Phylogenetic Relationship between the Fruit Bat (*Rousettus aegyptiacus*) and Lesser Tailed Bat (*Rhinopoma Hardwickei*) Inferred from G-Banded Chromosomes and Electrophoretic Protein Pattern Analysis

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Abstract: The present work is an attempt to find out both the genetic similarities and the divergences between the Fruit bat (*Rousettus aegytiacus*) and lesser tailed bat (*Rhinopoma hardwickei*) by using G-banding technique for bone marrow metaphase chromosomes and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for liver protein. The diploid chromosome number (2n) for each of these two species is 36 chromosomes and NFa is 68 arms. The chromosomes of both two species are grouped in 17 pairs, in addition to the sex chromosomes. The relative lengths of the X chromosome and the Y chromosome are 5.6 % and 6.4 % in the fruit bat respectively and The relative lengths of the X chromosome and the Y chromosome are 0.7 % and 1.2% in the lesser tailed bat respectively. The G-banding displays obvious alternations of white and dark bands; this facilitates ideogram construction. The sequence of banding pattern of chromosomes of the 2 species display fairly similar and different pictures. SDS-PAGE for liver soluble protein showed 23 and 19 protein bands in the, *Rousettus aegyptiacus* and *Rhinopoma hardwickei* respectively. The two species have 2 common bands. The molecular weight of the bands ranges from 118-26 kDa and from 118-24 kDa in the *Rousettus aegyptiacus* and *Rhinopoma hardwickei*, respectively. Bands of molecular weight 70,60,50,42,36,31,29 and 27 kDa are characteristic to the *Rousettus aegyptiacus* and are missed in the *Rhinopoma hardwickei.* Also, bands of molecular weight 113,105, 65 and 24 KDa are characteristic to the *Rhinopoma hardwickei*. Moreover, the unique band at molecular weight 113 is characteristic to the *Rhinopoma hardwickei*. The statistical analysis showed that the degree of similarity between the two species is 0.095(9.5%). As a conclusion, the Fruit bat (Rousettus *aegytiacus*) and the Lesser tailed bat (*Rhinopoma hardwickei*) are not identical and separated species.
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Key Words: the Fruit bat (*Rousettus aegyptiacus*) and the lesser tailed bat (*Rhinopoma hardwickei*), Chromosomes, G-banding, Protein electrophoresis, Phylogenetic relationship.

1. Introduction

Mammals of the order Chiroptera are widely distributed in Egypt. Fruit bats are serious pests of commercial fruit crops (**Albayrak *et al.*, 2008**). Fruit bats, *Rousettus spp*. are not only a cause of great economic loss in fruit crops but are also vectors of domestic animals and human transmissible ectoparasites and different diseases(**Reeves *et al.*, 2006; Albayrak *et al*., 2008 and Kuzmin *et al.*, 2008 ,Towner *et al*., 2009**). Also, *Rhinopoma spp* are known to be reservoirs for a variety of different diseases whereas, it feeds on insects (**Qumsiyeh, 1985**) and acts as a vector of different fungal diseases such as subcutaneous zygomycosis (**Gugnani, 1999**).

Earlier studies of the identification of bats in Egypt have included the taxonomic classification, local geographical distribution as well as the gross anatomical features (**Qumsiyeh, 1985**). *Rousettus aegyptiacus* is the only bat species of the all bats recorded in Egypt that belong to the megabats (**Qumsiyeh, 1985 and Wassif, 1995**). The *Rousettus aegyptiacus* is represented by the subspecies *Rousettus aegyptiacus aegyptiacus*, distributed in Israel, Lebanon, Jordan, Egypt, Cyprus, Turkey, Levant to middle of Arabia and Africa and the subspecies *Rousettus aegyptiacus arabicus* distributed in Oman ; Aden; and occurred from the South Arabia to Pakistan (**Bergmans, 1994 ; Benda and Horacek, 1998**). **Almeida *et al.*, 2011,** studied the phylogenetic relationship of the family Pteropodidae based on morphological diversity. The microbats of the family Rhinopomatidae contains a single genus with two species(*Rhinopoma hardwickei* and *Rhinopoma microphylum*) in Egypt (**Qumsiyeh,1985 and Wassif, 1995**). The lesser tailed bat, *Rhinopoma hardwickei* is represented by the two subspecies *Rhinopoma hardwickei arabium* and *Rhinopoma hardwickei cystops* in Egypt (**Qumsiyeh, 1985 and Wassif, 1995**). Rhinopomatidae was regarded as the most primitive group of microchiroptera close to the common ancestor of microbats and megabats (**VanValen, 1979 and Eicki *et al*., 2005**).

The analysis of genetic diversity between or within different species, populations and individuals is a central task for many disciplines of biological science. The systematic similarity and diversity between *Rousettus aegyptiacus* and *Rhinopoma*

hardwickei species was tackled according to chromosomal studies . Recently, biochemical data and molecular genetics were utilized for the Pteropodidae and Rhinopomatidae phylogenetic relationships detection.

Standard karyotypes have often been used as a measure of the magnitude of chromosomal variation (**Bengtsson, 1980**). The karyological studies showed that the chromosome number of the *Rousettus* SPP. is 36 chromosomes (**Ray-Chaudhuri, 1968; Harada *et al.*,1982; Hood *et al.*, 1988; Rickart *et al.*, 1989; Karatas *et al.*, 2003 and Albayrak *et al.*, 2008**). Also, the karyotype of the *Rhinopoma hardwickei* illustrated that the chromosomal number is 36 chromosomes (**Ray- Chaudhuri *et al.*, 1968 and Qumsiyeh and Baker, 1985**).

Definitely, the conventional unbanded chromosome karyotypes are not satisfactory for accurate cytogenetic studies and the individual chromosome identification could not be spotted precisely; this made their sorting out of uncertain validity. In general, the banding techniques made the comparisons more meaningful, and thus the structural changes in the chromosomes can be identified precisely.

The G-banding chromosomes have been examined for *Rousettus aegyptiacus* (**Haiduk *et al.*, 1981**and **Haiduk, 1983)** and *Rhinopoma hardwickei* (**Qumsiyeh and Baker, 1985**).

Moreover, the development of molecular markers which are based on polymorphism found in proteins or DNA has greatly facilitated research in a variety of disciplines such as phylogeny, ecology genetics and animal breeding. The electrophoretic investigation of serum proteins is a good indicator for evolutionary studies and to resolve the cladistic relationships of several species (**Ferguson, 1980**). **Juste *et al.*, 1996, 1997** used the electrophoretic investigation of proteins to resolve the cladistic relationships of several species of *Rousettus*. Also, the molecular level has been reviewed a general evolutionary in Pteropodidae and Rhinopomatidae which detecting of genetic differences among species (**Colgan and Flannery 1995; kirsch *et al.*, 1995; Alvarez *et al.*, 1999; Just, 2002; Hulva and Horacek, 2002; Hulva *et al.*, 2007 ., Levin, 2008; and Goodman *et al.*,2010**). Molecular data invalidated the traditional subdivision of bats into suborders Megachiroptera and Microchiroptera when **Teeling *et al.*, 2000, 2002, 2005** provided molecular evidence supporting sister position of one clade of microbats, Rhinolophoidea with megabats, Pteropodoidea within the suborder Yinpterochiroptera. According to molecular genetics, the family Rhinopomatidae is arranged among superfamily Rhinolophoidea (**Van Den Bussche and Hoofer, 2004; Eicki *et al.*, 2005 and Teeling *et al.*, 2005**) and it was regarded as the most primitive group of microchiroptera close to the common ancestor of microbats and megabats (**Eicki *et al.*, 2005**). Moreover, the taxonomic and molecular characteristics of Rhinopomatidae are shared with suborder Yangochiroptera and suborder Yinpterochiroptera (**Hulva *et al.*, 2007**).

The present study was mainly focused on the similarities and divergences between and within the *Rousettus aegyptiacus* and *Rhinopoma hardwickei* commonly present in the local environment by investigation of the standard, G-banded bone marrow chromosomes and the electrophoretic protein pattern analysis.

2. Material and Methods

Animals

The present work was carried out on the Fruit bat (*Rousettus aegyptiacus*) and lesser tailed bat (Rhinopoma *hardwickei*). The Fruit bat(*Rousettus aegyptiacus*) and the Lesser tailed bat (*Rhinopoma hardwickei*) were trapped from Abu-Rawash area, Giza governorate, Cairo, Egypt.

Chromosomal Preparations

Metaphase chromosomes were prepared from bone marrow cells by using the air-dried technique as described by **Hliscs *et al.* (1997)** with some modifications. Some slides were stained with conventional Giemsa stain to investigate the number and morphology of metaphase chromosomal spreads and other slides were stained with Giemsa-trypsin banded stain.

G-Banding Technique

Giemsa banding stain was carried out according to the procedure of **Burgos *et al.* (1986)** with some modifications.

Microscopic Examination and Karyotyping

From conventionally Giemsa stained slides, 30 metaphase spreads / animal were investigated to determine the regular diploid number. Photomicrographs were captured from conventionally Giemsa-stained and Giemsa-trypsin banded metaphase plates. Each individual chromosome was cut out and homologous chromosomes were then sorted out in pairs based on similarities of G-banding patterns and arranged in a descending order according to the relative length. Identification of the chromosomal morphology based on centromere location followed that proposed by **Levan *et al.* (1964).**

Protein Electrophoresis (SDS/PAGE):-

Protein electrophoresis was carried out with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Liver samples of six fruit bats (*Rousettus aegyptiacus*) and five lesser tailed bats (*Rhinopoma hardwickei*) were utilized in this study. Liver sample was grounded with 1 ml of 1X extraction buffer; (10% SDS, 10 ml glycerol, 1M Tris-HCl and 0.25M EDTA, pH 8.8) and left overnight in refrigerator. Then, the samples were centrifuged and the clear supernatants containing water-soluble proteins were used for electrophoresis. Liver soluble protein fractions were separated exclusively on a vertical slab gel (19.8 cm x 26.8 cm x 0.3 cm) using the gel electrophoretic apparatus (Manufactured by APPEX) according to the method of **Laemmli (1970)** as modified by **Payne (1976).** The final monomer concentration in the 0.75 mm-thick slab gels was 12 % (w/v) for the separating gel and 4% (w/v) for the stacking gel. Prior to loading, all samples were incubated in the presence of 1% (w/v) SDS and 100 mM DTT for 5 min at 100^{o}C. The samples were run at a constant voltage of 200 V applied for 45 min. The proteins were visualized by Coomassie brilliant Blue stain.

Gels and Statistical Analysis

Gels were documented using a digital camera (SONY®, 5 MP) and on the basis of the band mobility. The clear bands were scored using Totallab® 120 Gel analysis program (Nonlinear Inc., Durham NC, USA). As "1" for presence while "0" for absence in a binary data form, while the unclear unidentified bands were excluded automatically by the program.

Genetic similarity and genetic distance were estimated within and among species according to **Nei and Li, 1979**. The index of similarity between individuals was calculated using the formula: $S.I = 2n_{xy} / (n_x+n_y)$, where S.I.is the similarity index value, n_{xy} is the number of protein bands shared by individuals $_x$ and $_y$, n_x and n_y are the number of detected protein bands scored for each individual. G.D= 1- S.I, where G.D is the genetic distance (dissimilarity). The similarity coefficients were used to construct dendrogram using the unweighted pair group Methods with Arithmetic averages (UPGMA) method from NTSYS-pc package (**Rohlf, 2000**). Also, phylogenetic analyses were done by neighborjoining (NJ) method (**Saitou and Nei 1987**).

3. Results

In the present work, the standard karyotype and the G-banding technique were utilized for comparative karyotyping of metaphase chromosomes of the Fruit bat (*Rousettus aegyptiacus*) and lesser tailed bat (*Rhinopoma hardwickei*). As well as, SDS-PAGE for liver soluble protein was carried out to determine the similarity and divergence between and within the two species**.**

1-Standard karyotype of *Rousettus aegyptiacus*: (Fig.1)

The diploid number (2n) = 36 chromosomes and NFa= 68 without the sex chromosomes. The chromosomes are arranged in descending order according to the absolute length. The karyotype is composed of 9 pairs of metacentric, 4 pairs of submetacentric and 4 pairs of subacrocentric autosomes (Fig.1). The X-chromosome is a medium sized submetacentric which comprises about 5.6% of the female haploid complement and is about the size of the marker autosome (no.10). The Y-chromosome is a minute acrocentric which comprises about 0.7% of the female haploid complement (Table.1). A pair of medium sized metacentric autosome (No.10) is characterized by the presence of a secondary constriction near the centromere and this pair has variable size in the same genome (refer to Fig.1). Also sometimes pair No.7 and 8 shows polymorphism in size in the same genome. The smallest autosomes (No.17) show polymorphism in the type (2 autosomes are subacrocentric or one autosomes subacrocentric and the other is submetacentric) (Figs.1 & 2).

2- The characteristics of the G-banding pattern of each chromosome of the *Rousettus aegyptiacus* (Figs. 2 & Fig. 5):

The G-banded karyotype of *Rousettus aegyptiacus* is present in Fig.2 and idiogram Fig.5. Most chromosomal pairs are characterized by distinct banding patterns, and the homology of most arms can be determined with reasonable accuracy. Pairs No. 9, 14, 15, shows a G-negative band in the short arm. Pair No.10 contains a broad G- negative region around the centromere corresponding to the secondary constriction of the marker chromosome No.10 of standard karyotype in Fig.1. Pair No.17 shows completely heterochromatin and polymorphism in type which sometimes one autosome is subacrocentric and the other is submetacentric. Also Pairs No.5, 13 and 16 shows completely heterochromatin in short and long arms. Pairs No. 1,2,3,4,5,6,10,12,13 and 14 contains a broad G- negative region around the centromere .The X-chromosome is characterized by a G- negative band in the distal region of the long arm, which is bordered on each side by G- positive bands. The Y chromosome has completely heterochromatin.

3-Standard karyotype of *Rhinopoma hardwickei*: (Fig.3)

The diploid number 2n=36 chromosomes and FNa= 68 without sex chromosomes. The autosomes of this species consist of 13 metacentric pairs, 3

submetacentric pairs and one subtelocentric pairs (Fig.3) . The X chromosome is a medium- sized submetacentric which comprises relative length about 6.4% of the female haploid complement and it is equal in size to pair No. 5&6. The Y chromosome is acrocentric and comprises about 1.2% of the female haploid complement (Table.2, Fig.3).

4- The characteristics of the G-banding pattern of each chromosome of the *Rhinopoma hardwickei* (Figs.4 & 5):

Most chromosomal pairs are distinctly banded, and homologous pairs can be readily distinguished from other autosomes in the genome. Pairs No. 13 and 15 contains a broad G- negative region around the centromere and shows completely heterochromatin in short and long arm. Also, pair no.5 contains a broad G- negative region around the centromere. The Y chromosome has completely heterochromatin

5-Comparison standard karyotype between *Rousettus aegyptiacus* and *Rhinopoma hardwickei*:-

The non- banded karyotypes of *Rousettus aegyptiacus* and *Rhinopoma hardwickei* are quite similar in both diploid number (36) and FNa (68). *Rousettus aegyptiacus* and *Rhinopoma hardwickei* have almost some identical standard karyotype. Pair No. 7 in each *Rousettus aegyptiacus* and *Rhinopoma hardwickei* is subtelocentric and the relative length equal 6.3 and 6.1 respectively. Pairs No.6 (SM) in *Rousettus aegyptiacus* is similar to pair No.4 (SM) in *Rhinopoma hardwickei* and the relative length is 6.6 and 6.8 respectively. Pairs No. 13(SM) in *Rousettus aegyptiacus* is similar to pair No.14 (SM) in *Rhinopoma hardwickei* and the relative length is 3.4% and 4.1% in *Rousettus aegyptiacus* and *Rhinopoma hardwickei* respectively. Also pairs No. 12,14,15 and 16 (M) with relative length about 4,2.6,2.6 and 2.4 in *Rousettus aegyptiacus* are similar to pairs No. 12,13,15 and 16(M) with relative length about 4.7,4.4,3.7 and 3 in *Rhinopoma hardwickei* respectively. Also, pairs No.1,2,4 and 5 with relative length 10,9.3,8.7 and 6.6 in *Rousettus aegyptiacus* were equal to No.1,2,3 and 5 with relative length 8.8,8.5,6.8 and 6.4 in *Rhinopoma hardwickei* respectively.

6- The characteristics of the G-banding pattern of each chromosome of the *Rousettus aegyptiacus* and *Rhinopoma hardwickei*

The comparisons of the G-banding haploid genomes of the *Rousettus aegyptiacus* and *Rhinopoma hardwickei* are presented in Fig.5, which the results of these comparisons substantially differ with those presented from standard karyotypes in Fig.1and 3. By using G-band patterns found that standard karyotype similarities can be misleading. Regardless the size of the bands, the G-banded karyotypic comparison of Rousettus *aegyptiacus* (Figs.2&5) and *Rhinopoma hardwickei* (Figs.4&5) reveals homology of the chromosomal pairs No. 2, 16 and Y chromosome in both two species and the chromosome No. 13 of the *Rousettus aegyptiacus* is similar to the chromosome No. 15 of the *Rhinopoma hardwickei*; also chromosome No.5 of *Rousettus aegyptiacus* is similar to the chromosome No.13 of the *Rhinopoma hardwickei*. Moreover, they share in some pairs of the chromosomal arms.

7- SDS-PAGE analysis of liver soluble proteins for *Rousettus aegyptiacus*

The liver protein patterns of the 6 individuals from *Rousettus aegyptiacus* are demonstrated in Fig.6 and the analysis were summarized in table 3. According to the relative mobility of bands and molecular weight, a total of 23 bands were detected. Eleven common bands were observed among individuals at molecular weight of 118,60,50,46,42,40,36, 34, 30, 29 and 27 kDa. Band frequencies ranged from 0.17 to 1.00 with an average 0.86. The similarity index within this strain *Rousettus aegyptiacus* are represented in Table 4. Similarity coefficient value ranged from 0.76 to 1.00 with an average of 0.853 (85.3%) indicating a great homogeneity among individuals in this strain. The relationship among the individuals fruit bat, *Rousettus aegyptiacus* is presented in the form of dendrogram (Fig.7). Also, Fig.8 illustrates NJ tree with branch length values. The tree topology was presented the sister relationship of *Rousettus aegyptiacus* samples

8- SDS-PAGE analysis of liver soluble proteins for *Rhinopoma hardwickei*

The liver protein patterns of the 5 individuals from *Rhinopoma hardwickei* are demonstrated in Fig.9 and the analysis were summarized in table 5. According to the relative mobility of bands and molecular weight, a total of 19 bands were detected .Eight common bands were observed among individuals at molecular weight of 118,105,80,65,55,48,40,and 38 KDa. Band frequencies ranged from 0.20 to 1.00 with an average 0.67. The similarity matrix within this strain *Rhinopoma hardwickei* is represented in Table 6. Similarity coefficient value ranged from 0.67 to 0.96 with an average of 0.776 (77.6%). The relationship among the individuals *Rhinopoma hardwickei* is presented in the form of dendrogram (Fig.10). Fig.11 illustrates NJ tree with branch length values. The tree topology was presented the sister relationship of *Rhinopoma hardwickei* samples.

9-Comparaison SDS-PAGE analysis of liver soluble proteins for both two species

Liver soluble proteins of the fruit bat, *Rousettus aegyptiacus* and lesser –tailed bat, *Rhinopoma hardwickei* were separated by SDS-PAGE technique. The soluble muscle protein of the fruit bat are separated into 23 bands that ranged from 118 to 26 kDa (Fig.6,Table 3), while that of the lesser – tailed bat are separated into 19 bands that ranged from 118 to 24 kDa (Fig. 9, Table 5). Both strains share 2 common bands with molecular weights 118 and 40 kDa (Table 7). The gel analysis within and among the individuals of the two strains are presented in table 7. The present study revealed that the fruit bat soluble protein is characterized by 8 bands with molecular weights 70,60,50,42,36,31,29 and 27 kDa while ,the lesser tailed bat is characterized by 4 bands with molecular weights 113,105,65 and 24KDa (Table 7). *Rhinopoma hardwickei* is characterized by unique band at molecular weight 113. The similarity coefficient was calculated according to the total number of protein fractions and the number of sharing bands, where the similarity coefficient value is 0.095 (9.5%) and the polymorphic loci among the two species is 0.926 (92.6%). The relationship between the fruit bat, *Rousettus aegyptiacus* and lesser –tailed bat, *Rhinopoma hardwickei* is presented in the form of dendrogram (Fig.12). This dendrogram shows two main clusters which one is formed by the *Rousettus aegyptiacus* samples R.ae1- R.ae6, while the other cluster is formed by the *Rhinopoma hardwickei* samples R.ha 1- R.ha.5.

Table (1): Calculations for the relative length, arm ratio, centromeric index and classification of each metaphase chromosome derived from five karyotypes of bone marrow cells of the fruit bat, *Rousettus aegyptiacus*

Chromosome number	Relative length (%)			Centromeric index	Aram ratio	Classification
	Short arm	Long arm	Total			
1	4.76	5.28	10.04	47.37	1.11	M
2	4.62	4.62	9.25	50	1	M
3	2.38	6.61	8.98	26.5	2.78	SM
4	04.36	4.36	8.72	50	1	M
5	3.30	3.30	6.61	50	1	M
6	2.38	4.23	6.61	36	1.78	SM
7	1.32	5.02	6.34	20.83	3.8	ST
8	1.72	4.36	6.08	28.26	2.54	SM
9	1.06	4.23	5.55	19.05	4	ST
10	2.77	2.77	5.55	50	1	M
11	1.06	3.70	4.76	33.3	3.5	ST
12	1.72	2.25	3.96	43.3	1.31	M
13	1.19	2.25	3.43	34.62	1.89	SM
14	1.06	1.59	2.64	40	1.5	M
15	1.32	1.32	2.64	50	1	M
16	1.19	1.19	2.38	50	1	M
17	0.40 or 0.26	0.92 or 1.06	1.32	30 or 20	2.33 or 4	SM or SA
X	1.85	3.70	5.55	33.33	2	SM
Y	0	0.66	0.66	α	0	A

M= Metacentric, SM= submetacentric SA= Subacrocentric; A= Acrocentr

Table (2): Calculations for the relative length, arm ratio, centromeric index and classification of each metaphase chromosome derived from five karyotypes of bone marrow cells of the lesser tailed bat, *Rhinopoma hardwickei*

Chromosome number	Relative length (%)			Centromeric index	Arm ratio	Classification
	Short arm	Long arm	Total			
1	4.39	4.39	8.78	50	1	M
2	4.22	4.22	8.45	50	1	M
3	3.21	3.55	6.76	47.5	1.11	M
4	2.03	4.73	6.76	30	2.33	SM
5	03.04	3.38	6.42	47.37	1.11	M
6	3.21	3.21	6.42	50	1	M
7	1.35	4.73	6.08	22.22	3.5	ST
8	2.70	3.04	5.74	47.06	1.13	M
9	1.52	4.05	5.57	27.27	2.67	SM
10	2.52	2.53	5.07	50	1	M
11	2.20	2.70	4.90	44.83	1.23	M
12	2.36	2.36	4.73	50	1	M
13	2.03	2.36	4.39	46.15	1.17	M
14	1.18	2.87	4.05	29.17	2.43	SM
15	1.86	1.86	3.72	1.250	1	M
16	1.52	1.52	3.04	50	1	M
17	1.42	1.45	2.87	49.41	1.02	M
X	2.36	4.05	6.42	36.84	1.71	S.M
Y	0	1.18	1.18	0	0	A

M= Metacentric, SM= submetacentric SA,Subacrocentric; A= Acrocentr

Table(3): The binary data obtained from protein gel electrophoresis of *Rousettus aegyptiacus* species

BN	RF	MW	1	2	3	4	5	6	BF
1	0.027	118	1	1	1	1	1	1	1.00
2	0.095	100	1	1	0	0	0	1	0.50
3	0.130	96	1	1	1	1	0	1	0.83
4	0.156	80	1	1	0	1	0	1	0.67
5	0.171	75	1	1	1	0	1	0	0.67
6	0.206	70	1	1	0	1	1	1	0.83
7	0.253	60	1	1	1	1	1	1	1.00
8	0.301	55	1	1	0	0	1	1	0.67
9	0.339	50	1	1	1	1	1	1	1.00
10	0.374	48	1	1	0	1	0	0	0.50
11	0.400	46	1	1	1	1	1	1	1.00
12	0.472	42	1	1	1	1	1	1	1.00
13	0.516	40	1	1	1	1	1	1	1.00
14	0.550	38	1	1	0	0	1	1	0.76
15	0.622	36	1	1	1	1	1	1	1.00
16	0.666	34	1	1	1	1	1	1	1.00
17	0.705	32	1	1	1	1	0	1	0.83
18	0.732	31	1	1	1	0	1	1	0.83
19	0.766	30	1	1	1	1	1	1	1.00
20	0.813	29	1	1	1	1	1	1	1.00
21	0.856	28	0	0	0	0	0	1	0.20
22	0.878	27	1	1	1	1	1	1	1.00
23	0.926	26	1	1	1	1	1	0	0.83

BN= band number RF=relative front MW= molecular weight in kilo Dalton BF= band frequency

Table (4): The similarity index and genetic distance based on SDS-PAGE of liver protein data within *Rousettus aegyptiacus.*

G.I G.D	1	2	3	4	5	6
1	1	0	0.16	0.16	0.13	0.10
2	1	1	0.16	0.13	0.13	0.10
3	0.84	0.84	1	0.15	0.15	0.22
4	0.84	0.87	0.85	1	0.24	0.19
5	0.87	0.87	0.85	0.76	1	0.19
6	0.90	0.90	0.78	0.81	0.81	1

Table(5): The binary data obtained from protein gel electrophoresis of *Rhinopoma hardwickei* species.

BN	RF	MW	1	2	3	4	5	BF
1	0.029	118	1	1	1	1	1	1.00
2	0.050	113	0	0	0	1	0	0.20
3	0.086	105	1	1	1	1	1	1.00
4	0.117	100	1	0	1	1	0	0.60
5	0.135	96	0	1	0	0	1	0.4
6	0.155	80	1	1	1	1	1	1.00
7	0.1670	75	1	0	0	0	0	0.20
8	0.236	65	1	1	1	1	1	1.00
9	0.315	55	1	1	1	1	1	1.00
10	0.318	48	1	1	1	1	1	1.00
11	0.409	46	1	0	0	0	0	0.20
12	0.481	40	1	1	1	1	1	1.00
13	0.574	38	1	1	1	1	1	1.00
14	0.690	34	1	0	0	1	0	0.40
15	0.742	32	1	0	1	1	0	0.60
16	0.777	30	0	1	1	0	1	0.60
17	0.806	28	1	1	0	1	1	0.80
18	0.869	26	1	0	0	0	1	0.40
19	0.931	24	1	0	1	0	0	0.40

BN= band number RF=relative front MW= molecular weight in kilo Dalton BF= band frequency

Table (6) : The similarity matrix and genetic distance based on SDS-PAGE of liver protein data within *Rhinopoma hardwickei.*

S.I G.D	1	2	3	4	5
1	1	0.33	0.21	0.17	0.29
2	0.67	1	0.22	0.25	0.04
3	0.79	0.78	1	0.20	0.25
4	0.83	0.75	0.80	1	0.28
5	0.71	0.96	0.75	0.72	1

Table(7): Analysis for both individuals of two species (*Rousettus aegyptiacus & Rhinopoma hardwickei*).

BN	MW	R.ae1	R.ae2	R.ae3	R.ae4	R.ae5	R.ae6	R.ha1	R.ha2	R.ha3	R.ha4	R.ha5	BF
1	118	1	1	1	1	1	1	1	1	1	1	1	1.00
2	113	0	0	0	0	0	0	0	0	0	1	0	0.09
3	105	0	0	0	0	0	0	1	1	1	1	1	0.45
4	100	1	1	0	0	0	1	1	0	1	1	0	0.55
5	96	1	1	1	1	0	1	0	1	0	0	1	0.64
6	80	1	1	0	1	0	1	1	1	1	1	1	0.82
7	75	1	1	1	0	1	0	1	0	0	0	0	0.45
8	70	1	1	0	1	1	1	0	0	0	0	0	0.45

9	65	0	0	0	0	0	0	1	1	1	1	1	0.45
10	60	1	1	1	1	1	1	0	0	0	0	0	0.55
11	55	1	1	0	0	1	1	1	1	1	1	1	0.82
12	50	1	1	1	1	1	1	0	0	0	0	0	0.45
13	48	1	1	0	1	0	0	1	1	1	1	1	0.73
14	46	1	1	1	1	1	1	1	0	0	0	0	0.64
15	42	1	1	1	1	1	1	0	0	0	0	0	0.55
16	40	1	1	1	1	1	1	1	1	1	1	1	1.00
17	38	1	1	0	0	1	1	1	1	1	1	1	0.82
18	36	1	1	1	1	1	1	0	0	0	0	0	0.55
19	34	1	1	1	1	1	1	1	0	0	1	0	0.73
20	32	1	1	1	1	0	1	1	0	1	1	0	0.73
21	31	1	1	1	0	1	1	0	0	0	0	0	0.45
22	30	1	1	1	1	1	1	0	1	1	0	1	0.82
23	29	1	1	1	1	1	1	0	0	0	0	0	0.55
24	28	0	0	0	0	0	1	1	1	0	1	1	0.45
25	27	1	1	1	1	1	1	0	0	0	0	0	0.55
26	26	1	1	1	1	1	0	1	0	0	0	1	0.64
27	24	0	0	0	0	0	0	1	0	1	0	0	0.33

BN= band number RF=relative front MW= molecular weight in kilo Dalton
BF= band frequency R.ae= *Rousettus aegyptiacus* . R.ha= *Rhinopoma hardwickei* .

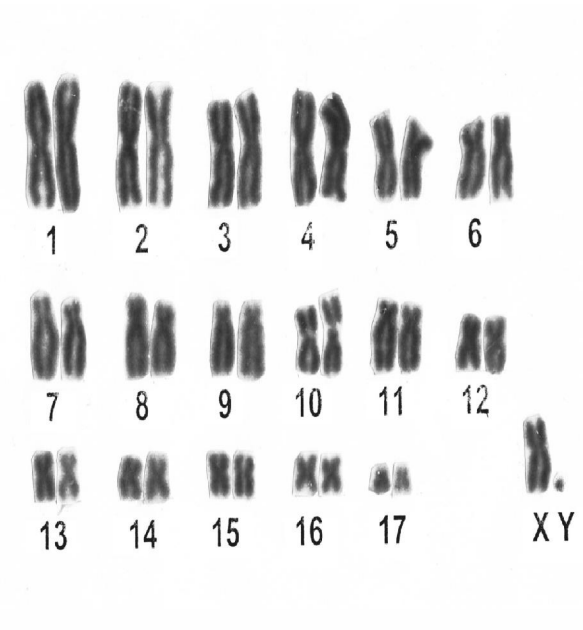


Figure (1): Standard chromosomal karyotype prepared from bone marrow cells of the fruit bat, *Rousettus aegyptiacus*

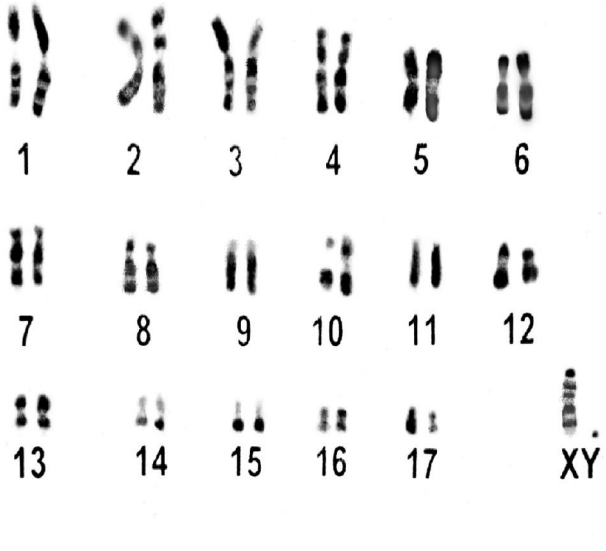


Fig Figure (2) : G-banded chromosomal karyotype prepared from bone marrow cells of the fruit bat, *Rousettus aegyptiacus* .

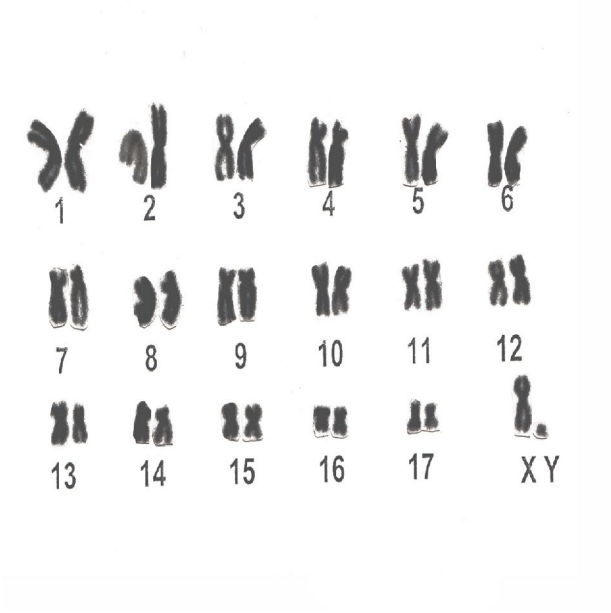


Figure (3): Standard chromosomal karyotype prepared from bone marrow cells of the lesser –tailed bat. *Rhinopoma hardwickei*

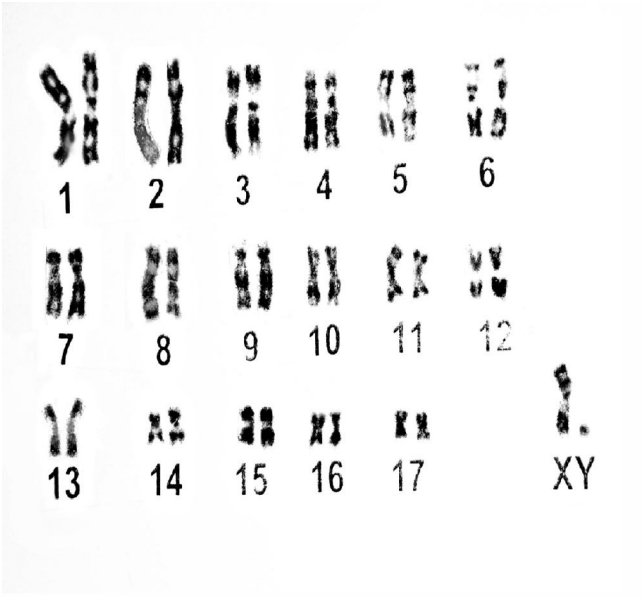
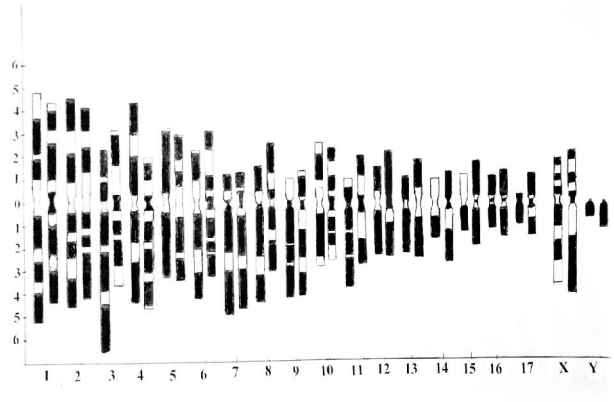


Figure (4): G-banded chromosomal karyotype prepared from bone marrow cells of the lesser –tailed bat. *Rhinopoma hardwickei*.



Com **Figure (5):** Comparative idiogram for G-banded chromosomes of the *Rousettus aegyptiacus* and *Rhinopoma hardwickei*.Note that: the chromosomes are arranged in pairs with the left handed chromosome for the fruit bat, *Rousettus aegyptiacus* and the right-handed chromosome for the lesser–tailed bat,*Rhinopoma hardwickei.*

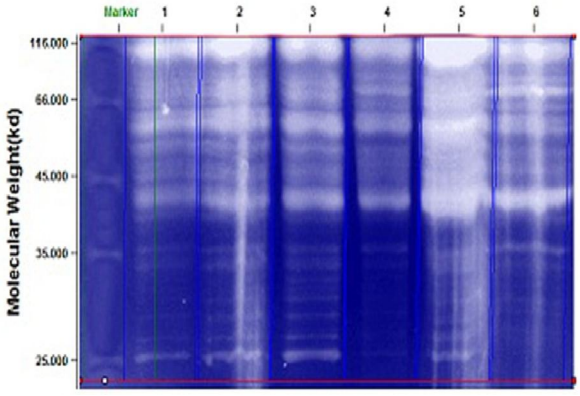


Figure (6): Liver protein profile bands of *Rousettus aegytiacus* separated on a SDS-PAGE. Lane 1 represents protein marker. Lanes 2-7 represent liver *Rousettus aegytiacus* samples from 1 to 6.

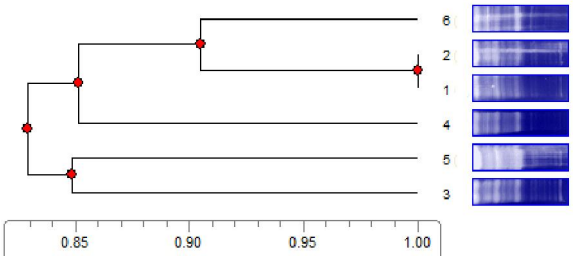
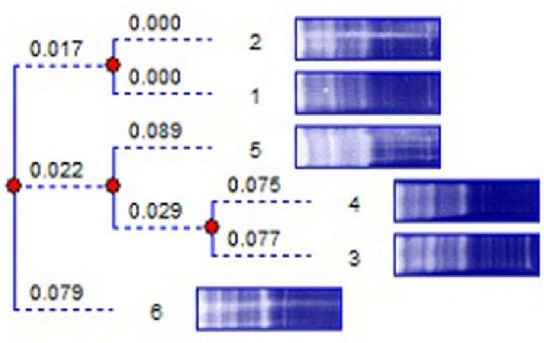


Figure (7):Dendogram on genetic relationship among the *Rosettus aegyptiacus* based on protein data.



Figure(8): Phylogenetic tree of the *Rosettus aegyptiacus* samples relationship as obtained by Neighbor-joining method.

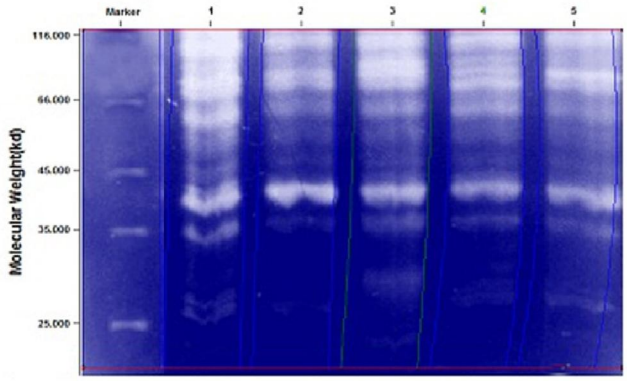


Figure (9): This gel shows a variety of different proteins being separated on a polyacrylamide gel. Lane 1 represents protein marker. Lanes 2-6 represent liver *Rhinopoma hardwickei* samples from 1 to 5

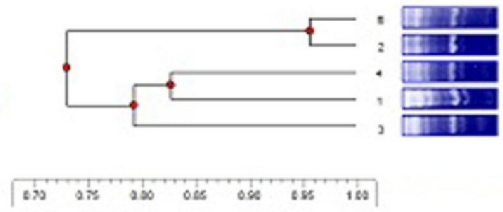


Figure (10): Dendogram on genetic relationship among the *Rhinopoma hardwickei* based on protein data.

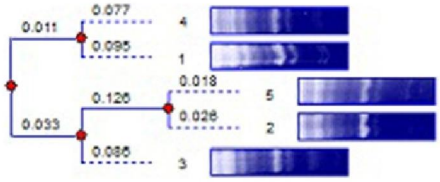


Figure (11): Phylogenetic tree of the *Rhinopoma hardwickei* samples relationship as obtained by Neighbor-joining (NJ) method.

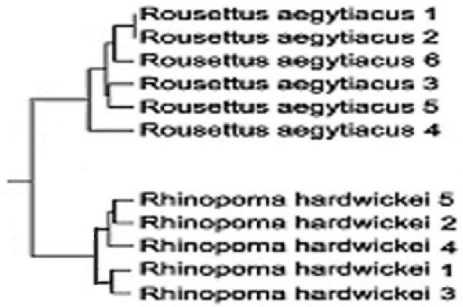


Figure (12): Dendrogram on genetic relationship within and among *Rousettus aegyptiacus* and *Rhinopoma hardwickei* species based on protein data.

4. Discussion

The order Chiroptera are the second most order of mammals, after the rodents (**Redi *et al*., 2005**). Several authors recorded that the families Rhinopomatidae (microbats) and pteropodidae (megabats) belong to the suborder Yinpterochiroptera (**Springer *et al.*, 2001 and Teeling *et al*., 2000, 2005**).

Chromosomal banding, Biochemical and molecular studies of the identification of bats have been examined previously by several authors. In the present work, the standard karyotype of *Rousettus aegyptiacus* (2n= 36, FNa=68, Fig.1) is identical to *Rousettus amplexicaudatus* and *Rousettus Leschenaulti* (**Ray- Chaudhuri *et al*., 1968; Harada *et al*., 1982; Rickart, 1989 and Mao *et al*., 2007**) although our terminology for those same chromosomes is slightly different (9 metacentric, 4 submetacentric, 4 subtelocentric, a medium submetacentric X-chromosome and a smallest acrocentric Y- chromosome). However, the reports of *Rousettus aegyptiacus* (2n=36, FNa=66) by **Karatas *et al*., 2003 and Albayrak *et al*., 2008** revealed the karyotypic similarity to the *Rousettus aegyptiacus* in the present work but differs in terminology of some chromosomes and in the smallest pair number 17 which is acrocentric rather than subacrocentric. Also, the standard karyotype of the fruit bat, *Eonycteris spelaea* (Peteropodidae) was similar to that of *Rousettus aegyptiacus* in the present work but differs in its subacrocentric Y- chromosome (**Harada *et al*., 1982**). Also, the standard karyotype of *Rousettus aegyptiacus* in the present work was reported as indistinguishable from the fruit bats, *Myonycteris torquata* and *Lissonycteris* (*Rousettus*) *angolensis* (**Haiduk *et al*., 1980**) but differ in its smallest pair of subacrocentric. A pair of medium sized metacentric autosome (No.10) is characterized by the presence of a secondary constriction near the centromere and this pair has variable size (polymorphism in relative

length) in the same genome (refer to Fig.1). This result confirms the earlier results on *Rousettus* Spp. of **Ray- Chaudhuri *et al*., 1968; Harada *et al.,* 1982 and Rickart *et al*., 1989.**

The standard karyotype of *Rhinopoma hardwickei* (2n= 36, FNa= 68) in the present work is indistinguishable from that described from specimens from India (**Ray- Chaudhuri *et al*., 1968**) although our terminology for those same chromosomes is slightly different (13 pairs metacentric, 3 pairs submetacentric, 1pair subtelocentric, medium sized submetacentric X- chromosome and a very small acrocentric Y-chromosomes.

In the present work, the *Rousettus aegyptiacus* and *Rhinopoma hardwickei have* the same 2n=36 chromosomes and FNa=68 chromosomal arms. This diploid numbers (36) is very near to the primitive diploid number of bats (2n=36) (**Baker, 1970**). Therefore *Rousettus aegyptiacus* and *Rhinopoma hardwickei* were considered to be ancestors of most bats (**Baker, 1970**). *Rousettus aegyptiacus* and *Rhinopoma hardwickei* have almost identical standard karyotype. These results are similar to **Ray-Chudhuri *et al*., 1968** which stated that such extensive similarities between the chromosomes complements of these two species cannot be just accidental, but should reflect close genetic relationships between pteropodidae and Rhinopomatidae. In the present work, the relative lengths of the corresponding chromosome pairs between the two species have showed some a relatively sharp variation. The differences between the two species can be explained by pericentric inversion (**Ray- Chudhuri *et al*., 1968**).

By using G-banding technique several phenomena of chromosomal evolution and rearrangements such as Robertsonian fusion and fission, increase in heterochromatin, inversion, and tandem translocation can be deduced (**Gibson, 1984**). The G- band karyotype of *Rousettus aegyptiacus* in the present work (Fig.2) is identical to *Rousettus amplexiacus* (**Haiduk *et al.,* 1981**). Also , the G-band karyotype of *Rousettus aegyptiacus* in this work appear homologous to *Myonycteris torquoto; Lissonycteris* (*Rousettus*) *angolensis* (**Haiduk *et al*., 1981**) with the exception of chromosomal rearrangements in some pairs and an additional of heterochromatin to pair 17 produced biarmed pair increasing the fundamental number. Also, the most chromosomes of the fruit bats *Epomops franquetti*, *Epomophorous wahlbergi, Epomophorous minor* and *Hyrsignathus monstrosus* (2n= 36 and FNa= 68) are homologous to those found in *Rousettus aegyptiacus* in the present work with the exception of chromosomal rearrangements in some pairs (**Haiduk *et al*., 1981**).

The G- band karyotype of *Rhinopoma hardwickei* (Fig.4) in the present work is identical to *Rhinopoma hardwickei* from Palestine (**Qumsiyeh and Baker, 1985**).

Moreover, the corresponding chromosomal pairs of both two species could be matched to detect the conserved chromosomes in both two species. To test the conclusion of standard karyotype in the present work, we have compared the G- banded karyotypes of *Rousettus aegyptiacus* and *Rhinopoma hardwickei* (Fig. 5). From the ideogram (Fig.5), our results demonstrate that the standard karyotype similarities between the two species can be misleading.

Regardless the size of the bands, the sequences of the banding pattern of the chromosomal pairs No. 2, 16 and the Y-chromosome are similar in both two species (Refer to Fig.5). Apart from the chromosomal size and the chromosome position in ideogram, the chromosome No. 13 of the *Rousettus aegyptiacus* is similar to the chromosome No. 15 of the *Rhinopoma hardwickei*; also the chromosome No.5 of *Rousettus aegyptiacus* is similar to the chromosome No.13 of the *Rhinopoma hardwickei*. This indicates that these chromosomes are conserved and did not show visible structural chromosomal rearrangements. Also, we found that they share in some pairs of the chromosomal arms. The X-chromosomes in the two species are dissimilar in the sequences of the banding pattern. This result confirms the earlier results of **Qumsiyeh and Baker, 1985** with some few differences.

In the present work, sharp and distinct protein bands could be obtained by using SDS-PAGE for the *Rousettus aegyptiacus* and *Rhinopoma hardwickei* soluble proteins (Figs. 6 and 9). In the present work, SDS-PAGE electrophoresis technique demonstrated little genetic variation among the samples of the fruit bat *Rousettus aegyptiacus* which the genetic distance showed significant 0.15 of variance among samples. The genetic variation among the samples may be referred to the migrations between the populations and panmictic between the individuals. The higher similarity coefficient 0.85 (85%) found in both samples studied of *Rousettus aegyptiacus*, may be attributed to the small phylogenetic distances between them. Little protein electrophoretic studies have been conducted to identify the genetic differences among *Rousettus species* all over the world. In this regard, **Webb and Tidemann,1996** found low Fst genetic variation among fruit bats, *Pteropus alecto* (0.023); *P. poliocephalus* (0.014) and *P. scapulatus* (0.028) by using protein electrophoresis. Also, **Sinclair *et al*., 1996** reported very little genetic variation (5%) among the samples of fruit bat *Pteropus scapulatus* by using Allozyme electrophoresis technique. Moreover 22 enzymes were examined to assess the

polymorphism among Rousettus species by using a starch-gel electrophoretic analysis (**Juste *et al*., 1997**). Also, cytochrome b data and genotypic data from microsatellite loci showed that *Rousettus madagascariensis* and *Rousettus obliviosus* are strongly supported as sister to each other relative to other Rousettus species (**Goodman *et al*., 2010**).

Furthermore, the difference in protein banding pattern between the *Rhinopoma hardwickei* samples indicate that they were not pure subspecies but they were a hybrid of *Rhinopoma hardwickei* subspecies. No literature studies were recorded on protein of *Rhinopoma hardwickei*. In the present work, there is high variation between the samples . **Hulva *et al*., 2007** which found deep genetic divergences within *Rhinopoma hardwickei* lineages of different localities by using cytochrome b technique. Moreover, the genetic distance between the Iranian species (*Rhinopoma hardwickei hardwickii*) and the Jordan, Syria, Yemen (*Rhinopoma hardwickei arabium*); Upper Egypt and Libya (*Rhinopoma hardwickei cystops)* species showed high variation in the genetic distance (8-9%). In the other hand, **Hulva *et al*.(2007)** and **Levin *et al*. (2008)** found a high genetic similarity (low genetic diversity) within the greater mouse-tailed bat, *Rhinopoma microphyllum* from several populations by using mitochondrial markers. On the other hand, **Akmali *et al*. (2011)** found no significant differences between the Iranian samples, *Rhinopoma microphylum harrisoni* (0.0021 – 0.0045), indicating a close relationship among the haplotypes but they found genetic variation (2.079) between Rhinopoma *microphylum harrisoni* from Iran and *Rhinopoma microphylum microphylum* from Levant and Morocco.

In the present work the two molecular weights 65 and 70 in *Rousettus aegyptiacus* and *Rhinopoma hardwickei* respectively are comparable with the vertebrate albumin (65-70KDa) (**Peters, 1996; Metcalf *et al*., 1998**) .The two species share 2 similar bands with molecular weights 118 and 40kDa. From this, the two species show low similarity coefficient of 0.905 (9.5%). Therefore, they were not closely related to each other. From the present dendrogram (Fig.12) two main clusters were presented; one is formed by the *Rousettus aegyptiacus* while the other cluster is formed by *Rhinopoma hardwickei*.

As a conclusion, the *Rousettus aegyptiacus* and *Rhinopoma hardwickei* were not identical. The results of the previously mentioned studies led to the conclusion that the G- banding chromosomes and protein banding patterns are species-specific and the taxonomical position of *Rousettus* and *Rhinopoma* species and subspecies are argued and questionable. Moreover, the uniform protein bands of the *Rousettus aegyptiacus* were clear and sharp which indicate the purity of their strain, while the *Rhinopoma hardwickei* showed variation in number of bands which may indicate the hybrid origin of this strain and alteration of their habitat. From these results, the two species is a separate from each other. Therefore, the G-banding technique and protein electrophoresis are a powerful tool in detecting the polymorphism and the genetic relationship within and between the different species. .

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