Sci. & Cult. 76 (7–8) 261-266 (2010)

Molecular Investigation in Wistar Rats by RAPD Typing

ABSTRACT : Sixty Wistar samples were used for developing applicability of twenty RAPD primers. A total of 1160 bands were scored. The percent polymorphism was 0.015 but this polymorphism was not statistically significant (P>0.05). Mean difference, Single Linkage Euclidean distances among ten groups (Wis1 to Wis10) were calculated. Our results suggest that the some primers may also be used as identifying markers with other polymorphic alleles and may satisfactorily be used for rapid and genetic characterization, evaluation and strain improvement by biotechnologists.

Key Words: Laboratory rats, Molecular characterization, Muridae, Phylogenetic Analysis, RAPD.

Wistar is the oldest laboratory rat strain (*Rattus norvegicus*), maintained at the Wistar Institute in Philadelphia, is the progenitor of most of the rat strains. The Wistar strain maintained since last century has not been typed genetically so far[1]. Rats are thought to have originated in the area of Asia currently occupied by southern Russia and northern China. *Rattus norvegicus* albino strain is commonly found in Europe. It is essential to characterize a breed for its conservation. Genetic variation between and within breed is described as diversity. Various stocks of Wistar strain are maintained in laboratories across the globe, are to be introduced in biomedical research and are proposed to be used extensively in various research applications like reproductive physiology, parasitology, microbiology, biochemistry, nutrition, pharmacology, toxicology, physiology, surgery and oncology[2].

The conventional methodology used for the determination of species origin has predominantly been based on immunochemical and electrophoretic analysis of protein. To maintain the genetic purity of these strains, as well as to avoid any accidental mix up with other strains, we decided to examine DNA fingerprinting of these strains so that a molecular signature, unique to them could be generated. Compared to the conventional method of DNA fingerprinting, which is quite elaborate and time consuming, PCR-based methods are easier to perform and analyze.

This popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated and applicability of the RAPD technique has captivated many scientists' interest. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of molecular characterization of the genome of the species in question. Nevertheless, RAPD markers can be used successfully as a routine method, being highly sensitive, rapid, simple and not expensive for species identification. Additionally, through the acquisition of sequence data, DNA can potentially provide more information than protein, due to the degeneracy of the genetic code and the presence of many non-coding regions[3]. RAPD markers have currently become the markers of choice for molecular characterization and identification. Therefore, in this study, twenty RAPD primers were tested to establish molecular characters and assist in rapid identification for examining DNA fingerprinting. These strains will provide a molecular signature unique to them to be used for more extensive studies on population genetics of this rodent group.

Materials and Methods : *Lineage Full :* Eukaryota; Metazoa; Bilateria; Deuterostomia; Chordata; Craniata; Vertebrata; Gnathostomata; Sarcopterygii, Amniota; Synapsida; Therapsida; Mammalia; Eutheria; Rodentia; Muroidea; Muridae; Murinae; *Rattus*; *norvegicus.*

Animals : Sixty Wistar rats (2n=42) aged 3 months were raised at animal facilities; both sexes were used for this experiment. These rats were collected from Indian Veterinary Research Institute, Izatnagar and used as tissue donors. The rats were housed in polypropylene cages at 24°±2°C with light-dark cycles of 12 h duration and 50±5% RH. The animals were fed 15 g pellet feed with millets and 1 g dry prawn, and water was provided *ad libitum* to the animals. Sterilized paddy husk was used as the bedding material and this was changed twice a week. The rats were randomly allocated into 10 groups; each group containing 6 rats was used for DNA extraction.

DNA extraction : Tail tissues were subjected to the isolation procedures according to protocol using phenol:chloroform extraction methods with 3M sodium acetate and Isopropanol. Air dried DNA pellet was rediluted with TE buffer (10 mM Tris-HCl and 1 mM EDTA, *p*H 8.0)

and incubated with RNase at a concentration of 100 µg/ml at 37°C for 1 hr. The concentration of the DNA was estimated spectrophotometrically at 260 nm using 100 µl of 1:100dilutions of DNA. Subsequently, quality of the DNA was checked by 1% agarose gel electrophoresis containing ethidium bromide and visualized on a UV transilluminator[4]. The power conditions were set to 50 volts and run at constant current for 2 hours at 20°C.

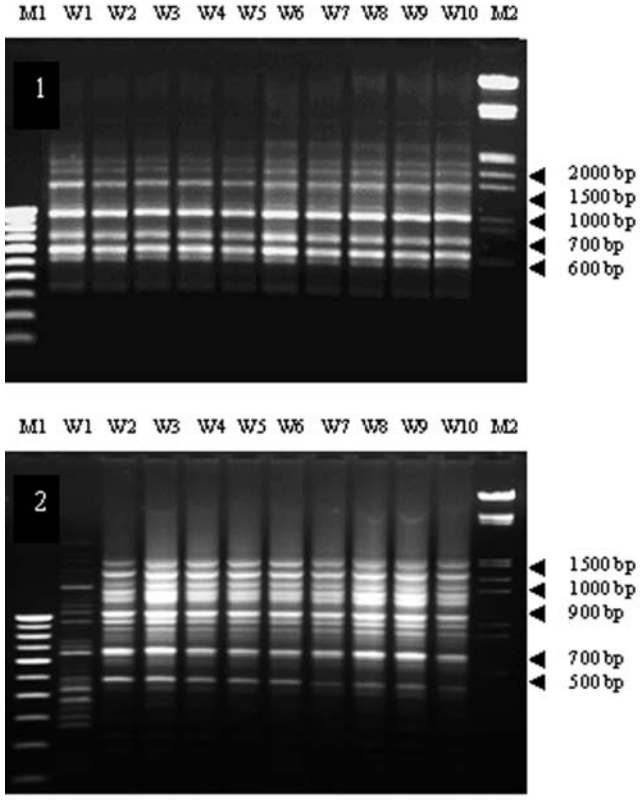
RAPD primers : Twenty oligomers RG 1- 5′ AGTCAGCCAC 3′ ; RG 2- 5′ AATCGGGCTG 3′ ; RG 3- 5′ AGGGGT CTTG 3′ ; RG 4- 5′ GAAACGGGTG 3′ ; RG 5- 5′ GTGACGTAGG 3′ ; RG 6- 5′ TCTGTGCTGG 3′ ; RG 7- 5′ TTCCGAACCC 3′ ; RG 8- 5′ AGCCAGCGAA 3′ ; RG 9- 5′ GACCG CTTGT 3′ ; RG 10- 5′ AGGTGACCGT 3′ ; RG 11- 5′ GTTGGTGGCT 3′ ; RG 12- 5′ GTCCACTG TG 3′ ; RG 13- 5′ TCGGCACGCA 3′ ; RG 14- 5 GTGAGGCGTC 3′ ; RG 15- 5′ CCGCATCTA C3′ ; RG 16- 5′ GTGGATGCGA 3′ ; RG 17- 5′ AGGACCAGG 3′ ; RG 18- 5′ AGGGCCCGGG 3′ ; RG 19- 5′ AT CGAGGAG 3′ ; RG 20- 5′ GGCAAGCT GGTGGGAGGTAC 3 (Bangalore Genei) were used for RAPD marker studies.

PCR Amplifications: (*Reactions and Conditions*) : The presence of DNA was determined by a PCR assay. RAPD primer sets were used in PTC-200, MJ Research Gradient Thermocycler, Version 4.0. RAPD-PCR was performed in 25 µl reaction volume containing 40 ng genomic DNA, 0.4 µl (5 pmole) primer, 2.0 µl dNTPs (2.5mM), 2.5 µl of 10X assay buffer with $MgCl_2$ (15mM), 0.5µl (3U/µl) of Taq DNA polymerase (Bangalore Genei Pvt.Ltd.). DNA was amplified to provide first denaturation of 5 min. at 94°C followed by 45 cycles of 1 min. at 94°C, 1 min. at 35°C followed by 2 min. at 72°C and final extension at 72°C for 5 min.

Gel Electrophoresis : Amplified DNA was separated by electrophoresis in 1.2 % horizontal agarose gels (wide range A 2790 of Sigma) containing ethidium bromide, illuminated with ultra violet light and gels were photographed using gel documentation system (Alfa Imager). The gel electrophoresis was run at 60 volts for 2 hours at 20°C.

Analysis of Molecular data : Band positions for RAPD were visually determined and pair wise comparisons of degree of band sharing were made. Similarity indices were calculated[5] as S.I. = 2Nab/Na+Nb. Dendogram was constructed by UPGMA method, (bootstrapped the data with 1000 replications using computer programme Statistica). Band positions for RAPD markers were visually determined and calculated 1 for presence and 0 for absence for a particular position. Genetic variations like percent polymorphism, similarity indices, and phylogenetic distance were calculated using statistical methods[6]. Comparative mean differences were also calculated by Newman-Keuls multiple comparison test using one way ANOVA.

Results : It was found that the largest amplified products were 3000bp and the smallest ones 100bp. The number of scorable bands for responding primers ranged from 0 to 10 with an average of 5 bands. A total of 1160 bands in Wistar were scored against 60 individuals of this strain (Table 1). RAPD method resulted in DNA banding pattern (Figs. 1 and 2) that clearly resolved differences among Wistar samples against different 20 oligonucleotides (RG-1 to RG-20), thus demonstrating the usefulness of these assays for strain identification as RAPD analyses differed among all Wistar samples. Additionally, RAPD banding patterns of Wistar samples constitute the beginning of Wistar strain database of DNA fingerprints with which other strains and field isolates can be compared. Identifying RG -1,2, 4, 10, 12 and 18 RAPD markers were amplified in all isolates with 3000bp, 2000bp, 1000bp, 700bp; 2000bp, 1000bp, 700bp, 300bp; 3000bp, 2500bp, 2000bp, 1000bp, 800bp, 400bp; 3000bp, 2500bp, 2000bp, 1000bp,



M1=100 bp DNA Ladder, M2=500 bp DNA Ladder,
W1-W10 = WISTAR Samples

Figs. 1 and 2 Allelic patterns of Wistar [*Rattus norvegicus*] based on (RG 2 and 10) RAPD markers DNA Fingerprinting

TABLE 1: Total number of alleles obtained using different 20 RAPD primers in genomic DNA of Wistar strains [*Rattus norvegicus*]

Product Size (bp)	RG 1	RG 2	RG 3	RG 4	RG 5	RG 6	RG 7	RG 8	RG 9	RG 10	RG 11	RG 12	RG 13	RG 14	RG 15	RG 16	RG 17	RG 18	RG 19	RG 20	Total no. of alleles in all primers
3000	9	5	-	6	6	7	6	9	8	-	-	-	-	-	-	-	-	-	1	2	**59**
2500	5	-	8	**10**	2	6	9	9	8	9	-	**10**	8	4	2	-	-	**10**	7	8	**115**
2000	9	**10**	-	**10**	4	9	9	**10**	8	9	7	-	2	4	5	4	8	-	2	-	**110**
1500	-	**10**	6	**10**	**10**	9	8	9	8	**10**	2	9	-	4	9	4	4	-	**10**	**10**	**132**
1400	-	-	-	-	-	-	-	-	-	-	2	-	-	5	3	-	-	-	2	2	**14**
1200	**10**	-	-	**10**	-	-	-	9	9	9	**10**	-	-	7	6	8	9	**10**	8	7	**112**
1000	-	**10**	**10**	**10**	**10**	**10**	9	9	9	**10**	-	**10**	**10**	4	4	**10**	**10**	1	3	1	**140**
900	-	-	9	1	**10**	-	-	-	-	**10**	-	**10**	4	3	-	-	-	-	-	1	**48**
800	9	-	-	**10**	**10**	-	-	9	9	1	**10**	**10**	8	3	2	2	-	1	2	-	**86**
700	-	**10**	-	9	**10**	**10**	-	-	-	**10**	-	-	2	3	4	-	-	9	3	-	**70**
600	**10**	**10**	-	9	8	**10**	**10**	9	**10**	-	-	-	-	5	6	3	1	-	7	2	**100**
500	-	-	6	4	-	-	1	-	9	**10**	7	-	5	5	1	-	1	1	6	8	**64**
400	-	-	-	**10**	4	-	1	9	-	1	-	-	-	2	7	-	-	-	2	4	**40**
300	-	6	-	5	6	-	-	-	-	1	5	-	4	3	3	2	-	9	1	2	**47**
200	-	-	-	3	-	-	-	-	-	-	-	9	-	5	2	-	-	-	2	-	**21**
100	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	**2**
Total no. of alleles in each primers	52	61	39	107	80	61	53	82	78	80	43	58	43	59	54	33	33	41	56	47	1160

800bp, 700bp, 600bp, 400bp; 1200bp, 900bp, 800bp, 700bp, 400bp; 1500bp, 900bp, 800bp respectively along with other polymorphic alleles. The percent polymorphism was 0.015 in Wistar in individual isolates but this polymorphism was not statistically significant among ten groups. This means that the sixty samples are almost similar, no mutation occurring amongst the isolates. As per statistical analysis of twenty RAPD primers, the average similarity indices ranged from 0.474 to 1.000 and the genetic similarity as well as mean differences Wis6 vs. Wis5 = 0.004, Wis2 vs. Wis4 = 0.033 and Wis10 vs. Wis8 = 0.2065 were calculated by Newman-Keuls multiple comparison test using one way ANOVA among Wistar strains. Fig. 3 shows the dendogram with 3 groups and 6 subgroups of Wistar. Three clusters of this sampling may be visualized: Wis1 plus Wis2, Wis4 plus Wis5 and Wis7 plus Wis8. The individuals of clustered Wis3 and Wis6 remained in a in between position and Wis10 was the more divergent sample. The constructed dendogram also revealed the Single Linkage Euclidean Distance among the ten groups such as Wis4 vs. Wis5 = 0.14, Wis2 vs. Wis7 = 0.54 and Wis2 vs. Wis10 = 0.97 but this Single Linkage Euclidean distance was not statistically significant (P>0.05). The RAPD affinities of Wistar standard genetic distances are depicted in the UPGMA dendogram.

The RAPD data generated were subjected to cluster analysis to generate a dendogram and to estimate the phylogenetic closeness and distance among the rat strains tested. It was observed that the Wistar samples are genetically more or less similar and phylogenetically separated from each other.

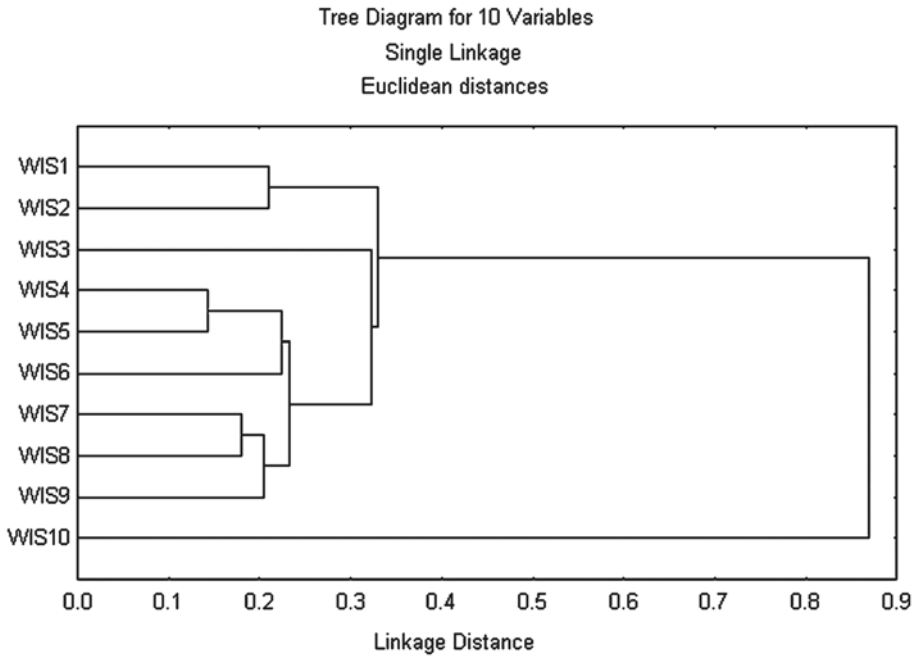


Fig. 3. Phylogenetic analysis of Wistar rat [*Rattus norvegicus*] based on Similarity Indices

Discussion : The great adaptability and hardiness of rat have made it a suitable model for a variety of different types of research. Sample preparation and gel electrophoresis protocols were standardized to ensure reproducibility of RAPD results. DNA fingerprinting is currently in vogue for genetic monitoring especially for rodents. Therefore, RAPD is the best tool to check percent polymorphism within the population. The reason for bands with high or lower intensity is still not known. Perhaps some primers do not perfectly match the priming sequence, amplification in some cycles might not occur and therefore bands remain fainter. The chance of these kinds of bands being sensitive to reaction conditions of course would be higher than those with higher intensity amplified with primers perfectly matching the primer sites. The most important factor for reproducibility of RAPD profile has been found to be the result of inadequately prepared template DNA. Differences between the template DNA concentration of two individual DNA samples result in loss or gain of some bands. It is known that the bands number in PCR reaction rather depends upon the peculiarity of the chosen sequence, as compared to its length. In this connection, it is clear that the more important stages are the experimental search and selection of primers to solve the problem if RAPD fingerprinting is to be applied. A simple methodology has been independently developed using primers about 10-20 nucleotides long having different amplification and electrophoretic conditions from RAPD and is termed as the arbitrarily primed polymerase chain reaction (AP-PCR) technique. Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. Over the last decade, Polymerase Chain Reaction (PCR) technology has gained popularity as a research technique and has led to the development of several genetic assays based on selective amplification of DNA[7]. RAPD markers are amplification products of anonymous DNA sequences using single, short arbitrary nucleotide primers. DNA

polymorphisms with RAPD-PCR and arbitrary primed PCR have proved to be advantageous as genetic markers in various species[8]. RAPD procedures have proved to be a useful tool for assessing genetic variability because band profiles with the twenty selected primers were reproducible and their patterns of inheritance proved to be Mendelian for a dominant marker. One disadvantage of RAPD markers is that they are dominant, hence the statistical information generated is less per marker in F_2 populations. Therefore, when mapping with dominant markers, it is necessary to use backcross or recombinant inbred populations, haploid or gametophytic tissue or alternatively an F_2 population where only RAPD markers amplified from a single parent were mapped[9].

RAPD was developed for analyzing genetic polymorphism in Wistar rats. The RAPD markers might be a potential tool for the study of rats. Biochemical genetics and immunological evidence of genetic heterogeneity in Wistar Kyoto rats were frequently employed in experimental studies of hypertension[10]. The spontaneously hypersensitive rat (SHR) and nonmotensive Wistar-Kyoto (WKY) are most frequently employed in experimental studies of hypertension. WKY fatty rats (Wistar) were also used as a model of obesity and non-insulin dependent diabetes mellitus[11]. The original Wistar colony from SHR and WKY rats were probably highly polymorphic for nuclear genes[12]. A genetic map for rat chromosome 2 that includes five candidate genes for blood pressure regulation was constructed in a region containing a quantitative trait locus (QTL) for blood pressure in Wistar rats[13]. RAPD technique based on polymerase chain reaction (PCR) has been one of the most common molecular techniques to develop DNA markers[14]. The reliability of RAPD markers for the estimation of genetic structure of natural populations of the murid rodent *Calomys musculinus*, the heritability of RAPD bands in parents and in their offsprings was also analysed[15]. The identification of genes was preferentially expressed in the oviduct during early Wistar rat embryo development using RNA-arbitrarily primed PCR[16]. Variability along with the above mentioned RAPD-PCR markers for evolutionary and biomedical research purposes will pave way for investigations on population genetics with a major advantage of increasing the number of polymorphic DNA markers which may enable us to find linkage relationships with interesting phenotypes in rats. The rat pink eyed (p) mutation is an identical intragenic deletion in pink eye dilute coat strains and several Wistar derived albino strains[17]. Routine genetic monitoring based on physical (skin colour, mandible analysis) and biochemical traits (isoenzyme, immunological marker) is practiced by several breeding and research centers to maintain genetic quality of laboratory animals under their care as in WNIN rat[18]. Genetic variation and differentiation of the *Apodemus agrarius* (striped field mouse) whose range consists of two large isolates were examined using RAPD-PCR analysis[19]. Contrary to the small interspecies diversity of genus *Oligoryzomys* reported in other genetic investigations, RAPD analysis performed depicted strong genetic differences among the taxa of the genus and the genetic diversity estimates obtained were significantly different[20]. The potential adverse biological effects of sub chronic exposure of male rats to low intensity static magnetic field on the biophysical properties of hemoglobin molecules as well as possible genotoxic effects on DNA stability using RAPD technique have been analysed[21].

In conclusion, DNA research brings the prospect of better diagnosis, with tests for a variety of diseases, both inherited illnesses and those that arise through mutations in body cells. Some DNA testing potential goes unrealized. Therefore, in order to prevail over this, these RAPD marker studies ensure that the levels of genetic variation are maintained through time and allow comparison of heterozygosity among species.

The RAPD data generated were subjected to cluster analysis to generate a dendogram and to estimate the phylogenetic closeness and distance among the rat strain tested. It was observed that the Wistar samples are genetically more or less similar and phylogenetically separate from each other. Genetic variation between and within breed is described as diversity. It is essential to characterize a breed for its conservation and rat exhibits a number of characterstics that make a useful model of human characterization.

In the present study, we report the successful amplification of twenty RAPD genetic markers which can be used as potential genetic markers for Wistar strain identification. Thus, RAPD primers can act as easy and reliable source of genotyping of laboratory animals or any other molecular genetic research work where DNA is required as an experimental material for study. We have found that RAPD analyses with single primers has a major advantage in increasing the number of polymorphic DNA markers, which may enable us to find linkage relationships with interesting phenotypes in Wistar rats.

Acknowledgement

The authors would like to express their gratitude to Dr. Rajesh Rathor, Senior Scientist for granting the

permission to conduct the study at Bacteriology Laboratory, CADRAD, Indian Veterinary Research Institute, Izatnagar. ❐

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Received : 9 March, 2009

Revised : 5 October, 2009

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