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The hybrid zone between the Italian and Northern Crested Newts (*Triturus carnifex* and *T. cristatus*) reaches Germany

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Abstract. While it is generally assumed that only the Northern Crested Newt (*Triturus cristatus*) occurs in Germany, there are early reports of Crested Newts with features typical of the Italian Crested Newt (*T. carnifex*) from the Berchtesgadener Land (Bavaria). We study a panel of eight nuclear DNA SNP markers for 26 individual Crested Newts to test if *T. carnifex* alleles occur in this region. All but two of the studied individuals contain alleles diagnostic for *T. carnifex*, with the largest percentage of *T. carnifex* alleles observed in a single individual being 37.5%. The sampled individuals show morphological characteristics typically associated with *T. carnifex*. We conclude that the natural hybrid zone between the Northern Crested Newt and the Italian Crested Newt reaches further west than previously realized and extends into the extreme southeast of Germany.

Key words. Amphibia, Caudata, Bavaria, Berchtesgadener Land, gene flow, hybridization, introgression, KASP genotyping.

Introduction

The Italian Crested Newt, *Triturus carnifex* (Laurenti, 1768), and the Northern Crested Newt, *T. cristatus* (Laurenti, 1768), are morphologically and genetically distinct and are not each other's sister species (Arntzen 2003, Fahrbach & Gerlach 2018, Wielstra et al. 2019). *Triturus carnifex* is known to have been introduced in Germany, near Isen in Upper Bavaria (Franzen et al. 2002), inside the native range of *T. cristatus*. Similarly, *T. carnifex* has been introduced inside *T. cristatus* territory in multiple other places in Europe, where the two species now hybridize (Brede et al. 2000, Arntzen 2001, Maletzky et al. 2008b, Brede 2015, Meilink et al. 2015, Dufresnes et al. 2016, 2019, Wielstra et al. 2016).

These two Crested Newt species also meet at a natural hybrid zone, but the position of this zone has been incompletely documented (Maletzky et al. 2008a, Mikulíček et al. 2012, Arntzen et al. 2014, Wielstra et al. 2014, Mačát et al. 2019). Schmidtler (1976) reported five different localities in the Berchtesgadener Land, a district in Bavaria in the extreme southeast of Germany (Fig. 1), where Crested Newts possessed *T. carnifex*-like morphological characters, such as a yellow vertebral line, a rather washed-out belly pattern and relatively sparse lateral white speckles (Fig. 2). Schmidtler (1976) assumed these newts were genetically admixed between *T. cristatus* and *T. carnifex*, suggesting that the hybrid zone between the two species protrudes into Germany.

Maletzky et al. (2008b) studied the morphology of Crested Newts in Bavaria based on the Wolterstorff Index (the ratio between forelimb length (PaL) and interlimb distance (LiE), defined as WI = PaL × 100 / LiE; Wolterstorff 1923). Maletzky et al. (2008b) found no evidence for a natural occurrence of *T. carnifex* in Germany based on the WI. However, the diagnostic value of WI is rather limited and this issue will only be exacerbated by hybridization (Arntzen & Wallis 1994, 1999). The number of rib-bearing vertebrae (NRV) has been proposed as a more reliable marker for species identification in Crested Newts (Arntzen & Wallis 1994, 1999). Still, the diagnostic value of NRV breaks down at hybrid zones (Arntzen et al. 2014), also making it suboptimal to test the potential occurrence of *T. carnifex* in southeast Germany.

Maletzky et al. (2008b) also genotyped mitochondrial DNA and only identified haplotypes typical of *T. cristatus* in southeast Germany. However, mitochondrial DNA is a notably unreliable marker for species identification in general and particularly so near hybrid zones, where the marker often shows introgression (Toews & Brelsford 2012). Mitochondrial DNA introgression is so common in Crested Newts that it is unreliable for species identification across vast areas of Crested Newt distribution, even at relatively large distances from the hybrid zones (Wielstra et

al. 2017a, 2017b). To determine potential genetic admixture between the two species, multiple nuclear DNA markers would need to be consulted.

Presently, the general consensus seems to be that the natural hybrid zone between *T. carnifex* and *T. cristatus* is positioned outside of Germany (Franzen 2020). We genotype a panel of eight nuclear DNA SNP markers from Berchtesgadener Land to test the hypothesis that *T. carnifex* alleles occur naturally inside the national boundary of Germany.

Methods

Sampling and DNA extraction

In 2020/2021 we tried to revisit the five ponds in Berchtesgadener Land described in Schmidtler (1976). Two of these could be relocated. One pond, near Sillersdorf (1/a in Table 1), was sampled. The other pond, near Schönau am Königssee (e in Table 1), had a dense fish population and no *Triturus* (or other) newts could be found. Instead we sampled a nearby pond ca. 300 meters distant (2 in Table 1). The two sampled ponds are positioned 27 kilometres apart as the crow flies (Fig. 1). We took mouth swabs for two newts from Sillersdorf (two adult males) and 14 from Schönau am Königssee (two adult males, four adult females and eight juveniles), using 4N6FLOQSwabs (Copan). For each pond, we also collected five eggs. Material was stored in 96% ethanol. DNA was extracted using a salt extraction protocol (Sambrook & Russell 2001) with the Wizard® Genomic DNA Purification Kit (Promega).

Mitochondrial DNA sequencing and analysis

We Sanger sequenced one mitochondrial DNA marker (ND4) following the protocol described in Wielstra et al. (2013). Sequences were edited in Geneious Prime 2020.2.2

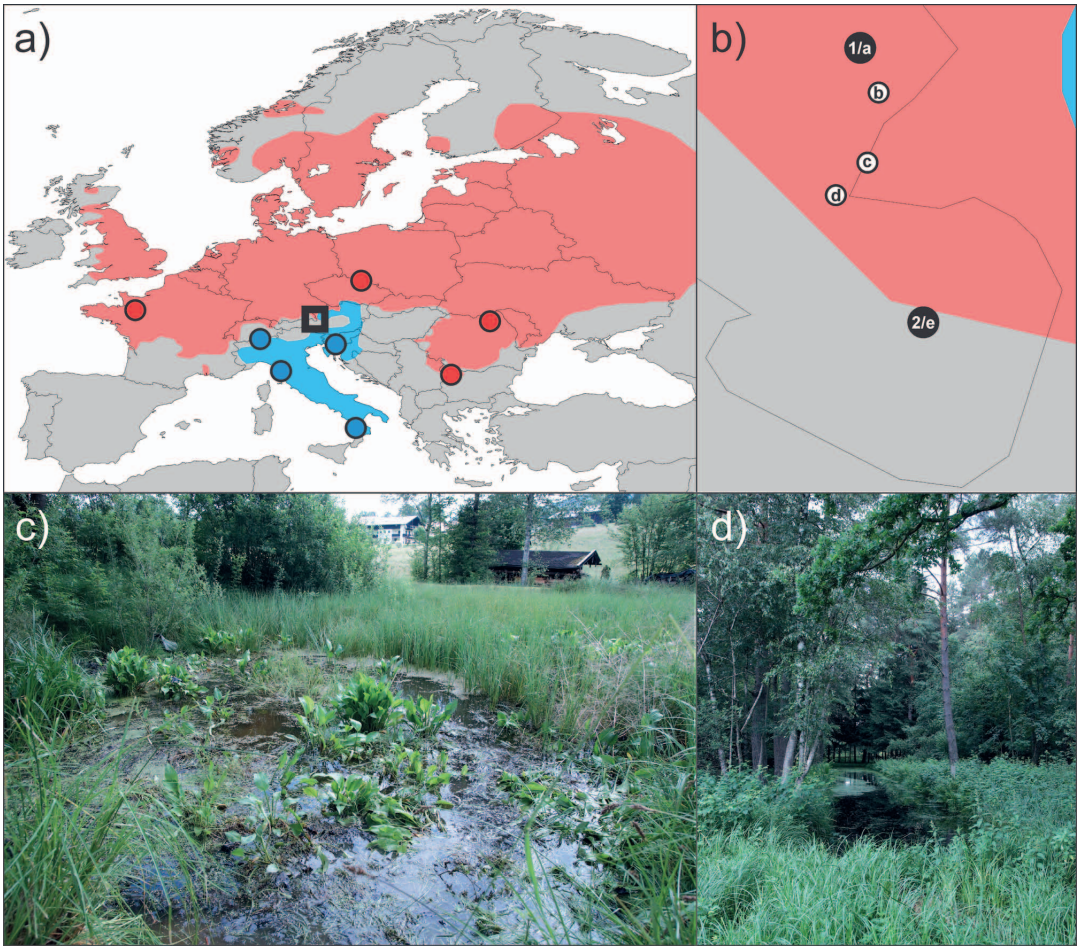

Figure 1. Sampled crested newt localities: (a) rough outlines of the ranges of the northern crested newt (*Triturus cristatus*) and the Italian crested newt (*T. carnifex*) in red and blue, respetively, based on Wielstra et al. (2014); red and blue dots are the reference populations for the two species and the square shows our study area lifted out in b; (b) study area in Berchtesgadener Land in extreme southeast Germany, with localities 1–2 examined in this study and a–e presented in Schmidtler (1976). The photographs are from the localities (c) Schönau am Königssee (locality 2 in b) and (d) Sillersdorf (locality 1 in b). For details of localities see Table 1.

Table 1. Sampling details for crested newts localities: 1–2 are examined in this study, a–e are described in Schmidtler (1976) and reference populations are pure *Triturus cristatus* and *T. carnifex* populations originally used to confirm that the studied markers are species diagnostic (see Fig. 1). For localities marked with an * the coordinates of the ponds could not be traced, so we use the coordinates for the place names in Google Earth instead.

Code as in Fig. 1	Locality	Latitude	Longitude
	Localities examined in this study		
1	Germany: Sillersdorf	47.854	12.924
2	Germany: Schönau am Königssee	47.615	12.978
	Localities from Schmidtler (1976)		
a	Germany: Sillersdorf	47.854	12.924
b	Germany: Ainring*	47.815	12.942
c	Germany: Marzoll*	47.755	12.930
d	Germany: Bayerisch Gmain	47.725	12.903
e	Germany: Schönau	47.617	12.981
	Reference localities *T. carnifex*		
	Switzerland: Locarno	46.167	8.800
	Slovenia: Kramplje	45.733	14.500
	Italy: Pisa	43.717	10.400
	Italy: Fuscaldo	39.417	16.033
	Reference localities *T. cristatus*		
	France: Mayenne	48.300	-0.617
	Poland: Tłumaczów quarry	50.558	16.434
	Romania: Brădătel	47.491	26.178
	Bulgaria: Montana	43.416	23.222

(https://www.geneious.com) and aligned against the database of Crested Newt haplotypes from Wielstra et al. (2013). With the 'Haplotypecollapser' function in FaBox 1.5 (Villesen 2007) we determined to which haplotype(s) our new mtDNA sequences were identical.

Nuclear DNA SNP genotyping and analysis

We genotyped ten previously described nuclear DNA SNP markers that are diagnostic for *T. carnifex* versus *T. cristatus* across the natural range of the species (Wielstra et al. 2016). Genotyping was conducted using a protocol involving Kompetitive Allele Specific PCR (KASP) technology (LGC genomics, UK) at the SNP genotyping facility of the Institute of Biology, Leiden University. KASP genotyping involves fluorescence-based genotyping using allele specific primers that also contain a unique tail sequence. Different fluorescently labelled primers present in the KASP master mix correspond to each tail sequence and are activated when incorporated during subsequent PCR cycles, with further cycling causing signal intensity to increase (Semagn et al. 2014). Hence, for each marker for each individual, the presence of one or the other SNP variant, or both in the case of heterozygosity, can be determined. Full details of the approach can be found in (Wielstra et al. 2016).

For each individual we determined the hybrid index, i.e. the proportion of alleles present from a particular parental species (Barton & Gale 1993). We focused on the proportion of *T. carnifex* alleles, so the hybrid index runs from 0 (pure *T. cristatus*) to 1 (pure *T. carnifex*). We visualized the KASP genotyping data for the nuclear markers using the R package HIest (Fitzpatrick 2012), which determines the genomic composition of individuals based on ancestry (the fraction of alleles derived from each parental species) and heterozygosity (the fraction of loci heterozygous for alleles from each parental species).

Morphology

Newts were checked for three external characters that are considered helpful in telling *T. cristatus* and *T. carnifex* apart (Fig. 2):

1. Presence/absence of a yellow vertebral line: In juveniles, the presence of a bright yellow vertebral line, which continues on the upper edge of the tail, is highly indicative of *T. carnifex*. While adult *T. carnifex* females may retain this feature, it fades with time and they rarely show a pronounced yellow vertebral line. If there is any yellow colouration visible at all in juvenile *T. cristatus*, it is usually limited to the upper edge of the tail.

2. Density of white speckles along the lateral sides: While adults of *T. cristatus* usually have many white spots along the lateral sides, *T. carnifex* typically have none or only a few. This character was not checked in juveniles as these usually have a lot of white speckling in both species.

3. Qualities of the ventral pattern: The ventral pattern of adult *T. cristatus* usually shows rather small and sharply demarcated dark spots in the abdominal region, with the throat coloration consisting of yellowish, white and black elements. In adult *T. carnifex*, on the other hand, the ventral pattern typically shows larger and washed-out spots on the abdomen, and the throat a black base colour with white spots. In freshly metamorphosed juveniles the ventral pattern is not yet fully developed and hence not considered informative.

It should be taken into account that there is large variation for these three characters within species and this is exacerbated in hybrid zones, so species identification based on external characteristics alone is unreliable (Arntzen 2003, Fahrbach & Gerlach 2018).

Results

We recover a single *Triturus cristatus* mitochondrial DNA haplotype (Tcri02, GenBank Accession Number GU982384) across all 26 individuals. Two out of ten nuclear DNA SNP markers did not perform well and were discarded. Therefore, eight nuclear DNA SNP markers (16 alleles)

were genotyped in total. The number of *T. carnifex* alleles present in the 26 individuals ranged from 0 to 6 (average 2.4) and the hybrid index ranged from 0 to 0.375 (average 0.151). Twenty-four out of 26 individuals possessed at least one *T. carnifex* allele. Locality Sillersdorf (1 in Fig. 1) has a higher average hybrid index than Schönau am Königssee (2 in Fig. 1), namely 0.223 versus 0.125 (Fig. 3; Supplementary document 1).

Regarding external characteristics, none of the newts corresponds fully to either *T. carnifex* or *T. cristatus*. All four adult females lack the yellow vertebral line (and in adult males this line is never present). Six out of eight juveniles do show the yellow vertebral line though, in agreement with the findings of Schmidtler (1976). However, this line does not continue all the way from the neck to the tailtip in most cases (Fig. 4). Lateral speckling and ventral

Figure 2. Dorsal, ventral and lateral views of crested newts, showing the morphological characters discussed in the main text. On the left is the northern crested newt (*Triturus cristatus*) and on the right the Italian crested newt (*T. carnifex*).

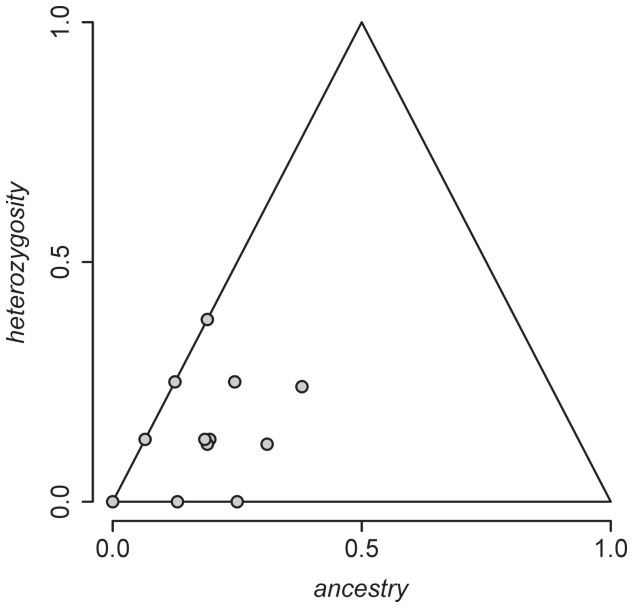

Figure 3. Genetic composition of crested newts from Berchtesgadener Land based on eight nuclear DNA markers. Ancestry is the fraction of alleles derived from *Triturus carnifex* and heterozygosity the fraction of loci that is heterozygous (possessing both a *T. carnifex* and a *T. cristatus* allele). Therefore, the lower left corner of each triangle corresponds to a pure *Triturus cristatus* genotype, the lower right to a pure *T. carnifex* genotype, and the top corner to a pure F1 hybrid. Note that some individuals overlap.

patterning in juveniles is not considered informative and therefore we only focus on the eight adults examined. They show only sparse white speckles on the flanks, as is characteristic for *T. carnifex* (Fig. 4). The basic colour of the abdominal region appears a deep orange, on which there are variably pronounced dark spots. Although spots are washed-out in some individuals, these are of smaller size than is typical for *T. carnifex* (Fig. 4). All but one show a throat coloration that clearly contrasts with the belly and is mostly dark, which is typical of *T. carnifex*. However, four out of eight individuals also show (slight) orange on the throat, which in turn is more typical of *T. cristatus*.

Discussion

The position of the natural hybrid zone between the two Crested Newt species *T. carnifex* and *T. cristatus* has been incompletely documented (Maletzky et al. 2008a, Mikulíček et al. 2012, Arntzen et al. 2014, Wielstra et al. 2014, Mačát et al. 2019). We study the genotype and morphology of Crested Newts from two populations in Berchtesgadener Land, to test the hypothesis, posed by Schmidtler (1976), that genetic admixture between *T. carnifex* and *T. cristatus* occurs in southeastern Germany. While only a *T. cristatus* mitochondrial DNA haplotype is present in the examined populations, our panel of eight nuclear DNA SNP markers provides clear evidence in support of Schmidtler's hypothesis of genetic

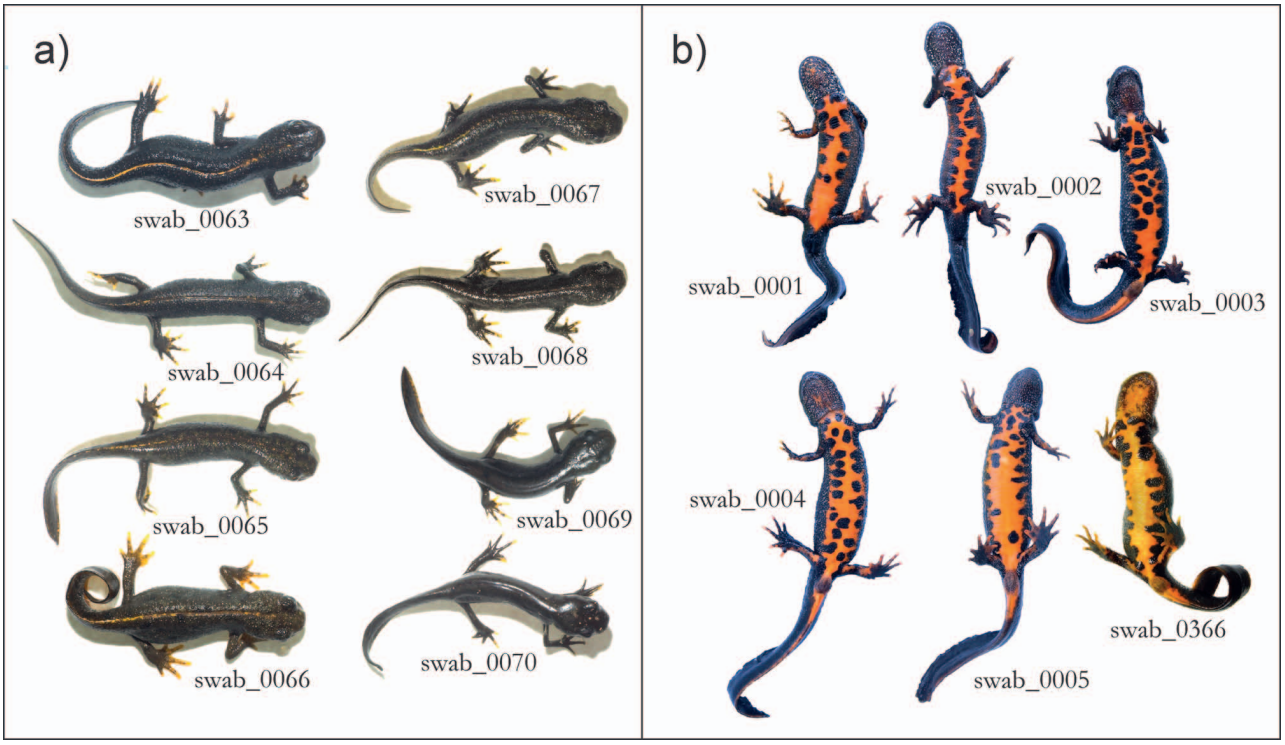

Figure 4. Pictures of crested newts from Schönau am Königssee in Berchtesgadener Land: (a) dorsal sides of eight juveniles, (b) ventral sides of six adults. Individuals within but not between panels are approximately to scale. Labels correspond to Appendix 1.

admixture with *T. carnifex*. Although they are in the minority in the sampled populations, there are *T. carnifex* alleles present (up to six out of a total of 16 genotyped alleles, so up to 37.5%). The majority of alleles belongs to *T. cristatus*.

We also confirm the presence of morphological features characteristic for *T. carnifex*. While external characteristics are notoriously variable within Crested Newt species, and cannot solely be used for species identification, the presence of a yellow vertebral line in most of the sampled juveniles (first described by Schmidtler 1976) is highly indicative of *T. carnifex* influence. We did not see a clear morphology-genotype relationship: while two out of three juveniles with the highest number of *T. carnifex*-alleles (3 out of 16) also had the clearest yellow vertebral line, the third individual did not show this line at all (see Fig. 4 and Supplementary document 1). The sparse lateral speckling observed in the six adults is more akin to *T. carnifex* as well. The abdominal pattern cannot be unequivocally assigned to one of the two species in the six adults, but again shows features typical of *T. carnifex*.

Based on our genetic and morphological results, we conclude that the hybrid zone between *T. carnifex* and *T. cristatus* protrudes into the extreme southeast of Germany. Given that *T. cristatus* is the genetically prevalent species, we hit upon the westernmost section of the *T. carnifex*–*T. cristatus* hybrid zone here (Arntzen et al. 2014, Wielstra et al. 2014). We cannot say at this stage if the hybrid zone is stable or moving. Perhaps the hybrid zone is located at the place where the two species initially obtained secondary contact, after they independently expanded from their glacial refugia. Or it might be that the *T. carnifex* alleles represent a 'shadow' of a formerly more extensive range of this species, that receded because *T. cristatus* expanded at its expense, leaving a genomic footprint of hybrid zone movement (as shown in other Crested Newt hybrid zones, see Wielstra et al. 2017a, 2017b). Finally, we could be looking at the front of a westward moving hybrid zone, with *T. carnifex* outcompeting *T. cristatus* and moving into Germany. We know that *T. carnifex* has already expanded into Central Europe from the Balkan Peninsula, eastwards around the Alps, at the expense of the Danube Crested Newt *T. dobrogicus*, because *T. carnifex* has captured *T. dobrogicus* mtDNA and transported it outside of the lowland range to which *T. dobrogicus* is restricted (Mikulíček et al. 2012, Mačát et al. 2019, Wielstra et al. 2021). To distinguish between these biogeographical scenarios, it is key to determine if gene flow across the hybrid zone is symmetrical (as expected for a stable hybrid zone) or is biased towards either *T. carnifex* or *T. cristatus* (the expanding species is expected to show introgression in the area where it has outcompeted the other species; Wielstra 2019). This requires a broader sampling in Germany, Austria and the Czech Republic, including a much larger number of markers than studied here. Meanwhile, we recommend, using the technique in the present paper, to test if genetically (mostly) pure *T. carnifex* naturally occur further east in Germany.

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Supplementary data

The following data are available online:

Supplementary document 1. Details on sampled crested newts.