THE DISTRIBUTION OF CRESTED NEWTS IN SERBIA: AN OVERVIEW AND UPDATE

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Four, morphologically similar species of crested newts (genus *Triturus*) meet and hybridize in Serbia, making a complex system with several hybrid zones. The puzzling and rapidly changing taxonomic status of crested newts has been a major issue in getting hold of their distribution. We provide an overview of the current knowledge on *Triturus* taxonomy and compile available faunistic data into a distribution map, highlighting the species contact and hybrid zones. At the present state of faunistic and genetic research, the north-western, north-eastern, eastern and south-eastern parts of Serbia transpire as areas that are exceptionally important for the diversity of crested newts.

Key words: distribution map, faunistic data, hybrid zones, *Triturus*

INTRODUCTION

Large-bodied newts (genus *Triturus* Rafinesque 1815) are tailed amphibians (Urodela: Salamandridae) widely distributed throughout Europe and adjacent Asia. *Triturus* is a monophyletic genus that is approxi-

mately 24 million years old (Steinfartz *et al.* 2007, Wielstra *et al.* 2019). Biogeographical patterns of species distribution largely reflect Pleistocene refugia patterns and postglacial range expansions (Wielstra *et al.* 2014a). Species have clearly distinct distributions with parapatric ranges, but in contact zones hybridization may be more or less frequent (Arntzen *et al.* 2014, Wielstra *et al.* 2014b).

Over the last 40 years, the puzzling and rapidly changing taxonomic status of *Triturus* species has been a major issue in getting hold of their distribution. In Serbia, four of altogether nine species meet and hybridize, making a complex system with several hybrid zones, showing features like spatial species replacement and asymmetric mitochondrial DNA (mtDNA) introgression (e.g. Wielstra & Arntzen 2012, Arntzen *et al.* 2014, Wielstra *et al.* 2017).

Historically, the determination of species and their distribution ranges in Serbia was challenging because of the great morphological similarity among them, let alone that of interspecific hybrids. Moreover, the first phylogeographic studies were based on mtDNA analyses alone, which enabled identification of potentially cryptic species, but could also be misleading due to possible mtDNA introgression. This problem emerged in *Triturus* newts in Serbia (Wallis & Arntzen 1989, Wielstra & Arntzen 2012, Wielstra *et al.* 2017). In many populations individuals that were morphologically recognized as a single species were, based on mtDNA, identified as another crested newt species, which suggested possible introgression. At the time, the studies that combined morphology and allozyme data contributed to a better understanding of species distributions, but only to already recognized taxa (e.g. Kalezić & Hedgecock 1980, Kalezić *et al.* 1990, Kalezić *et al.* 1997). A more profound multilocus exploration of nuclear DNA was needed to clarify a complicated situation. Continuous work on resolving the *Triturus* phylogeny lasted over several decades (Rafinski & Arntzen 1987, Giacoma & Balleto 1988, Arntzen & Sparreboom 1989, Arntzen & Wallis 1999, Litvinchuk *et al.* 1999, Zajc & Arntzen 1999, Arntzen *et al.* 2007, Steinfartz *et al.* 2007, Themudo *et al.* 2009, Wiens *et al.* 2011, Wielstra & Arntzen 2011, Wielstra *et al.* 2012, Wielstra *et al.* 2013a). Recent application of new molecular methods and approaches in systematic research (e.g. KASP genotyping and Next-generation sequencing) enabled the comparison of large number of nuclear markers and many individuals at the same time and this allowed species delineation and analysis of interspecific hybridization (Wielstra *et al.* 2014c, Wielstra *et al.* 2017, Wielstra *et al.* 2019).

Here we provide a brief historical overview of the taxonomy of the genus *Triturus*. The first classifications of the family Salamandridae were

based on different morphological (Bolkay 1928, Wake & Özeti 1969) or behavioural characteristics (Halliday 1977). These studies suggested a monophyletic origin of the genus *Triturus*. However, at that time, the genus comprised species of large-bodied, small-bodied, alpine, and banded newts. The genetic analyses, which included molecular methods exploring allozymes, microsatellites and mtDNA (e.g. Titus & Larson 1995, Weisrock *et al.* 2006), led to a rejection of the monophyly of these species groups as members of a singular genus (Steinfartz *et al.* 2007, Zhang *et al.* 2008).

García-París *et al.* (2004) suggested separation of small-bodied species in the genus *Lissotriton* Bell, 1839 and species *Ichtyosaura alpestris* (formerly *T. alpestris* and *Mesotriton alpestris*) in the genus *Mesotriton* Bolkay 1928. Litvinchuk *et al.* (2005) additionally separated the species *Ommatotriton vittatus* (former *T. vittatus*) in the genus *Ommatotriton* Gray 1850.

Today, the genus *Triturus* comprises nine large-bodied newt species, with a basal division in marbled and crested newts (e.g. Wielstra *et al.* 2019). Marbled newts (*T. marmoratus* and *T. pygmeus*) have a specific, marbled dorsal coloration. The crested newts on the other hand are named after the large, prominent crest on the back of the males, which is expressed during the mating season. They have a uniform, dark dorsal colouration and a vivid orange belly with black dots. Initially the crested newt was seen as a single species with four subspecies: *T. c. cristatus*, *T. c. carnifex*, *T. c. dobrogicus* and *T. c. karelinii*. It was noted though that various forms differed in body proportions and, as Wolterstorff (1923) showed, could be told apart by the ratio of the limb length to the interlimb distance. This morphometric index was proposed to bear Wolterstorff's name (Arntzen & Wallis 1994).

A comparative analysis of particular genomic characters and the results of cytogenetic studies of interspecific hybrids led Bucci-Innocenti *et al.* (1983) to recognize the four *T. cristatus* subspecies as full species: *T. cristatus* (Laurenti 1768) with the most northern distribution (from Great Britain, Sweden and Norway in the north to the northern part of the Balkan peninsula in the south, and from central Russia and the Ural mountains in the east to the Atlantic shore in France in the west); *T. dobrogicus* (Kiritzescu 1903) that occurs in the wider Danube river valley (from the city of Vienna to the Black sea, all over the Pannonian plains); *T. carnifex* (Laurenti 1768) that inhabits the Apennine peninsula and the northern Balkans (from the Alps in Italy at the south to the Dinaric Alps in the east and reaching into the south of the Czech Republic); and *T. karelinii* (Strauch 1870) that occurs from the Balkan peninsula to the southern shores

of the Caspian sea, including Crimea, Caucasus, Anatolia as well as some isolated pockets in Serbia. These species are often considered as *T. cristatus* superspecies.

Over the decades, the taxonomic status of *T. cristatus* and *T. dobrogicus* remained unchanged, although Borkin and Litvinchuk (2000) proposed two *T. dobrogicus* subspecies: western, *T. d. macrosomus* and eastern, *T. d. dobrogicus* based on species disjunctive distribution. Several genetic studies (Vörös & Arntzen 2010, Wielstra *et al.* 2016, Vörös *et al.* 2016) and morphological analysis (Naumov & Biserkov 2013) rejected this subdivision. On the contrary, *T. carnifex* and *T. karelinii* were split up into several new species. Based on morphology, allozyme and mtDNA research, two subspecies of *T. carnifex* were recognized – *T. c. carnifex* and *T. c. macedonicus* (Crnobrnja *et al.* 1989, Kalezić *et al.* 1997, Arntzen & Wallis 1999). Arntzen and Wallis (1999) classified species originally described as *Molge karelinii* var. *macedonica* Karaman 1922 as subspecies *T. c. macedonicus*. Further, based on mtDNA analysis and deep genetic differentiation as well as differences in ventral coloration patterns, Arntzen *et al.* (2007) suggested that two subspecies should be considered as species – *T. carnifex* (Apennine Peninsula) and *T. macedonicus* (Balkan Peninsula).

Wallis and Arntzen (1989) noticed a deep geographic structuring in mtDNA within *T. karelinii*, even within the limited sampling over the western part of the range. Litvinchuk *et al.* (1999), based on genome size, protein variation, mtDNA and morphological characters, suggested that *T. karelinii* populations in Serbia represent a separate subspecies which was named *T. k. arntzeni*. Steinfartz *et al.* (2007) confirmed the paraphyletic grouping within *T. karelinii*. Themudo *et al.* (2009), based on data from mtDNA and nuclear DNA analysis, proposed that *T. k. karelinii* and *T. k. arntzeni* should be considered as two separate species – *T. karelinii* (Strauch 1870) with eastern distribution and *T. arntzeni* (Litvinchuk *et al.* 1999) with a western distribution. Furthermore, Arntzen and Wielstra (2010) analysed nuclear DNA by protein electrophoresis and concluded presence of western and eastern group which correspond to *T. arntzeni* and *T. karelinii*. The extensive phylogeographic research based on mtDNA sequencing by Wielstra *et al.* (2010) revealed that *T. karelinii* group is composed of three geographically coherent clades: an eastern one (Caucasus, Crimea and the southern shores of the Caspian sea), a central clade (north Turkey and the southern shores of the Black sea) and a western clade (western Turkey – Asian part and the Balkan Peninsula). By applying spatial ecology methods, used to quantify ecological niche differences between *Triturus* species, including species candidates, Wielstra *et al.* (2012) concluded that three *T. karelinii* phylogenetic lineages are actually three cryptic species. Further multilocus approach confirmed that three

mtDNA phylogenetic lines also represent differentiated pools of nuclear DNA genes (Wielstra *et al.* 2013a). Furthermore, the authors suggested that individuals previously assumed as *T. arntzeni* from its type locality in Serbia (Vrtovac, near Knjaževac) are *T. macedonicus*. They recommended that species name *T. arntzeni* should be considered as a *T. macedonicus* synonym and be used for the western lineage of *T. karelinii*. Considering the western and central *T. karelinii* phylogenetic lines, Wielstra *et al.* (2013b) described *T. ivanbureschi* as a species distributed in the south-eastern part of the Balkan Peninsula (Bulgaria, eastern parts of Greece, Macedonia, Serbia), European part of Turkey and Asian part of Turkey (near shores of Aegean sea, Sea of Marmara, and Black sea). Detached from main distribution range, there is *T. ivanbureschi* enclave in Serbia (Arntzen 2003), possibly formed by spreading of *T. macedonicus* and bordered by two other newt species, *T. cristatus* in the east and *T. dobrogicus* at the north (Wielstra & Arntzen 2012, Wielstra *et al.* 2017).

Next-generation sequencing with the development of 52 nuclear markers (Wielstra *et al.* 2014c) confirmed that *T. ivanbureschi* mtDNA is widespread, outside the distribution borders as defined by nuclear DNA. Also, individuals from *T. arntzeni* type locality are genetically admixed *T. macedonicus* × *T. ivanbureschi* hybrids. Wielstra and Arntzen (2014) proposed that due to hybridization, the name *T. arntzeni* should be considered as synonym for *T. macedonicus* as was already suggested, but also as a synonym for *T. ivanbureschi* (Wielstra & Arntzen 2014). Further multilocus nuclear DNA sequencing showed that *T. ivanbureschi* species actually represent two separated gene pools, which should be considered as two different, morphologically cryptic species: the western *T. ivanbureschi* (south-eastern Balkan Peninsula) and the eastern, new species, *T. anatolicus*, distributed in Anatolia (Wielstra & Arntzen 2016).

Such dynamic of constant, subsequent changes in taxonomy produced inconsistency in assigning populations of crested newts to particular taxa in various legislative documents and the scientific literature, as well as in some faunistic databases. Our aim is to make a compilation of the current knowledge of *Triturus* distribution in Serbia, as follows:

Collect and systematize faunistic data of *Triturus* species in the Republic of Serbia available in the literature and faunistic databases;

Synthesize data into a distribution map [georeferenced at the 10 × 10 km standardized UTM (Universal Transverse Mercator) grid];

Highlight the species contact and hybrid zones;

Provide a guideline for further faunistic research.

MATERIAL AND METHODS

The list of all recorded *Triturus* populations, with the geographical information for each population and the sources from which the record was taken is provided in Appendix 1. The species distribution was defined in relation to the genetic data published by Wielstra *et al.* (2017), which were accommodated for the purpose of this study. We defined contact zones and hybrid populations by the proportion of species contribution to the gene pool. If two or three species contribute with at least 20% in the gene pool, we defined the population genetically admixed. The populations in which the contribution of other species in the gene pool was less than 20%, we considered population as “pure”. The remaining populations are designated based on morphology and their position relative to genetically known composition of surrounding populations. Most of new faunistic data were collected in year 2020, as results of the projects mentioned in the acknowledgements and from the public internet faunistic database BIOLOGER (Popović *et al.* 2020).

All faunistic records were plotted on the 10×10 km National Grid UTM (Universal Transverse Mercator) reference for the Republic of Serbia, by using an application created in Visual Basic 6.1 in the program WinWord 2003 (Niketić 1999).

RESULTS

We collected a total of 495 records of *Triturus* species in Serbia, of which literature data constituted 87.5% ($n = 433$), unpublished field data made up 10.1% ($n = 50$) and internet data constituted 2.4% ($n = 12$) of the records (Appendix 1). The genetic information was available for 73 records, which is 14.7% of total records of *Triturus* species in Serbia. As expected, *T. macedonicus* was the most represented species, accounting for 197 records (39.8%), followed by *T. dobrogicus* with 153 records (30.9%), *T. cristatus* with 50 records (10.1%) and *T. ivanbureschi* with 48 (9.7%) records. Admixed populations were the least represented, with altogether 47 records (9.5%) (Appendix 1).

Triturus dobrogicus is distributed in the Pannonian and partly Peripannonian part of Serbia, with disjunct population in the north-eastern part (Ključ region) (Fig 1). *Triturus cristatus* exists in north-eastern and eastern Serbia, with disjunct populations in the south-eastern part of the Banat region. The distribution of *T. ivanbureschi* includes two separate parts, in the Šumadija and Pomoravlje regions and in eastern and south-eastern Serbia. *Triturus macedonicus* has the widest distribution, occupying the largest part of the Mountain-valley region, with a distribution gap in the western and south-western geographic regions of Serbia.

Fig. 1 – Distribution (UTM grid 10 x 10 km) of *Triturus* species in the Republic of Serbia. Species colour codes are: yellow – *T. dobrogicus*, red – *T. cristatus*, blue – *T. ivanbureschi* and purple – *T. macedonicus*. Morphological data are shown by unicolor round symbols and genetic data are shown by round symbols with a black interior dot. Concentric coloured circles represent hybrids identified from genetic data, with the outer circle corresponding to the species with the highest contribution to the population gene pool and the inner dots showing the species with the smaller contribution to the population gene pool.

A hybrid status was found for *T. dobrogicus* × *T. cristatus* at the Vršački breg Mt. (EQ29 – south-eastern Banat) and near Kladovo (FQ14 – north-eastern Serbia); *T. dobrogicus* × *T. ivanbureschi* in Šumadija (DQ72 – foothills of Kosmaj Mt.); *T. cristatus* × *T. ivanbureschi* in north-eastern Serbia (EQ63 – near Kučevo and EP – near Žagubica). The most frequent were *T. ivanbureschi* × *T. macedonicus* hybrids and these were predominantly distributed in the wide area of eastern and south-eastern Serbia around Dimitrovgrad, Pirot, Babušnica, Sokobanja, Sićeavačka klisura gorge, as well as at the Stara planina Mt., the Suva planina Mt. and at the Vlasina Plateau (Fig. 1). Based on the mentioned criteria, admixed population of three species were also recorded, namely *T. ivanbureschi* × *T. macedonicus* × *T. dobrogicus* near Mionica (DQ20 – north-western Serbia) and *T. ivanbureschi* × *T. macedonicus* × *T. cristatus* around Boljevac and at the foothills of the Rtanj Mt. (EP63 and EP74 – eastern Serbia) (Fig 1.).

DISCUSSION

Although the distribution of crested newts in Serbia was generally known from the previous publications (Arntzen 2003, Wielstra *et al.* 2014b, Kalezić *et al.* 2015, Džukić *et al.* 2016), this study provides an updated account on species distribution, including interspecific hybrid zones (Arntzen *et al.* 2014, Džukić *et al.* 2016).

However, as is clear from Fig. 1, there are still relatively large gaps apparent in the recording of some species. There are only a few records of *T. macedonicus* in western and south-western Serbia. For example, this species has not yet been recorded at following mountains: Mokra Gora Mt., Zlatibor Mt., Zlatar Mt., Kamena Gora Mt., Ozren Mt., Javor Mt., Mučanj Mt. and others, although *T. macedonicus* is expected in the mountain areas (Džukić *et al.* 2016). The absence of records could be due to the lack of suitable habitats, but also (and more likely) because of the lack of the research in these regions. The same stands for a large part of eastern Serbia (the surroundings of Zaječar, Knjaževac and Svrlijig) where three species (and their hybrids) could occur: *T. cristatus*, *T. ivanbureschi* and *T. macedonicus*. More systematic faunistic research is also needed in Pomoravlje (around Požarevac, Svilajnac, Despotovac and Jagodina) and north-eastern Serbia (the surroundings of Kučevo, Majdanpek and Donji Milanovac), in order to obtain a more precise view on the distribution of *T. cristatus*, *T. ivanbureschi* and *T. dobrogicus*, as well as the occurrence of hybrid zones between them. Future studies on the distribution of *T. dobrogicus* in the Peripannonian region, as well as of occurrence of hybrid populations of *T. dobrogicus*, *T. ivanbureschi* and *T. macedonicus* should

be done in north-western Serbia (surroundings of Valjevo, Mionica, Ljig, Ub, Koceljeva, Šabac and Bogatić).

The frequency of hybrid populations reported in this study is probably largely underestimated. First, we set high threshold for defining hybrid population based on genetic data. Second, for most populations considered in this study only morphological data were available, which makes the recognition of hybrid populations almost impossible (Arntzen *et al.* 2018). Actually, a high level of gene flow and introgression characterises *Triturus* populations in Serbia (Wallis & Arntzen 1989, Wielstra & Arntzen 2012, Arntzen *et al.* 2014, Wielstra *et al.* 2017). In Serbia all *T. macedonicus* populations, except at Kosovo and Metohija, carry *T. ivanbureshi* mtDNA. The genetically admixed populations are almost all populations at the species contact zones, as well as the populations of *T. ivanbureshi* within the recognized enclave and populations of *T. macedonicus* took over from *T. ivanbureshi*. At the present state of faunistic and genetic research, the north-western, north-eastern, eastern and south-eastern parts of Serbia transpire as areas that are exceptionally important for the diversity of crested newts.

Further research is needed on the distribution and genetics of *T. ivanbureschi* populations in Šumadija region, which is crucial for further delineation of the *T. ivanbureschi* enclave, particularly the *T. ivanbureschi* – *T. macedonicus* contact zone along the Zapadna Morava river valley, which we presume is the natural barrier between these two species. The same holds for north-western Serbia where also three species come into contact. Another interesting problem is the possible spreading of the lowland species, *T. dobrogicus*, to the south along Velika Morava River valley and its hybridisation with other species as suggested by genetic data (Wielstra *et al.* 2017). More data for this species is required, from both Pannonian and Peripannonian plane, taking into account the population position relative to large rivers (Danube, Sava and Tisza) which were recognised as important geographical elements in shaping evolutionary history of amphibians (Vörös *et al.* 2016).

According to the IUCN criteria, *T. cristatus*, *T. ivanbureschi* and *T. macedonicus* are considered as least concerned species (LC), whereas *T. dobrogicus* is considered as near threatened (NT). All four species of crested newts are listed in Annex II of the Habitat Directive. The conservation status of the crested newts in Serbia is given in the Red Book of Fauna of Serbia I – Amphibians (Kalezić *et al.* 2015). Although all four *Triturus* species are strictly protected by law in the Republic of Serbia (Anonymous 2010a, b), the designation of areas of species distribution and the recognition of populations of special importance is the first and obligatory step for the improvement of the conservation statuses of crested

newts in Serbia. The improvement of the conservation statuses of crested newts is extremely important due to rapid loss of suitable habitats. International scientific cooperation and mutual conservation actions are required for populations at the species contact zones which lay along the administrative borders of The Republic of Serbia with Romania (*T. dobrogicus* and *T. cristatus*), Bulgaria (*T. ivanbureschi* and *T. macedonicus*) and at the borders with Bosnia and Herzegovina, and Croatia (*T. dobrogicus* and *T. macedonicus*).

Active conservation measures such as strict control in the field (by rangers and inspectors) and well-designed educational activities (from schoolchildren and laypeople to decision-makers) must also be undertaken, in order to achieve effective protection of all *Triturus* species as well as their natural habitats.

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SUPPORTING INFORMATION

Online Appendices:

Appendix 1. – Distribution records of four *Triturus* species and their hybrids (Tcri – *T. cristatus*, Tdob – *T. dobrogicus*, Tiva – *T. ivanbureschi*, Tmac – *T. macedonicus*) in the Republic of Serbia with: regions, broad locations, exact localities, toponyms, UTM 10×10 km squares, longitudes, latitudes, altitudes, legators and dates of observations; L – literature sources, I – internet sources, F – field data; * – available genetic data; r – river, v – village.

**ДИСТРИБУЦИЈА ВЕЛИКИХ МРМОЉАКА У СРБИЈИ:
ПРЕГЛЕД И ДОПУНА**

ТИЈАНА ВУЧИЋ, ЉИЉАНА ТОМОВИЋ, АНА ИВАНОВИЋ

Р Е З И М Е

Род *Triturus*, чини група од девет врста великих мрмољака од којих четири живе у Србији: *Triturus cristatus*, *T. dobrogicus*, *T. ivanbureshi* и *T. macedonicus*. Ове врсте, са парapatричком дистрибуцијом, на местима контакта формирају комплексан систем хибридних зона. Због велике сличности, детерминација врста на основу спољашње морфологије је непоуздана, нарочито у хибридним зонама. Посебан проблем у утврђивању дистрибуције је неусаглашеност података доступних у литератури, базама података и законској легислативи, до којих је дошло услед низа узастопних промена у таксономији.

Циљ овог рада је да пружи кратак историјски преглед проблема-тике таксономије великих мрмољака; прикупи све доступне фауни-стичке податке, систематизује их и представи у виду UTM карте дистрибуције (10 × 10 km); да поред дистрибуције врста назначи зоне контакта и хибридне популације, као и да пружи смернице за будућа фаунистичка истраживања.

Прикупљено је укупно 495 фаунистичка податка. *Triturus macedonicus* је најзаступљенија врста са 39.8% уноса, затим *T. dobrogicus* са 30.9%, *T. cristatus* са 10.1% уноса и *T. ivanbureschi* са 9.7% уноса. Најмање су заступљене хибридне популације са 9.5% уноса.

На основу добијене дистрибуције јасно су уочљиве зоне од значаја за будућа фаунистичка истраживања. То су пре свега области у којима у потпуности недостају подаци о присуству великих мрмољака, као што су делови западне и југозападне Србије. Велике области северо-западне, североисточне и источне Србије, као и Поморавља, посебно су интересантне са аспекта хибридизације, генетичке варијабилности и диверзитета великих мрмољака. Додатна фаунистичка и генетичка истраживања у оквиру ових области су неопходна да би се прецизније утврдила дистрибуција врста, генетички диверзитет популација и омогућила њихова адекватна заштита.