BMC Plant Biology

RESEARCH | Article in Press

Whole-genome sequencing of eight grapevine clones of cv. Tribidrag (*Vitis vinifera* L.) reveals measurable differentiation not linked directly to phenotypic divergence

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Received: 22 May 2026 / Accepted: 1 October 2026
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Abstract

Vegetative propagation of grapevine ensures genetically uniform progeny. Tribidrag (syn. Zinfandel, Primitivo, Kratošija) is a red-berried grapevine cultivar that produces high-quality wines. Although clonal variation in Tribidrag has been described at both the phenotypic and molecular levels, the relationship between phenotypic traits and their underlying genomic basis is poorly understood. In this study, productive traits, including yield, yield components, and basic must chemical parameters, were evaluated in eight Tribidrag clones over three consecutive years. There was significant variability in clone productivity and phenotypic plasticity, and significant clone-by-year interaction. Primitivo clones FPS03 and FPS06 had lower productivity, smaller berry size, greater sugar content, and lower total acidity in 2021 and 2022 than the Zinfandel 1–24 clone, which consistently displayed greater productivity in all three years. Genetic variability among clones was assessed using short-read whole-genome resequencing. A high and relatively uniform level of genomic variation was detected, with ~3.8 million SNPs and ~300,000 InDel variants per sample. Most structural variants were located in intergenic regions. Pairwise SNP differences among eight clones in 100-kb genomic windows revealed candidate signatures of clonal selection. Principal component analysis identified three principal clusters with no clear geographic association among samples. The identified genetic groupings showed no direct association with phenotypic or productive traits of individual clones. These findings contribute to a more comprehensive understanding of the genomic basis of clonal variability in Tribidrag.

Keywords Intravarietal variability · Clonal differences · Tribidrag · Somatic mutation · DNA fingerprinting

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https://doi.org/10.1186/s12870-026-10110-x

Published online: 09 October 2026

Introduction

Intravarietal genetic variability is a fundamental prerequisite for implementing clonal selection, i.e., identifying mother vines with desirable agronomic traits. Clonal diversity is an important source of genetic variability and offers opportunities to improve traditional cultivars and to overcome abiotic stress under specific terroir conditions [1–2]. Vegetative propagation over prolonged periods promotes accumulation of mutations in a given cultivar, with the emergence of multiple distinct phenotypes within that variety. This phenomenon is particularly pronounced in old cultivars grown across extensive geographic areas. In viticulture, as with many other clonally propagated agricultural crops (fruit species, vegetables, ornamental plants), numerous advanced, registered clones that have surpassed the original varieties are available to growers. A representative example is Pinot noir, which has been cultivated for > 2,000 years and exhibits substantial clonal variation, including Pinot gris and Pinot blanc [3]. Clones are propagated vegetatively from a single mother vine, resulting in theoretically uniform progeny that express traits of the maternal plant. The primary drivers of clonal variation within cultivars are somatic mutations [4]. Such mutations affect agronomically important traits relevant to breeding programs and occur relatively frequently within the grapevine genome [5].

The traditional approach to identifying clonal variation is based on characterization of viticultural traits and metabolite profiles [6–8]. With the development of DNA polymorphism-based molecular techniques, various genetic markers have been employed to assess clonal genetic variability. Amplified fragment length polymorphism (AFLP) analysis, a highly promising molecular marker system to detect intravarietal variability, revealed only minor molecular differences among clones of the cultivars Primitivo, Zinfandel, and Crljenak kaštelanski (syn. Tribidrag) [9]. Intravarietal variability among clones of Primitivo, Zinfandel, and Tribidrag has also been investigated using SSR, IRAP, REMAP, and AFLP markers. Although the AFLP technique showed relatively good discrimination, its resolution was insufficient to reliably differentiate Tribidrag clones by their geographic origin and viticultural performance [10]. Integrating traditional clonal selection with modern molecular tools such as genomics is a promising strategy to better understand the genetic drivers of phenotypic variability among grapevine clones and to enhance the efficiency of clonal improvement and breeding programs [2].

The advent of next-generation sequencing (NGS) technologies and advances in genomics has enabled high-resolution, very accurate identification and analysis of structural variants [11]. A comprehensive understanding of structural variation in the grapevine genome is important for clonal selection and to develop novel clones that are better adapted to specific environmental conditions and market demands. Structural variants in the grapevine genome are large-scale genomic alterations involving substantial numbers of nucleotides and can markedly influence gene function, phenotypic expression, and plant adaptation. These variants include DNA sequence changes manifested as inversions, in which DNA segments are excised and reinserted in the reverse orientation; duplications, where specific genomic regions are replicated, potentially increasing gene copy number or enhancing gene expression; deletions, in which genomic segments that may encompass one or multiple genes are lost, affecting plant function; translocations, in which genomic segments are relocated from one locus to another, potentially disrupting gene regulation at both sites; and insertions, in which additional nucleotide sequences are incorporated into the genome, which may alter gene function or expression [12, 13]. Among these forms of genetic variation, single nucleotide polymorphisms (SNPs) and small insertions/deletions (InDels) are the most extensively investigated and are widely employed to determine the genetic basis of key agronomic traits in grapevine clones.

Zinfandel is a very old European cultivar. It shares an identical SSR profile with Primitivo in Italy, Tribidrag in Croatia, and Kratošija in Montenegro [14, 15]. Historic records indicate that this cultivar was once widely distributed across Adriatic regions extending to Germany [16].

The cultivation of Zinfandel in the United States began in 1852; however, the precise European origin from which it was introduced to America remains unclear [17]. It is presumed that the first cuttings were obtained from the Imperial Royal nursery (cultivar collection) in Vienna, which maintained many autochthonous cultivars from the Austro-Hungarian Monarchy. The Vienna nursery, along with many other European nurseries, served as

a source of planting material during the establishment of viticulture in the United States. At that time, the introduction of grapevine cultivars to America was unregulated and varieties arrived via multiple routes, most often along immigrant migration pathways. Historically, it was believed that Zinfandel was introduced by Agoston Haraszthy, as were many other cultivars. However, subsequent evidence demonstrated that Zinfandel was already present in the United States prior to Haraszthy's arrival [18].

The level of selection applied to these cultivars, under three different names in national varietal registers as distinct varieties, yet sharing an identical SSR genotype, has differed substantially among countries. In Italy and the United States, systematic genetic and sanitary selection programs have been implemented, while in Croatia, such selection efforts were conducted historically at a considerably lower intensity. Given the different selection pressures and the three distinct regions with a long tradition of cultivating this cultivar, Zinfandel-Primitivo-Tribidrag (ZPT) represents an excellent model for mutation research. In the United States, genetic selection of ZPT began in the 1960s, with a primary emphasis on vine health, which ultimately resulted in preferential propagation of only one or two clones. Among these, Zinfandel 1 A became the most widely used in commercial production [19, 20]. In Italy, clonal selection programs were also conducted, and new vineyards were established with certified clonal material. In contrast, ZPT remained a long-neglected cultivar in Croatia. At one point, ZPT was on the verge of extinction in Croatia, and its revitalization began in 2002 following the identification and genetic characterization of autochthonous cultivars [14]. Since then, cultivation of ZPT has been successfully revived in Croatia, with a steady increase in new vineyards and the market popularity of its wines growing rapidly [21].

The genetic variability of ZPT clones has recently been investigated using whole-genome sequencing (WGS) and SNP-based markers [11]. Genomic tools have also identified clonal variation in Primitivo [22]. A substantial level of genetic variability was detected among ZPT clones. Mutations and small insertions/deletions (InDels) were most frequently identified within intergenic regions and exons, and less often within coding sequences, suggesting a comparatively limited impact of these mutations on phenotypic traits. Furthermore, the identified clones did not exhibit clear geographic clustering [11]. Comparable genome-wide patterns of clonal variation were reported in other cultivars, including Cabernet Sauvignon, Sauvignon blanc, Chardonnay, and Merlot [23].

As part of the revitalization of ZPT in Croatia, the first virus-free clones of this variety were planted. A relatively high phenotypic variability was observed among the clones, motivating a more detailed investigation of their genetic variability using whole-genome sequencing (WGS) and SNP markers. Recently, the genomes of many traditional Croatian grapevine cultivars were sequenced [24], providing an opportunity to compare structural variants in ZPT clones.

This study investigated the phenotypic variability and viticultural traits of eight ZPT clones under Mediterranean climate conditions in Croatia, and examined their genetic variability using whole genome sequencing.

Materials and methods

Plant material

Eight clones belonging to V. vinifera cvs. Zinfandel, Primitivo, and Tribidrag were analyzed (Table 1). Three clones originated from Croatia (Tribidrag IP130, Tribidrag VV079, and Tribidrag VV101), three from California (Zinfandel 1–10, Zinfandel 1–24, and Zinfandel 1–31), and the last two from Italy (Primitivo FPS03 and Primitivo FPS06). The clones were sourced from the field collection of the Institute for Adriatic Crops and Karst Reclamation (IAC) in Split, Croatia. All clones underwent virus disease testing and therapy at the Foundation Plant Services (FPS) Grape Program at the University of California between 2002 and 2013. The cutting material was transferred from FPS to IAC in 2013. The buds were grafted onto SO4 rootstock clone 47 GM (Vitis berlandieri x Vitis riparia) at Hochschule Geisenheim University, Germany. Grafts were planted in 2014 at the grape germplasm repository of the Institute for Adriatic Crops and Karst Reclamation in Split, Croatia. Fruit and leaf samples were collected from the same vines for viticultural trait measurements and molecular analysis. Formal

Table 1 List of Zinfandel-Primitivo-Tribidrag clones used in this study

Clone code	Clone code at foundation plant services (FPS) UC Davis, California	Library ID	Cultivar	Country origin of selection
Tribi VV079	Zinfandel FPS43.1	CU13	Tribidrag	Croatia
Tribi VV101	Zinfandel FPS44.1	CU7	Tribidrag	Croatia
Tribi IP130	Zinfandel FPS42.1	CU14	Tribidrag	Croatia
Zin 1–24	Zinfandel FPS24	CU11	Zinfandel	USA
Zin 1–10	Zinfandel FPS10	CU8	Zinfandel	USA
Zin 1–31	Zinfandel FPS31	CU12	Zinfandel	USA
Prim FPS03	Primitivo FPS03	CU10	Primitivo	Italy
Prim FPS06	Primitivo FPS06	CU9	Primitivo	Italy

taxonomic identification of the plant material used in this study as Vitis vinifera L. was performed by Dr. Goran Zdunić based on the morphological characteristics of grapevine. The experimental material consisted exclusively of cultivated grapevine selections of the cultivar Zinfandel, with clone identities documented by the clone codes assigned by Institute for Adriatic Crops and Karst reclamation and the corresponding Foundation Plant Services, University of California, Davis, California, USA, codes provided in Table 1. No wild plants or plant material collected from natural populations were used in this study. As the experimental material consisted exclusively of cultivated grapevine selections, no herbarium voucher specimens were deposited.

Phenotypic observations were conducted using the OIV descriptor list, which encompasses shoot tips, mature leaves, and clusters [25].

Ten vines per clone were selected for viticultural trait measurements. There were only five vines of Tribidrag IP130, which therefore was not included in viticultural measurements. Clones were grown under the same viticultural practice. Vines were grown on the bilateral spur cordon system with spacing of 1.0 m between vines and 2.2 m between rows. Eight buds were left on each vine. The soil in the vineyard is clay-sand.

Vinicultural performance of clones

Viticultural performance was evaluated over three consecutive years (2021 to 2023). All vines, regardless of clone, were harvested on the same date each year. During harvest, clusters and yield per vine were determined. One hundred berries per vine were collected, weighed, and crushed to obtain juice. Sugar content (°Brix) was measured with a temperature-compensating digital refractometer (HI96814 Wine Refractometer, Hanna Instruments, RI, USA). Total titratable acidity (g/L) was determined by titration with 0.1 M NaOH and bromthymol blue indicator. Juice pH was measured with a pH meter (FiveGo F2 No C225315429, Mettler-Toledo GmbH, Switzerland). The clusters on each vine were harvested and weighed.

Data analyses of viticultural variables

All recorded viticultural and fruit composition data were analyzed using a two-way analysis of variance (ANOVA), with clone and year as fixed factors, including their interaction (clone × year). All standard ANOVA assumptions, including the normality of residuals and homogeneity of variances, were evaluated through diagnostic plots (Q-Q and residual vs. fitted plots) and were found to be satisfactorily met. When the two-way ANOVA indicated significant effects, differences among clone means within each year were further evaluated using Tukey's Honestly Significant Difference (HSD) post hoc test, which controls the family-wise error rate for multiple pairwise comparisons. To further characterize clone performance within specific seasons, one-way ANOVAs followed by Tukey's HSD tests ($P \leq 0.05$) were conducted for each year independently. All statistical analyses were performed using Statistica version 13 (TIBCO Software Inc., Palo Alto, CA, USA) [26]. Results are presented as mean ± standard deviation (SD) and visualized using OriginPro 9 (OriginLab, Northampton, Massachusetts, USA).

DNA extraction

Young leaves were collected in the spring from shoot tips of individual vines, freeze-dried, and DNA was extracted using the CTAB method [27]. Genomic DNA was cleaned once by sodium acetate precipitation and reconstituted in nuclease-free water (Ambion, Texas, USA).

Microsatellite analysis

The samples were genotyped at nine microsatellite markers: VVS2 [28]; VVMD5 and VVMD7 [29]; VVMD25, VVMD27, VVMD28 and VVMD32 [30]; and VrZAG62 and VrZAG79 [31] to verify the varietal identity of all accessions (Supplementary Table S1). The extracted DNA was quantified and used at a working DNA concentration of 1 ng/µL. All forward primers were 5' end-labeled with fluorescent dyes (FAM, HEX, TAMRA, or ROX). The KAPA Fast Multiplex PCR Kit (2x) (KapaBiosystems, USA) was used to prepare reaction mixtures containing master mix, 100 pmol of each primer, and ~ 1 ng of template DNA. Amplification was performed in ABI 9700 thermal cyclers (Applied Biosystems) using the following program: an initial denaturation at 95 °C for three min, followed by 30 cycles of denaturation at 95 °C (15 s), annealing at 60 °C (30 s), and extension at 72 °C (30 s). A final extension was performed at 72 °C for seven min. DNA from two certified reference cultivars, Muscat à petits grains and Cabernet franc, was amplified and used for data comparison. Amplified products were resolved by capillary electrophoresis on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Applied Biosystems, Foster City, California, USA) with GeneScan-LIZ 600 as an internal standard. Peaks were identified by size and height with GeneMapper 6.0 software (Applied Biosystems).

Population genomic analyses

The genomic data from the same eight clones in this study were reported in a previous study [24]. In brief, sequencing libraries with an insert size of ~ 150 to 250 bp were prepared with the NEBNext Ultra DNA Library Prep Kit (Illumina, USA) according to the manufacturer's directions. Paired-end sequencing (150 bp) was performed on an Illumina NovaSeq 6000 platform by both Novogene (Beijing, China) and Berry Genomics (Beijing, China). The target sequencing depth was 20× for each accession. Variant detection was carried out with GATK3 (v.3.8; https://github.com/broadinstitute/gatk) according to the recommended workflow using the VS-1 genome as a reference. Since the data contain information for 3,525 *Vitis* accessions [24], we extracted the SNPs and InDels for the eight clones from the final VCF file. We removed monomorphic and missing loci, but retained all 0/0 positions. These filtering criteria yield variants that differ among analyzed clones. SNPs and InDels were annotated by genomic location using ANNOVAR (version 2016-02-01; [32]. The distribution of SNPs and InDels was visualized with the CMplot package (version 4.5.1; [33]) in R (version 4.2.3). Whole-genome SNPs were used to construct the phylogenetic tree using SNPhylo (version 20140701; [34]). Principal component analysis (PCA) was performed with genome-wide complex trait analysis software (GCTA, version 1.94.1; [35]), and the first two eigenvectors were plotted. Relationship between each clone was verified using KING (version 2.2.5; [36]) with the option "--kinship".

The genome of VS-1 was divided into 100 kb windows. For each pairwise combination of the eight clonal accessions, the number of differential SNPs in each window was counted. Pairwise genetic differences were quantified as the number of differential SNPs divided by the window length. For each window, the pairwise genetic differences across all combinations were averaged. A Manhattan plot was generated based on these averaged values. Windows in the top 1% of the distribution were identified as significant. Genes located within these significant windows were further retrieved and KEGG enrichment was performed using TBtools (version 2.210; [37]).

Results

Phenotypic variability of ZPT clones

We examined the phenotypic characteristics of eight ZPT clones, including three clones originating from old vineyards in Croatia (Tribi VV079, Tribi VV101, and Tribi IP130), three clones from the USA (Zin 1–24, Zin 1–10, and Zin 1–31), and two clones from Italy (Prim FPS03 and Prim FPS06). The phenotypic traits of shoot tips, mature leaves, and grape clusters were highly uniform among the clones (Supplementary Figs. 1, 2, 3 and 4). Only very subtle differences were observed between clones in the density of prostrate hairs on the shoot tip (OIV 004), degree of petiole sinus opening (OIV 079), degree of resistance to Botrytis (OIV 459), and cluster compactness (OIV 204; Supplementary Table 2).

We compared the productive traits of the clones over three consecutive years. Two-way ANOVA revealed that clone significantly affected all analyzed variables ($p<0.001$; Table 2). Year significantly influenced most yield and grape composition parameters except cluster weight. In all three years, statistically significant differences were observed among clones in yield, cluster weight, number of clusters per vine, and berry weight ($p<0.05$). In 2021, clones Tribi VV101 and Zin 1–24 (3.3 kg) had the greatest yield, while in 2022 and 2023, Zin 1–24 had the greatest yield (2.1 kg and 3.1 kg, respectively). Cluster weight was greatest for Tribi VV101 in 2021, while in 2022 and 2023, the greatest cluster weights were in Zin 1–24 and Tribi VV079 (Fig. 1).

There were also significant differences in must chemistry among clones and years. Sugar content was greatest in 2022, reaching 25.3 °Brix in Zin 1–24, while total acidity varied across years, with higher values recorded in 2021 and 2023 than in 2022. pH values differed significantly among clones and vintages. Clone had a highly significant effect on all traits ($p<0.001$), while year had a strong effect on all parameters except cluster weight ($p=0.148$). The C × Y interaction was significant for all traits except the number of clusters per vine, indicating that individual clones respond differently depending on the growing season.

Genetic variability among ZPT clones

The amount of filtered data ranged from 7.01 to 8.90 GB, with sequencing depths of 14.46× to 18.14×, ensuring reliable variant detection (Supplementary Table 3). Quality scores Q20 (>95%) and Q30 (>89%), along with a low error rate (0.03%), confirm high sequencing accuracy. The mapping rate to the reference genome was also high (96.62 to 97.74%). GC content was stable (35.28 to 36.55%), and the duplication rate was moderate (13.19 to 17.92%).

A similar level of genomic variability was observed among the analyzed clones (Table 3). The total number of SNPs ranged from 3,796,699 in Prim FPS06 to 3,835,193 in Prim FPS03, while the number of InDel variants ranged from 302,688 to 309,789, with the highest values also found in Prim FPS03. The number of unique SNPs varied from 135 (Prim FPS06) to 365 (Prim FPS03), while the number of unique InDels ranged from 65 (Tribi VV101) to 146 (Prim FPS03). Notably, the Prim FPS03 clone consistently had the most total and unique variants, indicating more pronounced genetic differentiation than the other clones. The remaining samples showed

Table 2 Results of two-way analysis of variance (ANOVA) showing the effects of clone (C), year (Y), and their interaction (C × Y) on yield components and grape composition parameters of Tribidrag clones evaluated over three consecutive growing seasons. Values represent F-statistics for each source of variation

Source	Degree of freedom	Yield	Cluster weight	Berries per cluster	Clusters per vine	Berry weight	Total soluble solids	Total acidity	pH
Clone (C)	6	19.91***	19.29***	5.82***	9.02***	14.74***	4.58***	12.14***	4.03***
Year (Y)	2	25.29***	1.92ns	99.41***	33.68***	202.23***	134.91***	248.34***	11.99***
C x Y	12	5.71***	4.69***	4.12***	1.41ns	1.82*	2.01*	6.19***	2.59***

Significance levels are indicated as follows: ns, not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$

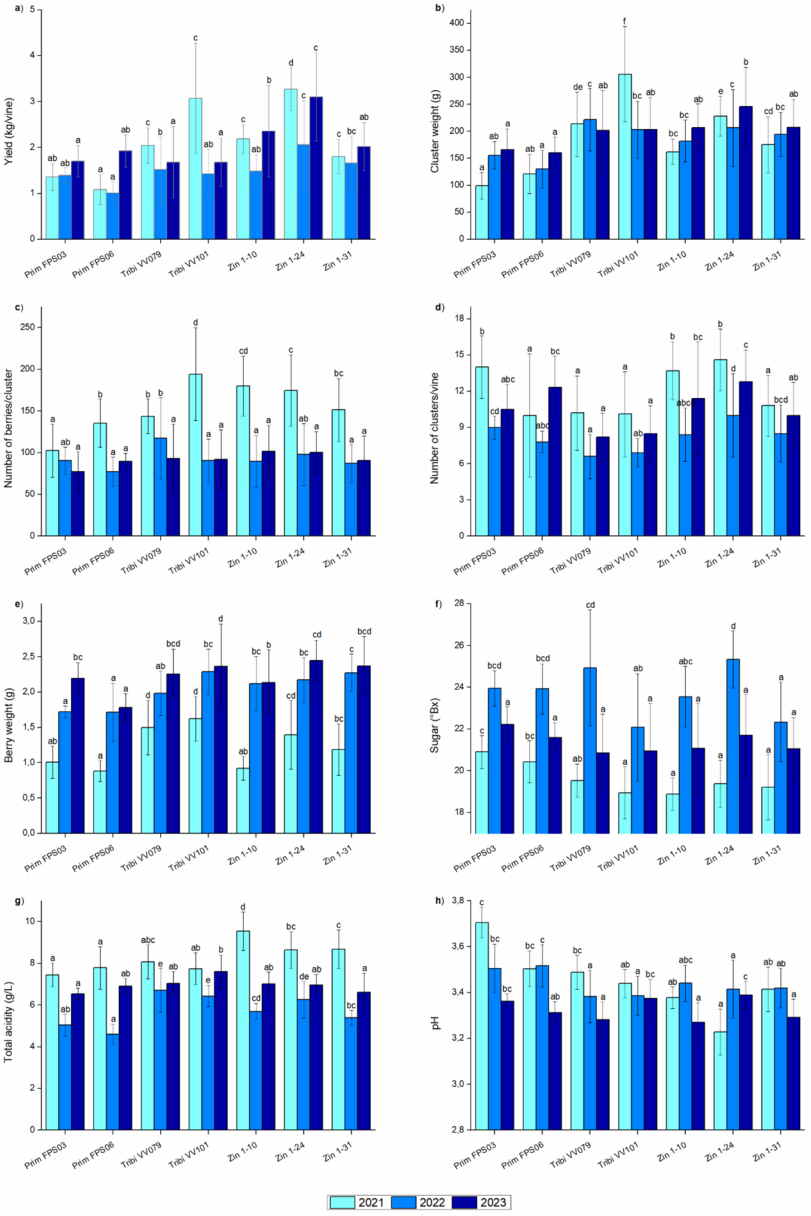


Fig. 1 Yield components and fruit composition among seven clones of Zinfandel-Primitivo-Tribidrag across three growing seasons (2021 to 2023). Bar charts show (**a**) yield (kg vine^{-1}), (**b**) cluster weight (g), (**c**) number of berries per cluster, (**d**) number of clusters per vine, (**e**) berry weight (g), (**f**) soluble solids (°Bx), (**g**) total acidity (g L^{-1}), and (**h**) juice pH. Bars represent mean values for each year (2021, 2022, and 2023), and error bars indicate standard deviation. Different letters above bars within the same year indicate significant differences among clones according to Tukey's HSD test ($p \leq 0.05$). One-way anova analyses were performed separately for each year

smaller deviations and a relatively narrow distribution of total variants, with moderate differences in the number of unique SNPs and InDel variants, suggesting a similar degree of overall genomic diversity among them.

For SNP variants, the largest proportion consisted of intergenic variants (~2.19 to 2.22 million), followed by intronic variants (~913,010 to 918,246), while the number of exonic SNPs was substantially lower (~241,351 to 243,768; Table 4). Variations in regulatory regions (upstream and downstream) were also abundant (~194,318 to 202,253), while splicing, 5′ UTR, and 3′ UTR variants were less frequent. The Prim FPS03 sample had the most intergenic, intronic, and upstream/downstream SNPs, consistent with its previously observed higher total variant count. A similar distribution pattern was observed for InDel variants. The majority of InDels were in intergenic regions (~58,075 to 59,960), followed by intronic regions (~29,685 to 30,009), while exonic InDels were

Table 3 Summary of variations in Zinfandel-Primitivo-Tribidrag clones

Note: The monomorphisms for all clones were removed, and for each clone, the missing loci (./.) were removed, but remained the 0/0 genotype

Sample	SNP number	Unique SNP	InDel number	Unique InDel
Tribi VV101	3,817,156	161	305,720	65
Zin 1–10	3,807,991	167	304,714	69
Prim FPS06	3,796,699	135	302,688	83
Prim FPS03	3,835,193	365	309,789	146
Zin 1–24	3,818,172	182	306,425	86
Zin 1–31	3,797,152	172	304,217	76
Tribi VV079	3,809,036	161	305,279	71
Tribi IP130	3,816,223	197	306,427	99

Table 4 Summary of SNP and InDel annotation

SNP	Exonic	Intergenic	Intronic	Splicing	Upstream	Downstream	UTR5	UTR3	Upstream; Downstream
Tribi VV101	243,756	2,206,767	916,894	1745	199,171	195,178	13,083	21,782	18,780
Zin 1–10	243,509	2,200,111	915,528	1731	198,565	194,925	13,101	21,782	18,739
Prim FPS06	243,768	2,191,757	914,501	1731	196,981	194,398	13,119	21,808	18,636
Prim FPS03	242,808	2,219,390	918,246	1726	202,253	196,807	13,099	21,808	19,056
Zin 1–24	243,305	2,207,221	916,693	1724	199,906	195,628	13,081	21,786	18,828
Zin 1–31	241,351	2,194,994	913,010	1716	198,357	194,318	13,063	21,730	18,613
Tribi VV079	243,437	2,200,863	915,333	1728	198,947	195,108	13,072	21,781	18,767
Tribi IP130	243,402	2,205,811	916,100	1726	200,048	195,471	13,087	21,783	18,795
InDel	**Exonic**	**Intergenic**	**Intronic**	**Splicing**	**Upstream**	**Downstream**	**UTR5**	**UTR3**	**Upstream; Downstream**
Tribi VV101	3213	58,837	29,848	82	8398	8049	690	1297	849
Zin 1–10	3189	58,619	29,788	83	8341	8017	690	1296	836
Prim FPS06	3224	58,075	29,733	84	8231	7926	690	1305	833
Prim FPS03	3182	59,960	30,009	84	8638	8127	688	1310	873
Zin 1–24	3193	59,041	29,872	84	8425	8050	692	1299	851
Zin 1–31	3133	58,596	29,685	83	8318	7986	684	1305	846
Tribi VV079	3191	58,694	29,822	83	8366	8057	682	1306	845
Tribi IP130	3199	59,003	29,822	83	8461	8067	690	1295	846

comparatively rare (~3,133 to 3,224). Variants in splicing and UTR regions were present in very low numbers. As with SNPs, Prim FPS03 had the most intergenic and regulatory InDel variants.

SNPs and InDels are distributed heterogeneously across the genome (0.5 Mb windows), with clearly defined regions of increased and decreased density across the 19 chromosomes (Fig. 2). For SNPs, local "hotspot" regions (yellow to red zones) with elevated variant frequency are particularly pronounced in segments of chromosomes Chr9, Chr13, Chr14, Chr17, and Chr18. In contrast, certain regions, especially pericentromeric or distal segments of some chromosomes, exhibit lower variant density (green zones). The number of SNPs per window reaches a maximum of ~10,470 variants per 0.5 Mb. A similar distribution pattern was observed for InDel variants, although with lower absolute values (up to ~720 variants per window). Regions of increased InDel density largely overlap with regions of elevated SNP density, suggesting a correlated distribution of different variant types along the genome.

Genetic relatedness among ZPT clones

PCA analysis clearly separates clones and indicates the presence of differentiated groups (Fig. 3). PC1 explains 14.55% and PC2, 14.42% of the total variability. Clones Tribi VV079 and Tribi VV101 cluster together in the same region of the plot, indicating their close genetic relationship, while Zin 1–31 is clearly separated along the PC2 axis, suggesting greater genetic distance relative to the other samples. Prim FPS03, Zin 1–24, Tribi IP130, and Zin 1–10 occupy intermediate positions and show relative proximity to each other, while Prim FPS06 differentiates along the PC1 axis. The phylogenetic dendrogram confirms these relationships (Fig. 4). Tribi VV101

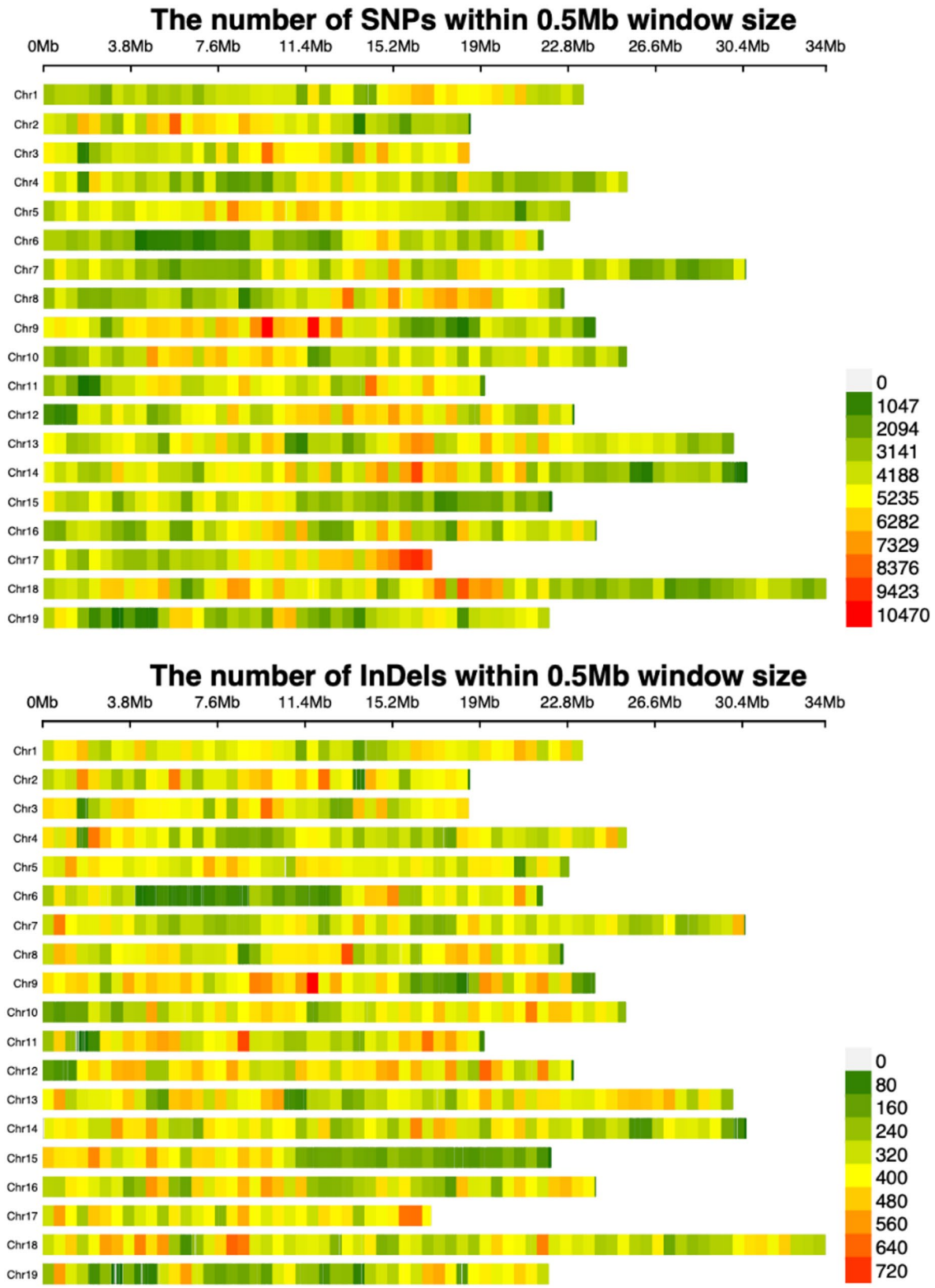


Fig. 2 Distributed characteristics of SNP and InDel across the VS1 chromosomes

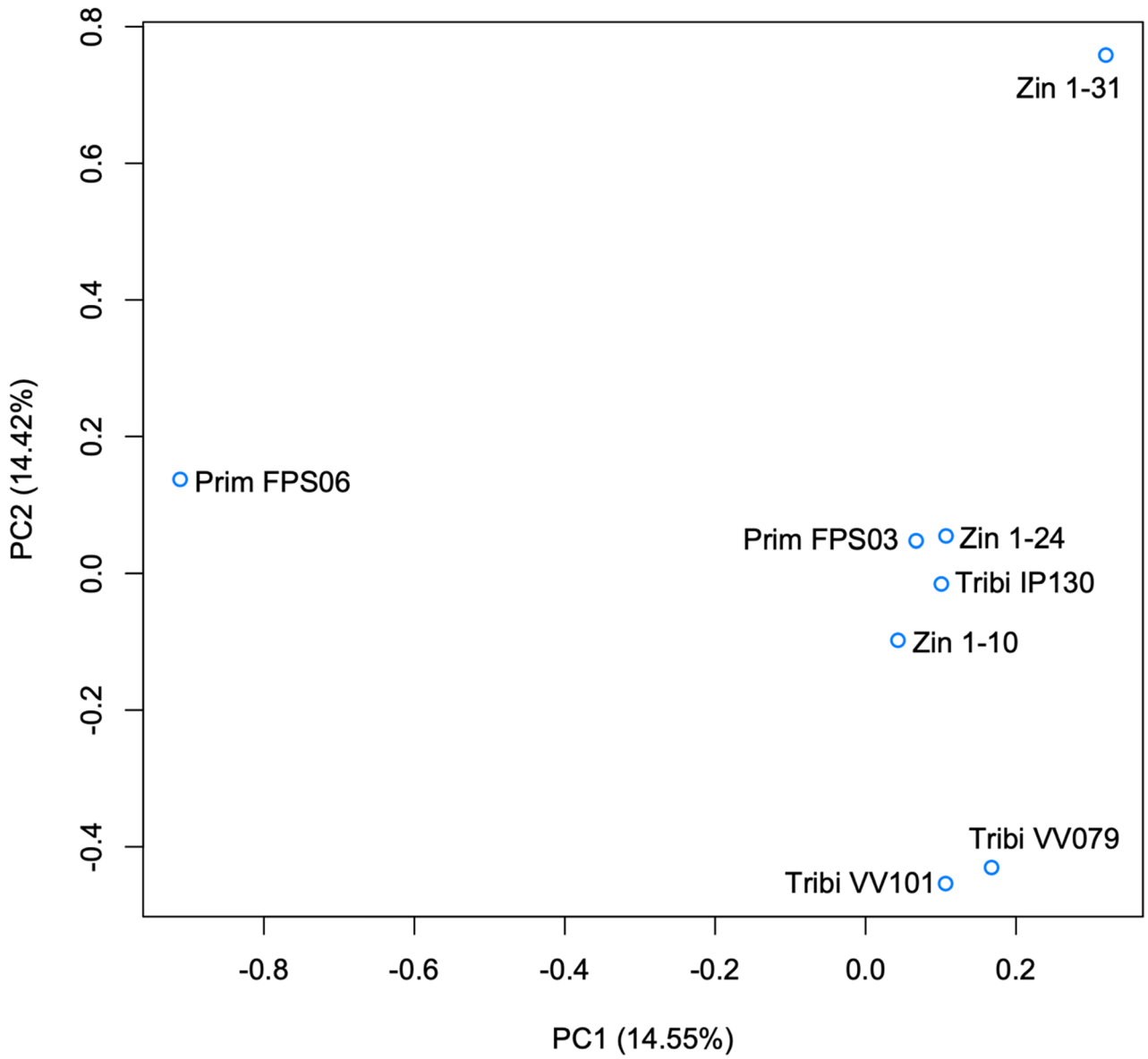


Fig. 3 Principal component analysis (PCA) of eight grapevine clones of Zinfandel-Primitivo-Tribidrag based on SNP markers

and Tribi IP130 form a close cluster, while Zin 1–24, Zin 1–31, and Prim FPS06 group within the same broader branch. Prim FPS03 and Tribi VV079 exhibit longer branch lengths, reflecting their relatively higher genetic differentiation. However, overall genetic distances are very small (scale 0.01), indicating that while the clones are highly similar, the differences are sufficient to distinguish subgroups. Kinship analysis further quantifies these relationships (Fig. 5). Kinship coefficients fall within a narrow range (0.410 to 0.422), reflecting generally high genetic relatedness among the clones. The greatest coefficients were observed between Prim FPS03 and the Tribi VV101 and Tribi IP130 clones, while Zin 1–31 shows relatively low relatedness values, corroborating its greater differentiation in PCA analysis.

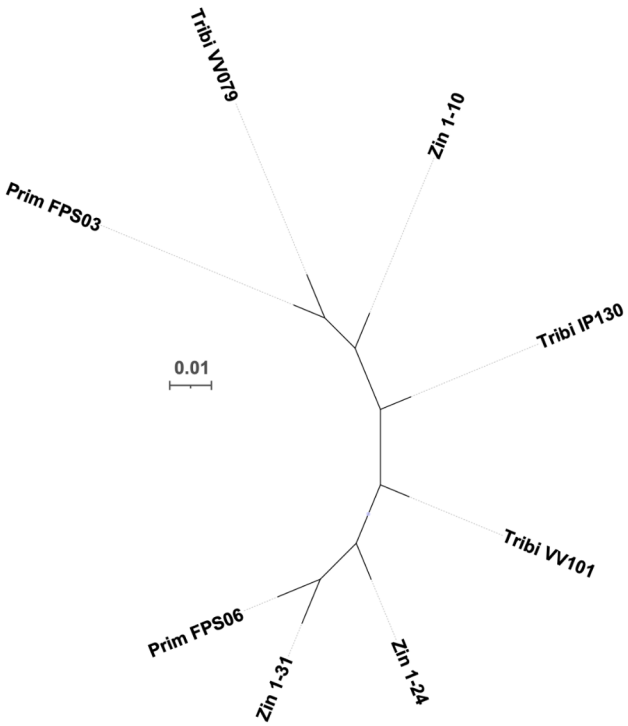


Fig. 4 Phylogenetic tree of eight grapevine clones of Zinfandel-Primitivo-Tribidrag based on SNP markers

Candidate genomic signatures potentially associated with clonal selection

Clonal propagation maintains the genetic identity of grapevine cultivars over thousands of years, but selection with its associated genetic changes allow clones to adapt better to environments at new locations. To investigate the genetic signatures of clonal selection, we compared pairwise local SNP differences among eight ZPT clones across the genome in 100-kb windows (Fig. 6). As expected, most regions showed < 0.2% nucleotide differences in pairwise comparisons, attesting to the genetic basis of clonality. However, there were also multiple regions with significantly elevated mean SNP difference (top 1%: > 0.456% nucleotide differences), notably on Chr9 and Chr18. Functional annotation of genes within these high-variability regions identified a cluster of candidate R genes (*RPS2*), LRR receptor-like genes, and ITN-like genes located in the peak at Chr9. Because these genes are well-known players in plant disease resistance and environmental adaptation (Supplementary Table 4), this result suggests that the top priorities of clonal selection were local adaptation of the cultivar. This functional profile was further supported by KEGG enrichment analysis of all genes in the top regions, which highlighted "plant–pathogen interaction" and "environmental adaptation" as significantly enriched terms (Supplementary Table 5). These annotated genes and enriched pathways are therefore considered biologically plausible candidate regions potentially associated with clonal selection.

Discussion

This study determined phenotypic and genomic differences among eight Zinfandel clones. To achieve this, we evaluated morphological traits, assessed key viticultural characteristics under field conditions, and performed whole-genome sequencing (WGS). The underlying hypothesis was that each clone represents a unique phenotype with distinct agronomic traits resulting from somatic mutations within the genome.

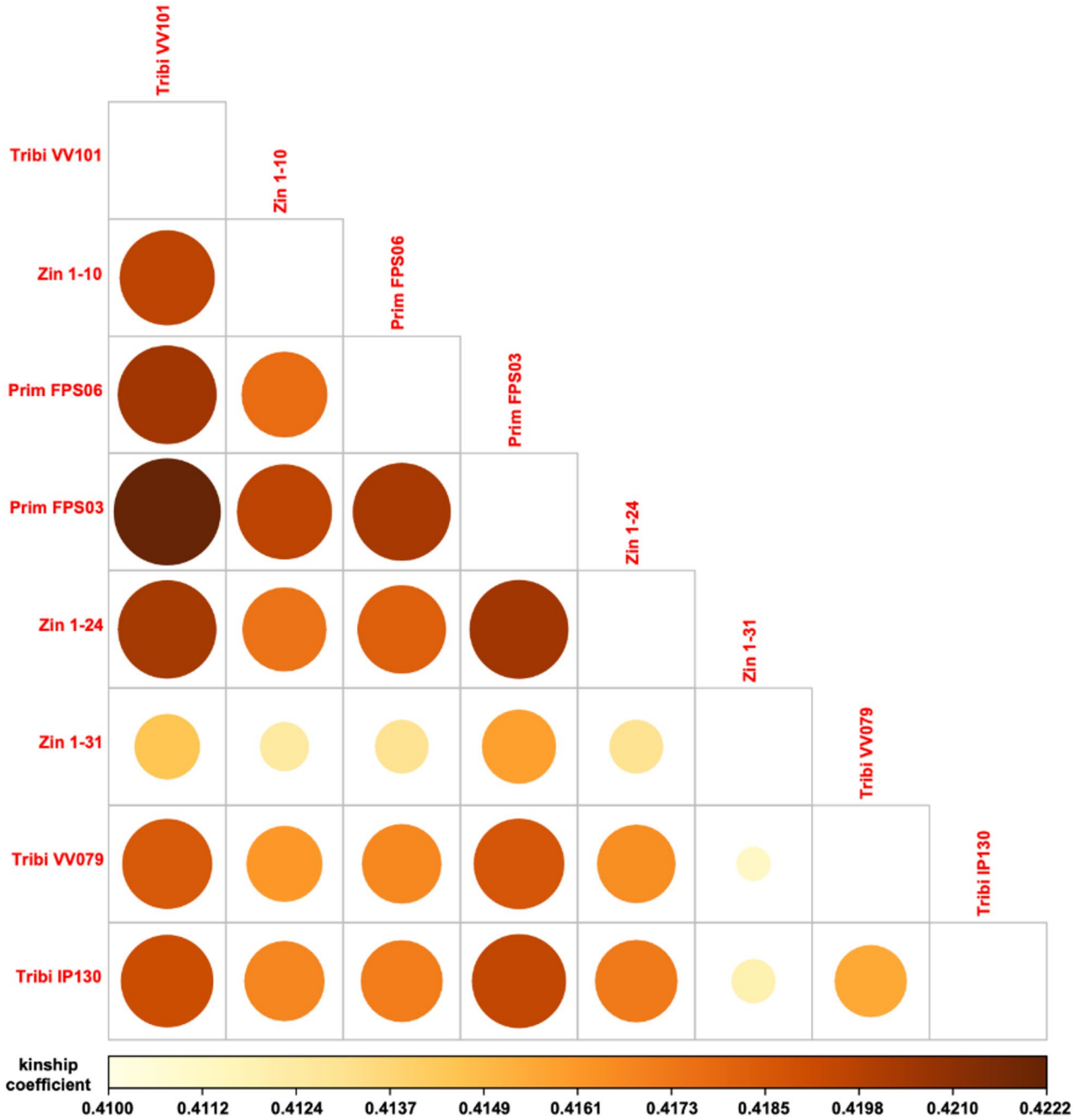


Fig. 5 Relationship inference with kinship coefficient for eight grapevine clones of Zinfandel-Primitivo-Tribidrag based on SNP markers

Phenotypic differentiation among clones

A two-factor analysis of variance (ANOVA) of the productive traits of Zinfandel clones revealed statistically significant effects of clone, growing season, and their interaction. There was a significant interaction for all productive traits, demonstrating that differences among clones were not consistent across growing seasons, i.e., individual clones responded differently to environmental variation. Such a pattern suggests differing degrees of phenotypic plasticity among the investigated Zinfandel clones and a strong dependence on climate conditions

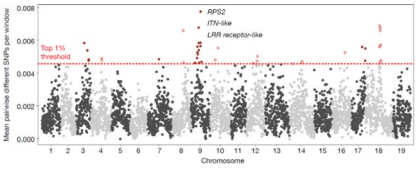


Fig. 6 Genome-wide distribution of local SNP differences calculated in consecutive 100-kb windows for all pairwise clonal comparisons. The Y-axis indicates the proportion of SNP differences per window across the grapevine genome (X-axis)

during key developmental stages of berry growth [38, 39]. In addition to environmental effects, epigenetic mechanisms such as DNA methylation may contribute to phenotypic variability among grapevine clones, as environmentally induced epigenetic modifications can influence gene expression and plant traits without altering the underlying DNA sequence [40, 41]. The matching of a specific clone to a particular terroir may therefore be an important adaptive strategy in the context of climate change, and our results indicate that clones should be evaluated across multiple terroirs. A limitation of this study is that phenotypic evaluations were conducted at a single experimental site over three consecutive growing seasons. While this approach allowed assessment of clonal performance across years, it did not permit evaluation of genotype × environment interactions. Therefore, the observed phenotypic differences should be interpreted within the context of the studied Mediterranean environment, and future multi-location trials will be necessary to validate the stability and broader applicability of these findings across diverse soil and climatic conditions. A given clone may exhibit superior performance in one environment, but not necessarily maintain this superiority under substantially different environmental conditions. Ripening dynamics and other phenological stages are particularly critical factors in aligning clone performance with environmental conditions. In our study, significant differences were detected among clones in basic grape quality parameters (sugar content, total acidity, and pH), suggesting that harvest timing should be optimized individually for each clone. A five-year evaluation of production traits in six Pinot blanc clones also reported a significant clone-by-growing-season interaction [42].

Our results across individual growing seasons indicate that the Primitivo clones FPS03 and FPS06 consistently had lower productivity, smaller berry size, greater sugar content, and lower total acidity during 2021 and 2022 compared, for example, with Zinfandel 1–24, which maintained high productivity throughout all three years. The observed differences in soluble solids (sugar content) among Tribidrag clones may not be solely attributable to structural genomic variation. Differences in yield and yield components can influence source–sink relationships and carbohydrate allocation, thereby affecting sugar accumulation in berries. These findings are consistent with previous research on clonal variants of Primitivo and Zinfandel, in which Primitivo clones demonstrated superior productivity, earlier ripening, greater sugar accumulation, and lower berry weight than Zinfandel clones [19, 20]. Although genetic differentiation was detected among the Zinfandel clones, genetic variation among individual vines within each clone should also be considered, as such within-clone variation may contribute to phenotypic differences. This possibility warrants further investigation using larger numbers of individually sampled vines from each clone.

Variability in viticultural performance is well documented among clones of different cultivars. For example, Malbec clones respond quickly to seasonal variation [43]. Clonal populations of eight important European cultivars had stable sugar content over a 14-year period in Pinot gris, Pinot noir, and Müller-Thurgau, while in Pinot blanc and Riesling, mean values and range varied significantly over time [44]. Sources of variability within vineyards may include soil heterogeneity, differences in bud load per vine, total leaf area, and training system [45, 46]. In the present study, all clones were evaluated within the single vineyard, with 10 vine replicates per clone. Nevertheless, there was a relatively high level of variability among replicates. The results, therefore, represent

preliminary estimates of the clones' productive performance. In future viticultural evaluation cycles, it would be advisable to include more replicates and multiple terroirs, which would likely improve the resolution of differences among clones.

Morphological evaluation revealed subtle differences among clones in the density of prostrate hairs on the shoot tip and susceptibility to *Botrytis cinerea*. Notably, Primitivo clones FPS03 and FPS06 had a looser cluster architecture, which was associated with greater resistance to gray mold than the other clones. Cluster compactness in grapevine is a highly relevant trait from a production standpoint and represents a primary target in clonal selection programs [47]. In the Tribidrag IP130 clone, slightly greater susceptibility to gray mold was observed, corresponding with its relatively more compact clusters. These results suggest that cluster compactness in the investigated clones should be analyzed further with respect to cluster architecture, flower morphology, and fertilization using advanced phenotyping methods to better understand the underlying mechanisms of this complex trait [48]. Clonal variation in cluster compactness has also been linked to atypical flower morphology, reduced pollen availability, and impaired gamete functionality in Tempranillo clones [49]. Similar patterns of variation have been observed among Malbec clones, affecting flower morphology, flower functionality and fruit set rates [50].

Careful evaluation of leaf morphology revealed a slightly smaller leaf area in Primativo FPS03 and FPS06, while mature leaves of Tribidrag VV079 and VV101 exhibited more pronounced and deeper lateral sinuses than the other clones. Leaf morphological traits in grapevine form the basis for varietal identification and the recognition of clonal variation [51]. Although the observed differences in leaf morphology are subtle and influenced by environmental factors, they cannot currently be linked directly to productive traits. However, these characteristics could serve as practical markers for clone identification in the field.

Genomic variability among clones

Analysis of relatedness among the clones did not reveal clustering according to phenotypic traits or geographic origin. Because formal genotype–phenotype association analyses were not feasible with the limited number of genetically distinct clones, the lack of correspondence between genome-wide differentiation and phenotypic variation should not be interpreted as evidence for the absence of specific genotype–phenotype associations, which require validation in larger datasets. Instead, there was a high and relatively uniform level of genomic variability among the analyzed clones, with ~ 3.8 million SNPs and around 300,000 InDel variants per sample, yet a very limited number of clone-specific (unique) variants. This pattern is consistent with an analysis of 15 Zinfandel clones, including Primitivo and Tribidrag, using whole-genome resequencing [11]. Many of these clones overlap with those examined in this study. In our research, the number of unique SNPs (135 to 365) and InDels (65 to 146) per clone further supports the conclusion that intravarietal variability in Zinfandel results from gradual accumulation of a limited number of somatic mutations during vegetative propagation. Similar findings were reported in an analysis of four Malbec clones [50]. However, in the present study, all clones underwent virus elimination via in vitro plant tissue culture, a process that itself can generate somatic mutations [52, 53]. Therefore, some of the observed mutations in the analyzed clones may not reflect historical accumulation during clonal propagation, but could also result from more recent mutational events. Future comparative genomic analyses of the original source material, combined with detailed propagation records, will be necessary to determine the evolutionary origin of the detected variants and to distinguish naturally accumulated somatic mutations from those potentially arising during historical propagation or virus sanitation procedures.

In this study, "hotspot" regions of elevated SNP and InDel density were observed on multiple chromosomes (Chr9, Chr13, Chr14, Chr17, and Chr18), alongside regions of reduced variability, particularly in pericentromeric areas. The overlap between high-density SNP and InDel regions suggests correlated mechanisms of mutation formation, consistent with observations that certain genomic regions are more prone to accumulating mutations and other genetic changes than others [50].

Genomic differences were primarily in intergenic and intronic regions, with a substantially smaller proportion located in exons, consistent with previous findings [11]. A similar pattern, in which structural variants are located predominantly in intergenic regions with largely neutral effects on phenotype, was reported for Cabernet Sauvignon, Sauvignon blanc, Chardonnay, and Merlot clones from Chilean nurseries [23]. In the present study, ~ 241,000 to 244,000 SNPs were identified within exons; however, these represent only a small fraction of the total variants compared with those in intergenic regions (~ 2.2 million). This confirms that most genomic diversity accumulates in regions with limited functional regulation. However, there are clear hotspots on chromosomes where SNPs accumulate, e.g., Chr9 and Chr18. We examined the genes in these regions and found that many are putative players in disease resistance and environmental adaptation. This indicates clearly that the studied Tribidrag clones underwent clonal selection throughout their history across different geographic regions of cultivation. The highly differentiated genomic regions identified in this study provide a valuable resource for future investigations aimed at identifying candidate genes underlying agronomically important traits through integrated genomic and functional analyses.

Relatedness analysis among the clones in this study revealed very small genetic distances, with kinship coefficients ranging from 0.410 to 0.422, yet sufficient to distinguish subgroups. This pattern aligns with findings that even a limited number of reliable somatic variants can enable precise identification and discrimination of clones when a large set of genome-wide markers is analyzed [23]. The clear differentiation of certain clones, as evidenced by the distinct placement of Zin 1–31 in the PCA analysis (Fig. 3), may reflect differences in propagation history, selection practices, or the accumulation of mutations. The Croatian Tribidrag clones VV101 and VV079, which exhibit high genomic similarity, were selected from the same vineyard at the Svinišće site and likely originate from the same mother vine. In contrast, Tribidrag IP130, selected from a different site in Kaštela, is positioned farther from them in the PCA, indicating a distinct propagation and selection history. Notably, the Prim FPS03 clone consistently has the most total and unique variants and shows more pronounced differentiation in phylogenetic analyses. This pattern likely reflects its longer cultivation history, greater exposure to environmental stresses, or less intensive selection during nursery propagation.

Conclusion

Overall phenotypic and genomic diversity among studied ZPT clones is low, yet sufficiently pronounced to allow reliable differentiation. Genome-wide genetic differentiation among the investigated Zinfandel clones was not clearly reflected in the major agronomic and quality traits evaluated over three growing seasons In Primitivo FPS03, a clear phenotypic divergence from the other clones was observed, likely reflecting more potentially functional structural variants. Further studies involving more clones and environments are needed to test specific genotype–phenotype associations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1186/s12870-026-10110-x.

Acknowledgements This research was supported by the Croatian Science Foundation under the project "Prospection and genomic analysis of endangered Croatian grapevine cultivars and wild grapevine – RemainGrape" (IP-2025-02-5376). WC is supported by the National Natural Science Foundation of China (grant 32070599) and Yunnan Fundamental Research Projects (grant 202402AE090004).

Author contributions GZ conceptualized the study with input from WC and wrote the first draft of the manuscript. KL, AM, LM, and MOZ managed plant material and conducted field trials. SD and CW developed the bioinformatics pipeline for genomic analyses. EM and IP contributed to data interpretation. All authors reviewed and commented on previous versions of the manuscript and approved the final version.

Funding This research was supported by the Croatian Science Foundation under the project "Prospection and genomic

analysis of endangered Croatian grapevine cultivars and wild grapevine – RemainGrape" (IP-2025-02-5376). WC is supported by the National Natural Science Foundation of China (grant 32070599) and Yunnan Fundamental Research Projects (grant 202402AE090004).

Data availability The data supporting the findings of this study are included in the manuscript. The WGS resequencing data is taken from publication [24], and data is available at the Genome Warehouse in the National Genomics Data Center, China National Center for Bioinformation, under accession number CRA006917.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

Key message Tribidrag clones show substantial phenotypic and genomic variation, with SNP differences among eight clones in 100-kb genomic windows revealing signatures of clonal selection.

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