Phylogeny and systematics of adders (*Vipera berus* complex)

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Zusammenfassung

*Phylogenie und Systematik der Kreuzottern (*Vipera berus-*Komplex).*

Die Einbeziehung molekularer Merkmale hat es in den letzten Jahren ermöglicht, Licht in die ungeklärte Phylogenie der Viperinae zu bringen. Die phylogeografischen Beziehungen innerhalb des *Vipera berus*-Komplexes wurden mit Hilfe mitochondrialer Gensequenzen (Cytochrom b, ribosomale 12S- und 16S-Sequenzen) analysiert. *V. berus* bildet gemeinsam mit *V. nikolskii* und *V. barani* sowie *V. pontica* aus der Schwarzmeerregion eine monophyletische Gruppe, deren Schwesterart *V. seoanei* aus den Pyrenäen darstellt. Die untersuchten Genmerkmale stimmen weiterhin darin überein, dass fünf eigenständige historisch-geografische Evolutionslinien abgrenzbar sind:

1. die nord-mitteleuropäischen und asiatischen Kreuzottern (einschließlich *V. b. sachalinensis* und einigen als *V. nikolskii* bestimmten Populationen);
2. die alpinen Kreuzottern (genaue geografische Abgrenzung noch zu ziehen);
3. die Balkankreuzotter (*V. b. bosniensis*);
4. „*V. nikolskii*" der Wolga-Region (auch hier geografische Abgrenzung noch unklar);
5. *V. barani* einschließlich „*V. pontica*".

Es ist noch zu früh, aus dieser phylogeografischen Aufspaltung taxonomische Konsequenzen zu ziehen. Morphologische Daten sprechen dafür, dass zwischen benachbarten Populationen der mitochondrialen Haplotypengruppen noch relativ rezent Genfluss stattfand (außer bei der geografisch isolierten *V. barani*). Alle fünf Populationsgruppen (einschließlich der bisher zur Nominat-Unterart *V. b. berus* gezählten alpinen Gruppe) verdienen allerdings zumindest Unterartrang.

Schlüsselwörter: *Vipera berus*, *Vipera barani*, *Vipera nikolskii*; mitochondriale DNA; Phylogenie; Taxonomie; Phylogeographie.

Abstract

Consideration of molecular characters has shed light on the hitherto unresolved phylogeny of viperine snakes. The phylogeographical relationships within the *Vipera berus* complex were analysed with mitochondrial gene sequences (cytochrome b, 12S and 16S RNA). *V. berus*, *V. barani*, *V. pontica* and *V. nikolskii*, together represent a monophyletic group, with *V. seoanei* from the Pyrenees as sister species. The analysed genes consistently delimit five historically and geographically separate evolutionary clades:

1. North-Central European and Asiatic adders (including *V. b. sachalinensis* and several populations which had been determined as *V. nikolskii*);
2. Alpine adders (exact geographical delimitation not yet certain);
3. Balkan adders (*V. b. bosniensis*);
4. "*V. nikolskii*" from the Volga region (geographical delimitation uncertain, too);
5. *V. barani*, including "*V. pontica*".

It would be premature to draw taxonomical consequences from this phylogeographical differentiation. Morphological data argue for a relatively recent gene flow between neighbouring populations of separate mitochondrial haplotype groups (except for the geographically isolated *V. barani*). However all five groups (among them the Alpine group which has been included in the nominate subspecies *V. b. berus* so far) deserve at least subspecies status.

Keywords: *Vipera berus*, *Vipera barani*, *Vipera nikolskii*; mitochondrial DNA; phylogeny; taxonomy; phylogeography.

1 Introduction

CAROLUS LINNAEUS, in his typological species conception, not only described *Coluber berus* L., 1758, but at the same time a reddish "species", *Coluber chersea* L., 1758 and little later a black variant, *Coluber prester* L., 1761. The last name survived in the literature as subspecies of *V. berus* until MERTENS & WERMUTH (1960) restricted its type locality, like that of the other two, to Sweden and made clear that *chersea* and *prester* were synonyms of *berus*. Also many other names, including a large number of adder forms described by REUSS in the twenties and thirties of the 20th century were just synonyms without deserving a nomenclatural status. The only three subspecies then remaining were *V. berus bosniensis* BOETTGER, 1889, from the Balkans, *V. b. sachalinensis* ZAREVSKY, 1917, from the Far East of Asia, and *V. b. seoanei* LATASTE, 1879, from the Pyrenees. Since then, the taxonomic situation at the southern margin of the distribution range of adders has been the focus of attention. *V. berus ornata* BASOGLU, 1947, from northeastern Turkey was synonymized with *V. kaznakovi* NIKOLSKY, 1916 (MERTENS & WERMUTH 1960). *V. berus dinniki* NIKOLSKY, 1913 was first synonymized with *V. kaznakovi*, too (MERTENS & WERMUTH 1960) and then recognized as a different species (VEDMEDERJA et al., 1986). SAINT GIRONS & DUGUY (1976) made clear that *V. seoanei* is a separate species. In 1983, a black adder was discovered in northern Turkey and described as *Vipera barani* BÖHME & JOGER, 1983. Another black *prester*-like viper was *Vipera nikolskii* VEDMEDERJA, GRUBANT & RUDAEVA, 1986, from Ukraine and southern Russia. Finally in northeastern Turkey, an adder with an *aspis*-like pattern was described as *Vipera pontica* BILLING, NILSON & SATTLER, 1990. Immunological distances between plasma albumins (HERRMANN et al. 1987, 1992a) showed that *berus* is clearly different from *seoanei*, whereas *sachalinensis* appeared nearly identical to *berus*.

JOGER et al. (1997), after having received additional specimens of *Vipera barani*, some of them black, but one having an "*aspis*" pattern, made a first DNA study using a part of the cytochrome *b* gene, and found *nikolskii* nearly identical with *berus*, whereas *barani* was clearly different and seemed more similar to *bosniensis*. This was reinforced by hemipenis comparisons. FRANZEN & HECKES (2000) recorded more specimens of *V. barani*. Finally BARAN et al. (2001) argued on the basis of additional specimens (one of them close to the type locality of *V.* "*pontica*"), that *pontica* should be regarded a synonym of *barani*.

The genus name *Vipera* had been introduced by LAURENTI (1768) for his *Vipera franciscireди* – a synonym of *Coluber aspis* LINNAEUS, 1758. SAINT-GIRONS (1978) and OBST (1983) reduced its usage to the "small" European *Vipera* species (*Vipera* s. str) while subsumizing the "large" "Oriental" *Vipera* species under the name *Daboia*. HERRMANN et al. (1992b) put the *"Daboia" xanthina* group back into *Vipera* while resurrecting the genus *Macrovipera* for the *"Daboia" lebetina* group. Recently the *xanthina* group was declared a subgenus *Montivipera* (NILSON et al., 1999). ZEROVA (1992) resurrected the name *Pelias* MERREM, 1820 for the *Vipera berus*, *V. kaznakovi* and *V. ursinii* groups. This was followed by NILSON & ANDRÉN (1987). Most authors retain *berus* in the genus *Vipera*, treating *Pelias* as a subgenus at most. Molecular data (HERRMANN et al. 1992a, HERRMANN & JOGER 1997, JOGER et al. 1997) however made clear that the *kaznakovi* and *ursinii* species groups are separate from the *berus* group.

Regarding the general phylogenetic position of *Vipera* within the Viperidae, HERRMANN et al. (1999) and LENK et al. (2001) produced a series of molecular trees which showed that *Vipera* s. l. is part of a monophyletic Eurasian group of vipers which also includes *Eristicophis* and *Pseudocerastes*. The anatomically based phylogenetic hypothesis of GROOMBRIDGE (1986) is thus partly confirmed whereas the alternative

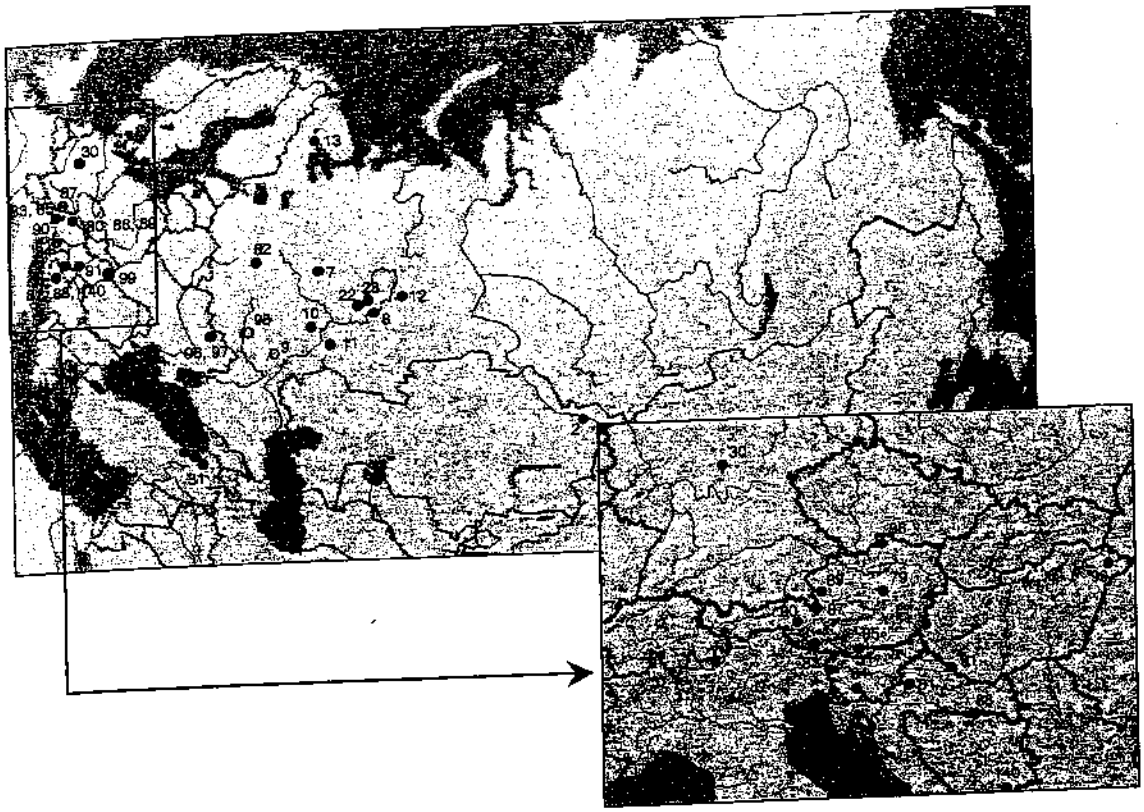

Fig. 1. Sampled localities of *V. berus* group. Colour code: haplotype groups.
Herkunft der untersuchten Proben. Farben bezeichnen Haplotypengruppen.

hypothesis of ASHE & MARX (1988), assuming polyphyly of both *Vipera* s. l. and *Vipera* s. str., is rejected. However GROOMBRIDGE assumed a paraphyletic *Vipera* s. str. whereas LENK et al. could prove that it is one of three monophyletic groups within *Vipera* s. l. if *Montivipera* is made a subgenus of *Macrovipera*.

The systematic position of the adders can currently be described as follows:

"*Vipera* s. l.": Genera *Daboia*, *Macrovipera*, *Vipera* s. str.

Genus *Vipera:* Subgenera *Vipera* (*aspis-ammodytes* group), *Pelias* (*berus* group = "adders", *ursinii* group, *kaznakovi* group),

Vipera (Pelias) berus group: *V.* (*P.*) *barani* (incl. synonym or subspecies *pontica*), *V.* (*P.*) *berus* (incl. subspecies *bosniensis*, *sachalinensis* and possibly *nikolskii*), *V.* (*P.*) *seoanei*.

This study focuses on the phylogeographical differentiation of the *Vipera berus* group only.

2 Materials and Methods

Materials

Figure 1 shows the geographic origin of the samples used for this study.

Vipera ammodytes, *V. ursinii*, *V. darevskii* and *V. kaznakovi* were used as outgroup taxa.

Laboratory procedures

As a source of DNA we used alcohol preserved tissue samples, some from museum specimens, or we used whole blood drawn from live animals and stored in 70-90 % ethanol or in an EDTA buffer (ARCTANDER 1988) until needed.

We used a standard method for obtaining total genomic DNA (SAMBROOK et al. 1989). Tissues were digested with proteinase K dissolved in lysis buffer (Tris HCl 100 mM at pH 8.0, EDTA 50 mM at pH 8.0, NaCl 10 mM, SDS 0.5 %) to a concentration of 60 µg/ml. Digestion was carried out for several hours at 50-65 °C with constant motion. Digestion was followed by extraction twice with phenol/$CHCl_3$ then once with $CHCl_3$ alone, alternatively, extraction with guanidine isothiocyanate. In either case, extraction was followed by precipitation of the DNA with two volumes of ice-cold 100 % ethanol. The precipitated DNA was washed with 80 % ethanol, dried and redissolved in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) to give a stock solution.

Template DNA for the polymerase chain reaction (PCR) was prepared by diluting the stock DNA with TE buffer to give a spectrophotometric absorbance reading of between 0.2 and 0.7 at A260. Amplification of target DNA was carried out in 100 µl reactions. Primers for amplification and sequencing, as well as conditions for the PCR were as described in LENK et al. (2001). Following clean-up of PCR products using the Promega Wizard® PCR Preps Purification System (Promega, Madison, WI, USA) according to manufacturer's instructions, cycle sequencing was performed and sequences were determined using either the ABI Prism Genetic Analyzer model 3100 (Applied Biosystems, Foster City, USA) or the ALFExpress II (Amersham Pharmacia Biotech, Uppsala, Sweden) automatic sequencer.

Phylogenetic analyses

Because there were no indels in the nucleotide sequences of the mitochondrial protein coding genes, alignment by eye was simple and was accomplished by using either of the computer programs ESEE (CABOT & BECKENBRACK 1989) or Sequencher 4.0 (Gene Codes Corporation, Inc., Ann Arbor, Michigan, USA). To infer phylogenies we used the methods of maximum parsimony (MP), and neighbor joining (NJ); these analyses were carried out using PAUP* 4.0b10. Maximum parsimony analyses were conducted with heuristic searches, tree-bisecting-reconnection (TBR) using a branch swapping algorithm and simple stepwise addition. In performing MP analyses we used equal weighting for all characters as well as with codon third positions removed. Although it is common practice to downweight codon third positions and in particular to weight differentially transitions and transversions (transversion parsimony), it seemed not necessary in this study where maximum nucleotide differences of 7 % occurred. Moreover a recent study showed that characters that have experienced higher rates of evolution relative to others have not necessarily produced greater levels of homoplasy (NAYLOR et al. 1995). Other studies have shown that downweighting of third codon positions in mitochondrial protein coding genes has a negative effect on the outcome of phylogenetic analyses (ALLARD & CARPENTER 1996, PHILIPPS et al. 1996, MILINKOVITCH & LYONS-WEILER 1998, BJÖRKLUND 1999, SAVOLAINEN et al. 2000, BAKER et al. 2001). In choosing to emphasize equal weighting for all nucleotide sites we are making the assumption that at the taxonomic level of our ingroup, the value of increased resolution at subterminal nodes provided by codon third positions will outweigh possible perturbations at deeper nodes.

Support for clades was assessed by bootstrapping (FELSENSTEIN 1985). In this study bootstrap values refer to 1000 replicates. In our interpretation of bootstrap values we follow FELSENSTEIN & KISHINO (1993). Accordingly, we define weak, moderate and strong support as 50-69 %, 70-89 % and 90-100 % respectively for all bootstrap values.

3 Results

We sequenced 924 base pairs (bp) of cytochrome *b* and 850 bp of alignable 12s and 16s ribosomal DNAs (combined). It was decided to produce two separate phylogenetic trees in order to facilitate comparison and to detect artefacts more easily (Fig. 2 and 3). Identical sequences were pooled; so the trees show each detected haplotype only once. *V. seoanei* was the sister species to all other members of the *berus* group (*Vipera berus* s.l.) in all bootstrapped calculations. Among cytochrome *b* sequences, 21 different haplotypes were found in *V. berus* s. l., in the rDNAs these were only 12. However both analyses detected an identical number of five haplotype groups, each of which contained the same individuals in both data sets. These five groups were:

1. *V. b. berus*, including European samples from north of the Alps, Hungary, Ukraine, Kasakhstan and Russia (several of them had been determined as *V. nikolskii*) and *V. b. sachalinensis*;
2. *V. b. bosniensis* from different Balkanian localities;
3. A subsample from the Russian range of "*V. nikolskii*".
4. *V. barani* including a specimen initially determined as *V. pontica*;
5. Alpine populations of *V. b. berus* (Berchtesgaden/Germany, several Austrian localities and the only Slovenian sample).

The nucleotide difference between those five groups amounted to 15-20 bases (1.6-2.2 %) in cytochrome *b*. The only significant association between any two groups was between *barani* and "*nikolskii*". The Russian *b. berus* appeared to be slightly different and more diverse then the central European *b. berus*. The individual from eastern Hungary had a haplotype of its own.

4 Discussion

The haplotype groups that could be identified denominate geographical and most probably evolutionary groups. Two of them, *bosniensis* and *barani*, are morphologically different from *berus* and have been suspected to represent different species. Another presumed species, *V. nikolskii*, is poorly defined morphologically and can easily be confounded with black *berus* (ZINENKO 2004). The identity of most "*nikolskii*", including specimens from north of the published range of *nikolskii* or at its presumed northern boundary (VEDMEDERJA et al. 1986), but also from the type locality, Kharkiv (Ukraine), with Russian *berus* confirms earlier results (JOGER et al. 1997). However, the discovery of a locally distributed unique haplotype in the Russian range of *nikolskii* in southern Russia (haplotype group 3) requires further study. The two specimens do not differ substantially from other "*nikolskii*" in their morphology. However *nikolskii* is generally difficult to diagnose. An interpretation is premature, but possible explanations could be:

- introgression of *berus* haplotypes into the range of *nikolskii*; the Russian *nikolskii* would thus be relicts of a once wider distributed taxon.
- a selective pressure for black colouration, responsible for colour identity of local *berus* with *nikolskii*.

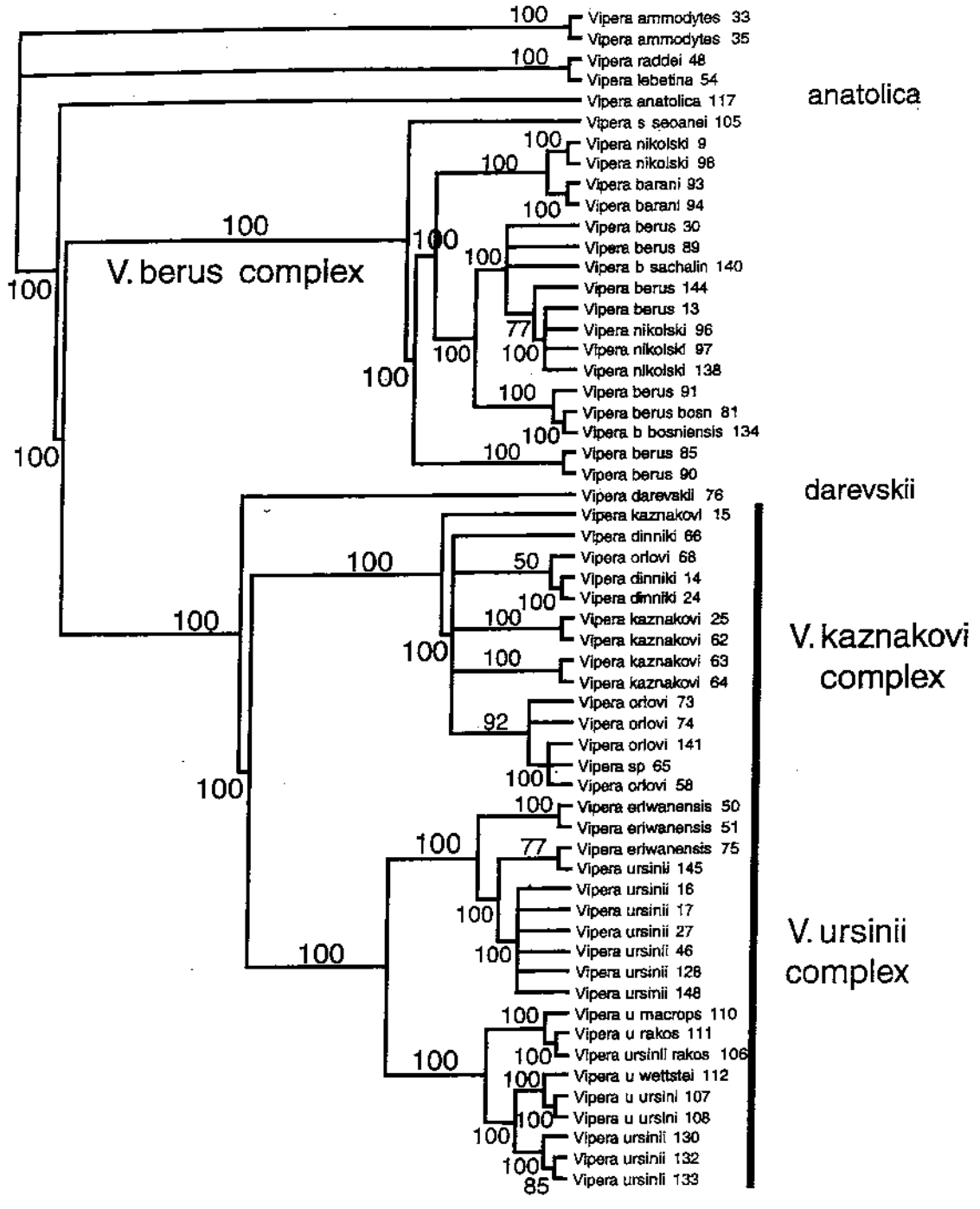

Fig. 2. 50 % Majority-rule consensus maximum parsimony tree of the *Vipera berus* group, derived from 200 equally parsimonious trees (cytochrome b gene, 924 nt).

50 % Majority-rule consensus Maximum-Parsimonie-Baum der *Vipera berus*-Gruppe, erstellt aus 200 Bäumen gleicher Parsimonie (Cytochrom b-Gen 924 Basenpaare).

Additional specimens from the area linking the differentiated *nikolskii* populations in Russia with other Russian and Ukrainian populations are needed. They should be checked for nuclear markers to identify hybrids between the two forms.

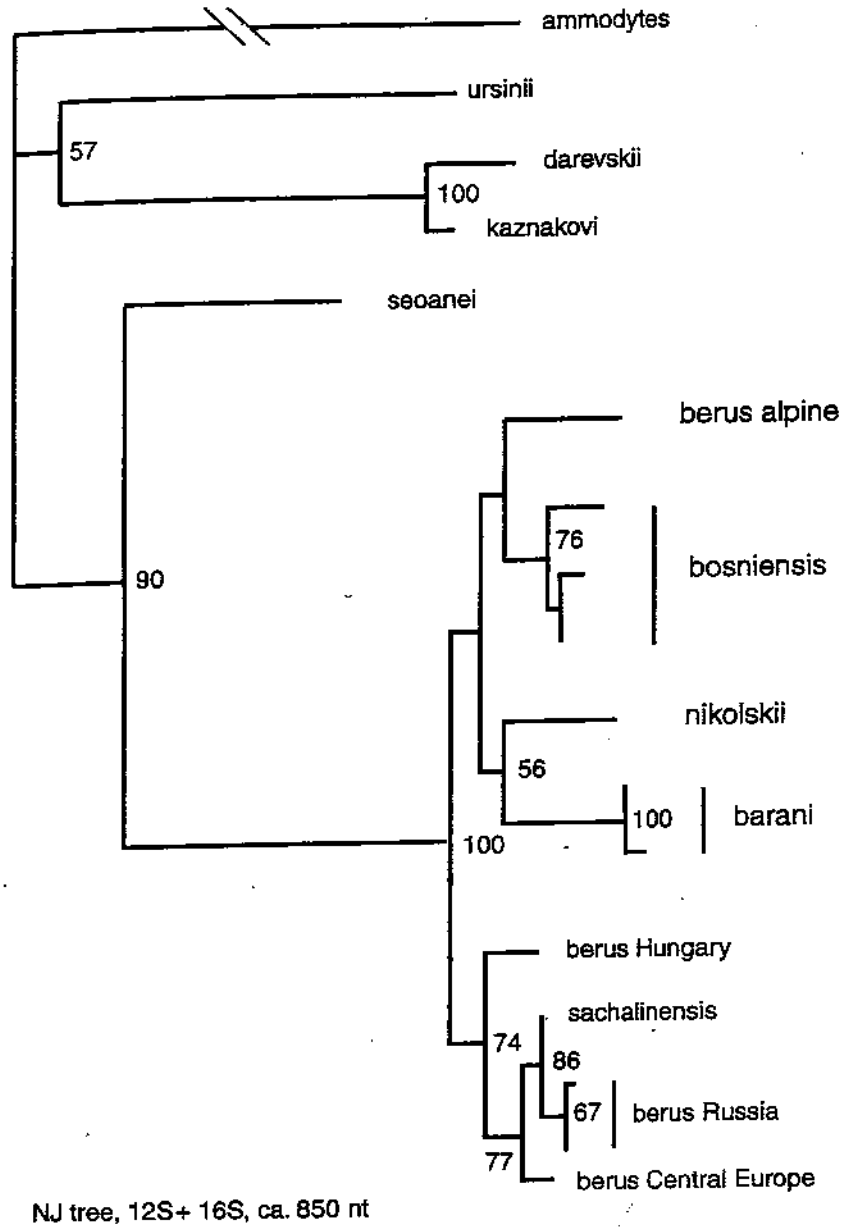

Fig. 3. Neighbor Joining tree of the *Vipera berus* group, derived from DNA sequences of ribosomal 12s and 16s genes (850 NT). Bootstrap values above 50 are indicated.

Neighbor-joining-Baum nach DNA der *Vipera berus*-Gruppe, erstellt nach DNA-Sequenzen der ribosomalen 12s- und 16s-Gene (850 Basenpaare). Bootstrap-Werte über 50 % werden angegeben.

Another unexpected result was the identification of a peculiar Alpine haplotype group. In Austria, this group is only found south of the Danube. Further south it extends into Slovenia.

The biogeographical situation reminds of the ecologically similar lizard *Zootoca vivipara* where a peculiar oviparous relict form exists in Slovenia and southern Austria (Mayer et al. 2000), whereas the typical viviparous form is found from Europe to the Pacific. It is obvious that the Alpine group of *berus* must have had a different (probably

southern) Pleistocene refuge area than *Vipera b. berus*. Italian *berus* should be checked for possible identity with that group.

It is tempting to draw taxonomic conclusions such as distinguishing the five haplotype groups as subspecies of *V. berus*. If a phylogenetic species concept was applied, they could even be regarded as different species. The significant but low genetic difference does not preclude nor suggest it. In order to apply the biological species concept, the contact zones between the groups should be particularly investigated for recent gene flow (nuclear markers).

In the case of *Vipera barani*, which is isolated from the other forms, a species status can neither be refuted nor confirmed. If *Vipera barani* alone would be recognized as a separate species, a paraphyletic *berus* would result. However, paraphyly is a normal stage in speciation (AVISE 1994) and monophyly at species level is not required (JOGER et al. 1998).

Other problems still to be solved concern the taxonomical recognition of *V. b. sachalinensis* (which is apparently very young but already differentiated morphologically) and of *V. nikolskii* (as the type locality harbours "typical" *berus* haplotypes).

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