

Genetic Diversity and Phylogenetic Relationship Among Eight Species of Order: Rodentia in Egypt Based on Random Amplified Polymorphic DNA (RAPD) Marker

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Abstract: Of all mammals the rodents have an importance and great diversity. The phylogenetic relationships between the different species of the order Rodentia has an interest and investigations for many researchers. The current study suggests the ability of Random Amplified Polymorphic DNA (RAPD)-PCR to estimate the genetic distance and reflect the genetic relations and variability among eight rodent species of order Rodentia in Egypt. After the use of five arbitrary short primers (OPA-4, OPT-7, OPG-2, OPN-4 and OPM-1) to generate polymorphic, reproducible and score able bands. The results explained that, the highest genetic distance 1 was pronounced between *Rattus norvegicus* and *Mus musculus praetextus* similarly between *Mus musculus domesticus* and *Rattus norvegicus*. This is followed by the genetic distance between *Mesocricetus auratus* and *Mus musculus domesticus* as well as between *Cavia porcellus* and *Rattus norvegicus*. Nonetheless, the lowest genetic distance 0.31 was noticeable between *Mus musculus praetextus* and *Mus musculus musculus*.

Keywords: Rodentia, *Mus Musculus*, *Rattus Rattus*, *Acomys Cahirinus*, *Mesocricetus Auratus*, *Cavia Porcellus*

1. Introduction

Rodents represent more than 40% of the all living mammalian species. About 5–10% of the worldwide spreading rodents are major pest species in agricultural and urban localities, consuming significant amounts of agricultural products. Rodents causes food contamination, crops destruction and acts as vectors for certain diseases resulting in dangerous effects on farm production [1-4].

Furthermore, muroide rodents are mostly associated with negative impacts on the surroundings as well as human income, they also contribute positively as models for biomedical research especially after the complete sequencing of the their genome. The classification and identification of rodents particularly within many mammalian species, are very difficult, they require examination of external and internal morphology (like as skull and dentition) which are

often biased by age and sex variations and lack of comparative material. The morphological approach used for identification which has usually undertaken by skilled mammalogists even by the use of microscopic analysis requires high-skilled experience to achieve a reliable identification and even so, they failed to go further from a general group of assumed species. Thus, the genetic analyses of rodents were in need to assess both their systematics and population structure [5-8].

Advances in DNA analysis enhanced the researches in mammals particularly rodent's identification, consequently it confirm the classification of specimens to establish a base for future research including the new biological existences. Such DNA analyses also reveal variation patterns in the use of arthropod as a food source by different bat species or diet variation during different physiological periods. DNA analysis even allows describing mixed contents of the

stomach or the samples of feces to distinguish the preys consumed by a given predator. Furthermore and in most cases, the DNA analysis help to confirm the appearance of certain species in areas out of their distributional range such as bats and Artiodactyls [9-17].

Moreover, DNA analysis is very valuable for identification of the traded wild animals (either live, hunted or accidentally killed), or their organs and other biological materials, ranging from body parts of the animal (e.g. skins, skeletons meat, teeth and horns); these must be identified molecularly with its containing DNA trace [18]. The introduction of DNA-based technologies offered the possibility for overcoming and improves the capability of detecting, monitoring and controlling the trade of animals.

Thus, the current investigation was aimed to analyze the genomic DNA structure of some rodent species which belonging to the Order: rodentia in the Egypt in order to clarify the genetic affinity between them using Random Amplified Polymorphic DNA (RAPD) Marker.

2. Materials and Methods

2.1. The Experimental Animals

4 of the 8 investigated rodent specimens were brought alive from its natural habitat in Egypt either from fields around Zagazig City of Sharkia Province; black or roof rats, (*Rattus rattus*), homes in Sheba village of Sharkia Province; gray house mice, (*Mus musculus domestics*) and black mouse, (*Mus musculus praetextus*) and from Abu-Rawash of Giza Province spiny mouse (*Acomys cahirinus*). But, the laboratory albino mice (*Mus musculus musculus*), albino rats (*Rattus rattus norvegicus*), Guinea pig (*Cavia porcellus*) and Syrian golden hamsters, (*Mesocricetus auratus*) which are inbred strains introduced to animal breeders in Egypt a few decades ago and were conveyed from experimental animal facility of the Faculty of Veterinary Medicine, Zagazig University, Egypt.

In the present work, 24 rodent specimens (3 from each species) were used to compare between their DNA structures. They were transported alive to the laboratory of the Zoology Department, Faculty of Science, Zagazig University and kept in well-aerated cages under controlled laboratory conditions supplied with sufficient food and water before analysis as recommended in the guidelines given by the Institutional Ethical Committee /IRB approval in Zagazig University.

2.2. DNA Analysis of the Studied Rodent's Species

2.2.1. DNA Extraction

In the present studies, heart tissues sample from each of the studied rodent's species (*Mus musculus*, *Mus musculus praetextus*, *Mus musculus domesticus*, *Acomys cahirinus*, *Rattus norvegicus*, *Rattus rattus*, *Mesocricetus auratus* and *Cavia porcellus*) were taken off immediately after animals had been sacrificed. The collected heart tissues sample stored in freezer till analysis of all take places.

Total genomic DNA was extracted from heart tissues using

the DNeasy Tissue Kit (QIAGEN), following the manufacturer's instructions as described below:

Spin Protocol for DNA Extraction from Heart Tissue:

Buffer AW1, Buffer AW2, and QIAGEN Protease have been ensured to be properly prepared and no precipitate has formed in Buffer AL. Samples was equilibrated to 25°C, water bath was heated to 56°C for use in step 4 and Buffer AE was equilibrated to room temperature for elution in step 11.

1- 25mg tissue were weighted and added to 1.5 ml micro-centrifuge tube. 2- 180 µl Buffer ATL were added to the sample.

3- 20 µl QIAGEN Protease (or proteinase K) was pipetted into the bottom of micro-centrifuge tube, and tissue cut into small pieces.

4- Homogenate was mixed by pulse vortexing for 15 s first and then thoroughly to yield a homogeneous solution.

5- Incubation at 56°C for 2hours until tissue completely digested or incubation overnight.

6- Briefly centrifuged to remove drops from the inside of the lid.

7- 200 µl Buffer Al were added to sample, then incubation at 70°C for 10 minutes.

8- 200 µl ethanol (96–100%) were added to the sample, mixed again by pulse-vortexing for fifteen second, and then briefly centrifuged to remove drops from the inside of the lid.

9- The mixture from step eight was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap then closed, and the sample was centrifuged at 8,000 rpm for one min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

10- The QIAamp Mini spin column is then carefully opened and 500 µl Buffer AW1 was added without wetting the rim. Cap was closed and the sample was then centrifuged at 8,000 rpm for 1 min. QIAamp Minispin column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded.

11- The QIAamp Mini spin column was then carefully opened and 500 µl Buffer AW2 was added without wetting the rim. The cap was then closed, and the sample was centrifuged at 14,000 rpm for 3 min.

12- The QIAamp Mini spin column was then placed in a clean 1.5 ml micro-centrifuge tube, and the collection tube containing the filtrate was discarded.

13- Finally, the QIAamp Mini spin column is carefully opened and 200 µl Buffer AE was added, incubated at 25°C for 1 min., and then centrifuged at 8,000 rpm for 1 min. DNA samples were stored at -20°C until use.

2.2.2. Random Amplified Polymorphic DNA (RAPD)

For RAPD analysis, five of randomly selected primers (Operon Technologies, USA) oligonucleotide primers having GC content 60-70% (Table 1).

The formula for primer Tm calculation: $T_m = 4(G + C) + 2(A + T) = ^\circ C$

PCR reactions were also carried out by Techne PCR mini

thermal cycler. Each PCR reaction mixture of 12.5 µl consisted of PCR GoTaq Green Master Mix, 1 µl of each oligo primer (10 pmol/µl) and 5 µl DNA extract and the volume was brought to 25 µl by deionized water, all in the rmowell® GOLD 0.2 ml Polypropylene PCR Tubes. PCR conditions were as follows: initial denaturation (1 cycle) on 94°C for 5 min followed by 45 cycles, each one consisting of a DNA denaturation step (1 min at 94°C), one primer annealing step (1 min at 36°C) and a DNA extension step (2 min at 72°C) and an extra final extension step was performed for 10 min. at 72°C to complete amplification as recommended by [19]. PCR products were kept in 4°C until being electrophoresed and analyzed.

Table 1. The five RAPD Primers and their sequences.

NO	PRIMERS	Sequence (5'–3')	G+C (%) content	Tm value °C
1	OPN4	5'GAC CGA CCC A-3'	70%	34°C
2	OPM1	5'GTT GTT GGC T -3'	50%	30°C
3	OPT7	5'GGC AGG CTG T -3'	70%	34°C
4	OPA4	5'AAT CGG GCT G-3'	60%	32°C
5	OPG2	5'GGC ACT GAG G -3	70%	34°C

2.2.3. Agarose Gel Electrophoresis

Reagents and Chemicals

1-Agarose powder (Fisher Scientific, UK)

2-Tris Borate EDTA buffer (TBE): for 1 litter of working 1X TBE, 10.8gm Tris base, 5.5gm boric acid (United Co., Egypt), and 0.74gm of EDTA (WINLAB, USA) were dissolved and the volume is brought to 1 litter by ddH₂O.

3-Ethidium Bromide (EthBr) stain: (Promega, USA) ready for use.

2.2.4. Pre-stained 1.0% Agarose Gel Preparation for Small Unit Used for separation of PCR Product

The PCR reactions were done as mentioned by [20] with slide modification of [21]. Since, 0.5 g of agarose was added to 50 ml TBE buffer into a 100 ml bottle, and dissolved in the microwave oven (Kelvinator®, USA) for about 40 seconds. Solution was mixed twice during the microwaving, cooled down to ~ 50°C, and then 1.0 µl of the Ethidium Bromide was added and mixed. The horizontal gel apparatus was sealed, and the comb was inserted until its base is 2 mm from the base of the gel to make pores. Molten agarose was poured onto a gel plate while avoiding bubbles. Gel tray was then inserted into the proper position in the electrophoresis chamber, after that gel stand was filled with buffer TBE until it covers the gel completely. Comb was removed and gel became ready to receive samples for electrophoresis.

2.2.5. DNA Loading Buffer (6X) Preparation

30% (v/v) glycerol was added to 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF and stored at 4°C.

Loading Samples: 5 µl of 6X loading dye were added to PCR product samples, mixed well and then the mixture was injected into the gel pores (wells).

Running Agarose Gel: Gel was run in Minicell horizontal electrophoretic unit 8 wells (UNITEC, EC-370M, USA) at a

constant current 100V for ~1 hour until bromophenol blue passes ~two third the gel distance.

Gel Visualization and Analysis: The gels were then visualized using UV transilluminator (Upland CA.91786, M-20, USA) and were photographed using Digital Camera. Images were formatted and analyzed by “Gel-Pro®3.1” analyser software. The molecular weight (size) was determined by using 100 base pair DNA ladder marker (Solis BioDyne, Riia, Tartu, Estonia) from 100-3000 bp MW as a standard (3µl loaded), which is loaded and migrated the same gel with samples.

3. Results

The current study applied using RAPD-PCR where, five random decamer RAPD primers were screened to produce banding patterns in the eight samples of genomic DNA constituted of albino mouse, house mouse, black mouse, spiny mouse, albino rat, black rat, hamster and guinea pigs. So universal primers fitting to rodents were selected, since the RAPD profile of the different studied species of mice, rats, hamster and guinea pigs were greatly dissimilar.

The primers OPA-4, OPT-7, OPG-2, OPN-4 and OPM-1 appeared to be the most useful for detection of genus specific population. These five arbitrary short primers to differentiate between the different studied species produced 74 polymorphic, reproducible and mark-able bands. The maximum number of bands 23 was produced with primer OPA-4 (Figure 1) but the minimum number of bands 9 was created with primer Opt7 (Figure 2). However, the primers OPN-4, OPG-2 and OPM-1 generated 19, 11 and 12 reproducible bands, respectively (Figures 3, 4, 5, 6).

All of these scored bands are polymorphic (100%) and none of them showed mono-morphism. The used primers; OPA-4, OPG-2 produced the highest percentage (100%) of polymorphic bands, meanwhile short primers OPT-7, OPN-4 and OPM-1 produced only 89.0%, 94.7%, 92.0% of polymorphic bands, respectively. The unique fragments were produced by OPT-7, OPN-4 and OPM-1 primers.

The molecular weight of the amplified bands was ranged from 50 bp to 650 bp. The maximum size-range of amplified products 200-650 bp for a single primer was found with OPA-4, whereas the minimum 100-300 bp was as obtained with Opt-7 (Table 2, Figure 7).

Nei's Genetic Distance (D)

We can calculate genetic distance by the equation of Ni and Li [22]

$$GD = 1 - [2Nab / (Na + Nb)]$$

Since: Na=number of bands in species a, Nb= number of bands in species b and Nab = number of common bands in species a and b [23].

The highest genetic distance (1) was found between albino rat (*R. r. norvegicus*) & black mouse (*M. m. praetextus*) and between house mouse (*M. m. domesticus*) & albino rat (*R. r. norvegicus*) distantly related. This is followed by the genetic distance between Syrian hamster (*Mesocricetus auratus*) and

house mouse (*M. m. domesticus*) as well as between guinea pig (*Cavia porcellus*) and albino rat (*R. r. norvegicus*). Nonetheless, the lowest genetic distance 0.31 was noticeable between black mouse (*M. m. praetextus*) and albino mouse (*M. m. musculus*) close related (Table 3).

Phylogenetic Relationships Using Euclidean Distance:

The dendrogram for the studied eight rodents subspecies based on Euclidean genetic distance were showed in (Figure 8).

There were Five Separate Clusters:

1-Black mouse (*M. m. praetextus*) and albino mouse (*M.*

m. musculus) clustered together in the first one.

2-Guinea pig (*Cavia porcellus*) clustered separately in second cluster.

3-Spiny mouse (*Acomys cahirinus*) and house mouse (*M. m. domesticus*) clustered together in third cluster,

4-Syrian hamster (*Mesocricetus auratus*) and albino rat (*Rattus norvegicus*) clustered together in fourth cluster.

5-Black rat (*Rattus. rattus rattus*) clustered separately in fifth cluster.

Table 2. Characteristic of fragments generated by the primers selected for the RAPD analysis. Number of Amplified Bands per Morphotype (NA Bands/ Morphotype), Total Number of Amplified Bands (TNA Bands), Band Frequency per Primer (Band Freq. / Primer), Number of Polymorphic Bands (NP Bands), Number of Monomorphic Bands (NM Bands), Polymorphic Percentage (%POL) and Range of Amplified Fragment in base pair (RAF bp).

Primer code	NAB/ animal species								TNAB	Band				% POL	RAF (bp)
	Black mouse	Albino mouse	House mouse	Spiny mouse	Albino rat	Black rat	Hamster	Guinea pig		Freq. / Primer	NUB	NPB	NMB		
OPA4	5	5	1	2	0	5	3	2	23	0.3108	0	23	0	100%	200-650
OpT7	1	1	1	1	1	1	1	2	9	0.1216	1	9	0	89.0%	100-300
OPN4	1	2	2	2	1	5	3	3	19	0.2567	1	19	0	94.7%	50-450
OPG2	2	2	1	0	1	2	1	2	11	0.1486	0	11	0	100%	250-400
OPM1	3	1	2	2	0	2	1	1	12	0.1621	1	12	0	92.0%	100-450
Total	12	11	7	7	3	15	9	10	74						
Band Freq. / morphotype	0.16	0.15	0.09	0.09	0.04	0.20	0.12	0.13							

Table 3. Genetic distances between rodent species using RAPD data.

	Black mice	Albino mice	House mice	Spiny mice	Albino rat	Black rat	Hamster	Guinea pig
Black mice	0	0.31	0.79	0.8	1	0.63	0.81	0.41
Albino mice		0	0.81	0.78	0.72	0.62	0.5	0.53
House mice			0	0.43	1	0.55	0.87	0.65
Spiny mice				0	0.8	0.82	0.63	0.77
Albino rat					0	0.78	0.5	0.85
Black rat						0	0.5	0.52
Hamster							0	0.68
Guinea pig								0

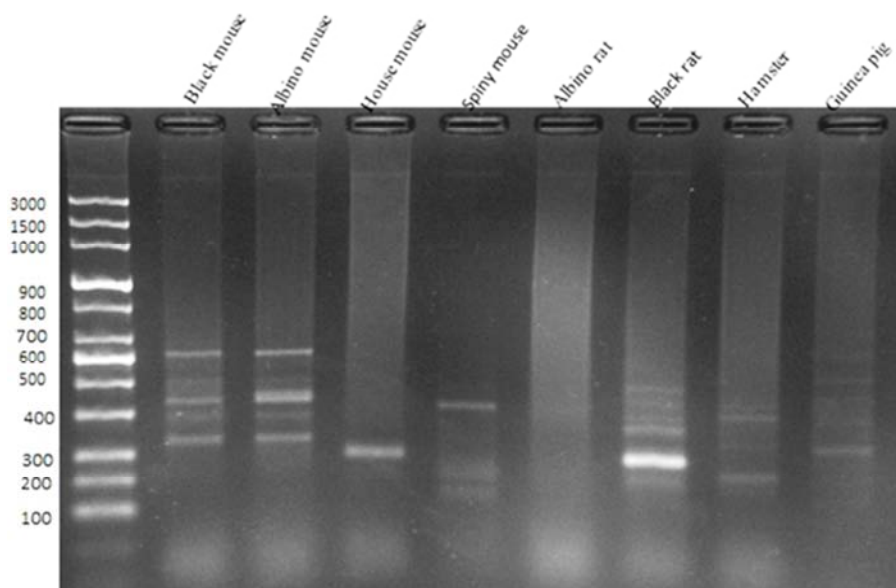


Figure 1. Gel electrophoresis for PCR- product amplified using primer OPA4.

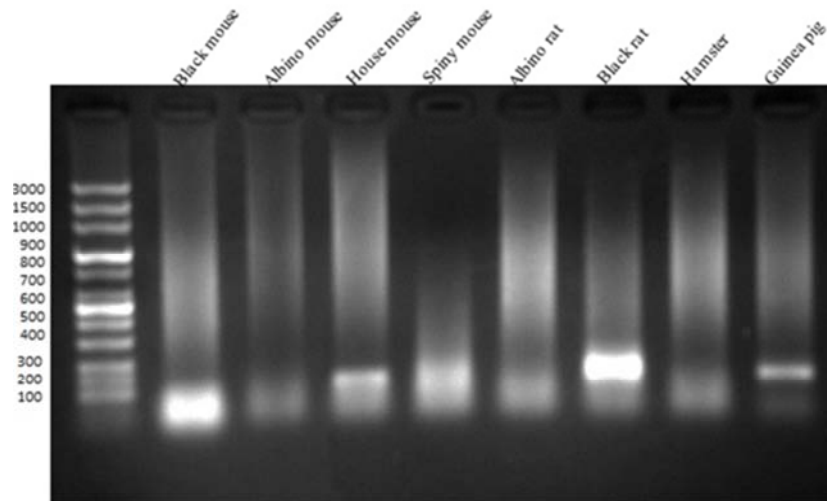


Figure 2. Gel electrophoresis for PCR- product amplified using primer OpT7.

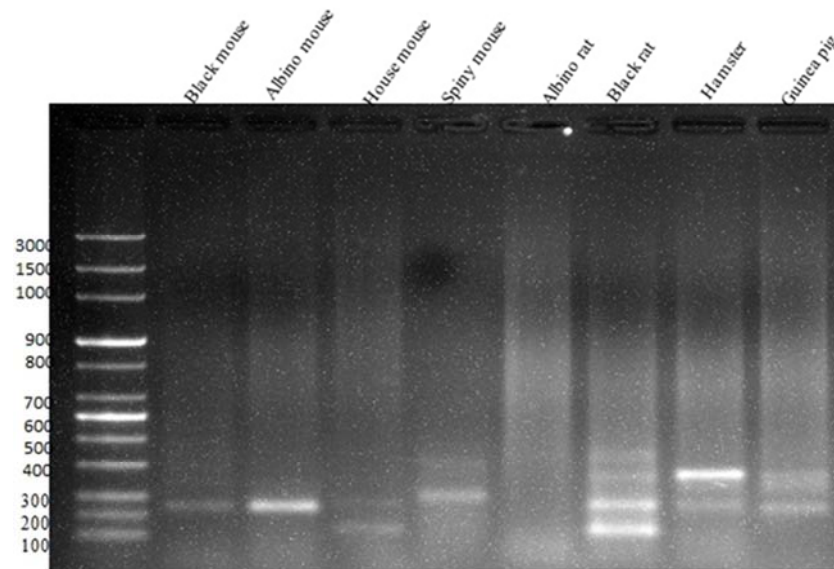


Figure 3. Gel electrophoresis for PCR- product amplified using primer OpN4.

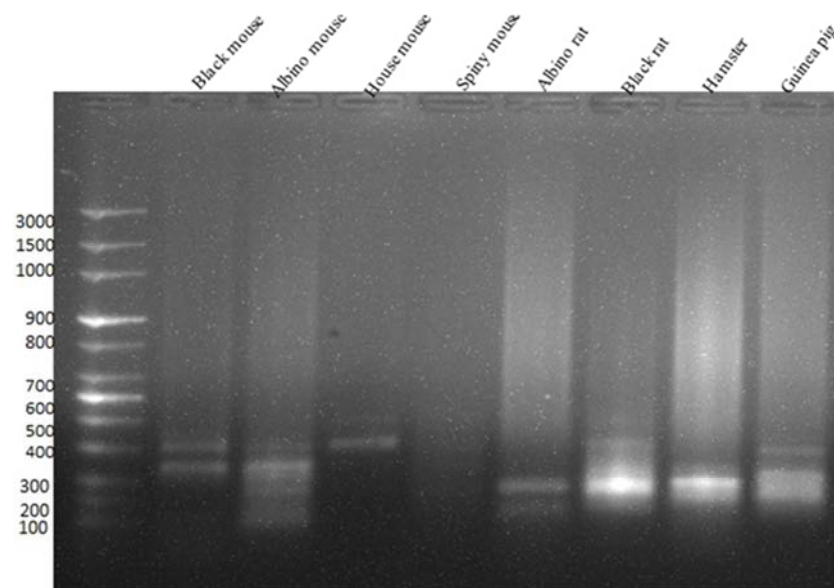


Figure 4. Gel electrophoresis for PCR- product amplified using primer OpG2.

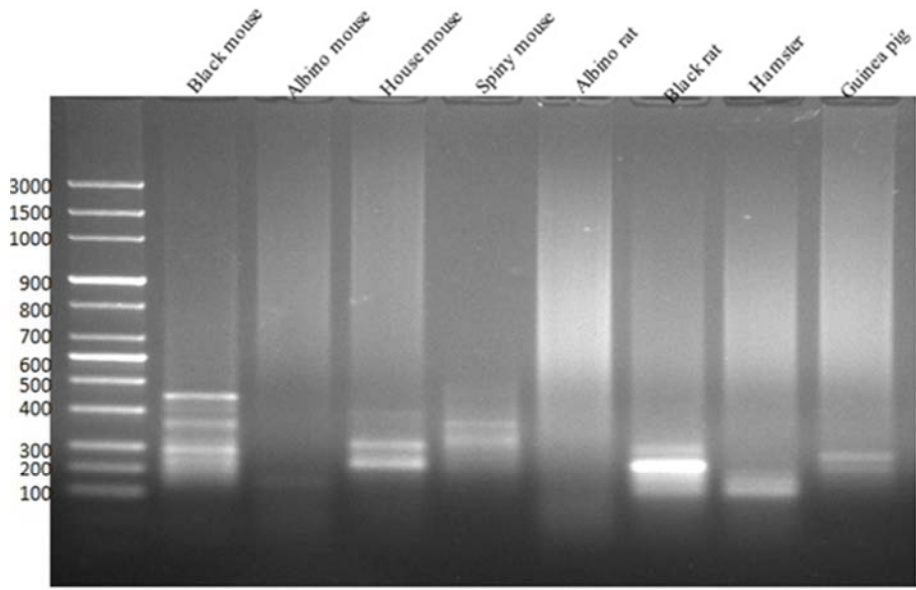


Figure 5. Gel electrophoresis for PCR- product amplified using primer OpM1.

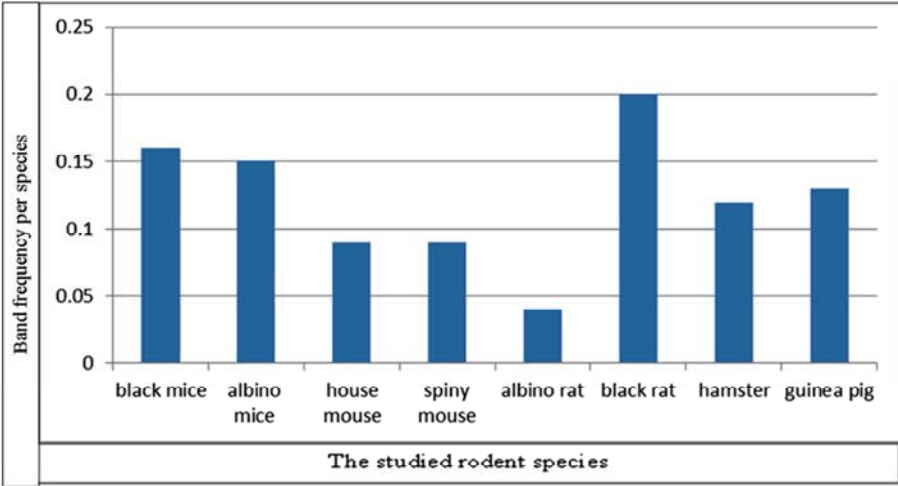


Figure 6. Band frequencies recorded for the rodent species.

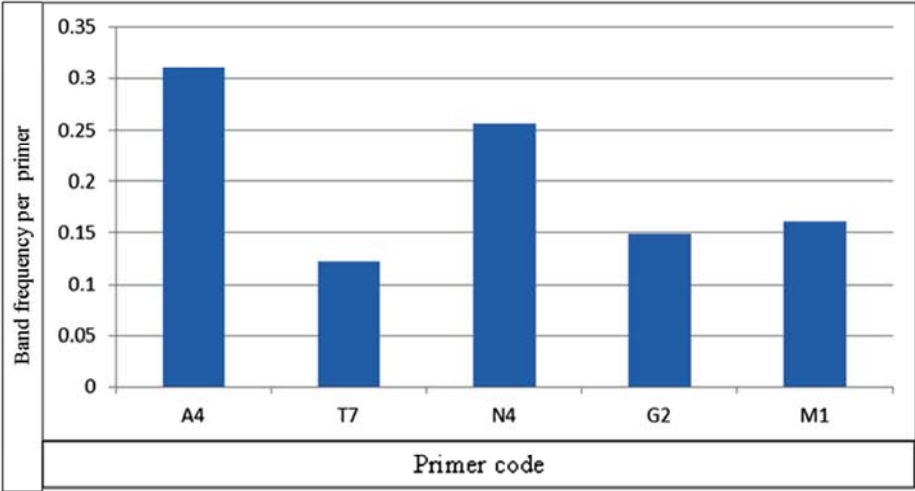


Figure 7. Band frequencies recorded per the 5 deca-nucleotide primers.

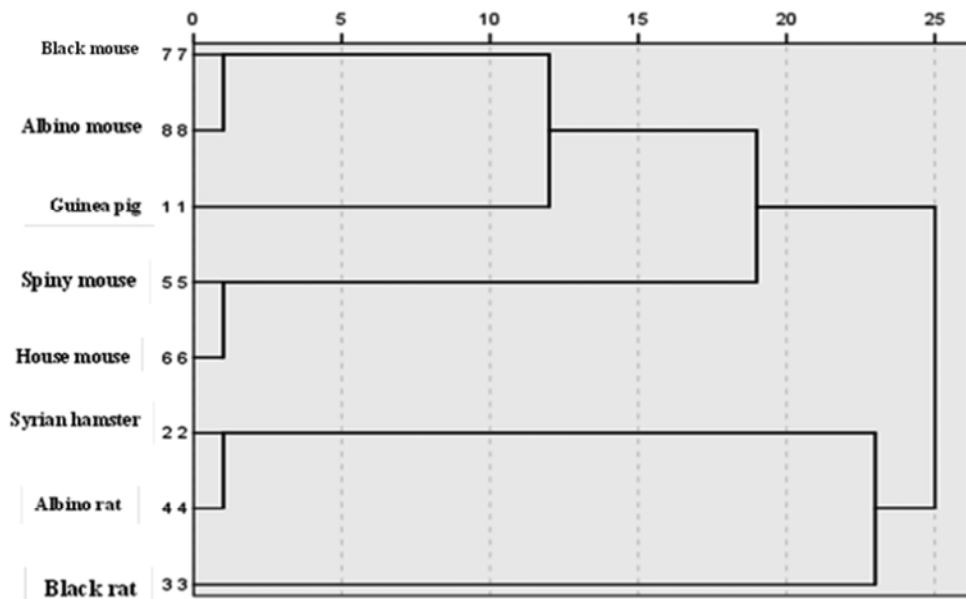


Figure 8. Dendrogram of studied rodents based on genetic distances among them.

4. Discussion

In the present study, the genomic DNA sequences for *Mus musculus*, *Mus musculus praetextus*, *Mus musculus domesticus*, *Acomys cahirinus cahirinus*, *Rattus norvegicus*, *Rattus rattus*, *Mesocricetus auratus* and *Cavia porcellus* were analyzed.

This was supported [24, 25] who recorded that it is possible to use of DNA analyses to compare chromosomes directly at the DNA level. Using cladistics analysis rearrangements that have differentiated the rodent's karyotype were then more exactly mapped and placed in a phylogenomic outlook. Comparative chromosomes are a new term that was used to define the field of cytogenetic dealing with recent molecular approaches although chromosomes were originally defined the chromatin dynamics and morphological changes in interphase chromosome structures.

In the modern taxonomy, classification and identification many processes are applied. In this context, DNA analysis moved forward from just identification and becoming a service technique important for aspects of taxonomy and origin, even being relevant in other biological areas, ranging from distribution to conservation and behavior. DNA analysis is a universal molecular identification system for living organisms. Its effectiveness has been confirmed recently, since it is common to link DNA specific to the animal's species with phylogenetic reconstruction, which probably led to discovering of new animal species. Mammals are one of the important animal groups achieved this purpose, since Mammals are represent with about 5564 species listed in the Catalogue of Life, ITIS database [26, 27].

Herein, RAPD markers were used to detect genetic differences between the eight rodent subspecies. The RAPD and other molecular assay showed to be manifold for taxonomic relationships and genetic diversity studies. All the used five decanucleotide random primers produced at test

(100%) polymorphic fragments and none of them showed monomorphic bands indicating the successful PCR use here far revealing instructive RAPD banding pattern and indicated that in genetic relatedness among the eight rodent subspecies.

It was found that, from all five used primers, primer OPA4 exhibited highest primer band frequency is 0.3108 while the lowest value is 0.1216 recorded from primer OPT7 interesting the high molecular fragment of about 650 recorded for black and albino mouse with primer OPA4. This band was not observed in others species which indicated the presence of internal genetic variation, and closed relation between the two subspecies.

Also the two subspecies (black & albino mouse) recorded 4 bands variation between 300 and 500. This confirms a strong intragenic relationship between the two subspecies. These data were supported by [28-30] since they observed the same intragenic relation between nuclear and mitochondria data within *Mus* of Asian group.

Nonetheless, the present data for the studied eight rodent species showed the presence of molecular fragment 400 bp in (Black and Albino mouse, spiny mouse and Syrian hamster) also fragment 200bp in (Spiny mouse, Black rat and Hamster) and fragment 300 bp in (Black and albino mouse also Black rat and Guinea pig).

All data revealed an intragenic relation between the eight rodent species, which are agreement with several result recorded by [31] who stated that there are strong phylogenetic and divergence for major Rodent groups within multiple genes.

Nonetheless, [32] concluded that, the primers with G+C content generates more number of polymorphic bands in the three subspecies (mice, rat and guinea pig).

The maximum number of polymorphic band was recorded in guinea pig while mice scored minimum number of polymorphic bands. An average band frequency value the maximum heterogeneity and homogeneity were recorded in

black rat, Black & Albino mice. This indicated high rate of homogeneity and heterogeneity (0.20, 0.16 & 0.15) and the lowest rate were recorded in albino rat (0.04).

These data were contrasted with data recorded by [33, 34] recorded a very low level of genetic diversity in house mice and high genetic diversity in guinea pig i.e. the heterogeneity more in guinea pig than rat and mice.

The designed dendrogram depicted unambiguous grouping of samples consistent with their genus, the high genetic distance existed between (Albino rat, Black mice & Hamster) followed by (Hamster & house mouse as well as Guinea pig & Albino rat similarly Spiny mouse & Hamster). Nonetheless, the low genetic distance is existed between (Black mice & Albino mice).

These results donated to the taxonomy of the eight rodent subspecies, in which Black, House & Albino mice under the same species *Mus musculus* while Albino rat & black mice under the same sub family Murinae. Also albino rat, black mice and hamster belong to super family Muridae. In addition Albino rat, Spiny mouse & Hamster are under the same super family Muridae.

These results are in agreement with many authors who supported the utility of genetic distance to categorize and explain the evolutionary link between populations, degree of genetic variation in addition to history of animals. Also, the significance of using the genetic distance measures to calculate biodiversity, population structure and genetic changes in white Pekin and Muscovy duck [35- 37].

5. Conclusion

According to the results of the present investigation, dendrogram between the eight studied rodents species spread in the Egyptian climate based on Euclidean genetic distance explained that, there were five separate clusters; Black mouse (*M. m. praetextus*) and albino mouse (*M. m. musculus*) clustered together in the first one, Guinea pig (*Cavia porcellus*) clustered separately in second cluster, Spiny mouse (*Acomys cahirinus*) and house mouse (*M. m. domesticus*) clustered together in third cluster, Syrian hamster (*Mesocricetus auratus*) and albino rat (*Rattus norvegicus*) clustered together in fourth cluster, Black rat (*Rattus. rattus rattus*) clustered separately in fifth cluster.

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