

Electrophoretic characterisation of the venom samples obtained from various Anatolian snakes (Serpentes: Colubridae, Viperidae, Elapidae)

Hüseyin ARIKAN¹, Bayram GÖÇMEN^{1*}, Yusuf KUMLUTAŞ²,
Nurşen ALPAGUT-KESKİN¹, Çetin ILGAZ² and Mehmet-Zülfü YILDIZ^{1,3}

1- Ege University, Faculty of Science, Department of Biology, Zoology Section, 35100 Bornova, Izmir, Turkey

2 - Dokuz Eylül University, Faculty of Education, Department of Biology, Buca, Izmir, Turkey

3 - Harran University, Faculty of Art and Science, Department of Biology, Zoology Section,
Osmanbey Campus, Sanliurfa, Turkey

* Corresponding author: Ege University, Faculty of Science, Department of Biology, Zoology Section, 35100 Bornova,
Izmir, Turkey, Tel:+90 (232) 388 40 00/1795, E-mail: bayram.gocmen@ege.edu.tr

Abstract. The venoms extracted from a colubrid snake [*Malpolon monspessulanus* (Hermann)], seven viperids [*Montivipera xanthina* (Gray), *Montivipera wagneri* Nilson &Andrén, *Vipera ammodytes* (Linnaeus), *Vipera kaznakovi* (Nikolsky), *Vipera eriwanensis* (Bonaparte), *Vipera barani* Böhme & Joger, *Macrovipera lebetina* (Linnaeus)] and an elapid snake [*Walterinnesia aegyptia* (Lataste)] collected from various regions of Anatolia were compared using polyacrylamide gel disc electrophoresis and densitometry analysis methods. The electrophoretic patterns (protein bands) of the examined venom snakes were demonstrated. The resulting electropherograms showed important qualitative differences amongst the colubrid snake *Malpolon monspessulanus*, the elapid species *Walterinnesia aegyptia* and the viperid snakes. In *M. monspessulanus* and *W. aegyptia* samples the total protein fraction numbers were 11 and 12, respectively, while in viperid samples the number was between 10 and 14, indicating a higher venom complexity in viperids compared to that of opisthophylcolubrid and proteroglyph-elapid snakes. Electrophoretic data support the phylogenetic argument previously outlined for the family Viperidae. Moreover, it is concluded that the *Macrovipera wagneri* and *Walterinnesia aegyptia* are closely related taxa with front-fanged delivery systems in the light of protein band analogies.

Key words: Colubrid, viperid and elapid snake venoms, polyacrylamide gel disc electrophoresis, densitometry.

Introduction

The venomous snakes have been widespread in tropical and subtropical regions of the world (Edstrom 1992, Arikan et al. 2005). Studies related to snakes in Anatolia often directly deal with the taxonomy and distribution of species. According to studies conducted in Anatolia (Baran 1976,

Basoglu & Baran 1980, Nilson et al. 1988, Nilson et al. 1999, Nilson & Andrén 2001, Ugurtas et al. 2001, Budak & Göçmen 2005, Joger 2005) 15 venomous snake species [14 in the family Viperidae (Solenoglypha) and one in the family Elapidae (Proteroglypha)] and four semi-venomous snake species of the family Colubridae (Opisthoglypha) live in Anatolia.

The polyphyletic family Colubridae produces venom in a specialised cephalic and oral gland, a Duvernoy's gland, located in the temporal region (Mackessy 2002). Although the Duvernoy's gland is a homologue of the venom glands of viperid and elapid snakes, it is anatomically and functionally distinct (Kardong 2002). Venoms obtained from snakes of the Viperidae, Colubridae and Elapidae families in various parts of the world, have been used for research in the pharmacological, biochemical, immunological and toxicological fields (Hageman 1961, Mebs 1968, Tu & Ganthavorn 1968, Basu et al. 1969, Bonilla & Horner 1969, Robertson & Delpierre 1969, Young & Miller 1974, McKinstry 1983, Kochva 1987, Minton & Weinstein 1987, Tun-Pe et al. 1995, Kardong 2002, Mackessy 2002, Arikan et al. 2005). Snake venoms are mixtures of biologically active substances most of which are enzymes or non-enzymatic polypeptides. They affect victims in different ways depending on their enzyme ingredients. They can be neurotoxic or haemolytic. In the light of the conducted studies mentioned above, it has been reported that venoms of solenoglyph (Viperidae and Crotalidae) and opisthoglyph (Colubridae) group snakes are mainly haemolytic-proteolytic while the venoms of proteroglyph (Elapidae and Hydrophiidae) group snakes are fundamentally neurotoxic. Lenk et al. (2001) have stated that the evolutionary radiation at least in Viperidae might have been driven by possession of an effective venom apparatus and a

foraging strategy (sit-wait-strike). According to Lenk et al. (2001), Eurasian vipers could be unambiguously divided into four monophyletic groups.

Only a few electrophoretic studies have been conducted on venom proteins of different viperid and colubrid snakes from Anatolia as well as their clinical, physiological and serological effects on human (Arikan et al. 2005, Arikan et al. 2006, Göçmen et al. 2006).

The present study deals with the venom proteins of some viperid snakes, a colubrid species (*Malpolon monspessulanus*) and a single elapid species (*Walterinnesia aegyptia*) living in Anatolia. The aim of the present study was to establish the venom electrophoretic patterns of various venomous snakes from different parts of Turkey and, to make proper comments on the phylogenies and taxonomies of the examined taxa in the light of obtained findings.

Materials and Methods

The viperid, colubrid and elapid specimens used in the study were collected from different parts of Anatolia at different dates [*Montivipera xanthina* - Gumuldur (Izmir Province), *Montivipera wagneri* - Karakurt (Kars Province), *Vipera ammodytes* - Persembe (Ordu Province), *Vipera kaznakovi* - Hopa (Artvin Province), *Vipera eriwanensis* - Ardahan (Ardahan Province), *Vipera barani* - Sakarya (Sakarya Province), *Macrovipera lebetina* - Ceylanpinar (Sanliurfa Province), *Walterinnesia aegyptia* - Tektek Mountain (Sanliurfa Province), *Malpolon monspessulanus* - Cigli (Izmir Province)]. All specimens captured were mature. The specimens were taken to the laboratory alive and their venoms were extracted without applying any pressure

on their venom glands, as described by Tare *et al.* (1986). To remove dead cells, venom extracts were centrifuged for 5 minutes at 600 g and stored at -20°C until electrophoretic separation.

During electrophoretic analysis, a 5 µl venom sample was used for each separation. The venom proteins were separated according to Davis (1964), slightly modified by Özeti and Atatür (1979). In detail, a pH 6.7 stacking gel was layered above the pH 9 separation gel of 7.5% polyacrylamide, together with a pH 8.3 tris-glycine buffer system. The "loading gel" of Davis (1964) was omitted. Electrophoretic separations were run at room temperature (approx. 20-25°C) using a Canalco Model 1200 disc electrophoresis apparatus. Gels containing separated proteins were stained with 0.5% Amido Black (Naphtol Blue Black 10-B) and excess stain was removed passively in 7% acetic acid baths. Then, the stained gels were photographed. Qualitative evaluation of the gels was done directly from the electropherograms and the densitometric curves of the separations were obtained by means of a Gelman ACD-15 Model 39430 densitometer at 500 nm.

Results

The venom secretions of both *Malpolon monspessulanus* and *Walterinnesia aegyptia* which have an opisthoglyph and a proteroglyph venom apparatus, respectively, were colorless. In contrast, the venom extracts of the viperid snakes, which have a solenoglyph venom apparatus were light yellow in color and had a higher viscosity than those of the colubrid and elapid venom secretion.

Gel photographs of the venom protein samples of the seven viperid species belonging to three different genera (*Vipera*, *Montivipera* and *Macrovipera*), one colubrid species

(*Malpolon monspessulanus*) and one elapid species (*Walterinnesia aegyptia*) were given in Fig.1. As seen in Figure 1, each specimen has a peculiar electrophoretic pattern. Significant differences in fraction numbers (Table 1), electrophoretic mobilities and densities of the venom proteins were observed among the taxa.

Gel photographs showing the electrophoretic separation of each venom sample together with their densitometric tracing curves, are given in Figs. 2 to 10. As can be seen in Fig. 2, in the colubrid species *Malpolon monspessulanus*, protein fractions could only be divided into 11 fractions, one of which was in the albumin zone and 10 were in the globulin zone.

The electrophoretic patterns of the viperid venom protein samples from Anatolian specimens (Fig.3-10) showed qualitative differences among them; which suggest that all of the taxa have clearly distinct venom proteins. These venom proteins could be separated into 10-14 fractions. Among the seven vipers examined, it was found that the total protein fraction number was lowest in *Montivipera wagneri* and highest in *Montivipera xanthina*. The venom protein fractions were found to be 10 in *Montivipera wagneri*, 12 in *Vipera kaznakovi*, *Macrovipera lebetina* and *Walterinnesia aegyptia*, 13 in *Vipera ammodytes*, *Vipera eriwanensis* and *Vipera barani*, and 14 in *Montivipera xanthina*.

The venom protein distribution patterns of two *Montivipera* species (*wagneri* and *xanthina*) are distinctly different. The main qualitative diffe-

rences were: (a) in *wagneri* the number of discernible globulin fractions was 8 compared to 12 in *xanthina*, (b) there was a discernible pre-albumin fraction in *xanthina* compared to a discernible post-albumin fraction in *wagneri*.

The venom proteins of four species of the genus *Vipera* (Caucasian *kaznakovi*

and *eriwanensis*, Euro-Asian *ammodytes* and Anatolian *barani*) consist of a total of 12-13 electrophoretic fraction (Figs. 5-8), with 11-12 fractions found in the globulin and 1-2 fraction(s) in the albumin zone. A discernible pre-albumin fraction before the main albumin fraction was only seen in *V. eriwanensis*.

Table 1. Distribution of the numbers of fractions obtained from the venom protein electrophoresis of different Anatolian snakes. **G:** Globulin fraction number, **PoA:** Post-albumin fraction number, **A:** Albumin fraction number, **PreA:** Pre-albumin fraction number.

Species	G	PoA	A	PreA	Total
<i>Malpolon monspessulanus</i>	10	-	1	-	11
<i>Montivipera xanthina</i>	12	-	1	1	14
<i>Montivipera wagneri</i>	8	1	1	-	10
<i>Vipera kaznakovi</i>	11	-	1	-	12
<i>Vipera ammodytes</i>	12	-	1	-	13
<i>Vipera eriwanensis</i>	11	-	1	1	13
<i>Vipera barani</i>	12	-	1	-	13
<i>Macrovipera lebetina</i>	9	-	2	1	12
<i>Walterinnesia aegyptia</i>	10	1	1	-	12

The other viperid species which belongs to the different genera, *Macrovipera lebetina* is clearly distinct from the all other investigated viperid species by the presence of 9 fractions in the globulin zone and three albumin fractions which one of them is pre-albumin (Fig. 9).

The densitometric curves of venom proteins of Afro-Asiatic elapid species, *Walterinnesia aegyptia* were observed for the first time and had a total of 12 fractions (Fig.10). The numbers of globulin/albumin fractions were detected as 10/2. As seen in *Montivipera*

wagneri (Fig. 4), a post-albumin fraction was also present, but was more prominent in *Walterinnesia aegyptia*.

Discussion

In many previous studies (Tu & Ganthavorn 1968, Basu et al. 1969, Bonilla & Horner 1969, Young & Miller 1974), it has been reported that the secretions of Duvernoy's gland in various colubrid snakes have important characteristic electrophoretic patterns and more similar complexities

compared to elapid and viperid venoms. Minton and Weinstein (1987) pointed out that the colubrid venoms they analyzed using SDS-PAGE electrophoresis, contained 7-10 protein bands and that the Duvernoy's gland secretion was as complex as most proteroglyph (Elapidae & Hydrophiidae) venoms. However, the total number of protein bands was lower than those of viperid snakes. Our results, based on the polyacrylamide gel electrophoresis, indicated that the Duvernoy's gland secretion of the colubrid snake *Malpolon monspessulanus*

and the venom gland secretion of the elapid species *Walterinnesia aegyptia* had a total of 11 and 12 fractions (protein bands) respectively, while in the viperid samples they numbered between 10-14. Therefore, it can be concluded that the venom of viperid snakes is more complex compared to the Duvernoy's gland secretion in *Malpolon monspessulanus* (a colubrid snake) and the venom gland secretion in *Walterinnesia aegyptia* (an elapid snake). This finding is in accordance with those of Minton and Weinstein (1987) and Mackessy (2002).

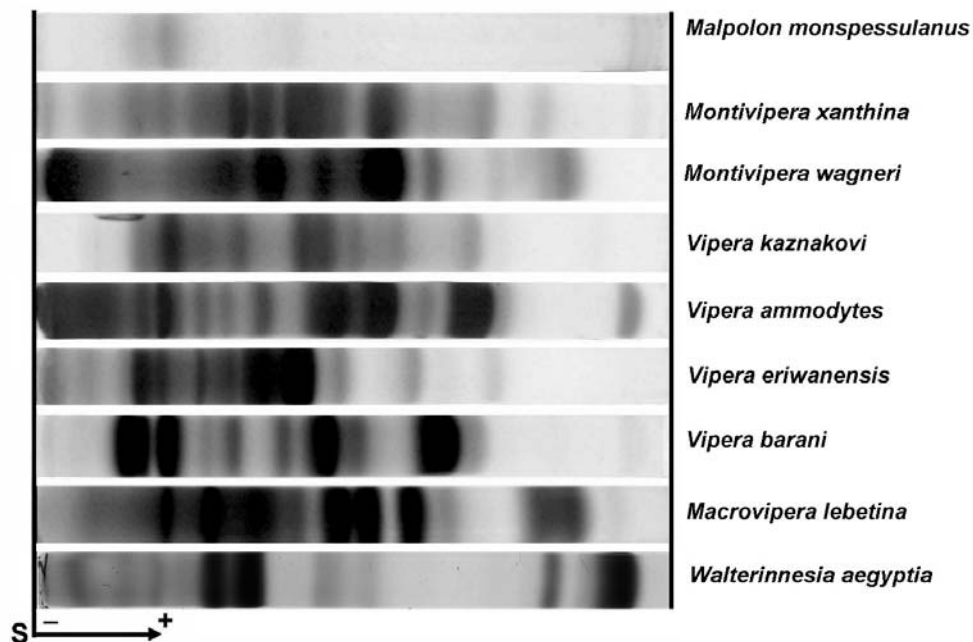


Figure 1. Venom protein electropherograms of a colubrid (*Malpolon monspessulanus*), eight viperid and an elapid (*Walterinnesia aegyptia*) snakes (S: Start, junction between the stacking and separation gels).

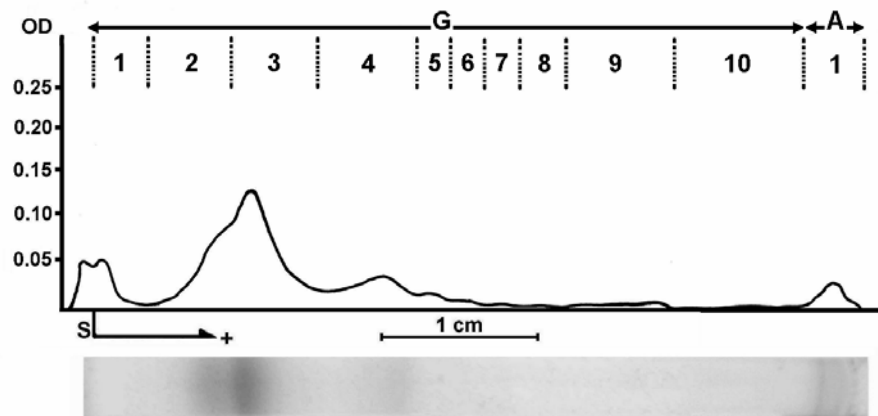


Figure 2. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from an Afro-Euro-Asiatic *Malpolon monspessulanus*, together with its densitometric tracing curve. OD: Optical density, S: Start (junction between the stacking and separation gels). A: Albumin fraction zone, G: Globulin fraction zone.

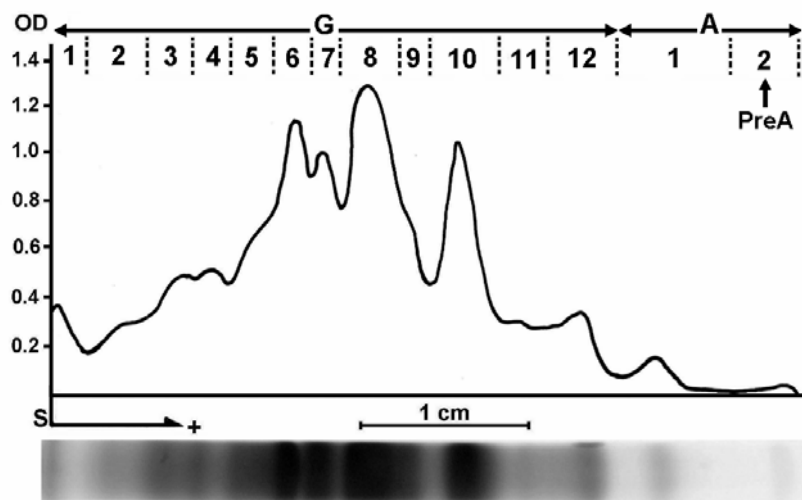


Figure 3. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Ottoman Viper, *Montivipera xanthina*, together with its densitometric tracing curve. PreA: Pre-albumin fraction. For further explanation, see legend to Fig. 2.

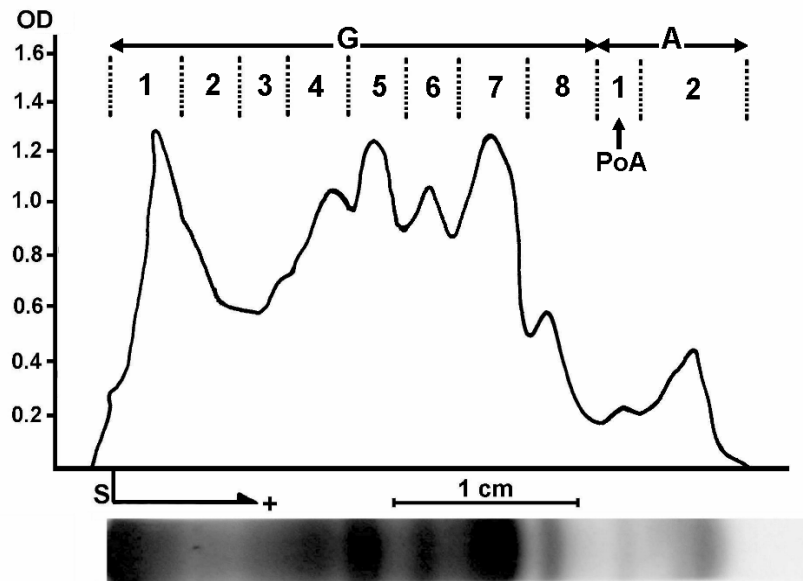


Figure 4. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Irano-Anatolian Wagner's Adder, *Montivipera wagneri*, together with its densitometric tracing curve. PoA: Post-albumin fraction. For further explanation, see legend to Fig. 2.

Nilson & Andrén (1986) have proposed a phylogenetic relationship tree based on the morphological characters amongst the mountain vipers (*Montivipera* species, formerly known as *Vipera xanthina* complex) of the Middle East. According to this theory *Montivipera wagneri* is more primitive than *M. xanthina*. Our results obtained from the gel electrophoresis of venom proteins support this theory since the total fraction number of venom proteins was very low in *M. wagneri*.

Tun-Pe et al. (1995) studied Russell's viper (*Daboia russelli siamensis*) venoms using SDS-PAGE electrophoresis in different sized specimens. These

investigators showed that the venoms from the youngest (smallest) snakes have fewer protein bands, the number of bands increased as the snakes aged, the venom of young snakes had a high lethal potency and as snakes aged, this potency decreased. Similar results were obtained for Ottoman Viper, *Montivipera xanthina* by Arikan et al. (2006). All specimens used in the present study had already reached sexual maturity. So, the study material did not allow us to make a comparative study on the intra-specific variations based on age or size. However, we detected remarkable differences among the taxa examined, both in the total number of protein bands and the density of each

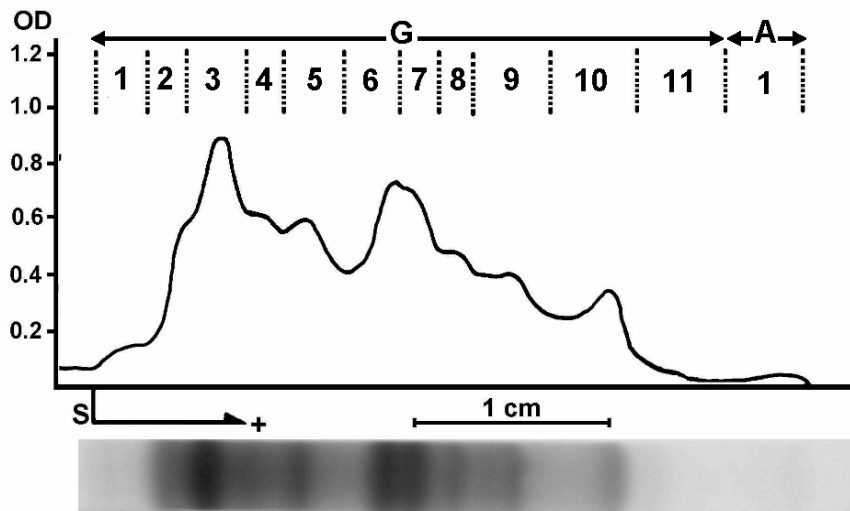


Figure 5. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian-Caucasian Viper, *Vipera kaznakovi*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.

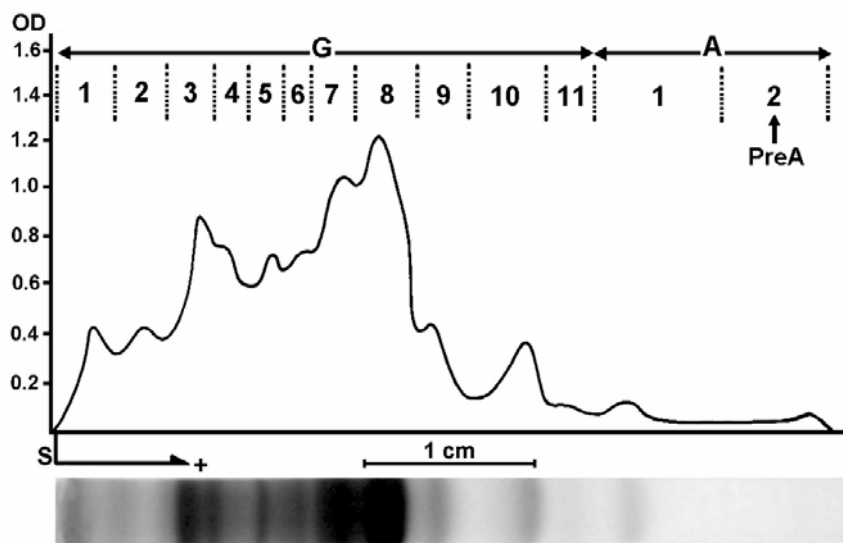


Figure 6. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Euro-Anatolian-Caucasian Nose-horned Viper, *Vipera ammodytes*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.

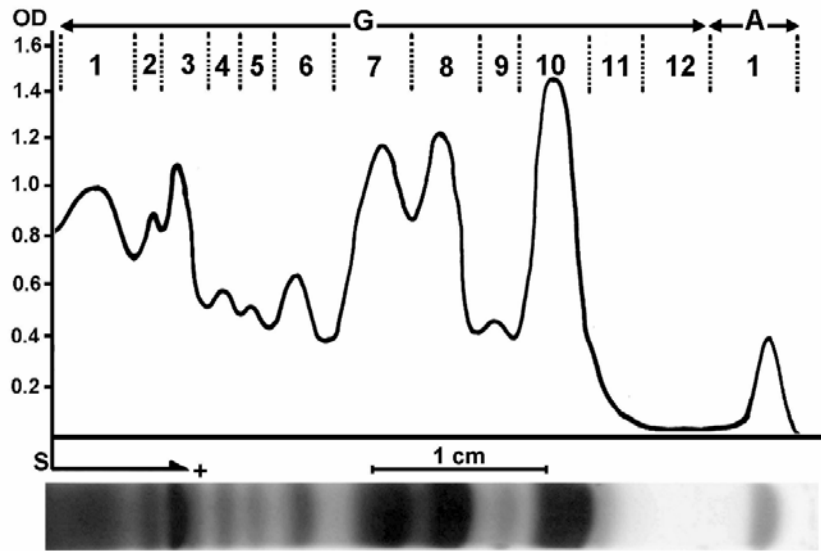


Figure 7. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian-Caucasian Small Viper, *Vipera eriwanensis*, together with its densitometric tracing curve. PreA: Pre-albumin fraction. For further explanation, see legend to Fig. 2.

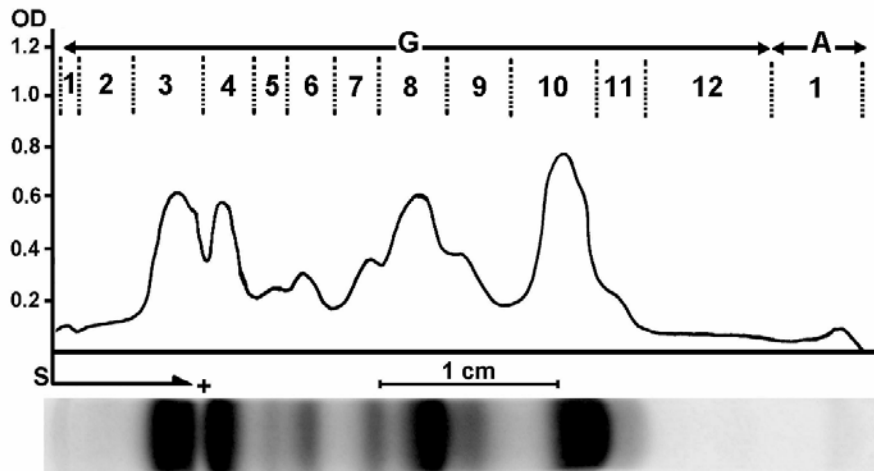


Figure 8. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Baran's Viper, *Vipera barani*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.

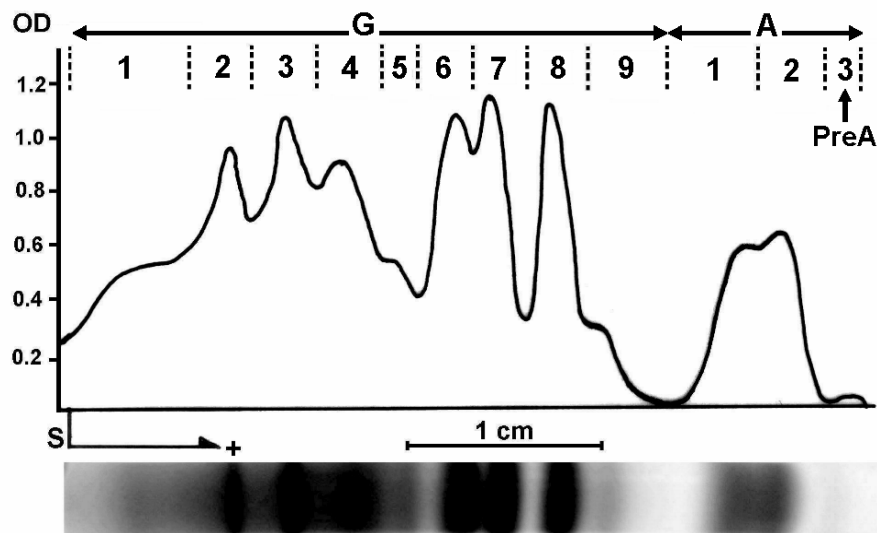


Figure 9. Photograph showing the electrophoretic separation of the venom protein sample obtained from one Afro-Asiatic Levantine Viper, *Macrovipera lebetina*, together with its densitometric tracing curve. PreA: Pre-albumin fraction. For further explanation, see legend to Fig. 2.

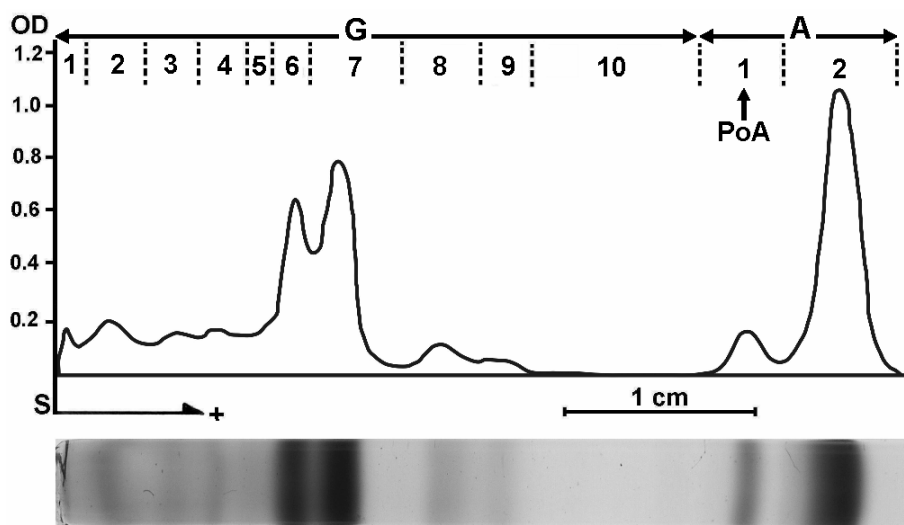


Figure 10. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Afro-Asiatic Desert Cobra, *Walterinnesia aegyptia*, together with its densitometric tracing curve. PoA: Post-albumin fraction. For further explanation, see legend to Fig. 2.

protein band. *Montivipera wagneri* was found to have the lowest total number of proteins with 10 fractions, while *Montivipera xanthina* had the highest with 14 fractions. Accordingly, the venom of the Ottoman viper, *Montivipera xanthina* appears said to have a more complex composition from the viewpoint of venom proteins compared to all the other viperid, colubrid and elapid taxa examined in this study.

Similarities such as the low number globulin fractions and the presence of a post-albumin fraction between *Montivipera wagneri* and *Walterinnesia aegyptia*, which is recognized as the most primitive elapid species (Keogh 1998), indicate a close relationship between the two snake families; Viperidae and Elapidae, both with front-fanged venom delivery systems.

Lenk et al. (2001) used the nucleotide sequences of mitochondrial cytochrome b and 16S rRNA genes to reconstruct a molecular phylogeny of the family Viperidae. According to their analysis, Eurasian vipers have been divided into four monophyletic groups. The radiation might have been driven by the possession of an effective venom apparatus. Our results indicate the Eurasian vipers living in Anatolia could be divided into four groups in terms of venom protein bands: (a) Irano-Caucasian *Montivipera wagneri* (10 bands); (b) Middle East-Caucasian *Macrovipera lebetina* plus *V. kaznakovi* (12 bands); (c) Euro-Anatolian-Caucasian *V. ammodytes* plus *Vipera* s.str [*Vipera barani* and *V. eriwanensis*] (13 bands) and (d) Anatolian

Montivipera xanthina (14 bands). These electrophoretic venom protein patterns are consistent with the findings of Lenk et al. (2001).

Although there was a general consensus in the literature that the Duvernoy's "venom" gland in colubrids is homologous to the venom glands of viperid snakes, Kardong (2002) and Mackessy (2002) have stated that it is functionally and anatomically distinct. In addition it has many other physical (viscosity, color) and chemical (composition, toxicity, enzymatic activity) differences in its venom. There is no information available on the biochemistry and pharmacology of venoms and also on the anatomy, histology and cytology of the venom glands from the species distributed in Anatolia. Future studies should focus on the above mentioned fields in order to gain more information and a deeper understanding of both the functional and evolutionary relationships between the venom components and the snakes that produce them.

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