RESEARCH ARTICLE Agricultural Science Digest, Volume 42 Issue 3: 358-364 (June 2022)

Multiplexing: An Efficient Way of Genetic Monitoring of Laboratory Mice using Microsatellite Markers

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10.18805/ag.D-5470

ABSTRACT

Background: Inbred strains of laboratory mice have been widely used in biomedical research. An inbred strain is the one which has been maintained by sibling mating for twenty or more consecutive generations. Except for the sex difference, an inbred strain is homozygous at all loci. Most of the traits are consistent, do not vary in offspring and respond to experimental procedures uniformly. This enables researchers to produce reliable data for therapeutic purposes, hence inbred strain must be genetically pure over the generation.

Methods: A total of 20 mice samples from 10 inbred strains *i.e.*, A/J, BALB/c, C3H/J, CD1, C57BL/6, DBA/2, FVB/NJ, ICRC, Swiss Webster and Swiss/Ba were screened for their genetic purity with the help microsatellite markers. Tail DNA was isolated using Proteinase/K-Phenol-chloroform extraction method and quantified by Nanodrop. DNA obtained was used for microsatellite marker analysis by standardizing multiplex-PCR. Seven multiplex (duplex) panels were successfully established by optimizing various reaction conditions to analyse any deviation in the genetic profile.

Result: Upon comparing genetic profiles obtained in multiplex PCR with the information available on Mouse Genome Informatics database, it was concluded that most of the inbred mice are genetically consistent as they showed zero divergence in observed base pair size mainly due to stringent breeding protocol and optimum living conditions. The work done also signifies a cost-effective, time saving, high throughput and robust method of genetic monitoring of laboratory mice strains.

Key words: Genetic monitoring, Mice, Microsatellite markers, Multiplex PCR.

INTRODUCTION

Animal models have been longstanding used to address diverse range of scientific questions ranging from basic to translational sciences. Animal models have played a crucial role in bridging the gap between human understanding of underlying mechanisms of clinically significant processes and discovery of therapeutic targets. Number of vital breakthrough discoveries in medical sciences have been made possible on account of experimentation on animal models. Most vaccines, which save millions of lives every year, have been successfully developed using animal models. Therapies for tissue regeneration using stem cells were engineered, tried and tested in animals (Han *et al*. 2020). Animal species serves as model to design and improvise surgical techniques, therapies and treatment prior to human application. The anatomical and physiological similarities between humans and rodents make them an excellent candidate to study the role of various metabolites in biochemical processes. Researchers interrogate the pathogenesis of disease in healthy and diseased states, but each level of investigation require a well characterized and controlled animal model for testing the hypothesis and validation of human data, which brings genetic quality assurance programme into the frame.

Strains of laboratory rodents are of two kinds, first kind comprises of genetically uniform population *i.e.*, Inbred strain and other kind is of genetically heterogenous population *i.e.*, random-bred and outbred population. Inbred strain is an artificial population representing a specific allele from an allelic pool. An inbred strain is maintained by continuous sibling mating for twenty or more consecutive generations. The main challenge in maintaining an inbred strain colony is the stable inheritance of allele in subsequent generations without any alteration. The factors that lead to genetic alteration of inbred strains are genetic drift, mutation and contamination by accidental outcross (Guenet and Benavides, 2010). These deviations may produce different set of conclusions amongst researchers working on similar objective. Hence, to produce reliable outcomes in scientific community, inbred strains must be genetically pure. Besides maintaining the genetic purity of the laboratory rodents,

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How to cite this article: Naseem, A., Ahire, S. and Ingle, A. (2022). Multiplexing: An Efficient Way of Genetic Monitoring of Laboratory Mice using Microsatellite Markers. Agricultural Science Digest. 42(3): 358-364. DOI: 10.18805/ag.D-5470.

Submitted: 04-08-2021 **Accepted:** 24-02-2022 **Online:** 30-03-2022

Facility Managers also struggle to keep the Facility running, no matter there is a shortage of feed, bedding, staff or the extreme weather conditions or the unusual pandemic. It needs years of experience to develop logistics of the manpower, supply and maintenance of critical strains (Thorat and Ingle, 2021). It is also enviable to maintain the baseline data of production efficient, litter size, birth weight, weaning weight, as well as haematological and biochemical baseline data of the strains maintained as a reference to monitor the breeding performance of a given strain (Thorat and Ingle, 2020).

Genetic monitoring of inbred strains and background characterization of genetically altered animal models are an essential component of Genetic Quality Assurance programmes run in any Animal Facility (Benavides *et al*. 2020). Quality control program carried out in animal facilities play a crucial role in assessing the purity of inbred strains. Genetic monitoring is the examination of genetic composition of laboratory rodents to scan for any deviation of allele in subsequent generations. Purity is checked at various loci, genes and molecular markers. It is essential to perform genetic monitoring at regular intervals to detect genetic contamination in resident colonies. Although genetic monitoring program helps the researchers to find out the contamination, all efforts must be to prevent the strains from contamination. This can be achieved by having a stringent breeding program in place, record keeping as well as maintaining stringent environmental condition. Cryopreservation of the important or all strains in use may also be one of the means to prevent loss of important strains as well as avoid the hassles of import of new strains (Thorat and Ingle, 2012; Thorat and Ingle, 2017). Using these cryopreserved embryos, it is very helpful to retrieve the lost strains (Thorat *et al*. 2013). Consistent monitoring constructs a unique genetic profile for each strain (Bryda and Riley, 2008) and assists in identifying plausible aberration in the genome of inbred animal colonies. Methods of genetic monitoring ranges from phenotype based assessments to DNA based techniques. Phenotype based methods takes into consideration the evaluation of external features, reproductive performances, skin grafting and protein analysis by PCR. DNA based techniques involves exploitation of genetic markers for monitoring purpose. Genetic marker is a DNA sequence with known location within a genome and is present ubiquitously in all living organisms (Abdul-Muneer, 2014). Any alteration in the sequence of nucleotides in genetic marker region can be detected using molecular techniques. Diverse types of polymorphism serves as genetic markers, including single nucleotide polymorphism (SNP's), simple sequence length polymorphism (SSLPs) and restriction fragment length polymorphism (RFLPs). SSLPs include variations termed as minisatellites (Variable number of tandem repeat or VNTR) and microsatellites (short tandem repeats or STRs) (Abdul-Muneer, 2014). DNA based molecular techniques provides more comprehensive and robust analysis of genetic markers and yields more conclusive results. These tools include RFLPs, amplification of microsatellite marker by PCR, Single-stranded conformation polymorphism analysis (SSCP) and detection of SNPs by DNA sequencing. Purity of the laboratory mice can also be checked by use of conventional methods (Ingle and Ahire, 2013).

In this study, we have undertaken microsatellite markers amplification by multiplex PCR. The term microsatellite was coined by Litt and Luty, 1989. Microsatellites are 1 to 6 nucleotide long repeats, inherited in Mendelian pattern, co-dominant and highly polymorphic (Abdul-Muneer, 2014). They are distributed many times in the genome depending upon the type of species. They are present within transcriptionally active and inactive regions of nuclear and organellar DNA but their role in non-coding regions have not been explored yet (Perez-Jimenez *et al*. 2013). The repetitive nature of these sequences leads to accumulation of mutation that are responsible for variations among individuals. These repeat sequences can be amplified and genotyped easily in a cost-effective manner which makes them an excellent candidate for screening of background strains and to undertake genetic monitoring programs.

The aim of this study is to generate profile of microsatellite markers using multiplex PCR in mice strains that could be used in a time saving and cost-effective way of genetic monitoring of laboratory animals.

MATERIALS AND METHODS

Sample collection and DNA isolation

Laboratory Animal Facility (LAF) of ACTREC (https://actrec.gov.in/index.php/cri-research-support-facility-detail/70) is a home for rodents that are maintained in polysulfone make IVC cages under 12-hour dark and light cycles, with temperature (20-22°C), humidity (40-60%) and air pressure controlled to meet regulatory specifications using a heating, ventilation and air conditioning (HVAC) system. During the sample collection process of quality control schedule, randomly collected two mice from each strain of A/J, BALB/c, C3H/J, CD-1, C57BL/6, DBA/2, FVB/NJ, ICRC, Swiss Webster and Swiss/Ba were humanely sacrificed using CO_2 chambers in the month of February, 2021. Tail samples were aseptically collected in an Eppendorf tubes. Genomic DNA was aseptically isolated from these tail samples by Proteinase/K - Phenol-Chloroform method (Ghatak *et al*, 2013). The proposal for checking genetic integrity of laboratory rodents maintained at ACTREC Animal Facility was approved by the Institutional Animal Ethics Committee (IAEC) of ACTREC, Navi Mumbai which is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

DNA integrity and quantitation

Each isolated DNA sample (5 µl) was mixed with 1 µl of 6X loading dye and loaded on Agarose gel (1%) for DNA

integrity. Quantitation was done using Nanodrop and samples were further diluted to 100 ng/µl.

Microsatellite marker

Oligonucleotide primers for microsatellite marker were chosen, which were located throughout the mouse genome. These markers were selected from the Mouse Genome Informatics Database (MGI) http://www.informatics.jax.org/marker. In the critical subset, one marker from each of the chromosome was selected to cover the whole genome in such a way that it will have different product sizes of each primers permitting identification from the other strain maintained at the LAF. These primers were obtained purified from the Merck Life Science Pvt. Ltd., Bangalore, India. Table 1 enlists the primer name, their chromosomal location and the primer sequences.

Multiplex PCR

DNA samples (1 µl = 100 ng) was taken in PCR tubes. Master mix was made using 2X Taqmix, Nuclease free water and

Table 1: List of microsatellite markers along with their chromosomal location and primer sequences.

Marker	Chromosome nos.	Primer sequences
D1Mit3	1	F- TTTTTGTTTTCTTTTCTTTTCCC
		R- CCCTCTTCTGGTTTCCACAT
D2Mit30	2	F- CATCCAAGCAGTAACGTAGACG
		R- AAATGTTACACCCTCTGCGG
D3Mit18	3	F- GAACAGTTCCCAGGTCCTCA
		R- CTGCCTTTAAATTCTGTCACCC
D4Mit15	4	F- AGGAATACTGAATGTGGACTTTCC
		R- TCCCTTGATTAACAGAAGACCTG
D5Mit1	5	F- AATAAAGCTGTGAGGTAAACCCC
		R- GAAACAAATGATTGTTTTGAGCC
D6Mit8	6	F- TGCACAGCAGCTCATTCTCT
		R- GGAAGGAAGGAGTGGGGTAG
D7Nds1	7	F- GAGATCTTCCATACTCATATT
		R- TAGATAGTGTTAACAGTGACC
D8Mit4	8	F- CCAACTCATCCCCAAAGGTA
		R- GTATGTTCAAGGCTGGGCAT
D9Mit11	9	F- GCCTTCATGTGTACCTGAATGCAC
		R- GGCTCTGTAATCACTGAAGCTGGT
D10Mit3	10	F- GTTGATAGTCCCACCTCACTCA
		R- TGAGAAATTCCATCTGTGGC
D11Mit5	11	F- TTCTGTGAGCCTGGAGGAGT
		R- TACAGGACTAGTTTCCATTTGGG
D12Mit5	12	F- CACATAGACCAGACAGGCATGCGT
		R- CAAGGTCACGTTGCTAGCTAGGAA
D13Mit9	13	F- GGGTTCCAGATTGAGTGGAA
		R- TTGCCAAAGTGTCAAAATCA
D14Mit5	14	F- CACATGAACAGAGGGGCAG
		R- GTCATGAAGTGCCCACCTTT
D15Mit12	15	F- ATGGACACCTGACACTGCAA
		R- AAGGGCTTTTACCTGGGAAT
D16Mit 5	16	F- CGGGGATCATCCCTAAAAAC
		R- TCCCCAATTCCTCTTGTGTC
D17Mit11	17	F- TGAATTTATGAGGGGGGTCA
		R- TGTCCCATATCTCTCTTTATACACA
D18Mit40	18	F- GGTAGGAGTCACTTTCCGTCC
		R- TTTTGTGAGCATTTTTATACCATT
D19Mit1	19	F- AATCCTTGTTCACTCTATCAAGGC
		R- AATCCTTGTTCACTCTATCAAGGC
DXMit16	X chromosome	F- CTGCAATGCCTGCTGTTTTA
		R- CCGGAGTACAAAGGGAGTCA

*Source: Mouse genome informatics database (MGI). http://www.informatics.jax.org/marker.

two set of different primers (forward and reverse primer). 12 µl of master mix was added to PCR tubes to make up the volume to 13 µl. PCR tubes containing reaction mixture was placed in the wells of thermal cycler ensuring absence of oxygen bubbles in the tubes. Program used for setting up the PCR reaction is given in Table 2. The product was run on 2% Agarose gel and visualised in Gel Doc system.

RESULTS AND DISCUSSION

After the animals are sacrificed, tail samples were harvested in cryo-tubes. DNA isolation of tail samples was performed by Proteinase/K -Phenol Chloroform extraction method and quantitation of DNA was done using Nanodrop system. The DNA samples of 10 inbred mice strains were genotyped by multiplex PCR to check if these strains were pure for an allele within the microsatellite region. Products amplified by multiplex PCR were run on Agarose gel electrophoresis and visualized under UV exposure. Size of bands amplified by oligonucleotide primer were manually recorded. Fig 1 displays the results from the amplification of seven primer pairs. Two sets of primers were used in each panel for 20 DNA samples of ten inbred strains. Table 3 shows the products sizes of the microsatellite markers of inbred strains used in this study.

Various combinations of markers were run together to standardise the multiplex. Accordingly, we could standardise 7 possible combinations of 2 markers in each reaction. Microsatellite marker no. D1Mit3 was run in combination with D10Mit3 which yielded the expected product sizes in case of all strains. Microsatellite marker no. D2Mit30 was run in combination with D8Mit4 which yielded the expected product sizes in case of all strains for both the markers except for C57BL/6 strain where it generated product size of 380 bp for D2Mit30 instead of standard 320 bp and product size of 195 bp for D8Mit4 instead of 157 bp. Microsatellite marker no. D3Mit18 was run in combination with D19Mit1 which yielded the expected product sizes in case of all strains. Microsatellite marker no. D5Mit1 was run in combination with D11Mit5 which yielded the expected product sizes in case of all strains. Microsatellite marker

Table 2: General PCR conditions used for all reactions.

Cycle no.	Process	Temperature	Time
Cycle 1	Initial denaturation	94°C	5 mins.
Cycle 2	Denaturation	94°C	20 sec.
Cycle 3	Annealing	Variable for different sets	30 sec.
Cycle 4	Extension	72°C	20 sec.
	Back to cycle 2 × 35		
Cycle 5	Final extension	72°C	3 mins.
Cycle 6	Hold	4°C	Infinite time

Table 3: Product sizes of the microsatellite markers used in this study.

Strains →	A/J	BALB/c	C3H/J	CD-1	C57BL/6(J)	DBA/2	FVB/NJ	ICRC	Swiss	Swiss/Ba
Marker ↓	Product sizes (bp)									
D1Mit3	185	185	185	185	160	160	185	160	185	185
D2Mit30	136	136	136	120	380	136	120	136	136	136
D3Mit18	212	212	212	189	231	231	212	212	212	231
D4Mit15	329	329	329	279	279	279	279	279	279	279
D5Mit1	135	135	135	135	135	127	135	127	135	127
D6Mit8	190	190	164	190	164	164	190	190	190	190
D7Nds1	260	270	265	265	238	260	260	270	260	260
D8Mit4	200	200	195	200	195	195	200	170	195	195
D9Mit11	116	116	116	116	74	104	104	104	104	137
D10Mit3	245	245	215	245	245	215	215	245	215	245
D11Mit5	212	186	186	212	219	187	187	187	187	219
D12Mit5	160	160	160	176	176	160	176	176	176	160
D13Mit9	126	126	145	145	126	145	126	126	126	126
D14Mit5	178	178	164	178	178	164	178	164	178	164
D15Mit12	190	190	190	190	190	190	190	190	190	190
D16Mit5	132	132	158	158	156	132	158	132	158	158
D17Mit11	160	150	176	150	176	150	176	176	176	176
D18Mit40	132	132	132	136	142	142	142	142	142	132
D19Mit1	142	142	142	142	121	142	142	121	142	142
DXMit1	84	84	84	84	97	84	84	84	84	84

no. D6Mit8 was run in combination with D13Mit9 which yielded the expected product sizes in case of all strains. Microsatellite marker no. D9Mit1 was run in combination with D15Mit12 which yielded expected product sizes in case of D9Mit11 for all strains but did not yield expected product sizes in case of A/J, BALB/c, C3H/J, C57BL/6 and DBA/2 strains for D15Mit12. In case of D15Mit12, it yielded 190 bp band in A/J instead of 150, 190 bp band in BALB/c instead of 150, 190 bp band in C3H/J instead of 150, 190 bp band in C57BL/6 instead of 150 and 190bp band in DBA/2 mice instead of 160 bp. Microsatellite marker no. D12Mit18 was run in combination with DXMit1 which yielded the expected product sizes in case of all mice strains.

For some of the strains such as ICRC and Swiss/Ba which are originated from Swiss mice in the past at our institute, the results obtained are considered as standard for them. We observed the differences in D3Mit18, D5Mit1, D9Mit11, D10Mit3, D11Mit5, D12Mit5, D14Mit5 and D18Mit40 for Swiss/Ba mice when compared to the original strain of Swiss mice. In case of CD1, FVB/NJ, ICRC, Swiss Webster and Swiss/Ba mice, since the product sizes are not available, we have considered them as observed in our case.

The use of animals in biomedical research field puts up variety of concern, ethical aspects of experimenting on live animals, cost of experiments and the type of facility provided in maintaining the animals. Animals such as drosophila melanogaster, spider, jelly fish, squids, dogs, monkeys, apes and rodents are very commonly exploited in research. Amongst all, rodents such as mice and rats provide an advantage over other animals as they have shorter maturation period, greater number of offspring, easy to maintain and genetically closer to humans.

Analysis of microsatellite markers is done conventionally by using single primer and then data analysis

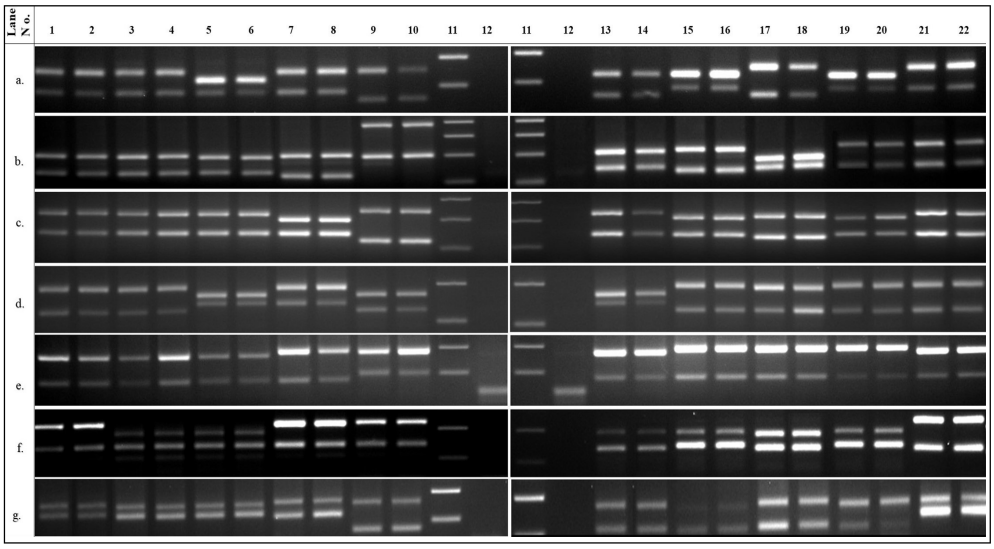

Fig 1: Multiplex primers used in panel (a) D1Mit3 and D10Mit3; (b) D2Mit30 and D8Mit4; (c) D3Mit18 and D19Mit1; (d) D6Mit8 and D13Mit9; (e) D12Mit5 and DXMit1; (f) D5Mit1 and D11Mit5; (g) D9Mit11 and D15Mit12.
Strain in Lane 1 and 2- A/J; 3 and 4- BALB/c; 5 and 6- C3H/J; 7 and 8- CD-1; 9 and 10- C57BL/6; 11- 100 bp Ladder; 12- Negative control; 13 and 14- DBA/2; 15 and 16- FVB/NJ; 17 and 18- ICRC; 19 and 20- Swiss Webster; 21 and 22- Swiss/Ba strain of mice.

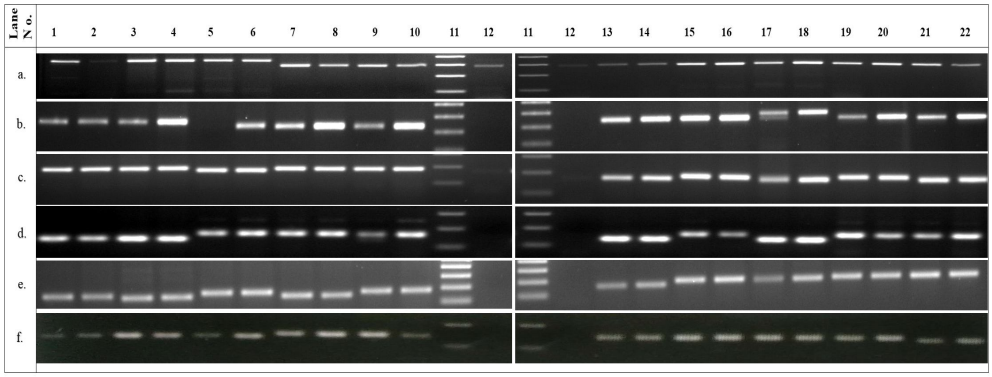

Fig 2: Single primers used in panel (a) D4Mit15; (b) D7Nds1; (c) D14Mit5; (d) D16Mit5; and (e) D17Mit11 (f) D18Mit40.
Strain in Lane 1 and 2- A/J; 3 and 4- BALB/c; 5 and 6- C3H/J; 7 and 8- CD-1; 9 and 10- C57BL/6; 11- 100 bp Ladder; 12- Negative control; 13 and 14- DBA/2; 15 and 16- FVB/NJ; 17 and 18- ICRC; 19 and 20- Swiss Webster; 21 and 22-Swiss/Ba strain of mice.

but this method is time and reagents consuming. Hence, our blueprint was to combine multiple markers in each reaction and thereby reducing the number of reaction and the reagents used. The efficiency and specificity of each set of primer were tested by optimizing the annealing temperature. Non-specific bands were cleared by increasing the annealing temperature in a graded manner to get final results. Six primers did not produce proper results when run in duplex. However, they yielded expected product sizes when run singly as shown in Fig 2.

Analysis revealed consistent genotype at loci D1Mit3, D3Mit18, D4Mit15, D5Mit1, D6Mit8, D7Nds1, D9Mit11, D10Mit3, D11Mit5, D12Mit5, D13Mit9, D14Mit5, D16Mit5, D17Mit11, D18Mit40, D19Mit1 and DXMit1, depicting homogeneity of inbred strains. At the same time, we also noticed few genetic differences at loci D2Mit30 and D8Mit4 for C57BL/6 strain and differences for D15Mit12 in A/J, BALB/c, C3H/J, C57BL/6 as well as DBA/2 strains. In case of other strains like CD1, FVB/NJ, ICRC, Swiss Webster and Swiss/Ba, we considered the observed product sizes as standard bands for them as there are no reported results in the literature. Primers D2Mit30 and D8Mit4 were coupled together to perform duplex PCR. Results of all strains were consistent when compared with the literature except for C57BL/6J. The expected product size of C57BL/6J for primers combination D2Mit30:D8Mit4 was 320:157 bp, but the observed product size was 380:195. We did not get the expected product sizes for D15Mit12 marker for A/J, BALB/c, C3H/J, C57BL/6 as well as DBA/2 strains. These three markers were repeated several times even with increased annealing temperature or modulating the enzyme and primer concentrations to check if there is any non-specific bands. However, they consistently yielded similar product sizes. At extreme temperature, the product sizes disappeared indicating that the results are true results. In the past, communication with the Jackson Laboratory, USA have confirmed that it is likely that the Mouse Genome Informatics (MGI) have not fully characterized all the markers and hence we may get the consistent deviated results for some of the markers. In this situation, it is construed that if we get consistently similar deviated results, we may consider them as true product sizes for these markers and may be replaced with the reported product size in the future. However, in such cases one need to be careful for the setting up of fool proof PCR reaction to confirm the consistent deviated product sizes. Development of non-specific product sizes of the unexpected bands may be the possibility when appropriate temperature, enzyme and DNA templates are not used in the reaction. Reports from other laboratories who are also running these three markers may support the finding in this study. Furthermore, sequencing of the product size must be the confirmatory test to ascertain the strain/ species specificity.

CONCLUSION

Upon comparing genetic profiles obtained in multiplex PCR with the information available on Mouse Genome Informatics (MGI) database, it was concluded that the most of the inbred animals are genetically consistent as they showed zero divergence in observed base pair size. Further it demonstrates that in-house animal models are being bred with stringent breeding protocol and maintained at optimum living conditions. However, the deviation in three microsatellite markers which may arise due to wrongly characterization of these markers in the database. The work done also signifies a cost-effective, time saving, high throughput and robust method of genetic monitoring of laboratory mice wherein, instead of using single marker in standard PCR reaction, we can simultaneously amplify two loci by adding two primers in the same reaction mixture followed by gel electrophoresis.

ACKNOWLEDGEMENT

The authors acknowledge the support of Dy. Director, CRI-ACTREC and Director, ACTREC for providing infrastructure support to undertake this study.

Conflict of interest

The authors declare no conflict of interest.

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