

The cytonuclear interactions during grapevine domestication^{oo}

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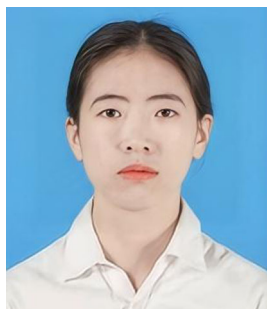
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ABSTRACT

DNAs from the cytoplasmic genomes often communicate with the nuclear genome during regulation, development, and evolution. However, the dynamics of cytonuclear interaction during crop domestication have still been rarely investigated. Here, we examine cytonuclear interactions during grapevine domestication using pan-mitogenome, pan-plastome, and haplotype-resolved nuclear genomes, all assembled

from long-read sequences across 33 wild and domesticated grapevine accessions. Structural variation shaped the mitogenomic variation in gene contents, leading to duplications of three specific genes during grapevine domestication (one *cox* and two *rpl* genes). Extensive genomic signals of cytonuclear interactions were detected, including a total of 212–431 nuclear–mitochondrial segments (NUMTs) and 95–205 nuclear–plastid segments (NUPTs). These results showed that NUMTs were under strong selection and were more abundant in cultivated grapes, whereas NUPTs dominated in wild grapes, indicating the evolutionary trajectories of cytonuclear interactions during grape domestication. Through Genome-Wide Association Study (GWAS), we identified 84 candidate genes associated with mitochondrial–nuclear genome interactions. Among these, the *PFD1* gene acts as a signaling regulator, modulating specific signaling pathways regulated by the mitochondria. Interestingly, there are significantly more cytonuclear interaction genes near NUMTs than in other genomic regions, suggesting NUMT-mediated interactions between the nuclear and

mitochondrial genomes. Overall, our study provides evidence that NUMTs promote cytonuclear interaction during grapevine domestication, offering new insight into the impact of cytonuclear interactions on plant evolution, genetics, and breeding.

Keywords: grapevine, mitochondrial structural variation, NUMTs, NUPTs, viticulture

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INTRODUCTION

In plants, there has been a substantial and ongoing exchange of genetic material between the endosymbiotic-originated organellar genomes (mitochondria and chloroplasts) and the nuclear genome (Timmis et al., 2004; Noutsos et al., 2005; Kleine et al., 2009; Keeling, 2010; Roger et al., 2017; Zhang et al., 2023, 2024). This dynamic interplay has led to the transfer of organellar DNA segments into the nuclear genome, resulting in the formation of nuclear–mitochondrial segments (NUMTs) and nuclear–plastid segments (NUPTs) (Leister, 2005). A substantial amount of organellar DNA has been found in the nuclear genome of flowering plants (Adams et al., 2000, 2001). Notable examples include the 620 kb NUMT in *Arabidopsis* (Lin et al., 1999; Stupar et al., 2001) and the 131 kb NUPT in rice (Consortium, 2003), which are the two largest organellar DNA insertion fragments reported to date. While the identification of human NUMTs has reached a relatively mature stage (Dayama et al., 2020), the identification of plant NUMTs remains a challenge. Traditional approaches rely primarily on sequence alignment strategies, such as using tools like BLAST to compare nuclear genome sequences with mitochondrial or plastid genome sequences. However, these methods are subject to homologous sequence interference (Richly and Leister, 2004; Noutsos et al., 2005; Michalovova et al., 2013), which can lead to incomplete identifications and numerous errors. Recent advances in sequencing technology, particularly the availability of long-read sequencing technologies such as PacBio and Nanopore, have provided longer read lengths and higher accuracy, effectively overcoming the limitations of short-read sequencing. As a result, the identification of NUMTs and NUPTs using PacBio HiFi sequencing technology has proven to be the most appropriate and efficient method for understanding their formation and evolutionary mechanisms.

Nuclear–cytoplasmic interaction is essential for the proper functioning of plant cells and plays a crucial role in plant evolution by maintaining the coordination between the nuclear and organellar genomes, thereby ensuring metabolic balance and genetic stability (Woodson and Chory, 2008). In plants, cytoplasmic male sterility (CMS), a condition characterized by pollen infertility, results from perturbations in the complex interplay between the mitochondrial and plastid genome and the nuclear genome. However, nuclear-encoded restorer of fertility (Rf) genes can effectively suppress or overcome this sterility phenotype, demonstrating their co-evolutionary relationship with CMS (Chase, 2007; Caruso et al., 2012). In several crops, including

citrus, pepper, rice, and maize, the interaction between the *PPR* gene family and the mitochondrial genome has driven the evolution of CMS and fertility recovery (Kim et al., 2007; Toriyama, 2021; Wang et al., 2022; Yang et al., 2022). Furthermore, incompatibility between the nuclear and cytoplasmic genomes can lead to hybrid breakdown, as normal cellular function requires harmonious interactions between these two components (Leon et al., 1998; Levin, 2003). In the genus *Pisum*, interspecific hybrids between wild *Pisum sativum* ssp. *elatius* and the cultivated variety Sprint-1 exhibit leaf development abnormalities and pollen sterility, mainly due to CMS resulting from nuclear–cytoplasmic conflicts (Bogdanova and Galieva, 2009). In *Rhododendron*, intersubgeneric crosses between evergreen azaleas (EV) and yellow-flowered deciduous azaleas (JP) can result in genome–plastome incompatibility, particularly when EV plants are used as maternal parents. This incompatibility can result in albino traits in hybrid offspring due to the mismatch between the EV plastids and the JP nuclear genome (Ureshino et al., 2010). Nuclear–cytoplasmic interactions are pivotal in the mechanisms of reproductive isolation, genomic co-adaptation, and the emergence of new species (Burton and Barreto, 2012; Barnard-Kubow et al., 2016; Sloan et al., 2017), as evidenced by their role in creating barriers that restrict gene flow, sometimes leading to speciation (Baack et al., 2015), and their implications in the evolution of reproductive isolation, as highlighted by the varied evolutionary forces, such as post-pollination barriers and genomic conflicts, that shape plant reproductive systems (Aalto et al., 2013). For example, in *Petunia*, the nuclear-encoded RNA editing enzyme is unable to edit nucleotides in the tobacco plastid *atpA* gene, leading to the production of albino hybrids. In contrast, backcross hybrids exhibit normal phenotypes, underlining the critical role of genomic interactions in maintaining hybrid adaptability (Schmitz-Linneweber et al., 2005).

As a source of genetic variation, NUMTs can also affect gene function, genome structure, and genetic diversity during domestication (Purugganan and Fuller, 2009). During the domestication of rice (*Oryza sativa*), NUMTs are shown to affect the expression of key domestication genes. For instance, mutations in the *SOP10* gene have been associated with reduced levels of reactive oxygen species (ROS), allowing rice to exhibit greater resistance to cold-induced bleaching (Zu et al., 2023). While NUMTs occasionally have a positive effect on domestication, they can also present challenges. Research indicates that NUMTs may disrupt the coordination between mitochondrial and nuclear genes, leading to phenotypic abnormalities. For example, in *Arabidopsis*, the mismatched nucleocytoplasmic combination of Cvi-0 and

Sha can impair mitochondrial function and may be linked to metabolic disorders. This highlights the complex interactions between mitochondrial and nuclear DNA in plants (Roux et al., 2016). In addition to their influence on gene function, NUMTs and NUPTs can also affect haplotype structure. NUMTs/NUPTs accumulate gradually during polyploid evolution in *Triticum/Aegilops*, and these dynamic accumulation processes can affect haplotype structure (Zhang et al., 2024). Although studies on the effects of NUMTs/NUPTs on haplotypes in plants are limited, this study aims to investigate their potential impact on haplotype structure in grapevine accessions, thereby contributing to a deeper understanding of their role in plant domestication.

Grapes are widely cultivated and consumed worldwide as an important horticultural crop (Zhou et al., 2017; Grassi and De Lorenzis, 2021). Significant progress has been made in the study of grapevine and its domestication history, with well established genetic pathways (Zhou et al., 2017, 2019; Dong et al., 2023; Xiao et al., 2023). A substantial amount of high-quality genomic and resequencing data has been published in grapevine (Shi et al., 2023; Peng et al., 2025), including pan-genome studies (Liu et al., 2024; Guo et al., 2025), which capture the full spectrum of genetic diversity across cultivars. Expanding beyond the nuclear genome, recent advances in pan-mitogenome and pan-plastome research (Wang et al., 2022; Magdy et al., 2019) have demonstrated how organellar genome variation can influence traits and domestication. These comprehensive genomic resources, combined with grapevine's well-characterized domestication history, make grapevine an ideal model system for investigating the impact of NUMTs and NUPTs on crop domestication (Xiao et al., 2025). The purpose of this study is to investigate the following three questions. First, how common are SVs in the mitochondrial genome of *Vitis*? Second, what are the distribution characteristics of NUMTs/NUPTs in the grape genome and are there any differences between NUMTs and NUPTs? Finally, do NUMTs affect the domestication and cytonuclear interactions of grapes? In this study, we constructed pan-mitogenome and pan-chlorogenome based on 33 grape samples and found that three genes (*infA*, *rpl14*, and *rpl36*) were lost in the East-Asian wild grapevine mitogenomes. A *Vitis*-specific structural variation (SV) in selective sweeps was identified based on the assembled mitochondrial genomes and population data across 100 grapevine genomes. In addition, we accurately identified NUMT and NUPT phenomena in 13 grapevine accessions and detected 84 candidate genes significantly associated with cytonuclear interactions through Genome-Wide Association Study (GWAS) analysis.

RESULTS

The pan-mitogenome and pan-chlorogenome for grapevine

We utilized 33 long-read sequencing data samples to assemble mitogenomes and plastid genomes from four populations

(wine, table, *Vitis vinifera* ssp. *sylvestris* (Syl), *Vitis* wild relatives (Vwr); Table S1), resulting in a pan-mitogenome (Grapepan_mt v.1.0, 1.28 Mb) (Figure 1A) and a pan-chlorogenome (Grapepan_cp v.1.0, 138 kb) (Figures S1, S2). The pan-mitogenome is about twice the size of a single mitochondrial genome, and contains 144 continuous segments from the Bmng mitochondrial genome, which serves as the reference genome in this study. The pan-chlorogenome, about the same size as a single plastid genome, contains 31 continuous segments. The assembled mitochondrial genomes ranged in size from 663.16 kb (*Vitis rotundifolia*: Vmu) to 817.37 kb (*Vitis vinifera* cv. Carmenere: Vca) and contained 108–129 genes. Reverse repeats were rare in most Vwr grapes, while forward repeats were more common (Table 1). The GC content of the mitochondrial genomes has remained relatively consistent, with an average of approximately 44%. The proportion of TEs in the mitochondrial genome is about 22% (Tables S2, S3). For plastid genome assembly, all 33 grape genomes were successfully assembled into the typical four-part ring structure, ranging in size from 159,011 bp (*Vitis vinifera* ssp. *sylvestris*: Vs) to 163,619 bp (*Vitis vinifera* cv. Cabernet Franc: Vcf). The average GC content of the plastid genomes was 37.38%, while TEs accounted for about 87% (Tables S4, S5).

To study the SV and its impact on cytoplasmic genes, we further performed gene copy number analysis and detected a total of 55 protein-coding genes that were universally present in all 33 grape mitochondrial genomes. Compared with domesticated grapes, four protein-coding genes (*infA*, *rpl14*, *rpl36* and *rps19*) were lost in nine wild grapes and one cultivated grape (Figure 1B). In addition, other genes such as *psaC*, *psaJ*, *rpl2*, *rpl33*, *rps18*, *psbM* and *rpl32* were lost in various grape species. The replication of the *petL* gene occurred in almost all grapes, while the replication of the *rps19* gene was observed in all grapes. For the plastid genome, most protein-coding genes are single copies. Unlike the mitochondrial genome, the plastid genome has a maximum copy number of two genes, among which five genes, *ndhB*, *pafl*, *rpl23*, *rps7* and *ycf2*, each have two copies in the 33 grape genomes (Figure S3). In addition, we selected 13 representative grapes to observe the rearrangement phenomenon among different grape mitochondrial genomes and revealed rearrangements among different grape mitochondrial genomes. Specifically, more rearrangement events took place in the Vwr group than in the other groups, while fewer rearrangement events were observed within the table grape group, ranging from two to six. Although the Vwr population had a greater number of rearrangement events, the mitochondrial genome structure of *Vitis heyneana*, *Vitis piasezkii*, and *Vitis rotundifolia* was similar. Throughout the evolution of grapes, the Vwr population experienced more genetic rearrangement events than the other three populations (Figure S4).

SV and artificial selection on mitogenomes during grape domestication

To examine the potential impact of selective sweeps on mitochondrial genetic diversity, we conducted selective

Table 1. Summary of the 33 mitochondrial assemblies

Species name	Sample	Group	Genome size (bp)	Gap	Number of genes	Number of repeat sequences		
						F	P	R
<i>Vitis vinifera</i>	Ved	Wine	817,057	1	116	27	21	2
<i>Vitis vinifera</i>	Ves	Wine	817,062	1	116	26	22	2
<i>Vitis vinifera</i> cv. Chardonnay	Vch	Wine	816,446	0	116	25	21	4
<i>Vitis vinifera</i> cv. Zinfandel	Vzc	Wine	809,242	0	115	25	20	5
<i>Vitis vinifera</i> cv. PN40024	Pn	Wine	773,298	0	119	23	23	4
<i>Vitis vinifera</i> cv. Pinot Noir	Pnzh	Wine	773,300	0	119	24	22	4
<i>Vitis vinifera</i> cv. Cabernet Franc	Vcf	Wine	816,200	2	117	27	21	2
<i>Vitis vinifera</i> cv. Carmenere	Vca	Wine	817,368	1	115	27	21	2
<i>Vitis vinifera</i> cv. Cabernet Sauvignon	Vcs	Wine	810,760	1	108	30	20	0
<i>Vitis vinifera</i> cv. Merlot	Vme	Wine	816,568	0	116	25	20	5
<i>Vitis vinifera</i> cv. Thompson Seedless	Whb	Table	816,566	0	116	25	20	5
<i>Vitis vinifera</i> cv. Black Monukka	Whz	Table	817,063	1	116	27	21	2
<i>Vitis vinifera</i>	Bmng	Table	816,336	0	116	26	19	5
<i>Vitis vinifera</i>	Hmng	Table	816,566	0	116	25	20	5
<i>Vitis vinifera</i> cv. Manicure Finger	Mrz	Table	816,565	0	116	25	20	5
<i>Vitis vinifera</i> cv. Julian	Zla	Table	816,565	0	116	25	20	5
<i>Vitis vinifera</i> cv. Muscat Hamburg	Mgx	Table	817,057	1	116	29	19	2
<i>Vitis labrusca</i> × <i>Vitis vinifera</i>	Ql	Table	755,036	0	114	26	24	0
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vsyb	Syl	817,077	1	116	27	21	2
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vsyb	Syl	817,072	1	116	26	21	3
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vsya	Syl	773,074	0	118	22	22	6
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vs	Syl	790,627	0	117	26	19	5
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vsyc	Syl	816,500	0	116	28	22	0
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Vsyd	Syl	816,463	0	116	28	22	0
<i>Vitis vinifera</i> ssp. <i>sylvestris</i>	Hey	Vwr	757,300	0	114	30	20	0
<i>Vitis adenoclada</i>	Xzpt	Vwr	784,133	0	115	29	21	0
<i>Vitis piasezkii</i>	Bypt	Vwr	791,319	1	114	32	18	0
<i>Vitis davidii</i>	Cm	Vwr	738,535	1	112	31	19	0
<i>Vitis amurensis</i>	Spt	Vwr	774,208	0	115	29	21	0
<i>Vitis rotundifolia</i>	Mmpt	Vwr	762,981	0	115	29	21	0
<i>Vitis riparia</i>	Vri	Vwr	755,294	0	113	27	23	0
<i>Vitis rupestris</i>	Vrp	Vwr	795,535	5	110	39	11	0
<i>Vitis rotundifolia</i>	Vmu	Vwr	663,157	0	117	33	17	0

F, P and R in the table indicate forward repeats, palindromic repeats, and reverse repeats, respectively.

sweeps scans using resequencing data from 100 grapevine samples, representing four distinct populations: 13 from table, 28 from wine, 26 from Syl, and 33 from Vwr grapevine (Table S6). Our analysis identified 5.82% (46.00 kb out of 790.627 kb) of the mitochondrial genome under selection, which included three protein-coding genes (*nad3*, *rps12*, *ccmFC*), one tRNA, and one *orf* gene (Figure 2A, B; Table S7). Within these selected regions, we identified a ~30-kb deletion (SV: 665.448–695.774 kb) among six East-Asian wild grapes (Hey, Xzpt, Bypt, Cm, Spt, Mmpt), which belong to the Vwr species (Figure 2C). This SV was further divided into three parts (SV1, SV2, and SV3). SV1 and SV3 were mitochondria-specific sequences, while SV2 shared high homology (over 99% BLASTN) with a plastid genome sequence (Table S8). Thus, our analysis focused on the frequencies of SV1 and

SV3 across table, wine, Syl, and Vwr grapevines. Our results showed that 96.43% (27/28) of wine grapes contained SV1 at a significantly higher frequency compared to Vwr grapes (18.18%) (Figure 2D) (chi-squared test, P -value = 4.814474e-09). Similarly, SV3 was also more prevalent in wine grapes than in Vwr (chi-square test, P -value = 4.209265e-06) (Figure 2E).

Among the *Vitis* mitogenomes, large sequence fragments of the SV first appeared in Vmu grape and subsequently spread to domesticated grapes (Figure 2F). Notably, all cultivated grape mitochondrial genomes possessed large SV fragments. Regarding nuclear sequence matching, most wine grapes (except for Vcs and Vcf) shared over 50% of the SV sequences with their nuclear genomes, with proportions of 46.38% and 48.38%, respectively. Syl and Vwr grapes exhibited a broader range of sequence sharing, ranging from

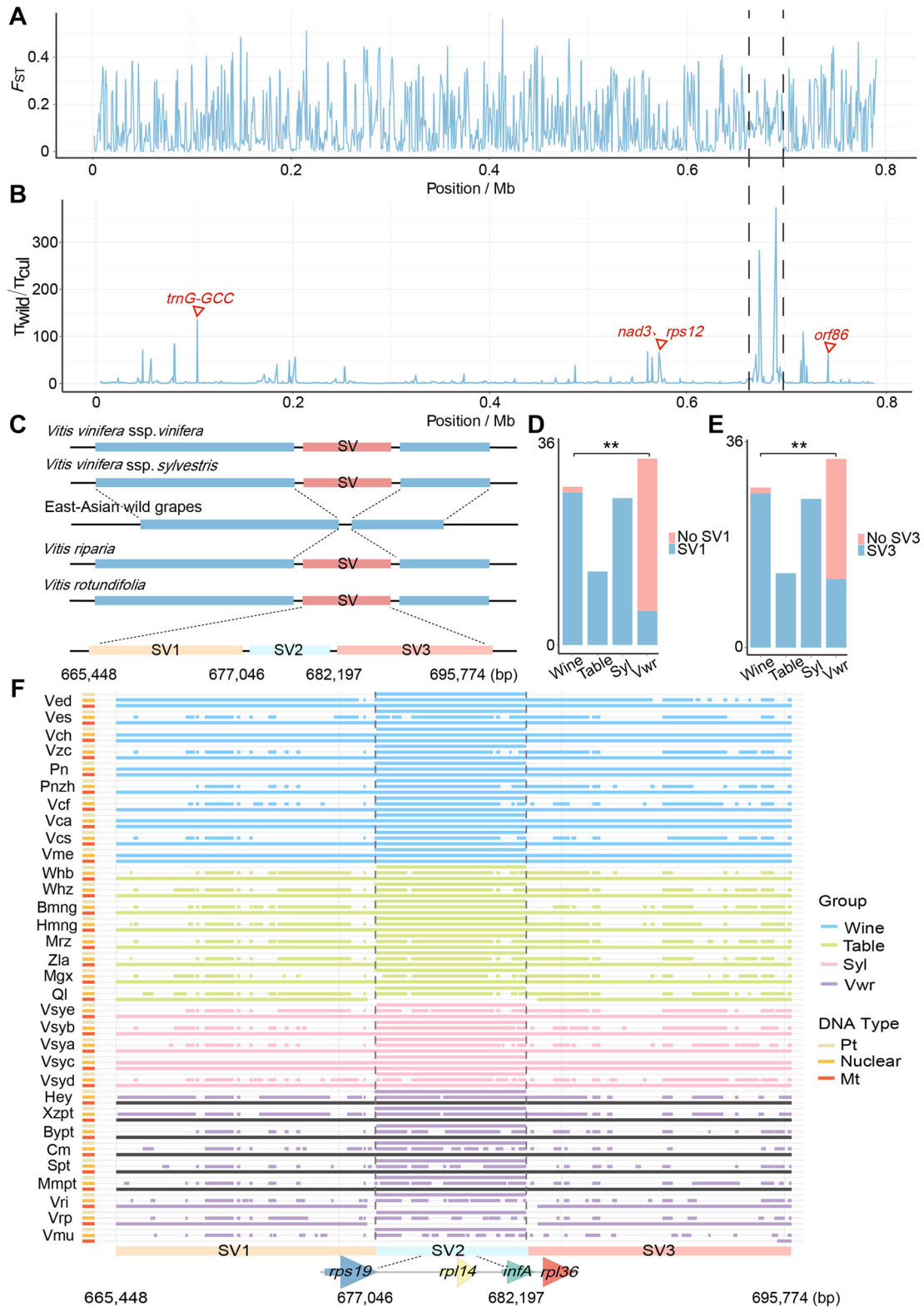


Figure 2. SV analysis of the mitogenomes of grape

(A) Distribution of F_{ST} values in four grape populations. (B) Distribution of π_{wild}/π_{cul} ratios; cul means cultivated grape population; dotted regions represent the SV (from 665,448 to 682,197 bp) in East-Asian wild grapes. (C) A SV was identified in East-Asian wild grapes (*V. heyneana*, *V. adenoclada*, *V. piasezkii*, *V. davidii*, *V. amurensis*, *V. rotundifolia*). The SV sequence is further divided into three parts (SV1, SV2, and SV3). (D, E) Appearance times of SV1 (D) and SV3 (E) in the four grape groups. Syl is *Vitis vinifera* ssp. *sylvestris* and Vwr is *Vitis* wild relatives. Red represents the samples not containing the SV, and blue represents the samples containing the SV. (F) Homologous sequences of SV1, SV2, and SV3 in 32 mitogenomes, plastid genomes and nuclear genomes. The gray bar indicates no information was available.

33% to 100%, whereas table grapes displayed a narrower range, from 47.46% to 65.57% (Table S9). These results indicate that SV changed repeatedly and continued to evolve during grape domestication.

Asymmetric accumulations of NUMTs/NUPTs in grapevine nuclear genomes

To explore whether the four genes (*infA*, *rpl14*, *rpl36* and *rps19*) lost in East-Asian wild grape mitochondria were transferred to NUMTs/NUPTs, and to examine the patterns of NUMTs/NUPTs during grapevine evolution, we successfully identified 212 to 431 high-confidence NUMT and 95 to 205 NUPT fragments in 13 grape samples using PacBio HiFi data, following the application of a series of quality control filters (Figure S5; Tables S1, S10). First, the four genes (*infA*, *rpl14*, *rpl36* and *rps19*) were found in their NUPTs (Table S11). Additionally, the most abundant NUMTs were found in the range 200–399 bp, whereas NUPTs were predominantly found in the range 400–599 bp (Figure 3A, B). The number of NUMTs and NUPTs varied in different species. Cultivated grapes typically contained more NUMTs, whereas wild species tended to have a higher abundance of NUPTs (Figure S6). However, haplotype variation within species showed minimal differences in the numbers of NUMTs and NUPTs (Table S10). The distribution of NUMTs and NUPTs within the nuclear genome varied by species, chromosome location and gene. Some NUMTs were functional genes before being transferred to nuclear DNA, but lost their gene function after transfer, either due to mutations during the transfer process or because they are no longer needed in their new environment. Some NUMTs were not genes before transfer but formed neo-functionalized genes after integration into the nuclear genome. This suggests that these DNA fragments may have acquired new functions after being transferred. In most grapevine species, NUMTs were less abundant on chromosomes 3 and 17, while NUPTs were less frequent on chromosomes 2 and 18 (Figure 3C, D). Conversely, NUMTs were more abundant on chromosome 18, and NUPTs were more abundant on chromosome 17 (Figures S7–S10).

We further investigated the frequency and distribution of mitochondrial and plastid sequences transferred into the nuclear genome, finding the highest transfer rates in intergenic regions. NUMTs and NUPTs were differentially associated with transposable elements (TEs), with NUMTs being mostly non-TE sequences and NUPTs having a higher proportion of TE-associated sequences, including DNA, LINE, LTR, MITE, SINE, and rRNA elements (Figure 3E, F). Interestingly, in some cultivated grapes (e.g., Whb, Whz, Bmng), NUMTs in promoter regions were more frequently associated with LINE elements, while wild grapes (e.g., Hey, Bypt) showed a predominance of LTR elements. Regarding the distribution of NUMTs and NUPTs within the nuclear genome, there were generally few differences between haplotypes, except for Hey and Mrz, where Hey showed significant differences in the exon and intergenic regions, while Mrz exhibited more differences in the intergenic region

(Figure S11). For NUPTs, the proportions of each region and TE were generally similar between hap1 and hap2, with LTR having the highest proportion. However, some cultivars, such as Whb, Whz, Mgx, and Ql, showed significant differences in the exon regions, and the differences in the proportions of Hey and Vs (wild grapes) across regions were more pronounced (Figure S12). These results highlight species-specific and haplotype-level variations in NUMTs and NUPTs accumulation, suggesting distinct evolutionary patterns shaping the transfer and retention of organelle-derived sequences in grapevine genomes.

Homozygous NUMTs drive the genomic evolution of grapevine

To investigate the potential impact of homozygous and heterozygous NUMTs/NUPTs on grape evolution, we observed an interesting pattern: homozygous NUMTs were more prevalent in wild grapes, while heterozygous NUMTs, especially heterozygous NUPTs, became increasingly abundant in domesticated grapes (Figure 4A). In total, 834 genes were captured in hap1 and 775 genes in hap2 of NUMTs, of which 598 and 580 genes had UniProt IDs, respectively. For NUPTs, 763 genes were captured in hap1 and 559 in hap2, with 537 and 425 genes having UniProt IDs (Tables S12, S13). Furthermore, we predicted 352 deleterious variation sites in 119 genes. Interestingly, one *MIK2*-like gene (*Vitvi031348*) plays a dual role in sensing the conserved SCOOP feature MOBS in plants and microorganisms, and triggering plant immunity (Hou et al., 2021; Stahl et al., 2022). This gene underwent NUMT and NUPT events in three table grape varieties, suggesting that defense-related genes in the nuclear genome are more susceptible to “attacks” by mitochondrial and plastid gene fragments. The newly inserted NUMT and NUPT fragments into the *MIK2* gene increased harmful variations, which may adversely impact the adaptability of grapes (Tables S14, S15).

Functional annotation revealed that NUMTs were significantly enriched in biological processes related to defense responses, such as defense response to bacteria, plant-type hypersensitive response, highlighting the critical role of these homozygous genes in grapevine survival and adaptation. We also discovered unique processes, such as the regulation of response to salt stress, are unique to certain haplotypes, which helps the organism adapt to and resist salt stress, thereby ensuring survival and normal growth. In contrast, heterozygous genes contributed to novel biological functions (Figures 4B, S13; Table S16). For NUPTs, processes like photosynthesis and photosynthetic electron transport in photosystem II were shared in both haplotypes, supporting basic metabolism. Notably, we observed that *Q0ZIY9* (associated with photosynthesis)—a gene expressed in cultivated grapes (Mgx) but absent in wild grapes (Cm)—may represent a domestication-selected gene retained during grape evolution. Haplotype-specific processes like proteolysis were found to be crucial for cell function and organism survival (Figures 4C, S14; Table S17).

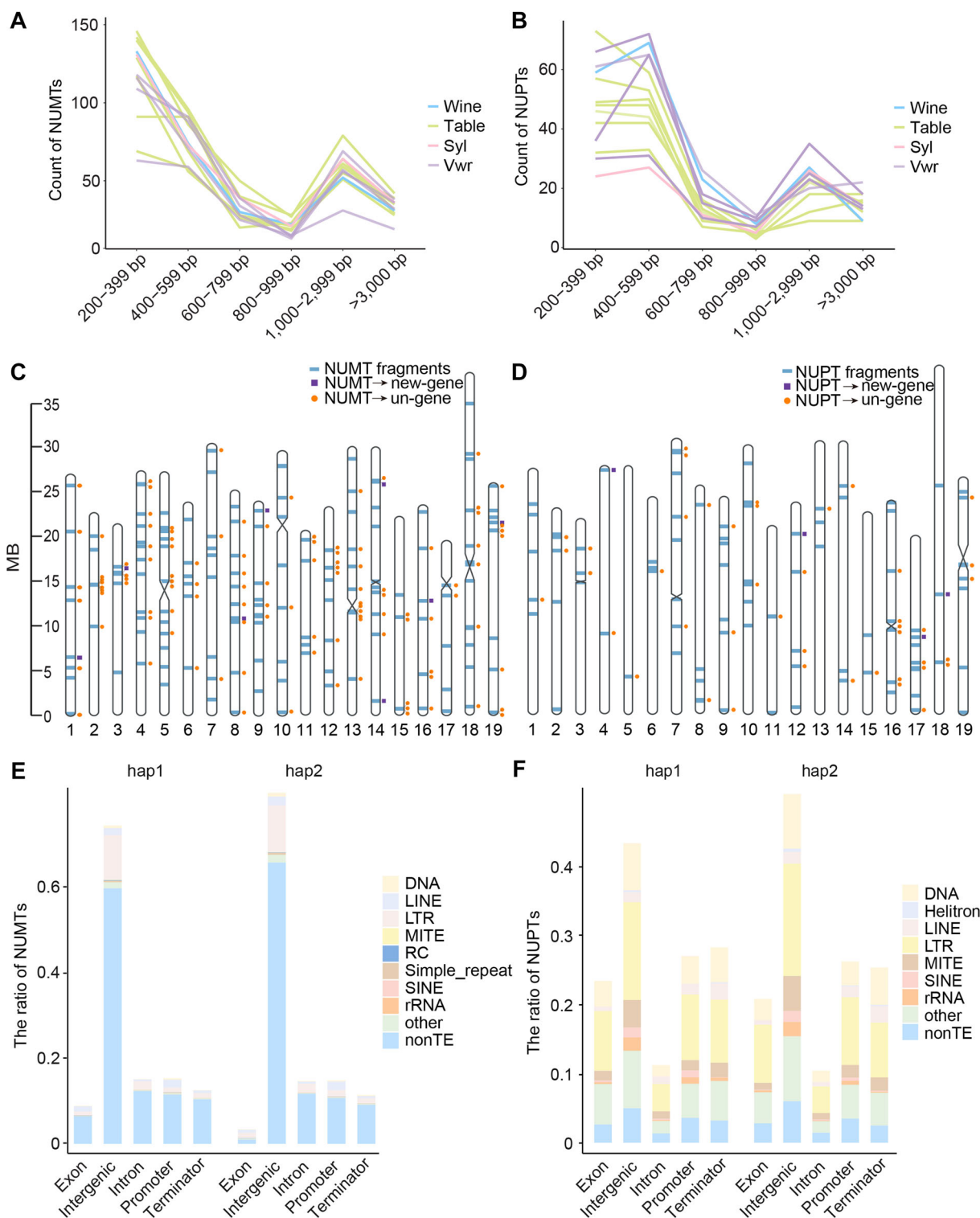


Figure 3. Characteristic of NUMTs and NUPTs in grapes

(A, B) The length and number of NUMTs (A) and NUPTs (B) of four grape populations. The line chart shows the number distribution of NUMTs and NUPTs for different grape varieties at different length intervals. (C, D) The distribution of NUMTs (C) and NUPTs (D) in the nuclear chromosomes of Whzhap1. Blue indicates the location on nuclear chromosomes. The purple box indicates that the mitochondrial/plastid genome did not form genes before transfer to the nucleus, and formed new genes after transfer; The orange circle indicates that the mitochondrial/plastid genome was a gene before it was transferred to the nucleus and is not a gene after it is transferred. (E, F) Composition of NUMTs (E) and NUPTs (F). The histogram shows the ratio distribution of NUMTs and NUPTs in transposable elements (TE) and non-TE regions of different gene structures in the two haplotypes. Intergenic region: the region of the genome other than promoter, terminator, and gene regions; Promoter region: within 2 kb upstream of genes; Terminator region: within 2 kb downstream of genes.

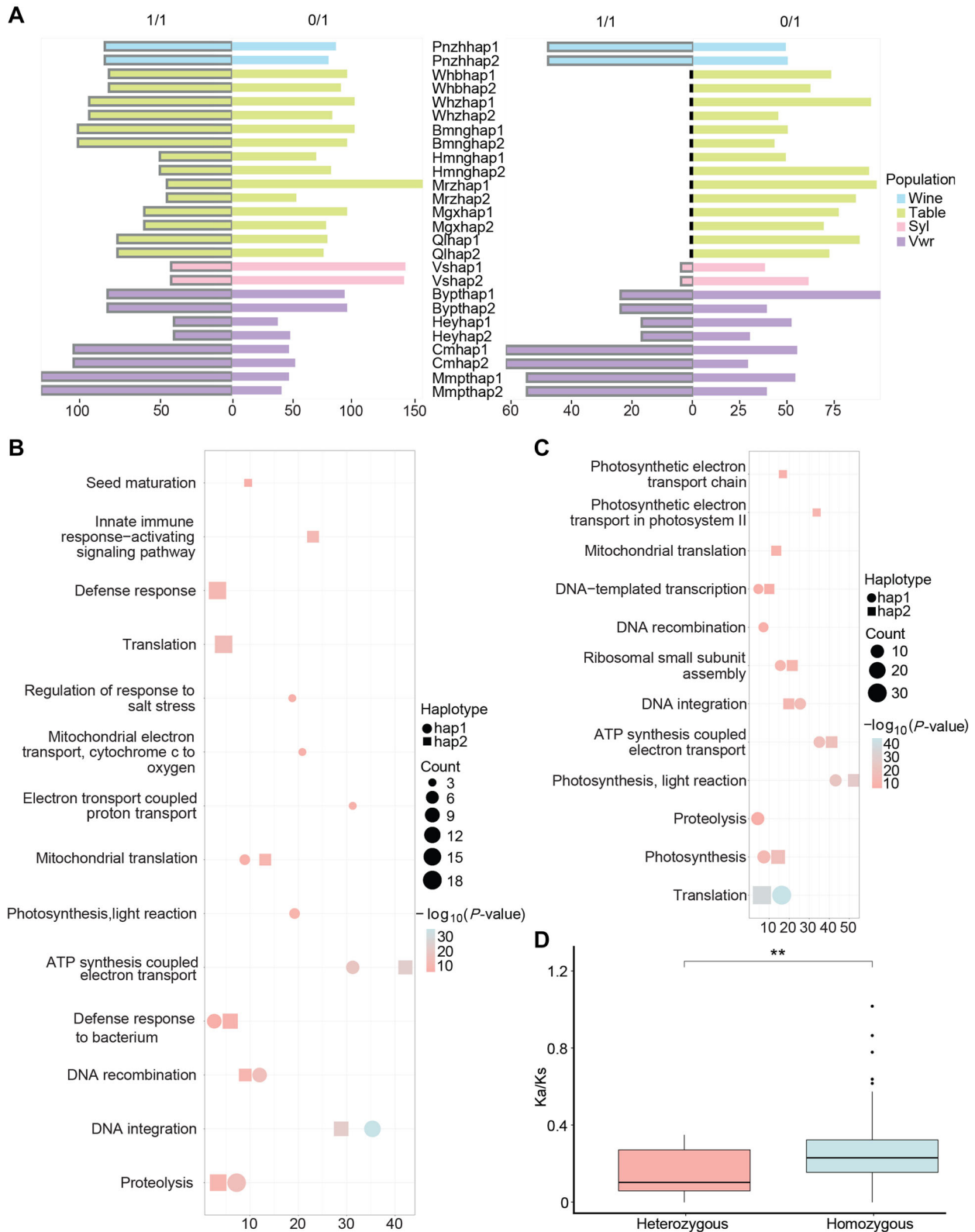


Figure 4. Comparative analysis of heterozygous and homozygous genes of NUMTs and NUPTs during the domestication of grapes (A) Frequency plots of NUMTs (left) and NUPTs (right) in both haplotypes of four grape groups. 0/1 = heterozygous, 1/1 = homozygous. (B, C) GO enrichment (BP, biological process) of hap1 and hap2 genes captured exon and promoter of NUMTs (B) and NUPTs (C). In the diagram, the circles represent hap1, while the squares represent hap2. The size of the circles indicates the number of genes, with larger circles indicating a greater number of genes. (D) Ka/Ks of heterozygous and homozygous NUMTs genes in wild and cultivated grapes. Significant P -value for difference: $**P < 0.01$.

Differential NUMT genes were significantly enriched in metabolic pathways, oxidative phosphorylation, and photosynthesis, all of which are equally important in both haplotypes, indicating their indispensability in grape physiology. However, aminoacyl-tRNA biosynthesis was only significantly enriched in hap2 (Figure S15), enhancing protein synthesis efficiency in heterozygotes under specific conditions. Differential gene enrichment analysis of NUPTs revealed that several metabolic pathways, including photosynthesis and RNA polymerase activity, critical for grapevine metabolism, along with a unique pathway in carbon metabolism (Figure S16), which is central to plant life and involves energy conversion and substance synthesis. Furthermore, Ka/Ks ratio analysis showed significantly higher values for homozygous NUMT genes in wild grapes compared with cultivated grapes (Figure 4D). This marked difference suggested that homozygous genes have experienced stronger positive selection pressure and may have influenced the evolution of grapevine.

NUMTs facilitate cytonuclear interactions in grapevines

To reveal the potential co-evolution between the grapevine cytoplasmic genome and nuclear genome, we adopted the approach described by Lian et al. (2024), using two populations (50 cultivated and 50 wild grapes; Table S6), and detected significant mitonuclear and plastonuclear association signals (Figures 5A, S17–S19). GO enrichment of 84 candidate genes from mitonuclear GWAS revealed their involvement in DNA integration, recombination, and biosynthesis (Figure S20). Similarly, 133 candidate genes from nuclear–plastid interactions were enriched in proteolysis, defense response and protein export from the nucleus (Figure S21). Among the candidate genes, *Prefoldin subunit 1* (*PFD1*, *Vitvi020593*), which is closely associated with mitochondrial variation, was identified on chromosome 11 of grapevine (Figure 5B). The PFD subunit, also known as the gene involved in microtubule synthesis (GIM), forms a heterohexameric PFD complex involved in the folding of microtubules and actin. Notably, the accumulation of PFDs is associated with the degradation of HY5, which plays a key role in low temperature response, suggesting that PFDs may be involved in the plant's response to abiotic stress (Perea-Resa et al., 2017). Furthermore, the coverage of the *PFD1* gene in the Integrative Genomics Viewer (IGV v2.12.3) was significantly higher than the surrounding nuclear genome sequences, confirming its localization in the mitochondrial genome (Figure 5C).

The heatmap revealed a pattern in the distribution of heterozygous and homozygous sites, showing notable differences between cultivated and wild grapes at these loci. Cultivated grapes tended to have a higher frequency of homozygous sites at core-mitochondrial interaction loci. This pattern may be attributed to the selection of beneficial genotypes during domestication in cultivated grapes. In contrast, wild grapes retained a higher proportion of heterozygous

sites, likely contributing to greater genetic diversity and environmental adaptability (Figure 5D, E). Finally, we found that NUMT events were significantly enriched near significant nuclear and mitochondrial association sites compared to non-significant association sites (Figure 5F), suggesting that NUMTs play a facilitating role in cytonuclear interaction and contribute to the co-evolution of the grapevine cytoplasmic and nuclear genomes.

DISCUSSION

The primary objectives of this study were to use grapevines as a model to explore cytoplasmic genome variation, patterns of cytonuclear interaction, and the impact of NUMTs and NUPTs on nuclear genome evolution. We successfully assembled the mitochondrial and plastid genomes of 33 grapevine samples and identified NUMTs and NUPTs. By examining their insertion modes in detail, we reveal their distribution patterns in the grape genome and propose possible mechanisms for their insertion. These findings provide new insight into the dynamics of cytonuclear transfer and confirm the role of NUMTs and NUPTs in grape domestication (Figures 3, 4). Genome-wide association analysis further identified significant nuclear genes associated with mitochondrial and plastid variation, suggesting a complex signaling network in cytonuclear interaction. Additionally, nuclear mutations may influence the pattern of cytonuclear interactions. Notably, we observed differences in variable loci between wild and cultivated grapes, suggesting that certain loci may have facilitated cytonuclear co-evolution. Although our study has yielded meaningful results, there are still areas for improvement. For instance, in the assembly of the mitochondrial genome, we focused primarily on the main circular structure, which limited our ability to fully explore the potential conformational diversity of mitochondria. This may have restricted a more comprehensive understanding of the genome's complexities. Meanwhile, the formation mechanisms of NUMTs/NUPTs remain unclear.

Plant mitochondrial genomes exhibit variable genome sizes and structures (Gualberto and Newton, 2017; Cole et al., 2018). Here, we assembled and annotated the mitochondrial genomes of 33 grapevine species, providing new insight into this variability. Previous reports have indicated that the larger size of grapevine mitochondrial DNA is not due to gene content but rather the expansion of its intergenic regions (Goremykin et al., 2009). We propose that copy number variations within the same clade may influence mitochondrial genome size, with larger genomes often being associated with lower copy numbers (Figure S22). This phenomenon may be because large insertions cannot be effectively corrected by homologous repair when the number of mitogenome copies is low. Plant mitochondrial genomes undergo significant structural changes during the co-evolution of endosymbionts and hosts, leading to

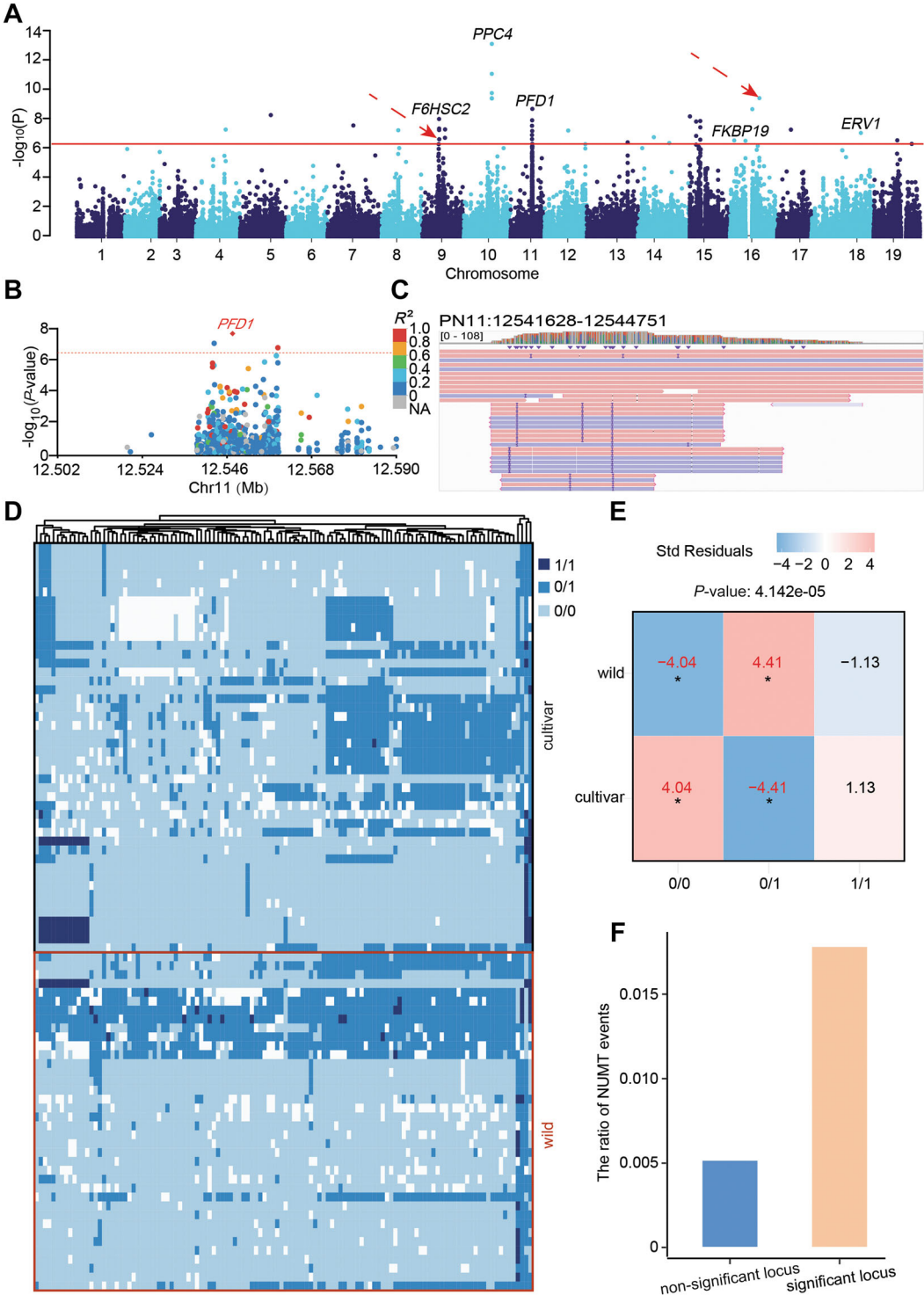


Figure 5. Genomic insights into mitonuclear interactions: candidate genes and variant analysis

(A) Manhattan plot of GWAS analysis for mitochondrial single nucleotide polymorphism (SNPs) in grapevine. The horizontal line represents the significance threshold. The dotted red arrows indicate that a nuclear-mitochondrial segments (NUMT) event occurred at that site. (B) SNPs surpassing this threshold are highlighted in the zoomed-in plot, with the most significant SNP marked by a diamond. (C) Depth map of the *Vitis020593* gene and its surrounding sequences in IGV. The color differentiation is based on the read strand orientation, where pink signifies a match between the read direction and the gene's orientation, and purple indicates the opposite read direction and gene's orientation. (D) Heatmap of significant associated variation sites in the nucleus and mitochondria between wild and cultivated grapes. Colors represent allelic state: light blue = homozygous reference allele, dark blue = heterozygous, and ink blue = homozygous alternative allele. Each row illustrates the genotype of one sample. (E) Correlation analysis of nucleoplasmic significant association loci with genotype in wild and cultivated grapes. Significant P -value for difference: $*P < 0.05$. (F) The ratio of NUMT events at nucleocytoplasmic significant association sites and non-significant sites.

increased genomic complexity. Compared with the highly conserved plastid genome, the mitochondrial genome exhibits greater SV. In grapes, we observed significant rearrangements within mitochondrial genomes of the same genus, with a more than 10-fold difference in rearrangement rates among 11 genera of the Rosaceae family (Sun et al., 2022). Moreover, several studies have shown that nuclear gene variants (such as *MSH1*) (Gualberto and Newton, 2017) can also contribute to mitochondrial genome rearrangements. The mitochondrial genome of the QI, a cultivated variety, showed three gene loss events that are common in wild grapes. This change is likely to have been due to its breeding background, as it is a cross between an American species and a European subspecies. These complex structures pose challenges for accurate genome assembly, especially as most mitochondrial genomes are simplified to the main circular structure. As a result, many important structural features may be overlooked. Therefore, future studies should focus on the complete assembly of mitochondrial genomes to better reveal their complex structural characteristics and gain a deeper understanding of their role in plant evolution and function.

To better understand the role of NUMT and NUPT fragments in domestication, we examined their insertion patterns. Previous studies have shown that organelle DNA is preferentially inserted into centromeric regions, and then propagated via transposition (Michalovova et al., 2013). Nevertheless, our analysis revealed that, in some varieties, insertions did not occur in the centromeric regions (Tables S18, S19), possibly due to limitations in assembly quality. Similar to findings in *Sorghum* (Zhang et al., 2023), we observed an asymmetric deposition pattern for NUMTs and NUPTs in grapevines, with different distributions among cultivars and transfer regions. Despite these similar patterns, we found that the insertion rates of NUMTs and NUPTs were not constant. The number of NUMTs increased, while NUPTs decreased during grapevine domestication. This may be due partly to assembly quality or the potential benefits of transferring mitochondrial function genes to the nucleus for improved environmental adaptation or domestication selection. Additionally, no homozygous NUPTs were found in table grapes, suggesting stronger selective pressures on cultivated varieties (Figure 4A). Homozygous plastid insertions into the cultivated nuclear genome may lead to lethal effects or produce traits unfavorable for domestication and, thus, these insertions may have been eliminated by natural selection. Regarding NUMTs, we observed that their sequences retained a GC content similar to that of the source mitochondrial genome, but the GC content of these transferred sequences was lower than that of the surrounding nuclear genome, indicating that they have been less influenced by the nuclear genome during evolution and exhibit an independent evolutionary pattern. In contrast, NUPT sequences not only have a GC content similar to that of the source plastid genome but also resemble the surrounding

nuclear genome, suggesting that these fragments are more structurally stable.

Cytonuclear interactions, which involve the cooperative interplay between nuclear and cytoplasmic-encoded gene products, are essential for the maintenance of plant physiology (Sloan et al., 2018; Kan et al., 2024). These interactions are key to speciation and biological evolution, exerting significant influence on genome stability and function (Chase, 2007; Sharbrough et al., 2017; Zupok et al., 2021). For example, hybridization experiments in *Helianthus* species have shown that cytonuclear interactions between different ecotypes profoundly impact hybrid fitness (Lexer et al., 2003; Sambatti et al., 2008). Moreover, Patrick Chinnery's team discovered that some children had mitochondrial DNA "insertions" within their nuclear DNA, which were not present in their parents. These insertions were occasionally associated with rare diseases, highlighting the dynamic interplay between mitochondrial and nuclear DNA (Wei et al., 2022). Our research further supports this notion. Through GWAS in grapes, we identified genes (*RUN1* and *RPV1*) associated with disease resistance that are potentially located at the junction of the nuclear and mitochondrial genomes. Ka/Ks analysis of these genes revealed that they underwent functional selection after integration into the nuclear genome (Figure S23), consistent with Patrick Chinnery's findings. This suggests ongoing co-evolution between the nuclear and mitochondrial genomes. In conclusion, cytonuclear interactions are important in biological evolution and speciation. Our findings further support this notion and provide new insight into the role of cytonuclear interactions in crop domestication.

MATERIALS AND METHODS

Assembly and annotation of grapevine pan-mitochondrial and pan-plastid genomes

To investigate the evolution dynamics of mitochondrial and plastid genomes during *Vitis* domestication, we collected 33 samples from four groups (Table S1). Thirty-four grapevine mitochondrial and plastid genomes were assembled using long-read sequencing data. The average coverage of reads was downsampled to ~10 Gb. First, the long-read sequencing data were compared with the reference mitochondrial genome *Vitis vinifera* (NC_012119.1) using minimap2 v2.26 (Li, 2021), and the mapped reads were subsequently extracted to assemble the mitochondrial genome with Flye v2.9.2-b1786 (Kolmogorov et al., 2019). A similar strategy as the mitochondrial assembly was employed for assembling the plastid genomes. The pan-genomes of the grapevine mitochondrial genome and plastid genome were constructed in minigraph-0.13 (r397) with an incremental graph generation mode (<https://github.com/lh3/minigraph>). Reference genomes for plastid assembly included *Vitis amurensis* (NC_031383.1), *Vitis heyneana* (NC_039796.1), *Vitis piasezkii* (NC_039789.1), *Vitis rotundifolia* (NC_039788.1), *Vitis riparia*

(NC_039680.1), *Vitis rotundifolia* (NC_023790.1), *Vitis rupestris* (NC_036764.1) and *Vitis vinifera* (NC_007957.1). Mitochondrial and plastid genomes were annotated with the Geseq tool (Tillich et al., 2017), and the final annotations were manually checked to ensure the correct positions of the start and stop codons.

The forward repeats, reverse repeats and palindromic repeats within the 33 grapevine mitochondrial genomes were identified using REPuter (Kurtz et al., 2001) from BiBiServ2 (<https://bibiserv.cebitec.uni-bielefeld.de>) with parameters set to “Edit distance” of 3 and “Minimal Repeat Size” of 30 bp. NUCmer v3.1 (Kurtz et al., 2004) was used for the collinearity analysis of the 13 grape mitochondrial genomes, and the results were visualized using Mummerplot (Marçais et al., 2018). To visually observe gene loss in the mitochondrial genome, we use `gene_content.R` script for visualization.

Population genetic analysis

We utilized published genome resequencing data for 100 grape accessions (Table S6). The “Vs” mitogenome served as the reference for single nucleotide polymorphism (SNP) and insertion/deletion (INDEL) calling. Raw data was mapped to the reference genome using Burrows–Wheeler Alignment v0.7.17-r1188 (BWA) (Li and Durbin, 2009). SAMtools v1.13 (Li et al., 2009) was used to convert the resulting SAM files into BAM format. Variant identification was performed using GATK v4.2.3.0 (McKenna et al., 2010). The individual gVCF files from the 100 accessions were consolidated into a single VCF file using GLnexus v1.2.7 with the DeepVariantWGS config (Yun et al., 2021). Finally, SNPs and INDELs with depths (DP) greater than 2 and maximum missing rates less than 0.1 were extracted for further analysis.

The nucleotide diversity (π) and fixation index F_{ST} were calculated by VCFtools v0.1.16 (Danecek et al., 2011) with a 1,000-bp sliding window and 500-bp steps in grapes. To further identify the regions with signals of selective sweeps in cultivated grapes, a criterion was established: the top F_{ST} must be greater than 0.1, and the π_{wild}/π_{cul} ratio must be greater than 1 based on common SNPs in the grape mitogenomes.

The genomic databases with SIFT predictions were generated using the tool `make-SIFT-db-all.pl`, which took genomic sequence data in FASTA format and a gene annotation file in GTF format as input (Vaser et al., 2016). The required protein database was downloaded from UniProt (2021) (<https://www.uniprot.org/>). Subsequently, SNP data in VCF format, along with the genomic databases, were input into SIFT 4G to assess deleterious mutations.

SV frequency assessment and origin of the grape mitogenome

To assess the SV frequency across 100 grape accessions, BEDTools v2.18 (Quinlan, 2014) was used to compute SV mapping coverage. The SV was segmented into three parts (SV1, SV2, and SV3), with their respective read depths (ldep) and whole-genome depths (Wdep) calculated. To correct for sequencing depth variations, the ldep/Wdep

ratio was used to determine SV presence or absence. SV1 and SV3 ratios were categorized into low (absent) and high (present) levels. Notably, SV2, homologous to plastid sequences, was excluded from further analysis. The frequencies of SV1 and SV3 in various grape populations were then compared using a chi-square test to identify significant differences.

To determine the origin of the SV sequences, we performed a BLASTN search to detect the homologous sequence in 33 *Vitis* mitochondrial and plastid and nuclear genomes. The inferred putative origin of the intracellular transfer and nuclear-shared sequences were identified by performing BLASTN searches of mitogenomes against nuclear DNA, with an e-value cutoff lower than $1e-10$, identity greater than 90 and hit length more than 100 bp, and the ggplot2 package v3.4.3 (Villanueva and Chen, 2019) (<https://cran.r-project.org/web/packages/ggplot2/index.html>) was used to visualize the results.

Identification of NUMTs and NUPTs for 13 grape species

First, Blastn v2.11.0+ (Camacho et al., 2009) was used to identify potential transfer events from organelles to the nuclear genome with the filter parameter “identity greater than 80% and alignment length greater than 100 bp.” Second, the average depths of the nuclear genome, mitochondrial genome and plastid genome were calculated, preliminary NUMT and NUPT fragments were identified based on their calculated depths. Specifically, the theoretical value of the depth of the NUMTs region = average depth of the nuclear genome + average depth of the mitochondrial genome, and similarly the theoretical value of the depth of the NUPTs region = average depth of the nuclear genome + average depth of the chloroplast genome. Finally, minimap2 v2.26 was used to map the 2 kb upstream and downstream sequences of NUMT and NUPT fragments to PacBio HiFi reads. The results were visualized in IGV v2.12.3 to see if there were long reads linking NUMTs and NUPTs with their surrounding nuclear genomes, providing further evidence of NUMT and NUPT authenticity (Figures S24, S25).

NUMTs and NUPTs dynamic insertion analysis

The distribution.R script was used to visualize the distribution of NUMTs and NUPTs on the nuclear genome. BEDtools helped to annotate the distribution patterns of NUMTs and NUPTs within different chromosomes and genomic regions (i.e., exon, intron et al.) based on the nuclear genome annotation file. The genome-wide annotation pipeline and the identification of centromeres were adapted from previous research (Shi et al., 2023; Yue et al., 2023). Transposable element families were identified by RepeatModeler2 (Flynn et al., 2020) with a pan-*Vitis* database of repeat families (Shi et al., 2023) and annotated all repetitive sequences of the combining reference genome using RepeatMasker v4.1.2 (Tarailo-Graovac and

Chen, 2009). The ggplot2 package v3.4.3 was used to visualize the results.

We then conducted a blastn search for two haplotypes of the same variety to identify both heterozygous and homozygous fragments of NUMTs and NUPTs. To detect the function of genes captured in the two haplotype regions of NUMTs and NUPTs, we downloaded the protein sequence library of Swiss-Prot (<https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/>) for a local blast. After this, we extracted the NUMTs and NUPTs protein sequences for each haplotype of 13 grape varieties and blasted using diamond v.2.0.15 (parameter: `-k 1 -e 0.00001`, <https://github.com/python-diamond/Diamond>). We further extracted all genes of PN_T2T through diamond as background genes for GO and KEGG analysis. Then we uploaded the Swiss-Prot ID to DAVID (<https://david.ncifcrf.gov/tools.jsp>) and completed GO enrichment and KEGG analysis. The results were further selected with the filter parameter *P*-value threshold of less than 0.05. Finally, data visualization was completed by GO.R and KEGG.R scripts.

In addition, the One Step MCScanX tool within TBtools (Chen et al., 2023) was used to identify homologous gene pairs between wild and cultivated grapes. TBtools was also employed to calculate the Ka/Ks ratio (non-synonymous to synonymous substitution ratio) for both heterozygous and homozygous NUMT genes between wild and cultivated grapes, and then a *t*-test was used to test whether there were significant differences.

The mapping of candidate genes associated with cytonuclear interactions in grapevine

We selected the samples used in the above artificial selection analysis to conduct GWAS analysis. There are two populations: cultivated ($n = 50$) and wild ($n = 50$). The pipeline for detecting nuclear genome variation was similar to that of the mitochondrial genome. Fastp v.0.23.4 (Chen, 2023) was used to filter low-quality reads with default parameters, and BWA was used to compare the filtered data with the PN_T2T reference genome. The filtered BAM files were further processed by sorting with SAMtools v1.13 and marking the PCR duplicates with Picard v2.23.0 (<http://broadinstitute.github.io/picard/>), respectively. Subsequently, SNPs and InDels were identified using GATK v.4.2.6.0. This was followed by the consolidation of the genotyping from the gVCF files into a VCF file. High-quality variants were obtained using PLINK with $MAF \geq 0.05$ and missing < 0.2 , resulting in 147,035 variants (137,077 SNPs, 9,958 InDels) for further analysis.

To eliminate the risk of false positives arising from the presence of multiple copies, we assessed the depth of coverage for each mitochondrial variant and compared it to the average depth across the whole mitochondrial genome. Variants with coverage depths exceeding 1.5 times the mean depth were excluded (Lian et al., 2024). Furthermore, we filtered out mitochondrial SNPs that were supported by fewer than four reads. Subsequently, we

calculated the reference allele frequency by dividing the count of reads that support the reference allele by the total number of reads, and we term this value the “phenotype.” For the plastid, the phenotypic value is obtained in a similar way to the mitochondrial, but the unique plastid sequence is used as the reference, and the rest of the steps are the same as calculating the mitochondrial phenotypic value.

A mixed linear model in GEMMA v.0.98.3 (Zhou and Stephens, 2012) was employed for GWAS analysis. Variant positions and *p*-wald test statistics (*P*-value) were extracted to generate Manhattan and Q-Q plots using R scripts. A Python script was utilized to identify the associated genes within 10 kb windows of the significant variants, which were above the significance threshold of $-\log_{10}(0.05/\text{Variant Numbers})$. GO enrichment analysis was employed using the web tool DAVID and the results were visualized with the GO.R script. The zoomed-in plots of GWAS signals were visualized using LDBlockshow v.1.40 (Dong et al., 2021).

The salient sites associated with nuclear and mitochondria for each sample were screened out using VCFtools and the results were plotted with the pheatmap.R script. The chi-squared test was used to test whether there were significant differences between wild grapes, cultivated grapes, and genotypes. The significantly associated loci and the non-significantly associated loci were each screened out, and the proportion of NUMT events occurring at these sites was calculated.

Data availability statement

The PacBio data used for mitogenome and plastid genome assembly, along with the raw WGS data for grape accessions, were downloaded from NCBI. BioProject IDs are provided in Tables S1, S6, respectively. Mitochondrial and plastid genome assemblies and annotations of genome and variation maps were uploaded to Zenodo (<https://zenodo.org/record/14489445>). Custom scripts and workflows are available in the GitHub repository (https://github.com/zhouyiflab/Nuclear_Cytoplasmic_Interactions/).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Y.Z. and C.L. conceived and designed the study, and L.T. also provided assistance for the project. T.H. and C.L. performed experiments and data analysis. Y.X. and T.Z. collected the samples. Y.D., L.Z., C.C., Y.Z., X.S., and Y.L. assembled and annotated the haplotype-resolved nuclear genomes. Y.P. and H.X. provided the suggestions for genome selection and classification of gene structures, respectively. X.X., J.Y., Y.Z., X.S., J.L., M.D., X.F., S.Y., W.W., Z.C., S.Q., B.A., Z.J., and X.L. performed data analysis. T.H., C.L., and Y.X. wrote the manuscript. Y.Z. supervised the project. All authors provided critical feedback and revised the manuscript.

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SUPPORTING INFORMATION

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