Truly invasive or simply non-native? Insights from an artificial crested newt hybrid zone

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Abstract

Introductions of non-native species can pose serious threats to native populations and ecosystems. However, the impact of introduced species depends on intrinsic characteristics, local habitat conditions, and the interaction with native species. Case-specific management strategies may therefore be required. Using phenotypic characters and molecular markers for species identification, we provide insights into an artificial hybrid zone between two closely related newt species, the native *Triturus cristatus* and the introduced *T. carnifex*, near Tübingen, south-west Germany. Our analyses revealed a central Italian origin of the non-native *T. carnifex* and suggested their sustained presence in the study area for at least six years, probably much longer. In some ponds, extensive hybridization with native *T. cristatus* was detected. However, we found no evidence for a displacement of the native species by its non-native congener. The gradient from pure *T. carnifex* to pure *T. cristatus* currently extends over 7 km. A future expansion of the hybrid zone and swamping of a neighboring *T. cristatus* meta-population appears unlikely under the local configuration of breeding ponds. We propose to monitor the hybrid zone using genetic markers for evaluating the direction and speed of gene flow, complemented by capture-recapture studies to reveal trends in species-specific population sizes. To protect the native *T. cristatus*, we recommend practitioners to maintain their habitats, for example, by preventing illegal release of gold fish, by counteracting early drying of the breeding ponds, and by regularly cutting back trees and shrubs along the shoreline.

Keywords

amphibians, genetic monitoring, introgressive hybridization, management, non-native, *Triturus*

Henri A. Thomassen and Alexander Kupfer are to be considered joint senior author.

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1 INTRODUCTION

Non-native species play a major role in global environmental change. Several recent studies emphasize the detrimental effects that non-natives can have on native ecosystems (Gallardo et al., 2016; Jernelov, 2017; Lavergne et al., 1999; Molnar et al., 2008), and call for a rapid eradication to avert ecological and economic damage (Pluess et al., 2012). Up to 25% of all species that have been introduced outside their native range become invasive (Jeschke & Strayer, 2005; Williamson, 1997), meaning that they spread rapidly and reach dominance in the new environment (Valéry et al., 2008). The percentage of introduced species causing some sort of negative impacts on native systems is smaller, because their effects on native species and ecosystems can also be neutral or even positive. For example, non-native species can provide prey or habitat to endangered native species or may increase the stability of ecosystems under climate change (Cattau et al., 2016; Dickie et al., 2014; Lampert et al., 2014; Schlapefuer et al., 2011; Sogge et al., 2008). For the management of introduced species, it may therefore be appropriate to distinguish between “non-native,” “invasive”, and “harmfully invasive” species. We consider a species non-native to an area where it did not historically occur and where it arrived by human-mediated ways of dispersal. Among non-native species, invasive species are those acquiring a competitive advantage in the new environment, enabling rapid spread and population dominance (Valéry et al., 2008). Hararmfully invasive species are invasives with a strong and predominantly negative effect on the invaded ecosystems.

Besides habitat loss and overexploitation, harmfully invasive species are globally among the most important threats to many taxa, including amphibians (Bax et al., 2003; Falaschi et al., 2020; Pereira et al., 2012). Competition, predation, and the transmission of pathogens (Miaud et al., 2016; Remon et al., 2016; Richter-Boix et al., 2013) but also introgressive hybridization can have severe effects on native species (Dufresnes et al., 2015; Falaschi et al., 2020; Fitzpatrick & Shaffer, 2007; Kraus, 2015; Meilink et al., 2015; Riley et al., 2003). When individuals from two distinct species with incomplete pre- or postzygotic isolation interbreed, the genetic integrity of the parental species can be compromised due to backcrossing (Allendorf et al., 2001; Dufresnes et al., 2016; Rhym & Simberloff, 1996). In amphibians, numerous examples of introgressive hybridization exist from natural and artificial hybrid zones. For example, under field conditions, hybrids were observed in European pond frogs (Pelophylax, Arioli et al., 2010), tree frogs (Hyla, Dufresnes et al., 2015), tiger salamanders (Ambystoma, Riley et al., 2003), and many newt species (Triturus, Lissotriton, Arntzen & Hedlund, 1990; Arntzen et al., 2021; Arntzen et al., 1998; Dufresnes et al., 2016; Wielstra & Arntzen, 2010; Wielstra & Arntzen, 2020; Zielinski et al., 2019).

Hybridization appears to be particularly common along contact zones between members of the Triturus genus, for example, the Northern crested newt, Triturus cristatus (Laurenti 1768), and the Italian crested newt, Triturus carnifex (Laurenti, 1768) (Wielstra et al., 2014). The natural hybrid zone between the two species is relatively narrow (approximately 15–30 km) with a predominance of pure parental genotypes at the edges and hybrids in the center (Maletzky et al., 2008; Mikulicek et al., 2012). Apart from their natural hybrid zone, the two species co-occur along artificial contact zones caused by human translocations of T. carnifex into the native range of T. cristatus. Such introductions have been reported from several European countries (UK: Brede, 2000; Brede, 2015; Griffiths, 1996, Switzerland: Arntzen & Thorpe, 1999; Dufresnes et al., 2016; Dufresnies et al., 2019, the Netherlands: Bogaerts, 2002; Meilink et al., 2015; Wielstra et al., 2016, and Germany: Franzen et al., 2002; Maletzky et al., 2008). These introductions deserve special attention by conservationists, because both species face a strong decline throughout their native ranges, and introgressive hybridization between the two species may potentially lead to “genetic pollution” (Butler, 1994). The effect may, however, not be bi-directional, because it appears that alleles mainly introgress from the native into the non-native gene pool in the wake of an outwardly expanding hybrid zone (Currat et al., 2008).

Based on their external morphology, T. cristatus and T. carnifex are relatively hard to distinguish, especially when hybrids are present (Maletzky et al., 2008; Brede, 2015; Figure 1). Several external morphological traits have been proposed for species discrimination (Arntzen & Wallis, 1994; Brede, 2000; Wolterstorff, 1923). External characters, however, turned out to be unreliable when backcrossing of hybrids with one of the parental species occurs, and genetic tools are therefore required for studying hybrid zones (Brede, 2015; Dufresnies et al., 2016; Wielstra et al., 2016).

Here, we study an artificial hybrid zone between T. cristatus and T. carnifex near Tübingen (Germany). The studied waterbodies cover a gradient from a forested site (100% forest cover within a radius of 100 m) and two sites at the forest edges (79% and 44% forest cover in the surroundings) to several ponds in an agricultural matrix (no forest cover in the surroundings). In the north-west of the study area, a meta-population of T. cristatus with high conservation relevance is located. To distinguish between species, identify hybrids, and examine the
geographic origin of the non-native newts, we employ external morphological criteria commonly used for species identification and molecular markers consisting of microsatellites and mitochondrial DNA fragments (Canestrelli et al., 2012; Dufresnes et al., 2016; Dufresnes et al., 2019; Wielstra et al., 2013; Wielstra et al., 2021). We incorporate information on pond age to develop a likely scenario for the dispersal of crested newts in the study region.

2 MATERIAL AND METHODS

2.1 Study species

Triturus cristatus is distributed north of the Alps, spanning Great Britain and most of central Europe from western France to the Ural Mountains in the east (e.g., Thiesmeier & Kupfer, 2000; Wielstra et al., 2014). Typically, the species inhabits medium-sized waterbodies with dense aquatic vegetation and strong insolation (Thiesmeier & Kupfer, 2000). Triturus cristatus has a relatively slender build, a dark, warty skin, and a yellow to orange belly with sharply edged black ventral dots (Arntzen, 2003; Arntzen & Thorpe, 1999). In contrast, T. carnifex has a relatively stout body, a smooth grayish skin with only few white lateral stippling, and round, blackish, diffusely edged dots on the venter (Arntzen & Thorpe, 1999; Brede, 2000, Figure 1). Female, juvenile, and subadult T. carnifex can often be identified by a yellow vertebral stripe (Franzen et al., 2002). The native range of T. carnifex covers most parts of Italy and the southern Alpine area (e.g., Andreone & Marconi, 2006; Arntzen, 2003). Habitat requirements of both crested newt species are similar, although T. carnifex might thrive in a broader range of habitats, including anthropogenically impacted waterbodies (Arntzen, 2003; Arntzen & Thorpe, 1999; Melink et al., 2015).

Native populations of T. cristatus and T. carnifex are legally protected under annexes II and IV of the European Habitats Directive (92/43/EEC, Arntzen, Kuzmin, et al., 2009; Romano et al., 2009). In Germany and in the state of Baden-Württemberg, T. cristatus is considered vulnerable and endangered in the national and regional Red Lists, respectively (Geiger et al., 2020; Laufer, 1999). Along a small contact zone in northern Austria and the Czech Republic, the native ranges of the two species overlap and hybrids were found (Maletzky et al., 2008; Mikulíček et al., 2012). The occurrence of native T. carnifex in the very south-east of Germany was suspected based on morphological criteria (Schmidtler, 1976). A recent molecular study confirmed the presence of T. carnifex alleles in this region, but no genetically pure individuals of T. carnifex were found (Fahrbach et al., 2021).

2.2 Study area and sampling sites

Our study area is located near Tübingen, Germany (48.52°N, 9.05°E, 345 m–469 m a.s.l.; Figure 2), and is characterized by a forested hill and an adjacent river valley. The area is ecologically well-studied and among the most species-rich regions in Germany (Gottschalk, 2019). Even if waterbodies are rare, eleven different native amphibian species were recently found (Bamann, 2019). Triturus cristatus, which had been quite abundant in former times, has drastically decreased in the south-eastern part of the study area, where it likely disappeared during the mid-1900s (Löderbusch, 1987; Schmid, 1966). A single crested newt record is available from 1987 (Srauba, 2013; site B, Figure 2). More recently, crested newts were resighted in several ponds, and habitat management actions were set in place (Straub, 2013).

FIGURE 1 Phenotypic appearance of typical male crested newts. Triturus cristatus (left), Triturus carnifex (right), and T. cristatus × carnifex hybrid (middle). Note that hybrid phenotypes are highly variable and, if at all, are difficult to distinguish from parental phenotypes. The shape of the male newts’ crest is not a suitable indicator of species membership.
We first observed putative *T. carnifex* at site B in May 2015. The site is located on top of the hill, enclosed by forest, and consists of one medium-sized old quarry pond (≈ 300 m²) and a few small temporary waterbodies nearby. In contrast, sites A and C are located at the forest edges approximately 1.1 and 0.8 km to the east and north-west from site B, and sites D–H are embedded in an agricultural matrix (Figure 2). We started collecting genetic samples in 2016 at sites B, D, and H, for which the presence of crested newts was known. During 2017–2021, we checked a further 16 ponds for the presence of crested newts. We collected genetic samples at sites A, C, and E in 2017–2019. A few additional samples were collected from site B in 2017 and 2018, and site D was sampled again in 2019. In 2021, we surveyed sites F and G for the first time and morphotyped the crested newts from these sites without collecting genetic samples. The surveyed ponds include all potentially suitable breeding ponds within a radius of 5 km from site B, the nearest known population of pure *T. cristatus* (site H, Figure 2), and all waterbodies (except for garden ponds and private fish ponds) that could potentially serve as “stepping stones” from pure *T. carnifex* or admixed populations towards the nearest *T. cristatus* site.

| site | A | B | C | D | E | F | G | H |
|------|----|----|----|----|----|----|----|----|
| n    | 4  | 38 | 15 | 19 | 1  | -  | -  | 21 |
| mean ancestry | 0.00 | 1.00 | 0.77 | 0.27 | 0.43 | -  | -  | 0.01 |

**Figure 2** Occupied and potential crested newt sites (*Triturus cristatus/T. carnifex*) and interpond distances in the study area. Sites that were considered largely unsuitable for crested newt breeding include those with fish and those strongly shaded, or heavily anthropogenically impacted. We present the number of individuals that were sampled for genetic analyses between 2016 and 2019 (n) and the genetic ancestry of the populations. A mean ancestry of 0 indicates a population of pure *T. cristatus*, 1 indicates pure *T. carnifex*, and values in between represent admixed populations. At site A, four pure *T. cristatus* were included in the genetic sampling. However, we observed phenotypic *T. carnifex* at the site a few weeks before our genetic sampling campaign. No genetic samples were available from sites F and G. Note the gradual transition from non-native *T. carnifex* at site B, over admixed populations at sites C–G, towards native *T. cristatus* at site H.

2.3 | Field methodology

Surveys of crested newts were conducted with funnel traps (floating, 47 × 23 × 23 cm) and dip-netting. Based on phenotypic criteria, we distinguished *T. cristatus*,
T. carnifex, and hybrids in the field. We refrained from species identification of larvae using phenotypic traits, because no clear external characters are available to discriminate between the species in early to mid-aged larval stages. Phenotypic species classification was later compared to genetic classification.

A total of 101 crested newts from six different sites were sampled for genetic analyses. Except for 18 individuals (15 larvae/juveniles and 3 adults from ponds A, B, and C), which were preserved as museum specimens, newts were sampled noninvasively using skin swabs (Prunier et al., 2012). We used conventional cotton buds to swab newts several times gently over the back, flanks, and tail. After swabbing, cotton buds were stored in SDS buffer or immediately dried with silica gel and brought to the laboratory.

2.4 Laboratory procedure and data analysis

DNA was extracted from cotton swabs using standard protocols, including a proteinase-K treatment and filtration with QIAamp® spin columns (Qiagen, Hilden, Germany). For cotton buds stored in SDS buffer, we modified the protocol by refraining from using the buffer ATL, adding the proteinase K directly into the SDS buffer, and consequently increasing the amount of the reagents (proteinase K, buffer AL, ethanol) threefold.

Following the procedure outlined in Dufresnes et al. (2016), we genotyped the sampled newts with nuclear (nDNA) and mitochondrial (mtDNA) genetic markers, allowing the detection of both current and historical hybridization events. We used a set of 11 microsatellite loci (nDNA) and one marker of the mitochondrial control region (CR, primer pair L-CR-Uro/H-tRNAPhe-Uro; Dufresnes et al., 2016). The microsatellite primers were originally designed for T. cristatus and/or T. carnifex and have proven useful for studying hybridization between the two species (Dufresnes et al., 2016; Dufresnes et al., 2019; Krupa et al., 2002). Mitochondrial genes have a strong tendency to introgress, in the wake of an outwardly expanding hybrid zone mainly from the native into the non-native species (Currat et al., 2008). This means that native mtDNA is usually retained in the gene pool of a population, even if the native nDNA may become entirely replaced (“cyto-nuclear discordance,” Toews & Brelsford, 2012). To infer the geographic origin of the non-native T. carnifex, we additionally sequenced the mitochondrial ND4-marker (primer pair ND4carnF1/ND4carnR2, Canestrelli et al., 2012; Table 1) in eleven newts with mtDNA of T. carnifex, six individuals from site B, four from site C, and one from site D.

Microsatellite polymerase chain reactions (PCRs) were carried out in three multi- and one singleplex reactions with a volume of 10 μl (Table 1). Fluorescent dye-labeled primers were prepared according to the manufacturer’s instructions and combined for multiplex PCRs. Each reaction contained 5 μl of Qiagen 2 × Master-Mix, 2 μl of primer mix, 1 μl of PCR-grade H2O (0.8 μl of H2O + 0.2 μl of BSA in the singleplex and the multiplex 2 reaction), and 2 μl of template DNA. The temperature profile for the PCRs included a denaturation for 5 min at 95°C, followed by 10 cycles of annealing with tₐ being the primer-specific annealing temperature: 30 s at 95°C, 90 s touchdown from (tₐ + 5°C) to (tₐ + 0.5°C), and 30 s at 72°C. The extension step was performed with 28 cycles (25 for multiplex 3) with 30 s at 95°C, 90 s at tₐ, and 30 s at 72°C, followed by 30 s at 60°C.

For the mtDNA-analysis, amplicons were amplified in two separate 25 μl PCRs with 2.5 μl of 10 × Qiagen PCR buffer, 2.5 μl of 25 mM MgCl₂, 3 μl of dNTPs, 1 μl of BSA, 1 μl of each primer (10 pmol/μl), 2 μl of template DNA, 0.15 μl of Taq polymerase, and 11.85 μl of PCR-grade H2O (CR) / 0.17 μl of Taq polymerase, and 11.83 μl of PCR-grade H2O (ND4). After an initial denaturation for 3 min at 94°C, PCRs included 35 cycles with 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by a final step with 72°C for 5 min. Clean-up of PCR-products was performed using the Promega Wizard® DNA clean-up system (Promega, Fitchburg, US) and amplicons were sequenced with the forward primers and Big Dye v. 3.1 reaction chemistry. All amplicons were run on an ABI 3730 48-capillary sequencer at the LMU Sequencing Service, Munich.

We aligned sequences of 312 bp (CR) and 555 bp (ND4) with publicly available sequences from NCBI GenBank®. Haplotypes were called based on full matches of the candidate sequences with the references. Microsatellite alleles were scored using GeneMarker® V2.4.0, and manually checked. We used GenePop in R (R Core Team, 2007; Rousset, 2008) to check for Hardy–Weinberg equilibrium and linkage disequilibrium in the pure T. carnifex population (site B) and nine reference populations with >5 sampled T. cristatus. P-values were adjusted with the Benjamini–Hochberg correction. The presence of potential null alleles was assessed with Micro-Checker (van Oosterhout et al., 2004).

To identify pure parental individuals and hybrids from the genetic data, we used three different approaches, that is, Bayesian cluster assignment with STRUCTURE 2.3.4 (Pritchard et al., 2000) and NewHybrids Version 1.1 beta (Anderson & Thompson, 2002) as well as coefficients of ancestry and interclass heterozygosity (Fitzpatrick, 2012). In STRUCTURE, we tested 1 to 16 genetic clusters (K) with 100,000 iterations after a burn-in of 10,000.
The maximum number of tested clusters (K = 16) corresponds to the total number of sampled ponds from within and outside the hybrid zone. We assumed correlated allele frequencies and the admixture model. We carried out ten replicate runs per K and determined the most likely K with the deltaK-method in Structure Harvester (Earl & von Holdt, 2012; Evan et al., 2005). For this particular K, we then repeated the STRUCTURE analysis in 25 independent replicates with 500,000 iterations each, after a burn-in of 50,000. We combined the replicates with Clumpp (Jakobsson & Rosenberg, 2007) and displayed ancestry coefficients graphically using the R-package strataG (Archer et al., 2017). Furthermore, individuals were assigned to six early-generation hybrid classes (parental species, first and second generation hybrids) using NewHybrids with 500,000 iterations after a burn-in of 50,000. Using the “z-option”, we invoked 163 individuals from 10 nearby populations (< 65 km from the study area) as references for T. cristatus and the individuals from site B (all identified as pure T. carnifex by STRUCTURE) as references for T. carnifex. We manually calculated coefficients of ancestry and interclass heterozygosity from a set of eight species-diagnostic markers (all but A8). Thereby, we considered an allele diagnostic for one species when it was frequently observed in that species but had a maximum of two occurrences in the other species. Species-diagnostic alleles for T. cristatus and T. carnifex were coded with 0 and 1, respectively. We averaged ancestry and heterozygosity coefficients across loci such that ancestry coefficients of 0 (T. cristatus) and 1 (T. carnifex) indicated pure parental genotypes and heterozygosity coefficients of 0 and 1, respectively, indicated a pure parental genotype and full interspecies heterozygosity. To infer the autochthonous status of T. cristatus in the study area, we ran STRUCTURE on a subset of our data, containing only pure T. cristatus from the study region and from the 10 reference populations (K = 1–13, 100,000 iterations, burn-in of 10,000, 10 replicates).

### RESULTS

We obtained high-quality DNA of sufficient quantity permitting microsatellite analyses and the sequencing of two distinct mitochondrial DNA markers in 98 and 84 out of 101 sampled crested newts from six different sites within

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**Table 1** Nuclear and mitochondrial genetic markers used in this study, references, annealing temperatures (t, in °C), and reaction names. We report ranges of fragment lengths and haplotypes observed in the reference populations and present the number of species-diagnostic alleles (n) for the microsatellite markers.

| Microsatellites | Reference | t, | Reaction | T. carnifex | T. cristatus |
|-----------------|-----------|----|----------|-------------|-------------|
| Marker name     | Reference |    |          | range n     | range n     |
| Tcri13          | Krupa et al. (2002) | 53 | Multiplex 1 | 88–100 3 | 109–131 7 |
| Tcri29          | Krupa et al. (2002) | 53 | Multiplex 1 | 250–258 3 | 290–339 8 |
| Tcri36          | Krupa et al. (2002) | 53 | Multiplex 1 | 201–221 4 | 234–314 15 |
| Tcri46          | Krupa et al. (2002) | 53 | Multiplex 1 | NA - | 269–314 11 |
| Tcri35          | Krupa et al. (2002) | 55 | Singleplex | 282–370 3 | 203–235 9 |
| A7              | Dufresnes et al. (2016) | 56 | Multiplex 2 | 175–199 3 | 215–252 9 |
| D5              | Dufresnes et al. (2016) | 56 | Multiplex 2 | NA - | 284–284 1 |
| A8              | Dufresnes et al. (2016) | 58 | Multiplex 3 | 284–320 - | 253–485 - |
| A126            | Dufresnes et al. (2016) | 58 | Multiplex 3 | 278–384 4 | 304–372 10 |
| D1              | Dufresnes et al. (2016) | 58 | Multiplex 3 | 192–192 1 | 214–282 7 |
| D127            | Dufresnes et al. (2016) | 58 | Multiplex 3 | 229–229 1 | 185–185 1 |

| Mitochondrial markers | Reference | t, | Region | T. carnifex haplotype | T. cristatus haplotype |
|-----------------------|-----------|----|--------|-----------------------|-----------------------|
| Primer                | Reference |    |        |                       |                       |
| L-CR-Uro/H-tRNA Phe-Uro | Dufresnes et al. (2016) | 55 | control region | CAR3a | CR12/2a/3 |
| ND4carnF1/ND4carnR2    | Canestrelli et al. (2012) | 55 | ND4    | CIII10/11 | - |
the study area (A–E, H, Figure 2). The genetic analyses confirmed that both crested newt species, that is, the native *T. cristatus* and the introduced *T. carnifex*, co-occur in the study area and hybridize in several ponds.

### 3.1 Phenotypic identification

Based on external morphological criteria and coloration, all individuals from site B were classified as *T. carnifex*. In contrast, all sampled individuals from ponds A, E, and H were assigned to the native *T. cristatus*. At pond C, traits of both parental species were observed with some individuals exclusively possessing traits of one of the species and others showing intermediate phenotypes. At pond D, newts were sampled twice at an interval of three years with all but one newt being classified as pure *T. cristatus* in 2016, but two newts classified as *T. carnifex* and nine as hybrids in 2019. Interestingly, one newt, first captured in 2016 and recaptured in 2019, showed remarkable phenotypic plasticity, that is, a blackish dorsal coloration and a sharply edged belly pattern in 2016 but a more grayish skin and diffuse ventral dots in 2019 (Figure 3). As a consequence, the exact same individual was phenotypically identified as *T. cristatus* in 2016 but as a hybrid in 2019. Genetic analyses suggested an F2-like nuclear genetic status for this individual. At sites F and G, two out of four and three out of eight captured individuals showed a hybrid phenotype, whereas the others were classified as *T. cristatus*.

### 3.2 Nuclear genetic status

Data on the newts' nuclear genetic status were obtained from a set of 11 microsatellites. In a few instances, we detected more than two allele peaks in individuals from pond C. As the origin of the additional allele peaks was unclear, we removed the data from our analysis. The markers Tcri46 and D5 failed to amplify in our putative *T. carnifex* samples and were therefore also excluded. Finally, individuals with missing data in more than four out of the nine remaining markers were removed from the dataset. We observed significant deviations from Hardy–Weinberg equilibrium (HWE) in two markers (Tcri29: *T. carnifex*; A126: six out of nine tested *T. cristatus* populations) and potential null-alleles for Tcri35 (*T. carnifex*) and A126 (some *T. cristatus* populations). However, deviations from HWE and potential null-alleles were not consistent across populations; consequently we decided to keep the markers for the analysis. The marker D1 was monomorphic in the pure *T. carnifex* population and D127 was monomorphic, though species-specific, in both species. Tests for linkage disequilibrium did not indicate any significant associations between markers.

We analyzed the microsatellite dataset with three different approaches and obtained highly concordant results. The STRUCTURE analysis with nine polymorphic microsatellite loci suggested a clear segregation into two clusters, representing introduced *T. carnifex* and native *T. cristatus* (Figure S1). Ancestry coefficients from STRUCTURE were strongly correlated with those calculated from diagnostic loci ($R = 0.99, p < .001$). Only individuals with intermediate STRUCTURE ancestry coefficients were identified as hybrids, that is, F2 or backcross to *T. carnifex*, by NewHybrids (Figure 4).

The STRUCTURE analysis confirmed the morphological identification of crested newts as pure *T. carnifex* at site B and pure *T. cristatus* at site H. All three methods suggested extensive hybridization at sites C, D, and E with a high percentage of *T. carnifex* alleles at pond C and a predominance of the *T. cristatus* gene pool at site

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**FIGURE 3** Strong phenotypic plasticity in the belly pattern of a hybrid crested newt (*Triturus cristatus × T. carnifex*). In 2016, the belly pattern showed sharply edged, black dots, whereas dots were diffusely edged and grayish in 2019.
D. While F2-like individuals and backcrosses to *T. carnifex* were abundant, no F1-hybrids and no backcrosses to *T. cristatus* were observed at the studied ponds. “Pure *T. cristatus*” was the most likely early-generation hybrid category for all sampled newts from site H. Discriminating between six discrete hybrid classes is, however, likely to be an oversimplification because ancestry-heterozygosity plots indicated a large variability in the hybrid genotypes at ponds C, D, (and E), suggesting hybridization over multiple generations (Figure 5). At pond D, where newts were sampled in 2016 (*n* = 8) and 2019 (*n* = 11), no significant shift of newt ancestry was detected within the three years (mean ancestry coefficient 2016: 0.269, mean ancestry coefficient 2019: 0.282, F$_{1.18}$ = 0.013, *p* = 0.912). At least some *T. cristatus* from sites A and D clustered with individuals from site H and other populations from central Baden-Württemberg (Figure S2, Figure S3).

### 3.3 Mitochondrial genetic status

The studied CR marker allowed a clear discrimination between the species. Within our study area, we found one CR haplotype each for *T. carnifex* (CAR3a) and *T. cristatus* (CR12/2a/3). The two lineages differed clearly (8 bp). All sampled newts at sites A and H possessed the mtDNA of *T. cristatus*, whereas newts from site B exclusively carried mtDNA of *T. carnifex*. Mitochondrial DNA from both species was found at sites C and D, with a strong predominance of the *T. carnifex* mtDNA at pond C and the *T. cristatus* mitotype at pond D (Figure 4).

In a subset of eleven *T. carnifex*/hybrids from the study area, we consistently observed the ND4-haplotype CIII10/11. This haplotype has previously been reported from central Italy (Mulino di Pianoro, Emilia-Romagna; Minucciano, Tuscany; Canestrelli et al., 2012) and the
DISCUSSION

Our study confirmed a new record of introduced Italian crested newts (*T. carnifex*) near Tübingen (Germany) with a central Italian origin. Their low allelic richness indicates that the population was founded by a limited number of individuals, theoretically by as few as a single breeding pair. *Triturus carnifex* and hybrids with the native Northern crested newt (*T. cristatus*) occur in the study region in several ponds.

4.1 | Phenotypic and molecular species identification

Nuclear and mitochondrial genetic markers confirmed the phenotypic species identification at sites with pure populations of either *T. cristatus* or *T. carnifex*. Conversely, at ponds with hybrids (sites C, D, E), phenotypic classification was only partially in line with the newts’ genetic status. The genetic analyses confirmed the phenotypic classification as “hybrid” in 50% of individuals, whereas in the other 50% genetically pure *T. cristatus* were erroneously considered hybrids. In admixed populations, pure *T. cristatus* were correctly identified by their phenotype in only 40% of the cases. The newts that were erroneously considered *T. cristatus* turned out to be hybrids (Figure 4). Even though we never misclassified genetically pure *T. cristatus* as *T. carnifex* and vice versa, the high degree of misclassifications observed in our study indicates that phenotypic traits are not useful for a reliable species identification in hybrid zones, confirming previous findings (Brede, 2015). The large differences in the number of morpho-/genotyped individuals between sites can be explained by differences in the local population sizes, differences in pond characteristics (e.g., water depth, vegetation cover) affecting capture probability, and different sampling effort.

4.2 | Spread and dominance

During the past century, several amphibian and reptile species were introduced into the study region (Bamann, 2019).
The site of introduction of *T. carnifex* and the pattern of spread cannot be inferred with certainty from our data. However, multiple pieces of evidence are in favor of the scenario that *T. carnifex* was originally released at site B or C and, while living undetected in the area for many years, its alleles have dispersed over 3 to 4.5 km.

Our data show a clear gradient from pure non-native *T. carnifex* to pure native *T. cristatus* within 7 km distance between sites B and H. In between (sites C, D, and E), crested newts showed variable degrees of admixture, specifically a gradually decreasing frequency of *T. carnifex* alleles with increasing geographic distance from site B. We found no alleles of *T. cristatus* at site B and therefore consider as factual that *T. cristatus* was absent immediately before the arrival of *T. carnifex*. At site C, we found mtDNA of *T. cristatus* in 1 out of 16 genotyped individuals and we consider it likely that *T. carnifex* also was the first crested newt at this site, while alleles of *T. cristatus* may have reached the population with immigrating *T. cristatus* and/or hybrids from site D or E. If a vital population of *T. cristatus* had been existing prior to the arrival of *T. carnifex* at site C, we would expect to still find the *T. cristatus* mitotype at high frequencies (Currat et al., 2008). Alternatively, strongly asymmetric mate choice, that is, preferably ♀ *T. carinifex* × ♂ *T. cristatus* mating, or asymmetric incompatibilities could explain the observed pattern (Arntzen, Jehle, et al., 2009). However, we observed many hybrids with the *T. cristatus* mitotype at site D, what makes this explanation rather unlikely.

Apart from the sites where *T. carnifex* has likely been the dominant crested newt species since its arrival, the species did not reach dominance at the studied waterbodies. Ancestry-heterozygosity plots clearly revealed extensive hybridization between the two species at sites C, D, and E for more than two generations. This means that alleles of both species occurred at the sites for at least six years prior to our study (see Cvetković et al., 1996 for the generation time of *T. carnifex*). Our data do not support any factual statement going beyond six years, but it appears likely that *T. carnifex* has thrived in the study area for much longer. Schmid (1966) and Löderbusch (1987) systematically surveyed amphibian breeding ponds in the south-eastern part of our study area (including sites A, B, C, and E) but did not observe crested newts. Crested newts can be overlooked when present in low densities and when standard monitoring methods such as dip-netting are used (e.g., Kupfer, 2001). Nevertheless, the lack of records despite two independent amphibian surveys points to the absence of crested newts from this part of the study area during the 1960s to early 1980s. The first more recent record dates back to 1987, when Straub (2013) observed a crested newt (unfortunately without determining the species) at site B. It seems well possible that this individual was one of the first *T. carnifex* being introduced, because sites E–G have been only constructed after 1980 (E: 1983, Löderbusch, 1987; F: 1987, Kienzler pers. communication; G: 2018, Stoltze pers. communication) and site D is at least not visible on aerial images dating back to 1968. It is therefore hard to imagine from where *T. cristatus* could have immigrated into site B. Four probably suitable breeding ponds for the species during the mid-1900s were in the surroundings of site H, 7 km apart from site B. Three of the waterbodies still exist and are nowadays part of a *T. cristatus* metapopulation (Figure 2).

Considering the lifetime dispersal capacity of *T. carnifex* of approximately two kilometers (Mori et al., 2017) and its likely presence in the study area for a couple of years, it is not surprising that its gene pool has spread. Our data suggest that both crested newt species and/or their hybrids may occasionally disperse 2.2 km (inter-pond distance C–E). Relatively strong dispersal of introduced *T. carnifex* was reported from the Veluwe (Netherlands, Meilink et al., 2015), from the Geneva Basin (Switzerland) after multiple independent releases (Arntzen & Thorpe, 1999; Dufresnes et al., 2016), and from São Miguel (Azores) where congeners are absent and *T. carnifex* was released more than 100 years ago (Machado et al., 1998; Svanberg, 1975). In contrast, the population at Beam Brook (UK, Brede, 2015) has not spread considerably in the 80 years after its introduction and the hybrid zone near Basel (Switzerland) is currently restricted to a few ponds within two kilometers from the putative introduction site (Dufresnes et al., 2019).

### 4.3 Impact on native species

In contrast to Meilink et al. (2015) and Dufresnes et al. (2016), who reported strong introgression of native mtDNA into individuals with a predominance of non-native nDNA, we did not observe substantial cyto-nuclear discordance. Interestingly, all genetically sampled newts from site A (including three subadult newts) were *T. cristatus*, although we observed phenotypic *T. carnifex* a few weeks before our genetic sampling campaign (Bamann, 2019). We hypothesize that *T. carnifex* has either arrived very recently at the site or that the two species have clear assortative mating preferences (see Michalak & Rafinski, 1999 for *Lissotriton*), because a considerable introgression could be expected even with a very low frequency of interspecies mating (Currat et al.,...
Invasiveness and future invasive potential

Recently, *T. carnifex* was considered “one of the most successful invasive amphibians in Europe” (Dufresnes et al., 2019). However, although introductions of *T. carnifex* may be relatively frequent (Arntzen & Thorpe, 1999; Bogaerts, 2002; Brede, 2000; Brede, 2015; Dufresnes et al., 2016, Dufresnes et al., 2019; Franzén et al., 2002; Machado et al., 1998; Maletzky et al., 2008; Malkmus, 1995; Meilink et al., 2015; Svanberg, 1975; this study), the appraisal “invasive” may need to be reconsidered (see also Brede, 2015). Valéry et al. (2008) characterize an invasive species by “a competitive advantage following the disappearance of natural obstacles to its proliferation”, which enables the species to spread rapidly and become dominant. Our genetic data suggest that dispersal distances of introduced *T. carnifex* are similar to distances reported from the species’ native range (Mori et al., 2017) and similar to dispersal distances of local *T. cristatus*. *T. carnifex* did not become dominant at any site that has previously been occupied by *T. cristatus*. Hence, our data do not provide evidence for a replacement of the native species. At pond D, where *T. carnifex* has likely immigrated into an existing population of *T. cristatus*, we observed extensive hybridization but still a dominance of *T. cristatus* alleles. Our current data do therefore not provide any support for *T. carnifex* being an “invasive” or even a “harmfully invasive” species and we propose to term *T. carnifex* in our study region “non-native.” Nevertheless, we acknowledge that our study can only provide very limited information about the speed and the direction of gene flow and a follow-up study would be necessary to complement our snap-shot, also because predicting future trajectories of introduced populations is difficult and a certain time lag without signals of an invasion can be followed by population explosions (Crooks & Soulé, 1999).

Management implications

Visser et al. (2016) claimed that the lack of waterbodies serving as “stepping stones” can lead to the stabilization of a moving hybrid zone. Furthermore, they suggested that species-specific habitat preferences may shape hybrid zone dynamics. Both apply to the situation in Tübingen. There are currently no suitable breeding ponds for crested newts in the center of our study area and a small river may impede the dispersal of *T. carnifex* towards the northwest (Figure 2). Consequently, the chance of *T. carnifex* alleles reaching site H and adjacent waterbodies without human assistance is small. In turn, the preference of *T. cristatus* for open habitats could prevent its immigration into site B. Therefore, we predict the hybrid zone to stabilize approximately at its current extent. In case that alleles of *T. carnifex* may nevertheless reach the *T. cristatus* meta-population in the north-west by single immigrants, high-density blocking would likely hamper their establishment as long as the non-native alleles do not provide a selective advantage (De Meester et al., 2016; Waters et al., 2013). A capture-recapture study at site H suggested a population size of several hundred *T. cristatus*. In contrast, the population of pure

Apart from genetic replacement, native *T. cristatus* may potentially suffer from a loss in fitness after hybridization with the non-native congener (e.g., Muhlfeld et al., 2009). Although hybridization has apparently no effect on the survival of larvae (Wyssmüller, 2007), it remains unclear how different degrees of introgression may affect the fecundity of crested newts and the hatching rate of embryos (compare to Vucic et al., 2020). Yet, disrupted meiosis and dysfunctional gametes were reported for hybrids (Callan & Spurway, 1951), and relatively many malformations were observed in a natural hybrid zone of the two species (Mačát et al., 2015). We observed foot malformations (missing and supplementary toes) in two phenotypic *T. cristatus* from site G. However, the degree of malformations at our study sites is not significantly increased compared to >10 other *T. cristatus* populations that we studied systematically since 2016 (Hinneberg, unpublished data) or to values from the literature (Arntzen et al., 2021).

When the phenotype of the non-native species and the hybrids is significantly different from the native phenotype, hybridization can have severe impacts on community members (Ryan et al., 2009), that is, species that are not genetically affected by hybridization. Crested newts may occasionally prey on other amphibians, in particular their eggs and larvae. However, amphibians are not the main prey items for either *T. cristatus* or *T. carnifex* (Careddu et al., 2020; Dolmen & Koksvik, 1983; Fasola & Canova, 1992; Iannella et al., 2020; Rošca et al., 2013), meaning that it may make little difference to other amphibians whether the native or the non-native species, or hybrids co-occur in the pond. The limited impact of *T. carnifex* and hybrids on native amphibians may also be evidenced by the rich amphibian fauna at our study sites, with up to seven species per pond, including threatened species such as *Bombina variegata* and *Pelophylax lessonae*.

4.5 Management implications

Visser et al. (2016) claimed that the lack of waterbodies serving as “stepping stones” can lead to the stabilization of a moving hybrid zone. Furthermore, they suggested that species-specific habitat preferences may shape hybrid zone dynamics. Both apply to the situation in Tübingen. There are currently no suitable breeding ponds for crested newts in the center of our study area and a small river may impede the dispersal of *T. carnifex* towards the northwest (Figure 2). Consequently, the chance of *T. carnifex* alleles reaching site H and adjacent waterbodies without human assistance is small. In turn, the preference of *T. cristatus* for open habitats could prevent its immigration into site B. Therefore, we predict the hybrid zone to stabilize approximately at its current extent. In case that alleles of *T. carnifex* may nevertheless reach the *T. cristatus* meta-population in the north-west by single immigrants, high-density blocking would likely hamper their establishment as long as the non-native alleles do not provide a selective advantage (De Meester et al., 2016; Waters et al., 2013). A capture-recapture study at site H suggested a population size of several hundred *T. cristatus*. In contrast, the population of pure
T. carnifex at B was estimated at approximately 70 individuals in 2016 (Hinneberg, unpublished data).

Under the precautionary principle, it may be desirable to remove any non-native plant and animal population, irrespective of its current “invasiveness” to prevent future invasions (Pluess et al., 2012). Removing aliens is, however, often difficult, involves a certain risk of failure, and can have unintended side-effects (Pluess et al., 2012; Sogge et al., 2008). Considering the time to reach maturity for individual newts and the potential presence of “skipping breeders” (Cvetković et al., 1996; Hagström, 1979), attempting removal of non-native T. carnifex alleles would require extensive trapping at all sites occupied by T. carnifex or hybrids and during a minimum of four consecutive years. Moreover, an eradication attempt could be harmful to both non-native and native crested newts, because hybrids cannot be reliably differentiated from pure native T. cristatus without determining the genotype of each individual newt (Brede, 2000, this study). Instead, we recommend a targeted monitoring of the hybrid zone, including genetic tools, and we encourage the use of skin swabbing for DNA sample collection. Limited DNA quantities were a major challenge in our study. To ensure sufficient DNA quantity and quality in future studies, we propose swabbing multiple times with a gentle pressure over the newts’ back, tail sides, and venter, to dry the cotton tip immediately after swabbing with silica gel and to extract the DNA within four weeks after swabbing. We suggest an elution volume of 30–50 μl. Furthermore, we recommend the assessment of (species-specific) population sizes for all waterbodies in the study region with capture-recapture. The monitoring should be repeated in regular time intervals, for example, after 5–8 years (approximately two newt generations), thus allowing speed and direction of gene flow to be determined and trends in species-specific population sizes to be detected. To protect the native T. cristatus, we suggest specific measures of habitat maintenance at sites which are currently unaffected by hybridization but also at sites with a low frequency of non-native alleles. For example, managers should prevent the illegal release of gold fish, counteract the drying out of breeding ponds before newt larvae reach metamorphosis, and cut back trees, shrubs, and reeds that shade the waterbodies. A vital population of T. cristatus is key to the species’ conservation and very likely to be the best protection against a future, potentially more harmful invasion of T. carnifex.

AUTHORS’ CONTRIBUTIONS

Heiko Hinneberg conducted the fieldwork, the laboratory work, and the data analysis. Heiko Hinneberg contributed to the acquisition of funding and led with writing the original manuscript. Thomas Bamann contributed to fieldwork and provided funding. Julia C. Geue substantially contributed to the development of the laboratory protocol and supported laboratory work. Katharina Foerster contributed to the design of the study and provided funding. Henri A Thomassen supervised the laboratory work and supported data analysis. Alexander Kupfer contributed to the design of the study, supported fieldwork, and provided funding. Henri A Thomassen and Alexander Kupfer supported writing of the original manuscript. All authors critically revised the manuscript. Henri A Thomassen and Alexander Kupfer should be considered joint senior author.

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CONFLICT OF INTEREST

We declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The full dataset, containing phenotypical and genetic data, is accessible as supporting information.

ETHICS STATEMENT

The permits for capturing protected crested newts were issued by the Regierungspräsidium Tübingen, Referat 55 (55–6/8852.15-3/Hinneberg, Heiko and 55–6/8852.15-3/SMNS). 18 newts (15 larvae/juveniles and 3 adults) were secured as museum records, under the permission 55–6/8852.15-3/SMNS. All other individuals were sampled for DNA using noninvasive skin swabs and were released unharmed immediately after sampling.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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