Comparative proteomic analysis reveals functional and evolutionary diversity in five Montivipera snake venoms

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Abstract

Proteomic characterization of snake venoms is essential for understanding the molecular basis of their evolution and for identifying bioactive compounds of therapeutic interest. The *Montivipera* species endemic to the Near and Middle East region remain poorly studied despite their interesting biological activities. Previous analyses of *Montivipera* venoms have revealed only partial proteomic profiles, with notable discrepancies between studies. To address this gap, we conducted a proteomic analysis of five *Montivipera* species, including *M. bornmuelleri*, *M. bulgardaghica*, *M. albizona*, *M. raddei* and *M. xanthina*. We also analyzed the venom of *Macrovipera lebetinus* to provide a broader comparative framework. These venoms were analyzed using an integrated approach combining SDS-PAGE, RP-HPLC and shotgun proteomics, using both trypsin and multi-enzymatic limited digestions to maximize protein identification and coverage. SDS-PAGE and RP-HPLC analyses revealed the remarkable complexity and diversity of *Montivipera* venoms, which were further confirmed by shotgun proteomics, identifying between 129 and 179 proteins and peptides per species. The major protein families detected included snake venom metalloproteinases, phospholipases A_2, venom serine proteases, C-type lectins, venom vascular-endothelial growth factors, and disintegrins. Notably, the relative abundance of these protein families varied across species, suggesting interspecific differences in envenomation profiles. Comparative analysis revealed a high degree of similarity among *Montivipera* species, with 39 shared proteins across all five venoms. Our findings confirmed the major toxin families previously reported in *Montivipera* venoms and revealed the presence of several low-abundance protein families that were not previously identified. Thus, this study highlights both the conserved and unique features of *Montivipera* venom proteomes, offering a valuable foundation for future functional and evolutionary investigations.

Introduction

Snake venom (SV) serves as a potent chemical weapon, enabling snakes to subdue their prey or to defend against predators and humans. These venoms are complex cocktails containing hundreds of biologically active proteins and peptides that selectively target vital organs [1]. The molecular composition of SV varies at different taxonomic levels, defined as interfamily, intergeneric, interspecific, and intraspecific variability [2]. Such differences have a significant impact on the curative effects of antivenoms, which remain the most effective treatment against snakebite to date [3]. Thus, the determination of SV composition at the species level is essential to develop specific antivenoms, ensuring effective management of snakebites. Additionally, characterizing SV is crucial for the discovery of novel bioactive proteins and peptides with putative therapeutic potential [4]. Furthermore, SV proteomic characterization can help understand the evolutionary mechanisms driving venom diversity, adaptation, and speciation [5].

The *Montivipera* genus has a relatively recent history of discovery, and according to different authors 8 to 10 species have been described so far [6]. These mountain vipers are endemic to the Near and Middle East region encompassing Türkiye, Armenia, Azerbaijan, Iran, Syria, and Lebanon and are found at altitudes between 1000 and 2000 m above sea level (a.s.l.) (IUCN red list, https://www.iucnredlist.org)

(Fig. 1). They can be identified by their distinct colored dorsal patterns and body lengths ranging from 20 to 80 cm, in combination with other morphometric characters. The venoms of *Montivipera sp.* remain scarcely studied so far. To address this knowledge gap, this study proposes a comprehensive venom investigation from five *Montivipera* species: *M. bornmuelleri*, *M. bulgardaghica, M. xanthina, M. raddei*, and *M. albizona*. For the sake of comparison, we also included the more documented venom of the sister-genus *Macrovipera lebetinus.*

Since the *Montivipera* species inhabits high altitudes –hence their name "Mountain-viper – human snakebites are rare. The recorded clinical manifestations upon *Montivipera* envenomation include local symptoms such as swelling, ecchymosis, tissue damage and local haemorrhage, while systemic symptoms are hemostatic alterations, neurotoxicity and hypotension [7–9]. Importantly, it has been shown that in the absence or delay of antivenom administration, a *Montivipera* snakebite can lead to long-term morbidity or even mortality [8, 10]. Up till now, no antivenoms are available for *Montivipera* species except for a polyvalent antivenom developed against *M. xanthina* among other Eurasian vipers [11]. Therefore, a deep proteomic characterization of *Montivipera* venoms can allow the exploration of the potential cross-reactivity of existing antivenoms, which is particularly relevant given the limited clinical need and lack of commercial interest in developing species-specific antivenoms.

At the functional level, the *Montivipera* venoms are poorly studied. However, a few investigations of their biological activities have shown promising biomedical potential. Specifically, the venoms of *M. raddei* and *M. bulgardaghica* exhibit cytotoxic effects on both cancerous and non-cancerous cell lines, suggesting potential anti-proliferative properties [12]. In addition, *M. xanthina* venom has both cytotoxic and antimicrobial effects on cancer cell lines and on fungi and bacteria, respectively [13, 14]. Among the investigated species, *M. bornmuelleri* venom has been the most studied, revealing a range of interesting biological activities, including antimicrobial, pro- and anticoagulant, cytotoxic, immunomodulatory, neurotoxic, and vasorelaxant effects [15–21]. These activities evidence the diversity of *Montivipera* venom components, underscoring the importance of their identification and characterization for both therapeutic development and research applications.

Recently, the venom proteomic content of four *Montivipera* species, including *M. xanthina, M. bulgardaghica, M. raddei* and *M. albizona*, has been evaluated [12, 22–24]. These studies yielded variable results regarding both the number of proteins identified in each venom and the different protein families detected. Thus, the characterization of the proteomic composition of these four venoms remains to be performed to validate and complete previous reports. Moreover, the composition of *M. bornmuelleri* venom has never been analyzed and is still representing an important knowledge gap.

SV proteomes can be analyzed through different workflows, each having its advantages and disadvantages [25]. Shotgun proteomics is however the current gold standard for venom proteome deciphering as it offers a comprehensive, high-throughput approach to venom analysis, enabling the simultaneous identification (and relative quantification) of a broad range of proteins without the need for prior toxin separation. Unlike traditional gel-based or targeted methods, it allows for deeper coverage of

low-abundance components and post-translational modifications, which are critical for understanding venom complexity. This makes it particularly well-suited for profiling poorly characterized venoms, such as those of *Montivipera* species. The current work, additionally to shotgun proteomics, proposes to combine the classical trypsin digestion with the more recent Multi-Enzymatic Limited Digestion (MELD) [26]. Unlike conventional approaches, MELD minimizes sample loss and biases toward abundant proteins, enabling more accurate identification of low-abundance venom components. By generating a higher number of overlapping peptides for each toxin, it improves the protein sequence coverage and leads to more confident assignment of venom toxins. The integration of both trypsin and MELD digestion for analyzing SVs has been recently demonstrated to be effective for obtaining a comprehensive SV composition [27, 28].

Therefore, this study aims to analyze the venom composition of the five previously mentioned *Montivipera* venoms by employing SDS-PAGE, RP-HPLC and shotgun proteomics using Trypsin and MELD digestions. This allows us to determine the proteomic composition of *M. bornmuelleri* for the first time, in addition to complementing and validating the previously published venom proteomes of *M. bulgardaghica, M. albizona, M. raddei* and *M. xanthina.* Besides uncovering the proteomic profiles of the *Montivipera* venoms, we aimed to perform a comparative analysis of their proteomes to provide information about venom evolution among species of the *Montivipera* genus that live in non-overlapping geographical habitats. For comparison purposes, the *Macrovipera lebetinus* venom was analyzed as an outgroup, providing a phylogenetic reference point that helps distinguish lineage-specific traits among *Montivipera* venoms.

Materials and Methods

2.1 Reagents and chemicals

Coomassie Brilliant Blue (CBB) R250 (C.I 42660, Sigma-Aldrich Merck), Acetonitrile (ACN, 412412000-CER, Carlo Erba), Ultrapure water (412142-CER, Carlo Erba), Trifluoroacetic acid (TFA, 152005, Sigma Aldrich Merck), dithiothreitol ultrapure molecular biology grade (DTT, Thermo scientific), iodoacetamide bio-ultra pure (IAA, Sigma aldrich), Trypsin (Thermo scientific, MS grade), Chymotrypsin (Thermo scientific, MS grade), Glu- C (Thermo scientific, MS grade), formic acid (FA, Biosolve, ULC-MS/GC-SFC 99%).

2.2 Venoms

Venom samples of *M. bornmuelleri* were purchased from Latoxan (Portes-lès-Valence, France). The venoms of *M. bulgardaghica, M. raddei, M. albizona, M. xanthina* and *Macrovipera lebetinus* were purchased from Alphabiotoxine (Montroeul-au-bois, Belgium). Venoms were sourced from animals bred and kept in captivity. Animals originated from different geographical locations as follows: *M. bornmuelleri* (Lebanon), *M. bulgardaghica* (Türkiye), *M. raddei* (Türkiye), *M. xanthina* (Türkiye), *M. albizona* (Türkiye) and *Macrovipera lebetinus* (Türkiye).

2.3 SDS-PAGE analysis

Montivipera venom samples were separated using 12% polyacrylamide gels in the presence of SDS (SDS-PAGE). For this, 10 µg of the venom samples were mixed with Laemmli 5x buffer under reducing conditions (12.5% β-mercaptoethanol) or non-reducing conditions [29]. Samples were then heated at 95°C for 5 min and loaded onto the gel. The Precision Plus Protein Standard Kaleidoscope™ protein ladder was used as a molecular weight (MW) marker. Protein bands were visualized by CBB R250 staining for 1h at RT. Gel images were captured using the ChemidocTM XRS + imaging system. A linear regression logarithmic plot of molecular mass versus relative migration distance served as a calibration curve to calculate the MW, corresponding to each band.

2.4 Chromatographic Profiling

Lyophilized *Montivipera* venoms were dissolved in ACN/H_2O (5:95, v/v) at concentrations of 0.5 mg/mL. Venom samples (100 µg) were centrifuged at 10,000 rpm for 10 minutes at 4°C to eliminate impurities. The supernatants were applied onto a C18 Vydac® column 218TP54 (250 x 4.6mm, 5 µm, 300 Å) for fractionation. The chromatographic run was performed on a Waters HPLC system coupled to a Waters 600 controller and a Waters 2487 dual wavelength UV detector. Venoms were eluted using a segmented three-step gradient of solvent B in solvent A, going from 5 to 30% B in 8 min, 30 to 75% B in 27 min and 75 to 100% B in 5 min. The total run duration was 40 min at a flow rate of 1 mL/min. Solvent B consisted of 0.1% TFA in ACN, and solvent A was composed of 0.1% TFA in ultrapure water. The UV-absorbance of the eluted venom components was monitored at 214 and 280 nm. The data acquisition was performed using Waters Empower 3 software. Peak integration was performed automatically by Empower 3 software and manually adjusted when necessary.

2.5 Proteomics analysis

2.5.1 Sample preparation

Lyophilized venoms (10 µg) were dissolved in 2 µL of 50 mM NH_4HCO_3 at pH 7.8. Reduction of the venom protein and peptide disulfides was performed using 2 µL of 30 mM DTT for 40 min at 56°C while shaking at 650 rpm. The reduced cysteines were then alkylated using 3 µL of 60 mM IAA for 30 min at RT at dark. A quench of the alkylation reaction was performed by incubating the samples in 11 mM DTT for 10 min at RT in the dark. For the digestion of the samples, two different protocols were employed: the conventional trypsin digestion and the MELD, where 3 proteolytic enzymes are used simultaneously to digest the venom samples, including trypsin, chymotrypsin, and Glu-C.

Trypsin digestion. A first trypsin digestion was performed by adding proteomic grade enzyme to the reduced and alkylated venom samples to reach a final trypsin/substrate ratio of 1/50. Samples were then incubated overnight at 37°C, while shaking at 650 rpm. A second tryptic digestion was performed the following day to a trypsin/substrate ratio of 1/100 in ACN at a final concentration of 80% (v/v) for 3h at 37°C. The enzymatic digestion was quenched by acidification of the medium using TFA at a final

concentration of 0.5%, to reach pH 3.0. The digested samples were speed-dried and reconstituted in 20 µL of 0.1% TFA for desalting on ZipTip® C18 resin pipette tips Pierce™ according to the manufacturer's protocol. The elution was performed using 20 µL of 50.0% ACN, 49.9% H_2O, 0.1% TFA (v/v). The resulting eluates were lyophilized and reconstituted to 15 pmol/9 µL in H_2O containing 0.1% TFA, then submitted to mass spectrometry analysis.

MELD digestion. The multi-enzymatic limited digestion involved two parallel digestions of 10 µg of lyophilized venoms with the same enzymes but at different ratios. A MELD concentrated digestion was performed at enzyme/substrate ratios of 1/85, 1/85, and 1/55 for trypsin, Glu-C, and chymotrypsin, respectively. Simultaneously, a MELD diluted digestion was performed at enzyme/substrate ratios of 1/750, 1/750, and 1/500 for trypsin, Glu-C, and chymotrypsin, respectively. Enzyme mixtures were prepared on ice in 25 mM NH_4HCO_3 and 5 mM $CaCl_2$ solution. Digestions were performed at 37°C for 2 h while shaking at 650 rpm. The reactions were stopped using TFA at a final concentration of 0.5%. Both concentrated and diluted MELD digests were pooled, and 3.5 µg of venom-digested samples were desalted using ZipTip® C18 resin pipette tips Pierce™ according to the manufacturer's protocol. The resulting digests were lyophilized and reconstituted to 15 pmol/9 µL in H_2O with 0.1% TFA for mass spectrometry analysis.

2.5.2 Mass spectrometry analysis

Digested samples were analyzed using an Acquity M-class ultra-high-performance liquid chromatography (UPLC) system (Waters, Corp., Milford, CT, USA) coupled with a Q-Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA) in nano-electrospray positive ion mode. The trap column used ahead of the analytical column was a Symmetry C18 5µm (180 µm x 20 mm). Venom digests separation was performed using an analytical HSS T3 C18 1.8 µm (75 µm x 250 µm) (Waters, Corp., Milford, CT, USA) chromatographic column. Samples were loaded at 20 µL/min on the trap column in 98% solvent A and 2% solvent B for 3 min and were subsequently separated on the analytical column at a flow rate of 500 nL/min using a linear gradient as follows: initial condition 2% solvent B hold for 2 min; 2 to 7% B in 5 min; 7 to 30% B in 55 min; 30 to 40% B in 10 min; 40 to 90% B in 3 min; maintained at 90% for 5 min before the column was reconditioned to initial conditions. The mobile phase composition was as follows: solvent A was made of 0.1% FA in water and solvent B was made of 0.1% FA in ACN.

The nano-HPLC was connected to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer method is a TopN-MSMS method where N was set to 12, meaning that the spectrometer acquires one Full MS spectrum, selects the 12 most intense peaks in this spectrum (singly charged and unassigned charge precursors excluded) and makes a Full MS2 spectrum of each of these 12 compounds. Precursor mass was measured by an Orbitrap mass analyzer at the resolution of 70,000 (at m/z 200) and a mass range of 400 to 1600 m/z. AGC target of 3×10^6 or maximum injection time of 200 ms. Data was collected in data-dependent acquisition (DDA) mode. For MS^2 analysis, the 12 most intense precursor ions were selected for high-energy collision-induced dissociation with an isolation window of 2.0 m/z. The fragmented ions were scanned

at a resolution of 17,500 (at m/z 200) with an AGC target of $1x10^5$ or maximum injection time of 50 ms. Normalized collision energy (NCE) set at 27. The main parameters for Q-Exactive tune were spray voltage of 2.3 kV, capillary temperature of 270°C and S-Lens RF level of 50.0.

Protein identification by automated *de novo* sequencing was performed using the software Peaks Studio X + version 10.5 against the database created by the deposits related to “Snake + venom” in the UniProt repository (117,665 sequences, April 2024). Carbamidomethylation was set as a fixed modification, and oxidation (M) and Deamidation (NQ) were set as variable modifications, with a maximum missed cleavage number limited to 2. Precursor mass and fragment mass error tolerances were set at 5 ppm and 0.015 Da, respectively. A false discovery rate (FDR) of 0.1% and unique peptide ≥ 2 with significant peptides were used to filter out inaccurate proteins for the PEAKS search algorithms and “*De novo* only” analysis with a − 10lgP > 20 for the database match with high confidence. To achieve confident results, at least one unique peptide is needed for a protein group. The top proteins were used for classification; these proteins are the components supported by the most unique peptides in the group.

2.5.3 Relative abundance

To estimate the relative abundance of venom proteins, we employed a label-free quantification approach based on the intensity of the three most intense peptide ions detected by mass spectrometry. This method relies on the assumption that the most abundant peptides in each protein contribute significantly to its overall signal intensity. By summing up the intensities of the three most intense ions corresponding to each protein, we obtained a semi-quantitative measure of protein abundance, allowing for comparative analysis of venom composition across samples.

2.6 Protein Similarity Coefficient score

To estimate the similarity of venom composition between *Montivipera* species, we used the Protein Similarity Coefficient (PSC) as described by Calvete et al. [30]. We calculated the PSC for two given species, “a” and “b”, using the following formula:

$$PSCab = 2\,x\,\frac{nb.\ of\ proteins\ shared\ between\ a\ and\ b}{total\ nb.\ of\ distinct\ prot.\ in\ a + total\ nb.\ of\ dictinct\ prot.\ in\ b}\,x\,100$$

Since protein identification was based on sequence homology, we considered two proteins to be similar between venoms if they matched the same reference protein, indicated by an identical accession number. It is important to note that these measures provide only minimal estimates of similarity between the venom profiles. It is likely that some proteins we currently consider identical may, in fact, differ in one or more aspects if complete sequences were available.

2.7 Phylogeny

Mitochondrial cytochrome b (88 sequences, 1062 bp) was aligned using the MAFFT algorithm with default parameters (http://mafft.cbrc.jp/alignment/server/ accessed May 1–10, 2025). The best evolutionary model was determined using MEGA X (https://www.megasoftware.net/ accessed May 1–

10, 2025) [31, 32]. The maximum likelihood (ML) method and Bayesian inference (BI) procedure were performed using PHYML [33] and MrBayes [34], respectively, available on the NGPHYLOGENY website (https://ngphylogeny.fr). Codon positions included were as follows: 1st + 2nd + 3rd + Non-coding. The final dataset comprised a total of 1,062 positions. Bayesian inference analysis was performed for 750,000 generations, 50% of which were eliminated as burn-in. For the maximum likelihood method, the reversible General Time model (BIC: 9460.207, AICc: 7741.886, lnL: -3688.55) and invariant sites ([+ I], 67.616% of sites) were applied. The approximate likelihood ratio test (aLRT) was of the SH type. The bootstrapping procedure (number of bootstraps: 1000) and a posterior Probability were applied for ML and BI, respectively. *Macrovipera lebetinus* (KJ415300-301) was used as an outgroup.

2.8 Graphs and statistics

Proteomic data was visualized using Microsoft Excel to generate pie charts and bar charts. The Venn diagrams were generated using the multiple list comparator tool from the molecular biology tools (molbiotools) server.

Results

For clarity and consistency, the following abbreviations for SV components will be used throughout this study: **SVMP**: snake venom metalloproteinase, **PLA_2**: phospholipase A_2, **SVSP**: snake venom serine protease, **svVEGF**: snake venom endothelial growth factor, **CTL**: C-type lectin, **CRiSP**: cysteine-rich secretory protein, **SVMPi**: SVMP inhibitor, **LAAO**: L-amino acid oxidase, **Pep**: peptidase, **5'-NT**: 5'-nucleotidase, **PLB**: Phospholipase B, **GC**: Glutaminyl cyclase, **PDE**: phosphodiesterase, **NGF**: nerve growth factor, **NP**: natriuretic peptide, **KSPI**: Kunitz-type serine protease inhibitor, **Dis**: Disintegrins

3.1 Phylogenetic analysis

Phylogenetic analysis, ML and BI, involving 88 CYTB mitochondrial sequences from *Montivipera* species, constructed trees with similar typologies confirming the *xanthina complex*, including the *xanthina* and *bornmuelleri* clades, and the *raddei* complex established by Stümpel et al. [6]. Phylogenetic tree typology showed a monophyletic clade, "*raddei* complex", including *Montivipera raddei* (*M. r. raddei, M. r. kurdistanica*), *M. albicornuta* (with two evolutionary lineages), *M. kuhrangica*, and *M. latifii*. This cluster reflects the geographical proximity of these species, as indicated by Stümpel et al. [6]. Another monophyletic clade containing *M. albizona*, *M. bulgardaghica, M. wagneri*, and *M. bornmuelleri* is also supported. This clade, named "*bornmuelleri*" by Stümpel et al. [6], is geographically located in the Middle East. Interestingly, individual spec1 named "*M. bulgardaghica*" in Fig. 2 has probably been misidentified as it is grouped with individuals of the *M. albizona* species. In parallel, individuals identified as *Montivipera xanthina* constituted two distinct evolutionary branches independent of geographical origin. Stümpel et al. indicates that lines comprising *M. xanthina* individuals coded xt5, xt6, xt8, and xt9 would reflect a Taurus morphotype [6]. The *xanthina* group seems to originate from the East.

3.2 SDS-PAGE profile analysis of *Montivipera* venoms

The SDS-PAGE analysis of *Montivipera* venoms revealed that their components belong to a wide range of molecular weights ranging from 9 to ~ 125 kDa (Fig. 3**)**. The banding pattern suggests the presence of protein families commonly found in viperid venoms, including SVMPs, SVSPs, LAAOs, CRiSPs, PLA_2, svVEGFs, CTLs and Disintegrins. The venoms exhibited varying levels of complexity in terms of band number. Under non-reducing conditions, the least diverse venoms displayed 14 bands (*M. bornmuelleri* and *M. bulgardaghica*) while the most diverse venom displayed 19 bands (*M. raddei*) **(**Fig. 3a**)**. Notably, the number of bands decreased in reducing conditions highlighting the presence of intermolecular interactions between venom components **(**Fig. 3b**)**. Overall, the migration pattern of *Montivipera* venoms revealed a significant resemblance between venoms with 12 bands being commonly present in all studied *Montivipera* venoms. On the other hand, the venom of the sister genus *Macrovipera lebetinus* revealed major differences in the migration profile compared to the *Montivipera* venoms.

3.3 RP-HPLC analysis of *Montivipera* venoms

Next, we analyzed the venoms using analytical C18 RP-HPLC. Using our separation protocol, the *Montivipera* venom profiles displayed between 23 and 33 chromatographic peaks (Fig. 4). The venom chromatograms shared common major chromatographic peaks notably at retention times of ~ 10.5, 11.3, 15.7 and 19 min (shaded areas in Fig. 4). It is worth noting that these common peaks exhibited different intensities among *Montivipera* species suggesting that the components within these peaks have different abundances in the venoms. According to previous proteomic analyses of *Montivipera* venoms, the early-eluting fractions likely contain NPs, KSPI and Disintegrins (shaded in yellow), followed by PLA_2s and SVSPs (shaded in green). The more hydrophobic, late-eluting peaks might be predominantly composed of SVMPs and LAAOs (shaded in red) [24, 35].

Interestingly, certain *Montivipera* venoms exhibited distinct major peaks that were not detected in other venoms, such as the peak eluted at 18.2 min in *M. xanthina* and another at 20.4 min in *M. bulgardaghica* (indicated by red arrows in Fig. 4). Overall, the RP-HPLC profiles of *Montivipera* venoms showed a high degree of similarity, whereas the profile of *Macrovipera lebetinus* venom appeared more atypical. For instance, the chromatogram of *Macrovipera lebetinus* did not exhibit the major peaks present in all the *Montivipera* venoms, such as the peaks at retention times 10.5, 15.7 and 19 min (shown in shaded areas).

3.4 Proteomics Analysis of *Montivipera* Venoms

3.4.1 Integration of Trypsin and MELD digestions in venom proteome analysis

To evaluate the efficacy of integrating both Trypsin and MELD digestion protocols in the analysis of *Montivipera* venoms, we compared the total number of proteins identified using each digestion protocol separately. Table 1 summarizes the total number of proteins identified by each digestion protocol, highlighting variations in the number of venom components detected across protocols.

Table 1
Comparison of the total number of proteins using trypsin and MELD digestion protocols

Species	*M. bornmuelleri*	*M. bulgardaghica*	*M. xanthina*	*M. r. raddei*	*M. albizona*	*M. lebetinus*	*Total*
# identified proteins by the trypsin approach	81	119	93	117	83	60	553
# identified proteins by the MELD approach	93	134	104	120	138	92	681
% variation	**+ 14.8%**	**+ 12.6%**	**+ 11.8%**	**+ 2.6%**	**+ 66.3%**	**+ 53.3%**	**+ 23.1%**

Using Venn diagrams to illustrate the overlap, we found that MELD digestion identified between 45 and 84 unique proteins that were not detected by Trypsin digestion alone (Fig. 5). On the other hand, the Trypsin digestion led to the identification of 13 to 59 proteins that were not identified by the MELD technique. This highlights the importance of combining both digestion protocols to get a more complete proteomic profile of the studied venoms.

Using both digestion protocols, we identified between 129 and 179 proteins in the *Montivipera* venoms, all classified into 16 to 18 protein families. The complete list of identified proteins and peptides for the studied species is provided in **Supplementary Table S1**. On the other hand, the number of proteins found in the sister-genus *Macrovipera lebetinus* was significantly lower, with 105 proteins grouped into 17 families. Table 2 summarizes the diversity of protein families and indicates the number of isoforms detected within each family. For classification purposes, the TOP proteins, defined as those supported by the highest number of unique peptides within each group, were selected to represent the corresponding protein families.

Table 2
Diversity and number of proteins and protein families identified in *Montivipera* venoms and *Macrovipera lebetinus* using shotgun proteomics

	M. bornmuelleri	**M. bulgardaghica**	**M. xanthina**	**M. raddei**	**M. albizona**	**M. lebetinus**
Nb. of proteins	129	174	145	179	167	105
Nb. of protein families	17	16	18	16	17	17
Id. protein families	**Nb. of isoforms**					
SVMP	35	36	23	32	40	17
PLA_2	8	27	35	51	35	5
CTLs	24	27	33	22	37	32
SVSP	9	16	11	6	11	6
svVEGF	2	7	5	11	5	5
LAAO	3	3	3	3	3	5
Disintegrin	6	4	3	3	6	5
Peptidase	4	7	4	6	5	16
NGF	5	1	3	3	3	3
KSPI	5	10	2	8	1	-
5'NT	1	1	1	1	1	1
PLB	1	1	1	1	1	1
CRiSP	4	-	11	9	8	1
SVMPi	-	-	1	-	2	1
NPs	2	1	1	-	-	1
GC	14	15	-	15	2	-
PDE	2	-	1	-	1	1
Hyaluronidase	-	1	4	5	-	1
Uncharacterized	4	17	3	3	6	4

3.4.2 Diversity of enzymatic vs. non enzymatic venom components

Our proteomics analysis revealed a higher diversity of enzymatic components compared to non-enzymatic components for the five *Montivipera* venoms (Fig. 6). Notably, the enzymatic components found in *Montivipera* venoms include PLA_2s, SVMPs, SVSPs, LAAOs, peptidases, hyaluronidases, PLB, PDE, and 5'-NT. On the other hand, the identified non-enzymatic components include families such as svVEGFs, CTLs, CRiSPs, Disintegrins, NGF, NPs, KSPIs, and SVMP inhibitors. *M. bornmuelleri* venom contains 53 enzymatic toxins and 40 non-enzymatic toxins. *M. bulgardaghica* and *M. albizona* venoms shared similar numbers of enzymatic components (81 and 82, respectively), while fewer non-enzymatic components were identified (53 and 56 isoforms, respectively). *M. xanthina* venom contains 62 enzymatic components and 42 non-enzymatic components. The enzymatic and non-enzymatic components of *M. raddei* were 75 and 45, respectively. As a result, all *Montivipera* venoms exhibit a higher diversity of enzymatic toxins than their non-enzymatic compounds. Interestingly, almost equal amounts of enzymatic and non-enzymatic isoforms (49 and 43, respectively) were identified in the venom of *Macrovipera lebetinus*.

3.4.3 Protein families and their relative abundance in *Montivipera* venoms

The proteomics analysis of *Montivipera* venoms revealed the presence of the predominant protein families described in the viperid venoms. Based on their relative abundance (Fig. 7), these protein families can be classified as major, secondary, and minor components. The major protein families constituting the *Montivipera* venoms were SVMPs, PLA_2s, SVSPs, svVEGFs, CTLs and Disintegrins. *M. bornmuelleri* venom showed the highest SVMP abundance among all venoms, being the most abundant protein family in this venom, followed by svVEGFs, SVSPs, Disintegrins, CTLs and PLA_2s. Alternatively, CTLs are the most abundant component in *M. bulgardaghica* venom, followed by SVSPs, svVEGFs, PLA_2s, SVMPs and Disintegrins. Notably, the major constituents of *M. xanthina* venom were PLA_2s and svVEGFs followed by SVMPs, SVSPs, CTLs and Disintegrins. Similarly, the most abundant protein family in *M. r. raddei* venom is PLA_2, followed by CTLs, SVMPs, Disintegrins, svVEGFs and SVSPs. Regarding the *M. albizona* venom, its most abundant component is the CTL family, followed by svVEGFs, SVMPs, PLA_2s, and SVSPs. The venom of the closely related genus *Macrovipera lebetinus* has PLA_2s and SVMPs as its major components, followed by CTLs and Disintegrins. The main difference observed in this venom is its low abundance of svVEGFs (0.58%) as compared to the *Montivipera* venoms (7.84 to 22.12%). In addition, *Macrovipera lebetinus* venom exhibits an exceptionally high abundance of peptidases (17.6%) that was not evidenced in *Montivipera* venoms.

The secondary protein families constituting the *Montivipera* venoms include CRiSPs, peptidases, NGFs and LAAOs. Notably, CRiSPs were found only in the venoms of *M. albizona, M. raddei* and *M. xanthina* at abundances of 7.69%, 6.32%, and 2.2%, respectively. The abundances of NGFs, LAAOs and peptidases in *M. bornmuelleri* venom are 2.44%, 2.02% and 0.18%, respectively. *M. bulgardaghica, M. raddei, M. xanthina* and *M. albizona* exhibit lower abundances of NGFs, LAAOs and peptidases, having less than 1% of each.

Various minor components are identified in the *Montivipera* venoms, having abundances of less than 1% each. These components include 5'-NT, SVMP inhibitors, PDE, PLB, KSPI, NPs, peptidases, glutamyl cyclase, and hyaluronidases. Altogether, these components account for no more than 3.5% of the total venom compositions, and their presence is inconsistent across species, being found in some venoms but absent in others.

Our results revealed a conserved venom composition across *Montivipera* species characterized predominantly by high abundance of SVMPs, CTLs, PLA_2s, SVSPs, svVEGFs and disintegrins. While the relative abundances of these toxin families varied among species, they consistently represented the major components in all five *Montivipera* venoms analyzed. Remarkably, all *Montivipera* venoms displayed a high abundance of svVEGFs, highlighting a potential conserved role for these proteins within the genus. In contrast, the venom of *Macrovipera lebetinus* exhibited a markedly different composition, characterized by lower levels of svVEGFs and SVSPs, and a higher representation of peptidases, distinguishing it from the *Montivipera* venoms.

3.5 Comparative proteomics analysis of *Montivipera* venoms

To evaluate the similarity between the five *Montivipera* venoms, we calculated the PSC shown in Table 3. As we expected, the venom of the sister genus *Macrovipera lebetinus* shared the fewest proteins with the five *Montivipera* venoms investigated here (PSC = 30.3–44.4). On the other hand, we estimated that the *Montivipera* species share varying levels of similarity between their venom proteomes (PSC = 46.4–71.8). The highest similarities were found between the venoms of *M. bulgardaghica* and *M. albizona*, sharing 113 proteins (PSC = 66.3) and between the venoms of *M. xanthina* and *M. albizona*, sharing 112 proteins (PSC = 71.8). The lowest estimated levels of similarity were seen with the venom of *M. bornmuelleri*, which seems to have the least similar venom to other *Montivipera* species.

Table 3
Protein Similarity Coefficient (%) of the *Montivipera* venom proteomes and *Macrovipera lebetinus*

	M. bornmuelleri	***M. bulgardaghica***	***M. raddei***	***M. albizona***	***M. xanthina***	***M. lebetinus***
M. bornmuelleri	–	**56.8**	**51.3**	**48.6**	**46.7**	**44.4**
M. bulgardaghica	56.8	–	**60.6**	**66.3**	**58.3**	**37.3**
M. r. raddei	51.3	60.6	–	**56.1**	**57.4**	**30.3**
M. albizona	48.6	66.3	56.1	–	**71.8**	**35.3**
M. xanthina	46.7	58.3	57.4	71.8	–	**36.8**
M. lebetinus	44.4	37.3	30.3	35.3	36.8	–

Values in bold and italics are repeated values for same species

To identify the common and unique protein isoforms among the five *Montivipera* venoms, we performed a Venn diagram analysis (Fig. 8). It revealed the presence of 39 common proteins across the five *Montivipera* venoms. These common proteins belonged to 11 protein families (**Supplementary Table S2**). The common proteins among *Montivipera* venoms included 10 common SVMPs, identified based on sequence homology with SVMPs isolated from the venoms of *Macrovipera lebetinus* (1 isoform), *Vipera ammodytes* (4 isoforms), *Crotalus adamanteus* (3 isoforms), and *Echis carinatus* (2 isoforms). We also identified 8 common CTLs among *Montivipera* venoms identified based on sequence homology with CTLs previously isolated from the venoms of *Macrovipera lebetina* (2 isoforms), *Vipera ammodytes* (3 isoforms), *Daboia siamensis* and *Daboia russelii* (1 isoform each). Additionally, 3 common PLA_2 isoforms were identified based on sequence match with two acidic PLA_2s from *Daboia russelii* and *Daboia siamensis* venoms and a basic PLA_2 from *Vipera berus* venom.

Discussion

In this study, we analyzed and compared the proteomic composition of five *Montivipera* species using trypsin and MELD digestion protocols followed by a shotgun proteomics approach. Accordingly, this work presents the first comprehensive proteomic characterization of the *M. bornmuelleri* venom and completes the previously published venom proteomes of *M. bulgardaghica*, *M. xanthina*, *M. raddei* and *M. albizona* [12, 22–24]. We included in this study the analysis of the venom of *Macrovipera lebetinus*, the sister-genus of *Montivipera*, as an outgroup providing a broader phylogenetic perspective and allowing the identification of lineage-specific venom traits. The evaluation of these venoms using the same experimental approach allowed an accurate comparison of their proteomic composition.

The venom composition of *Montivipera* species was characterized using a multi-technique approach that included SDS-PAGE, RP-HPLC and shotgun MS. First, the SDS-PAGE analysis demonstrated a rich protein profile within *Montivipera* venoms (13 to 19 distinct protein bands). This is higher than the number of bands typically reported for other viperid genera, which generally range from 5 to 10 [36–38]. Consistently, RP-HPLC chromatograms displayed numerous well-resolved peaks (23–33 peaks), further reflecting the biochemical complexity of these venoms. The high number of chromatographic peaks supports the presence of a wide array of peptides and proteins in these venoms. This complexity was also confirmed by shotgun proteomics analysis, which identified between 129 and 179 distinct proteins per venom sample. This is significantly higher than what has been reported for other viperid genera analyzed by the same technique, including species from *Vipera*, *Bothrops*, and *Deinagkistrodon* [38–40]. Similarly, the analyzed *Macrovipera lebetinus* venom in this study exhibited a lower number of identified venom components (105 components) as compared to *Montivipera* venoms. Collectively, these methods revealed the complexity and high diversity of venom components in *Montivipera* species. This result is in line with a previous comparative proteomic study of various viperid genera, which identified *M. xanthina* venom as the most diverse and complex among those evaluated, further supporting the exceptional molecular diversity observed in *Montivipera* venoms [22]. The molecular diversity of *Montivipera* venoms is largely driven by the presence of multiple protein isoforms, which result from evolutionary processes.

These isoforms are likely to enhance venom efficacy and versatility, as members of the same protein family may exhibit variations in target specificity, binding affinity, or mechanism of action [2].

Our investigation revealed that the *Montivipera* venoms are predominantly composed of SVMPs, PLA_2s, SVSPs, svVEGFs, CTLs and Dis. These protein families are commonly found in viperid venoms and contribute to their toxicity [1]. Their presence indicates that *Montivipera* venoms can exhibit various clinical manifestations. First, SVMPs are key contributors to the hemorrhagic activity of viperid venoms [41]. The presence of SVMPs and their high abundance in all *Montivipera* venoms suggests a conserved hemorrhagic potential across the genus. Among them, *M. bornmuelleri* venom exhibited the highest SVMP content, consistent with clinical reports of local haemorrhage at the bite site in envenomed patients [9]. This hemorrhagic effect was further demonstrated in zebrafish embryos, where the venom induced visible pericardial bleeding [21]. Second, PLA_2s, another major component of *Montivipera* venoms, are known to exhibit an array of biological activities including neurotoxicity, cytotoxicity, myotoxicity, inflammation, and hemostatic alterations [42]. All analyzed *Montivipera* venoms showed a high abundance of PLA_2s, with *M. xanthina* and *M. raddei* venoms being particularly enriched in this toxin family. This suggests that following envenomation by these species, more pronounced local and systemic inflammatory or myotoxic symptoms might be observed. CTLs are known to induce hemostatic alterations by interfering with platelet activity and vascular integrity. These peptides can exert either procoagulant or anticoagulant effects, potentially contributing to bleeding disorders or thrombotic events during envenomation [43, 44]. Among the venoms analyzed, *M. bulgardaghica* and *M.* albizona exhibited the highest CTL abundance, suggesting a greater capacity to disrupt coagulation homeostasis in bites from these species. svVEGFs are known to increase vascular permeability and promote angiogenesis, thereby facilitating the diffusion of other venom components through tissues [45]. While svVEGFs are usually present in low abundance in viperid venoms, all the analyzed *Montivipera* venoms displayed high abundance of this toxin family. This enrichment appears to be a specific feature of the *Montivipera* lineage, as evidenced by the low abundance of svVEGFs in the venom of the closely related genus *Macrovipera lebetinus*. Such a pattern may reflect an evolutionary adaptation of *Montivipera* venoms to facilitate the dissemination of venom components.

The main protein families identified in our study were similar to those reported by other research groups. Previous proteomic studies on *Montivipera* venoms have reported inconsistencies in the identified protein families across different studies, with each capturing a partial venomics profile of each species [12, 22, 24, 35, 46]. In our study, we demonstrated the presence of all the protein families identified by previous studies although with different abundances. In addition, we detected low-abundance proteins that were not identified by other research groups, such as peptidase, 5'-nucleotidase, phospholipase B, aminopeptidases, glutamyl cyclase, Kunitz-type serine protease inhibitors, natriuretic peptides, and hyaluronidases. Thus, the proteomic profiles identified in this study aligned with and complemented the previously published venom proteomes of four *Montivipera* species, including *M. bulgardaghica, M. xanthina, M. raddei* and *M. albizona*. Importantly, this report provided the first characterization of *M.*

bornmuelleri venom which helped better understand the clinical manifestations induced upon envenomation.

The discrepancies observed between venom proteomic studies, especially in low-abundance components, could be attributed to two main reasons: venom intraspecific variation and the choice of our proteomic approach. Venom composition can vary at the species level based on geographic origin, diet, age, sex of the specimen and environmental influences [47, 48]. In our study, we used pooled venom from vipers bred and kept in captivity, whereas the previously mentioned studies used venom from wild-caught specimens. Thus, this can lead to variability in venom composition revealed at the proteomic level. On the other hand, variability in results can arise from factors related to the employed proteomic strategy, including sample preparation, workflow, mass spectrometry parameters, and data analysis. In this study, we used the shotgun proteomics approach, which was not previously used to analyze any of the *Montivipera* venoms. Notably, the main advantage of the shotgun technique is that it does not require a decomplexation step before mass spectrometric analysis, reducing the risk of protein and peptide loss [25]. This technique proved to be effective for the analysis of *Montivipera* venoms, as it enabled the detection of low-abundance proteins that were missed by previously used techniques.

The similarity of *Montivipera* venom proteomes was evaluated using the protein similarity coefficient (PSC). Our analysis revealed a high overall similarity in venom composition across the studied species. The highest proteomic similarity was observed among *M. bulgardaghica*, *M. albizona*, and *M. xanthina*. These three species are phylogenetically classified within the *xanthina* complex (Fig. 2), which may account for their close venom profiles [6]. Interestingly, although *M. bornmuelleri* also belongs to the *xanthina* complex, its venom proteome exhibited the lowest similarity to the three previously mentioned species. In contrast, *M. raddei*, a member of the phylogenetically distinct *raddei* complex, showed a high PSC score with *M. bulgardaghica*, *M. xanthina*, and *M. albizona* venoms. Notably, all four species with higher similarity (*M. bulgardaghica*, *M. xanthina*, *M. albizona*, and *M. raddei*) have adjacent geographic ranges within Türkiye [6]. Conversely, *M. bornmuelleri* is geographically isolated, being endemic to Lebanon [49]. These findings suggest that environmental and ecological factors associated with geographic distribution may exert a stronger influence on venom composition than phylogenetic relatedness alone.

Furthermore, the Venn diagram analysis revealed a high number of shared proteins between *Montivipera* venoms. The venoms that share most proteins were also shown to be *M. bulgardaghica, M. albizona* and *M. xanthina.* This agrees with a previous proteomic study showing high similarity between the venoms of *M. bulgardaghica, M. xanthina and M. albizona* [24]. The Venn diagram revealed that most of the shared proteins among *Montivipera* species belonged to the SVMPs, CTLs, PLA_2s and SVSPs families. The latter protein families are typically conserved in viperid venoms to ensure effective predation and defense [1]. This suggests that envenomation by different *Montivipera* species may produce comparable clinical manifestations. Furthermore, the presence of structurally and functionally similar toxins implies a potential for cross-reactivity with the same antivenom, particularly those targeting the common components responsible for the pathophysiological effects during an envenomation. On the other hand,

each venom exhibited unique proteins that were not identified in other species, suggesting that the *Montivipera* venoms have diverged enough to adapt their venom profiles to their specific environments or prey. These results are consistent with the SDS-PAGE and RP-HPLC profiles of *Montivipera* venoms, showing that they share a significant number of protein bands and chromatographic peaks. However, each venom also displayed unique differences, suggesting species-specific variations in composition.

This balance between conservation and diversity reflects how venom composition evolves to suit ecological niches while retaining core functional elements typical of the *Montivipera* genus. Variability in SV often arises as an adaptation to specific environmental pressures and prey availability [2]. In the case of *Montivipera* species, these snakes inhabit diverse and geographically distant regions, including the coastal areas of Türkiye, the Anatolian and Armenian mountains, the Lebanese mountains, as well as parts of Iran and Iraq (IUCN, https://www.iucnredlist.org/). Specifically, *Montivipera* species are found at different elevations and therefore, they may encounter different prey and experience diverse environmental pressures, which can drive venom evolution to optimize their hunting and survival strategies. Additionally, evolutionary adaptations allow these vipers to develop highly specialized venoms that are fine-tuned to the ecological niche they occupy, targeting specific prey and ensuring efficient predation in their respective environments.

It is important to note that the PSC provides only a minimal estimate of the similarity between venom profiles. In the absence of transcriptomic data from the venom glands of *Montivipera* species, protein identification was conducted through sequence homology matching against the UniProt “Serpentes” database. The database lacks a comprehensive inventory of venom proteins, particularly protein isoforms, that may correspond to those present in *Montivipera* venoms. Thus, our analysis was based solely on the identification of peptide fragments, without access to complete protein sequences. Consequently, proteins considered identical may differ, highlighting the potential for overestimation of true proteomic similarity.

Conclusion

The proteomic characterization of five *Montivipera* species in this study highlighted the exceptional diversity and complexity of their venoms. This report provides the first publication of the *M. bornmuelleri* venom composition and allows us to validate and complete the previously published venom proteomes of *M. bulgardaghica, M. albizona, M. raddei*, and *M. xanthina*. Most importantly, the analysis of these venoms with the same proteomic approach allowed a reliable comparison of their composition. Our comparative analysis revealed a high degree of similarity among *Montivipera* venoms, with certain species exhibiting closer compositional profiles than others. In contrast, the venom of the sister genus *Macrovipera lebetinus* displayed the least similarity, underscoring its phylogenetic distance and supporting its role as an appropriate outgroup in comparative studies. Thus, this study provides a better understanding of the clinical manifestations associated with *Montivipera* envenomation. Given the high degree of shared toxin families and the limited availability of species-specific antivenoms, our findings

shed light on the potential use of existing antivenoms to treat envenomation by other *Montivipera* species, offering valuable guidance in contexts where therapeutic options are scarce.

Declarations

Author Contributions: CS, ZF, LQ, CM, CL: conceptualization; CS, DR, TC, LQ: formal analysis; CS, DR, TC, VL: investigation, methodology; CS: writing original draft; AV, RF, VL, ZF, LQ, CM, CL: review and editing; CM, CL, LQ: Supervision. All authors read, agreed, and approved the final version of the manuscript.

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Figures

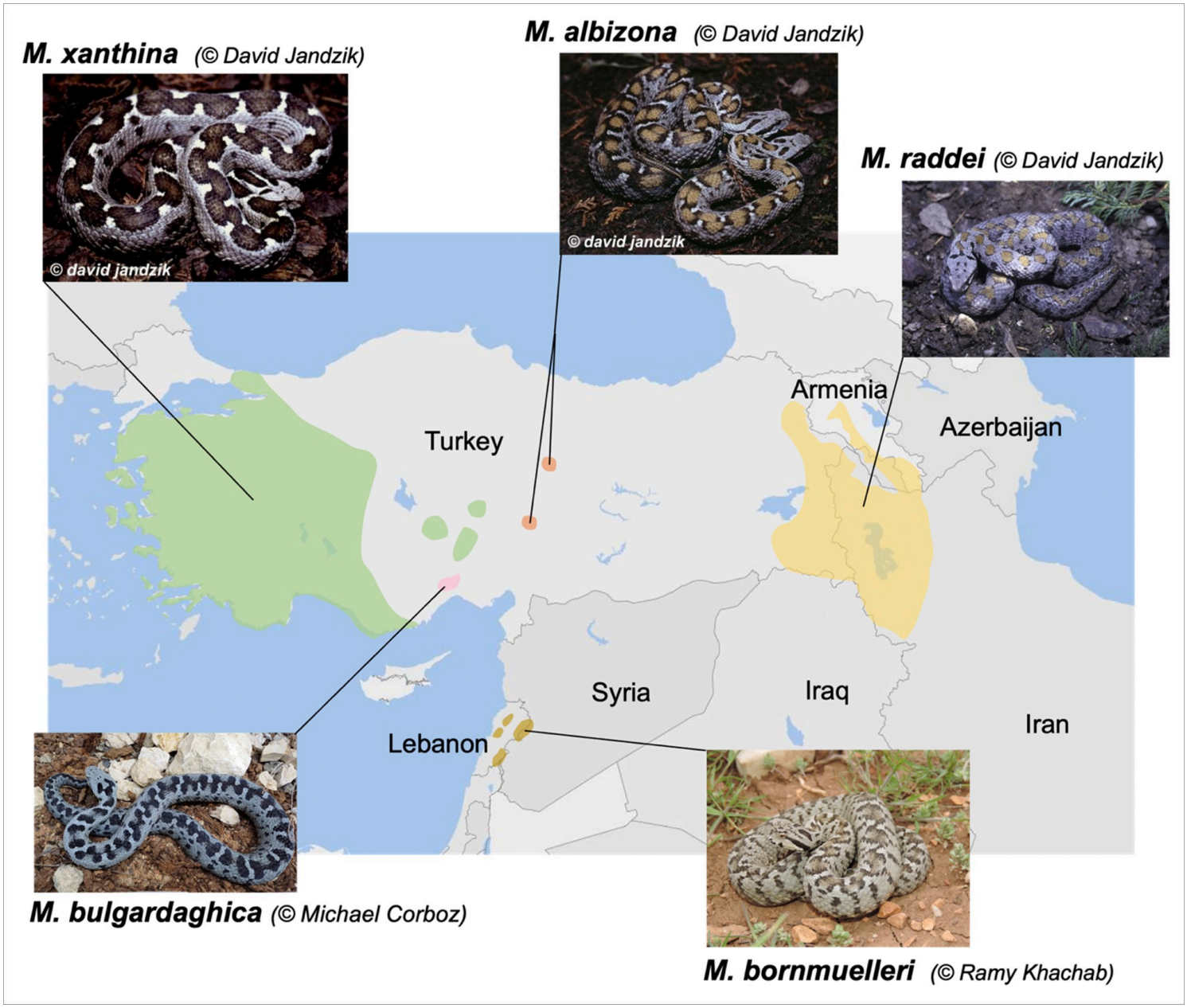

Figure 1

Geographical distribution of Montivipera species. M. bornmuelleri lives in Lebanese mountains (1000 to 2000 m a.s.l.), M. xanthina is found in Türkiye and Greece (0 to 2000 m a.s.l.), M. bulgardaghica is found in Turkish Taurus mountains (2000 m a.s.l.), M. raddei is present in Armenia, Türkiye, Iraq, Iran and Azerbaijan (1000 to 2000 m a.s.l.), M. albizona is found in the Anatolian diagonal in Türkiye (1500 and 1800 m a.s.l.) (The map was from Google (without copyright) and constructed using Powerpoint 2019 based data from the IUCN red list, https://www.iucnredlist.org) – © Snake photographs were used with the permission of the respective photographers.

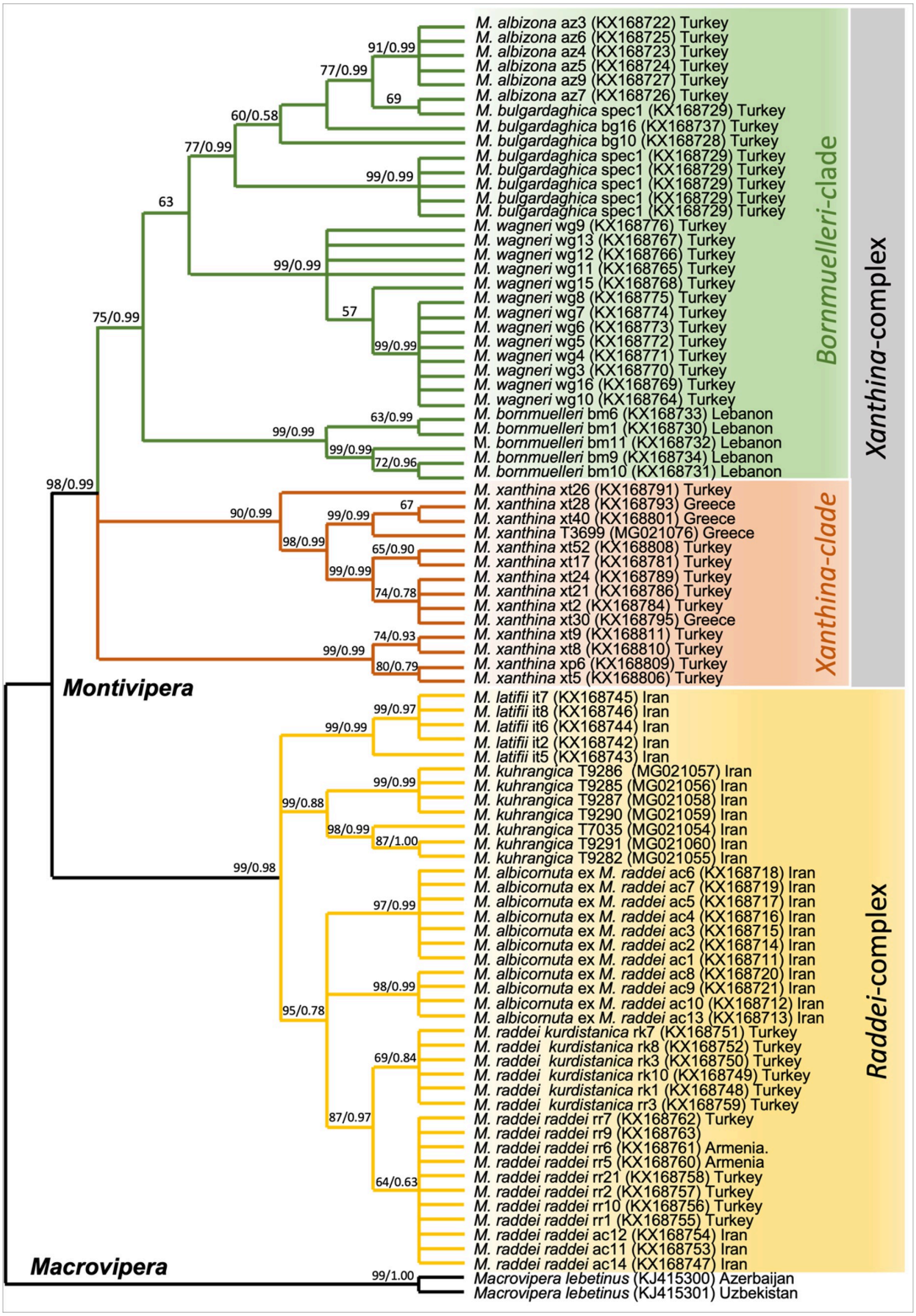

Figure 2

Evolutionary analyses, based on 88 mitochondrial cytochrome b sequences (CYTB: 1062 bp) from nine species of *Montivipera* (Viperidae, Colubroidea), were carried out using the maximum likelihood method (PhyML) and Bayesian inference (MrBayes). The maximum likelihood tree with the highest likelihood (-3688.55) is shown, and the rate-variation model resulted in some sites being evolutionarily invariant

([+I], 67.62% of sites). Bootstrap values based on 1,000 iterations have been indicated when greater than 50%. A posterior probability is also shown.

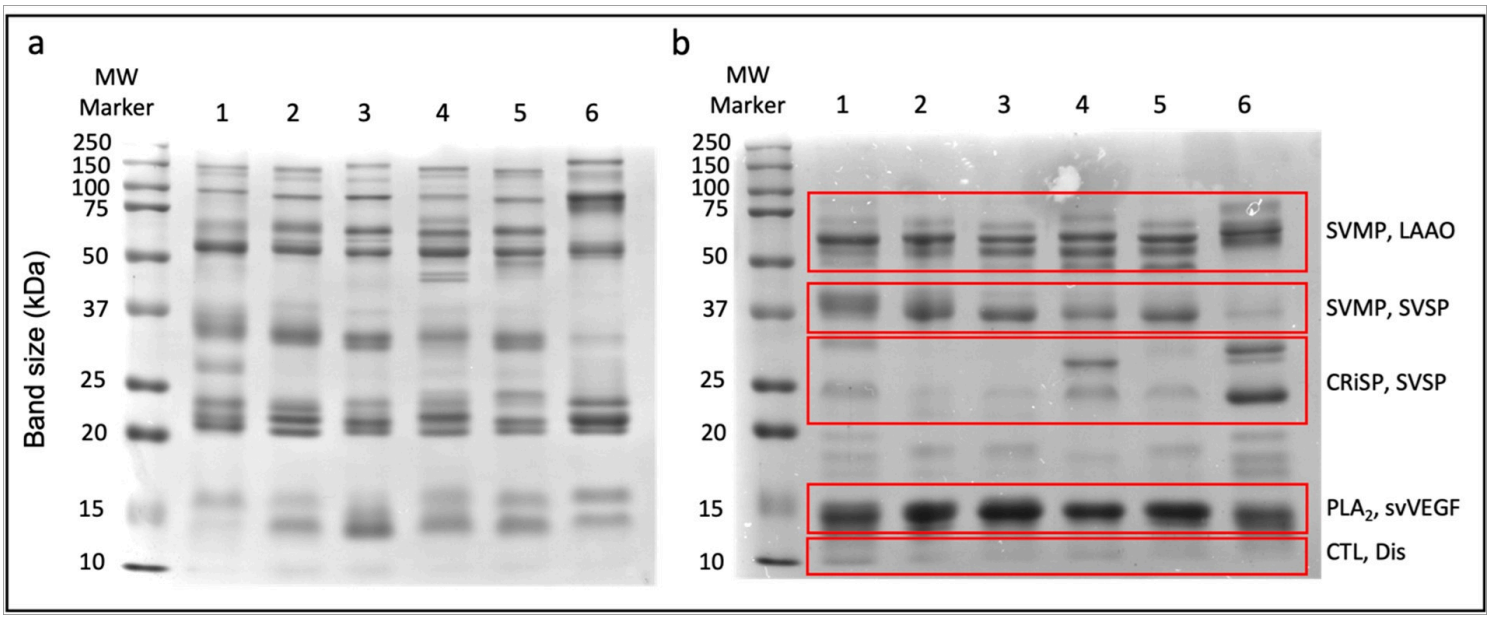

Figure 3

SDS-PAGE profiles of *Montivipera* venoms under **(a)** non-reducing and **(b)** reducing conditions stained with CBB R250. 1: *M. bornmuelleri*, 2: *M. bulgardaghica*, 3: *M. xanthina*, 4: *M. raddei*, 5: *M. albizona*, 6: *Macrovipera lebetinus*. The red boxes indicate clusters of protein bands within specific molecular weight ranges, corresponding to distinct protein families

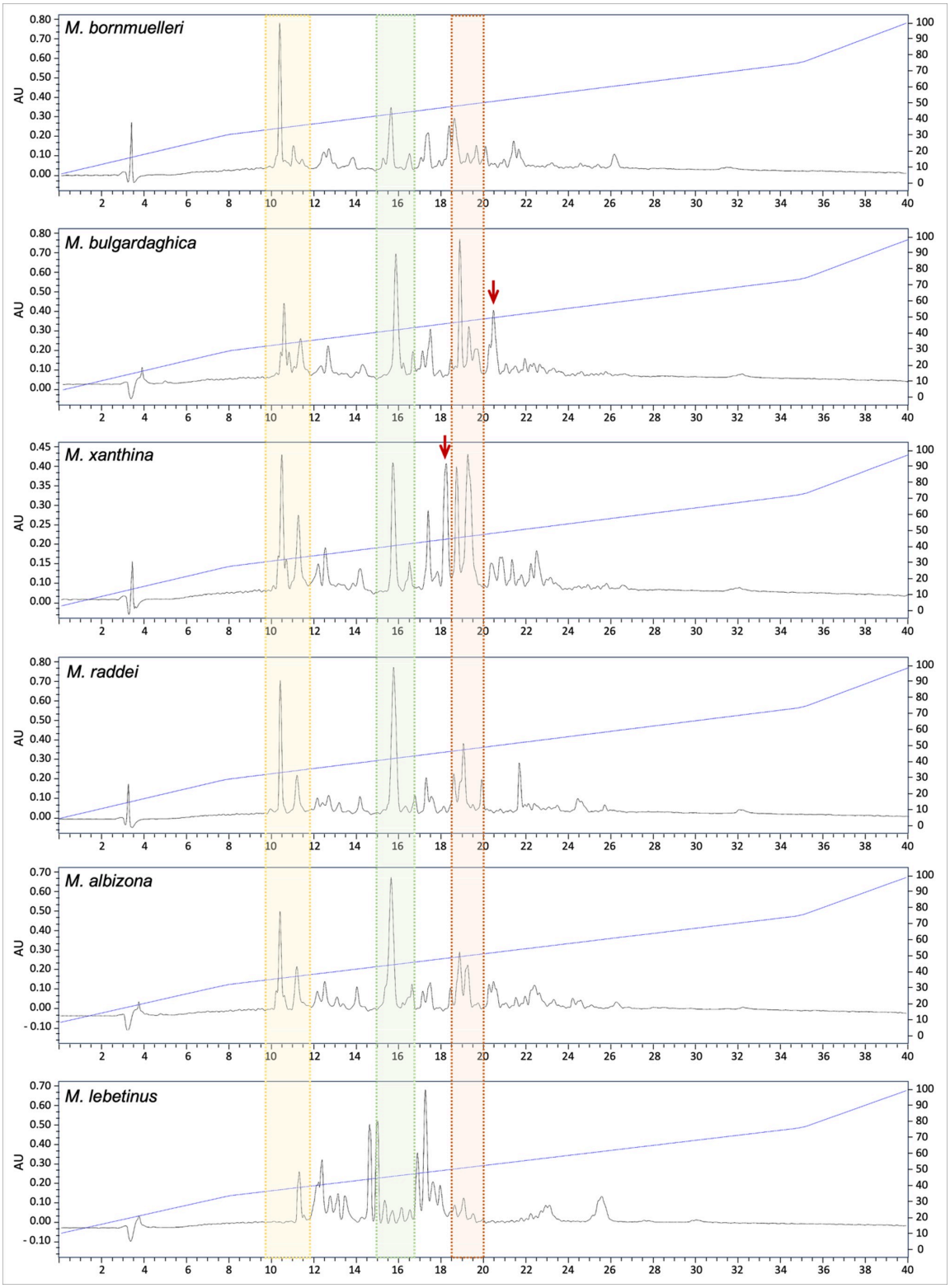

Figure 4

Chromatographic profiles of *Montivipera* venoms and *Macrovipera lebetinus* obtained by analytical C18 RP-HPLC. Chromatograms of *M. bornmuelleri, M. bulgardaghica, M. xanthina, M. r. raddei, M. albizona* and *Macrovipera lebetinus* (from top to bottom). Venoms were applied to a C18 Vydac analytical column equilibrated with 5% solvent B in solvent A, and components were eluted using a segmented linear gradient (blue line) going from 5 to 100% solvent B over a total run time of 40 min. The run was

performed at a flow rate of 1 mL/min and monitored at 214 nm. Shaded areas reveal shared peaks between the studied venoms, and the red arrows indicate the unique peaks in the venoms

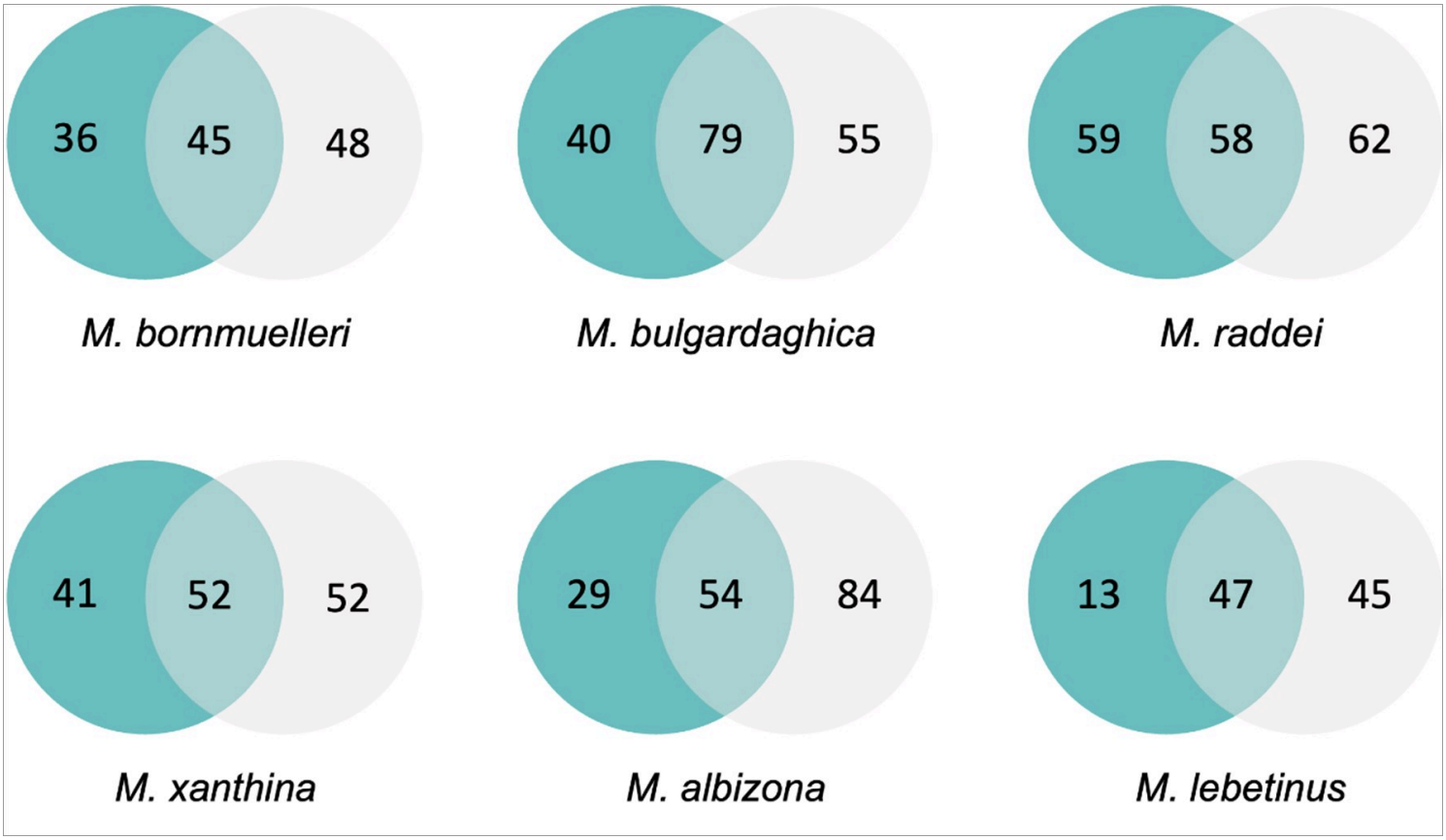

Figure 5

Venn diagrams illustrating the number of proteins identified by the shotgun proteomics using each digestion protocol (Trypsin vs. MELD) and the overlap between both protocols

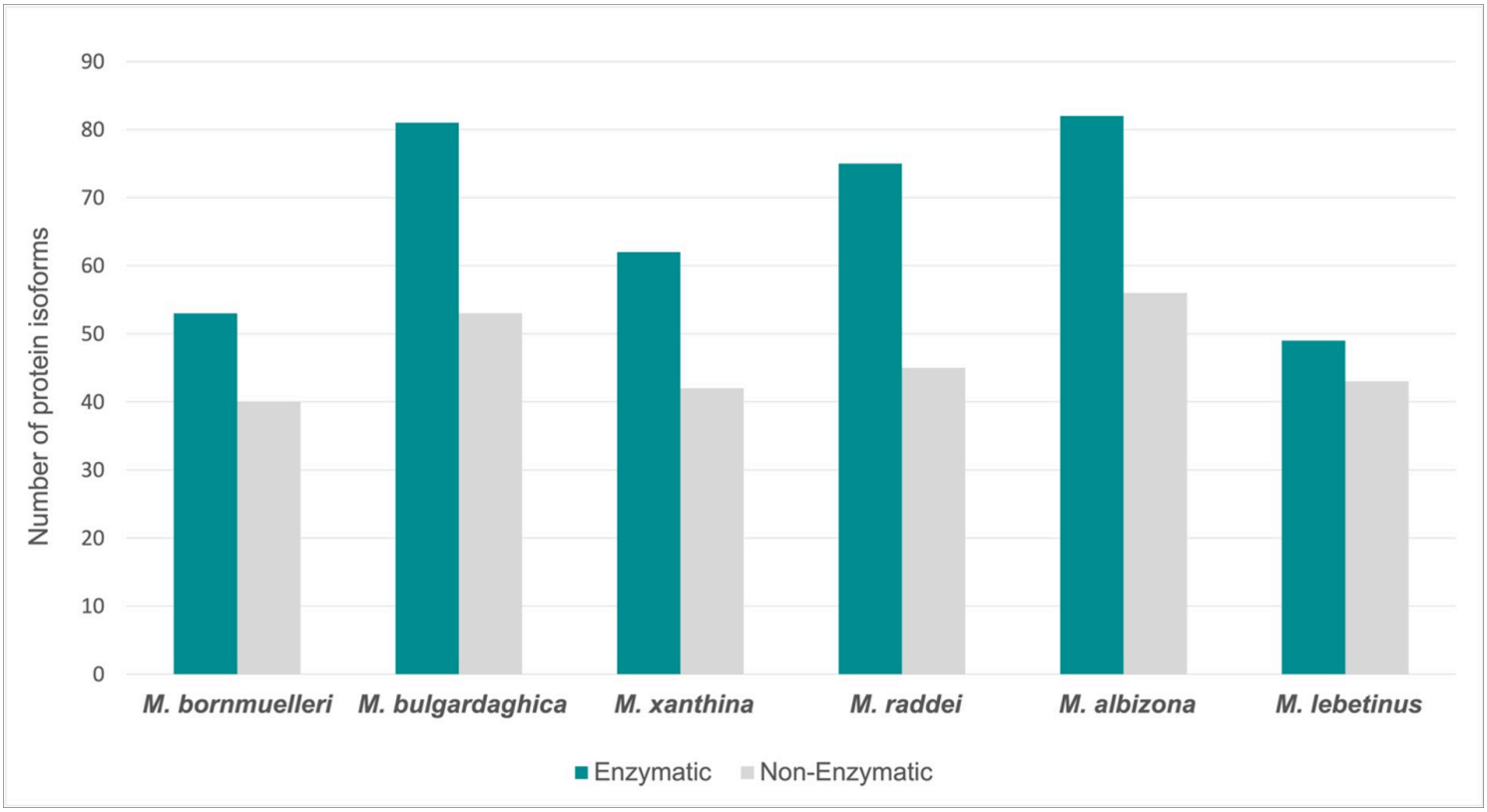

Figure 6

Bar chart showing the number of protein isoforms for enzymatic and non-enzymatic components in each of the *Montivipera* venoms and the *Macrovipera lebetinus* venom

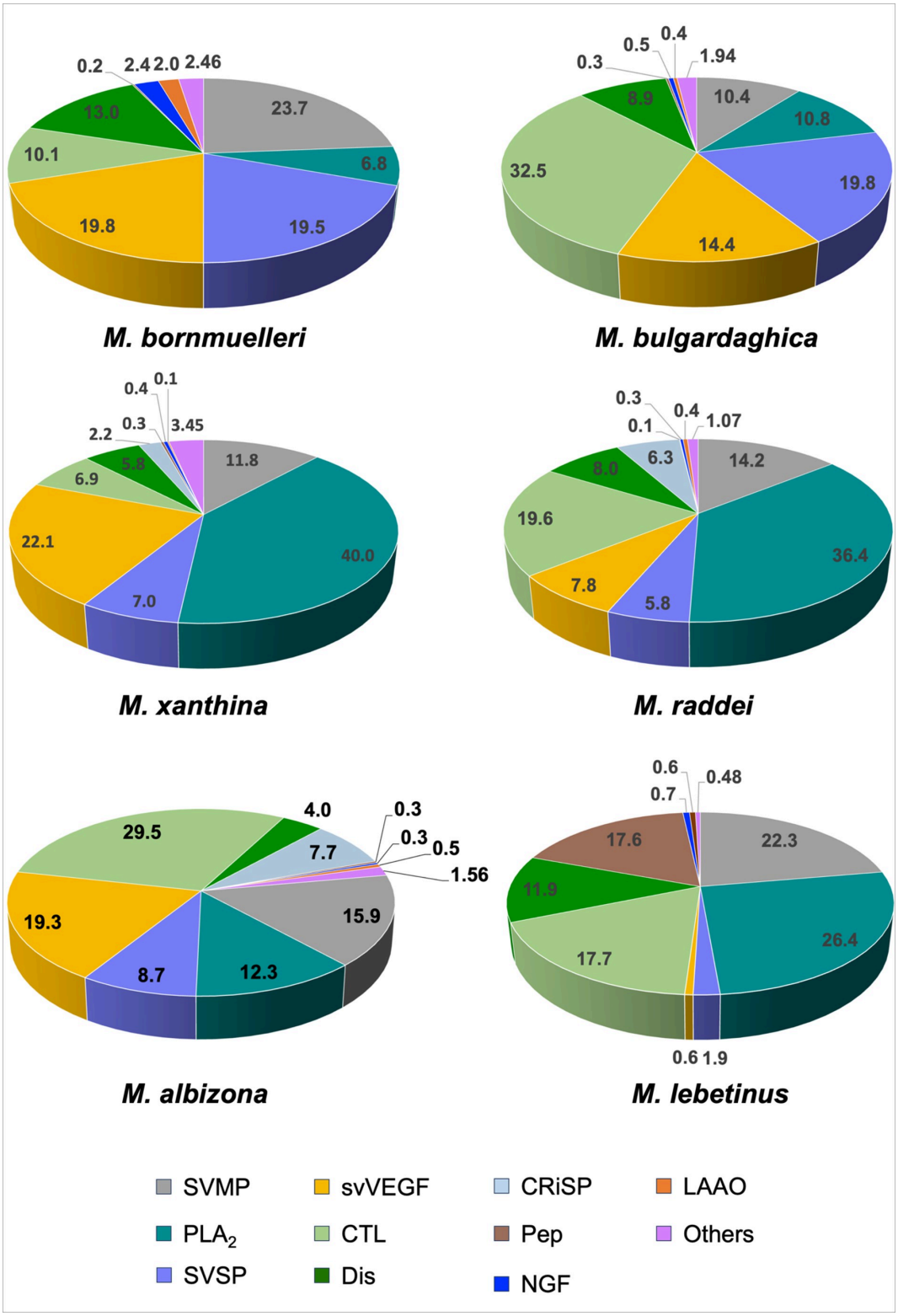

Figure 7

Pie charts showing the relative abundances of protein families in *Montivipera* and *Macrovipera lebetinus* venoms. Each section represents the relative abundance of a single protein family within the venom (the values represented are percentages). “Others” include the following minor protein families 5’-nucleotidase, SVMP inhibitor, phosphodiesterase, phospholipase B, Kunitz-type serine protease inhibitors, aminopeptidase, glutamyl cyclase, natriuretic peptide, and hyaluronidase.

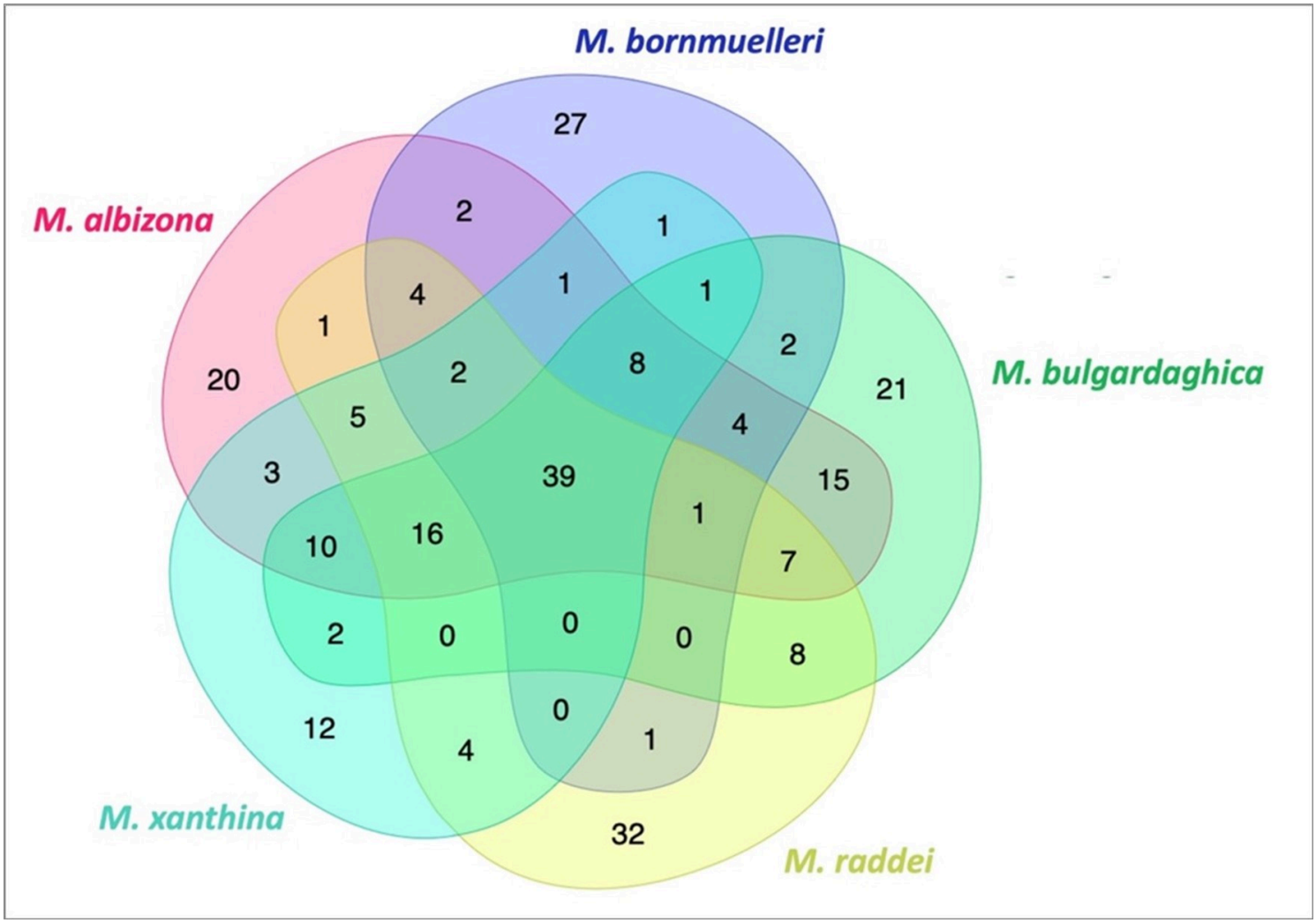

Figure 8

Venn diagram illustrating the common and distinct proteins between the venoms of *Montivipera* species, including *M. bornmuelleri, M. bulgardaghica, M. xanthina, M. r. raddei and M. albizona.* The values represented are the number of identified proteins.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx
- SupplementaryTableS2.pdf