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Research Article

SINGLE NUCLEOTIDE POLYMORPHISMS APPROACH FOR GENETIC BACKGROUND OF ICR MICE USING MASSARRAY

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ABSTRACT: Single nucleotide polymorphisms (SNPs) are an effective method for determining the genetic background of ICR outbred mice in the National Laboratory Animal Center, Mahidol University. We screened 20 SNPs on all autosomes and X chromosome. This method provides fast, accurate and reliable with high confidence for assessing the genetic background of the mice used in research. The SNP markers used in this study were obtained from publicly available SNP databases of inbred mice in the ICLAS Monitoring Center and were subsequently designed using MassARRAY assay design software. Tail samples from ICR outbred mice were collected for genomic DNA extraction. We amplified gene fragments using multiplex PCR and analyzed them through allele-specific primer extension, based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). We described the 20 multiplexes PCR assays for detection with the Sequenom MassARRAY® system. The genetic monitoring reference DNA samples from inbred mice were analyzed for quality control, demonstrating 100% accuracy. The MassARRAY® TYPER 4.0 genotyping software automatically determined phenotypes based on peak heights and mass. Among the analyzed SNPs, nine SNPs provided a heterozygous genotype frequency. No deviation from Hardy – Weinberg equilibrium (P_{HWE} > 0.05) was detected for all SNPs. All nine SNPs exhibited heterozygote deficiency, as indicated by positive F_{IS} values. These polymorphic SNPs offer in this study provide preliminary insights into genetic variability and may serve as candidate markers for future validation and refinement of genetic monitoring within outbred strains.

Keywords: SNP, Genetic background, MassARRAY, ICR mice.

INTRODUCTION

The management of outbred animals has become increasingly important due to their rising popularity in both academic research and commercial applications. These populations provide the genetic diversity necessary for modeling complex traits and studying natural genetic variation, making their effective management essential. Maintaining heterozygosity is a critical goal in managing outbred populations, as it ensures their health, adaptability, and long-term sustainability. Genetic monitoring plays a central role in this process, helping to preserve genetic integrity and guide breeding programs. By tracking allele frequencies and detecting deviations from genetic expectations, genetic monitoring addresses the challenges posed by genetic drift—a stochastic process that causes changes in allele frequencies due to chance rather than natural selection. As Hartl explains, genetic drift is particularly impactful in small populations, where random sampling can lead to significant fluctuations, reduced genetic diversity, and fixation of alleles, thereby compromising the population's adaptability and viability [1].

To counter these effects, robust genetic monitoring strategies must be employed to assess genetic diversity and population structure. The FELASA guidelines emphasize the importance of genetic quality assessment in maintaining the reliability and reproducibility of research outcomes, as well as the effectiveness of breeding programs [2]. Evaluating genetic diversity using tools like observed heterozygosity (*Ho*)

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and expected heterozygosity (*He*) is essential for understanding trends over generations and identifying any concerning patterns. The Hardy-Weinberg equilibrium (HWE) provides a theoretical framework for assessing genetic stability within populations. Deviations from HWE can signal inbreeding, genetic drift, or selection pressures, which are critical factors in maintaining the genetic health of outbred populations. Hartl underscores the importance of analyzing these metrics over successive generations to detect changes and ensure populations remain representative of their genetic pool [1]. Typically, HWE is assessed using the Chi-square test (X^2-test), which evaluates deviations from expected genetic distributions under specific assumptions. The Hardy-Weinberg principle stipulates that large populations with random mating, no selection, and no genetic drift or migration should maintain stable allele frequencies. Consequently, any significant deviations from HWE predictions may indicate violations of its assumptions, such as population stratification or genotyping errors [3]. In 1979, the ICLAS Monitoring Center established the concept of a genetic profile and a monitoring system for genetic testing, utilizing biochemical and immunologic markers for inbred strains of mice and rats [4]. Biochemical and immunological markers are complex to analyze and often require specialized equipment and techniques, which can lead to increased costs and testing times. Furthermore, some markers exhibit low genetic variability, making it challenging to effectively differentiate closely related strains [2].

Practical approaches for managing outbred populations combine genetic monitoring with advanced molecular techniques, such as genotyping-by-sequencing (GBS) and single nucleotide polymorphism (SNP) analysis. These methods enable precise assessments of genetic diversity and the identification of markers linked to desirable traits. Computational models further enhance these efforts by simulating genetic drift and predicting allele frequency distributions, providing critical insights for designing effective breeding programs. Integrating these technologies with established guidelines allows researchers and breeders to preserve the genetic diversity and integrity of outbred populations, ensuring their sustainability and utility in both research and commercial applications. SNPs, which represent single nucleotide variations in DNA sequences with typically two common alleles, have proven particularly valuable in genetic studies. Along with microsatellites, they are widely used for quality control in laboratory animals such as mice and rats. SNP analysis simplifies genotyping and facilitates high-throughput genetic assessments, making it an indispensable tool for effective population management [5]. The first published database used high-density oligonucleotide arrays to collect 2,848 SNPs located in 1,755 sequences tagged sites (STSs) identified across eight mouse strains, representing a first-generation mapping on the mouse genome [6,7]. Another experiment also developed a murine database of SNP [8]. The genotyping panel comprises 87 microsatellite markers that are polymorphic among commonly used inbred rat strains, serving applications that range from assessing genetic contamination within rat colonies [9]. Moreover, there is a genetic mapping substrains of C57BL/6 and BALB/c mice using SNPs characterization to identify the causative mutations of phenotypes induced by N-ethyl-N-nitrosourea mutagenesis [10]. The Jackson Laboratory provided baseline phenotypic data for the most common inbred mouse strains through The Mouse Genome informatics (https://www.informatics.jax.org/home/strain) which includes 420 inbred mouse and 230 inbred rat strains, complied by Dr. Micharl FW feasting. However, SNPs have not been widely utilized for genotyping outbred mice, as the data available in public databases are relatively limited compared to the extensive number of SNPs identified in inbred mouse strains. SNP markers are specific DNA sequences located at known positions on chromosomes, serving as crucial tools for genetic quality control. Outbred colonies are screened for genetic heterogeneity to identify contamination, monitor the progress of breeding programs and select future breeders.

The SNP genotyping was conducted using the MassARRAY® system coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer to provide rapid analysis of large multiplex PCR and single base extension (SBE) multiplex assays. MALDI-TOF MS is a superior technology for SNP detection, consisting of real-time TYPER software on the MassARRAY® analyzer system. This system integrates the robust multiplexed primer extension of the iPLEX® assay, achieving high accuracy in measuring molecular weight, high sensitivity for detecting both homozygosity and heterozygosity and offers a reasonable price [11]. Much research has been focused on SNP for various applications, including the identification and characterization of gene expression modulation [12], the analysis of genes that affect drug metabolism [13] and the development of human identification and sample tracking [14] by MassARRAY® system. Overall, the

combination of SNP analysis with the MassARRAY® system provides high throughput, accuracy, precision, multiplexing capability and enhanced efficiency in genetic monitoring. This study, identifies the single nucleotide polymorphism relevant to genetic quality in breeding programs. To investigate the genetic background of Institute of Cancer Research (ICR) outbred colonies at the National Laboratory Animal Center, Mahidol University (Mlac: ICR) population, we genotyped 20 SNPs located on chromosomes 1 through 19, including the X chromosome using the MassARRAY (Agena Bioscience™) platform, which combined competitive PCR with MALDI-TOF mass spectrometry. Our finding indicated that the identified SNPs exhibit susceptibility and are beneficial for genetic monitoring in outbred mice colonies.

MATERIALS AND METHODS

Samples, DNA isolation and DNA quantification

A total of 306 tail samples were collected to represent the genetic background of ICR outbred mice at the National Laboratory Animal Center, Mahidol University (Mlac: ICR). The experiment cohort was derived from parental stock maintained under a Maximum Avoidance of Inbreeding (MAI) mating scheme, yielding multiple litters with variable sizes and sex ratios. To control for litter effects and ensure balanced representation of both sexes, a sex – stratified random sampling procedure was executed within each litter across three consecutive generations. Each generation was defined as the interval between the establishment of breeders and the collection of their offspring for genotyping. Randomization was then performed within each litter, where two individuals of each sex were randomly selected to achieve a sex – balanced sampling design. In cases where all offspring in a litter were of the same sex, two individuals from that sex were randomly selected, maintaining consistency in sample size while controlling for potential litter bias. Tail samples, approximately 0.5–1 cm in length, were collected and stored in sterile 1.5 ml centrifuge tubes. DNA was isolated using the phenol-chloroform method, following the protocol described by Rupali *et al.,* 2023 [15]. The extracted DNA was eluted in 50 µl of DNase/RNase-free water, and its concentration was quantified using a NanoDrop spectrometer. The DNA concentrations ranged from 20–30 ng/µl, making the samples suitable for use with the MassARRAY® system. This sampling and extraction process ensured the representation of the genetic diversity within the ICR outbred population.

Selection of SNPs

In this study, SNPs were selected based on specific criteria to ensure accuracy and informativeness in genetic monitoring. Key factors included the distribution of SNPs across multiple chromosomes for broad genomic coverage, their polymorphic characteristics in laboratory mouse strains, and their relevance to genetic variability in both inbred and outbred populations. Priority was given to SNPs with well-documented allelic diversity and those commonly utilized in genetic studies of laboratory mice. These considerations ensured that the selected markers reliably represented the genetic variation within the Mlac: ICR population and enabled the detection of allele frequency changes across generations. Both autosomal and X-linked SNPs were included to facilitate a comprehensive analysis of genetic patterns throughout the genome. The genetic profiles of inbred mice preciously reported by the Center Institute for Experimental Medicine and Life Science (CIEM) using SNP analysis [15], this study further developed and adapted these SNP loci to assess the genetic quality of outbred mouse strains. A subset of SNP markers was selected to ensure coverage across all chromosomes. For genetic monitoring of Mlac:ICR mice, SNP databases from autosome and the X chromosome of inbred mice maintained at CIEM were utilized. The 20 selected SNPs included rs13476308, rs1347682, rs13477311, rs13477625, rs6234655, rs30716377, rs13479101, rs13479815, rs6313405, rs6208271, rs13481075, rs13481497, rs13481762, rs13482084, rs13482609, rs4217297, rs13482900, rs13483256, rs13483687, and rs13484117. These SNPs were verified using the Mouse Genome Informatics database. Primers for the Mass Extend multiplex assays were designed using MassARRAY Designer software version 4.0 (Agena Biosciences™). The SNP IDs and their corresponding extension primers are detailed in Table 1.

SNP detection

The genotype analysis was performed using iPLEX® Sample ID Plus Panel (Sequenom). The multiplex PCR of 20 SNPs, the following reaction mixture was prepared in a total volume of 5 µl per well in 96-well plates containing: 0.8 µl nuclease-free water, 0.5 µl 10X PCR buffer, 0.4 µl 25 mM $MgCl_2$, 0.1 µl 25 mM dNTP mix, 1.0 µl forward/reverse primer mix, 0.2 µl PCR enzyme and 2 µl DNA (5 – 25 ng/µl). PCR was carried out in a Bio-Rad T100 Thermal Cycler with the following conditions: initial denaturation at 95°C for 2 min followed by 45 cycles of 95°C for 30 sec, 56°C

for 30 sec, 72°C for 1 min and final step of 72°C for 5 min. The PCR products were treated with a cocktail of 1.53 µl nuclease-free water, 0.17 µl 10X SAP buffer and 0.3 µl shrimp alkaline phosphatase (1.7U/µl) (SAP) to remove excess dNTPs. The PCR cocktail was performed in the Bio-Rad T100 Thermal Cycler of 37°C for 40 min and 85°C for 5 min. The PCR products were then used as templates for the iPLEX® Pro extension reaction containing 0.62 µl nuclease-free water, 0.20 µl 10X iPLEX buffer plus, 0.20 µl 10X iPLEX termination mix, 0.94 µl extend primer mix (5 – 15 µM) and 0.04 µl iPLEX proenzyme (32 U/µl). The extension reaction cocktail was performed in the Bio-Rad T100 Thermal Cycler with the following conditions: initial denaturation at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, followed by 5 cycles of 52°C for 5 sec and 80°C for 5 sec and a final step at 72°C for 3 min. The samples were added with 29 µl nuclease-free water and then treated with clean resin. All plates were included a negative control (no template) to confirm that there was no contamination. All the final products were spotted onto SpectroCHIP® Array using the Nano dispenser RS1000 and then transferred into the MassARRAY Compact System. Genotyping analysis was performed using the MassARRAY® analyzer 4 with an autorun system, and results were reported using Typer software.

Data analysis

The separation of the mass detector records the relative time of flight from each extension product. The results from MassARRAY Typer 4.0 genotyping software were shown in cluster analyses with signal-to-noise ratios and peak heights in real time. The Typer software generates reports indicating whether SNPs are homozygous or heterozygous. The data from the SNP call was divided into 4 groups; conservative, moderate, aggressive genotyping and low probability calls (low signal-to-noise ratios). The genotype call shows no allele when no extended primers were detected at that locus.

Statistical analysis

Genetic variation at each SNP locus was categorized into three genotypes, depending on the alleles inherited from both parents. Hardy Weinberg Equilibrium (HWE) is described by the binomial relationship $(p + q)^2 = 1$, where p and q represent the frequencies of alternative alleles [17]. In this study, the quality of all nine SNPs was assessed using genetic parameters including HWE, the inbreeding coefficient within individuals relative to their subpopulation (F_{IS}), observed heterozygosity (*Ho*), and expected heterozygosity (*He*) in PLINK 1.9 [18 - 20]. Comparison between observed and expected genotype distributions was performed to identify potential genotyping errors. Genotyping quality control (QC) included calculation of per – SNP and per – sample call rates to assess data completeness. SNPs with call rates below 95% and samples with genotyping success below 95% were excluded by comparing genotype calls across technical duplicates to ensure reliability. Principal component analysis (PCA) was performed on the genotype matrix, treating each SNP as an individual variable. This algorithm summarizes the covariance structure among SNPs and generates a series of orthogonal principal components (PCs). Each PC represents a linear combination of SNP genotypes and captures a decreasing proportion of the overall genetic variance, the first PCs reflect the major axes of genetic differentiation among individuals. SNP loadings were examined to determine the relative contribution of each locus to the observed population structure. PCA scores for each individual were visualized in R to evaluate clustering patterns and potential substructure. All QC procedures, including filtering, summary statistics, and visualization of missingness patterns, were primarily conducted in PLINK 1.9, while additional data inspection, graphical summaries, and confirmation of QC metrices were performed in R 4.3.0.

RESULTS AND DISCUSSION

Genotyping analysis using MALDI-TOF MS

In total, 306 samples from three generations of Mlac: ICR mice were successfully genotyped in this study using the MassARRAY® system. A set of 20 SNP markers, distributed across autosomes and the X chromosome, was selected from the inbred mouse genetic monitoring database of the ICLAS Monitoring Center and applied to outbred mice genetic monitoring. DNA samples were purified, genotyped, and analyzed using the MassARRAY TYPER 4.0 genotyping software. The genotyping process achieved a high average call rate of 94–100%, with only 6% of samples being classified as “no call,” indicating excellent assay performance.

The PCR extension products for the 20 SNPs were analyzed and categorized using TYPER 4.0 software, which generated outputs for various genotype classifications, including conservative, moderate, aggressive, low-probability, and no alleles. The intensities plotted against the mass signals are shown in Fig. 1. The data displayed clear and distinct peak

Table 1. PCR primers and extension primers of SNP marker using MassARRAY Design Software.

SNP ID	Chromosome	Forward primer (5' → 3')	Reverse primer (5' → 3')	UEP sequence
rs13476308	1	ACGTTGGATGCGCTGG-GTGCTCAATTCTA	ACGTTGGATGAGTCTA-CACTGATCCCCAAG	CCCAAGTAAGGATCTG
rs13476824	2	ACGTTGGATGGTG-CATTTATCTTGGTAGGG	ACGTTGGATGAAAA-CAGGCATTTGTGTGG	AAATTTAGACACTGG-TAGT
rs13477311	3	ACGTTGGATGGGCAT-GATGCGATGTTTGTG	ACGTTGGATGGGGCA-CAGTTAGAAAACAGC	ACAGTTAGAAAA-CAGCTCAATG
rs13477625	4	ACGTTGGATGTTGCT-CACTCAAAACCAGCC	ACGTTGGATGCCATGGT-GCAAGAAAAGAGC	CAGCCAAATTTCT-GTCTTCTTTCC
rs6234655	5	ACGTTGGATGTTTCA-CAGTCACACCTCTGC	ACGTTGGATGCATG-GATTGCAACTTGCCTC	TACACATACTCACTTC
rs30716377	6	ACGTTGGATGGGGAAG-GATATATTGAGCTG	ACGTTGGATGCAGCTCT-GCTATCAATGCC	CAATGCCCTCCTCA
rs13479101	7	ACGTTGGATGGACAAG-GCCACTTTTGAAG	ACGTTGGATGTCAACGT-GACCTTGACTTGC	ACTTTTGAAGAGTG-CAAACCGCCAGGCAG
rs13479815	8	ACGTTGGATGCATACAG-GCCTTTACTCAAC	ACGTTGGATG-GCTTGTTGCTTGTTTA-ATGC	CTTTACTCAA-CAATATCTTT
rs6313405	9	ACGTTGGATGTCCAT-TCTAGGTATGGTGAC	ACGTTGGATGTCATC-CCAGCTCTCCTTTAC	ATGGTGACCACATAATG-CATTATC
rs6208271	10	ACGTTGGATG-TAGCTTGTCCCCTCAGT-TAG	ACGTTGGATG-TACTTCATGTGGGAAG-ACAG	GCAGATGATAATAGGTAG
rs13481075	11	ACGTTGGATGGGTG-TAGTGGTTTTTGGAGG	ACGTTGGATGGAAA-GAGAAACCATCTCGGC	CGATACCTCTCCCAAA-GAGAACAC
rs13481497	12	ACGTTGGATGGACCTT-GAAGGCTCATAGTG	ACGTTGGATGCTGT-TATAGTACCAACAAGG	AAGAGTTAATGAAA-CAAAAGG
rs13481762	13	ACGTTGGATGAGT-TAGACCTGTGTAGGGAC	ACGTTGGATGTAATCT-CACTCTGGACTGGG	ATATATGCT-CACTTTCAACCAA
rs13482084	14	ACGTTGGATGTT-GAAACTAGCAAGTAG-CCG	ACGTTGGATGGAAGTTA-AAGAAACAGGAG	ACAGGAGTTGACAT
rs13482609	15	ACGTTGGATGGAAGAA-CAAGTGAGACGATG	ACGTTGGATGGTCAGCT-CACCTTTCAGTAG	ACGATGGTTTGTAATA
rs4217297	16	ACGTTGGATGGATA-AAAGCTGGAGTGACCG	ACGTTGGATGGACAATC-CGGCATACAGAG	CCAGTACCCTTTAGAA-CA
rs13482900	17	ACGTTGGATGTG-GCTTTTTGGTTGGTTCC	ACGTTGGATGCCAATCT-GTGGCAATAATAG	GTGATTAGGAAAACACT-TACAAAAA
rs13483256	18	ACGTTGGATGCACG-CAGCTATCATTCTTTG	ACGTTGGATGGACAGT-CAGAGTGTGGAAAG	CAAGACTCTG-CACTTGTTTCCAAGATGC
rs13483687	19	ACGTTGGATGGAAGT-GTCTGTCTCAACGTC	ACGTTGGATGCAGGGA-CATACATTACCCAC	ACGTCAGCGG-CAACTTCAGAA
rs13483842	X	ACGTTGGAT-GTTTCAGAAT-GTTCCTTGCC	ACGTTGGATGGCCA-GAGAAGAAGCAATAGG	TTCCTTGCCTTTTAAT-GAACTAGTAT

Table 2. Genotype frequencies for Mlac:ICR population (N = 306).

SNP ID	Chromosome	Allele	Genotype frequency (%)		
rs13476308	1	A/G	AA	AG	GG
			-	-	100
rs13476824	2	C/T	CC	CT	TT
			19.3	46.4	34.3
rs13477311	3	A/G	AA	AG	GG
			47	42.8	10.2
rs13477625	4	A/G	AA	AG	GG
			-	-	100
rs6234655	5	C/T	CC	CT	TT
			-	0.3	99.7
rs30716377	6	C/G	CC	CG	GG
			100	-	-
rs13479101	7	A/G	AA	AG	GG
			100	-	-
rs13479815	8	C/T	CC	CT	TT
			100	-	-
rs6313405	9	C/G	CC	CG	GG
			69.6	26.1	4.3
rs6208271	10	A/C	AA	AC	CC
		C/T	-	-	100
rs13481075	11		CC	CT	TT
		A/T	-	-	100
rs13481497	12		AA	AT	TT
		G/T	-	-	100
rs13481762	13		GG	GT	TT
		C/A	-	-	100
rs13482084	14		CC	CA	AA
		C/T	66.5	21.4	12.1
rs13482609	15		CC	CT	TT
		A/G	100	-	-
rs4217297	16		AA	AG	GG
		A/C	87.8	11.3	0.9
rs13482900	17		AA	AC	CC
		A/G	1.0	19.7	79.3
rs13483256	18		AA	AG	GG
		C/G	26.8	50.7	22.5
rs13483687	19		CC	CG	GG
		C/T	100	-	-
rs13483842	X		CC	CT	TT
			80.8	8.25	11

heights with strong signal-to-noise ratios, demonstrating the accuracy and reliability of the SNP detection process. Additionally, the genotyping data provided insights into allele frequencies and heterozygosity levels across the three generations of Mlac: ICR mice. The results demonstrated consistency in genetic variation, aligning with expectations for naturally bred, randomly mating outbred populations. These findings validate the utility of the selected SNP markers and the MassARRAY® platform for comprehensive genetic monitoring in outbred mice populations.

Table 3. Association analyses of the variants in heterozygosity population. [*Ho* (observed heterozygosity), *He* (expected heterozygosity), P_{HWE} (Hardy – Weinberg equilibrium test), and F_{IS} (inbreeding coefficient). The reported value of N/A indicates that the statistical calculation could not be performed.]

SNP ID	*Ho*	*He*	P_{HWE}	F_{IS}
rs13476824	0.46 ± 0.03	0.49 ± 0.03	0.35	0.24
rs13477311	0.43 ± 0.03	0.43 ± 0.03	0.90	0.25
rs6234655	0.01 ± 0.01	0.01 ± 0.01	1.00	1.00
rs6313405	0.26 ± 0.03	0.28 ± 0.03	0.10	0.28
rs13482084	0.21 ± 0.02	0.23 ± 0.02	0.20	0.42
rs4217297	0.11 ± 0.02	0.12 ± 0.02	0.13	0.37
rs13482900	0.20 ± 0.02	0.19 ± 0.02	1.00	0.32
rs13483256	0.51 ± 0.03	0.50 ± 0.03	0.82	0.23
rs13483842	0.12 ± 0.02	0.15 ± 0.02	0.06	N/A

Genotype frequencies

The SNPs including rs13476308, rs1347682, rs13477311, rs13477625, rs6234655, rs30716377, rs13479101, rs13479815, rs6313405, rs6208271, rs13481075, rs13481497, rs13481762, rs13482084, rs13482609, rs4217297, rs13482900, rs13483256, 13483687 and rs13484117 were genotyped in Mlac:ICR samples. Table 2 presents the allele and genotype frequencies for these SNPs. The SNPs rs13476308 and rs13477625, all genotypes across the three generations were homozygous GG genotypes. Similarly, rs1347901 consistently displayed a homozygous AA genotype. The rs30716377, rs13479815, rs13482609, rs13483867 and rs6208271 also showed homozygous CC genotype. The rs13481762, rs13481075 and rs13481497 were severally homozygous TT genotypes. In terms of heterozygosity, rs13476824 exhibited both TT and CC genotypes, resulting in a peak height ratio of CT. The rs13477311 displayed genotypes AA and GG, leading to a peak height ratio of GA. For rs13482084, the observed genotypes were AA and CC, with a peak height ratio of CA. Similarly, rs6313405 showed GG and CC genotypes with a peak height ratio of CG. The rs4217297 revealed AA and GG genotypes, resulting in a peak height ratio of GA. The genotypes for rs13482900 were AA and CC, corresponding to a peak height ratio of CA. The rs13483842 exhibited TT and CC genotypes, with a peak height ratio of CT. Lastly, rs13483256 showed AA and GG genotypes, leading to a peak height ratio of GA. The rs6234655 demonstrated a distribution of genotyping frequencies of 99.7% TT and 0.3% CT. Overall, the genotyping frequencies for rs13476308, rs13477625, rs30716377, rs13479101, rs13479815, r6208271, rs13481075, rs13481497, rs13481762, rs13482609, rs13473687 indicated a predominance of homozygous genotypes. Conversely, rs13476824, rs13477311, rs6234655, rs6313405, rs13482084, rs4217297, rs13482900, rs13483256 and rs13483842 exhibited heterozygosity within the studies population. The samples were grouped based on genotyping frequencies, and the results were analyzed across different generations for each SNP. The results are illustrated in Fig 2.

Statistical analysis

A total of nine SNPs and 306 samples were subjected to genotyping quality control. All SNPs and samples exhibited a 100% call rate, and therefore, no markers or individuals were excluded. The retained dataset provided a complete genotyping profile. Replicate concordance among technical duplicates ranged from 0–1, indicating high reliability and internal consistency of the genotyping data. PCA based on PC1 through PC3 demonstrated that samples were broadly dispersed across all three axes, indicating notable genetic variability within the dataset. The first three PCs explained 18.28%, 16.39% and 13.43% of the total genotypic variance, respectively. PCA plots showed a relatively scattered sample distribution, with most samples forming a diffuse cluster (Fig 3). A few samples fell outside this main cluster, representing outlying genotypic profiles. These patterns reflect underlying variability within the dataset rather than well – defined population substructure. Genetic parameters calculated from the SNP markers were summarized in Table 3. All nine SNPs exhibited $P_{HWE} > 0.05$, indicating that observed genotype frequencies did not significantly deviate from Hardy – Weinberg equilibrium. The overall observed heterozygosity (*Ho*) was comparable to expected heterozygosity (*He*), resulting in an inbreeding

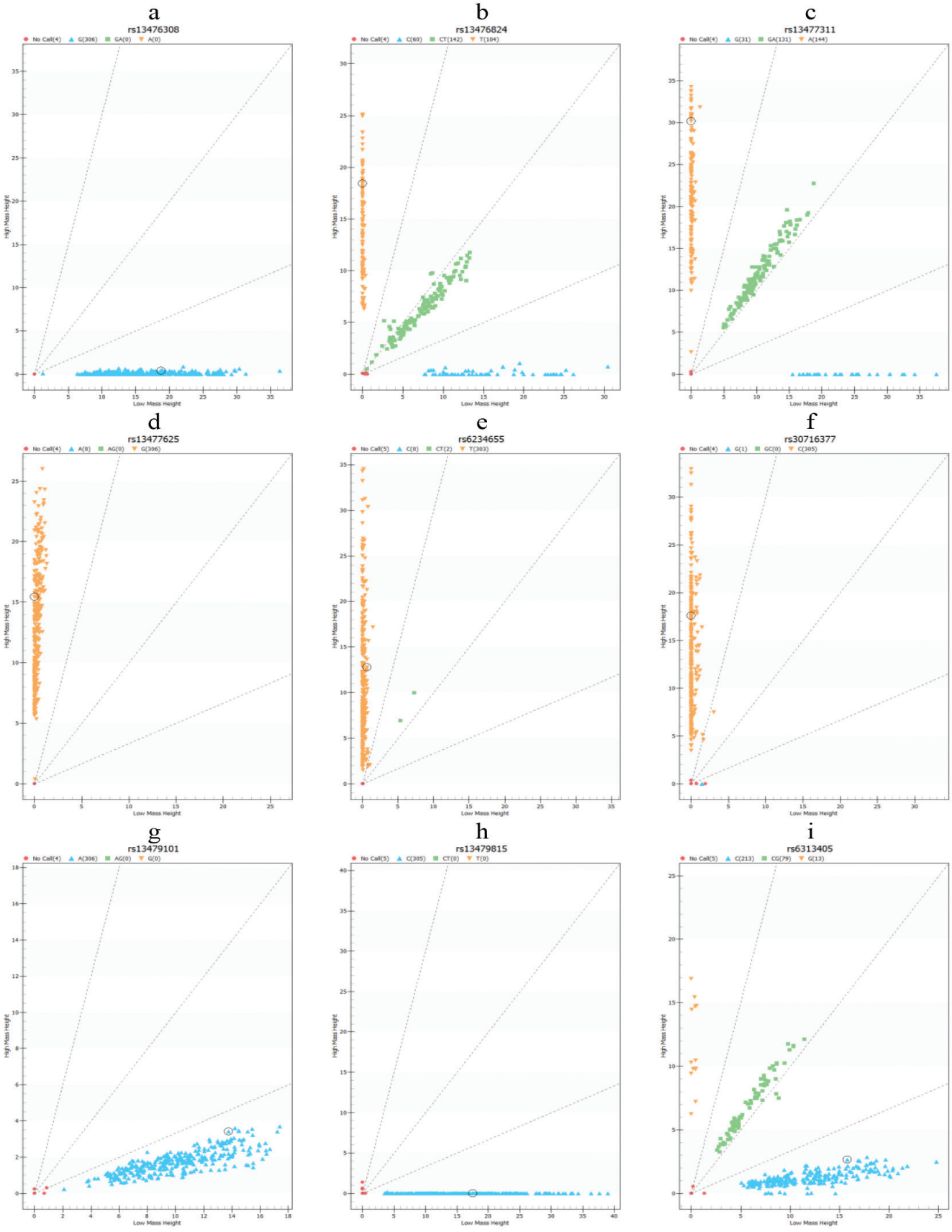

Fig. 1. Distribution of genotype calls from TYPER 4.0 software analysis. a – t show difference SNP detection. [Red spot represents No alleles, orange and blue spot represent homozygous allele and green spot represents heterozygous alleles.]

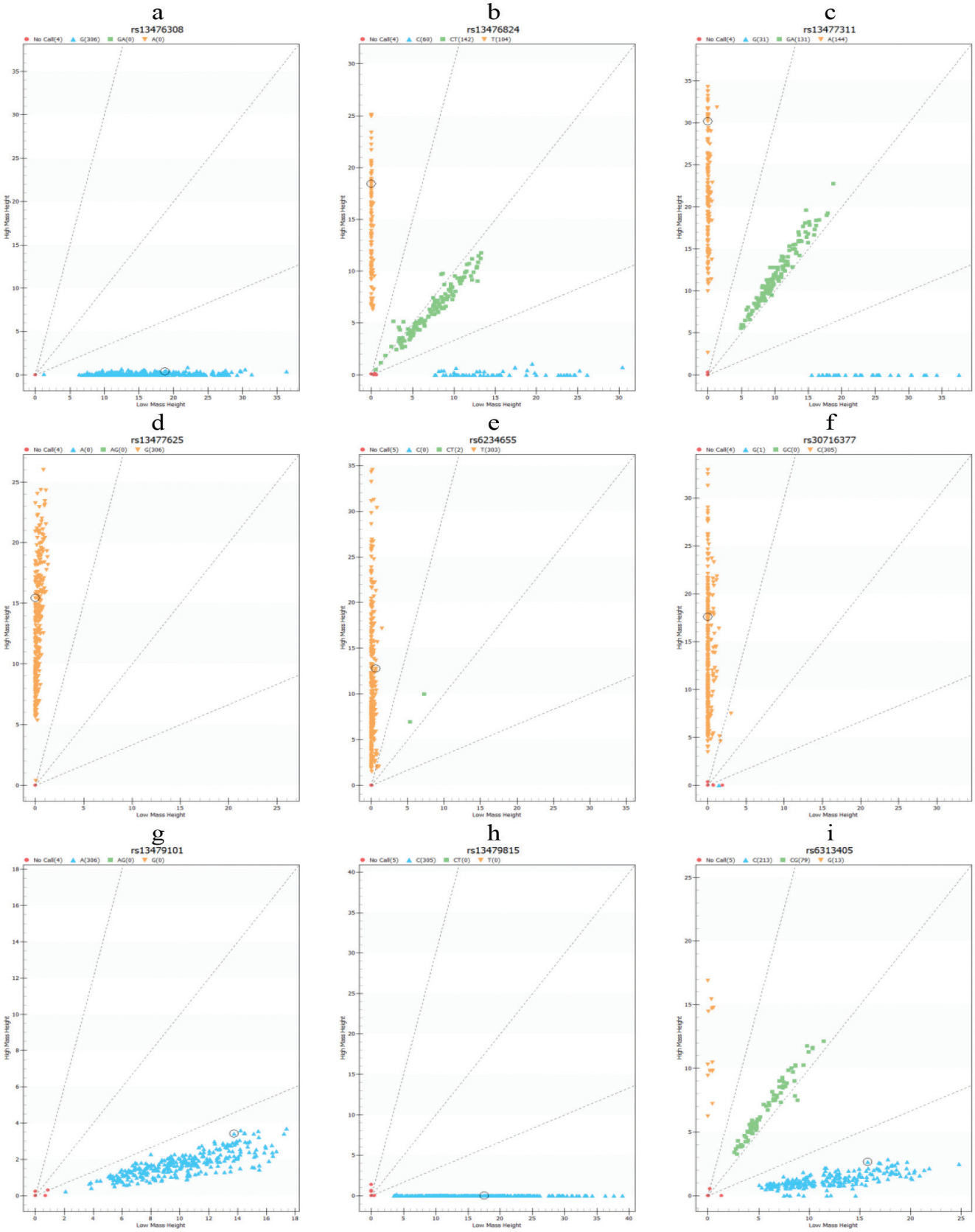

Fig. 1. (continued) Distribution of genotype calls from TYPER 4.0 software analysis. a – t show difference SNP detection. [Red spot represents No alleles, orange and blue spot represent homozygous allele and green spot represents heterozygous alleles.]

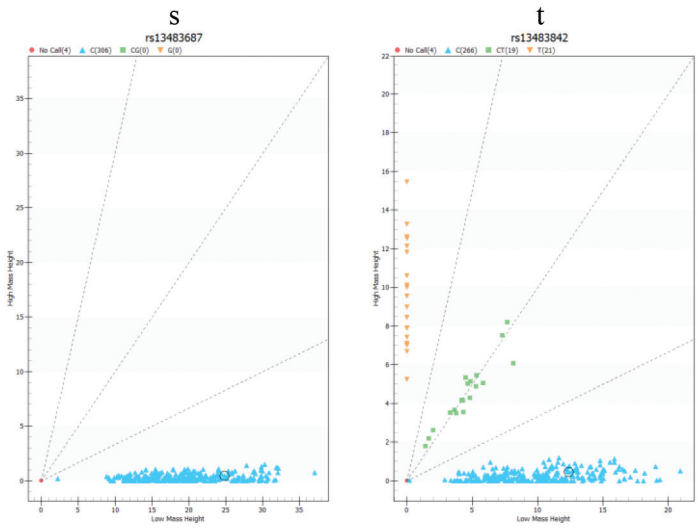

Fig. 1. (continued) Distribution of genotype calls from TYPER 4.0 software analysis. a – t show difference SNP detection. [Red spot represents No alleles, orange and blue spot represent homozygous allele and green spot represents heterozygous alleles.]

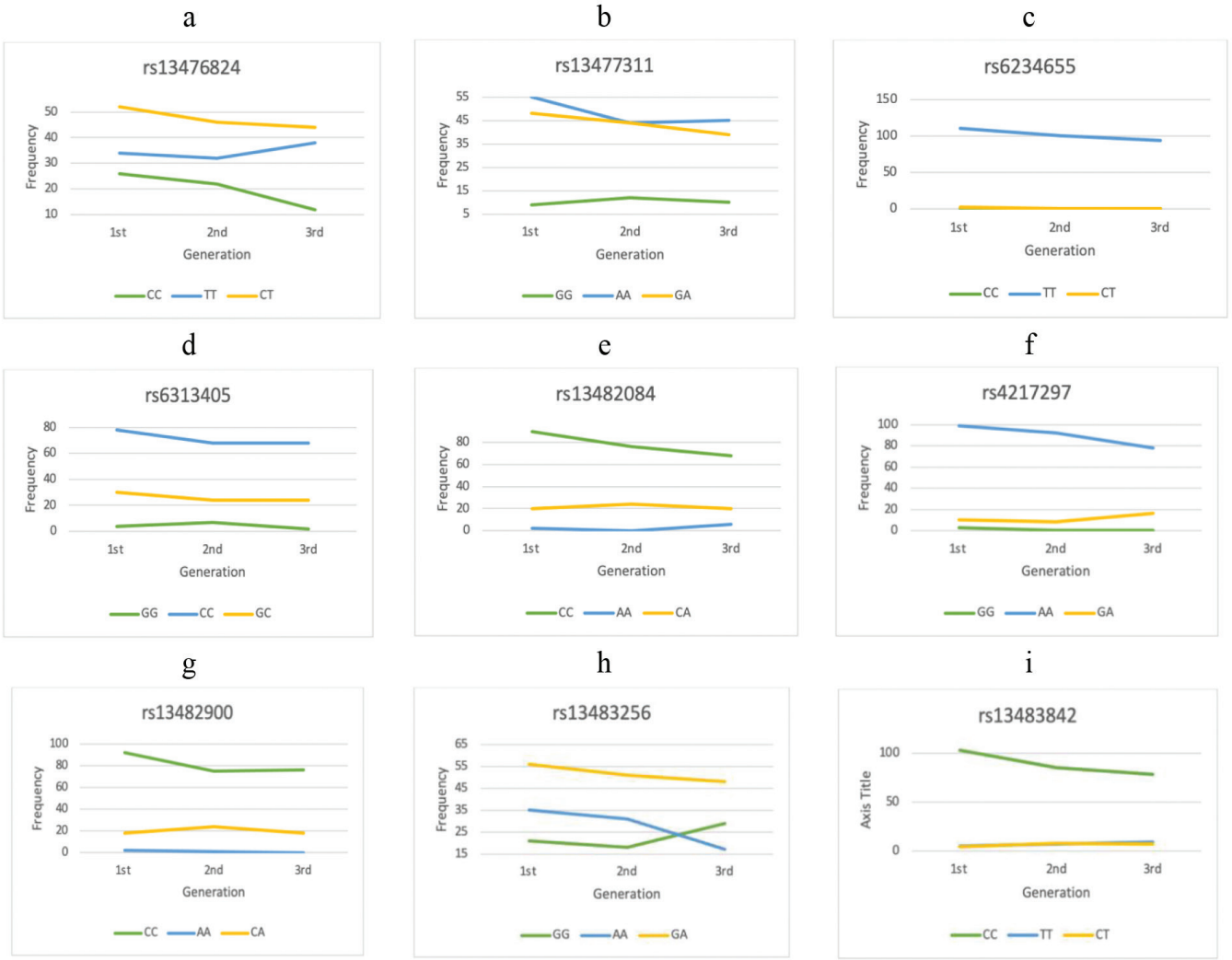

Fig. 2. Frequency of population between 1st, 2nd and 3rd generation in different genotypes of SNPs studies. a – i show difference SNP detection.

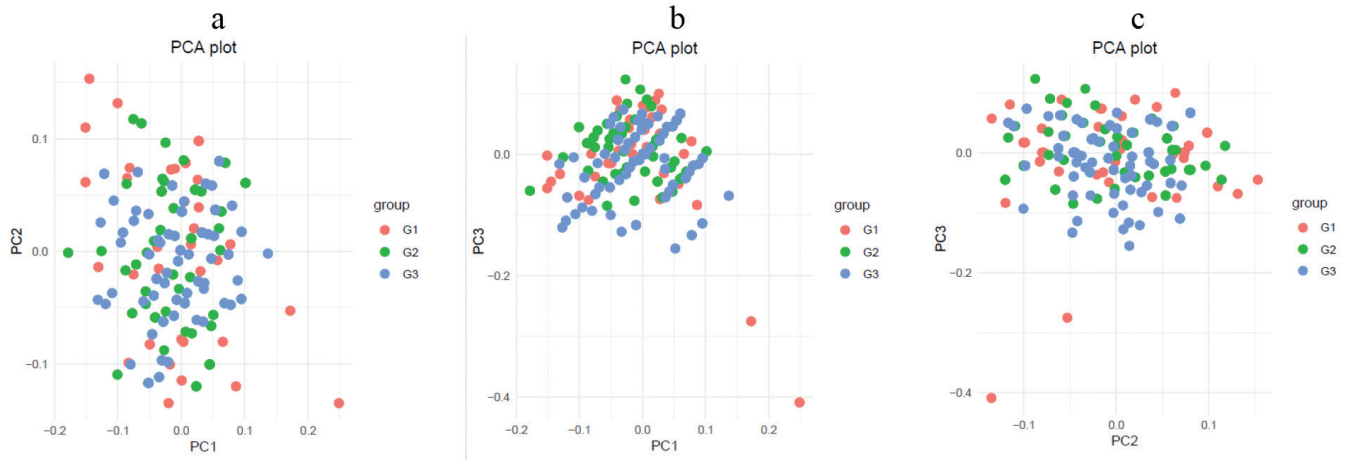

Fig. 3. Pairwise PCA scatter plots showing sample distribution along PC1 (18.28%), PC2 (16.39%) and PC3 (13.43%). a – c show PC1 vs PC2, PC1 vs PC3 and PC2 vs PC3 respectively.

coefficient (F_{IS}) of positive values for all loci. This positive F_{IS} value indicates a lower of heterozygotes in the population, suggesting high levels of inbreeding in population.

CONCLUSION

The study successfully applied a Single Nucleotide Polymorphism (SNP) – based approach to characterize the genetic background of ICR outbred mice maintained at the National Laboratory Animal Center, Mahidol University (Mlac:ICR). By utilizing twenty SNP markers distributed across all autosome and the X chromosome, the genetic quality and population structure of the Mlac:ICR stock were systematically evaluated across three generations. Genotyping was performed using the Sequenom MassARRRAY® platform integrated with matrix–assisted laser desorption/ionization time–of–flight mass spectrometry (MALDI–TOF MS), a robust and high–throughput technology that demonstrated exceptional accuracy, sensitivity, and efficiency, achieving genotyping call rates of 94–100%. Of the twenty analyzed SNPs, nine markers exhibited heterozygous genotypes, providing informative loci for assessing genetic variation. Statistical analyses confirmed that all nine loci were in Hardy – Weinberg equilibrium ($P_{HWE} > 0.05$), indicating the absence of genotyping bias, population stratification, or selection pressure. Principal component analysis (PCA) further supported these findings, revealing a coherent yet dispersed clustering pattern that reflected natural genetic variability without strong sub - structuring within the population.

Although the dataset exhibited a slight excess of homozygosity, as indicated by positive inbreeding coefficient (F_{IS}) values, this trend was not statistically significant and may reflect early signals of genetic drift or limited breeding diversity. The results emphasize the importance of ongoing genetic monitoring to preserve heterozygosity and maintain the genetic integrity of outbred colonies such as Mlac:ICR. The combination of multiplex PCR with MALDI – TOF MS genotyping proved to be a highly effective strategy for detecting genetic impurity, monitoring populations structure, and ensuring the authenticity of laboratory mouse stocks. In conclusion, the selected panel of polymorphic SNPs and the MassARRAY® genotyping system together establish a powerful and reproducible framework for routine genetic quality control in outbred mice. These findings provide a strong foundation for refining genetic monitoring strategies, enhancing colony management practices, and supporting the sustainable production of genetically diverse and reliable animal models for biomedical research.

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