Genetic Diversity among Eight Egyptian Snakes (Squamata-Serpents: Colubridae) Using RAPD-PCR

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Abstract: Genetic variations between 8 Egyptian snake species, *Psammophis sibilans sibilans, Psammophis Sudanensis, Psammophis Schokari Schokari, Psammophis Schokari aegyptiacus, Spalerosophis diadema, Lytorhynchus diadema, , Coluber rhodorhachis, Coluber nummifer* were conducted using RAPD-PCR. Animals were captured from several locality of Egypt (Abu Rawash-Giza, Sinai and Faiyum). Obtained results revealed a total of 59 bands which were amplified by the five primers OPB-01, OPB-13, OPB-14, OPB-20 and OPE-05 with an average 11.8 bands per primer at molecular weights ranged from 3000-250 bp. The polymorphic loci between both species were 54 with percentage 91.5 %. The mean band frequency was 47% ranging from 39% to 62% per primer .The similarity matrix value between the 8 Snakes species was ranged from 0.35 (35%) to 0.71 (71%) with an average of 60%. The genetic distance between the 8 colubrid species was ranged from 0.29 (29%) to 0.65 (65%) with an average of 40 %. Dendrogram showed that, the 8 snake species are separated from each other into two clusters .The first cluster contain *4 species* of the genus *Psammophis*. The second cluster includes the 4 species of the genera, *Spalerosophis; Coluber* and *Lytorhynchus*. *Psammophis sibilans* is sister to *Psammophis Sudanensis* with high genetic similarity (71%) and *Psammophis Schokari Schokari* is sister to *Psammophis Schokari aegyptiacus* with high genetic similarity (70%). The *Coluber rhodorhachis* are clustered and closer to *Spalerosophis diadema* (70%) than to *Coluber nummifer* (57%). Therefore, the evolutionary history of snakes still remains controversial. It is concluded that, the similarity coefficient and the genetic distance value between the 8 snake species indicates that, the 8 snake species are not identical and separated from each other.

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1. Introduction

The squamates are the most diversified group containing the lizards and snakes **(Vidal and Hedges, 2009)**. Several investigations have been recorded on the fauna of Egypt reptiles **(Anderson 1898; Marx, 1958 and 1968; Werner, 1983; Goodman and Hobbs, 1994)**. The suborder Serpents is distributed all over the world **(McDowell, 1987; Zug *et al*., 2001)**. The Superfamily Colubroidea represents nearly 2500 species of extant snakes **(Gasperetti, 1988; Pough *et al*., 2004)**. The monophyletic Colubroidea Snakes species were established primarily on basis of external and taxonomical features **(Zaher, 1999)**. Also, the herpetological studies are subdivided Colubroidea into the families Viperidae, Elapidae, Atractaspididae, Colubridae **(Pough *et al*., 2004)**, while **Dowling and Jenner (1988)** reserved the superfamily Colubroidea to the families Colubridae and Natricidae. Morever, **Zaher *et al.* (2009)** classified the family Psammophidae within the superfamily Elapoidea and the families Colubridae and Natricidae was kept within the superfamily Colubroidea. Also, colubroids have been studied in historical biogeography **(Pyron and Burbrink, 2009; Daza *et al*., 2010)**. 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 **(Cadle, 1988; Dowling *et al.,* 1996)** and molecular sequence analysis **(Lawson *et al*., 2005; Burbrink and Pyron, 2008; Wiens *et al.*, 2008; Kelly *et al*., 2009; Vidal *et al*., 2009; Zaher, *et al*., 2009; Pyron, *et al*., 2011)** to resolve the cladistic relationships among snakes and to clarify their phylogeny. Major changes to colubroid taxonomy have been proposed based on molecular studies **(Lawson *et al*., 2005; Burbrink *et al*., 2007; Zaher *et al*., 2009)**. Also, the molecular RAPD-PCR technique has been used as an important tool in genetic studies of snakes **(Prior *et al*., 1997; Jaggi *et al*., 2000; Dutra, *et al.,* 2008)**.

Although their morphology has been investigated previously, there are still major gaps in our knowledge of the relationships of these animals. These gaps may hide important differences between ancient taxonomies and molecular phylogenies which yet, the few species and genera were included in these phylogenies , leaving the classification of many genera in question **(Lawson *et al*., 2005; Kelly *et al*., 2009; Zaher *et al*., 2009)**.

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**) have been recorded the distribution of colubrid species of the family colubridae in the northern portion of the Egyptian Eastern Desert. These include: *Coluber florulentus, C. rhodorhachis, C. rogersi, Lytorhynchus diadema, Malpolon moilensis, Psammophis schoknri, P. aegyptius,* and *Spalerosophis diadema.* There are areas within Egypt where *P. aegyptius* and P. *schokari* are sympatric and both have been collected in the Egyptian Eastern Desert **(Goodman *et al.*, 1985)**. The classification of this group into subfamilies remain dissenting Topics **(McDowell, 1987; Vidal and Hedges, 2002; Kelly *et al.*, 2003; Nagy *et al.*, 2003)**. The Colubridae comprise 12 subfamilies, Xenodermatinae, Pareatinae, Calamariinae, Homalopsinae, Boodontinae, Pseudoxyrhophiinae, Colubrinae, Pseudoxenodontinae, Natricinae, Dipsadinae, and Xenodontinae **(Zaher, 1999)**. The monophyly of the subfamilies Colubrinae, Natricinae, Psammophiinae, and Xenodontinae appears to be common to several molecular studies **(Cadle, 1988; Dowling *et al.*, 1996; Gravlund, 2001; Kelly *et al.*, 2003)**. While, **Dowling and Jenner (1988)** reserved the subfamily Colubrinae and Natricinae within the families Colubridae and Natricidae, respectively. 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 molecular phylogenetic relationships of the colubrid species (subfamily Colubrinae) were recorded by several authors **(Lawson *et al.*, 2005; Gravlund, 2001; Kelly *et al.*, 2003)**.

Kelly, *et al* (2008) found that the family Psammophiidae includes eight genera and about 50 species. Phylogenetic studies of the family Psammophiidae has been establish based on immunological data **(Cadle, 1994)** and mitochondrial DNA sequences **(Gravlund, 2001; Vidal and Hedges, 2002; Nagy *et al.*, 2003; Kelly *et al.*, 2008)**. The generic diagnosis for Psammophis carried out by **Broadley (2002) and Kelly *et al.* (2008)**. **Bons and Geniez (1996)** found that the genus *Psammophis* of the subfamily Psammophiinae includes 24 species, most of them with an African origin, but some also occur in the Middle East and Asia. *Psammophis schokari* is widespread in North Africa having a Saharo-Sindian distribution; it is also found in the Middle East, Arabia, Iran, a large part of Afghanistan, Uzbekistan and northwest India **(Geniez *et al.* 2004)**. *Psammophis aegyptius* Marx, 1958, was formerly considered as a subspecies of *Psammophis schokari* but is currently recognized as a distinct species **(Schleich *et al.* 1996)**. In Morocco/Western Sahara, three distinct morphotypes have been recorded for *P. schokari:* the striped form; the unicoloured and the Western-Sahara form with a slightly less slender body, weakly striped pattern and greyish belly **(Bons and Geniez 1996; Rato, *et al.*, 2007)**. The occurrence of striped and unicoloured morphotypes has also been recorded in Israel and Sinai **(Kark *et al.* 1997; Rato *et al.*, 2007)**. **Broadley (1977, 2002)** involved the species *schokari, aegyptius, punctulatus, elegans* and *trigrammus* in the ‘‘Psammophis schokari group”. **Kelly *et al.* (2008)** noted that the *P. cf. sibilans* (Ethiopian), *P. rukwae*, *P. subtaeniatus, P. sudanensis* and *P. orientalis* are involved in the one group. The RAPD technique has been used as a important tool in genetic studies of snakes **(Prior *et al.*, 1997; Jaggi *et al.*, 2000; Dutra, *et al.*, 2008)**. Also, **Broadley (1977)** reported data of *Psammophis sibilans*.

This investigation aimed to illustrate the genetic diversity between some common Egyptian colubrid snakes of the family Colubridae by using RAPD- PCR technique.

2. Materials and Methods

Animal dealer collected eight Egyptian colubrid species (snakes) from different localities of Egypt. The eight species are belonging to four genera. Morphological identification and classification of the animals as well as scientific and common names of these species was carried out according to previous works **(Anderson, 1898; Marx, 1968; Goodman and Hobbs, 1994)**. The studied species are present in Table 1.

Genomic DNA extraction

Muscle tissue from the snakes were taken and stored at -20 ^{o}c. DNA extracted according to the method of **Sambrook (1989)** with slight modifications. DNA quality and concentration determined by spectrophotometric analysis and run in 0.7 % agarose gel. Each sample of DNA was examined by optical density values at 260 and 280 nm. Optical density ratios evaluated and only good quality DNA samples were used in PCR.

RAPD-PCR reaction

15 primers from Kits OP-B , OP-E and OP-O (Operon Technologies, Alameda, CA, USA) used for RAPD-PCR analysis (OPB-01, OPB-05, OPB-09, OP-10, OPB-12, OPB-13, OP-B14, OPB-17, OPB-19, OP-B20, OPE-01, OPE-05 OPE-10, OPO-01 and OPO-03). Only 10 primers (OPB-01, OPB-09, OPB-12, OPB-13, OPB-14, OPB-17, OPB-19, OPB-20, OPE-05 and OPO-03) were reacted well and used to amplify DNA from all species (table 2). It selected five primers (OPB-01, OPB-13, OPB-14, OPB-20 and OPE-05) (fig. 1-5) which had shown some variation among eight snake species. RAPD-PCR reactions carried out as described by Williams *et al*., (1993). PCR cycles

performed with 60 s, 94°C initial denaturation and 35 cycles of 20 s, 94°C; 20 s 35°C; and 30 s 72°C. Final extension performed at 72°C for 5 min. PCR amplifications were carried out in 96 well Thermal cycler (Eppendorf Master Cycler) and all amplifications were carried out at two times. A PCR mixture without template DNA placed in each analysis as a control. The PCR products separated on 1.5 % agarose gels (Sigma) containing ethidium bromide in 0.5 X TBE buffer at 100 V constant voltages. For evaluating the base pair length of bands, DNA ladder (Fermentas) was loaded with each gel.

Data and statistical analysis:

The RAPD banding patterns scored for the presence (1) and absence (0) of bands for each sample. The scores obtained using all primers in the RAPD analysis combined to create a single data matrix. The statistical analysis of the data included the calculation of allele frequencies according to **Nei (1987)** and the number and percentage of polymorphic loci according to **Nei (1973)**. Genetic similarity and genetic distance were estimated among species according to **Nei and Li (1979)**. Based on the genetic similarity matrix, the species were clustered by the unweighted pair group method with arithmetic averaging (UPGMA) using the program NTSYS-pc version 2.1 **(Rohlf, 1999)**.

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

No.	Scientific name	Common name	locality
1	*Psammophis sibilans sibilans*, **(Linnaeus, 1758)**	African Beauty snake, Abu Essuyur	Abu Rawash-Giza
2	*Psammophis Sudanensis*	Sudanensis snake	Faiyum- Cairo
3	*Psammophis Schokari Schokari*	Schokari Sand snake	Abu Rawash-Giza
4	*Psammophis Schokar aegyptius*, **(Marx, 1858)**	Egyptian Sand snake, Saharan Sand snake, Harseen	Egyptian Sahara, Faiyum
5	*Spalerosophis diadema*, **(Schlegel,1837)**	Clifford's Royal snake, Arqam Ahmar	Abu Rawash-Giza
6	*Lytorhynchus diadema*, **(Dumeril, Bibron and Dumeril, 1854)**	Diademed Sand Snake, Bisbas	Abu Rawash-Giza
7	*Coluber rhodorhachis rhodorhachis*, **(Jan,1865)**	Azrude Gabaly, **Jan' s** Desert Racer	Sinai
8	*Coluber nummifer*, **(Reuss, 1834)**	Coin Marked Snake, Arqam Baity	Sinai

Table 2: Sequence of primers employed in molecular phylogenetic relationship among eight snake species

Primers	Sequence	
B-01	5'-GTTTCGCTCC-3'	60%
B-09	5'-TGGGGGACTC-3'	70%
B-12	5'-CCTTGACGCA-3'	60%
B-13	5'-TTCCCCCGCT-3'	70%
B-14	5'-TCCGCTCTGG-3'	70%
B-17	5'-AGGGAACGAG-3'	60%
B-19	5'-ACCCCCGAAG-3'	70%
B-20	5'-GGACCCTTAC-3'	60%
E-05	5'-TCAGGGAGGT-3'	60%
O-03	5'-CTGTTGCTAC-3'	50%

3. Results

In the present study, only selected five primers (OPB-01, OPB-13, OPB-14, OPE-05 and OPB-20) out of the 15 random primers produced a PCR product for the investigation of the genetic variation between the eight studied serpents (colubrid) species. The primer B-13 produces much more of amplified fragments for the genomic DNA of the 8 colubrid species in comparison to the other primers. The five primers established 59 different bands scored for the presence or absence of bands among the eight snake species. The results of the RAPD analysis are present in the table (3) in which a total of 59 scorable amplified bands with an average 11.8 bands/primer at molecular weights ranged from 3000 to 250 bp between the eight colubrid species. Out of them 54 (91.5%) polymorphic bands were recorded with an average 10.8 bands/ primer. The numbers of RAPD bands are ranged from 10 to 17 bands/primer with polymorphic bands ranging from 7 to 16 per primer. The RAPD profile generated from these primers (Figs. 1, 2, 3, 4 and 5) and the RAPD scoring bands have utilized to estimate the band frequency. The mean band frequency was 47% for all snakes ranging from 39% to 62% per primer. The unique band ranged from 0 to 3.

The similarity matrix among the eight species is presented in table (4) which was estimated based on RAPD bands scored. The mean similarity coefficient value between the eight snake species was ranged from

0.35 (35 %) to 0.71 (71 %) with an average of 0.60 (60%).The genetic distance between the eight species was ranged from 0.29 (29 %) to (65 %) with an average of 0.40 (40 %). The species of *Psammophis sibilans sibilans and Psammophis sudanensis,* are closer to each other which have low genetic variation and high genetic similarity (71%). Also, *Psammophis schokar schokar* and *Psammophis schokari aegyptiacus* are nearer to each other with high genetic similarity (70%). *Psammophis sibilans* is more similar to *Psammophis sudanensis* than *Psammophis schokar schokar* (56 %) and *Psammophis Schokari aegyptiacus* (57%). The Colubrid, *Coluber rhodorhachis* is closer to the *Spalerosophis diadema* (70 %) than the *Coluber nummifer* (58 %). Moreover, The *Lytorhynchus diadema* in the separate clade of the subfamily colubrinae is more similar to *Coluber nummifer* (54 %) than to *Coluber rhodorhachis* (52 %) and *Spalerosophis diadema* (50%).

The UPGMA dendrogram was constructed to show phylogenetic relationships among the 8 snake species based on genetic similarity (Fig. 5). The phylogenetic tree constructed using an unweighted pair group method with arithmetic (UPGMA) method and similarity matrix indicates that the eight snakes are clustered into two main clusters. The first cluster contains 4 snake species belong to the subfamily Psammophinae. Within the subfamily Psammophinae, the four species are collected in two clades which *Psammophis sibilans* is sister to *Psammophis sudanensis* in the first clade with high genetic similarity (71%) and *Psammophis schokar schokar* is sister to *Psammophis schokari aegyptiacus* in the second clade with high genetic similarity (70%). The second cluster includes 4 colubrid species belong to the subfamily Colubrinae. Within the subfamily Colubrinae, the four species are grouped in two major clades, the *Lytorhynchus diadema* first clade and *Spalerosophis diadema, Coluber rhodorhachis* and *Coluber nummifer* second clade. The *Spalerosophis diadema* and *Coluber rhodorhachis* are sister clade to each other with high genetic similarity (70%) and the two species form a common branch which clustered with *Coluber nummifer*.

Table 3: Total number of bands, polymorphic bands, % of polymorphic bands, Mean band frequency, Unique bands and their size range (bp) for different primers of eight colubrid species.

Primers	Total No. bands	No. polymorphic bands	% of polymorphic bands	Band frequency	Mean sharing band frequency	Unique band	Size range (bp)
OP-B13	17	16	94.1%	0.13-1	0.41	3	3000-250
OP-B14	12	12	100%	0.25-0.75	0.40	1	2400-220
OP-B1	10	7	70%	0.13-1	0.62	2	2600-250
OP-E5	10	9	90%	0.25-1	0.58	0	1400-250
OP-B20	10	10	100%	0.13-0.63	0.39	0	1300-250
Total (average)	59(11.8)	54(10.8)	91.5 %		0.47		3000-250

Table 4: The similarity matrix and genetic distances among the eight snake species (according to **Nei and Li, 1979**).

	1	2	3	4	5	6	7	8
1		0.290	0.443	0.429	0.520	0.542	0.538	0.556
2	0.710		0.429	0.442	0.538	0.516	0.52	0.607
3	0.557	0.571		0.302	0.649	0.564	0.593	0.509
4	0.571	0.586	0.698		0.538	0.600	0.481	0.464
5	0.480	0.462	0.351	0.462		0.500	0.300	0.360
6	0.458	0.484	0.436	0.400	0.500		0.478	0.458
7	0.462	0.480	0.407	0.519	0.700	0.522		0.423
8	0.444	0.393	0.492	0.536	0.640	0.542	0.577	

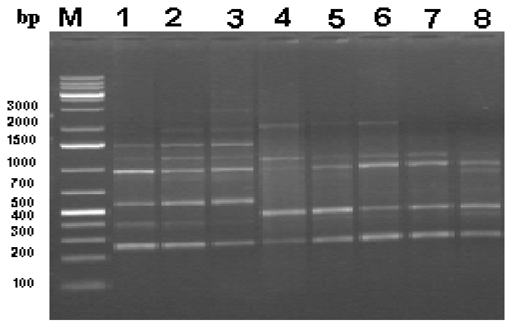

Figure 1: Gel electrophoresis represents RAPD -PCR products for DNA from eight colubrid species (Lanes 1 to 8) with OPB-01 primer. M, DNA marker with molecular size, (1kb plus, Fermantas). 1, *Psammophis sibilans sibilans;* 2, *Psammophis Sudanensis;* 3, *Psammophis Schokari Schokari;* 4. *Psammophis Schokari aegyptiacus;* 5, *Spalerosophis diadema;* 6, *Lytorhynchus diadema;* 7, *Coluber rhodorhachis rhodorhachis;* 8, *Coluber nummifer.*

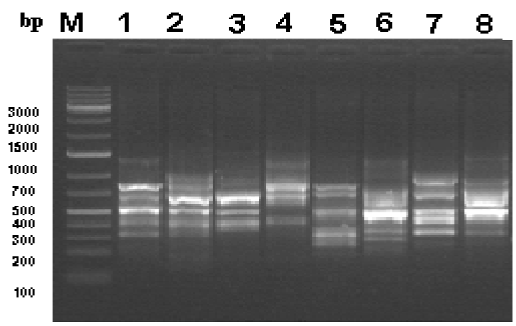

Figure 2: Gel electrophoresis represents RAPD -PCR products for DNA from eight colubrid species (Lanes 1 to 8) with OPE-5 primer. M, DNA marker with molecular size (1kb plus, Fermantas). 1, *Psammophis sibilans sibilans;* 2, *Psammophis Sudanensis;* 3, *Psammophis Schokari Schokari;* 4. *Psammophis Schokari aegyptiacus;* 5, *Spalerosophis diadema;* 6, *Lytorhynchus diadema;* 7, *Coluber rhodorhachis rhodorhachis;* 8, *Coluber nummifer.*

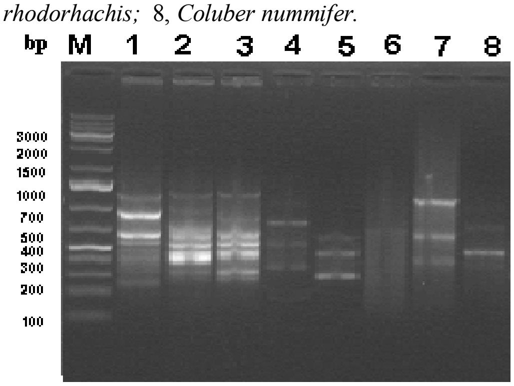

Figure 3: Gel electrophoresis represents RAPD -PCR products for DNA from eight colubrid species (Lanes 1 to 8) with OPB-20 primer. M, DNA marker with molecular size, (1kb plus, Fermantas). 1, *Psammophis sibilans sibilans;* 2, *Psammophis Sudanensis;* 3, *Psammophis Schokari Schokari;* 4. *Psammophis Schokari aegyptiacus;* 5, *Spalerosophis diadema;* 6, *Lytorhynchus diadema;* 7, *Coluber rhodorhachis rhodorhachis;* 8, *Coluber nummifer.*

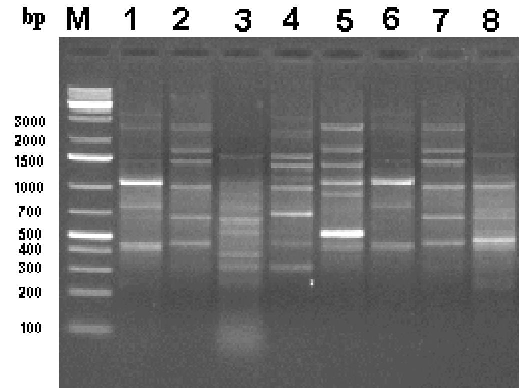

Figure 4: Gel electrophoresis represents RAPD -PCR products for DNA from eight colubrid species (Lanes 1 to 8) with OPB-13 primer. M, DNA marker with molecular size, (1kb plus, Fermantas). 1, *Psammophis sibilans sibilans;* 2, *Psammophis Sudanensis;* 3, *Psammophis Schokari Schokari;* 4. *Psammophis Schokari aegyptiacus;* 5, *Spalerosophis diadema;* 6, *Lytorhynchus diadema;* 7, *Coluber rhodorhachis rhodorhachis;* 8, *Coluber nummifer.*

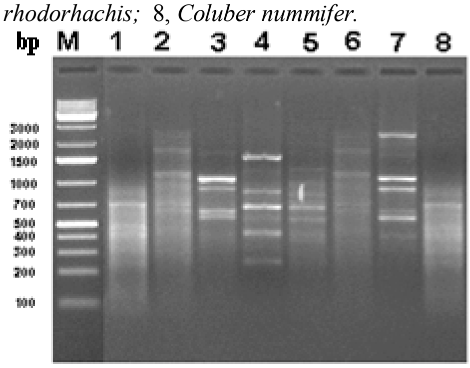

Figure 5: Gel electrophoresis represents RAPD -PCR products for DNA from eight colubrid species (Lanes 1 to 8) with OPB-14 primer. M, DNA marker with molecular size (1kb plus, Fermantas). 1, *Psammophis sibilans sibilans;* 2, *Psammophis Sudanensis;* 3, *Psammophis Schokari Schokari;* 4. *Psammophis Schokari aegyptiacus;* 5, *Spalerosophis diadema;* 6, *Lytorhynchus diadema;* 7, *Coluber rhodorhachis rhodorhachis;* 8, *Coluber nummifer.*

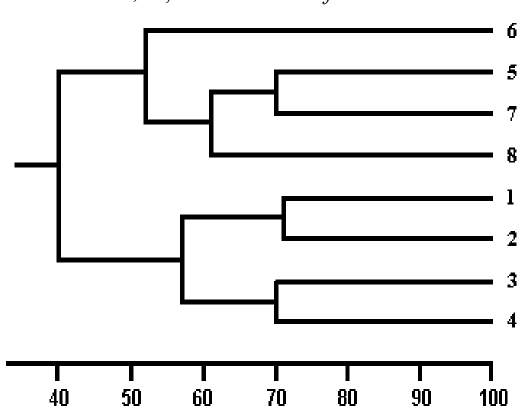

Figure (6): UPGMA based Dendrogram showing phylogenetic relationships among the eight colubrid species (1-8) based on RAPD-PCR by OPB-O1, OPB-13 OPB-14, OPE-05 and OPB-20 primers.

4. Discussion

1. The higher-level classification of Colubroidea has been in change as new molecular results contradict traditional taxonomy, and new phylogenies and taxonomies contradict each other **(Burbrink *et al.*, 2007; Wiens *et al.*, 2008; Zaher *et al.*, 2009)**. In the present work, the family colubridae is separated into two subfamilies, Psammophiinae and Colubrinae. These divisions were similar to those mentioned by **Vidal and Hedges (2002)**, **Kelly *et al.* (2003)** and **Lawson *et al.* (2005)**. **Vidal *et al.* (2007)** recognized Lamprophiidae as a single family, including Psammophiinae subfamily and genus *Psammophis* which is supported by **Gravlund (2001)**. The snake *Psammophis schokari* has a widespread distribution across North Africa, Morocco and Western Sahara and is represented by three different morphotypes: striped, unicoloured and the Western-Sahara morphology **(Bons and Geniez 1996)**. The three Moroccan/Western Sahara color morphotypes form one genetic lineage, indicating that colour pattern and does not reflect a different phylogenetic history, and is probably an ecological adaptation to the local environment **(Rato *et al.*, 2007)**. **Broadley (2002)** and **kelly *et al.* (2008)** studied the morphology of *Psammophis schokari* group which included the species *schokari, aegyptius*, *punctulatus, elegans* and *trigrammus*. *P. schokari* shows a genetic diversity ranging from 4–5%, in four different localities (Morocco/Western Sahara, Mauritania and Algeria). Surprisingly, Moroccan/Western Sahara and Algerian lineages are the most divergent ones. This geographic substructuring may be due to severe climate changes in the Sahara desert between the Miocene and Pleistocene associated with expansion/contraction phases of this desert. *Psammophis aegyptius* is sister taxon of *Psammophis schokari* with a high level of genetic divergence between them (10.7%) supporting the recognition of *P. aegyptius* as a distinct species **(Schleich *et al.* 1996; Rato *et al.*, 2007)**. Also, in the present work *Psammophis schokari aegyptius* is sister taxon to *Psammophis schokari schokari* with high genetic diversity between them (30.2%). **Largen and Rasmussen (1993)** and **Rato*, et al.*, (2007)** examined a large samples of *Psammophis sibilans* and found the vast majority to agree with Egyptian *P. sibilans* in their infralabial arrangement. **Kelly *et al.* (2008)** found that the *P. sudanensis* and *P. sibilans* are established in the same clade. These results are similar to our results which we found that the two species *P. sibilans* and *P. sudanensis* are presented in the same clade and the genetic similarity is 71% between them. In addition, the northern stripe- bellied sand snake, *P. sudanensis* is synonym to P.subtaeniatus **(Howell, 2000)**.
2. The Colubrinae is the largest subfamily within the family Colubridae. **Lawson *et al.* (2005)** show that the genus *Lytorhynchus* is sister to a clade composed of the genera Spalerosophis and Coluber. This result is similar to that found in the present work. Additionally, the *Spalerosophis diadema* and *Coluber rhodorhachis* are monophyletic (sister) to each other in one clade and these group of the two species is sister to clade contains *Coluber nummifer* . These results are similar to that recorded by **Lawson *et al.* (2005)**, **Nagy *et al.* (2004)** and **Pyron *et al.* (2011)**. The closely related species, *Platyceps (Coluber) rhodorachis* , *Platyceps (Coluber) rogersi* and *Platyceps (Coluber) florulentus* have high genetic diversity (7%) with *Spalerosophis diadema* and the morphological and molecular studies DNA were indicated a common origin between the genera *Platyceps (coluber)* and *Spalerosophis* **(Schatti and Utiger, 2001)**. Also, in the present work there is a close relation between *Coluber rhodorachis* and *Spalerosophis diadema* but the genetic diversty between them is 30%. Surprisingly, *Coluber rhodorhachis* and *Coluber nummifer* are the most divergent ones but *Coluber rhodorhachis* and *Spalerosophis diadema* are the most similarity ones. Therefore, the evolutionary history of snakes still remains controversial.
3. The similarity matrix between the eight varieties ranged from 35% to 71% with an average 60% (table 5). In conclusion, the similarity coefficient between the eight snake species indicates that the 8 snake species are not identical and separated from each other.

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