Genetic Diversity among Five Egyptian Non-Poisonous Snakes Using Protein and Isoenzymes Electrophoresis

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Abstract: The present work is an attempt to discover the genetic diversity among five Egyptian non-poisonous snakes; *Psammophis sibilans sibilans, Psammophis schokari aegyptius, Spalerosophis diadema, Lytorhynchus diadema and Coluber rhodorachis* by using SDS-PAGE electrophoresis for two water soluble isoenzymes as well as protein of liver samples. Obtained results revealed that, protein samples showed a total of 21 bands with molecular weight ranged from 250-18 kDa. 12 common bands were recorded in all species. Also, the genetic similarity is 83.8% among all species. The *Spalerosophis diadema* is closer to the *Coluber rhodorachis* (90%) than to *Lytorhynchus diadema* (89%) while the highest similarity is present between *Lytorhynchus diadema* and *Coluber rhodorachis* (92%). Moreover, there is a high similarity between *Psammophis sibilans sibilans* and *Psammophis schokari aegyptius* (91%). The two isoenzymes; *α*-esterase (*Est*) and peroxidase (*Px*) yielded 9 heterogeneous alleles arranged in six loci. The genetic similarity is 27.2% between all species. The high similarity observed between *Spalerosophis diadema* and *Coluber rhodorachis* (67%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (50%) and the similarity between *Lytorhynchus diadema* and *Coluber rhodorachis* is 50%. It is concluded that, the Species in the same subfamily have high similarity coefficient. The phylogenetic tree showed that, the *Psammophis* species are grouped in one cluster and the other colubrid species are grouped in other cluster.
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1. Introduction:

Extensive molecular genetic diversity has been discovered within and among populations and species whoever its first discovery in proteins (**Zuckerkandl and Pauling, 1965**), isozymes/allozymes (**Lewontin, 1974**) and DNA (**Kimura, 1983**). The technique of protein electrophoresis has contributed greatly to resolving systematic problems in many groups of organisms (**Avise, 1994; Mishra *et al*., 2010**). The usefulness of allozyme data to identify phylogenetic relationships has long been recognized as genetic markers (**Murphy *et al*., 1990 and 1996**). The suborder Serpents is distributed in the entire world (**McDowell, 1987; Zug *et al*., 2001**).The family Colubridae is the most diverse, widespread, and contains greater than 1800 species within all of Serpents (**Pough*et al*., 2004**). **Goodman and Hobbs (1994**) recorded the distribution of colubrid species of the family Colubridae in the northern portion of the Egyptian Eastern Desert which include: *Coluber florulentus,* C. *rhodorachis,* C. *rogersi, Lytorhynchus diadema, Malpolon moilensis, Psammophis schokari, P. aegyptius,* and *Spalerosophis diadema.* There are as within Egypt where *P. aegyptius* and *P. schokari* are sympatric and both have been collected in the Egyptian Eastern Desert (**Goodman *et al*., 1985**). Previous descriptions of the external and taxonomical features of some snakes have been ambiguous and unreliable. Therefore, several authors used the karyological studies (**Pinou and Dowling, 1994**), biochemical electrophoresis (**Dowling *et al.*, 1983 and 1996; Murphy and Crabtree, 1985; Dessauer*et al*., 1987; Cadle, 1988; Highton, *et al*., 2002**) and molecular sequence analysis (**Zaher *et al*., 2009; Pyron *et al*., 2011**) to resolve the cladistic relationships among snakes and to clarify their phylogeny. Snakes classification into subfamilies remains dissenting subjects (**McDowell, 1987; Kelly *et al*., 2003**). The monophyly of the subfamilies Colubrinae, Natricinae, Psammophinae, and Xenodontinae appears to be common to several molecular studies (**Dowling *et al*., 1996; Kelly *et al*., 2003**). Family Colubridae is now represented by twelve genera (*Dolichophis*, *Eirenis*, *Hemorrhois*, *Lytorhynchus*, *Malpolon, Natrix*, *Platyceps*, *Psammophis*, *Rhagerhis, Rhynchocalamus*, *Spalerosophis* and *Telescopus*) including 24 species **(Amr and Disi, 2011)**. The generic diagnosis for *Psammophis sp* carried out by **Kelly *et al.* (2008)**. *Psammophis aegyptius* (**Marx, 1958**) was formerly considered as a subspecies of *Psammophis schokari* but later on it is currently recognized as a distinct species **(Schleich *et al.* 1996).**

This research aimed to illustrate the genetic diversity between and within some common Egyptian colubrid snakes of the family Colubridae by using the electrophoresis analysis of two isoenzymes and SDS-protein. Moreover, an attempt was carried out to

verify the traditional morphological classification of the species of this study.

2. Materials and Methods:

2.1. Species:

Five Egyptian colubrid species were collected from different localities of Egypt (Table 1). Morphological identification and classification of the animals as well as scientific and common names of these species was carried out according to previous studies **(Anderson, 1898; Marx, 1968; Goodman and Hobbs, 1994)**. The work is carried out on two samples of *Psammophis sibilans sibilans, Psammophis Schokari aegyptius* and *Spalerosophis diadema* and one sample of Coluber *rhodorachis* and *Lytorhynchus diadema*. The five species are belonging to four genera and two subfamilies.

2.2. Tissue preparation:

Liver sample of each animal was taken and homogenized in phosphate buffer and centrifuged with high speed centrifuge (10,000 rpm for 10 min). Supernatants (water soluble proteins and isoenzymes) were stored at -20^{o}C for further electrophoretic analysis.

2.3. SDS-Protein:

SDS-polyacrylamide gel electrophoresis was performed in 11 % acrylamide slab gels following the system of **Laemmli (1970)**. A volume of 20 µL of the mixture was loaded in the gel. After electrophoresis, the gel was stained by Coomassie brilliant blue. The gel was distained and after the appearance of the bands is photographed.

2.4. Isoenzymeselectrophoresis:

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical system, thermostated at 4–6°C by a circulator cooling bath. Enzyme samples were loaded on to 21 x 22.8 cm plates with 4 mm thickness. Reservoir buffer was cold tris–glycine (pH 8.3) while bromophenol blue was used as marker front–dye. Gels were electrophoresed in 10% native-polyacrylamide gel as described by **Stegemann *et al.* (1985)** and at a constant current of 10 mA and 20-30 volts. For the two enzymes, 50 µl extract per well was loaded. Each isoenzyme was run in a separate gel.

2.5. Isoenzymes staining:

The gel was stained for α-esterase (α-*Est*) according to **Desborough *et al.* (1967)** with some modifications. The gel was stained in a freshly prepared mixture composed of 100 mg fast blue stain; dissolved in 100 ml phosphate buffer (1.3 g NaH_2PO_4 and 0.3 g Na_2HPO_4) and 125 mg α-naphthyl acetate; dissolved in 1 ml acetone and diluted with 10 ml phosphate buffer, filtered). The gel is kept in staining mixture at 37 °C overnight in dark place.

The gel was stained for peroxidase isoenzyme (*Px*) according to **Van Loon (1971)** with some modifications. The gel was stained in a freshly prepared mixture composed of (200 mg benzidine dissolved in 100 ml dis. water, 1 ml glacial acetic acid and few drops of H_2O_2) The gel is kept in staining mixture at 37°C overnight in dark place.

Gel fixation was carried as follow; the gel was washed two or three times with tap water; fixed in equal volumes of glycerol and water, after 24 h the gel is washed with tap water and photographed.

2.6. Statistics:

All gels of protein and isoenzyme electrophoresis 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. For isoenzymes, the bands of enzyme activity were designated using the known system of nomenclature (**Allendorf and Utter, 1978**). An abbreviation which corresponds to the name of the enzyme designated each locus. When multiple loci were involved, the fastest anodal protein band was designated as locus one, the next as locus two and so on. The allozyme and the specific locus was designated by numerical numbers superscripting the enzyme and the locus number e.g., *EST*–2^{a} means allele no. (a) at *EST* locus–2 and *PX*–1^{b} means allele no. (b) at *PX* locus–1. Genetic similarity and genetic distance were estimated within and among species according to **Nei and Li (1979)**. 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**).

3. Results

1-Comparison SDS-PAGE analysis of liver soluble proteins for the five serpent species:

Liver soluble proteins of five Egyptian snake species were separated by SDS-PAGE technique. Table (2) shows the gel analysis within and among five species. The soluble liver proteins are separated into 21 bands that ranged from 250 to 18 kDa (Fig.1 and Table 2) and band frequency ranged from 0.25-1.00 with mean value equal 74%. Both species share 12 common bands. Table (3) shows that the similarity coefficient between the individual within the same

species is higher than the similarity coefficient between the different species. The similarity matrix was ranged from 73% to 92% with average 83.8% and the genetic distance was ranged from 8% to 27%with average 16.2% between all species. Table (4) demonstrates the similarity coefficient between *Psammophis sibilans sibilans and Psammophis schokari aegyptius* is high (91%). Moreover, similarity coefficient between *Spalerosophis diadema* and Coluber *rhodorachis and* is higher (90%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (89%). The highest similarity is present between *Lytorhynchus diadema* and *Coluber rhodorachis* (92%).As shown in figure (2), the dendrogram is divided into two main clusters; one for Psammophis species while, the other cluster is formed by the rest species. The *Coluber rhodorachis* is clustered to *Lytorhynchus diadema* and the two species are sister clade to *Spalerosophis diadema*.

2-Comparaison isoenzymes analysis for the five serpent species:

Two enzymes were examined by native-polyacrylamide gel electrophoresis; α -esterase (*α-Est*) and peroxidase (*Px*). Figures 3 and 4 show gel profiles and zymograms of isoenzyme α-esterase and peroxidase, respectively. Table (5) shows 9 heterogeneous alleles (bands) with 6 loci. *α-Est* shows four genotypes with seven alleles while *Px* isoenzyme demonstrates two genotypes and two alleles. All of these alleles did not express simultaneously in one sample and they showed different isoenzyme forms. All genotype loci are monomers with one allele except Est-1 in the sample 2 (*Psammophis sibilans sibilans*), *Est-2* in the sample 3 (*Psammophis schokari aegyptius*) and *Est*-4 in samples 1 and 2 (*Psammophis sibilans sibilans*) are dimers with two alleles. *Est*-1 is established in all species except *Lytorhynchus diadema* and *Coluber rhodorachis*. *Est*-2 and *Px*-1 were limited to *Psammophis* species and were not recorded in other species. *Est*-4 is recorded only in *Psammophis sibilans* and *Coluber rhodorachis* samples. Moreover, *Est*-3 is documented in *Spalerosophis diadema* and *Coluber rhodorachis* while *Px-2* is limited to Colubrinae species. Moreover, *Lytorhynchus diadema* showed no *Est* activity. The similarity matrix was calculated according to the total number of alleles and the number of sharing bands, within and among species. Table (6) shows the similarity coefficient between the members within the same species is higher than the similarity coefficient between the different species. As shown in table (7), the similarity coefficient was ranged from 0% to 67% with average 27.2% and genetic distance was ranged from 33% to 100% with an average 72.8% between all the species. The high similarity coefficient is presented between *Spalerosophis diadema* and *Coluber rhodorachis* (67%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (50%) and the similarity coefficient is 50% between *Lytorhynchus diadema* and *Coluber rhodorachis*. In addition to that, the similarity coefficient is 36% between *Psammophis sibilans sibilans* and *Psammophis schokari aegyptius*. In figure (5), the similarity matrix has been calculated according to the number of sharing bands which constructed the clustering of *Psammophis* species in same cluster and the other colubrid species in the other cluster. Moreover, the constructed tree displays that the *Coluber rhodorachis* is sister to *Spalerosophis diadema* and not to *Lytorhynchus diadema*.

Table 1.Scientific name, Common name, Arabic name and locality of five Egyptian snakes

No.	Scientific name	Common name	locality
1	*Psammophis sibilans sibilans* **(Linnaeus, 1758)**	African Beauty snake, Abu Essuyur	Abu Rawash-Giza
2	*Psammophis schokari aegyptius* **(Marx, 1858)**	Egyptian Sand snake, Saharan Sand snake, Harseen	Egyptian Sahara, Faiyum
3	*Spalerosophis diadema* **(Schlegel,1837)**	Clifford's Royal snake, ArqamAhmar	Abu Rawash-Giza
4	*Lytorhynchus diadema* **(Dumeril, Bibron and Dumeril, 1854)**	Diademed Sand Snake, Bisbas	Abu Rawash-Giza
5	*Coluber rhodorachis* **(Jan,1865)**	Azrude Gabaly, **Jan' s** Desert Racer	Sinai

Table (2): The binary data obtained from protein gel electrophoresis based on SDS-PAGE of liver protein data among and within five Egyptian snake species.

B.N	RF	MW	1	2	3	4	5	6	7	8	B.F
1	0.03	250	0	0	0	0	1	1	0	1	0.38
2	0.06	234	1	1	1	1	1	1	1	1	1.00
3	0.11	202	0	0	0	1	1	0	0	0	0.25
4	0.16	174	1	1	1	1	1	1	1	1	1.00
5	0.23	142	1	1	1	1	1	1	1	1	1.00
6	0.27	126	0	0	0	0	1	1	0	0	0.25
7	0.31	112	1	1	1	1	1	1	1	1	1.00
8	0.47	70	1	1	1	1	1	1	1	1	1.00
9	0.52	62	1	1	1	1	1	1	1	1	1.00
10	0.47	57	1	1	1	1	1	1	1	1	1.00
11	0.54	54	1	1	1	1	1	1	1	1	1.00
12	0.58	50	1	1	1	1	1	1	1	1	1.00
13	0.62	45	1	1	1	1	1	1	1	1	1.00
14	0.65	41	1	1	1	1	0	0	0	0	0.50
15	0.69	36	1	1	1	1	0	0	0	0	0.50
16	0.71	34	1	1	1	1	1	1	1	1	1.00
17	0.82	25	1	1	1	1	1	1	1	1	1.00
18	0.83	24	1	1	1	1	0	0	0	1	0.63
19	0.86	22	1	1	0	0	0	0	0	0	0.25
20	0.89	20	0	0	1	1	0	0	0	0	0.25
21	0.93	18	1	1	1	1	0	0	0	0	0.50

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

- columns 1-2 *Psammophis sibilans sibilans*; columns 3-4 *Psammophis schokari aegyptius*; columes5-6, *Spalerosophis diadema*; column 7, *Lytorhynchus diadema* and column 8, *Coluber rhodorachis*

Table 3: The similarity matrix and genetic distances based on SDS-PAGE of liver protein dataamong and within five Egyptian snake species.

G.D G.S	1	2	3	4	5	6	7	8
1	100	0	6	9	25	23	17	23
2	1	100	6	9	25	23	17	23
3	94	94	100	3	25	23	17	16
4	91	91	97	100	21	25	20	19
5	75	75	75	79	100	3	11	10
6	77	77	77	75	97	100	8	7
7	83	83	83	80	89	92	100	8
8	77	77	84	81	90	93	92	100

Table 4: The genetic similarity (upper) and genetic distance (lower) based on SDS-PAGE of liver protein data among the five Egyptian snake species.

G.S G.D	P. si. si.	P. sc. ae.	S. di.	L. di.
P. sc. ae	91 09			
S. di.	75 25	73 27		
L. di.	83 17	80 20	89 11	
C. rh.	84 16	81 19	90 10	92 08

P. si. si=*Psammophis sibilans sibilans*; ***P. sc. ae***=*Psammophis schokari aegyptius;* ***S. di***=*Spalerosophis diadema*; ***L. di***=*Lytorhynchus diadema* and ***C. rh***=*Coluber rhodorachis*

Table (5): The alleles; presence (1) and absence (0) data obtained from electrophoretic pattern of α-*Est* and *Px* isoenzyme within and among five Egyptian snakes.

B.N	RF	Alleles	1	2	3	4	5	6	7	8	B.F
1	0.336	*Est*-4^{b}	1	1	0	0	0	0	0	0	0.25
2	0.386	*Est*-4^{a}	1	1	0	0	0	0	0	1	0.38
3	0.619	*Est*-3	0	0	0	0	1	1	0	1	0.38
4	0.696	*Est*-2 ^{b}	1	0	1	0	0	0	0	0	0.25
5	0.736	*Est*-2 ^{a}	0	1	1	1	0	0	0	0	0.38
6	0.817	*Est*-1 ^{b}	1	1	1	1	1	1	0	0	0.75
7	0.850	*Est*-1^{a}	0	1	0	0	0	0	0	0	0.13
8	0.800	*Px*-2	0	0	0	0	1	1	1	1	0.5
9	0.848	*Px*-1	1	1	1	1	0	0	0	0	0.5

BN= band number RF=relative front BF= band frequency

α-*Est* = α-esterase *Px* =peroxidase

Columns 1-2, *Psammophis sibilans sibilans*; columns 3-4 *Psammophis schokari aegyptius*; columns 5-6, *Spalerosophis diadema*; column 7, *Lytorhynchus diadema* and lane column 8, *Coluber rhodorachis*.

Table (6): The similarity matrix and genetic distances based on isoenzyme electrophoresis data among and within five Egyptian snake species.

G.D G.S	1	2	3	4	5	6	7	8
1	100	27	33	50	75	75	100	75
2	73	100	40	33	78	78	100	78
3	67	60	100	71	71	71	100	100
4	50	67	86	100	67	67	100	100
5	25	22	29	33	100	0	50	33
6	25	22	29	33	100	100	50	33
7	0	0	0	0	50	50	100	50
8	25	22	0	0	67	67	50	100

Table (7): The genetic similarity (upper) and genetic distance (lower) based on isoenzyme electrophoresis data among five Egyptian snake species.

G.S G.D	**P. si. si.**	**P. sc. ae**	**S. di**	**L. di**
P.sc.ae	**36 64**			
S.di	**20 80**	**29 71**		
L.di	**0.00 100**	**0.00 100**	**50 50**	
C.rh	**20 80**	**0.00 100**	**67 33**	**50 50**

P. si. si=*Psammophis sibilans sibilans*; ***P. sc. ae***=*Psammophis schokari aegyptius;* ***S. di***=*Spalerosophis diadema*; ***L. di***= *Lytorhynchus diadema* and ***C. rh***= *Coluber rhodorachis*

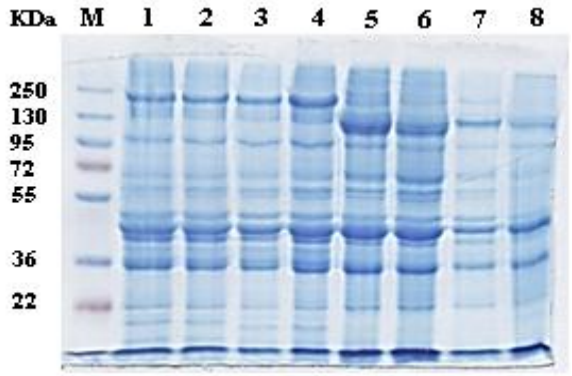

Figure (1): Liver protein profile bands of five Egyptian snakes separated on a SDS-PAGE. M represents protein marker. Lanes 1-2, *Psammophis sibilans sibilans*; lanes 3-4 *Psammophis schokari aegyptius*; lanes5-6, *Spalerosophis diadema*; lane 7, *Lytorhynchus diadema* and lane 8, *Coluber rhodorachis*.

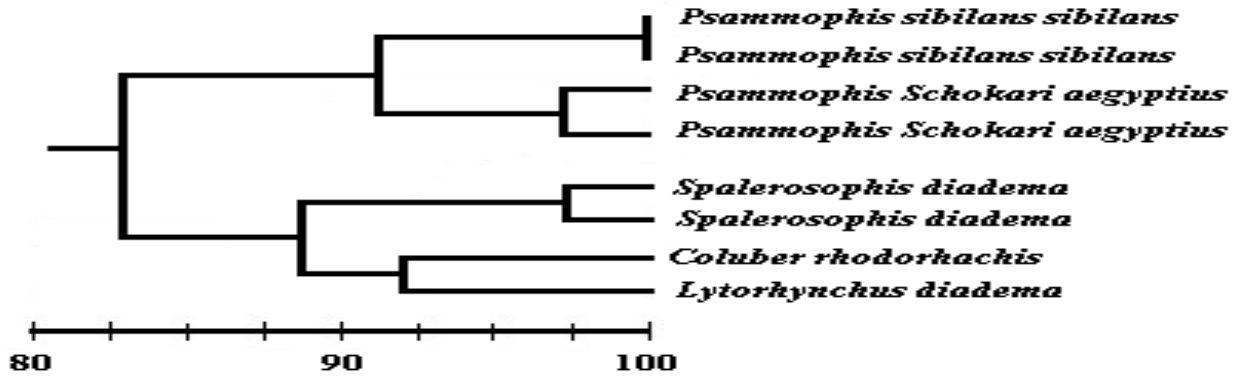

Figure (2): Dendrogram on genetic relationship within and among the five Egyptian snakes based on protein data.

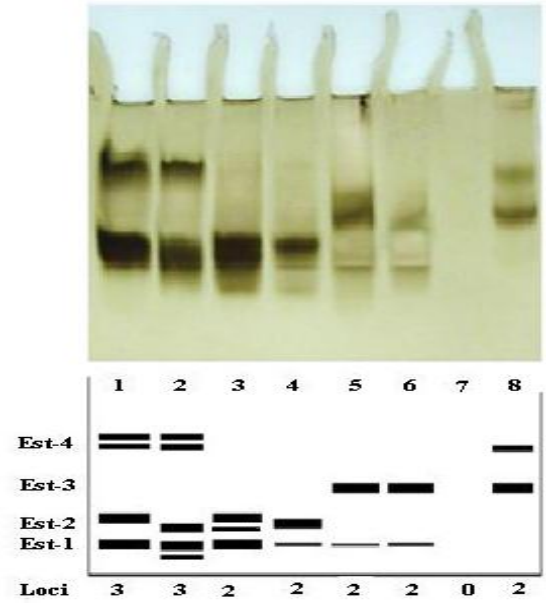

Figure (3): Profile and zymogram of α–esterase isoenzyme bands of five Egyptian snakes.

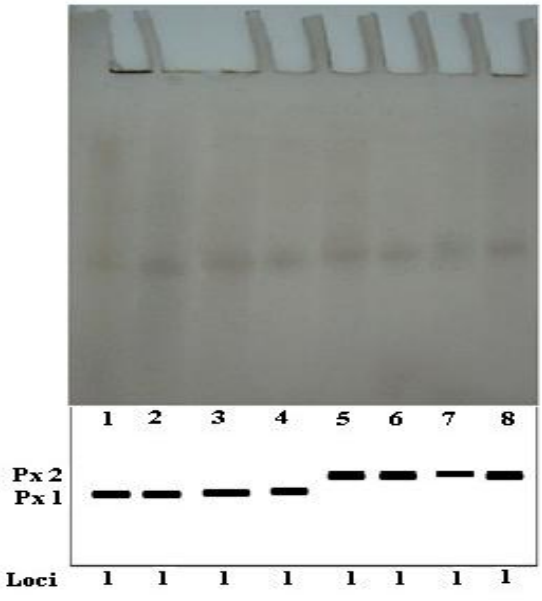

Figure (4): Profile and zymogram of peroxidase isoenzyme bands of five Egyptian snakes.

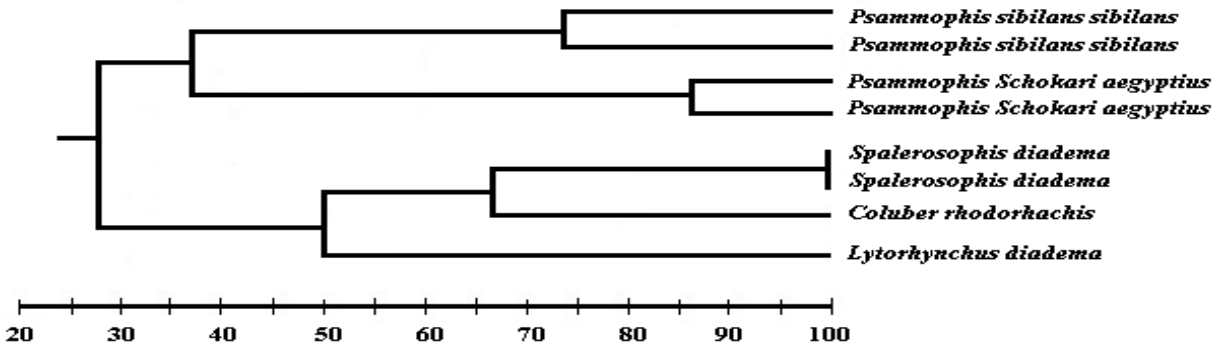

Figure (5): Dendrogram on genetic relationship within and among the five Egyptian snakes based on isoenzymes data.

4. Discussion:

Polymorphic proteins are useful markers for identifying different snakes and for studying their breeding forms, population genetics, and problems concerned with species development (**Dessauer *et al*., 1987**). Although many questions remain unsolved about the evolution of snakes, their genetic diversity, population structure, speciation, historical biogeography and phylogeny are evidently resolved through revising the protein structure. In the present study, the Colubridae snakes were clearly distinguished by using two electrophoretic parameters; the enzymes loci and the SDS-PAGE of liver protein into two clusters and several clades at different levels which are similar with previous studies suggesting paraphyly or polyphyly of this family (**Lawson and Dessauer, 1981**; **Dowling *et al.,* 1983**). However, the phylogenetic relationships among major clades of the Colubridae remain unresolved (**Dessauer *et al*., 1987**). In the present study, the electrophoretic banding of proteins indicated that the species of the family Colubridae have high sharing bands within the molecular weight of the range between 234;174-142;112 -45 kDa and in the range from 34-35 kDa. In the present work the high sharing bands between the colubrid species suggest that the subfamilies, Psammophinae and Colubrinae are belong to the family Colubridae. These results are similar to the previous studies (**Kelly *et al.,* 2003**; **Lawson *et al*., 2005**) by using DNA sequences.

In the present work, unexpected great close relationship between *Spalerosophis diadema*, *Coluber rhodorachis* and *Lytorhynchus diadema* was detected. This result supports the previous studies by **Lawson *et al.* (2005)** and **Pyron *et al*. (2011)** which presented that the genus *Lytorhynchus* is sister to a clade composed of the genera *Spalerosophis* and *Coluber*. In addition, the present work showed that, there is high similarity between *Spalerosophis diadema* and *Coluber rhodorachis* (90%) and the genetic distance between them is smaller (10%) by using SDS-PAGE of protein. This result is similar to that present by **Schatti and Utiger (2001)** which found 7% genetic diversity between *Spalerosophis diadema* and *Platyceps (Coluber) rhodorachis* by using molecular DNA sequences. Therefore these two species are considered to have the same monophyletic ancestor (**Schatti and Utiger, 2001**).

Spalerosophis diadema is more closer to *Coluber rhodorachis* (90%) than to *Lytorhynchus diadema* (89%) but *Coluber rhodorachis* is more related to *Lytorhynchus diadema* (92%) by using SDS-PAGE of protein. Moreover, *Spalerosophis diadema* is closer to *Coluber rhodorachis* (67%) than to *Lytorhynchus diadema* (50%) and *Coluber rhodorachis* has less similar to *Lytorhynchus diadema* (50%) by using isoenzymes of protein. Therefore, the evolutionary history of snakes still remains controversial and ambiguous. In addition to this, *Lytorhynchus diadema* has not any fragment with the other snakes in this work at *Est* loci. This result is similar to blind snakes (Typhlopidae-Colubrinae) which share no alleles with any other snake (**Dowling *et al*., 1996**). Then this study suggests that allozyme evolution among colubrid snakes has been conservative for some loci. The species in the subfamily Colubrinae shows more similarity to each other. These results were similar to **Dowling *et al.* (1983)** and **Lawson (1987)** which indicated that the members of Colubrinae are relatively closely related worldwide by using Albumin and transferrin immunological comparisons and electrophoretic studies.

However, phylogenetic studies using small allozymes and small sample sizes provide capable to distinguish relationships at the species level or at subfamilies level. This has shown similarity with other data (**Hillis, 1987**; **Crother *et al*., 1992**; **Crother, 1999**) which indicated that, the reduced

samples did not change inferred relationships found from larger samples and suggest that small sample sizes often are enough to positively estimate phylogeny. In spite of that, the small number of loci (two) and the sampling of only one and two illustrative of each species. Esterase enzyme profile for the eight snakes showed a variation between snakes of *Psammophis sibilans sibilans* and those of *Psammophis schokari aegyptius* that expect to be produced an identical band pattern. This variation in relative mobility and band numbers may be related to their physiological, biochemical and immunological processes to tolerate seasonal variation and environmental conditions (**Silva *et al*., 2011**; **Tosunoglu *et al*., 2011**).

In conclusion, the species in the same subfamily are more related to each other than between the species in the different subfamilies and displayed significant supports with some molecular and taxonomical studies.

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