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RESEARCH ARTICLE

# A new 10K liquid SNP genotyping array for wax gourd and its application in heterosis utilization and cultivars identification

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**Highlights**

- Developed the first high-density 10K SNP array for wax gourd using genotyping by target sequencing (GBTS) technology, comprising 10,722 genome-wide SNPs distributed across the genome, including 278 associated with functional trait loci.
- Demonstrated its utility in heterosis prediction, population structure analysis, and cultivar fingerprinting, demonstrating its applicability in genetic research and breeding programs.

**Abstract**

High-throughput single nucleotide polymorphism (SNP) arrays have emerged as essential genotyping tools, significantly accelerating breeding programs and advancing basic research. In this study, a high-throughput 10K SNP genotyping array for wax gourd was developed using genotyping by target sequencing (GBTS), featuring 10,722 SNPs evenly distributed across all 12 chromosomes, including 278 functional loci associated with key economic traits. To demonstrate its utility, genetic distances among 19 elite inbred lines were calculated from SNP data and correlated with heterosis for single fruit weight. The results revealed that greater genetic distance was associated with higher middle parent heterosis (MPH) for single fruit weight. Furthermore, 56 commercial wax gourd cultivars collected from eight regions were selected and genotyped. Population structure analysis, phylogenetic analysis, and principal component analysis (PCA) collectively indicated that these cultivars fall into two major groups. Group I, comprising black or dark green skinned wax gourds, exhibited lower genetic diversity than Group II, which includes green or light green skinned varieties, reflecting shorter genetic distances within Group I. Finally, 60 polymorphic SNPs were used to construct DNA fingerprints for distinguishing the 56 cultivars. As the first high-throughput genotyping platform for wax gourd, this SNP array provides an effective and powerful tool for genetic analysis.

**Keywords:** wax gourd, SNP genotyping array, heterosis, cultivar identification, DNA fingerprint

## 1. Introduction

Wax gourd (Benincasa *hispida* (Thunb) Cogn., 2n=2x=24) is an economically important vegetable crop widely distributed in China, India, and Southeast Asian countries (Xie *et al.* 2022). Similar to most cucurbit crops, the fruits of the wax gourd are the primary edible part and are rich in essential nutrients, such as proteins, carbohydrates, vitamins, minerals, and dietary fiber (Xie *et al.* 2019; Chang *et al.* 2024).

In recent years, diverse germplasm resources of wax gourd have been developed through long-term natural and artificial selection, including introduction and hybridization. Currently, most commercial varieties are $F_1$ hybrids cultivated to exploit heterosis (Yu *et al.* 2021; Liu *et al.* 2024). Effective utilization of heterosis relies on selecting parental lines with high combining ability to generate significant hybrid advantages (Yu *et al.* 2021). However, conventional breeding

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methods often depend on phenotypic selection guided by experience, rendering them susceptible to environmental variation (Yu *et al.* 2024). Additionally, field evaluation of numerous hybrid combinations demands significant manpower and material resources, resulting in prolonged breeding cycles and reduced efficiency. A positive correlation has been consistently observed between parental genetic distance and middle parent heterosis (MPH) for yield-related traits (Rahman *et al.* 2022). Therefore, conducting systematic clustering analysis and accurately determining the genetic distances among parental lines are critical for predicting heterosis and streamlining breeding strategies. Furthermore, accurate hybrid identification based on morphological traits remains challenging, primarily due to the close genetic relationship between parental lines (Jamali *et al.* 2019). These factors increase the risk of seed confusion and misidentification, disrupting the seed market order, infringing upon consumer rights, and posing significant challenges to variety resource protection and utilization (Zhang *et al.* 2022). These limitations underscore the urgent need for a reliable genotyping system to authenticate commercial wax gourd varieties, enhance seed market regulation, and strengthen intellectual property protection.

DNA markers are recognized as powerful tools for estimating genetic distance and identifying plant varieties, enabling robust assessment of genetic diversity based on DNA sequence variation (Yang *et al.* 2022). Various types of DNA markers, including restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP), have been widely applied in DNA fingerprinting, genetic diversity studies, population structure analysis, molecular identification, and modern breeding programs (Thomson *et al.* 2017; Li *et al.* 2019; Hu *et al.* 2022; Yang *et al.* 2022; Ting *et al.* 2023). Compared to other molecular markers, SNPs are abundant and widely distributed in plant genomes, offering several prominent advantages that make them a preferred choice for crop molecular breeding (Marrano *et al.* 2019). With the continuous advancement of next-generation sequencing (NGS) technologies, it is now feasible to generate large-scale SNP datasets for molecular research and genetic improvement (Luo *et al.* 2023). SNP arrays based on genotyping by target sequencing (GBTS) technology offer high quality, density, sensitivity, and operational simplicity, proving highly valuable for germplasm evaluation, variety identification, gene mapping, and whole-genome selection breeding (Zhou *et al.* 2018; Sun *et al.* 2020). The wax gourd genome has been sequenced and assembled, providing a foundational resource for studying its genetic diversity and facilitating cultivar identification (Xie *et al.* 2019; Luo *et al.* 2023). Nevertheless, the successful application of molecular markers in wax gourd breeding remains limited due to issues of quality, quantity, and density (Xie *et al.* 2019). To date, no liquid SNP array has been reported specifically for wax gourd.

In this study, we developed the first 10K liquid SNP genotyping array for wax gourd using whole-genome resequencing data from 93 inbred lines collected in this study and 146 previously reported accessions. We further evaluated its utility in heterosis prediction and cultivar identification. The integration of genomic resequencing with an SNP genotyping array provides high-quality molecular markers for distinguishing commercial wax gourd varieties and characterizing their genetic differences. The array demonstrates high marker density, polymorphism, and genotyping accuracy, serving as a robust platform for genome-wide association studies, germplasm resource characterization, and molecular breeding in wax gourd.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

To develop high-density genotyping arrays, SNPs were selected from high-throughput sequencing data of diverse wax gourd germplasm resources. A total of 93 representative wax gourd germplasms were collected from 15 geographical regions (Appendix A). These inbred lines exhibited distinct morphological and growth characteristics. The majority originated from various provinces/autonomous region in China, including Hunan, Guangdong, Guangxi, Hainan, Yunnan, Zhejiang, Sichuan, and Fujian, with one additional line sourced from the Lao People's Democratic Republic. To enhance SNP diversity and ensure genotyping accuracy, high-depth genome resequencing data from 146 wax gourd accessions, previously reported by Xie *et al.* (2019), were integrated into the selection process. Furthermore, the performance of the genotyping array was evaluated using 56 commercial wax gourd hybrids (Appendix B). These hybrid $F_1$ cultivars displayed phenotypic variation in skin color, fruit length, fruit size, and seed type. Collectively, these 56 hybrids were selected to assess the utility of the wax gourd SNP array for cultivar identification. All wax gourd materials were cultivated in field trials conducted in Hunan or Hainan.

### 2.2. SNP detection, selection, and array development

Fig. 1 details the workflow for developing the wax gourd SNP array. Genomic DNA was extracted from 93 wax gourd inbred lines using a magnetic bead-based method. DNA concentration and purity were assessed using a microplate reader and agarose gel electrophoresis, respectively. Samples meeting the following quality criteria were selected for DNA-seq library construction: concentration >10 ng $\mu L^{-1}$, 260/280 absorbance ratios between 1.6 and 2.2, and distinct high-molecular-weight bands on gels. Qualified libraries were sequenced on the BGI sequencing platform (MGI) using a 150 bp paired-end strategy (Higentec, Changsha, China) at approximately 10× genome coverage.

High-quality clean reads were generated by filtering raw resequencing data from the 93 inbred lines to remove low-quality sequences that could compromise downstream analyses. Subsequently, the filtered reads from these 93 lines and 146 previously reported germplasm accessions were aligned to the wax gourd reference genome (pf3_genome.v1.1) (Luo *et al.* 2023) using BWA (version 0.7.17-r1188) (Li *et al.* 2009) with default parameters. To generate genomic Variant Call Format (gVCF) files, GATK (version 4.2.0.0-1) (Van der Auwera *et al.* 2013) was employed to

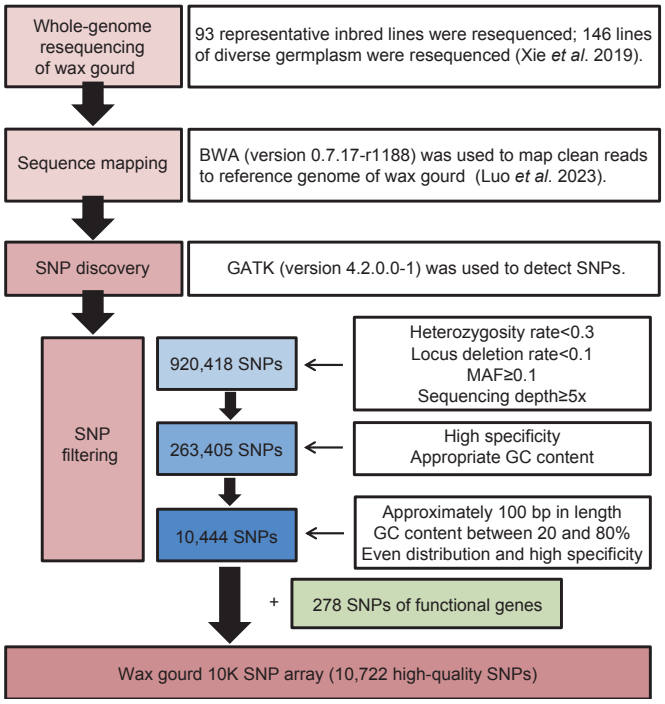



**Fig. 1 Design pipeline of SNP detection and selection for the wax gourd 10K SNP array.**

identify variant sites in each sample independently.

Initial hard filtering of SNPs was performed per sample using the following thresholds: quality by depth (QD)<2.0, Fisher strand (FS)>60.0, mapping quality (MQ)<40.0, strand odds ratio (SOR)>3.0, MQRankSum<–12.5, and ReadPosRankSum<–8.0. After applying additional filters for heterozygosity rate (<0.3), locus missingness (<0.1), minor allele frequency (MAF ≥0.1), and minimum sequencing depth (≥5×), a set of 920,418 high-confidence SNPs was retained. For each filtered SNP, 50-base sequences flanking both upstream and downstream regions were extracted. Following evaluation of sequence specificity and GC content, 263,405 SNPs suitable for probe design were identified. From this subset, 10,444 SNPs were selected based on optimal amplicon length (~100 bp), GC content of 20–80%, uniform genomic distribution, and high uniqueness, resulting in an average inter-SNP spacing of 88.83 kb (Appendix C). Additionally, candidate SNPs located within functional genes previously associated with key economic traits in wax gourd or other cucurbit crops were prioritized (Cheng *et al.* 2021; Liu *et al.* 2021; Wang *et al.* 2021; Cheng *et al.* 2022; Luo *et al.* 2022; Xie *et al.* 2022; Yan *et al.* 2022). To incorporate these loci into the array, their physical positions were confirmed *via* BLASTN alignment against the reference genome, and targeted capture probes were designed for the best-aligned SNPs. A total of 278 trait-associated SNPs were curated, including 135 markers linked to disease and abiotic stress resistance, and 143 related to fruit and seed development. As a result, 10,722 polymorphic SNPs were integrated into the wax gourd 10K liquid genotyping array using Genotyping by Pinpoint Sequencing of captured targets (cGPS) (Appendix D).

### 2.3. Population structure analysis and phylogenetic analysis

To evaluate the applicability of the SNP array for cultivar identification, 56 commercially available wax gourd hybrid cultivars were genotyped using the wax gourd 10K array. VCF files generated by the 10K array were processed with VCFtools for locus filtering and quality control. Loci were retained only if they exhibited a minor allele frequency greater than 0.05, a missing rate of no more than 10%, and exactly two alleles. After excluding loci with high missing data, low minor allele frequency, multiple alleles, or monomorphism, population structure, principal component analysis (PCA), and phylogenetic tree analyses were performed on the filtered dataset.

Using the quality-controlled SNP data, ancestry proportions were estimated using ADMIXTURE software (version 1.3.0), and the cross-validation error for assigning cultivars to *K* subpopulations was calculated. The optimal number of subpopulations (*K*) was determined based on the lowest cross-validation error, and results were visualized in R. PCA was conducted using the --pca parameter in PLINK software (version 1.90b4.6) (Purcell *et al.* 2007) and visualized using R. A genetic distance matrix among samples was computed using the --distance parameter in PLINK. A neighbor-joining (NJ) tree was constructed using the ape package in R and visualized *via* iTOL (version 6) (http://itol.embl.de/). For heterosis pattern validation, 19 elite inbred lines derived from 93 accessions were genotyped using the same array platform.

### 2.4. Selecting core SNP loci and constructing fingerprint for wax gourd

Based on genotyping data from 56 commercial wax gourd hybrid lines, 60 SNP markers exhibiting high MAF and polymorphism information content (PIC) value were selected to comprise a core SNP panel for DNA fingerprint construction. The remaining SNPs, each with MAF>0.40 and PIC>0.36, effectively distinguished diverse cultivars, averaging five SNPs per chromosome (Appendix E).

## 3. Results

### 3.1. Sequence alignment, SNP detection and selection, and array design

In this study, a total of 10,722 high-quality SNPs were included in the wax gourd 10K SNP genotyping array. A detailed description of the detection, filtering, and final selection of SNPs included in the array has been provided in Section 2.2 and Fig. 1. Combining resequencing data from 93 wax gourd inbred lines (details in Appendix A) and 146 previously published accessions in 2019, 10,444 highly reliable SNPs were selected through stringent filtering based on heterozygosity rate, locus deletion rate, minor allele frequency (MAF), and GC content. Additionally, 278 potentially functional SNP loci were identified and classified into two major categories. The first category comprised 135 loci associated with biotic and abiotic stress responses, such as powdery mildew resistance, low- and high-temperature tolerance, and salt stress adaptation. The second category

included 143 loci linked to agronomic traits, such as tendril development, leaf shape, plant height, male sterility, fruit skin color, fruit soluble solids content, fruit size and length, and seed shape and size. These functional SNPs were integrated into the array, resulting in a final set of 10,722 representative SNPs uniformly distributed across the 12 chromosomes of wax gourd (Appendices D and F). To validate the representativeness of the 10K SNP array markers, resequencing data from the 93 inbred accessions were analyzed. Genetic diversity analyses revealed high concordance in population structure between whole-genome SNPs and the 10K SNPs (Appendix G), with both datasets consistently assigning accessions to three major genetic clusters. These results confirm that the 10K SNP panel effectively captures genomic variation in wax gourd, enabling accurate genotyping and robust representation of population genetic diversity.

### 3.2. Characteristics of the wax gourd 10K SNP array

The array demonstrated high genomic coverage, with 10,722 SNPs uniformly distributed across all 12 chromosomes at an average density of 11.59 SNPs/Mb (Fig. 2-A and Table 1). Statistical analysis indicated that each chromosome contained over 600 SNPs, and SNP density varied minimally among chromosomes, ranging from 10.19 to 13.03 SNPs/Mb (Table 1). Notably, chromosome 10 exhibited the highest density at 13.03 SNPs/Mb, followed by chromosome 6 at 12.42 SNPs/Mb, whereas chromosome 7 had the lowest density at 10.19 SNPs/Mb. Chromosome 1, the longest, harbored the greatest number of SNPs. A strong correlation was observed between SNP number per chromosome and chromosome length (Appendix H), with Pearson's correlation coefficient (r) reaching 0.96. Additionally, physical interval analysis revealed an average distance of 86.63 kb between adjacent SNPs genome-wide. The spacing of 8,594 SNPs (80.24%) was less than 120 kb, while 2,116 (19.76%) exceeded this threshold (Fig. 2-B). Most SNPs were spaced within 30–120 kb, with approximately 43.06% of adjacent pairs separated by 30–60 kb and 34.62% by 60–120 kb.

The MAF values of these SNPs ranged from 0.1 to 0.5, with a mean of 0.26; 3,842 SNPs displayed high variability (MAF>0.30), accounting for 35.83% of the total (Fig. 2-C). PIC values spanned from 0.16 to 0.38, averaging 0.29, and 5,373 SNPs (50.11%) had PIC>0.30 (Fig. 2-D). Functional annotation showed that most SNPs were located in intergenic regions, followed by upstream and downstream regions (Fig. 2-E). Of the 10,722 SNPs, 5,469 (51.01%) were in intergenic regions, 1,933 (18.03%) in upstream regions, and 1,361 (12.69%) in downstream regions. The array also included 1,187 SNPs (11.07%) in exonic regions and 772 (7.20%) in intronic regions.

### 3.3. The 10K liquid SNP array for heterosis utilization in wax gourd

To further evaluate the utility of the SNP array for heterosis utilization, we genotyped 19 representative elite inbred lines selected from a panel of 93 wax gourd inbred lines and constructed a phylogenetic tree. This analysis grouped the

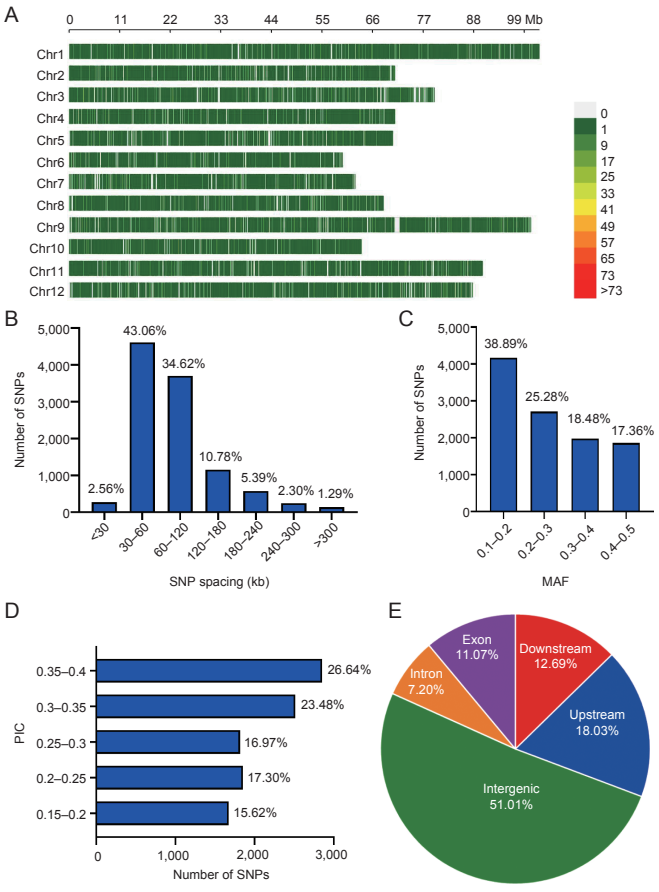



**Fig. 2 Characteristics of the wax gourd 10K SNP array.** A, distribution of 10K SNPs across the 12 chromosomes, with marker density shown in different bar colors; each bar corresponds to a 0.08 Mb genomic window. B, spacing distribution between adjacent SNP loci throughout the genome. C, minor allele frequency (MAF) distribution for the 10K SNPs. D, polymorphism information content (PIC) distribution for the 10K SNPs. E, genomic distribution of SNPs across functional regions.

accessions into three major clusters (Fig. 3-A). Among these, two lines exhibiting thick mature fruit cuticular wax (MFCW), BHD9 and BHA6, were selected and crossed to generate an $F_1$ hybrid. Additionally, two MFCW-lacking lines, BHD4 and BHE2, were similarly crossed to produce an $F_1$ hybrid. As shown in Fig. 3-A, BHD4 and BHE2 clustered together, whereas BHD9 and BHA6 were assigned to distinct groups. To examine the relationship between genetic distance and yield heterosis in wax gourd, single fruit weight data were collected from parental lines and their corresponding $F_1$ hybrids grown in two geographically distinct regions — Hunan and Hainan (Fig. 3-B). In both environments, the single fruit weight of each $F_1$ hybrid exceeded that of its respective parents, resulting in MPH for this trait across locations (Appendix I). The MPH value for the BHD9×BHA6 cross was 60.44% in Hunan and 65.41% in Hainan, surpassing that of the BHD4×BHE2 cross, which reached 45.89% in Hunan and 40.43% in Hainan. Genetic distances among the 19 elite inbred lines, calculated using PLINK, varied widely, ranging from 0.03 to 0.67 (Fig. 3-C and Appendix J). The genetic distance between BHD9 and BHA6 was 0.44, compared to

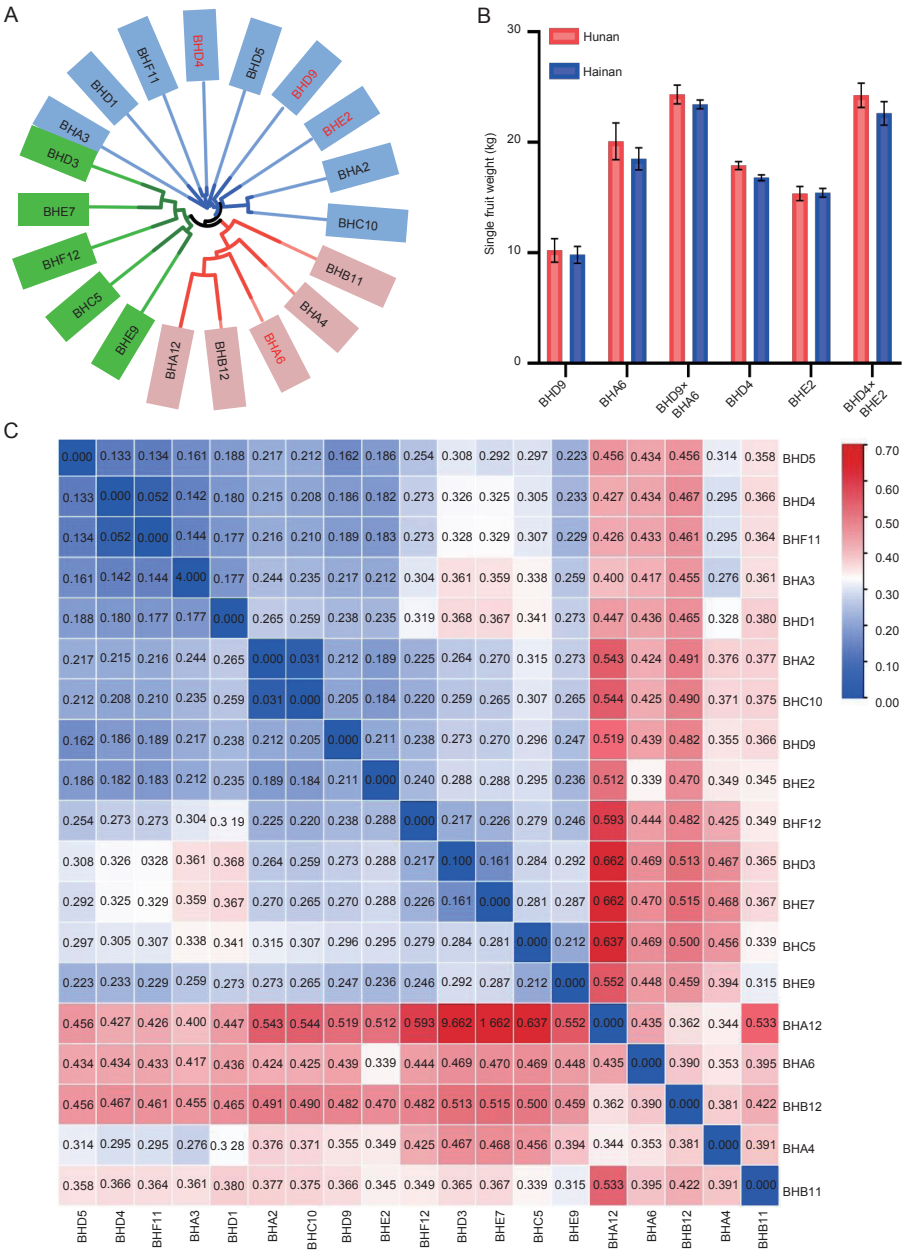



**Fig. 3 The application of wax gourd 10K SNP array in heterosis utilization.** A, phylogenetic relationships among 19 elite inbred lines. B, single fruit weight of parental lines and $F_1$ hybrids evaluated in Hunan and Hainan provinces. C, heatmap of genetic distances among 19 elite inbred lines.

**Table 1 Distribution of SNPs and inter-SNP distance across chromosomes**

| Chromosome | Length (Mb) | Number of SNPs | Density of SNP (SNPs Mb$^{-1}$) | Average distance (kb) |
|---|---|---|---|---|
| GWHBJVO00000001 | 102.24 | 1158 | 11.33 | 88.29 |
| GWHBJVO00000002 | 70.97 | 821 | 11.57 | 86.44 |
| GWHBJVO00000003 | 79.48 | 895 | 11.26 | 88.81 |
| GWHBJVO00000004 | 70.83 | 817 | 11.53 | 86.70 |
| GWHBJVO00000005 | 70.37 | 769 | 10.93 | 91.51 |
| GWHBJVO00000006 | 59.51 | 739 | 12.42 | 80.53 |
| GWHBJVO00000007 | 62.24 | 634 | 10.19 | 98.17 |
| GWHBJVO00000008 | 68.52 | 774 | 11.30 | 88.53 |
| GWHBJVO00000009 | 100.39 | 1124 | 11.20 | 89.32 |
| GWHBJVO00000010 | 63.56 | 828 | 13.03 | 76.76 |
| GWHBJVO00000011 | 89.89 | 1100 | 12.24 | 81.72 |
| GWHBJVO00000012 | 88.05 | 1063 | 12.07 | 82.84 |

0.18 between BHD4 and BHE2. These results demonstrate that the larger genetic distance between BHD9 and BHA6 corresponded to higher MPH for single fruit weight in their $F_1$ hybrid compared to the BHD4×BHE2 hybrid. Therefore, parental lines with a greater genetic distance may have an enhanced potential to form superior hybrid combinations with increased yield. Collectively, these findings confirm that the wax gourd SNP array enables accurate genotyping and reliable genetic distance estimation, thereby facilitating the effective exploitation of heterosis in breeding programs.

### 3.4. The 10K liquid SNP array for cultivar identification in wax gourd

**Population structure and genetic diversity analysis** To evaluate the utility of the array, 56 representative wax gourd cultivars were selected and genotyped using the platform. To assess its effectiveness in cultivar identification, the population structure of all samples was first analyzed. Using the Admixture software and high-quality SNP markers, population stratification was inferred. The optimal number of subpopulations (*K*) was determined by computing cross-validation errors for *K* values ranging from 1 to 12 (Appendix K), which plateaued at *K*=2, indicating this value as optimal. At *K*=2, the samples divided into two major groups (Fig. 4-A). Trait comparison revealed distinct peel colors between Group I and Group II: Group I exhibited black or dark green peel, whereas Group II displayed green or light green peel. This suggests that the classification was primarily driven by peel color. The grouping of the 56 cultivars was further supported by PCA (Fig. 4-B) and phylogenetic analysis (Fig. 4-C), indicating high genotyping accuracy with this array. In the PCA, cultivars were broadly separated into two clusters corresponding to dark-skinned (black or dark green) and light-skinned (green or light green) types (Fig. 4-B). Notably, Group II showed a dispersed distribution, while Group I formed a tightly clustered pattern. Additionally, a phylogenetic tree was constructed using PLINK, providing detailed insights into the genetic relationships among the sequenced lines. The tree topology was consistent with PCA results, clearly distinguishing the same two groups (Fig. 4-C). Group I included 33 commercially prevalent cultivars, such as E9 from Hunan and $F_1$ from Guangdong. Within Group I, cultivars D9, D10, and F9 from Hunan, along with A6 and B3 from Guangxi, formed distinct subclusters. Group II comprised 23 representative cultivars, including A9, C10, and E1, which clustered together, potentially due to their shorter fruit length. Collectively, population structure and phylogenetic analyses indicate that wax gourd cultivars with similar phenotypic traits, such as peel color, tend to cluster together genetically.

For a comprehensive assessment of genetic variation among the 56 commercial wax gourd cultivars, pairwise genetic distances were calculated using PLINK (Appendices L and M). The distances exhibited variable levels of divergence both within and between groups. Within Group I, genetic distances ranged from 0.00 to 0.27; within Group II, they ranged from 0.09 to 0.46; and between groups, distances varied from 0.24 to 0.53. Overall, individuals in

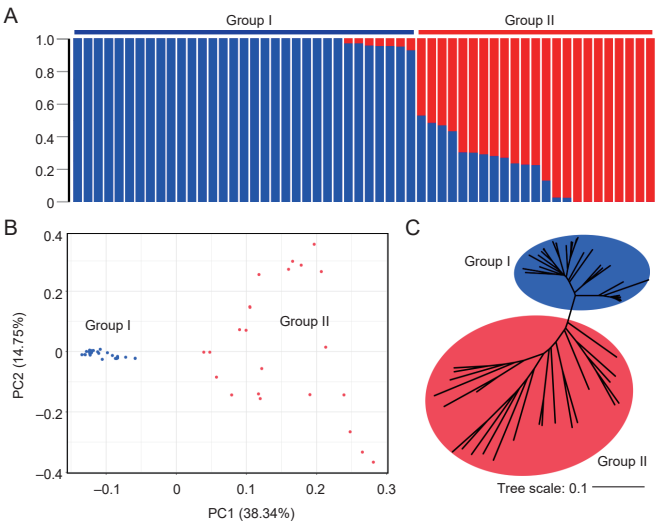



**Fig. 4 Population structure of 56 commercial cultivars based on the wax gourd 10K SNP array.** A, population structure distribution at *K*=2. Each vertical bar represents a hybrid line, with colors indicating distinct genetic groups. B, PCA of the 56 commercial cultivars, where each dot corresponds to a hybrid line and shared colors denote membership in the same genetic group. C, neighbor-joining tree of the 56 wax gourd hybrid lines, with samples from different genetic groups displayed in different colors.

Group I showed greater genetic relatedness than those in Group II, as evidenced by shorter intra-group distances. These findings demonstrate that the wax gourd 10K SNP array is highly effective for resolving population structure and assessing genetic diversity, even among closely related cultivars.

**The construction of SNP fingerprint** A core SNP set for population identification should enable discrimination among all samples with a minimal yet sufficient number of markers. To develop such a set for distinguishing wax gourd varieties, we identified a core panel of 60 SNP loci selected from the wax gourd 10K SNP array, with five highly polymorphic SNPs per chromosome (Appendices E and N). These SNPs exhibited a mean MAF of 0.45, with all values exceeding 0.4, and an average PIC of 0.37. Given their high polymorphism and discriminatory power, these markers provide sufficient resolution to differentiate all accessions. Therefore, a DNA fingerprint for the 56 wax gourd cultivars was constructed using this core set of 60 SNP markers (Fig. 5).

## 4. Discussion

DNA markers are increasingly utilized in plant breeding, particularly for assessing genetic similarities among genotypes (Daware *et al.* 2023). Nevertheless, their widespread application across many crops, such as SSR markers, remains limited by insufficient throughput, accuracy, and data standardization (Lin *et al.* 2023). Due to advantages including flexibility, efficiency, and high marker density, SNPs have emerged as the most extensively employed molecular marker type (Marrano *et al.* 2019). Moreover, with the advent of NGS technologies, SNP discovery through DNA sequencing has become more accessible and cost-effective

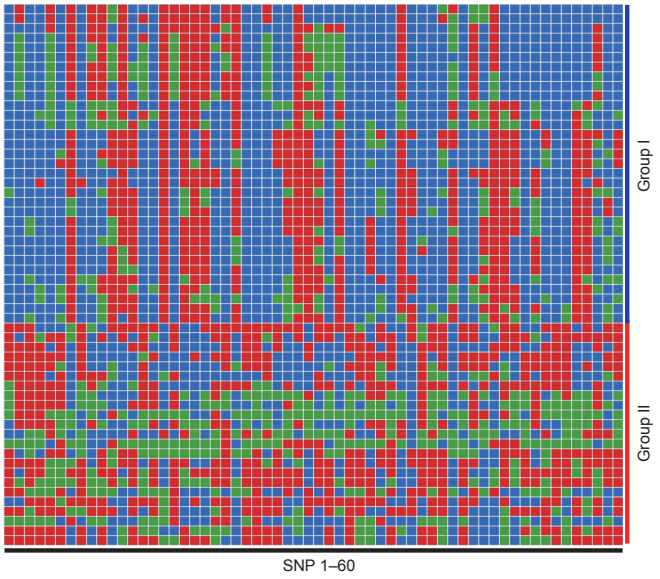



**Fig. 5 The SNP fingerprints of 56 wax gourd cultivars.** Each row displays the SNP genotypes at 60 loci for a single cultivar. Green indicates heterozygous genotypes (XY); red represents homozygous genotypes (XX); blue denotes homozygous genotypes (YY).

(Rasheed *et al.* 2017). Compared to various genotyping platforms, including whole-genome resequencing (WGR) (Jeon *et al.* 2024), genotyping-by-sequencing (GBS) (He *et al.* 2014), and kompetitive allele-specific PCR (KASP) (Ertiro *et al.* 2015), the use of high-density SNP arrays provides a robust and efficient tool for modern breeding programs. As a targeted sequence-capture strategy, SNP genotyping arrays have been successfully applied in rice (Kim *et al.* 2022; Daware *et al.* 2023), wheat (Sun *et al.* 2020; Fan *et al.* 2022), maize (Tian *et al.* 2021; Yu *et al.* 2023), cucumber (Zhang *et al.* 2020, 2022), watermelon (Yang *et al.* 2022), and melon (Yu *et al.* 2024). In this study, we developed a high-throughput and cost-effective 10K SNP array based on the GBTS system, establishing the first dedicated genotyping platform for wax gourd genetics and breeding. Furthermore, the array demonstrated utility and robustness in heterosis utilization and cultivar identification.

The SNP array was designed using resequencing data from 239 wax gourd accessions, comprising 93 newly reported in this study and 146 previously described (Xie *et al.* 2019) (Fig. 1 and Appendix A). A set of 10,722 representative SNPs was evenly distributed across the genome, with an average density of 11.59 SNPs/Mb (Fig. 2-A and Table 1). Analysis revealed a strong positive correlation between the number of SNPs per chromosome and chromosome length (r=0.96) (Appendix H), consistent with findings in soybean (Li *et al.* 2022) and eastern oyster (Xuereb *et al.* 2023). The average interval between adjacent SNPs was 86.63 kb, further indicating uniform distribution of core polymorphic sites across chromosomes and confirming comprehensive genome-wide coverage (Fig. 2-B and Table 1). High polymorphism is essential for QTL mapping in genome scans and for effective implementation of genomic selection (Gao *et al.* 2023). The array exhibited relatively high MAF and PIC, with mean values of 0.26 and 0.29, respectively (Fig. 2-C and D). Population structure analysis of the 93 newly sequenced accessions showed high concordance between the whole-genome SNP dataset and the subset derived from the 10K SNP array (Appendix G). This result confirms that the selected SNP loci are representative and effectively capture genomic variation in wax gourd, accurately reflecting population-level genetic diversity. Functionally, 118 SNPs (11.07%) were located in exonic regions, where they are more likely to influence gene function (Fig. 2-E) (Li *et al.* 2023). Overall, the 10,722 SNPs were polymorphic and suitable for genetic diversity analyses, consistent with performance observed in other genotyping arrays (Li *et al.* 2019; Marrano *et al.* 2019; Chao *et al.* 2024). Additionally, to enrich functional resolution, we incorporated 278 SNPs associated with disease resistance, abiotic stress tolerance, and plant and fruit morphology traits, curated from published studies (Appendices D and F). Compared to random genomic markers, these functional SNPs, which are derived from known functional genes or tightly linked loci, offer superior accuracy and stability in allele prediction across diverse genetic backgrounds. Their integration into breeding pipelines will enable precise marker-assisted selection (MAS) and efficient backcrossing strategies, accelerating the development of elite varieties. Therefore, leveraging this array for functional marker-driven breeding constitutes the primary objective of our subsequent work. Although the current array provides relatively broad coverage of the wax gourd genome, numerous functional genes and uncharacterized loci remain undetected, highlighting the need to expand probe content in subsequent versions of the liquid array.

Plant breeding effectively exploits heterosis, which has proven to be one of the most efficient approaches to enhancing grain yield in multiple crops (Boeven *et al.* 2020). SNP arrays offer unique advantages for investigating phylogenetic relationships and genetic diversity among germplasm collections (Burridge *et al.* 2024). In this study, cluster analysis was performed on 19 elite inbred lines using the 10K SNP array, and pairwise genetic distances were calculated. The parental lines were grouped into three major clusters: one containing nine parents and two others each comprising five parents, with genetic distances ranging from 0.03 to 0.67 (Fig. 3-A). Genetic distance serves as an effective metric for quantifying genetic divergence between parental lines (Liu *et al.* 2022). A significant correlation between genetic distance and heterosis has been documented in numerous crop breeding studies, where greater parental divergence generally leads to increased heterotic expression (Dapp *et al.* 2015). BHD4 and BHE2 clustered within the same group, while BHD9 and BHA6 were assigned to different groups, with genetic distances of 0.18 and 0.44, respectively. These lines were hybridized to generate $F_1$ hybrids. Both $F_1$ hybrids, BHD4×BHE2 and BHD9×BHA6, exhibited MPH for single fruit weight, surpassing their respective parents in both Hunan and Hainan provinces (Fig. 3-B and Appendix I). The MPH of BHD9×BHA6 exceeded that of BHD4×BHE2, with values of 60.44 and

65.41% recorded in the two locations, respectively (Appendix I). These results align with previous reports, suggesting a positive association between genetic distance and MPH for specific traits. Specifically, larger genetic distances between parents corresponded to higher MPH for single fruit weight. However, this study represents an exploratory investigation with certain limitations. The primary constraint is the small sample size, particularly the limited number of hybrid combinations evaluated. To strengthen the generalizability of these findings, subsequent research will prioritize expanding the number of hybrid combinations assessed. Additionally, supplementation with complementary phenotypic and genotypic data will facilitate more comprehensive analyses. Collectively, SNP arrays offer substantial advantages over conventional breeding methods in wax gourd, enabling broader applications in genetic diversity assessment and heterosis exploitation across diverse parental lines.

The protection of new plant varieties, which are products of human intellectual effort, plays a critical role in ensuring seed quality control, strengthening intellectual property rights, and improving agricultural productivity (Yu *et al.* 2024). For legal protection, a new plant variety must meet three technical criteria: distinctness, uniformity, and stability (DUS) (Zhang *et al.* 2022). However, due to the relatively recent initiation of wax gourd breeding programs and the close genetic relatedness among existing varieties, some commercial cultivars exhibit minimal phenotypic differences, making differentiation challenging using traditional DUS testing methods. This situation may lead to confusion in the seed market and potentially harm the interests of breeders and consumers alike. Molecular markers are widely used in variety identification due to their high accuracy and efficiency (Lin *et al.* 2019). In particular, DNA fingerprinting based on SNP arrays is increasingly employed for cultivar discrimination in several species, including cucumber (Zhang *et al.* 2020, 2022), melon (Yu *et al.* 2024), watermelon (Yang *et al.* 2022), and tea (Lin *et al.* 2019).

In this study, 56 commercial wax gourd cultivars were genotyped using the 10K SNP array. As shown in Fig. 4, population structure analysis clearly separated the 56 cultivars into two distinct groups, with significantly higher genetic diversity observed in Group II than in Group I. Groups I and II exhibited pronounced phenotypic divergence in peel color: black or dark green skin characterized Group I, while green or light green skin defined Group II. These observations were consistent with results from PCA and phylogenetic analyses, indicating high genotyping accuracy of the array in wax gourd. Moreover, individuals with similar phenotypic traits, such as fruit length, clustered together on the same branches. Cultivars bred in the same region also tended to cluster together, exemplified by D9, D10, and F9. However, some varieties from the same region were found in different clusters, which is likely attributable to the complex genetic backgrounds of their parental lines.

Furthermore, the genetic distances within Group II exhibited greater variability compared to those within Group I (Appendices L and M). In Group I, pairwise genetic distances among individuals were below 0.30, indicating close genetic relationships. In contrast, most individuals in Group II showed genetic distances exceeding 0.20, reflecting greater genetic divergence. These results revealed a greater genetic divergence among individuals within Group II, which reflects the complex genetic backgrounds of their parental lines. Overall, the utilization of the 10K SNP array can accurately depict the genetic diversity and relationships among wax gourd varieties. Low-density DNA fingerprinting plays an essential role in identifying, assessing, and utilizing germplasm resources (Li *et al.* 2023). Utilizing a minimal number of stable SNPs allows for effective reduction in identification costs and saves time. Finally, a total of 60 polymorphic SNPs were selected and found to fully distinguish all 56 cultivars investigated in this study (Fig. 5 and Appendix E). All selected SNPs exhibited a MAF>0.40 and PIC>0.36, averaging 5 SNPs per chromosome (Appendix N). Consequently, the SNP array is suitable for the efficient identification of wax gourd, offering extensive application prospects.

## 5. Conclusion

In this study, we developed and validated the first high-density liquid SNP array specifically for wax gourd. This array, which comprises 10,722 SNPs evenly distributed across the genome, has demonstrated its broad utility in population structure, genetic diversity, heterosis utilization, and cultivar identification. In summary, it provides a robust and versatile platform for advancing genetic research and breeding programs in wax gourd.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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