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

Christina Sahyoun, Damien Redureau, Thomas Crasset, Rudy Fourmy, Aude Violette, Vincent Leignel, Ziad Fajloun, César Mattei, Christian Legros, Loïc Quinton

PII: S0041-0101(26)00035-8

DOI: https://doi.org/10.1016/j.toxicon.2026.109017

Reference: TOXCON 109017

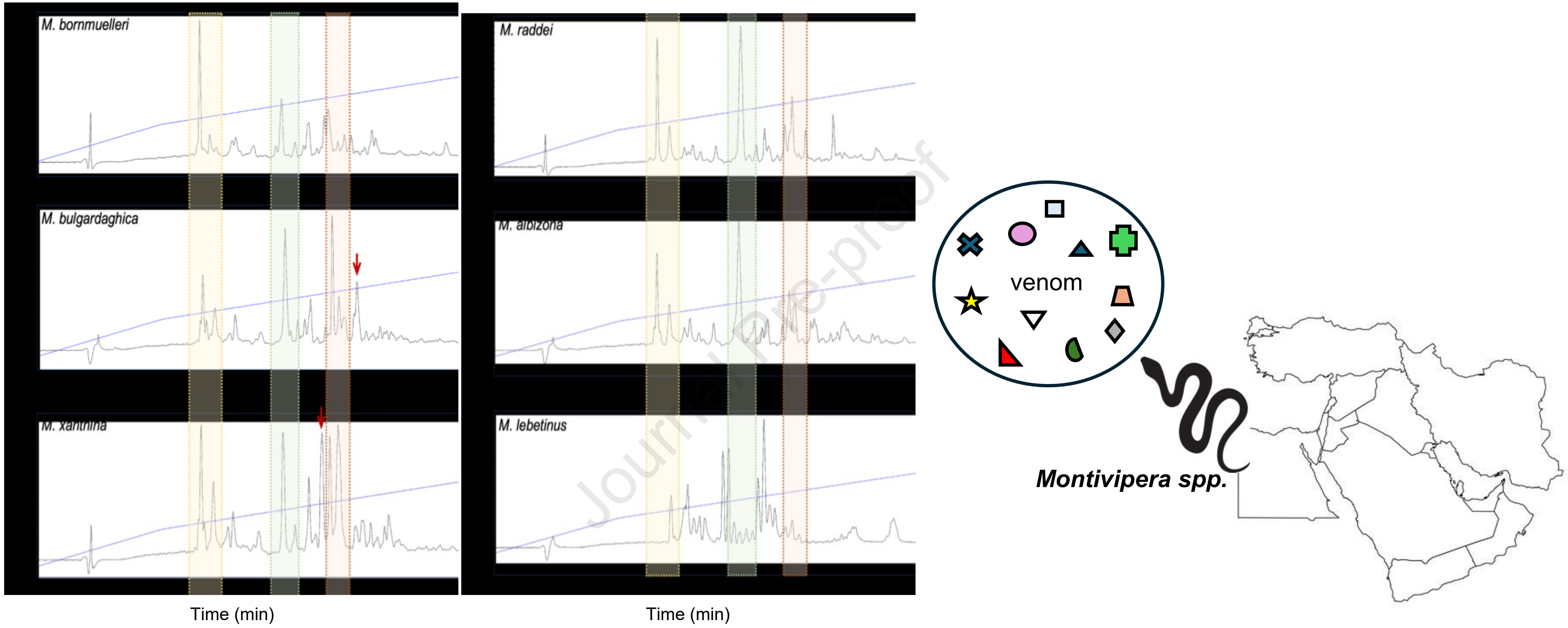
To appear in: *Toxicon*

Received Date: 8 December 2025

Revised Date: 20 January 2026

Accepted Date: 27 January 2026

Please cite this article as: Sahyoun, C., Redureau, D., Crasset, T., Fourmy, R., Violette, A., Leignel, V., Fajloun, Z., Mattei, C., Legros, C., Quinton, L., Comparative proteomic analysis reveals functional and evolutionary diversity in five *Montivipera* snake venoms, *Toxicon*, https://doi.org/10.1016/j.toxicon.2026.109017.


M. bornmuelleri
M. bulgardaghica
M. xanthina
Time (min)
M. raddei
M. albizona
M. lebetinus
Time (min)
venom
Montivipera spp.

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

Christina Sahyoun[1,2], Damien Redureau[3], Thomas Crasset[3], Rudy Fourmy[4], Aude Violette[4], Vincent Leignel[5], Ziad Fajloun[2,6], César Mattei[1*], Christian Legros[1*], Loïc Quinton[3*]

[1]Univ. Angers, INSERM, CNRS, MITOVASC, Equipe CarME, SFR ICAT, 49000 Angers, France.

[2]Laboratory of Applied Biotechnology (LBA3B), Department of Cell Culture, Azm Center for Research in Biotechnology and Its Applications, EDST, Lebanese University, Tripoli 1300, Lebanon.

[3]Laboratory of Mass Spectrometry, MolSys Research Unit, University of Liège, 4000 Liège, Belgium.

[4]Alphabiotoxine Laboratory srl, Barberie 15, 7911 Montroeul-au-bois, Belgium.

[5]Laboratoire BiOSSE, Le Mans Université, Avenue Olivier Messiaen, 72085 Le Mans, France

[6]Department of Biology, Faculty of Sciences III, Campus Michel Slayman Ras Maska, Lebanese University, Tripoli 1352, Lebanon

Corresponding authors: loic.quinton@uliege.be; cesar.mattei@univ-angers.fr; christian.legros@univ-angers.fr

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 provided 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 lebetina ssp.* to provide a broader comparative framework. These venoms were investigated 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.

1 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 comparative purposes, we also included the venom of the closely related sister genus *Macrovipera*, which belongs to the Old-World viper clade. Venoms from the genus *Macrovipera* have been well documented in the literature, particularly with respect to their proteomic composition and pharmacological activities [7–9]. In the present study, we specifically analyzed the venom of the *Macrovipera lebetina ssp.*, sourced from the same geographical region as the studied *Montivipera* species.

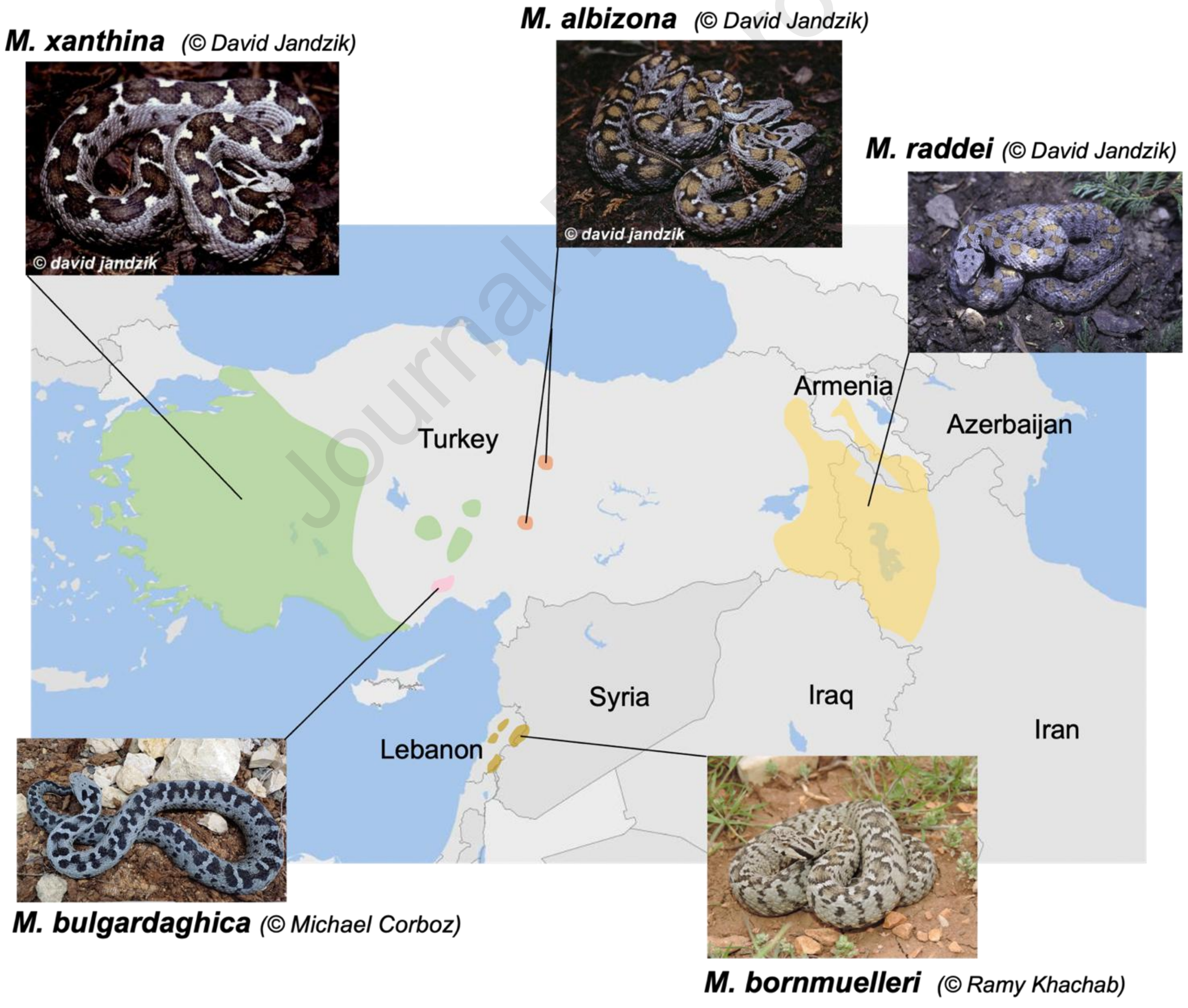


Fig. 1 Geographical distribution of *Montivipera* species. *M. bornmuelleri* lives in Lebanese mountains (1000 to 2000 m a.s.l.), *M. xanthina* is found in Türkiye and Greece (0 to 2000 m a.s.l.), *M. bulgardaghica* is found in Turkish Taurus mountains (2000 m a.s.l.), *M. raddei* is present in Armenia, 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.

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 include hemostatic alterations, neurotoxicity and hypotension [10–12]. 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 [11,13]. Up till now, no antivenoms are available for *Montivipera* species except for a polyvalent antivenom developed against *M. xanthina* among other Eurasian vipers [14]. 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 [15]. In addition, *M. xanthina* venom has both cytotoxic and antimicrobial effects on cancer cell lines and on fungi and bacteria, respectively [16,17]. Moreover, *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 [18–24]. These activities evidence the diversity of *Montivipera* venom components, underscoring the importance of their identification and characterization for both therapeutic development and research applications.

The venom proteomic content of four *Montivipera* species, including *M. xanthina, M. bulgardaghica, M. raddei* and *M. albizona,* has recently been evaluated. These studies show that the main protein families constituting these *Montivipera* venoms are venom metalloproteinases, venom serine proteases, phospholipase A_2, C-type lectins and venom vascular endothelial growth factor [15,25–27]. These studies yielded variable results regarding both the number of proteins identified in each venom and the different low abundance 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 still represents an important knowledge gap.

SV proteomes can be analyzed through different workflows, each having its advantages and disadvantages [28,29]. 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) [30]. 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 [31,32].

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* *lebetina ssp.* venom was analyzed as an outgroup, providing a phylogenetic reference point that helps distinguish lineage-specific traits among *Montivipera* venoms.

2 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 lebetina* were purchased from Alphabiotoxine (Montroeul-au-bois, Belgium). *Macrovipera lebetina* venom samples used in this study were obtained from specimens sourced from Mersin, southern Anatolia, Turkey, and are hereafter referred to as *Macrovipera lebetina* ssp., reflecting geographic origin without implying a definitive subspecies assignment. 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 lebetina ssp.* (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 [33]. Samples were then heated at 95°C for 5 min and loaded onto the gel. The Precision Plus Protein Standard Kaleidoscope^{TM} 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 xg 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. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [34] partner repository with the dataset identifier PXD073119 and DOI: 10.6019/PXD073119.

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. [35]. 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 (cytb) sequences were retrieved from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). These sequences were originally published in three independent studies [6,36,37]. Mitochondrial Cytb (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) [38,39]. The maximum likelihood (ML) method and Bayesian inference (BI) procedure were performed using PHYML [40] and MrBayes [41], 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 lebetin*a ssp. (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.

3 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, **AP**: Aminopeptidase, **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.

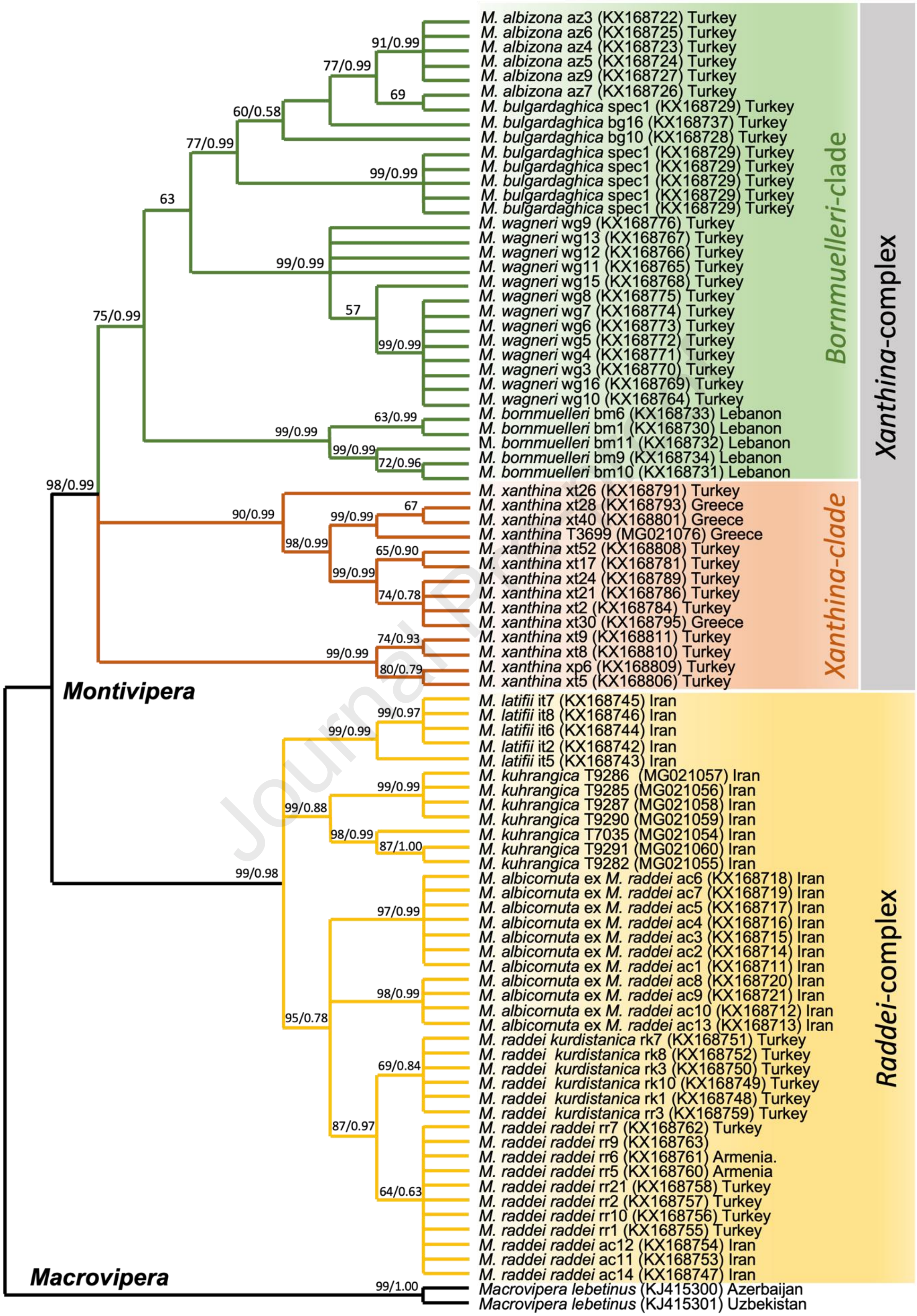

M. albizona az3 (KX168722) Turkey
M. albizona az6 (KX168725) Turkey
M. albizona az4 (KX168723) Turkey
M. albizona az5 (KX168724) Turkey
M. albizona az9 (KX168727) Turkey
M. albizona az7 (KX168726) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. bulgardaghica bg16 (KX168737) Turkey
M. bulgardaghica bg10 (KX168728) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. bulgardaghica spec1 (KX168729) Turkey
M. wagneri wg9 (KX168776) Turkey
M. wagneri wg13 (KX168767) Turkey
M. wagneri wg12 (KX168766) Turkey
M. wagneri wg11 (KX168765) Turkey
M. wagneri wg15 (KX168768) Turkey
M. wagneri wg8 (KX168775) Turkey
M. wagneri wg7 (KX168774) Turkey
M. wagneri wg6 (KX168773) Turkey
M. wagneri wg5 (KX168772) Turkey
M. wagneri wg4 (KX168771) Turkey
M. wagneri wg3 (KX168770) Turkey
M. wagneri wg16 (KX168769) Turkey
M. wagneri wg10 (KX168764) Turkey
M. bornmuelleri bm6 (KX168733) Lebanon
M. bornmuelleri bm1 (KX168730) Lebanon
M. bornmuelleri bm11 (KX168732) Lebanon
M. bornmuelleri bm9 (KX168734) Lebanon
M. bornmuelleri bm10 (KX168731) Lebanon
M. xanthina xt26 (KX168791) Turkey
M. xanthina xt28 (KX168793) Greece
M. xanthina xt40 (KX168801) Greece
M. xanthina T3699 (MG021076) Greece
M. xanthina xt52 (KX168808) Turkey
M. xanthina xt17 (KX168781) Turkey
M. xanthina xt24 (KX168789) Turkey
M. xanthina xt21 (KX168786) Turkey
M. xanthina xt2 (KX168784) Turkey
M. xanthina xt30 (KX168795) Greece
M. xanthina xt9 (KX168811) Turkey
M. xanthina xt8 (KX168810) Turkey
M. xanthina xp6 (KX168809) Turkey
M. xanthina xt5 (KX168806) Turkey
M. latifii it7 (KX168745) Iran
M. latifii it8 (KX168746) Iran
M. latifii it6 (KX168744) Iran
M. latifii it2 (KX168742) Iran
M. latifii it5 (KX168743) Iran
M. kuhrangica T9286 (MG021057) Iran
M. kuhrangica T9285 (MG021056) Iran
M. kuhrangica T9287 (MG021058) Iran
M. kuhrangica T9290 (MG021059) Iran
M. kuhrangica T7035 (MG021054) Iran
M. kuhrangica T9291 (MG021060) Iran
M. kuhrangica T9282 (MG021055) Iran
M. albicornuta ex M. raddei ac6 (KX168718) Iran
M. albicornuta ex M. raddei ac7 (KX168719) Iran
M. albicornuta ex M. raddei ac5 (KX168717) Iran
M. albicornuta ex M. raddei ac4 (KX168716) Iran
M. albicornuta ex M. raddei ac3 (KX168715) Iran
M. albicornuta ex M. raddei ac2 (KX168714) Iran
M. albicornuta ex M. raddei ac1 (KX168711) Iran
M. albicornuta ex M. raddei ac8 (KX168720) Iran
M. albicornuta ex M. raddei ac9 (KX168721) Iran
M. albicornuta ex M. raddei ac10 (KX168712) Iran
M. albicornuta ex M. raddei ac13 (KX168713) Iran
M. raddei kurdistanica rk7 (KX168751) Turkey
M. raddei kurdistanica rk8 (KX168752) Turkey
M. raddei kurdistanica rk3 (KX168750) Turkey
M. raddei kurdistanica rk10 (KX168749) Turkey
M. raddei kurdistanica rk1 (KX168748) Turkey
M. raddei kurdistanica rr3 (KX168759) Turkey
M. raddei raddei rr7 (KX168762) Turkey
M. raddei raddei rr9 (KX168763)
M. raddei raddei rr6 (KX168761) Armenia.
M. raddei raddei rr5 (KX168760) Armenia
M. raddei raddei rr21 (KX168758) Turkey
M. raddei raddei rr2 (KX168757) Turkey
M. raddei raddei rr10 (KX168756) Turkey
M. raddei raddei rr1 (KX168755) Turkey
M. raddei raddei ac12 (KX168754) Iran
M. raddei raddei ac11 (KX168753) Iran
M. raddei raddei ac14 (KX168747) Iran
Macrovipera lebetinus (KJ415300) Azerbaijan
Macrovipera lebetinus (KJ415301) Uzbekistan
Bornmuelleri-clade
Xanthina-clade
Xanthina-complex
Raddei-complex
Montivipera
Macrovipera
91/0.99
77/0.99
69
60/0.58
77/0.99
99/0.99
63
99/0.99
57
99/0.99
75/0.99
63/0.99
99/0.99
99/0.99
72/0.96
98/0.99
90/0.99
67
99/0.99
98/0.99
65/0.90
99/0.99
74/0.78
74/0.93
99/0.99
80/0.79
99/0.97
99/0.99
99/0.99
99/0.88
98/0.99
87/1.00
99/0.98
97/0.99
98/0.99
95/0.78
69/0.84
87/0.97
64/0.63
99/1.00

Fig. 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.

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 Dis. 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 lebetina ssp.* revealed major differences in the migration profile compared to the *Montivipera* venoms.

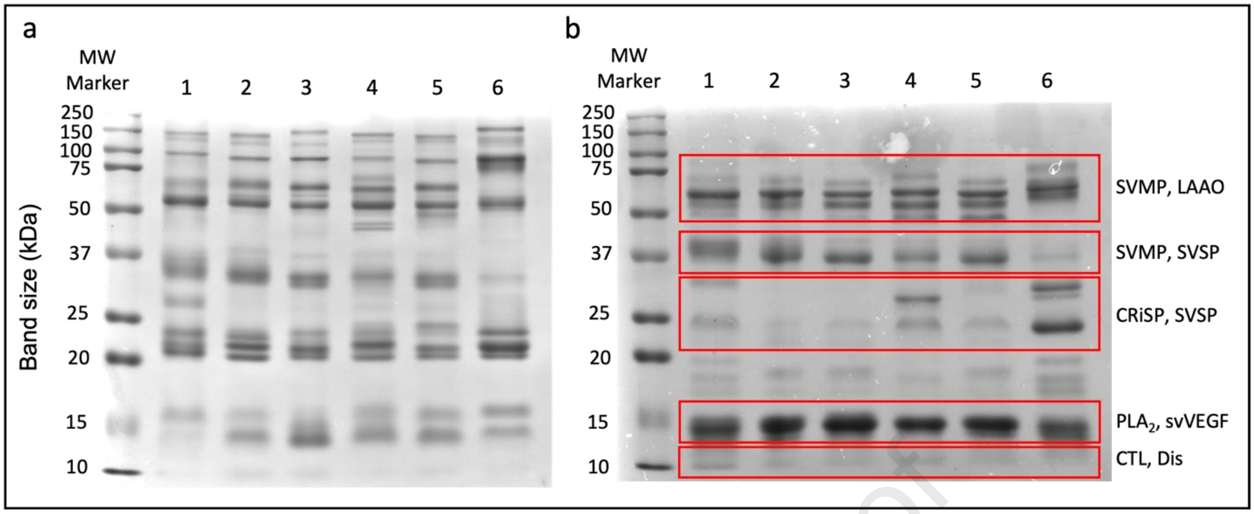


Fig. 3 SDS-PAGE profiles of *Montivipera* venoms under **(a)** non-reducing and **(b)** reducing conditions stained with CBB R250. 1: *M. bornmuelleri*, 2: *M. bulgardaghica*, 3: *M. xanthina*, 4: *M. raddei*, 5: *M. albizona*, 6: *Macrovipera lebetina ssp*. The red boxes indicate clusters of protein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

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) [27,42]. The putative identification of the HPLC peaks composition was inferred from known elution patterns of *Montivipera* venom components. Nevertheless, mass spectrometric characterization is necessary to confirm the precise protein composition of these peaks.

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 lebetina ssp.* venom appeared more atypical. For instance, the chromatogram of *Macrovipera lebetinus ssp.* 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).

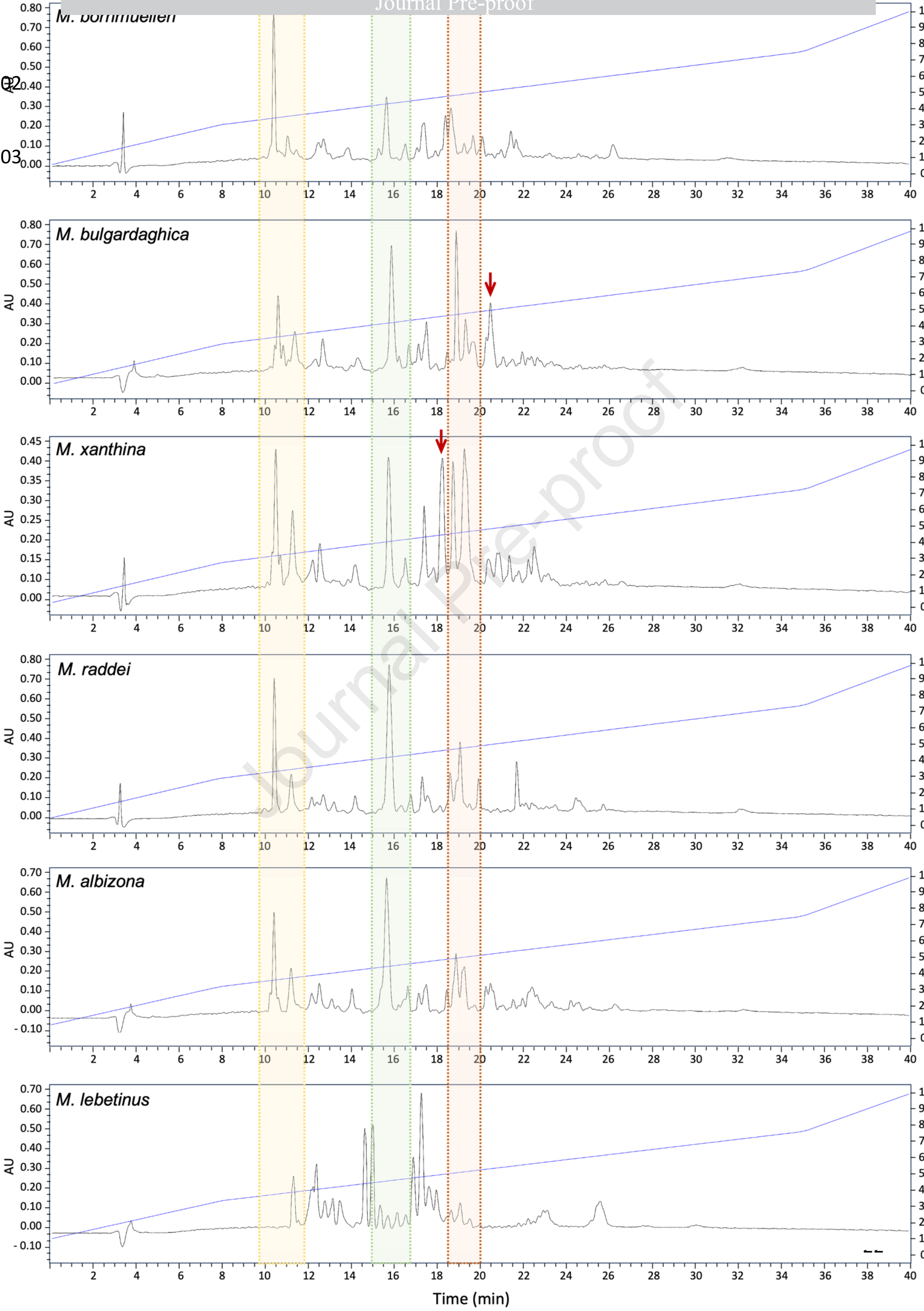

M. bornmuelleri
M. bulgardaghica
M. xanthina
M. raddei
M. albizona
M. lebetinus
AU
0.80
0.70
0.60
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.00
- 0.10
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Time (min)

Fig. 4 Chromatographic profiles of *Montivipera* venoms and *Macrovipera lebetina ssp.* obtained by analytical C18 RP-HPLC. Chromatograms of *M. bornmuelleri, M. bulgardaghica, M. xanthina, M. r. raddei, M. albizona* and *Macrovipera lebetina ssp.* (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. Shaded areas highlight the shared peaks among *Montivipera* venoms. Yellow: shared peaks at RT ~10.5 and 11.3 min, green: shared peak at RT ~15.7 min, red: shared peak at RT ~19 min

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 and we used Venn diagrams to illustrate the overlap. Our analysis revealed 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.

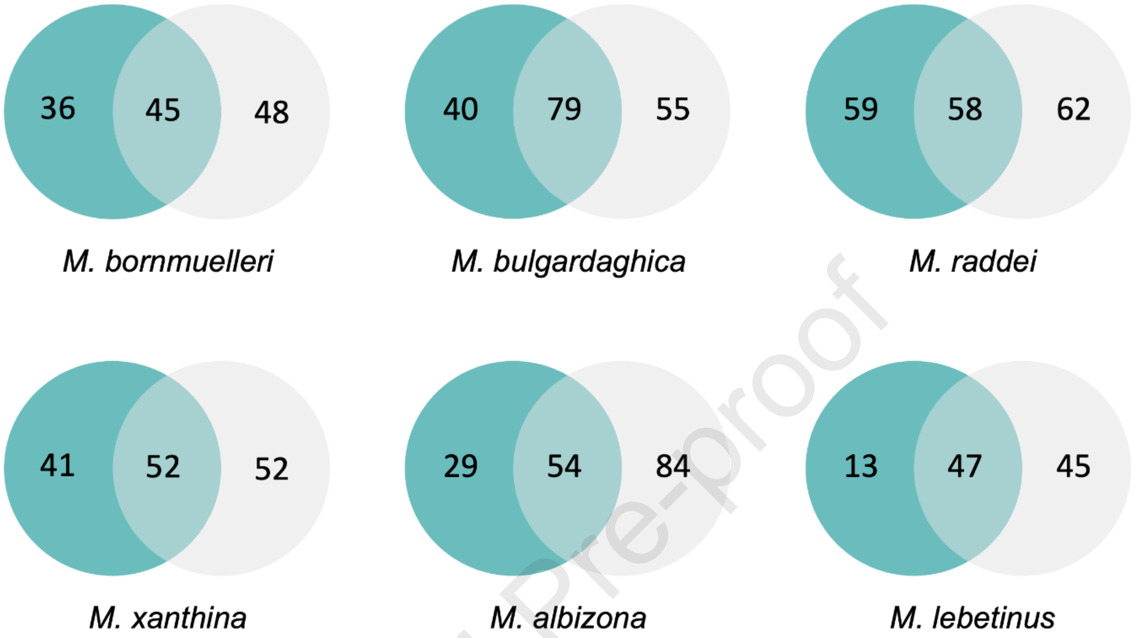


Fig. 5 Venn diagrams illustrating the number of proteins identified by the shotgun proteomics using each digestion protocol (Trypsin (blue) vs. MELD (grey)) and the overlap between both protocols

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 lebetina ssp.* was significantly lower, with 105 proteins grouped into 17 families. **Table 1** 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 1 Diversity and number of proteins and protein families identified in *Montivipera* venoms and *Macrovipera lebetina ssp.* using shotgun proteomics

	M. bornmuelleri	***M. bulgardaghica***	***M. xanthina***	***M. raddei***	***M. albizona***	***M. lebetina***
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	36	36	23	32	40	18
PLA_2	8	27	35	51	35	5
CTLs	24	27	33	22	37	33
SVSP	11	20	14	11	15	11
svVEGF	2	7	5	11	5	5
LAAO	3	3	3	3	3	5
Disintegrin	6	4	3	3	6	5
AP	1	3	1	1	1	9
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, APs, 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 lebetina ssp*.

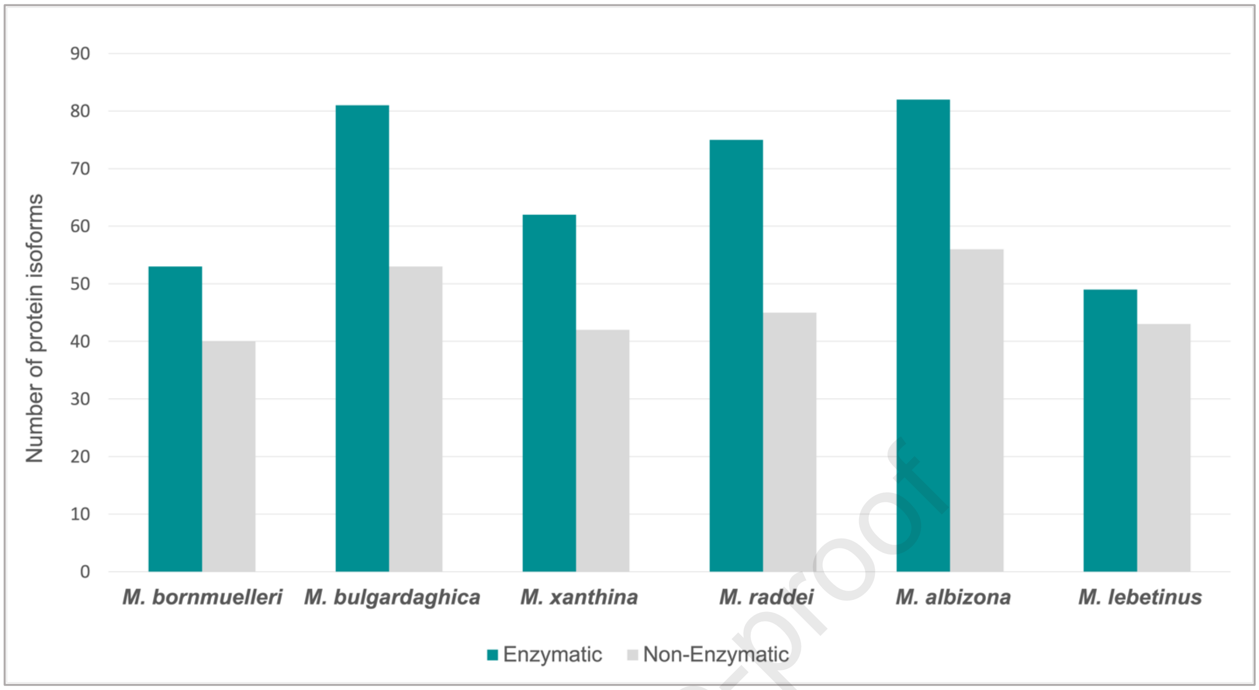


Fig. 6 Bar chart showing the number of protein isoforms for enzymatic and non-enzymatic components in each of the *Montivipera* venoms and the *Macrovipera lebetina ssp.* venom

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

The proteomics analysis of *Montivipera* venoms indicated 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 Dis. *M. bornmuelleri* venom showed the highest SVMP abundance among all venoms, being the most abundant protein family in this venom, followed by svVEGFs, SVSPs, Dis, CTLs and PLA_2s. Alternatively, CTLs are the most abundant component in *M. bulgardaghica* venom, followed by SVSPs, svVEGFs, PLA$_2$s, SVMPs and Dis. Notably, the major constituents of *M. xanthina* venom were PLA$_2$s and svVEGFs followed by SVMPs, SVSPs, CTLs and Dis. Similarly, the most abundant protein family in *M. r. raddei* venom is PLA$_2$, followed by CTLs, SVMPs, Dis, svVEGFs and SVSPs. Regarding the *M. albizona* venom, its most abundant component is the CTL family, followed by svVEGFs, SVMPs, PLA$_2$s, and SVSPs. The venom of the closely related genus *Macrovipera lebetina ssp.* has PLA$_2$s and SVMPs as its major components, followed by CTLs and Dis and SVSPs. 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%).

The secondary protein families constituting the *Montivipera* venoms include CRiSPs, 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 and LAAOs in *M. bornmuelleri* venom are 2.44% and 2.02%, respectively. *M. bulgardaghica, M. raddei, M. xanthina* and *M. albizona* exhibit lower abundances of NGFs and LAAOs 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, APs, 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 highlight a conserved venom composition across *Montivipera* species characterized predominantly by high abundance of SVMPs, CTLs, PLA$_2$s, 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 lebetina ssp.* exhibited a markedly different composition, characterized by lower levels of svVEGFs distinguishing it from the *Montivipera* venoms. However, it should be noted that the present analysis was based on relative quantification and, as such, comparisons between samples must be interpreted with caution. Despite this limitation, the approach remains well suited to reveal consistent trends in the distribution of toxin families across venoms [43]. Further refinement of quantitative comparisons could be achieved by incorporating a defined protein mixture as an internal reference standard. Alternatively, a more conventional but substantially more labor-intensive strategy would involve quantification by RP-HPLC–MS, gel densitometry and peptide-level quantification based on the Top 3 most intense ions obtained after tryptic digestion [44,45].

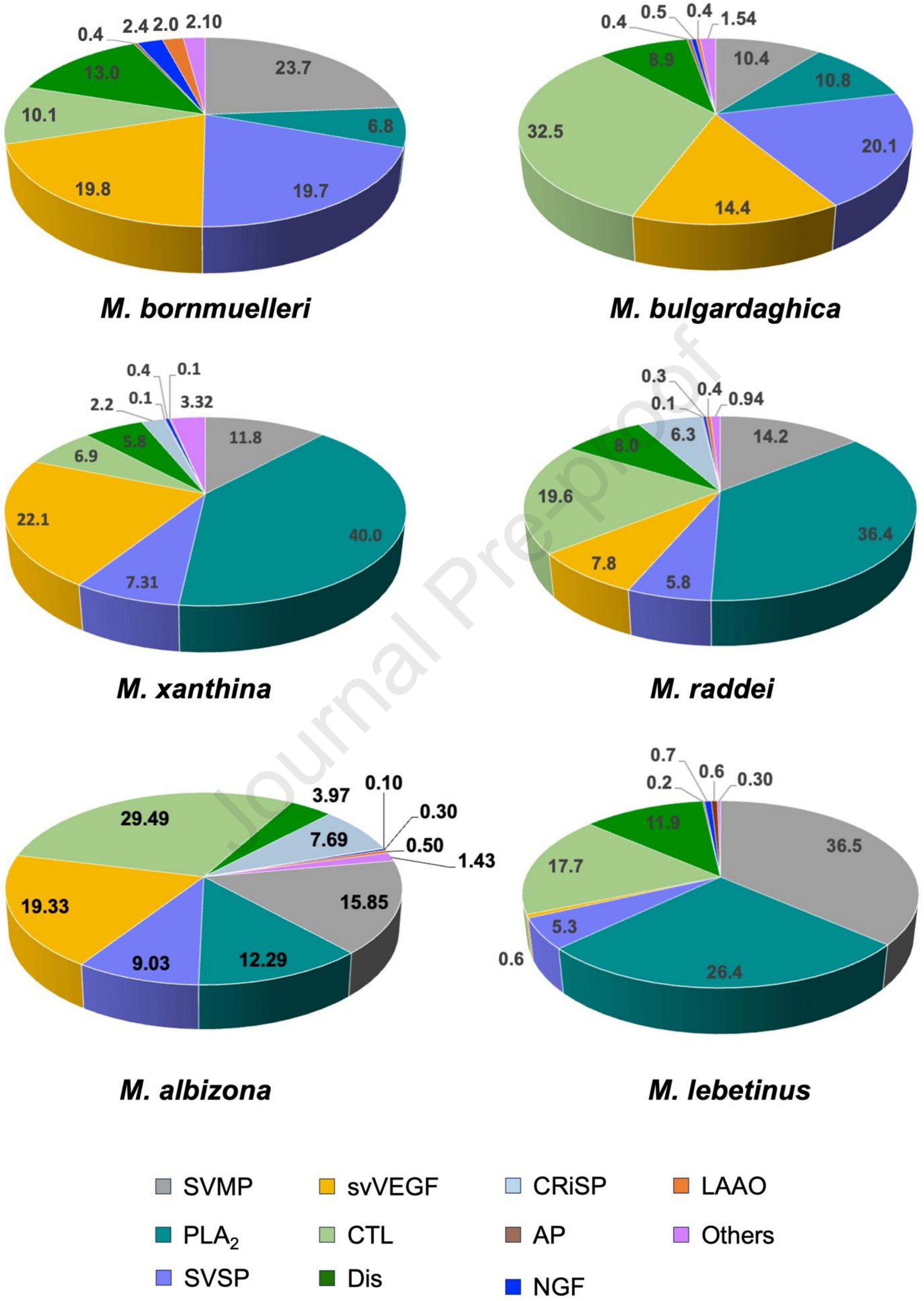

0.4
2.4
2.0
2.10
23.7
13.0
10.1
6.8
19.8
19.7
M. bornmuelleri
0.4
0.5
0.4
1.54
10.4
8.9
10.8
32.5
20.1
14.4
M. bulgardaghica
0.4
0.1
2.2
0.1
3.32
11.8
5.8
6.9
22.1
40.0
7.31
M. xanthina
0.3
0.4
0.1
0.94
6.3
14.2
8.0
19.6
36.4
7.8
5.8
M. raddei
3.97
0.10
0.30
7.69
0.50
1.43
29.49
19.33
15.85
9.03
12.29
M. albizona
0.7
0.6
0.2
0.30
11.9
36.5
17.7
5.3
0.6
26.4
M. lebetinus
SVMP
svVEGF
CRiSP
LAAO
PLA2
CTL
AP
Others
SVSP
Dis
NGF

Fig. 7 Pie charts showing the relative abundances of protein families in *Montivipera* and *Macrovipera lebetina ssp.* 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.

3.5 Comparative proteomics analysis of *Montivipera* venoms

To evaluate the similarity between the five *Montivipera* venoms, we calculated the PSC shown in **Table 2**. As we expected, the venom of the sister genus *Macrovipera lebetina ssp.* 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 2 Protein Similarity Coefficient (%) of the *Montivipera* venom proteomes and *Macrovipera lebetina ssp.*

	M. bornmuelleri	*M. bulgardaghica*	*M. raddei*	*M. albizona*	*M. xanthina*	*M. lebetina*
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. lebetina	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.

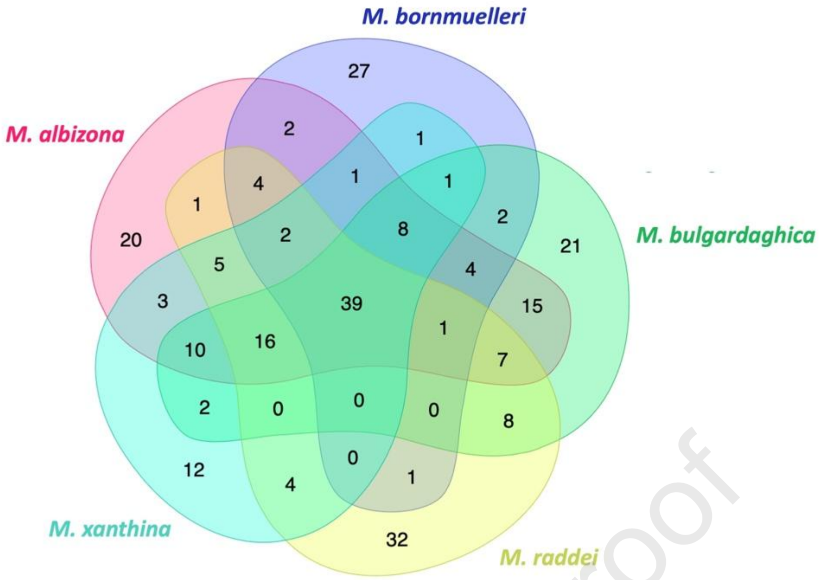


Fig. 8: Venn diagram illustrating the common and distinct proteins between the venoms of *Montivipera* species, including *M. bornmuelleri, M. bulgardaghica, M. xanthina, M. r. raddei and M. albizona.* The values represented are the number of identified proteins.

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* [15,25–27]. We included in this study the analysis of the venom of *Macrovipera lebetina ssp.*, the sister-genus of *Montivipera*, as an outgroup. This provides a broader phylogenetic perspective and allows 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 [46–48]. 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* [48–50]. Similarly, the analyzed *Macrovipera lebetina ssp.* 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 of *Montivipera* venoms [25]. This diversity is largely driven by the presence of multiple protein isoforms resulting 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 [1,51]. Their presence indicates that *Montivipera* venoms can exhibit various clinical manifestations. First, SVMPs are key contributors to the hemorrhagic activity of viperid venoms [52]. The presence of SVMPs and their high abundance in all *Montivipera* venoms suggests a conserved hemorrhagic potential across the genus, along with other biological functions attributed to SVMPs, such as tissue degradation, interference with hemostasis, and modulation of inflammatory processes. Among them, *M. bornmuelleri* venom exhibited the highest SVMP content, consistent with clinical reports of local haemorrhage at the bite site in envenomed patients [12]. This hemorrhagic effect was further demonstrated in zebrafish embryos, where the venom induced visible pericardial bleeding [24]. 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 [53]. 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 [54,55].

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 [56]. 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 *Macrovipera lebetinus ssp*. Such a pattern may reflect an evolutionary adaptation of *Montivipera* venoms to facilitate the dissemination of venom components.

It is important to note that the functional impact of toxin families could depend on factors other than their abundance, such as the enzymatic activity, target specificity and pharmacokinetic properties. In some cases, low abundance toxins could induce significant biological activity or even engage in synergistic interactions and thus exhibit strong biological outcomes upon envenomation [57]. Therefore, our abundance-based interpretations provide useful hypotheses regarding the potential biological activities of *Montivipera* venoms. However, they should be further confirmed by functional assays to confirm the biological relevance of specific toxins in the context of venom action.

The main protein families identified in our study were like 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 [15,25,27,42,58]. 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 aminopeptidases, 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 [59,60]. In our study, pooled venoms from vipers bred and kept in captivity were used to minimize individual variability. Consequently, the resulting proteomic profiles reflect venom composition under standardized conditions, in contrast to studies based on wild-caught specimens, which often capture population-level and ecologically driven variation. This could partly explain the 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 [28]. 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 [61]. 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 [27]. The Venn diagram revealed that most of the shared proteins among *Montivipera* species belonged to the SVMPs, CTLs, PLA$_2$s 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. Accordingly, future studies integrating venom gland transcriptomics, prey composition data, and appropriate multivariate statistical frameworks will be necessary to rigorously evaluate hypotheses of ecological selection.

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 lebetina ssp.* 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.

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.

Funding: This work was supported by the Eiffel Excellence program through a scholarship (EIFFEL-DOCTORAT 2022 / n°P850081J) awarded to CS to fund her PhD at the MITOVASC laboratory, University of Angers, France and by the Boehringer Ingelheim fonds through a Travel grant awarded to CS to fund a one-month research stay at the Mass spectrometry laboratory Liège-Belgium.

Data availability: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval: Not applicable.

Consent to Publish: Not applicable.

Consent to Participate: Not applicable.

References:

1. Oliveira, A.L.; Viegas, M.F.; Da Silva, S.L.; Soares, A.M.; Ramos, M.J.; Fernandes, P.A. The Chemistry of Snake Venom and Its Medicinal Potential. *Nat Rev Chem* **2022**, *6*, 451–469, doi:10.1038/s41570-022-00393-7.
2. Casewell, N.R.; Jackson, T.N.W.; Laustsen, A.H.; Sunagar, K. Causes and Consequences of Snake Venom Variation. *Trends in Pharmacological Sciences* **2020**, *41*, 570–581, doi:10.1016/j.tips.2020.05.006.
3. Gutiérrez, J.M.; Calvete, J.J.; Habib, A.G.; Harrison, R.A.; Williams, D.J.; Warrell, D.A. Snakebite Envenoming. *Nat Rev Dis Primers* **2017**, *3*, 17079, doi:10.1038/nrdp.2017.79.
4. Frangieh, J.; Rima, M.; Fajloun, Z.; Henrion, D.; Sabatier, J.M.; Legros, C.; Mattei, C. Snake Venom Components: Tools and Cures to Target Cardiovascular Diseases. *Molecules* **2021**, *26*, doi:10.3390/molecules26082223.
5. Fry, B.G. From Genome to "Venome": Molecular Origin and Evolution of the Snake Venom Proteome Inferred from Phylogenetic Analysis of Toxin Sequences and Related Body Proteins. *Genome Res.* **2005**, *15*, 403–420, doi:10.1101/gr.3228405.
6. Stümpel, N.; Rajabizadeh, M.; Avcı, A.; Wüster, W.; Joger, U. Phylogeny and Diversification of Mountain Vipers (Montivipera, Nilson et al., 2001) Triggered by Multiple Plio–Pleistocene Refugia and High-Mountain Topography in the Near and Middle East. *Molecular Phylogenetics and Evolution* **2016**, *101*, 336–351, doi:10.1016/j.ympev.2016.04.025.
7. Makran, B.; Fahmi, L.; Pla, D.; Sanz, L.; Oukkache, N.; Lkhider, M.; Ghalim, N.; Calvete, J.J. Snake Venomics of Macrovipera Mauritanica from Morocco, and Assessment of the Para-Specific Immunoreactivity of an Experimental Monospecific and a Commercial Antivenoms. *Journal of Proteomics* **2012**, *75*, 2431–2441, doi:10.1016/j.jprot.2012.02.022.
8. Schulte, L.; Damm, M.; Avella, I.; Uhrig, L.; Erkoc, P.; Schiffmann, S.; Fürst, R.; Timm, T.; Lochnit, G.; Vilcinskas, A.; et al. Venomics of the Milos Viper (Macrovipera Schweizeri) Unveils Patterns of Venom Composition and Exochemistry across Blunt-Nosed Viper Venoms. *Front. Mol. Biosci.* **2023**, *10*, 1254058, doi:10.3389/fmolb.2023.1254058.
9. Avella, I.; Damm, M.; Di Nicola, M.R.; Dresler, J.; İğci, N.; Kariş, M.; Kazemi, S.M.; Kreuels, B.; Paolino, G.; Sarigiannis, Y.; et al. The Biology and Toxinology of Blunt-Nosed Vipers. *npj biodivers* **2025**, *4*, 21, doi:10.1038/s44185-025-00090-w.
10. Anil, A.B.; Anil, M.; Kara, O.D.; Bal, A.; Özhan, B.; Aksu, N. Mannitol Therapy in Three Cases with Severe Edema Due to Snakebite: Case Report. *Turkiye Klinikleri J Med Sci* **2011**, *31*, 720–723, doi:10.5336/medsci.2009-13354.
11. Al-Musawi, M.; Chanbour, H.; El Masri, J.; Awad, R.; Armash, K. Erectile Dysfunction and Permanent Bladder Areflexia Following Montivipera Bornmuelleri Snakebite. *Cureus* **2021**, doi:10.7759/cureus.17968.
12. Tabbara, F.; Abdul-Nabi, S.; sADEK, R.; Kazzi, Z.; El Zahran, T. A Case Report of a Lebanon Viper (Montivipera Bornmuelleri) Envenomation in a Child. *Clinical Practice and Cases in Emergency Medicine* **2022**, *6*, 319–323, doi:10.5811/cpcem.2022.2.56176.
13. Hassanian-Moghaddam, H.; Monzavi, S.M.; Shirazi, F.M.; Warrell, D.A.; Mehrpour, O. First Report of a Confirmed Case of Montivipera Latifii (Latifi's Viper) Envenoming and a Literature Review of Envenoming by Montivipera Species. *Toxicon* **2022**, *207*, 48–51, doi:10.1016/j.toxicon.2021.12.020.

14. Lamb, T.; De Haro, L.; Lonati, D.; Brvar, M.; Eddleston, M. Antivenom for European *Vipera* Species Envenoming. *Clinical Toxicology* **2017**, *55*, 557–568, doi:10.1080/15563650.2017.1300261.
15. Nalbantsoy, A.; Hempel, B.-F.; Petras, D.; Heiss, P.; Göçmen, B.; Iğci, N.; Yildiz, M.Z.; Süssmuth, R.D. Combined Venom Profiling and Cytotoxicity Screening of the Radde's Mountain Viper (Montivipera Raddei) and Mount Bulgar Viper (Montivipera Bulgardaghica) with Potent Cytotoxicity against Human A549 Lung Carcinoma Cells. *Toxicon* **2017**, *135*, 71–83, doi:10.1016/j.toxicon.2017.06.008.
16. Yalcın, H.T.; Ozen, M.O.; Gocmen, B.; Nalbantsoy, A. Effect of Ottoman Viper (Montivipera Xanthina (Gray, 1849)) Venom on Various Cancer Cells and on Microorganisms. *Cytotechnology* **2014**, *66*, 87–94, doi:10.1007/s10616-013-9540-z.
17. Nalbantsoy, A.; Erel, Ş.B.; Köksal, Ç.; Göçmen, B.; Yıldız, M.Z.; Karabay Yavaşoğlu, N.Ü. Viper Venom Induced Inflammation with Montivipera Xanthina (Gray, 1849) and the Anti-Snake Venom Activities of Artemisia Absinthium L. in Rat. *Toxicon* **2013**, *65*, 34–40, doi:10.1016/j.toxicon.2012.12.017.
18. Accary, C.; Hraoui-Bloquet, S.; Hamze, M.; Mallem, Y.; El Omar, F.; Sabatier, J.-M.; Desfontis, J.-C.; Fajloun, Z. Protein Content Analysis and Antimicrobial Activity of the Crude Venom of Montivipera Bornmuelleri; a Viper from Lebanon. *Infect Disord Drug Targets* **2014**, *14*, 49–55, doi:10.2174/1871526514666140522114754.
19. Accary, C.; Rima, M.; Kouzahya, A.; Hleihel, W.; Sadek, R.; Desfontis, J.C.; Fajloun, Z.; Hraoui-Bloquet, S. Effect of the Montivipera Bornmuelleri Snake Venom on Human Blood: Coagulation Disorders and Hemolytic Activities. *Open J Hematol* **2014**, *5*, 1, doi:10.13055/ojhmt_5_1_4.140609.
20. Accary, C.; Hraoui-Bloquet, S.; Sadek, R.; Alameddine, A.; Fajloun, Z.; Desfontis, J.-C.; Mallem, Y. The Relaxant Effect of the Montivipera Bornmuelleri Snake Venom on Vascular Contractility. *J Venom Res* **2016**, *7*, 10–15.
21. Yacoub, T.; Rima, M.; Sadek, R.; Hleihel, W.; Fajloun, Z.; Karam, M. Montivipera Bornmuelleri Venom Has Immunomodulatory Effects Mainly Up-Regulating pro-Inflammatory Cytokines in the Spleens of Mice. *Toxicology Reports* **2018**, *5*, 318–323, doi:10.1016/j.toxrep.2018.02.011.
22. Sawan, S.; Yaacoub, T.; Hraoui-Bloquet, S.; Sadek, R.; Hleihel, W.; Fajloun, Z.; Karam, M. Montivipera Bornmuelleri Venom Selectively Exhibits High Cytotoxic Effects on Keratinocytes Cancer Cell Lines. *Experimental and Toxicologic Pathology* **2017**, *69*, 173–178, doi:10.1016/j.etp.2017.01.001.
23. Haddoub, C.; Rima, M.; Heurtebise, S.; Lawand, M.; Jundi, D.; Sadek, R.; Amigorena, S.; Fajloun, Z.; Karam, M.C. Cytotoxic Effect of *Montivipera Bornmuelleri's* Venom on Cancer Cell Lines: In Vitro and in Vivo Studies. *PeerJ* **2020**, *8*, e9909, doi:10.7717/peerj.9909.
24. Sahyoun, C.; Krezel, W.; Mattei, C.; Sabatier, J.-M.; Legros, C.; Fajloun, Z.; Rima, M. Neuro- and Cardiovascular Activities of Montivipera Bornmuelleri Snake Venom. *Biology (Basel)* **2022**, *11*, 888, doi:10.3390/biology11060888.
25. Abu Aisheh, M.; Kayili, H.M.; Numanoglu Cevik, Y.; Kanat, M.A.; Salih, B. Composition Characterization of Various Viperidae Snake Venoms Using MS-Based Proteomics N-Glycoproteomics and N-Glycomics. *Toxicon* **2023**, *235*, 107328, doi:10.1016/j.toxicon.2023.107328.

26. Jafari, Z.; Bandehpour, M.; Kaboli, M.; Kazemi, B. Partial Proteomics Analysis of Montivipera Raddei Venom: Partial Proteomics of Montivipera Raddei Venom. *Trends in Peptide and Protein Sciences* **2023**, *8*, 1-5 (e2), doi:10.22037/tpps.v8i1.41610.
27. Damm, M.; Karış, M.; Petras, D.; Nalbantsoy, A.; Göçmen, B.; Süssmuth, R.D. Venomics and Peptidomics of Palearctic Vipers: A Clade-Wide Analysis of Seven Taxa of the Genera *Vipera* , *Montivipera* , *Macrovipera* , and *Daboia* across Türkiye. *J. Proteome Res.* **2024**, *23*, 3524–3541, doi:10.1021/acs.jproteome.4c00171.
28. Sahyoun, C.; Rima, M.; Mattei, C.; Sabatier, J.-M.; Fajloun, Z.; Legros, C. Separation and Analytical Techniques Used in Snake Venomics: A Review Article. *Processes* **2022**, *10*, 1380, doi:10.3390/pr10071380.
29. Lomonte, B.; Calvete, J.J. Strategies in "snake Venomics" Aiming at an Integrative View of Compositional, Functional, and Immunological Characteristics of Venoms. *J Venom Anim Toxins Incl Trop Dis* **2017**, *23*, 26, doi:10.1186/s40409-017-0117-8.
30. Morsa, D.; Baiwir, D.; La Rocca, R.; Zimmerman, T.A.; Hanozin, E.; Grifnée, E.; Longuespée, R.; Meuwis, M.-A.; Smargiasso, N.; Pauw, E.D.; et al. Multi-Enzymatic Limited Digestion: The Next-Generation Sequencing for Proteomics? *J. Proteome Res.* **2019**, *18*, 2501–2513, doi:10.1021/acs.jproteome.9b00044.
31. Amorim, F.G.; Redureau, D.; Crasset, T.; Freuville, L.; Baiwir, D.; Mazzucchelli, G.; Menzies, S.K.; Casewell, N.R.; Quinton, L. Next-Generation Sequencing for Venomics: Application of Multi-Enzymatic Limited Digestion for Inventorying the Snake Venom Arsenal. *Toxins* **2023**, *15*, 357, doi:10.3390/toxins15060357.
32. Redureau, D.; Amorim, F.G.; Crasset, T.; Berger, I.; Schaffitzel, C.; Menzies, S.K.; Casewell, N.R.; Quinton, L. Dual Proteomics Strategies to Dissect and Quantify the Components of Nine Medically Important African Snake Venoms. *Toxins* **2025**, *17*, 243, doi:10.3390/toxins17050243.
33. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685, doi:10.1038/227680a0.
34. Perez-Riverol, Y.; Bandla, C.; Kundu, D.J.; Kamatchinathan, S.; Bai, J.; Hewapathirana, S.; John, N.S.; Prakash, A.; Walzer, M.; Wang, S.; et al. The PRIDE Database at 20 Years: 2025 Update. *Nucleic Acids Res* **2025**, *53*, D543–D553, doi:10.1093/nar/gkae1011.
35. Calvete, J.J.; Escolano, J.; Sanz, L. Snake Venomics of *Bitis* Species Reveals Large Intragenus Venom Toxin Composition Variation: Application to Taxonomy of Congeneric Taxa. *J. Proteome Res.* **2007**, *6*, 2732–2745, doi:10.1021/pr0701714.
36. Lenk, P.; Kalyabina, S.; Wink, M.; Joger, U. Evolutionary Relationships among the True Vipers (Reptilia: Viperidae) Inferred from Mitochondrial DNA Sequences. *Molecular Phylogenetics and Evolution* **2001**, *19*, 94–104, doi:10.1006/mpev.2001.0912.
37. Behrooz, R.; Kaboli, M.; Arnal, V.; Nazarizadeh, M.; Asadi, A.; Salmanian, A.; Ahmadi, M.; Montgelard, C. Conservation Below the Species Level: Suitable Evolutionarily Significant Units among Mountain Vipers (the Montivipera Raddei Complex) in Iran. *Journal of Heredity* **2018**, *109*, 416–425, doi:10.1093/jhered/esy005.
38. Nei, M.; Kumar, S. *Molecular Evolution and Phylogenetics*; Oxford University PressNew York, NY, 2000; ISBN 978-0-19-513584-8.

39. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution* **2018**, *35*, 1547–1549, doi:10.1093/molbev/msy096.
40. Guindon, S.; Dufayard, J.-F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* **2010**, *59*, 307–321, doi:10.1093/sysbio/syq010.
41. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian Inference of Phylogenetic Trees. *Bioinformatics* **2001**, *17*, 754–755, doi:10.1093/bioinformatics/17.8.754.
42. Sanz, L.; Ayvazyan, N.; Calvete, J.J. Snake Venomics of the Armenian Mountain Vipers Macrovipera Lebetina Obtusa and Vipera Raddei. *Journal of Proteomics* **2008**, *71*, 198–209, doi:10.1016/j.jprot.2008.05.003.
43. Krey, J.F.; Wilmarth, P.A.; Shin, J.-B.; Klimek, J.; Sherman, N.E.; Jeffery, E.D.; Choi, D.; David, L.L.; Barr-Gillespie, P.G. Accurate Label-Free Protein Quantitation with High- and Low-Resolution Mass Spectrometers. *J. Proteome Res.* **2014**, *13*, 1034–1044, doi:10.1021/pr401017h.
44. Lomonte, B.; Escolano, J.; Fernández, J.; Sanz, L.; Angulo, Y.; Gutiérrez, J.M.; Calvete, J.J. Snake Venomics and Antivenomics of the Arboreal Neotropical Pitvipers Bothriechis Lateralis and Bothriechis Schlegelii. *J. Proteome Res.* **2008**, *7*, 2445–2457, doi:10.1021/pr8000139.
45. Fusco, L.S.; Beraldo-Neto, E.; Calvete, J.J.; Sanz, L.; Lanari, L.C.; Lorente, C.; Leiva, L.C.; Pimenta, D.; De Roodt, A.R.; Pla, D. Venomic and F(Ab’)$_2$ Antivenomic Studies of Bothrops Alternatus Venom Proteins. *International Journal of Biological Macromolecules* **2025**, *318*, 144777, doi:10.1016/j.ijbiomac.2025.144777.
46. Lang Balija, M.; Leonardi, A.; Brgles, M.; Sviben, D.; Kurtović, T.; Halassy, B.; Križaj, I. Biological Activities and Proteomic Profile of the Venom of Vipera Ursinii Ssp., a Very Rare Karst Viper from Croatia. *Toxins* **2020**, *12*, 187, doi:10.3390/toxins12030187.
47. Kumkate, S.; Chanhome, L.; Thiangtrongjit, T.; Noiphrom, J.; Laoungboa, P.; Khow, O.; Vasaruchapong, T.; Sitprija, S.; Chaiyabutr, N.; Reamtong, O. Venomics and Cellular Toxicity of Thai Pit Vipers (Trimeresurus Macrops and T. Hageni). *Toxins (Basel)* **01 16**, *12*, doi:10.3390/toxins12010054.
48. Jiménez-Charris, E.; Montoya-Gómez, A.; Torres, J.K.; Gómez-Díaz, M.; Bolívar-García, W. First Functional and Proteomic Analysis of Bothrops Asper Snake Venom from Gorgona Island - Colombia, and Its Comparative Characterization with Two Colombian Southwest Ecoregions. *Biochimie* **2022**, *194*, 19–27, doi:10.1016/j.biochi.2021.12.005.
49. Gopcevic, K.; Karadzic, I.; Izrael-Zivkovic, L.; Medic, A.; Isakovic, A.; Popović, M.; Kekic, D.; Stanojkovic, T.; Hozic, A.; Cindric, M. Study of the Venom Proteome of Vipera Ammodytes Ammodytes (Linnaeus, 1758): A Qualitative Overview, Biochemical and Biological Profiling. *Comp Biochem Physiol Part D Genomics Proteomics* **3 AD**, *37*, 100776, doi:10.1016/j.cbd.2020.100776.
50. Nie, X.; He, Q.; Zhou, B.; Huang, D.; Chen, J.; Chen, Q.; Yang, S.; Yu, X. Exploring the Five-Paced Viper (Deinagkistrodon Acutus) Venom Proteome by Integrating a Combinatorial Peptide Ligand Library Approach with Shotgun LC-MS/MS. *Journal of Venomous Animals and Toxins including Tropical Diseases* **2021**, *27*.

51. Damm, M.; Hempel, B.-F.; Süssmuth, R.D. Old World Vipers-A Review about Snake Venom Proteomics of Viperinae and Their Variations. *Toxins (Basel)* **2021**, *13*, 427, doi:10.3390/toxins13060427.
52. Baldo, C.; Jamora, C.; Yamanouye, N.; Zorn, T.M.; Moura-da-Silva, A.M. Mechanisms of Vascular Damage by Hemorrhagic Snake Venom Metalloproteinases: Tissue Distribution and In Situ Hydrolysis. *PLoS Negl Trop Dis* **2010**, *4*, e727, doi:10.1371/journal.pntd.0000727.
53. Sampat, G.H.; Hiremath, K.; Dodakallanavar, J.; Patil, V.S.; Harish, D.R.; Biradar, P.; Mahadevamurthy, R.K.; Barvaliya, M.; Roy, S. Unraveling Snake Venom Phospholipase A2: An Overview of Its Structure, Pharmacology, and Inhibitors. *Pharmacol. Rep* **2023**, *75*, 1454–1473, doi:10.1007/s43440-023-00543-8.
54. Pereira, D.F.D.C.; Matias Ribeiro, M.S.; de Sousa Simamoto, B.B.; Dias, E.H.V.; Costa, J.O.; Santos-Filho, N.A.; Bordon, K.C.F.; Arantes, E.C.; Dantas, N.O.; Silva, A.C.A.; et al. Baltetin: A New C-Type Lectin-like Isolated from Bothrops Alternatus Snake Venom Which Act as a Platelet Aggregation Inhibiting. *J Chromatogr B Analyt Technol Biomed Life Sci* **2021**, *1173*, 122695, doi:10.1016/j.jchromb.2021.122695.
55. Castanheira, L.E.; Lopes, D.S.; Gimenes, S.N.C.; Deconte, S.R.; Ferreira, B.A.; Alves, P.T.; Filho, L.R.G.; Tomiosso, T.C.; Rodrigues, R.S.; Yoneyama, K.A.G.; et al. Angiogenenic Effects of BpLec, a C-Type Lectin Isolated from Bothrops Pauloensis Snake Venom. *International Journal of Biological Macromolecules* **2017**, *102*, 153–161, doi:10.1016/j.ijbiomac.2017.04.012.
56. Ferreira, I.G.; Pucca, M.B.; Oliveira, I.S.D.; Cerni, F.A.; Jacob, B.D.C.D.S.; Arantes, E.C. Snake Venom Vascular Endothelial Growth Factors (svVEGFs): Unravelling Their Molecular Structure, Functions, and Research Potential. *Cytokine & Growth Factor Reviews* **2021**, *60*, 133–143, doi:10.1016/j.cytogfr.2021.05.003.
57. Deshwal, A.; Phan, P.; Datta, J.; Kannan, R.; Thallapuranam, S.K. A Meta-Analysis of the Protein Components in Rattlesnake Venom. *Toxins (Basel)* **2021**, *13*, 372, doi:10.3390/toxins13060372.
58. Igci N; Petras D; Goçmen B; Süssmuth R; Yildiz M Z; Ozel-Demiralp FD Iğci N, Petras D, Göçmen B, Süssmuth R, Yıldız MZ, Özel-Demiralp FD. Comparative Venomic Characterization of Four Medicinally Important Turkish Vipers (Macrovipera Lebetina, Montivipera Xanthina, Vipera Ammodytes, V. Kaznakovi).; Berlin, Germany, March 22 2025; p. 3.
59. Montealegre-Sánchez, L.; Montoya-Gómez, A.; Jiménez-Charris, E. Individual Variations in the Protein Profiles and Functional Activities of the Eyelash Palm Pit-Viper (Bothriechis Schlegelii) Venom from the Colombian Southwest Region. *Acta Tropica* **2021**, *223*, 106113, doi:10.1016/j.actatropica.2021.106113.
60. He, Y.; Gao, J.; Lin, L.; Ma, X.; Ji, X. Age-Related Variation in Snake Venom: Evidence from Two Snakes (Naja Atra and Deinagkistrodon Acutus) in Southeastern China. *AHR* **2014**, *5*, 119–127, doi:10.3724/SP.J.1245.2014.00119.
61. Souad Hraoui-bloquet; Riyad Sadek; Claudine Accary; Walid Hleihel; Ziad Fajloun AN ECOLOGICAL STUDY OF THE LEBANON MOUNTAIN VIPER MONTIVIPERA BORNMUELLERI (WERNER, 1898) WITH A PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF ITS VENOM. *Lebanese Science Journal* **2011**, *13*.

Highlights

- Proteomic profiling of five *Montivipera* venoms using shotgun proteomics revealed extensive toxin diversity.
- Major toxin families were conserved, while their relative abundances varied across species.
- Comparative analysis revealed 39 conserved proteins across *Montivipera* venoms, highlighting strong interspecific similarity.
- Discovery of low-abundance toxin families expands current knowledge of *Montivipera* venom diversity.

Ethical approval and consent to participate

Not applicable.

Declaration of competing interest

The authors declare that they have no competing financial interests.