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Kicking *Triturus arntzeni* when it's down: large-scale nuclear genetic data confirm that newts from the type locality are genetically admixed

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Abstract

We collected nuclear DNA data (52 markers) with next-generation sequencing for nine *Triturus* newt specimens, including the holotype and two of the paratypes of *T. arntzeni*, from the type locality at Vrtovać in eastern Serbia. We compare these data to a reference set composed of the four crested newt species distributed in eastern Serbia namely *T. cristatus*, *T. dobrogicus*, *T. ivanbureschi* and *T. macedonicus* to determine to which of these species the newts from the type locality of *T. arntzeni* should be attributed. The majority of alleles in individuals from Vrtovać is derived from *T. macedonicus*, but a considerable number of *T. ivanbureschi* alleles is also present; alleles typical for *T. cristatus* and *T. dobrogicus* are found at low frequency. Accordingly, we interpret Vrtovać as a *T. macedonicus – T. ivanbureschi* hybrid population, albeit not composed of F1 hybrids but of genetically admixed individuals derived through multiple generations of backcrossing. The data support the notion that the name *T. arntzeni* should not be applied to a species newly distinguished in *T. karelinii sensu lato* (to which the name *T. ivanbureschi* has been given). We conclude that because of the hybrid nature of the individuals from Vrtovać, the name *T. arntzeni* should be placed not only in the synonymy of *T. macedonicus* but also in the synonymy of *T. ivanbureschi*. In this study we demonstrate that next-generation sequencing can provide high quality data for type material with degraded DNA and therefore can play an important role in taxonomy.

Key words: DNA degradation, Ion Torrent, Next-generation sequencing, *Triturus cristatus* superspecies, *Triturus ivanbureschi*, *Triturus macedonicus*

Introduction

In a recent paper in this journal, we showed that the crested newt species *Triturus karelinii* (Strauch, 1870) comprises (at least) one more species: *T. ivanbureschi* Arntzen and Wielstra 2013 (in Wielstra *et al.*, 2013c). The first hint of *T. ivanbureschi* representing a distinct species came from deep genetic divergence of mitochondrial DNA (Wielstra & Arntzen, 2011; Wielstra *et al.*, 2010). These lineages were subsequently found to differ in environmental space (Wielstra *et al.*, 2012) and were eventually confirmed to also represent discrete nuclear gene pools (Wielstra *et al.*, 2013a).

The name *T. arntzeni* Litvinchuk, Borkin, Džukić and Kalezić, 1999 (in Litvinchuk *et al.*, 1999), with Vrtovać, Serbia as type locality, has been applied to a species newly distinguished in *T. karelinii* sensu lato distributed on the Balkan Peninsula and western Asiatic Turkey (e.g. Arntzen & Wielstra, 2010; Espregueira Themudo *et al.*, 2009). However, the supposed differences of *T. arntzeni* from other newts (genome size, protein variation and morphological characteristics) have been put into question (Arntzen & Wielstra, 2010; Stoyanov *et al.*, 2011) and in a review of the species identity of crested newts from Vrtovać, the bulk of evidence (particularly genome size and three nuclear DNA markers) pointed towards *T. macedonicus* being the crested newt species occurring at this site (Wielstra *et al.*, 2013c). Wielstra *et al.* (2013c) concluded that the name *T. arntzeni* is a junior synonym of *T. macedonicus* (Karaman, 1922) and should not be applied to the newly distinguished species.

Wielstra *et al.* (2013c) failed to obtain genetic data for the type material of *T. arntzeni*, presumably because of DNA degradation. Nuclear data available for other individuals from Vrtovać concerned just three markers, which

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was insufficient for a detailed examination of the newts' genetic composition (Wielstra *et al.*, 2013a). The distribution of *Triturus* newts in eastern Serbia is intricate, but so much is clear that four species – next to *T. ivanbureschi* and *T. macedonicus* also *T. cristatus* (Laurenti, 1768) and *T. dobrogicus* (Kiritzescu, 1903) – are in parapatry (Fig. 1). Genetic admixture between crested newt species appears to be restricted to the species' contact zones (Arntzen JW, Wielstra B, Wallis GP, submitted). However, the type locality of *T. arntzeni* is positioned close to where the ranges of these four crested newt species meet (Wielstra *et al.*, 2013b), meaning the genetic ancestry of the Vrtovać newts might involve any and each of these four species.

We aim to examine the Vrtovać newts' genetic affinity in detail. Employing a recently developed next-generation sequencing protocol, we have now been able to obtain sequence data for 52 nuclear markers, from nine individuals from the type locality of *T. arntzeni*, including the holotype and two of the paratypes. This allows us to determine the genetic composition of these individuals with unprecedented accuracy and to determine to which of the four crested newt species in eastern Serbia they belong.

Material and methods

We sequenced 52 nuclear markers with Ion Torrent next-generation sequencing. A detailed description of the methodology is provided in Wielstra *et al.* (2014). In brief, we amplified markers of c. 140 bp in length (excluding primers), derived from transcriptome data and positioned in 3-prime untranslated regions, in five multiplex PCRs. We pooled the multiplexes for each individual and ligated unique tags to be able to recognize the product belonging to each individual. We sequenced all amplicons on the Ion Torrent next-generation sequencing platform and processed the output with a bioinformatics pipeline that filters out poor quality reads, identifies alleles and converts data to a format directly usable for population genetic analysis.

The investigated newt specimens were the holotype and two paratypes, collected in 1996 and stored in 70% at room temperature, three individuals for which tissue samples were collected in 2003 and stored in 95% alcohol at room temperature and three individuals for which tissue samples were collected in 2010 and stored in 100% alcohol at -80°C. We compared these individuals with a reference dataset of 48 newts taken from Wielstra *et al.* (2014), comprising four species, four populations per species and three individuals per population. The populations were chosen from across the species' ranges and were positioned away from documented contact zones (see Fig. 1 and Table 1 for sampling details).

We used BAPS v.5.3 (Corander *et al.*, 2008) and Structure 2.3.4 (Pritchard *et al.*, 2000) to assign individuals to distinct gene pools probabilistically. First we enforced BAPS and Structure to partition the individuals in four groups ($k = 4$), as we deal with four well-established reference species to which the Vrtovać newts could show genetic affinity. These analyses allowed us to determine the probability that the selected individuals from the *T. arntzeni* type locality would belong to each of the four Serbian crested newt species. Second we allow the two programs to determine the optimal value of k for a range of $k = 1$ to $k = 17$ (the upper limit defined by the total number of included populations, see Table 1). These analyses could reveal additional gene pools, which would reflect the presence of intraspecific population structure and/or strongly genetically admixed individuals. In BAPS we used ten replicates and tested for admixture between gene pools. In Structure we used the admixture model in combination with the correlated allele frequency model and ran five independent simulations with 100,000 iterations, after 50,000 iterations of burn-in.

Given the results of the BAPS and Structure analyses reported below, we compared the Vrtovać newts to only *T. ivanbureschi* and *T. macedonicus* with NewHybrids 1.1b3 (Anderson & Thompson, 2002). NewHybrids infers for every individual the probability with which it belongs to a purebred (either of the two parental species under consideration) or a hybrid class (F_1, F_2 or a backcross with either one of the two parental species). The program was run with a burn-in and formal run of 10,000 iterations each and the 12 *T. ivanbureschi* and 12 *T. macedonicus* individuals were set to belong to the two parental classes *a priori* (using the z option). Finally, we determined a hybrid index of the nine individuals from the *T. arntzeni* type locality by first determining which of the 52 markers were fully diagnostic for *T. ivanbureschi* and *T. macedonicus* based on the reference populations and subsequently counting the number of *T. ivanbureschi* and *T. macedonicus* alleles for these markers in the nine Vrtovać newts.

TABLE 1. Sampling details. We sampled three individuals in four populations each of the species *T. cristatus*, *T. dobrogicus*, *T. macedonicus* and *T. ivanbureschi* as a reference set to which we compared nine individuals, including the holotype and two paratypes, from the type locality of *T. arntzeni* at Vrtovać, Serbia. Population numbers correspond to those in Fig. 1. Individuals are identified with a code that refers to specimens in the alcohol collection (ID starting with ZMA) or tail tips tissue bank (remaining samples) of Naturalis Biodiversity Center, Leiden, the Netherlands. Tissue voucher 5030 concerns the holotype of *T. arntzeni*, for which the specimen is stored in the Zoological Institute, Saint Petersburg, Russia (ZISP.6121). Tissue vouchers 5031 and 5032 concern two of the paratypes of *T. arntzeni* (ZISP.6122).

Population number	Sample size	Sample ID	Species	Locality	Latitude	Longitude
1	3	1750, 1752, 1756	*Triturus cristatus*	France: Mayenne	48.300	-0.617
2	3	5049-5051	*Triturus cristatus*	Poland: Tłumaczów	50.558	16.434
3	3	4342-4344	*Triturus cristatus*	Romania: Brădătel	47.491	26.178
4	3	2616, 2617, 4485	*Triturus cristatus*	Bulgaria: Montana	43.416	23.222
5	3	ZMA8041-307-309	*Triturus dobrogicus*	Austria: Tadten	47.767	17.000
6	3	ZMA9083-512-514	*Triturus dobrogicus*	Hungary: Alap	46.800	18.683
7	3	ZMA9153-427-429	*Triturus dobrogicus*	Serbia: Senta	45.917	20.100
8	3	2427-2429	*Triturus dobrogicus*	Romania: Giurgeni	44.742	27.868
9	3	3245-3247	*Triturus macedonicus*	Montenegro: Bjeloši	42.374	18.907
10	3	3601-3603	*Triturus macedonicus*	Macedonia: Gostivar	41.817	20.899
11	3	2820-2822	*Triturus macedonicus*	Greece: Kounoupena	39.683	19.764
12	3	3775-3777	*Triturus macedonicus*	Greece: Kerameia	39.562	22.081
13	3	2492-2494	*Triturus ivanbureschi*	Bulgaria: Alexandrovo	42.601	25.093
14	3	2602-2604	*Triturus ivanbureschi*	Bulgaria: Alepu	42.348	27.714
15	3	2360-2362	*Triturus ivanbureschi*	Turkey: Keşan	40.917	26.633
16	3	1879-1881	*Triturus ivanbureschi*	Turkey: Bigadiç	39.351	28.217
17	9	2533-2535, 3621-3623, 5030-5032	*Triturus arntzeni*	Serbia: Vrtovać	43.404	22.450

Results

The total number of reads for the nine newts from the *T. arntzeni* type locality is 246,072 and the average number of reads per individual per marker is 526.0 ± 23.1 (standard error). For individual 5030 (the holotype of *T. arntzeni*) three of the 52 markers failed (of which two diagnostic between *T. ivanbureschi* and *T. macedonicus*, see below) and for individual 5031 (a paratype) one (non-diagnostic) marker failed; the other individuals from Vrtovać have a complete dataset. Datasets associated with this paper are available via the Dryad Digital Repository (see section 'Data accessibility').

The results of the BAPS and Structure analyses under $k = 4$ are shown in Fig. 2. The individuals in the reference set are allocated to their respective species with significant support ($p > 0.95$) in both analyses. With BAPS, seven individuals from the *T. arntzeni* type locality are allocated to *T. macedonicus* and two are allocated to both *T. macedonicus* and *T. ivanbureschi* (i.e. they are identified as genetically admixed). With Structure, all nine Vrtovać individuals are allocated to both *T. macedonicus* and *T. ivanbureschi* (with the probability of them belonging to *T. macedonicus* being higher) while one newt (the holotype of *T. arntzeni*) additionally shows a low probability of belonging to *T. cristatus* (Fig. 2). Structure also identifies $k = 4$ as the most optimal partitioning scheme for the dataset when k is allowed to vary. BAPS on the other hand suggests $k = 7$, partitioning *T. cristatus* into two gene pools instead of one and placing the *T. arntzeni* newts in two distinct gene pools rather than allocating them to both the *T. macedonicus* and *T. ivanbureschi* gene pools. The table with the BAPS and Structure scores is available from Dryad.

NewHybrids identifies all Vrtovać individuals as *T. ivanbureschi* and *T. macedonicus* F_2 hybrids with full support (a p-value of 1.0; Fig. 3). Of the 52 markers, 27 show no overlap in variants in the reference set for *T.*

macedonicus and *T. ivanbureschi*. The proportion of alleles typical for *T. macedonicus* and *T. ivanbureschi* present in the Vrtovać sample is shown in Fig. 3. For the nine Vrtovać individuals together, the majority of observed allelic variants is typical for *T. macedonicus* (55.6%), but a considerable number of alleles typical for *T. ivanbureschi* is also present (29.2%); the remaining alleles are not found in either reference species (14.4%) or have missing data (0.8%). The table with the distribution of *T. ivanbureschi* and *T. macedonicus* alleles in the newts from Vrtovać is available from Dryad.

In 18 instances (i.e. 1.9 % of the 9 x 52 x 2 = 936 alleles), concerning five markers and eight individuals, we find alleles diagnostic for *T. cristatus* and/or *T. dobrogicus* in the Vrtovać newts. The table with data in genotypic format is available from Dryad.

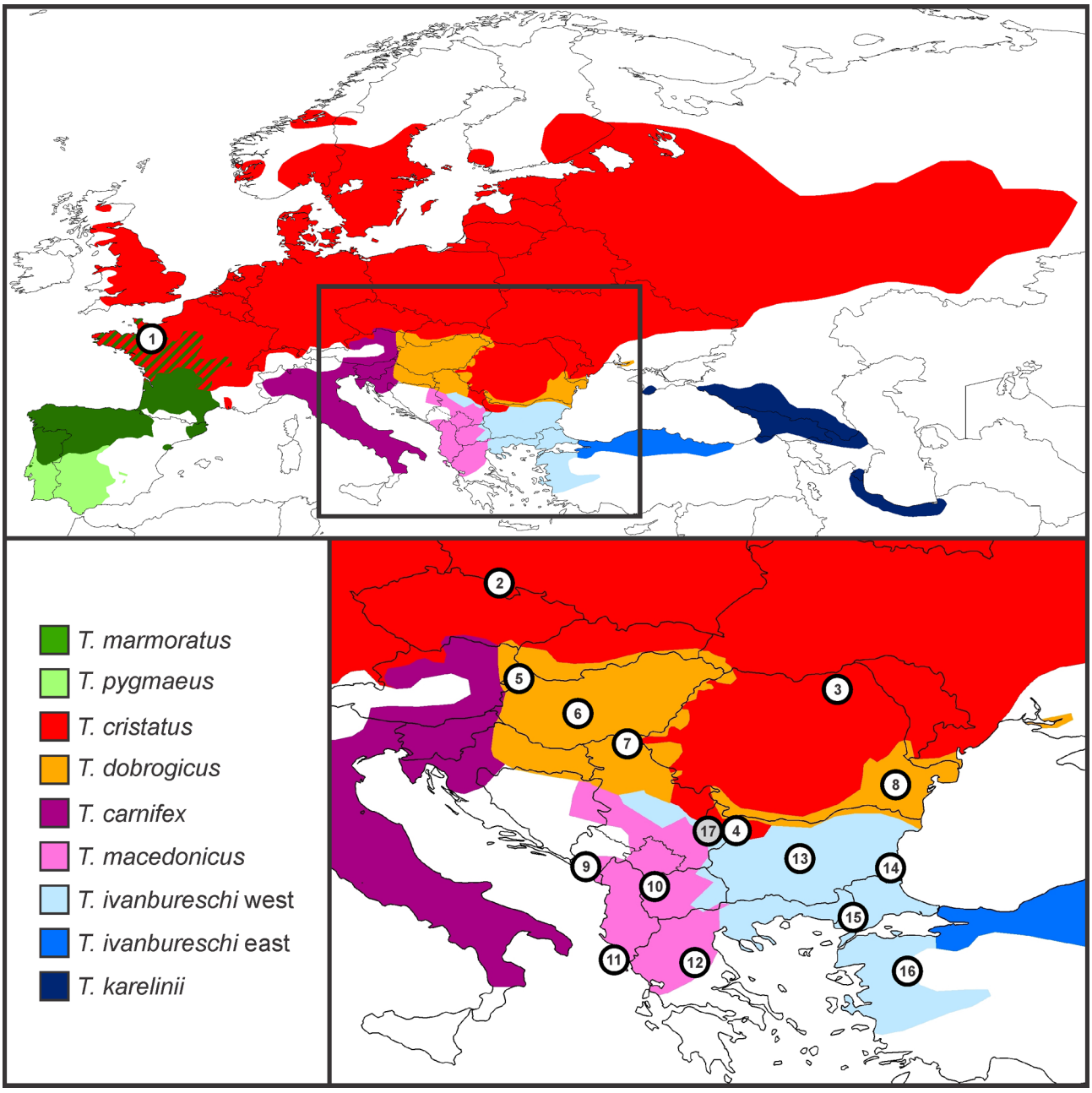

FIGURE 1. Sampling design. The distribution of *Triturus* species after Wielstra *et al.* (2013b) and the position of the sampled populations (details in Table 1).

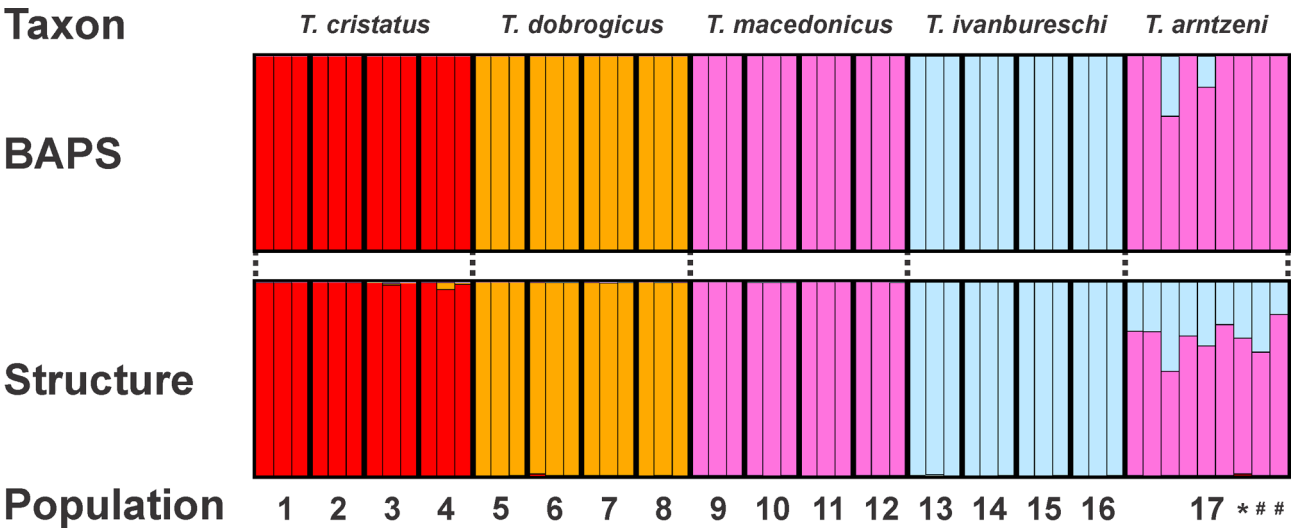

FIGURE 2. Clustering individual newts from the type locality of *Triturus arntzeni* to the four *Triturus* species from the Balkan Peninsula. In the BAPS and Structure analyses the individuals from the type locality of *T. arntzeni* are compared to the four *Triturus* species in the reference set (*T. cristatus*, *T. dobrogicus*, *T. macedonicus* and *T. ivanbureschi*). The *T. arntzeni* holotype is labelled with an asterisk (*) and the two paratypes with a hashtag (#). Bar plots were created with DISTRUCT (Rosenberg, 2004).

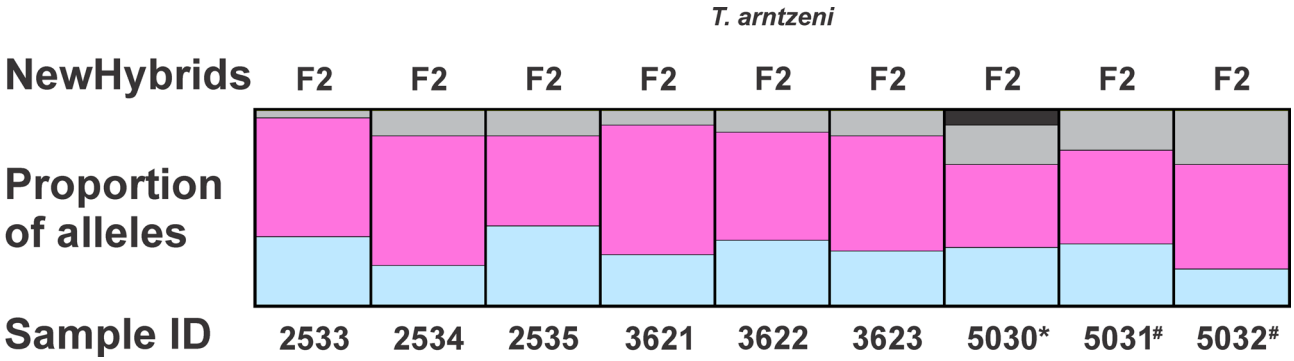

FIGURE 3. Quantifying the genetically admixed ancestry of *Triturus arntzeni*. In the NewHybrids analysis the individuals from the type locality of *T. arntzeni* are compared to *T. ivanbureschi* and *T. macedonicus*. For 27 markers with non-overlapping allele variants in the reference populations the proportion of *T. ivanbureschi* (blue) and *T. macedonicus* (pink) alleles is shown (see table available via Dryad for details). Alleles not observed in the reference populations are shown in grey and missing data are shown in black. The *T. arntzeni* holotype is labelled with an asterisk (*) and the two paratypes with a hashtag (#).

Discussion

Nomenclature. Our data show that newts from Vrtovać, including the holotype of *T. arntzeni*, are not genetically pure but represent *T. ivanbureschi* as well as *T. macedonicus*. This observation raises some nomenclatural issues. The International Code of Zoological Nomenclature (hereafter referred to as "the Code") defines a hybrid as the progeny of two individuals belonging to different taxa, in which the term taxon is used in a broad sense as "a population, or group of populations of organisms which are usually inferred to be phylogenetically related and which have characters in common which differentiate the unit". Under a strict interpretation, genetically admixed individuals derived through many generations of backcrossing would be left in limbo. This is because the parents of these individuals would themselves be genetically admixed and hence not qualify as taxa, as would their parents, and so on, back in time, up until the parents of the original F1 hybrid ancestor(s) involved.

In fact, most recognized species can be expected to show signs of introgression (Vences *et al.*, 2013). For example, mtDNA introgression has been regularly documented (Toews & Brelsford, 2012). In order to circumvent having to treat each and every species as a hybrid we interpret a hybrid here as an individual that possesses genetic

material derived from more than one species, at appreciable frequency and at a genome-wide level. We refrain from proposing a particular threshold as there are no objective grounds to do so. Considering that in individuals from the type locality of *T. arntzeni*, including the holotype, alleles of both *T. macedonicus* and *T. ivanbureschi* are present for most of the diagnostic markers scored (22 out of 27 markers; see table available via Dryad for details), at high frequency (on average over 55% *T. macedonicus* and almost 30% *T. ivanbureschi* alleles; Fig. 3), we consider the name *T. arntzeni* to having been given to a hybrid.

Article 1.3.3 of the Code states that a name proposed for a hybrid as such, that is, a specimen known to be of hybrid origin to the person describing it, is excluded from the provisions of the Code. However, the hybrid status of newts from Vrtovać was unknown to Litvinchuk *et al.* (1999) when they described them as a new taxon, so this rule does not apply. Article 23.8 of the Code states that a name established for an animal that at a later point in time was found to be a hybrid must not be used as the valid name for either of the parental species, even if it is older than all other available names for the parental species. The name *T. arntzeni* should hence not be applied to a species newly distinguished in *T. karelinii sensu lato*, corroborating the view expressed in Wielstra *et al.* (2013c). Although the name *T. arntzeni* is not valid, it is available. However, the suggestion of Wielstra *et al.* (2013c) that *T. arntzeni* is a junior synonym of *T. macedonicus* turns out to be incomplete, because it applies to *T. karelinii sensu lato* (i.e. *T. ivanbureschi*) equally well.

Similarly, *T. blasii* (de l'Isle du Dréneuf 1862) was found by Wolterstorff (1904) to represent a hybrid between the crested newt *T. cristatus* and the marbled newt *T. marmoratus* (Latreille, 1800) and is placed in the synonymy of both parental species, with the remark that it applies to the hybrid between the two (Dubois & Raffaëlli, 2009; Frost, 2013; Mertens & Wermuth, 1960). Whereas *T. blasii* concerns an F_1 hybrid (Arntzen *et al.*, 2009; Arntzen & Wallis, 1991; Wolterstorff, 1904), *T. arntzeni* is derived from an unknown number of generations of backcrossing in a full hybrid population. Accordingly, we place the name *T. arntzeni* in the synonymy of both *T. macedonicus* and *T. ivanbureschi*, with the remark that it concerns a backcross hybrid of these two species.

Historical biogeography – introgressed and rare alleles. The observed genetic admixture at population Vrtovać, and the overall distribution of *Triturus* species on the Balkan Peninsula, indicate that the type locality of *T. arntzeni* is positioned within the hybrid zone of *T. macedonicus* and *T. ivanbureschi*. From the geographical distribution of *T. ivanbureschi* mtDNA, we hypothesized that this hybrid zone has been moving ever since *T. macedonicus* and *T. ivanbureschi* obtained secondary contact following the Last Glacial Maximum (Wielstra & Arntzen, 2012, 2014). We consider the geographically wide-ranging asymmetric mtDNA introgression, from *T. ivanbureschi* into *T. macedonicus*, to be a genetic footprint left by *T. ivanbureschi*, when it was replaced by *T. macedonicus*. According to this scenario, backcrosses in the direction of the expanding species *T. macedonicus* would occur at the wave front of the moving hybrid zone. This is in line with the situation at Vrtovać, where genetic material of *T. ivanbureschi* is clearly present, but genetic material of *T. macedonicus* dominates.

The recording of allele variants at Vrtovać not found in the reference species may reflect limited sampling within the reference species. However, the observation is also in line with the 'rare allele phenomenon', in which allelic variants that are rare or virtually non-existent in parental species rise to high frequencies in the hybrid zone (Barton *et al.*, 1983; Lammers *et al.*, 2013; Woodruff, 1989). One explanation for this phenomenon is local adaptation to the selective environment of the hybrid zone itself, where any allele that reduces the degree of reproductive isolation and thus increases relative hybrid fitness is favored (Coyne & Orr, 2004). Less favored explanations are increased mutation rate or intragenic recombination (Lammers *et al.*, 2013).

We additionally found alleles typical for *T. cristatus* and *T. dobrogicus* at low frequency at Vrtovać. The presence of these alleles could reflect ancestral polymorphism and the presence of these alleles in *T. ivanbureschi* and *T. macedonicus* might be revealed with wider sampling. However, considering the relatively close proximity of both *T. cristatus* and *T. dobrogicus* in combination with hybrid zones shifting position, ancient hybridization and gene flow leading to these alleles being present at Vrtovać seems a likely explanation.

Modern DNA sequencing. Recent technological advances allow genome scale data to be obtained for non-model species (Ekblom & Galindo, 2011; this study). We were successful in sequencing nuclear DNA of the holotype and two paratypes of *T. arntzeni*, whereas an earlier attempt to obtain mtDNA and nuclear DNA sequences failed (Wielstra *et al.*, 2013c). We attribute the success to the short DNA fragments that we targeted in this study (c. 140 bp vs. 500-800 bp), because the success of PCR amplification of degraded DNA is inversely related to product size (Wandeler *et al.*, 2007). Fortunately the genetic variability and number of markers surveyed allowed us to establish the true identity of the Vrtovać newts. Museum specimens including type material older and more degraded than that used in the present study are within reach of genetic analysis and this is a promising

perspective for taxonomy as well as population genetics (Wandeler *et al.*, 2007). Several next-generation sequencing protocols actually involve a step in which high quality DNA is fragmented for library preparation, perhaps not dissimilar to what happens naturally when DNA breaks down.

Data accessibility

Data associated with this paper have been deposited in the Dryad online data repository under doi:10.5061/dryad.g3375 as follows. For the nine individuals from Vrtovać: 1) raw Ion Torrent reads in FASTQ format; 2) BWA alignments in SAM format; 3) raw SNP reports in VCF format; 4) filtered SNP report used to construct consensus sequences; and 5) number of reads per individual per marker. For the comparison with all *Triturus* species in the reference set: 6) table containing data in genotypic format; 7) allelic variants per marker; 8) FASTA files of reconstructed sequences; 9) BAPS input file (GENEPOP format); 10) Structure input file; and 11) the BAPS and Structure output. For the comparison with the species *T. ivanbureschi* and *T. macedonicus*: 12) NewHybrids input file; and 13) table with distribution of *T. ivanbureschi* and *T. macedonicus* alleles in newts from the *T. arntzeni* type locality.

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