

## **An electrophoretic comparison of the venoms of a colubrid and various viperid snakes from Turkey and Cyprus, with some taxonomic and phylogenetic implications**

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### **Abstract**

Venom extracts obtained from a colubrid snake [*Malpolon monspessulanus* (Hermann)] and eight viperids [*Vipera xanthina* (Gray), *Vipera ammodytes* (Linnaeus), *Vipera kaznakovi* (Nikolsky), *Vipera eriwanensis* (Bonaparte), *Vipera wagneri* Nilson & André, *Vipera barani* Böhme & Joger, *Macrovipera lebetina lebetina* (Linnaeus), *Macrovipera lebetina obtusa* Dwigubsky] distributed in Turkey and Cyprus were compared using polyacrylamide gel disc electrophoresis and densitometry analysis methods. The electrophoretic patterns of the examined snakes were demonstrated. The obtained electropherograms of the examined venom protein samples showed important qualitative differences between the colubrid snake, *Malpolon monspessulanus* and the viperid snakes; in the colubrid sample the total protein fraction number was 8, while in viperid samples they number between 10 and 14, indicating the venom complexity in viperids is higher than that of colubrid snakes. Electrophoretic data support the phylogenetic argument previously outlined of the family Viperidae. Moreover, in the light of the differences of the venom electrophoretic patterns, it is concluded that the southern Anatolian population of *Macrovipera lebetina* should not be identified as the nominate subspecies *M. l. lebetina*, which lives in Cyprus.

**Key words:** Colubrid and viperid snake venom, polyacrylamide gel disc electrophoresis, densitometry

### **Introduction**

Some of the earliest studies concerning snake venoms were on the secretions of serous type Duvernoys gland in various colubrid species (Hageman 1961; Mebs 1968; Robertson & Delpierre 1969; McKinstry 1983, Kochva 1987). The polyphyletic family Colubridae contains approximately 700 species, which produce venom in a specialised cephalic and oral gland, a Duvernoys gland, located in the temporal region (Mackessy 2002). Although

the Duvernoys gland is a homologue of the venom glands of viperid and elapid snakes, it is anatomically and functionally distinct (Kardong 2002). On different viperid, colubrid and elapid snake venoms; many electrophoretic studies were conducted (Tu & Ganthavorn 1968; Basu *et al.* 1969; Bonilla & Horner 1969; Young & Miller 1974; Minton & Weinstein 1987). A notable biochemical study on Russells viper belongs to Tun-Pe *et al.* (1995).

Studies related to snakes in Turkey often directly deal with the taxonomy and distribution of species. According to studies conducted in Turkey and Cyprus (Baran 1976; Basoglu & Baran 1980; Baran & Atatür 1998; Atatür & Göçmen 2001; Ugurtas *et al.* 2001; Budak & Göçmen 2005) nine venomous snake species living in Turkey belong to the family Viperidae (Solenoglypha) and four semi-venomous snake species (Opisthoglypha) to the family Colubridae. Another venomous snake, *Walterinnesia aegyptia* (Desert Cobra) belonging to the family Elapidae (Proteroglypha), was also recently reported from Turkey (Ugurtas *et al.* 2001).

Although Anatolian *Macrovipera lebetina* were attributed to the subspecies *obtusa* for a long time (Baran 1976; Basoglu & Baran 1980; Baran & Atatür 1998; Budak & Göçmen 2005), some authors (Billing & Schaetti 1984; Broodmann 1987; Ossenegg 1989; Mulder; 1995) stated that the *M. lebetina* specimens from the southern parts of Anatolia closely resemble those of Cyprus (*M. l. lebetina*). On the other hand, the most recent attempt at comprehensive investigation of the true vipers (family: Viperidae) relationships is that of Lenk *et al.* (2001) who used molecular methods. According to Lenk *et al.* (2001), Eurasian vipers could be unambiguously divided into four monophyletic groups.

The present study deals with the venom proteins of some viperid snakes and a colubrid species (*Malpolon monspessulanus*) living in Turkey and Cyprus, with some taxonomic and phylogenetic implications. All venom extracts obtained from different snakes were analysed utilizing polyacrylamide gel disc electrophoresis, and their venom electrophoretic patterns were compared.

## Material and Methods

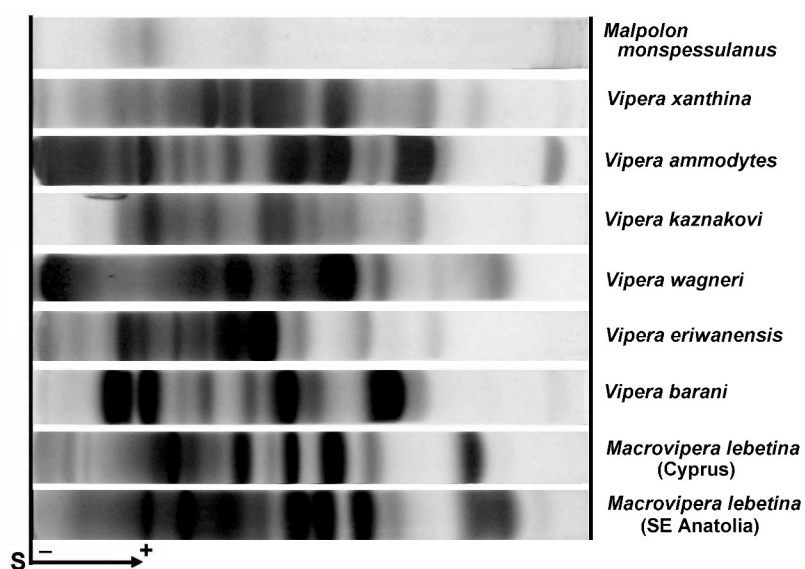
The viperid and colubrid specimens used in the study were collected from different parts of Turkey and Cyprus at different dates [*Vipera xanthina* Gumuldur (Izmir), *Vipera ammodytes* Persembe (Ordu), *Vipera kaznakovi* Hopa (Artvin), *Vipera eriwanensis* Ardahan, *Vipera wagneri* Karakurt (Kars), *Vipera barani* Sakarya, *Macrovipera lebetina obtusa* Ceylanpınarı (Sanliurfa), *Macrovipera lebetina lebetina* Dikmen (Nicosia, Cyprus), *Malpolon monspessulanus* - (Cigli, Izmir)]. All of these had already reached sexual maturity. 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). Due to the fact that the venom extracts contained some dead cells, they were centrifuged for 5 minutes at 600 g and stored at -20°C until electrophoretic separation.

In electrophoretic analysis, a 5  $\mu$ l venom sample was used for each separation. The venom proteins were separated according to Davis (1964), slightly modified by Özeti and Atatür (1979). Accordingly, 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

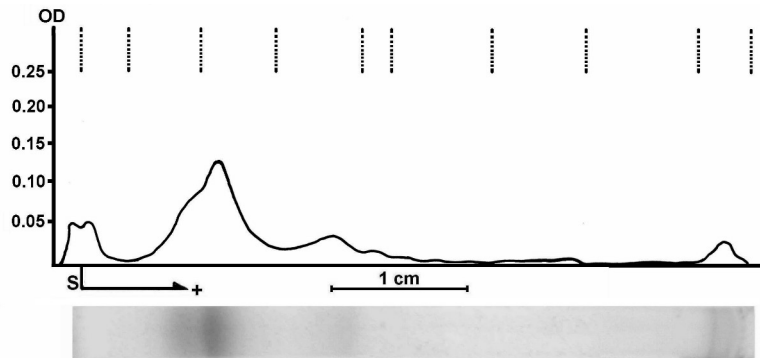
The venom secretion of *Malpolon monspessulanus*, which has an opisthoglyph venom apparatus (Duvernoys gland), was colourless, but the venom extracts of the viperid snakes, which have a solenoglyph venom apparatus were light yellow in colour and had a higher viscosity than that of the Duvernoys gland secretion.

Gel photographs of the venom protein samples of the eight viperids and one colubrid species (*Malpolon monspessulanus*) were given in Figure 1. It could be said that each specimen has a characteristics electrophoretic pattern. Significant differences were established among the taxa from the viewpoints of fraction numbers, electrophoretic mobilities and densities of the venom proteins.



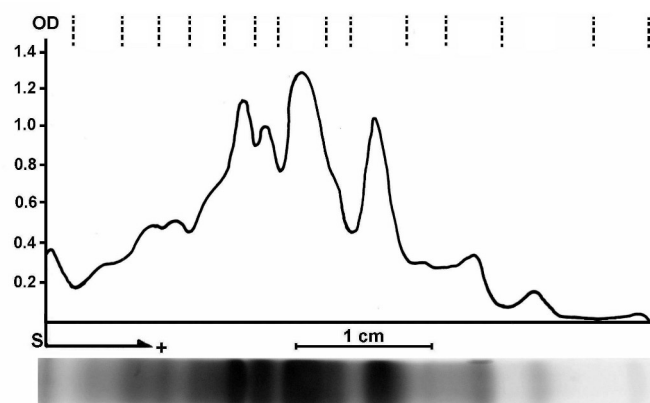
**FIGURE 1:** Venom protein electropherograms of a colubrid (*Malpolon monspessulanus*) and eight viperid snakes (S: Start, junction between the stacking and separation gels).

Gel photographs showing the electrophoretic separation of each venom sample, together with their densitometric tracing curves, are given in Figures 2 to 10. In the colubrid species, *Malpolon monspessulanus* (Figure 2), 8 protein fractions or fraction groups were detected.

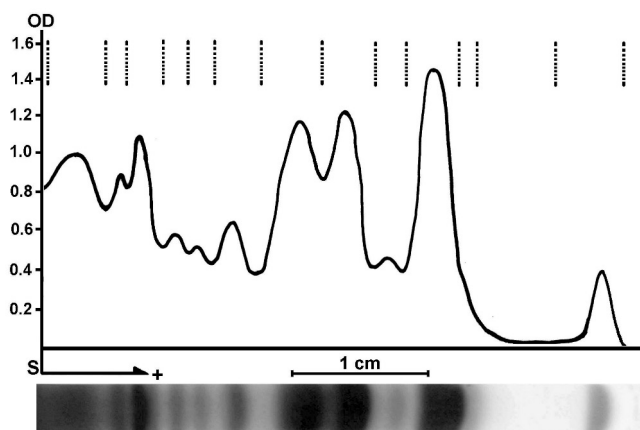


**FIGURE 2:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from an Anatolian *Malpolon monspessulanus*, together with its densitometric tracing curve. OD: Optical density, S: Start (junction between the stacking and separation gels).

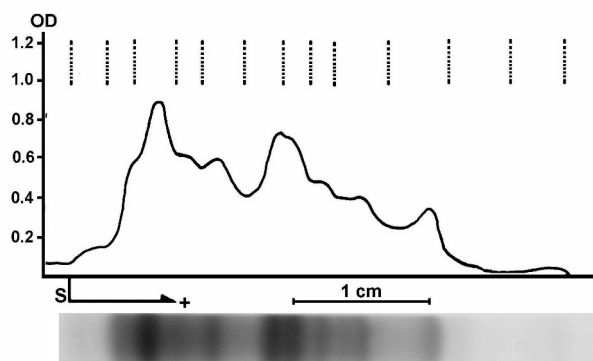
The electrophoretic patterns of the viperid venom protein samples from Turkish and Cypriote specimens (Figures 3–10) showed quite qualitative differences among them; which suggest that all of the taxa have clearly distinct venom proteins. These venom proteins could be separated into 10–14 fractions or fraction groups. Among the eight vipers examined, it was found that the total protein fraction number was lowest in *Vipera wagneri* and highest in *Vipera xanthina*. The venom protein fractions were found to be 10 in *Vipera wagneri*, 12 in *Vipera ammodytes*, *Vipera kaznakovi*, *Macrovipera lebetina obtusa* and *M. l. lebetina*, 13 in *Vipera eriwanensis* and *Vipera barani*, and 14 in *Vipera xanthina*.



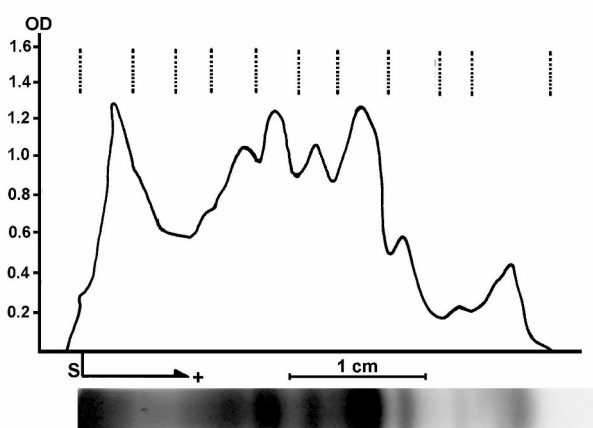
**FIGURE 3:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Ottoman Viper, *Vipera xanthina*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



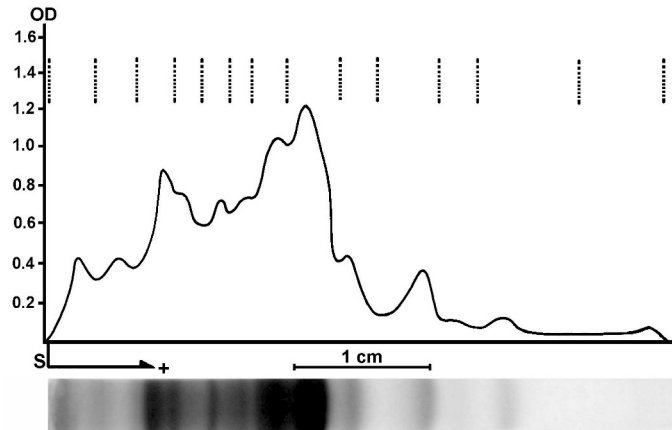
**FIGURE 4:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Nose-horned Viper, *Vipera ammodytes*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



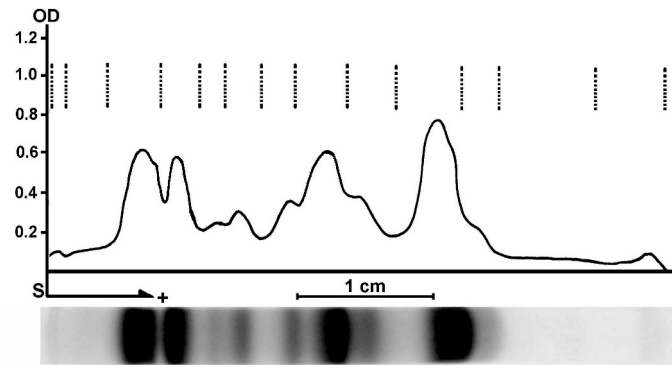
**FIGURE 5:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Caucasian Viper, *Vipera kaznakovi*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



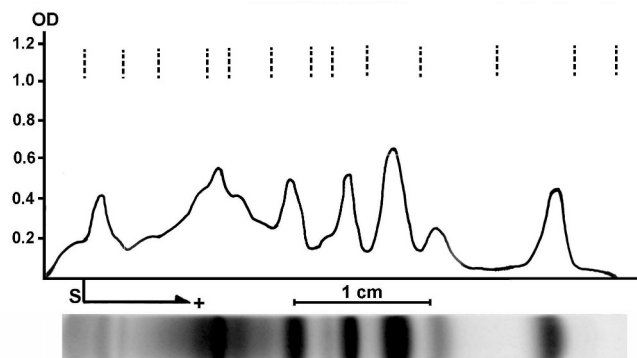
**FIGURE 6:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Wagners Adder, *Vipera wagneri*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



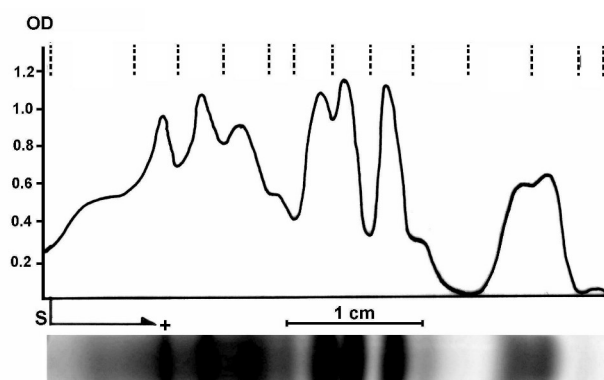
**FIGURE 7:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Small Viper, *Vipera eriwanensis*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



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



**FIGURE 9:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Cypriote Levantine Viper, *Macrovipera lebetina lebetina*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.



**FIGURE 10:** Gel photograph showing the electrophoretic separation of the venom protein sample obtained from one Anatolian Levantine Viper, *Macrovipera lebetina obtusa*, together with its densitometric tracing curve. For further explanation, see legend to Fig. 2.

## 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 Duvernoys gland in various colubrid snakes have important characteristic electrophoretic patterns and more similar complexities as compared to elapid and viperid venoms. Minton and Weinstein (1987) pointed out that the colubrid venoms they analysed using SDS-PAGE electrophoresis, contained 7–10 protein bands and that the Duvernoys gland secretion was as complex as most proteroglyph (Elapidae & Hydrophidae) venoms, but the total number of protein bands was lower than those of viperid snakes.

Our results based on the polyacrylamide gel electrophoresis indicated that the Duvernoys gland secretion of the colubrid snake, *Malpolon monspessulanus* had a total of 8 fractions or fraction groups (protein bands), while in the viperid samples they number 10–14. Therefore, it can be concluded that the venom of viperid snakes is more complex than that of the Duvernoys gland secretion in *Malpolon monspessulanus*, a colubrid snake. This finding is in accordance with those of Minton and Weinstein (1987) and Mackessy (2002).

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 also as snakes aged, this potency decreased. All specimens used in our study had already reached sexual maturity. So, the study material did not allow us to make a comparative study on revealing the intra-specific variations based on age or size. However, we detected remarkable differences among the taxa examined in both the total

numbers of protein bands and the density of each protein band. *Vipera wagneri* was found to have the lowest total number of proteins with 10 fractions or fraction groups, while *Vipera xanthina* had the highest with 14 fraction or fraction groups. Accordingly, the venom of Ottoman viper, *Vipera xanthina* could be said to have a more complex composition from the viewpoint of venom proteins compared to all other viper and colubrid taxa examined in this study.

Our data indicate that *Macrovipera lebetina* populations from southern Anatolia and Cyprus show significant difference regarding the venom electrophoretic patterns. Therefore we conclude that these populations are taxonomically distinct. Consequently, the population from southern Anatolia should not be allocated to the nominate subspecies *M. l. lebetina*, which lives in Cyprus, as previously suggested by Billing & Schaetti (1984), Broodmann (1987), Ossenegg (1989) and Mulder (1995).

Lenk *et al.* (2001) were 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 results, the true vipers originated in the Oligocene in Africa and successively underwent a radiation leading to five major basal groups. Of these, 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 Turkey and Cyprus could be divided into four groups in the viewpoint of the numbers of the venom protein bands: 1- Caucasian *Vipera wagneri* (10 bands); 2- Middle East-Caucasian *Macrovipera lebetina* plus *Vipera* s.str. [*V. ammodytes* and *V. kaznakovi*] (12 bands); 3- Anatolian *Vipera barani* plus Caucasian *V. eriwanensis* (13 bands) and 4-*Vipera 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 literature on that the Duvernoys “venom” gland in colubrids is homologue to the venom glands of viperid snakes, Kardong (2002) and Mackessy (2002) have stated that it is functionally and anatomically distinct in addition to many other physical (viscosity, colour) and chemical (composition, toxicity, enzymatic activity) differences of 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 species distributed in Turkey and Cyprus. Therefore, future works will be directed toward these research areas to understand both functional and evolutionary relationships between the venom components and the snakes that produce them.

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