terms of bioactive compounds, wine aroma volatiles, and antioxidant properties, and to optimize production processes, ensuring traceability, or verifying the trueness to type (Sabetta et al., 2022). Fewer studies have been conducted on 'Malvasia Nera' (Pesavento et al., 2008; Pollon et al., 2019; Dinu et al., 2021; Morcia et al., 2021) and 'Minutolo', which only recently has been investigated for sparkling wine production and for evaluating the effects of the use of a non-*Saccharomyces* starter on the chemical and sensory properties of wines (Fanelli et al., 2019; Capozzi et al., 2022; Paradiso et al., 2022).

A more in-depth analysis of the functional significance of the genetic variations found in 'Minutolo' compared to the other varieties will provide valuable insights into the regulation of biosynthetic pathways responsible for the synthesis and degradation of primary and secondary metabolite in grapes (Rienth et al., 2021).

'Minutolo' and 'Uva di Troia' were distinguished from the other varieties for SNPs in genes primarily related to plant growth, such as the WRKY transcription factors and *SAUR* genes.

The first group is essential for plant development by regulating genes that respond to carbohydrates (such as sucrose, glucose, and fructose) and hormones (including abscisic acid and auxins) in many species, including grape where their expression varies across different stages of berry development (Huang et al., 2021). The *SAUR* genes form the largest family of early auxin response genes (Spartz et al., 2012) and regulate various aspects of plant growth by promoting cell elongation. In grapevine, several *SAUR* members are known to influence berry size, making them potential candidates for grape selection (Li et al., 2021).

Finally, *MYB* genes also contain SNPs that are divergent between varieties. *MYB* genes form a large family of transcription factors involved in epidermal cell differentiation, stomatal opening, flavonoid synthesis and stress resistance (Kim et al., 2024). In grapevine, *MYBs* are central in regulating anthocyanin accumulation (Yan et al., 2021). In particular, the R2R3-MYB transcription factor regulates flavonol synthesis during berry development (Czemmel et al., 2009), while two anthocyanin-related *MYBA* clusters play overlapping and cooperative roles in influencing grape and wine quality traits such as color and astringency (Matus et al., 2017). Further understanding of the role and regulation of these enzymes would facilitate the development of strategies to enhance the sensory properties of wine by promoting the accumulation of secondary metabolites that positively contribute to wine aroma, ultimately improving both grape and wine composition.

Among the key loci, three showed divergences with a high impact on protein function. The *4-HYDROXYPHENYLPYRUVATE DIOXYGENASE* (*HPPD*) gene, which is divergent between 'Primitivo' and 'Negramaro', plays a role in the biosynthesis of tocopherols and plastoquinones. It also significantly influences the accumulation of anthocyanins, which are responsible for the coloration of grape berries (Mei et al., 2025). The color of grape berries is largely determined by the presence and concentration of anthocyanins, flavonoid compounds that are synthesized via the phenylpropanoid pathway. 'Primitivo' and 'Negramaro' have different anthocyanin profiles that contribute to their unique color and sensory properties (Negro et al., 2021; Sabetta et al., 2022). 'Primitivo' is known for its deep, intense color and robust fruity flavour profile, while 'Negramaro' tends to produce less colorful wines with more suitable secondary aromatic nuances. Two high-impact variants were identified in the *CALCIUM ION BINDING* gene and *CALCIUM-TRANSPORTING ATPASE CHLOROPLASTIC* gene, which are divergent between 'Minutolo Bianco' and 'Uva di Troia'. Some studies have shown that these genes are involved in the molecular mechanisms underlying the calcium-induced accumulation of anthocyanins (Yu et al., 2020). Since 'Minutolo Bianco' has a low anthocyanin content due to the white berry phenotype, these SNPs, which both generate stop-gain mutations, should be further investigated by functional studies to determine whether they are actually linked to the observed differences in anthocyanin content.

Overall, these findings emphasize the critical role of identifying outlier SNPs in understanding the genetic basis of grapevine diversity and its influence on wine quality. Outlier variants, especially those with significant functional relevance, enable the identification of key genes involved in essential processes such as anthocyanin biosynthesis, flowering regulation, and the production of aromatic compounds. By isolating SNPs that show significant divergence between varieties, we gain valuable insights into the genetic mechanisms that drive variation in grape composition, growth patterns and sensory attributes. This approach not only improves our understanding of grapevine biology but also opens up new opportunities for breeding programs to improve grape and wine quality. Further functional studies of these outlier SNPs could lead to the development of more precise strategies for enhancing specific traits and ultimately enable the cultivation of grape varieties with optimized aromatic profiles and resilience to environmental stresses.

Finally, the development of a panel of six SNPs which allows to discriminate between the analyzed Apulian varieties holds significant value for the region's viticulture. This genetic barcode offers a precise and reliable method for identifying specific grapevine varieties and will help to preserve the authenticity of regional wines and to prevent mislabelling or fraud. By ensuring that the correct variety is linked to a wine's origin, the SNP panel strengthens traceability and supports quality control in the wine industry. The ability to distinguish between these varieties also helps to preserve the integrity of Apulia's viticultural heritage and ensure the protection of traditional varieties. Moreover, it enhances legal and regulatory compliance by providing a tool for verifying that wines meet regional labelling standards, which is crucial for protecting both local producers and consumers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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