Quantitative Proteomic Analysis of Venoms from Russian Vipers of Pelias Group: Phospholipases A\(^2\) are the Main Venom Components

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Abstract: Venoms of most Russian viper species are poorly characterized. Here, by quantitative chromato-mass-spectrometry, we analyzed protein and peptide compositions of venoms from four Vipera species (V. kaznakovi, V. renardi, V. orlovi and V. nikolskii) inhabiting different regions of Russia. In all these species, the main components were phospholipases A\(^2\), their content ranging from 24% in V. orlovi to 65% in V. nikolskii. Altogether, enzyme content in venom of V. nikolskii reached ~85%. Among the non-enzymatic proteins, the most abundant were disintegrins (14%) in the V. renardi venom, C-type lectin like (12.5%) in V. kaznakovi, cysteine-rich venom proteins (12%) in V. orlovi and venom endothelial growth factors (8%) in V. nikolskii. In total, 210 proteins and 512 endogenous peptides were identified in the four viper venoms. They represented 14 snake venom protein families, most of which were found in the venoms of Vipera snakes previously. However, phospholipase B and nucleotide degrading enzymes were reported here for the first time. Compositions of V. kaznakovi and V. orlovi venoms were described for the first time and showed the greatest similarity among the four venoms studied, which probably reflected close relationship between these species within the “kaznakovi” complex.

Keywords: snake venom; viper; Vipera kaznakovi; Vipera nikolskii; Vipera orlovi; Vipera renardi; proteome; mass-spectrometry

1. Introduction

Venomous snakes inhabit all continents of the globe except Antarctica. They are particularly abundant in tropical areas of Asia, Africa, South America and Australia. Russia, despite its large territory, is inhabited by only a small number of poisonous snake species, which belong to three genera: Gloydius, Macrovipera and Vipera. The Vipera genus is the most speciose in Russia and includes more than ten species, the systematics within this genus being constantly updated [1,2]. The most abundant species is common (or European) adder Vipera berus, which has a very large habitat in Russia, ranging from its western borders to Sakhalin and the Ussuri region. V. berus is also spread throughout Europe—between 68 and 45 degrees north latitude. The venom of this species is fairly well studied. Biological activities of this venom were characterized and proteolytic, fibrinolytic, anticoagulant, and phospholipolytic ones were demonstrated by in vitro experiments [3]. Several toxic proteins were isolated from V. berus venom, including phospholipase A\(^2\) (PLA2) [4], metalloproteinase (SVMP) [5], L-amino acid oxidase (LAAO) [6] and several others. Recently, we have partially characterized the steppe viper V. renardi venom, the PLA2s and Kunitz type protease inhibitors were isolated from this venom and sequenced [7]. The isolated PLA2s were studied in more details and found to exert their
action both on lipid membranes [8] and on nicotinic acetylcholine receptor [9]. The venom of Nikolsky’s viper was also partially characterized and several proteins including heterodimeric neurotoxic PLA2s were identified [10,11]. The venoms of other Russian viper species are characterized very poorly. Thus, for Caucasian viper V. kaznakovi and Orlov’s viper V. orlovi, only the toxicity of venoms to insects was determined [12]. Here, we used proteomic chromato-mass-spectrometry analysis to obtain more detailed information on the composition of Russian viper venoms.

Modern proteomic analysis allows both qualitative and quantitative characterization of the venom proteins, leading to suggestions about venom biological effects. So far, among Vipera genus, the venoms of only three species, i.e., V. ammodytes, V. anatolica and V. raddei, were thus studied [13–15]. Semi-quantitative venom analysis of V. anatolica showed that the most abundant toxin family was SVMPs (41.5%), followed by two cysteine-rich secretory protein (CRISP) isoforms (15.9%); other proteins represented less than 10% per family [13]. SVMPs (31.6%) were also the most abundant in V. raddei venom, followed by PLA2s (23.8%), and, again, the contents of other toxin families did not exceed 10% each [14]. There is no quantitative analysis of the V. ammodytes venom, however monomeric and heterodimeric Group II PLA2s; serine proteinases (SVSPs); Group I, II, and III SVMPs; l-amino acid oxidases (LAAs); CRISP; disintegrins (Dis); and growth factors were found [15]. On the whole, the above data indicate that the composition of different viper venoms might be different. It should also be noted that V. raddei in some publications is classified as Montivipera raddei and attributed to Montivipera genus [16], thus some differences might be attributed to the discrepancy in classification. Using quantitative proteomic, we have studied the venoms from four Vipera species (V. kaznakovi, V. nikolskii, V. orlovi and V. renardi) that inhabit different regions of Russia. In contrast to the venoms of earlier studied Vipera species where the SVMP were found to be predominant [13–15], we have observed that the main components of the venoms studied are PLA2s, the content of which ranged between 24 and 65%.

2. Results

2.1. Venom Proteins Identification

In this work, venom proteomes and peptidomes for four species of Vipera snakes were analyzed. Venom proteomes were analyzed by LC-MS/MS after in-solution trypsin proteolysis. In total, for the four Vipera species venoms, the search against the Serpentes database resulted in the identification of 210 proteins (Tables 1 and 2 and Tables S1 and S2): 116 proteins were identified in V. kaznakovi, 124 in V. renardi, 135 in V. orlovi and 111 in V. nikolskii venoms. Most proteins could be matched to previously reported snake toxins. To minimize individual variations, venoms from several individual animals were pooled for analysis [12].

The proteins were categorized into 14 known venom protein families (Table 2). The most numerous classes were PLA2, SVMP, C-type lectin like (CTL) and serine protease (SP). Eleven families were represented in all viper venoms, while disintegrins (Dis) were absent in V. nikolskii. There were no Kunitz type proteinase inhibitors in V. kaznakovi, no hyaluronidase (Hya) in V. renardi and no bradykinin potentiating and C-type natriuretic peptides (B-NAP) in V. renardi and V. nikolskii. Besides, in the V. nikolskii venom, a single low abundance protein was identified belonging to Cysteine Proteases (CP), which are not common for snake venoms. Along with venom proteins, several Blood Proteins (BP) (up to 0.15% of the total protein abundance) and proteins with unclear family annotation (Other Proteins (OP)) (up to ~2% of the total protein content) were also found.

While the most numerous venom protein families were fairly similar in all snakes studied, individual protein composition was quite different (Figure 1). From 210 proteins only 46 were common for all four species and each species featured unique proteins: six in V. kaznakovi, 26 in V. renardi, eight in V. orlovi and 29 in V. nikolskii. These differences did not correlate with the total number of individual proteins identified in each venom.
| Protein No. in SuppData | Protein Name                  | Taxon                  | MW, KDa | Seq Cov, % | Prot Abun INT, % | Prot Abun INT, % | Prot Abun INT, % | Prot Abun INT, % | Prot Abun INT, % |
|-------------------------|-------------------------------|------------------------|---------|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1                       | Natriuretic peptide           | *Pseudonaja textilis*  | B-NAP   | 14         | 9.8             | 0.007           | 9.8             | 0.029           | 9.8             | 0.012           | 9.8             | -               | -               |
| 2                       | Hemoglobin subunit alpha      | *Vipera aspis*         | BP      | 15         | 6.4             | 0.000           | 6.4             | 0.000           | 6.4             | 0.000           | 6.4             | -               | -               |
| 3                       | Hemoglobin subunit beta-2     | *Naja naja*            | BP      | 16         | 7.5             | 0.000           | 7.5             | 0.000           | 7.5             | 0.000           | 7.5             | -               | -               |
| 4                       | Hemoglobin subunit beta       | *Erythrolamprus miliaris* | BP     | 15         | 23.3            | 0.001           | 19.2            | 0.000           | 12.4           | 0.003           | 9.0             | -               | -               |
| 33                      | Alpha globin                 | *Hydrophis melanocorphus* | BP     | 16         | 19.7            | 0.011           | 9.2             | 0.000           | 9.2             | 0.003           | 19.7            | -               | -               |
| 34                      | Alpha globin                 | *Elaphe climacophora*  | BP      | 11         | 25.2            | -               | -               | -               | -               | -               | -               | 0.002           | 25.2            |
| 116                     | Murrinoglobin-2              | *Ophiophagus hannah*   | BP      | 143        | 2.7             | -               | -               | 0.008           | 1.8             | 0.002           | 2.2             | -               | -               |
| 124                     | Serum albumin                | *Protobothrops flavoviridis* | BP    | 69         | 5.4             | 0.013           | 5.4             | 0.002           | 5.2             | -               | -               | -               | -               |
| 158                     | Hemoglobin subunit alpha      | *Hydrophis gracilis*   | BP      | 15         | 15.6            | 0.013           | 15.6            | 0.002           | 15.6            | 0.007           | 15.6            | 0.017           | 15.6            |
| 165                     | Hemoglobin subunit alpha      | *Crotalus horridus*    | BP      | 15         | 11.3            | 0.012           | 11.3            | 0.003           | 11.3            | 0.013           | 11.3            | -               | -               |
| 171                     | Hemoglobin subunit beta-1     | *Boiga irregularis*    | BP      | 16         | 21.8            | 0.006           | 19.2            | 0.000           | 19.2            | 0.003           | 19.2            | 0.004           | 19.2            |
| 177                     | Transferin                   | *Crotalus adamentaextus* | BP     | 77         | 3.1             | 0.011           | 3.1             | 0.004           | 3.1             | 0.009           | 3.1             | -               | -               |
| 199                     | Hemoglobin subunit beta-2     | *Thamnophis sirtalis*  | BP      | 16         | 19.7            | 0.002           | 19.7            | 0.000           | 19.7            | 0.006           | 19.7            | 0.020           | 19.7            |
| 206                     | Serum albumin-like            | *Thamnophis sirtalis*  | BP      | 57         | 8.0             | 0.012           | 8.0             | 0.008           | 8.0             | 0.006           | 8.0             | -               | -               |
| 180                     | Cathepsin B-like protein      | *Crotalus adamentaextus* | CP     | 37         | 5.0             | 0.011           | 5.0             | 0.002           | 5.0             | 0.003           | 5.0             | -               | -               |
| 4                       | Cysteine-rich venom protein   | *Philodryas patagoniensis* | CRISP | 1          | 64.3            | 0.013           | 64.3            | 0.002           | 64.3            | 0.002           | 64.3            | -               | -               |
| 118                     | Cysteine-rich secretory protein | *Daboia russelli*     | CRISP   | 26         | 24.7            | 0.019           | 24.7            | 0.017           | 24.7            | 0.017           | 24.7            | -               | -               |
| 195                     | Cysteine-rich secretory protein | *Daboia russelli*     | CRISP   | 25         | 17.6            | 0.018           | 17.6            | 0.006           | 17.6            | 0.018           | 17.6            | 0.017           | 17.6            |
| 89                       | Cysteine-rich venom protein   | *Protobothrops jerdoni* | CRISP  | 26         | 22.5            | 0.020           | 22.5            | 0.019           | 22.5            | 0.019           | 22.5            | -               | -               |
| 90                       | Cysteine-rich venom protein   | *Vipera nikolskii*     | CRISP   | 24         | 83.3            | 0.012           | 83.3            | 0.011           | 83.3            | 0.011           | 83.3            | 0.020           | 83.3            |
| 91                       | Cysteine-rich venom protein   | *Protobothrops flavoviridis* | CRISP | 26         | 7.7             | 0.035           | 7.7             | 0.035           | 7.7             | 0.035           | 7.7             | 0.020           | 7.7             |
| 92                       | Cysteine-rich venom protein   | *Protobothrops flavoviridis* | CRISP | 24         | 23.1            | 0.070           | 23.1            | 0.065           | 23.1            | 0.065           | 23.1            | 3.016           | 23.1            |
| 101                     | Snaclec 1                     | *Sistrurus catus*      | CTL     | 17         | 6.2             | 0.010           | 6.2             | 0.010           | 6.2             | 0.010           | 6.2             | -               | -               |
| 102                     | Snaclec VP12 subunit A        | *Daboia palustrinae*   | CTL     | 12         | 22.6            | 0.000           | 22.6            | 0.000           | 22.6            | 0.000           | 22.6            | -               | -               |
| 103                     | Snaclec VP12 subunit B        | *Daboia palustrinae*   | CTL     | 16         | 25.6            | 0.084           | 25.6            | 0.029           | 25.6            | 0.029           | 25.6            | -               | -               |
| 153                     | C-type lectin J              | *Echis coloratus*      | CTL     | 18         | 14.6            | 0.589           | 14.6            | 0.740           | 14.6            | 0.740           | 14.6            | 0.477           | 14.6            |
| 154                     | C-type lectin H              | *Echis coloratus*      | CTL     | 18         | 10.8            | 0.050           | 10.8            | 0.935           | 10.8            | 0.935           | 10.8            | 0.172           | 10.8            |
| 155                     | C-type lectin E              | *Echis coloratus*      | CTL     | 11         | 12.1            | 0.011           | 12.1            | 0.008           | 12.1            | 0.008           | 12.1            | 0.045           | 12.1            |
| 156                     | C-type lectin B              | *Echis coloratus*      | CTL     | 13         | 7.1             | 0.048           | 7.1             | 0.005           | 7.1             | 0.005           | 7.1             | -               | -               |
| 157                     | C-type lectin A              | *Echis coloratus*      | CTL     | 18         | 10.1            | 0.014           | 10.1            | 0.014           | 10.1            | 0.014           | 10.1            | -               | -               |
### Table 1. Cont.

| Protein No. in SuppData | Protein Name | Taxon | Protein Family | MW, KDa | Seq Cov, % | V. nikolskii | V. kaznakovi | V. orlovi | V. renardi |
|-------------------------|--------------|-------|----------------|---------|------------|--------------|--------------|------------|-------------|
| Protein Abun INT, %     | Seq Cov, %    | Protein Abun INT, % | Seq Cov, % | Protein Abun INT, % | Seq Cov, % | Protein Abun INT, % | Seq Cov, % | Protein Abun INT, % | Seq Cov, % |
| 159 Snaclec coagulation factor | Macrovipera lebetina | CTL | 18 | 6.3 | 0.039 | 6.3 | 0.199 | 6.3 | 0.340 | 6.3 | 0.051 | 6.3 |
| 173 C-type lectin-like protein 3B | Macrovipera lebetina | CTL | 17 | 42.6 | 2.457 | 42.6 | 2.758 | 42.6 | 2.500 | 42.6 | 1.545 | 42.6 |
| 174 C-type lectin-like protein 4B | Macrovipera lebetina | CTL | 17 | 14.7 | 0.018 | 12.0 | - | - | 0.017 | 12.0 | 0.353 | 14.7 |
| 175 Snaclec dabocetin subunit alpha | Daboia siamensis | CTL | 17 | 6.5 | 0.443 | 6.5 | 0.064 | 6.5 | 0.046 | 14.5 | 0.097 | 30.4 |
| 181 Snaclec tokaracetin subunit beta | Protobothrops tokarensis | CTL | 4 | 32.5 | 0.026 | 32.5 | - | - | - | - | - |
| 210 Snaclec anticoagulant protein | Deinagkistrodon acutus | CTL | 14 | 23.4 | - | - | - | - | - | - | - |
| 14 Disintegrin VB7A | Vipera berus berus | Dis | 7 | 76.6 | 0.111 | 23.4 | 0.008 | 23.4 | 12.932 | 76.6 |
| 15 Disintegrin VB7B | Vipera berus berus | Dis | 6 | 70.3 | 0.008 | 29.7 | - | - | - | 0.935 | 70.3 |
| 16 Disintegrin VLO4 | Macrovipera lebetina | Dis | 7 | 38.5 | 0.011 | 24.6 | 0.006 | 24.6 | 0.034 | 38.5 |
| 17 Disintegrin VA6 | Viperidae | Dis | 7 | 23.4 | - | - | - | - | - | - | - |
| 191 Disintegrin lebein-1-alpha | Macrovipera lebetina | Dis | 12 | 30.6 | 0.389 | 9.0 | 0.578 | 9.0 | 0.006 | 21.6 |
| 86 Hyaluronidase | Echis ocellatus | EHA | 52 | 13.6 | 0.007 | 13.6 | 0.111 | 13.6 | 0.004 | 2.7 | - |
| 176 Hyaluronidase | Crotalus adamanteus | Hya | 52 | 6.7 | 0.004 | 6.7 | 0.003 | 6.7 | 0.935 | 70.3 |
| 27 Inhibitor, chymotrypsin | Vipera ammodytes ammodytes | Dis | 7 | 30.6 | 0.389 | 9.0 | 0.578 | 9.0 | 0.006 | 21.6 |
| 191 Disintegrin lebein-1-beta | Macrovipera lebetina | Dis | 12 | 30.6 | 0.389 | 9.0 | 0.578 | 9.0 | 0.006 | 21.6 |
| 86 Hyaluronidase | Echis ocellatus | Hya | 52 | 13.6 | 0.007 | 13.6 | 0.111 | 13.6 | 0.004 | 2.7 | - |
| 176 Hyaluronidase | Crotalus adamanteus | Hya | 52 | 6.7 | 0.004 | 6.7 | 0.003 | 6.7 | 0.935 | 70.3 |
| 87 KP-Sut-1 | Suta fasciata | Kunitz | 13 | 9.4 | 0.072 | 9.4 | - | - | - | 0.077 | 9.4 |
| 142 Kunitz-type serine protease inhibitor kin-VN | Macrovipera lebetina transmediterranea | Kunitz | 10 | 34.7 | 0.610 | 34.7 | - | - | - | - | - |
| 5 l-amino-acid oxidase | Macrovipera lebetina | LAAO | 12 | 42.1 | 0.041 | 41.1 | 1.388 | 42.1 | 1.631 | 42.1 | 1.786 | 41.1 |
| 65 l-amino-acid oxidase | Macrovipera lebetina | LAAO | 6 | 6.0 | - | - | - | - | 0 | 6.0 | - |
| 66 l-amino-acid oxidase | Macrovipera lebetina | LAAO | 54 | 13.2 | - | - | - | - | 0.012 | 11.2 | 0.007 | 13.0 |
| 75 l-amino-acid oxidase | Daboia russelli | LAAO | 56 | 11.7 | 0.316 | 7.7 | 0.045 | 11.7 | 0.049 | 9.9 |
| 92 Kunitz-type serine protease inhibitor PIVL | Macrovipera lebetina transmediterranea | LAAO | 10 | 8.4 | - | - | - | - | 0 | 8.4 | 0.021 | 8.4 |
| 94 l-amino-acid oxidase | Gloydius halys | LAAO | 55 | 9.9 | - | - | - | - | 0 | 7.4 | - |
| 107 l-amino-acid oxidase | Ophiurus owinakensis | LAAO | 58 | 11.0 | 0.001 | 3.9 | 1.893 | 6.8 | 2.106 | 6.8 | 1.378 | 10.9 |
| 50 l-amino-acid oxidase | Protobothrops elegans | LAAO | 57 | 5.7 | - | - | 0 | 5.7 | - | - | - |
| 152 l-amino-acid oxidase | Echis coloratus | LAAO | 56 | 17.1 | - | - | 0.425 | 13.1 | 0.213 | 12.9 | 0.216 | 13.9 |
| 164 l-amino-acid oxidase | Crotalus harrisii | LAAO | 58 | 11.2 | 0.011 | 4.5 | 0.042 | 6.6 | 0.005 | 5.8 | 0 | 5.8 |
| 183 l-amino-acid oxidase | Bothrops moojeni | LAAO | 54 | 12.1 | 0.022 | 6.5 | 0.256 | 7.1 | 0.178 | 9.4 | 0.098 | 9.2 |
| 62 Venom nerve growth factor 2 | Daboia russelli | NGF | 27 | 14.4 | - | - | 0 | 14.4 | - | - | - |
| 63 Venom nerve growth factor | Vipera urinaria | NFG | 27 | 25.5 | 0.285 | 18.1 | 0.122 | 18.1 | 0.254 | 25.5 | 0.093 | 18.1 |
| 76 Snake venom S' nucleotidase | Gloydius bimaculatus | Nuc | 6 | 27.8 | 0.013 | 27.8 | - | - | - | - | - |
| 110 S'-nucleotidase | Ophiurus owinakensis | Nuc | 55 | 26.6 | 0.091 | 26.6 | 0.12 | 9.1 | 0.018 | 16.5 | 0.057 | 22.4 |
| 125 Phosphodiesterase | Macrovipera lebetina | Nuc | 96 | 35.4 | 0.243 | 35.4 | 0.103 | 21.4 | 0.088 | 20.7 | 0.038 | 19.5 |
| 126 S'-nucleotidase | Macrovipera lebetina | Nuc | 45 | 58.1 | 0.563 | 54.4 | 0.046 | 14.5 | 0.097 | 30.4 | 0.226 | 39.2 |
| 127 Venom phosphodiesterase 2 | Crotalus adamanteus | Nuc | 44 | 14.0 | 0.007 | 14.0 | - | - | - | - | - |
| 132 pyrophosphatase/phosphodiesterase family member 3-like | Python bivittatus | Nuc | 93 | 7.5 | 0.004 | 7.5 | 0.002 | 3.5 | 0.003 | 3.5 | - | - |
| Protein No. in SuppData | Protein Name | Taxon | Protein Family 1 | MW, KDa | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % |
|-------------------------|--------------|-------|-----------------|--------|-----------|----------------|-----------|----------------|-----------|----------------|-----------|----------------|-----------|
| 170                     | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | *Boiga irregularis* | Nuc     | 100    | 4.5       | -               | -         | 0.441          | 3.3       | 1.521          | 2.6       | -               | -         |
| 73                      | Proactivator polypeptide-like | *Crotalus adamanteus* | OP      | 58     | 19.7      | 0.036          | 19.7      | 0.011          | 5.2       | 0.024          | 8.7       | 0.006          | 2.5       |
| 104                     | ArfGAP with SH3 domain ankyrin repeat and PH domain 3 | *Micrurus fulvius* | OP      | 107    | 2.3       | -               | -         | 0.027          | 2.3       | 0.033          | 2.3       | -               | -         |
| 113                     | Uncharacterized protein | *Ophiophagus hannah* | OP      | 46     | 3.4       | -               | -         | 0.040          | 3.4       | 0.015          | 3.4       | -               | -         |
| 115                     | 78 KDa glucose-regulated protein | *Ophiophagus hannah* | OP      | 67     | 6.4       | 0               | 2.8       | 0.004          | 4.4       | 0              | 2.0       | -               | -         |
| 117                     | WD repeat-containing protein 67 | *Ophiophagus hannah* | OP      | 113    | 0.8       | -               | -         | -              | -         | -              | -         | 0.652          | 0.8       |
| 118                     | Putative adenylate cyclase-activating polypeptide type 1 receptor | *Ophiophagus hannah* | OP      | 7      | 18.2      | 0.008          | 18.2      | -              | -         | -              | -         | -              | -         |
| 119                     | Iron-responsive element-binding protein 2 | *Ophiophagus hannah* | OP      | 90     | 1.8       | -               | -         | 0.536          | 1.8       | 0.482          | 1.8       | 0.224          | 1.8       |
| 122                     | Glutathione peroxidase | *Ophiophagus hannah* | OP      | 29     | 21.6      | 0.078          | 18.9      | 0.047          | 16.7      | 0.095          | 21.6      | 0.081          | 16.7      |
| 128                     | PiggyBac transposable element-derived protein 5 | *Python bivittatus* | OP      | 69     | 2.7       | -               | -         | -              | -         | -              | -         | 0.067          | 2.7       |
| 129                     | Calmodulin-lysine N-methyltransferase | *Python bivittatus* | OP      | 14     | 12.3      | -               | -         | -              | -         | 0              | 12.3      | -              | -         |
| 130                     | Dipeptidase 2 | *Python bivittatus* | OP      | 46     | 7.0       | -               | -         | -              | -         | 0              | 7.0       | -              | -         |
| 131                     | Serine/threonine-protein phosphatase 6 regulatory subunit 1 isoform X3 | *Python bivittatus* | OP      | 92     | 1.9       | -               | -         | 0.199          | 1.9       | 0.117          | 1.8       | 0.279          | 1.8       |
| 133                     | E3 ubiquitin-protein ligase MARCS-like isoform X8 | *Python bivittatus* | OP      | 30     | 7.2       | -               | -         | -              | -         | -              | -         | 0.702          | 7.2       |
| 134                     | Nucleolar and coiled-body phosphoprotein 1 isoform X5 | *Python bivittatus* | OP      | 104    | 1.4       | -               | -         | -              | -         | 0              | 1.4       | -              | -         |
| 135                     | E3 ubiquitin-protein ligase UBR4 Extracellular matrix protein 1 | *Python bivittatus* | OP      | 555    | 0.2       | 0               | 0.2       | -              | -         | -              | -         | -              | -         |
| 136                     | Receptor-type tyrosine-protein phosphatase gamma-like | *Python bivittatus* | OP      | 102    | 1.9       | -               | -         | 0.026          | 1.9       | 0.018          | 1.9       | 0.018          | 1.9       |
| 138                     | Nurin-like | *Python bivittatus* | OP      | 32     | 7.7       | 0.010          | 7.7       | -              | -         | -              | -         | -              | -         |
| 162                     | Dickkopf-related protein 3-like | *Crotalus horridus* | OP      | 31     | 5.0       | -               | -         | 0.005          | 5         | 0.007          | 5.0       | -              | -         |
| 168                     | RNA binding motif protein 6 | *Boiga irregularis* | OP      | 59     | 3.5       | 0.007          | 3.5       | -              | -         | -              | -         | -              | -         |
| 172                     | Filamin-B isoform 15 | *Boiga irregularis* | OP      | 282    | 0.9       | -               | -         | 0.010          | 0.9       | -              | -         | -              | -         |
| 197                     | Leucine-rich repeats and immunoglobulin-like domains protein 1 | *Thamnophis sirtalis* | OP      | 48     | 1.4       | 0.010          | 1.4       | 0.028          | 1.4       | 0.026          | 1.4       | -              | -         |
Table 1. Cont.

| Protein No. in SuppData | Protein Name | Taxon | Protein Family | MW, KDa | Seq Cov, % | V. nikolskii Prot Abun INT, % | V. kaznakovi Seq Cov, % | V. orlovi Prot Abun INT, % | V. renardi Seq Cov, % |
|-------------------------|--------------|-------|----------------|---------|-----------|-------------------------------|------------------------|---------------------------|------------------------|
| 201 | Peroxiredoxin-4-like | Thamnophis sirtalis | OP | 31 | 8.7 | 0.002 | 8.7 | - | - | - | - |
| 202 | Obscurin | Thamnophis sirtalis | OP | 1024 | 0.5 | 0.109 | 0.4 | 0.107 | 0.2 | - | - | - |
| 203 | CCR4-NOT transcription complex subunit 3 | Calithrix jacchus | OP | 12 | 15.7 | - | - | 0.008 | 15.7 | 0.046 | 15.7 | - | - |
| 205 | Tyrosine-protein phosphatase non-receptor type 20 | Thamnophis sirtalis | OP | 50 | 4.0 | - | - | 0.019 | 4.0 | 0.020 | 4.0 | - | - |
| 208 | Protein BANP | Thamnophis sirtalis | OP | 40 | 4.4 | - | - | 0.064 | 4.4 | 0.076 | 4.4 | 0.119 | 4.4 |
| 209 | Microtubule-associated serine/threonine-protein kinase 1-like | Thamnophis sirtalis | OP | 94 | 0.7 | - | - | - | - | - | - | 0 | 0.7 |
| 10 | Basic phospholipase A\_1 chain \(\text{HDP-1P}\) | Vipera nikolskii | PLA2 | 13 | 86.9 | 20.289 | 86.9 | 5.460 | 9.0 | 0.230 | 9.0 | 0.288 | 27.0 |
| 13 | Basic phospholipase A\_2 B chain | Vipera aspis zinnikeri | PLA2 | 13 | 80.3 | 0.130 | 80.3 | - | - | - | - | - | - |
| 28 | Phospholipase A\_2 II | Vipera aspis | PLA2 | 5 | 40.4 | 0.018 | 19.2 | - | - | 0.009 | 21.2 | - | - |
| 29 | Phospholipase A\_2 III | Eristichopus macracionis | PLA2 | 13 | 22.3 | - | - | - | - | - | - | 0.419 | 22.3 |
| 30 | Acidic phospholipase A\_1 PLA-1 | Eristichopus macracionis | PLA2 | 13 | 29.8 | 0 | 28.9 | - | - | 0 | 21.5 | 0.388 | 29.8 |
| 31 | Acidic phospholipase A\_2 PLA-2 | Vipera ammodytes merakonias | PLA2 | 13 | 94.3 | 34.017 | 94.3 | - | - | - | - | 0.048 | 18.9 |
| 32 | Acidic phospholipase A\_3 homolog vipoxin A chain | Daboia russelli | PLA2 | 13 | 27.3 | - | - | - | 0.003 | 12.4 | 0.002 | 12.4 | 0.048 | 27.3 |
| 33 | Acidic phospholipase A\_4 homolog | Aethistrictus piscivorus | PLA2 | 13 | 27.6 | 0 | 16.3 | - | - | - | - | - | - |
| 58 | Phospholipase A\_2 homolog \(P\)-elapitoxin-Aa1 beta chain | Acanthophis antarcticus | PLA2 | 3 | 22.6 | - | - | 0.165 | 22.6 | 0.127 | 22.6 | 0.030 | 22.6 |
| 67 | Acidic phospholipase A\_2 RV-7 | Daboia siamensis | PLA2 | 13 | 45.1 | 1.078 | 45.1 | - | - | - | - | - | - |
| 78 | Basic phospholipase A\_2 Pla2Vb | Vipera aspis | PLA2 | 15 | 46.4 | - | - | 0 | 13.8 | 0.056 | 46.4 | - | - |
| 79 | Acidic phospholipase A\_3 Vur-PL3 | Vipera renardi | PLA2 | 15 | 69.3 | - | - | 12.956 | 67.2 | 12.705 | 67.2 | 7.113 | 58.4 |
| 82 | Acidic phospholipase A\_3 PL1 | Vipera renardi | PLA2 | 15 | 75.4 | 4.057 | 70.3 | 5.097 | 59.4 | 4.080 | 52.9 | 10.603 | 75.4 |
| 83 | Acidic phospholipase A\_4 Vur-PL2B | Vipera renardi | PLA2 | 15 | 72.3 | - | - | 0 | 19.7 | 0.041 | 19.7 | 14.999 | 72.3 |
| 84 | Basic phospholipase A\_5 homolog Vur-S49 | - | PLA2 | 15 | 63.0 | - | - | 0.009 | 18.8 | 0.055 | 18.8 | 7.676 | 63.0 |
| 85 | Basic phospholipase A\_2 vurtokxin | Vipera renardi | PLA2 | 15 | 50.0 | - | - | - | - | - | - | 4.220 | 50.0 |
| 95 | Ammodytin I1 | Vipera aspis | PLA2 | 15 | 56.5 | 0.032 | 56.5 | 0.048 | 56.5 | 0.040 | 44.9 | 0.026 | 39.9 |
| 96 | Ammodytin I2 | Vipera ammodytes montandoni | PLA2 | 15 | 75.4 | 1.389 | 75.4 | 1.428 | 75.4 | 0.524 | 63.8 | 1.710 | 59.4 |
| 97 | Ammodytin I2 | Vipera beras beras | PLA2 | 15 | 19.7 | - | - | - | - | - | - | 0 | 19.7 |
| 98 | Ammodytin I2 | Vipera urusinii | PLA2 | 15 | 36.5 | - | - | 0.021 | 31.4 | - | - | - | - |
| 99 | Ammodytin I2 | Vipera ammodytes montandoni | PLA2 | 15 | 45.3 | - | - | - | - | - | - | 0.017 | 45.3 |
| 100 | Ammodytin I1 | Vipera ammodytes montandoni | PLA2 | 15 | 14.5 | - | - | - | - | - | - | 0.009 | 14.5 |
Table 1. Cont.

| Protein No. | Protein Name | Taxon | Protein Family | MW, KDa | Seq Cov, % | V. nikolskii | V. kaznakovi | V. orlovi | V. renardi |
|-------------|--------------|-------|----------------|---------|------------|--------------|--------------|------------|------------|
|             |              |       |                |         |            | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % |
| 139         | Basic phospholipase A2 | Vipera ammodytes meridionalis | PLA2 | 13 | 80.3 | 0.086 | 80.3 | - | - | - | - | - | - |
| 151         | Phospholipase A2 Group IIE | Echis coloratus | PLA2 | 13 | 5.8 | - | - | - | - | 0.012 | 7.2 | 0.016 | 7.2 |
| 193         | Phospholipase A2-III | Daboia russelli | PLA2 | 13 | 13.1 | 0.019 | 13.1 | - | - | - | - | - | - |
| 195         | Phospholipase A2 ammodytin I | Vipera nikolskii | PLA2 | 15 | 75.4 | 4.779 | 75.4 | 4.663 | 75.4 | 4.289 | 63.8 | - | - |
| 196         | Basic phospholipase A2 chain | Echis coloratus | PLA2 | 15 | 69.6 | 0.068 | 69.6 | - | - | - | - | - | - |
| 109         | Phospholipase b | Ophiophagus hannah | PLB | 64 | 20.8 | - | - | 0.015 | 13.6 | 0.037 | 20.1 | 0.057 | 15.0 |
| 114         | Phospholipase B-like 1 | Ophiophagus hannah | PLB | 58 | 16.8 | 0.022 | 7.0 | 0.085 | 12.0 | 0.104 | 16.2 | 0.088 | 10.2 |
| 169         | Phospholipase B | Boiga irregularis | PLB | 64 | 26.9 | 0.080 | 16.5 | 0.218 | 15.9 | 0.321 | 21.0 | 0.334 | 12.7 |
| 179         | Phospholipase B | Crotalus adamanteus | PLB | 64 | 26.9 | 0.080 | 16.5 | 0.218 | 15.9 | 0.321 | 21.0 | 0.334 | 12.7 |
| 6           | Unassigned | Calloselasma rhodostoma | SP | 24 | 9.4 | - | - | 0.007 | 5.5 | 0.041 | 8.1 | 0.006 | 8.1 |
| 11          | Snake venom serine protease pallase | Gloydias halys | SP | 26 | 13.1 | 0.002 | 9.3 | 0.005 | 10.6 | 0.002 | 11.9 | 0 | 10.6 |
| 12          | Snake venom serine protease ussurase | Gloydias ussurasiensis | SP | 26 | 5.6 | 0.077 | 5.6 | - | - | - | - | - | - |
| 21          | Thrombin-like enzyme KN-BJ 2 | Bothrops jararaca | SP | 27 | 11.3 | 1.017 | 11.3 | 0.329 | 11.3 | 1.944 | 11.3 | 0.050 | 11.3 |
| 35          | Serine protease | Echis coloratus | SP | 28 | 8.5 | - | - | 0.043 | 8.5 | 0.172 | 8.5 | 0.087 | 8.5 |
| 36          | Serine protease | Echis ocellatus | SP | 24 | 6.3 | 0.515 | 6.3 | 1.980 | 3.6 | 2.401 | 6.3 | 0.694 | 6.3 |
| 37          | Serine protease | Echis coloratus | SP | 25 | 7.3 | - | - | 0.079 | 4.7 | 0.047 | 7.3 | - | - |
| 38          | Serine protease | Echis coloratus | SP | 25 | 12.0 | 0.004 | 12.0 | 0 | 9.4 | 0.007 | 12.0 | - | - |
| 39          | Serine protease | Echis coloratus | SP | 26 | 5.9 | 0.110 | 5.9 | 0.048 | 3.4 | 0.015 | 5.9 | 0.005 | 5.9 |
| 40          | Serine protease | Echis ocellatus | SP | 25 | 8.1 | 0.021 | 5.5 | - | - | - | - | 0.036 | 5.5 |
| 64          | Factor V activator RV-V gamma | Daboia siamensis | SP | 25 | 13.7 | 0.006 | 11.1 | 0.473 | 8.1 | 0.075 | 5.6 | - | - |
| 69          | Serine protease VLSF-3 | Macrovipera lebetina | SP | 28 | 15.5 | 3.526 | 15.5 | 2.548 | 12.0 | 3.429 | 14.3 | 1.303 | 14.3 |
| 70          | Beta-fibrinogenase | Macrovipera lebetina | SP | 28 | 18.3 | 0.017 | 18.3 | 0.008 | 9.3 | 0.078 | 11.7 | 0.224 | 11.7 |
| 71          | Chymotrypsin-like protease VLCTLP | Macrovipera lebetina | SP | 28 | 29.6 | 0.069 | 29.6 | - | - | - | - | - | - |
| 74          | Snake venom serine protease nikobin | Vipera nikolskii | SP | 28 | 43.2 | 12.595 | 43.2 | 2.334 | 34.2 | 8.515 | 32.3 | 1.732 | 28.0 |
| 77          | Snake venom serine protease pallabin | Gloydias halys | SP | 28 | 12.3 | 0.002 | 8.8 | - | - | - | - | 0.010 | 10.0 |
| 103         | Kalikrein-CohID-1 | Crotalus oreganus lindheimeri | SP | 28 | 10.8 | - | - | - | - | - | 0 | 10.8 |
| 105         | Serine protease | Protobothrops flavoviridis | SP | 18 | 17.4 | 0 | 17.4 | - | - | - | - | - | - |
| 106         | Serine protease | Ophiophagus hannah | SP | 28 | 19.8 | 0.049 | 13.9 | 0.776 | 11.6 | 0.088 | 11.2 | 0.005 | 5.0 |
| 141         | Venom serine proteinase-like protein 2 | Macrovipera lebetina | SP | 28 | 30.8 | 1.028 | 30.8 | 0.896 | 26.5 | 1.680 | 25.4 | 1.595 | 26.5 |
| 163         | Serine proteinase 1 | Crotalus horridus | SP | 28 | 13.2 | 1.023 | 10.9 | 0.274 | 4.3 | 2.042 | 6.6 | - | - |
| 187         | Snake venom serine protease HS112 | Bothrops jararaca | SP | 27 | 14.9 | - | - | 0.157 | 12.5 | 0.323 | 14.9 | - | - |
Table 1. Cont.

| Protein No. in SuppData | Protein Name | Taxon | Protein Family | MW, KDa | Seq Cov, % | V. nikolskii | V. kaznakovi | V. orlovi | V. renardi |
|------------------------|--------------|-------|----------------|---------|------------|-------------|-------------|-----------|-----------|
| 188                    | Snake venom serine protease KN6 | Trimeresurus stejnegeri | SP | 28 | 3.5 | 0.036 | 3.5 | - | - | - | - | - |
| 189                    | Snake venom serine protease 5 | Trimeresurus stejnegeri | SP | 28 | 11.6 | 0 | 11.6 | - | - | - | - | - |
| 190                    | Snake venom serine protease catroxase-2 | Crotalus atrox | SP | 27 | 17.4 | 0.352 | 17.4 | 0.162 | 8.5 | 0.531 | 10.9 | 0.012 | 10.9 |
| 198                    | Rho GTPase-activating protein 26-like | Thamnophis sirtalis | SP | 24 | 3.2 | - | - | 0.811 | 3.2 | 0.822 | 3.2 | 0.207 | 3.2 |
| 41                     | Metalloproteinase | Echis carinatus suchuruki | SVM | 69 | 14.6 | - | - | 2.987 | 6.5 | 2.973 | 6.5 | 1.419 | 6.5 |
| 42                     | Metalloproteinase | Echis carinatus suchuruki | SVM | 68 | 6.9 | - | - | - | - | - | - | 0.107 | 6.9 |
| 43                     | Metalloproteinase | Echis carinatus suchuruki | SVM | 27 | 6.5 | - | - | - | - | - | - | - | - |
| 44                     | Metalloproteinase | Echis coloratus | SVM | 56 | 6.3 | - | - | - | - | - | - | - | - |
| 45                     | Metalloproteinase | Echis coloratus | SVM | 56 | 11.2 | - | - | 0.109 | 9.4 | 0.080 | 9.4 | 0.116 | 9.0 |
| 46                     | Metalloproteinase | Echis coloratus | SVM | 66 | 10.7 | - | - | 0 | 9.2 | 0 | 9.2 | 0 | 10.7 |
| 47                     | Metalloproteinase | Echis coloratus | SVM | 69 | 3.1 | - | - | - | - | - | - | 0.008 | 3.1 |
| 48                     | Metalloproteinase | Echis coloratus | SVM | 69 | 7.4 | - | - | 0.208 | 7.4 | 0.435 | 4.7 | 0.058 | 4.9 |
| 49                     | Metalloproteinase | Echis coloratus | SVM | 68 | 8.8 | - | - | - | - | - | - | - | - |
| 50                     | Metalloproteinase | Echis coloratus | SVM | 68 | 13.8 | - | - | 0 | 8.9 | 0 | 5.2 | 0.488 | 4.9 |
| 51                     | Metalloproteinase | Echis coloratus | SVM | 61 | 5.4 | - | - | 2.479 | 5.4 | 2.296 | 5.4 | 1.071 | 5.4 |
| 52                     | Metalloproteinase | Echis coloratus | SVM | 68 | 6.4 | - | - | 0.019 | 6.4 | 0.012 | 5.6 | 0.021 | 6.4 |
| 53                     | Metalloproteinase | Echis pyramidium shakeshi | SVM | 62 | 8.1 | 0.009 | 2.5 | - | - | - | - | - | - |
| 54                     | Metalloproteinase | Echis pyramidium shakeshi | SVM | 46 | 5.4 | - | - | 0.019 | 5.4 | 0.014 | 5.4 | - | - |
| 55                     | Metalloproteinase | Echis carinatus suchuruki | SVM | 54 | 5.6 | - | - | - | - | - | - | 0.010 | 5.6 |
| 59                     | Group III snake venom metalloproteinase | Echis ocellatus | SVM | 62 | 10.7 | 0 | 3.1 | 1.254 | 6.0 | 1.744 | 9.0 | 0.651 | 10.7 |
| 61                     | Snake venom metalloproteinase VMP1 | Agkistrodon piscivorus leucosoma | SVM | 54 | 6.6 | - | - | - | - | - | - | 0.333 | 6.8 |
| 72                     | Snake venom metalloproteinase | Crotalus adamanteus | SVM | 68 | 9.0 | - | - | 0.005 | 3.4 | 0.016 | 4.7 | 0.085 | 7.7 |
| 93                     | H3 metalloproteinase 1 | Vipera ammodytes ammodytes | SVM | 68 | 43.5 | 0.611 | 32.0 | 3.415 | 33.3 | 2.970 | 35.9 | 2.737 | 37.3 |
| 108                    | P-III metalloproteinase | Orphis okinensis | SVM | 16 | 12.1 | - | - | 2.110 | 12.1 | 1.999 | 12.1 | 0.945 | 12.1 |
| 112                    | Metalloproteinase H4-A | Vipera ammodytes ammodytes | SVM | 68 | 14.2 | 0.022 | 11.4 | 0.342 | 4.2 | 0.006 | 4.2 | - | - |
| 143                    | Snake venom metalloproteinase lebetase-4 | Macrovipera lebetina | SVM | 26 | 19.4 | - | - | - | - | 0.008 | 12.4 | 0.091 | 19.4 |
| 146                    | Zinc metalloproteinase-disintegrin-like | Daboia russelli | SVM | 69 | 6.2 | - | - | - | - | - | - | 0.112 | 6.2 |
| Protein No. in SuppData | Protein Name | Taxon | Protein Family | MW, KDa | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % | Prot Abun INT, % | Seq Cov, % |
|------------------------|--------------|-------|---------------|---------|-----------|----------------|-----------|----------------|-----------|----------------|-----------|----------------|-----------|----------------|-----------|
| 147                    | Coagulation factor X-activating enzyme heavy chain | Debroa siamensis | SVMP | 69 | 10.0 | 0.003 | 2.9 | 0.473 | 7.3 | 0.088 | 7.1 | 0.010 | 7.3 |
| 160                    | Coagulation factor X-activating enzyme heavy chain | Vipera kaznakovi | SVMP | 68 | 11.4 | 0.013 | 5.4 | 1.243 | 11.4 | 0.105 | 6.5 | 0.020 | 5.4 |
| 166                    | Metalloproteinase F1 | Vipera ammodytes | ammodytes | SVMP | 68 | 25.7 | - | - | - | - | 0.001 | 4.1 | 0.498 | 25.7 |
| 182                    | Anti-hemorrhagic factor cHLP-A | Gloydius brevicaudus | SVMP | 36 | 4.0 | - | - | - | - | - | 0.008 | 4.8 | 0.027 | 8.8 | 0.675 | 7.9 |
| 184                    | Zinc metalloproteinase/disintegrin Zinc | Macrovoiper lebetina | SVMP | 53 | 11.9 | - | - | - | - | 0.001 | 4.8 | 0.027 | 8.8 | 0.675 | 7.9 |
| 185                    | metalloproteinase-disintegrin-like VLAIP-B | Macrovoiper lebetina | SVMP | 68 | 9.3 | 0.001 | 4.6 | - | - | - | - | 0.052 | 6.5 |
| 186                    | metalloproteinase-disintegrin-like VLAIP-A | Macrovoiper lebetina | SVMP | 68 | 25.2 | 0.005 | 17.7 | 0.138 | 14.9 | 0.110 | 17.5 | 0.048 | 19.0 |
| 192                    | Group III snake venom metalloproteinase | Echis ocellatus | SVMP | 69 | 10.7 | - | - | 0 | 7.9 | 0 | 10.7 | - | - |
| 194                    | metalloproteinase-disintegrin-like bothrojarin-2 | Bothrops jararaca | SVMP | 24 | 11.9 | - | - | 0.015 | 11.9 | 0.017 | 11.9 | 0.089 | 11.9 |
| 1                    | Renin-like aspartic protease | Echis ocellatus | TBP | 43 | 9.4 | 0.009 | 4.6 | 0.040 | 4.8 | 0.039 | 4.8 | 0.055 | 4.8 |
| 8                    | Aminopeptidase A | Gloydius brevicaudus | TBP | 110 | 7.0 | 0 | 2.1 | 0.004 | 2.5 | 0.007 | 2.5 | 0.029 | 7.6 |
| 20                   | Aminopeptidase N | Gloydius brevicaudus | TBP | 106 | 1.3 | - | - | - | - | - | 0 | 1.3 |
| 68                   | Glutaminyl-peptide cyclotransferases | Dabeia russellii | TBP | 42 | 37.8 | 0.109 | 37.8 | 0.092 | 29.1 | 0.055 | 32.1 | 0.085 | 37.8 |
| 111                 | Glutaminyl-cyclase | Orophea okinavensis | TBP | 40 | 33.2 | 0.012 | 33.2 | - | - | - | - | - |
| 120               | Cathepsin D | Ophiophagus hannah | TBP | 30 | 16.3 | 0.013 | 16.3 | - | - | - | - | - |
| 121            | Endoplasmic reticulum aminopeptidase 1 | Ophiophagus hannah | TBP | 91 | 1.6 | 0.003 | 1.6 | - | - | - | - | - |
| 123                | Renin | Ophiophagus hannah | TBP | 40 | 5.5 | 0.016 | 2.2 | 0.059 | 5.5 | 0.048 | 5.5 | 0.023 | 5.5 |
| 149               | Prostate-specific antigen-like 1 | Echis coloratus | TBP | 12 | 23.9 | - | - | - | - | 0.013 | 23.9 | - | - |
| 167              | Xaa-Pro aminopeptidase 2 | Crotalus adamanteus | TBP | 76 | 25.7 | 0.420 | 25.7 | 0.041 | 17.3 | 0.114 | 23.6 | 0.042 | 17.3 |
| 178             | Peptidyl-prolyl cis-trans isomerase | Bothrops jararacussu | TBP | 22 | 12.9 | 0.006 | 12.9 | - | - | - | - | - |
| 204           | Dipetidase 2-like | Bothrops jararacussu | TBP | 33 | 7.4 | 0.002 | 7.4 | - | - | - | 0.003 | 7.4 | 0.019 | 7.4 |
| 207               | Xaa-Pro aminopeptidase 2-like | Bothrops jararacussu | TBP | 27 | 19.4 | 0.057 | 19.4 | 0.011 | 11.3 | 0.016 | 19.4 | - | - |
| 60               | Snake venom vascular endothelial growth factor toxin vammin | Vipera ammodytes ammodytes | VEGF | 16 | 49.7 | 5.315 | 31.0 | 4.239 | 44.1 | 5.109 | 36.6 | 2.446 | 32.4 |
| 88               | Snake venom vascular endothelial growth factor toxin HF | Vipera aspis aspis | VEGF | 12 | 65.5 | 0.083 | 65.5 | 0.002 | 58.2 | 0.002 | 48.2 | - | - |
| 148           | Vascular endothelial growth factor A | Echis coloratus | VEGF | 22 | 34.4 | 0.017 | 34.4 | - | - | 0.004 | 17.2 | - | - |
| 150             | Vascular endothelial growth factor F | Echis coloratus | VEGF | 16 | 28.5 | - | - | 0.390 | 28.5 | 0.640 | 28.5 | 0.030 | 18.8 |

1 B-NAP: Bradykinin potentiating and C-type natriuretic peptides; BP: Blood protein; CP: Cysteine Proteases; CRISP: Cysteine-rich secretory protein; CTL: C-type lectin like; Dis: Disintegrin; Hya: Hyaluronidase; Kunitz: Kunitz type proteinase inhibitor; LAAO: l-amino acid oxidase; NGF: Nerve growth factor; Nuc: Nucleic acid degrading enzymes; OP: Other protein; PLA2: Phospholipase A2; PLB: Phospholipase B; SP: Serine proteinase; SVMP: Metalloproteinase; TBP: Toxin biosynthesis proteins (including aminopeptidases); VEGF: Vascular endothelial growth factor.
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Table 2. Protein families found in the venoms of Russian vipers.

| Protein Family | # of Identified Proteins | Protein Abundance \(^1\) LFQ/INT, % (# of Identified Proteins \(^2\)) | V. nikolskii | V. kaznakovi | V. orlovi | V. renardi |
|----------------|--------------------------|------------------------------------------------|--------------|-------------|-----------|-------------|
| PLA2           | (29)                     | 64.68/65.96 (14)                                | 0.00/0.00    | 41.03/36.43 | 24.21/27.27 | 44.05/47.64 |
| SVMP           | (32)                     | 0.66/0.66 (8)                                   | 16.15/16.31  | 14.77/13.92 | 11.98/10.53 | 9.52/8.36   |
| CTL            | (18)                     | 4.01/3.9 (9)                                    | 12.48/13.44  | 11.12/9.46  | 3.46/3.24   | 8.12/9.86   |
| SP             | (27)                     | 19.34/20.51 (20)                                | 10.79/10.96  | 23.97/22.61 | 7.87/6.03  | 10.56/11.56 |
| CRISP          | (7)                      | 0.66/0.41 (2)                                   | 9.72/10.89   | 12.2/12.3   | 7.98/8.26   | 7.98/8.26   |
| LAAO           | (11)                     | 0.08/0.07 (5)                                   | 3.99/4.33    | 4.59/4.19   | 4.21/3.56   | 8.12/9.86   |
| VEGF           | (4)                      | 7.57/5.42 (3)                                   | 3.96/4.63    | 4.2/5.76    | 2.92/2.48   | 2.92/2.48   |
| Dis            | (5)                      | 0.0/0.0                                         | 0.53/0.52    | 0.56/0.59   | 13.43/14.04 | 13.43/14.04 |
| OP             | (28)                     | 0.17/0.28 (11)                                  | 0.49/1.13    | 0.95/0.96   | 1.8/2.15    | 1.8/2.15    |
| PLB            | (4)                      | 0.12/0.1 (2)                                    | 0.32/0.34    | 0.52/0.5    | 0.54/0.51   | 0.54/0.51   |
| Nuc            | (7)                      | 0.88/0.92 (6)                                   | 0.21/0.6     | 2.12/1.73   | 0.47/0.32   | 0.47/0.32   |
| TBP            | (13)                     | 0.68/0.65 (11)                                  | 0.17/0.25    | 0.3/0.3   | 0.3/0.3   | 0.3/0.3   |
| NGF            | (2)                      | 0.33/0.28 (1)                                   | 0.14/0.12    | 0.25/0.25   | 0.12/0.09   | 0.12/0.09   |
| Hya            | (2)                      | 0.01/0.01 (2)                                   | 0.01/0.01    | 0.01/0.01   | 0.06/0.1   | 0.06/0.1   |
| BP             | (14)                     | 0.15/0.12 (12)                                  | 0.01/0.01    | 0.01/0.01   | 0.01/0.01   | 0.01/0.01   |
| B-NAP          | (1)                      | 0.01/0.01 (0)                                   | 0.03/0.03    | 0.01/0.01   | 0.0/0.0    | 0.0/0.0    |
| Kunitz         | (5)                      | 0.66/0.7 (4)                                    | 0/0/0        | 0.15/0.12   | 0.79/0.8   | 0.79/0.8   |
| CP             | (1)                      | 0/0/0                                           | 0/0/0        | 0/0/0       | 0/0/0       | 0/0/0       |
| total          | (210)                    | (111)                                           | (116)        | (135)       | (124)       | (124)       |

\(^1\) Protein abundance was calculated on the basis of peptide abundances for the peptides identified by MS/MS, as well as the peptides identified by MSI matching between chromatograms. Protein abundances were calculated either on the basis of the MaxLFQ (Label-Free Quantification) algorithm (LFQ) or on the basis of the comparison of total protein intensities (sums of peptide intensities were calculated for each protein) within a single venom (INT). \(^2\) Numbers of proteins were calculated on the basis of the peptides identified by MS/MS only (no MSI matching hits were used). Therefore the protein might not be listed as identified, but it would still be quantified by Matching with non-zero abundance value (e.g., B-NAP in V. nikolskii venom). \(^3\) B-NAP: Bradykinin potentiating and C-type natriuretic peptides; BP: Blood protein; CP: Cysteine Proteases; CRISP: Cysteine-rich secretory protein; CTL: C-type lectin like; Dis: Disintegrin; Hya: Hyaluronidase; Kunitz: Kunitz type proteinase inhibitor; LAAO: L-amino acid oxidase; NGF: Nerve growth factor; Nuc: Nucleic acid degrading enzymes; OP: Other protein; PLA2: Phospholipase A2; PLB: Phospholipase B; SP: Serine proteinase; SVMP: Metalloproteinase; TBP: Tissue biosynthesis proteins (including aminopeptidases); VEGF: Vascular endothelial growth factor.

**Figure 1.** The number of common proteins in four *Vipera* species studied. The number in bracket under each species name indicates the total number of proteins identified in this species venom.

2.2. Composition of Russian Viper Venoms

As a result of venom protein quantification, it was found that the main venom components were PLA2s; their content ranged from about 24% in *V. orlovi* venom to more than 60% in *V. nikolskii* (Table 2, Figure 2). The overwhelming majority of PLA2s belonged to D49 subgroup of group IIA as it might be expected for the snakes from Viperidae family. The venom of *V. nikolskii* contained PLA2s only from this group. One PLA2 of S49 subgroup was highly represented in *V. renardi*. One PLA2 of group IA
was observed in small amounts in three venoms and a low quantity of group IIE PLA2 was detected in *V. renardi* venom.

**Figure 2.** Relative abundance of venom proteins that were identified by LC MS/MS in Russian viper venoms. B-NAP: Bradykinin potentiating and C-type natriuretic peptides; BP: Blood protein; CRISP: Cysteine-rich secretory protein; CTL: C-type lectin like; Dis: Disintegrin; Hya: Hyaluronidase; Kunitz: Kunitz type proteinase inhibitor; LAAO: l-amino acid oxidase; NGF: Nerve growth factor; Nuc: Nucleic acid degrading enzymes; OP: Other protein; PLA2: Phospholipase A2; PLB: Phospholipase B; SP: Serine proteinase; SVMP: Metalloproteinase; TBP: Toxin biosynthesis proteins (including aminopeptidases); VEGF: Vascular endothelial growth factor.

Altogether, the enzyme content in venom of *V. nikolskii* reached about 85%, however this venom was characterized by a very low content of SVMPs (less than 1%) and LAAO (less than 0.1%). PLA2s accounted for more than 40% in *V. kaznakovi* and *V. renardi* venoms. While the content of SVMPs was less than 1% in the *V. nikolskii* venom, they comprised 12%–16% in *V. kaznakovi*, *V. orlovi* and *V. renardi*. The highest content of SPs was in *V. orlovi* venom (24%) and the lowest in *V. renardi* (8%). LAAO was at the level of 4%–5% in all the analyzed venoms with the exception of *V. nikolskii*. Nucleic acid degrading enzymes (Nuc) represented about 2% in *V. orlovi* venom and less that 1% in all the others. Phospholipase B (PLB) was found in all venoms (less than 1%) and very low amount of Hya (0.01%) was detected in three venoms. Among the non-enzymatic proteins, Dis (13%) in the *V. renardi* venom, CTL (12%) in *V. kaznakovi*, CRISPs (12%) in *V. orlovi* and vascular endothelial growth factors (VEGF, 8%) in *V. nikolskii* were the most abundant ones in the venoms studied. The total amount of non-enzymatic proteins was about 13% in the *V. nikolskii* venom and about 27%–28% in all the others. In addition to the proteins mentioned above, nerve growth factor (NGF) and Kunitz were present in all the venoms (less than 1%) with exception of *V. kaznakovi*, where Kunitz was absent.
Interestingly, comparison of both the nature of the identified proteins and their abundance showed very close venom compositions for the species \textit{V. kaznakovii} and \textit{V. orlovi}. The Pearson correlation coefficient for individual protein abundance LFQ was 0.83, while for the rest of pairs the correlation coefficient varied from 0.16 to 0.34 (Figure 3).

![Figure 3](image_url)

**Figure 3.** Protein number and abundance distributions for four \textit{Viper} species. (The panels under the diagonal showing the species names) Individual protein abundance label-free quantification (LFQ) pairwise comparison. Proteins unique for a single species in a pair are highlighted in the corresponding color and for better visualization in logarithmic scale are assigned 0.001% abundance instead of real 0%. (The diagrams above the diagonal showing the species names) Pairwise Venn diagrams showing the number of common and unique proteins for each pair of the venoms.

### 2.3. Identification of Endogenous Peptides in the Venoms

It is well known that snake venoms may contain various peptides: several peptide families including bradykinin-potentiating peptides, natriuretic peptides, sarafotoxin, \textit{etc.} were identified [17]. Moreover, the venoms studied in this work contain proteases, therefore their proteins may undergo proteolysis leading to generation of peptides. To study endogenously generated peptides in the venoms of interest, high molecular weight (MW) proteins were separated by ultrafiltration (10 KDa cut-off). The peptide fractions obtained were analyzed by LC-MS/MS in the same fashion as proteins, but without preliminary proteolysis. Peptides were searched at first against a full NCBI Serpentes database by Mascot search engine with 10% protein FDR (False Discovery Rate). A fusion database containing the peptidogenic proteins from Mascot search and the SwissProt Serpentes database was used for the final search in MaxQuant. Full NCBI database search with unspecific digestion failed in MaxQuant due to internal software limitations. In summary, 512 endogenous peptides from 80 proteins belonging to 13 protein families were found (Table S3). As expected, most of the peptides (462 peptides) belonged to proteins (48 proteins) which were earlier found in proteome, thus most likely representing venom protein degradation \textit{in vivo} as a result of proteases and peptidases activity. At the same time, 50 peptides (Table S4) belonged to 32 unique proteins from nine protein families (Table 3).
Among these, proteins in six families mostly had one peptide per protein, which can explain their identification only in the peptidome analysis as a result of a very low concentration of original proteins before degradation, so they were missed in the shotgun MS/MS selection in proteome analysis (or were beyond the taken FDR cut off). The largest number of unique peptides was found in proteins belonging to Dis and SVMP/Dis families: 25 peptides from 14 proteins were found. The peptides identified were mainly fragments of larger venom proteins. However, we found 12 peptides from proteins belonging to B-NAP family (Figure 4). These peptides may represent real endogenous peptides and possess their own biological activity. This is the first indication for the presence of bradykinin-potentiating and natriuretic peptides in venoms of vipers from the Pelias group.

| Bradykinin-potentiating peptides |
|----------------------------------|
| QQGLPQPLPEPPP Vn                  |
| QQGLPQPLPEPPP P85169 (BPDB_BOTJA) |
| MPKVPPPP Vn, Vo                   |
| P0DJK3 BPPAE_BOTCO                |
| MPKVPPPP Vn, Vo                   |
| QQPLPPPP Vn, Vo                   |
| QQPLPPPP Vn, Vo                   |

| Natuuretic peptides |
|---------------------|
| SGDIKX20CFWPEPIEIPQ CRIKX21 (VNPE_OXYSA) |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |
| CRX21 Vn, Vo, Ve     |

Figure 4. Bradykinin-potentiating and natriuretic peptides found in four viper venoms. P85169 (BPDB_BOTJA)—Bradykinin-potentiating peptide 13b from Bothrops jararaca, P0DJK3 (BPPAE_BOTCO)—Bradykinin-potentiating peptide 10e from Bothrops cotiara, Q90Y12 (BNP_CRODU)—Bradykinin potentiating and C-type natriuretic peptides from Crotalus durissus terrificus, P83231 (VNPE_OXYSA)—Natriuretic peptide TNP-c from Oxyuranus scutellatus canni (Papuan taipan), and P0DMD5 (VNPE_BUNMU)—amino acid sequence fragment 81–100 of Natriuretic peptide BM026 from Bungar us multicinctus. Vn, Vk, Vo, and Vr indicate V. nikolskii, V. kaznakovi, V. orlowi, and V. renardi, respectively.

Table 3. Snake venom protein families for which peptides were found in peptidome only.

| Family | Number of Proteins | Number of Peptides |
|--------|--------------------|--------------------|
| CTL    | 4                  | 5                  |
| Dis    | 3                  | 7                  |
| Kunitz | 2                  | 2                  |
| LAAO   | 2                  | 3                  |
| NAP    | 6                  | 14                 |
| PLA2   | 1                  | 1                  |
| SP     | 2                  | 2                  |
| SVMP   | 11                 | 22                 |
| VEGF   | 1                  | 1                  |

3. Discussion

We have analyzed venom proteomes and peptidomes for four species of Vipera, for which there is no genomic or transcriptomic data published. For each species, the venoms of at least 15 individual animals were pooled for the analysis. Protein identification for such “non-sequenced” species is
problematic for inherently database oriented bottom-up LC-MS/MS-based proteomics [18]. A possible solution is to use the protein sequences of closely related species, based on the assumption of their high homology level [18]. Thus, when the exact protein sequence is missing in the database, the protein might still be identified by partial/full homology with a known protein of another species. Here, we searched LC-MS/MS data against the database containing all the proteins from the taxon Serpentes in the NCBI database on the date of the experiment (the results are given in Table 1).

In the bottom-up proteomics, there are two major approaches for the quantitative analysis: (a) relative quantification of a single protein across samples; and (b) comparison of different proteins within a single sample. Principle (a) is based on the measurement of all the peptides belonging to a protein in several samples (and pair-wise peptide Fold Changes estimation) followed by protein Fold Change calculation as, e.g., mean or median value of the peptide fold changes. Principle (b) is based on the assumption that the sum of peptide peak areas (either all or just some of them, like in the top 3 theory [19,20]) for a given protein is proportional to its absolute abundance. Thus, comparison of these sums for two proteins is supposed to give the difference in their content within one sample. What is the most important, when making a comparison between several samples, the two approaches (a) and (b) are supposed to give consistent results.

In case of protein analysis of “non-sequenced” species, both these approaches encounter significant albeit different problems arising from the incomplete peptide identification due to the lack of adequate protein amino acid sequences in the search database. Principle (a) works best when as many as possible shared peptides per protein are identified and quantified for a pair of samples, since individual peptide measurements are prone to err due to possible post-translational modifications or isoforms. When it comes to different species, the number of shared peptides between samples goes down just because of different protein sequences. Besides, this approach works only when there are shared peptide sequences identified and quantified in both samples (recommended number of shared peptides for a reliable quantitation is 2). Thus, if a protein is unique for a sample, it cannot be quantified this way at all. Besides, it provides no data for concentration comparison between different proteins within a single sample.

Principle (b) was developed and verified for systems (artificial protein mixtures) where all the best flyer peptides for a protein (the peptides which have the best proportion between peptide concentration and intensity and thus have the maximum impact on the summed protein intensity) can be easily identified and quantified [19]. For “non-sequenced” species it would mess the final results through protein abundance underestimation if the missed peptides were among the best flyers for the given protein of some particular species but were overlooked because their amino acid sequence was missing in the database. For that, peptide MS/MS sequencing de novo might help a bit, but many peptides would still be missed for the reasons that are not clarified.

Here, we used two approaches to quantify proteins. First, we used MaxLFQ approach [21] which is basically principle (a), but it also uses absolute peptide intensities in addition to peptide FC comparison between samples (such results are labeled LFQ in Table 2). Second, we used direct comparison of sums of peptide intensities to make quantitation within each sample (such results are labeled INT in Table 2). The results of protein quantitation made by different methods gave quite similar results (Table 2, Table S1), especially when potential errors in individual protein contents were compensated by consolidation of proteins into families.

There is also a question of which types of peptides should be used for protein quantitation. Protein identification process deals not with separate proteins, but with protein groups, which are sets of individual proteins (at least partially homologous) sharing a set of identified peptide sequences. In the absence of unique specific peptides, no distinction between these proteins within a group can be made. A standard approach is to take as a hit the protein from a protein group which has a maximum number of assigned identified peptides. Thus, there are three types of peptides for a single protein group in the identification list: unique, razor and other (shared) peptides (MaxQuant terminology) [22,23]. Usually, protein groups have some unique peptides to pinpoint them as “correct” hits, but it is also possible
that the number of unique peptides for a protein is zero. Absence of unique peptides means that all the peptides from the current protein group are shared and can be just as successfully assigned to some other protein groups. In such situation, the final set of protein groups shown in the identification list is the minimal one sufficient to explain all the identified peptides (Occam’s razor principle). Shared peptides are named “razor” when they belong to the protein group with the maximum total number of peptides among other possible protein groups. These razor peptides are used for quantitation (along with unique peptides), both LFQ and intensity based [24]. A shared peptide, which is “razor” for some particular group, is counted in “all peptides” in all the protein groups to which it can be potentially assigned, and “all peptides” list is used to calculate Sequence Coverage.

Importantly, any analytical method may prove only that the amount of the compound under investigation is below the method sensitivity, rather than show the absolute absence of the compound in the sample. This is specifically applicable for the LC-MS/MS-based shotgun identification principle which selects peptide ions pseudo-randomly, sometimes missing the peptides with very low intensities just because of a wrong choice. Thus, quantitation is much more reliable for showing the absence of the compound (or, more accurate, the concentration being lower than its Low Limit of Detection). MaxQuant features chromatogram alignment and the possibility to quantify peptides on the basis of similarity of their retention time and \( m/z \) in the sample, where they were identified by MS/MS and in another sample where this particular \( m/z \) signal got lost during the shot-gun selection for the MS/MS analysis (proteins with such peptides are marked “By matching” in “Identity Type” column in Tables S1 and S2). In this work the protein is considered to be identified (and considered as present) in the sample only if it has an MS/MS spectrum identified in this particular sample. However, for quantitation both MS/MS identified peptides and the peptides identified on the basis of the above described similarity were used. This might lead to apparent contradictions when there are no proteins identified, but the protein abundance is non-zero (like natriuretic peptides (B-NAP) in the \( V. nikolskii \) venom—0.01/0.01 (0) in Table 2).

At the present time, the genus \( Vipera \) includes 22 species, however it is not homogenous. Molecular phylogeny studies showed that this genus comprises the \( V. aspis \) group, the \( V. ammodytes \) complex, and the \( Pelias \) group as separate clades [25]. Of these clades, only snakes from the \( Pelias \) group inhabits Russia. The \( Pelias \) was further classified into two subgroups, one comprising \( V. dinniki \), \( V. kasnakovi \), and \( V. ursinii \), and another including \( V. berus \), \( V. barani \), \( V. nikolskii \), and \( V. seoanei \) [25]. The first subgroup was further subdivided into the “\( kaznakovi\)” complex, including \( V. kasnakovi \), \( V. orlovi \) and some other closely related species, and the “\( ursinii\)” complex, in which \( V. renardi \) was included [26,27]. Earlier, for the vipers of the \( Pelias \) group, we have studied the venom toxicity towards crickets Gryllus assimilis [12] and found that it differed depending on feeding preferences. The snakes from the \( V. renardi \), \( V. lotievi \), \( V. kaznakovi \), and \( V. orlovi \) species feed on a wide range of animals including insects, whereas the snakes from \( V. berus \) and \( V. nikolskii \) species do not include insects in their diet. The venom from vipers which hunt insects was found to possess a greater toxicity towards crickets. This suggests that the venom composition may greatly differ among these species. As concerns the toxicity to other animals, it was shown that the venom of \( V. nikolskii \) was more toxic than that of \( V. berus \) to frogs (9–11 \( \mu g/g \) vs. 30–52 \( \mu g/g \) and mice (0.93 vs 1.58 \( \mu g/g \)) at intraperitoneal injection [28]. The venom of \( V. renardi \) was less toxic to mice (2.96 \( \mu g/g \)) than that of \( V. berus \) [28]. We were not able to find any data about toxicity of \( V. orlovi \) and \( V. kaznakovi \) venoms.

Regarding the danger to humans, the data about bites by these snakes are sparse. Most of the documented cases refer to steppe viper \( V. renardi \) and report that it usually has calm and timid behavior, is reluctant to bite, and seeks to escape. This viper bites only when it is in danger, for example, if the snake is suddenly stepped on or picked up. \( V. renardi \) is considered less dangerous to humans than common adder. The human fatalities as a consequence of steppe viper bites are not reliably known [29], though there are some cases of the death of horses and small ruminants. A picture of human envenomation is characterized mainly by local signs which include severe pain at the site of
the bite, redness, swelling that spreads far beyond the site of the biting. In severe cases, drowsiness, dizziness, nausea, increase of heart rate, and reduction in body temperature may be observed [30].

Records of the bites of humans by the Caucasian viper V. kaznakovi and Nikolsky’s viper V. nikolskii are practically absent. However, V. kaznakovi may be dangerous. Solitary human deaths and livestock losses after Caucasian viper bites were mentioned [30]. We were able to find only one report about human fatalities after the Nikolsky’s viper bites [31]. No information on the V. orlovi bites is available.

It should be noted that the venoms of not all Pelias species were studied equally well. The venom of V. berus is the best characterized. As mentioned earlier, the V. berus venom displayed in vitro proteolytic, fibrinolytic, anticoagulant, and phospholipolytic activities. In mice, significant local tissue-damaging effects, including edema, hemorrhage and myonecrosis were observed for this venom [3]. Several proteins involved in manifestation of those effects were isolated from V. berus venom. These proteins included basic PLA2 [4], SVMP [5], LAAO [6] and some others.

The V. nikolskii species is phylogenetically very close to V. berus and is included in the same subgroup within the Pelias group. It is regarded as a V. berus subspecies in some publications. However, the analysis of the V. nikolskii venom has shown it to differ greatly from that of V. berus. Thus, two heterodimeric PLA2s were isolated from the V. nikolskii venom [10], but similar proteins are absent in V. berus. The data obtained in the present work are in good agreement with the published results; the basic and acidic PLA2 subunits forming heterodimeric enzymes account for more than 50% of the V. nikolskii venom (Table 1). Earlier, cDNA encoding SP nikobin and Kunitz type inhibitor in the V. nikolskii venom gland was cloned and sequenced [11]. In this study we have found that nikobin is the main SP in the V. nikolskii venom (more than 12% of the total protein content, Table 1) and Kunitz-type serine protease inhibitor ki-VN was also the main protein of the Kunitz family in this venom (about 0.66%, Table 1). CRISP, the sequence of which was also deduced from cDNA analysis [32], was found in the venom in fairly low amount (0.66%, Figure 2). Interestingly, the content of CRISPs was much higher in other venoms studied and accounted for 8%, 10% and 12% in V. renardi, V. kaznakovi and V. orlovi venoms, respectively (Figure 2).

The steppe viper V. renardi is included in the “ursinii” complex [33] while the other two vipers, V. kaznakovi and V. orlovi, belong to the “kaznakovi” complex. Among these vipers only the composition of the V. renardi venom was in some way studied [7]. The amino acid sequences for several PLA2s and Kunitz-type inhibitor were deduced from the cloned cDNA of venom gland. Some PLA2s and Kunitz protein were isolated from the venom. The most abundant was ammodytin I2d analogue. In this work we have found all the PLA2s described by Tsai et al. [7] in the V. renardi venom, Vur-PL2 having the highest content (Table 1). Interestingly, this viper venom has very high content of disintegrins which accounts for about 13% of total protein, while the V. kaznakovi and V. orlovi venoms contain less than 1% and in the V. nikolskii venom no disintegrins were detected.

There are no published data on the composition of the V. kaznakovi and V. orlovi venoms and they are characterized for the first time in this work. These two venoms have the highest similarity among the four ones studied (Figure 3) that confirms the inclusion of V. orlovi in the “kaznakovi” complex. They have a fairly high content of SVMPs (15%–16%), CTL (11%–12%) and CRISPs (11%–12%) (Figure 3). The V. orlovi venom has the highest amount of SP (24%) among the four venoms studied (Figure 3) and only V. kaznakovi contains a small quantity of hyaluronidase (Hya) at the level of 0.01%. However no Kunitz type proteins were detected in the latter venom.

Although a limited number of B-NAP proteins (one in V. kaznakovi and one in V. orlovi, Table 2) were detected in the proteome analysis, several peptides derived from proteins of this family were found in the peptidomes of all the venoms studied (Figure 4). The mature bradykinin-potentiating peptide QGGLPRPGPEIPP was observed in the V. nikolskii venom and several fragments of similar peptides were detected in the other analyzed venoms. Several fragments of C-type natriuretic peptides were found in all four venoms as well (Figure 4). It should be noted that no bradykinin-potentiating and C-type natriuretic peptides from the vipers of Pelias group were reported so far.
In total, 210 proteins (Table 1) and 512 endogenous peptides (Table S3) were identified in four viper venoms. The overwhelming majority of the proteins (98%–99% of the total protein content) and the peptides represented 14 snake venom protein families (Table 2). The comparison of our results with those for other snakes of the Vipera genus shows higher representation of venom protein families in our data (Table 4). For example, while Nuc and PLB were found in all venoms studied in this work, no proteins of these families were reported for other venoms from the Vipera species (Table 4).

### Table 4. Snake venom protein families represented in viper venoms.

| Snake Venom Protein Family | V. kaznakovi Venom | V. renardi Venom | V. orlovi Venom | V. nikolskii Venom | V. anatolica Venom | V. raddei Venom | V. a. ammodytes Venom | V. a. meridionalis Venom |
|---------------------------|-------------------|-----------------|----------------|-------------------|-----------------|----------------|----------------------|------------------------|
| PLA2                      | +                 | +               | +              | +                 | +               | +              | +                    | +                     |
| SP                        | +                 | +               | +              | +                 | +               | +              | +                    | +3                    |
| Dis                      | +                 | +               | +              | −                 | +               | +              | +                    | +3                    |
| CRISP                    | +                 | +               | +              | −                 | +               | +              | +                    | −                     |
| Kunitz                   | −                 | +               | +              | +                 | +               | −              | −                    | +3                    |
| LAAO                     | +                 | +               | +              | +                 | +               | +              | +                    | +3                    |
| SVMP                     | +                 | +               | +              | +                 | +               | +              | +                    | +3                    |
| NGF                      | +                 | +               | +              | −                 | +               | +              | +                    | +3                    |
| CTL                      | +                 | +               | +              | +                 | +               | −              | −                    | −                     |
| PLB                      | +                 | +               | +              | +                 | +               | +              | +                    | +3                    |
| VEGF                     | +                 | +               | +              | −                 | +               | +              | +                    | +3                    |
| Nuc                      | +                 | +               | +              | −                 | −               | +              | +                    | +3                    |
| RNAP                     | +                 | +               | +              | −                 | −               | −              | −                    | −                     |
| Hya                      | +                 | +               | +              | −                 | −               | −              | −                    | −                     |

1 Taken from [13]; 2 Taken from [14]; 3 Taken from [15]; 4 Taken from [34].

Hya was observed in three of the studied venoms and this is also the first indication for the presence of this enzyme in the venoms of the Vipera species. We have found that the main components of all venom studied are PLA2s, while SVMPs were prevailing in venoms of V. anatolica [13] and V. raddei [14].

### 4. Conclusions

In this work, quantitative proteomic and peptidomic characterization of venoms from four vipers inhabiting Russia was done; the compositions of the venoms from V. kaznakovi and V. orlovi, which showed the highest similarity among the four studied species, were analyzed for the first time.

More than 200 proteins and over 500 peptides were detected in total in all four venoms. They represented 14 snake venom protein families. In all venoms studied, over 70% of the total proteins were enzymes, the highest enzyme content (85.7%) being in the V. nikolskii venom. The main components of the venoms were PLA2s, which accounted for 65% of total protein content in the V. nikolskii venom. For the first time, bradykinin-potentiating and C-type natriuretic peptides were reported for vipers of the Pelias group. Nucleic acid degrading enzymes and phospholipase B were found in the venoms of Vipera species for the first time.

Due to the low toxicity of the steppe viper, or a limited habitat of the Caucasian and Orlov’s vipers, these snakes do not pose an epidemiological threat to Russian population. However, the envenomation by Nikolsky’s viper, the venom of which was shown in this study to contain a considerable amount of neurotoxic phospholipase A2, may represent certain danger. An antiserum “Antigadyuka” (“Antiviper”) produced by Russian company “Allergen” is based on the venom of the common viper and may not be effective against the Nikolsky’s viper bites due to strong differences in the composition of the venoms. The need to consider the differences in the composition of the venoms in the antivenom production is discussed in recent publications [28,35] and should be taken into account by antiserum manufacturers.
5. Materials and Methods

The venoms of *V. kaznakovi*, *V. nikolskii*, *V. orlovi* and *V. renardi* vipers were obtained as described earlier [12]. The venoms from several individual animals were pooled as described in [12]. Snakes were captured in their natural habitat: *V. kaznakovi* in Krasnodar Territory near Adler, *V. nikolskii* in Penza region near Zubrilovo village, *V. orlovi* in Krasnodar Territory at Mikhaylovskiy mountain pass and *V. renardi* in Krasnodar Territory near Beysugskiy firth.

5.1. In-Solution Trypsin Digestion of Venom Samples

Lyophilized venom sample (100 µg each) was dissolved in 10 µL of a buffer containing 100 mM ammonium bicarbonate (ABC), 5% sodium deoxycholate (SDC) and 5 mM dithiothreitol (DTT) and incubated for 40 min at 60 °C to reduce cysteine residues. Then, 5 µL of 50 mM iodoacetamide (IAA) water solution was added and the mixture was incubated 30 min at RT, in the dark. Residual IAA was neutralized by 5 µL of 50 mM DTT and sample was diluted with 50 µL 50 mM ABC and trypsin was added in a 1:100 (enzyme/protein) ratio to the final volume 100 µL and the protein concentration ~1 mg/mL. Samples were incubated overnight at 37 °C. Trypsin was deactivated by addition of 5 µL of 10% TFA. Tryptic peptides were desalted using reverse-phase solid extraction cartridges Discovery DSC-18 (100 mg) (Supelco, Bellefonte, PA, USA) according to the manufacturer protocol. Final peptide solution was dried in vacuum and stored at −80 °C prior to LC-MS/MS analysis.

5.2. Endogenous Venom Peptides Isolation

Endogenous peptides from venom samples were isolated using C_{18} StageTips [36]. To make StageTips, two pieces of C_{18} Empore extraction disk were cut using blunt-ended 16-gauge needle and packed into a P200 pipette tip. Membranes were conditioned by 20 µL of methanol and equilibrated by 20 µL of 0.1% aqua TFA. Venom solutions were applied onto the conditioned tips, followed by membrane washing with 20 µL of 0.1% aqua TFA. Peptides were eluted by 20 µL of 80% ACN, 0.1% TFA. Eluates were dried in vacuum and stored at −80 °C prior to LC-MS/MS analysis.

5.3. LC-MS/MS Analysis

Analysis was performed on the QExactive HF mass-spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to the Dionex 3000 RSLCnano HPLC system (Thermo Scientific, Waltham, MA, USA). The HPLC system was configured in a trap-elute mode. An analytical column (75 µm × 150 mm) and a precolumn (100 µm × 10 mm) were in-house packed with Aeris Peptide C{18} 2.6 µm sorbent (Phenomenex, Torrance, CA, USA). Samples were loaded on the precolumn for 10 min at 3 mL/min with buffer A (3% AcN, 96.9% H_{2}O, 0.1% FA), followed by separation at 300 nL/min with the 4%-55% gradient of buffer B (80% AcN, 19.9% H_{2}O, 0.1% FA).

Mass-spectrometer experiment consisted of one full survey MS1 scan followed by 20 dependent MS2 scans for the most intense ions. MS1 spectra were acquired in the profile mode in mass range 350–1400 m/z, maximum IT time 100 ms, AGC target 3e6, resolution 60000. Dependent MS2 scan were performed at resolution 15000 for 200–2000 m/z mass range, AGC target 1e5, maximum IT 25 ms, isolation window 1.4 m/z. Dynamic exclusion was set to 30 s.

5.4. LC-MS/MS Data Analysis

Data analysis was performed in the MaxQuant software (V. 1.5.3.30, Max Planck Institute of Biochemistry, Martinsried, Germany, 2016). Proteomic LC-MS/MS data was searched with the Andromeda search engine incorporated in the MaxQuant software against NCBI Serpentes DataBase exported from the NCBI web-site [37] for the Taxon Serpentes 2015/11/17 and containing 134677 entries with the following parameters: digestion Trypsin/P; max number of miscleavages 2; include contaminants; fixed modification: carbamidomethyl (Cys); variable modifications: Oxidation (Met), Acetylation (N-term), Deamidation (Asn, Gln); min peptide length 6; max peptide MW 5500; PSM
FDR 0.01; protein FDR 0.05; decoy mode: revert; min number of peptides for identification 1; razor protein FDR; second peptide; match between runs; LFQ quantitation with minimum 2 peptide pairs; and stabilize large LFQ ratios. Full set of MaxQuant parameters for the analysis can be found in the Supplementary Data file mqpar_proteins.xml.

Peptidomic LC-MS/MS data were searched with the Mascot search engine against the same full NCBI Serpentes data base with the following parameters: MS tolerance 5 ppm; MS/MS tolerance 0.01 Da; charge: +1, +2, +3; fixed modification: carabamidomethyl (Cys); variable modifications: Oxidation (Met), Deamidation (Asn, Gln); enzyme none. Mascot results were reprocessed in the Scaffold software and identified peptidogenic proteins (protein FDR 10%) for all four venoms were added to the SwissProt Serpentes database exported from the NCBI web-site on 2015/11/30 and contains 2567 sequences to generate a fused database. This database was used for Andromeda search in MaxQuant software with the digestion parameter set to unpecific. Peptide length for unespecific digestion search was from 6 to 50 amino acids. The rest of the parameters were the same as for proteome data analysis, however in the peptidogenic protein features in the in the “Number of Unique and Razor Peptides (NoURP)” column, the number of peptides corresponds to that before PEP-based filtration.

Results were processed in the Perseus (V. 1.5.2.6, Max Planck Institute of Biochemistry, Martinsried, Germany, 2016) and Excel software (V. 12.06743.5000, Microsoft Corporation, Redmond, WA, USA, 2007) and with the use of R.

Supplementary Materials: The following materials are available online at www.mdpi.com/2072-6651/8/4/105/s1, Table S1: The detailed list of proteins identified in Russian viper venoms; Table S2: The detailed peptide identification list for proteome results (contains all peptide-related data from MaxQuant proteome analysis); Table S3: Endogenous peptides found in four viper venoms: “endogPeptidesWithProteins”—combined peptide and protein data, “endogenousPeptides”—peptide only data, “endogenousPeptidogenicProteins”—protein only data; Table S4: Endogenous peptides identified in proteins unique for peptidome analysis.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

| Abbreviation | Description |
|--------------|-------------|
| FC           | Fold Changes |
| FDR          | False Discovery Rate |
| B-NAP        | Bradykinin potentiating and C-type natriuretic peptides |
| BP           | Blood protein |
| CP           | Cysteine Proteases |
| CRISP        | Cysteine-rich secretory protein |
| CTL          | C-type lectin like |
| Dis          | Disintegrin |
| Hya          | Hyaluronidase |
| Kunitz       | Kunitz type proteinase inhibitor |
| LFQ          | label-free quantification |
| LAAO         | L-amino acid oxidase |
| NGF          | Nerve growth factor |
| Nuc          | Nucleic acid degrading enzymes |
| OP           | Other protein |
| PLA2         | Phospholipase A2 |
PLB  Phospholipase B
SP   Serine proteinase
SVMP Snake venom metalloproteinase
TBP  Toxin biosynthesis proteins (including aminopeptidases)
VEGF Vascular endothelial growth factor

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