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RESEARCH ARTICLE

Massive genetic introgression in threatened northern crested newts (*Triturus cristatus*) by an invasive congener (*T. carnifex*) in Western Switzerland

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Abstract Genetic pollution through introgressive hybridization of local species by exotic relatives is a major, yet neglected aspect of biological invasions, particularly in amphibians where human introductions are frequent. In Western Switzerland, crested newts make an interesting case: the Italian species *Triturus carnifex* was introduced at least a century ago within the range of the native and threatened *T. cristatus*. To understand the genetic consequences of this introduction and inform wildlife management authorities, we conducted a genetic survey on the remaining northern crested newt populations known in the area, using newly-developed species-diagnostic nuclear (microsatellites) and mitochondrial (control region) DNA markers. We documented massive nuclear introgression by the *T. carnifex* genome, which has completely replaced *T. cristatus* in most populations, especially in the Geneva area where the introduction was originally reported. However, many of these individuals retained the ancestral *T. cristatus* mtDNA, which could be explained by asymmetric introgression between the two species, stemming from demographic and/or selective processes. Analyses of genetic diversity support multiple events of *T. carnifex* releases, most-likely of proximate North Italian origin. We pinpointed the last indigenous populations in the region and recommend to prioritize their protection. Our study demonstrates the invasive potential of introduced taxa through introgressive hybridization, alerts about the underestimated rate of illegal amphibian translocations, and emphasizes the need for genetic analyses to monitor such invasions.

Keywords Conservation · Asymmetric reproductive isolation · Hybridization · Invasive species · Amphibian · Microsatellites · mtDNA

Introduction

The artificial introduction of non-native species raises serious concerns for the conservation of local biodiversity (Allendorf et al. 2001; Laikre et al. 2010). These threats may be ecological, i.e. interspecific competition with local taxa, alteration of trophic chains, and transmission of exotic diseases. In parallel, threats may also be genetic, as introduced individuals can potentially hybridize with local relatives and compromise their genetic integrity through introgression (Allendorf et al. 2001). This process is a sensitive issue for wildlife managers, the status of introgressed populations being usually controversial (Allendorf et al. 2001). Moreover, the consequences of introgression may greatly differ depending on the genetic and environmental factors involved. Introgressive hybridization with a distant relative may lower the fitness of individuals (i.e. outbreeding depression; as both species can be, at least partly, reproductively isolated), potentially precipitating

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the decline of local populations (e.g. Dufresnes et al. 2015b). Alternatively, ecological and genetic factors may interact to promote the invasion of exotic genes, and thus the rapid replacement of the native genome (Fitzpatrick et al. 2010). These effects may be exacerbated in the case of multiple introductions and when local populations are small, which is typically the case in most threatened species (Laikre et al. 2010).

Well-documented cases of massive genetic introgression raising important conservation issues mostly comes from mammals (e.g. red deers by sikas, Senn et al. 2010; minks by polecats, Cabria et al. 2011; wolves by domestic dogs, Godinho et al. 2011; Randi and Lucchini 2002; Randi et al. 2014; wild by domestic cats, Oliveira et al. 2015; between marten species, Kyle et al. 2003), and to some extent birds (Barilani et al. 2007) and fish (in particular due to large-scale commercial releases of non-native salmonids, Laikre et al. 2010). The matter has received less attention in amphibians, where intentional or accidental human translocations are frequent (Smith and Sutherland 2014), thus raising many opportunities for genetic pollution in native populations (Johnson et al. 2010; Austin et al. 2011; Dufresnes et al. 2015b; Meilink et al. 2015). Given the unpredictability of this process, case-by-case surveys using genetic tools are essential to understand the genetic consequences of human introductions and inform managing authorities (Fitzpatrick et al. 2010; Laikre et al. 2010).

The northern crested newt *Triturus cristatus* is one of the most threatened, widespread amphibians in Europe, and owing to population declines it is red-listed in the majority of countries/regions where it occurs (Edgar and Bird 2006; Dufresnes and Perrin 2015). It is known to naturally come into contact with its southern relative, the Italian crested newt *T. carnifex*, across a narrow hybrid zone in central Europe (Maletzky et al. 2008, 2010), whereas both species are geographically isolated by the Alpine arc in the rest of their ranges (Wielstra et al. 2013, cf. Fig 1). However, human introductions of *T. carnifex* have been documented throughout the 20th century in several Western-European countries, and cases of hybridization with local *T. cristatus* were reported in the United Kingdom (Brede et al. 2000), Southern-Germany (Franzen et al. 2002), and the Netherlands (Meilink et al. 2015).

In Switzerland, *T. cristatus* occurs north of the Alps across the Swiss Plateau (cf. Fig 1), but populations have been dramatically collapsing over the past decades (Schmidt and Zumbach 2005). The species is now listed as endangered by national authorities (Schmidt and Zumbach 2005). As for most Swiss amphibians, habitat degradation and loss are mainly responsible for this sharp decline, even more so for *T. cristatus* which requires large and deep sunny ponds with rich vegetation and is very sensitive to pollutants (Meyer et al. 2009). Moreover, in Western Switzerland, *T. carnifex* was presumably introduced multiple times in the area of Geneva, at least since the beginning of the 20th century (Arntzen and Thorpe 1999; Arntzen 2001), raising additional conservation concerns. Most probably, the species was originally imported for zoological experiments and released at the University of Geneva (Wittenberg 2005); in this part of range, the Alps form an impassable dispersal barriers and both species always had strongly disruptive natural distributions (Fig. 1; *Triturus* newts are not found above 1100 m in Switzerland, Grossenbacher 1988; Meyer et al. 2009). Genetic analyses based on allozyme polymorphism hence indicated that Geneva crested newts clustered with *T. carnifex*, suggesting that native *T. cristatus* had already been replaced in this area (Arntzen and Thorpe 1999; Arntzen 2001). Population monitoring based on morphological criteria also suggested that *T. cristatus* morphotypes had disappeared from the Geneva area and that hybrid forms now predominate (Ferrantin 2007). However, distinguishing between these two species in the field is difficult, but more so because hybrids show intermediate characters (Brede et al. 2000); therefore, monitoring this introduction requires genetic tools to infer species assignments and the degree of introgression.

In order to understand the genetic consequences of this introduction and its extent in the region, we conducted a comprehensive genetic survey of the last known crested newt populations in Western Switzerland. Based on cross-amplifying and newly-developed fast-evolving nuclear and mitochondrial markers, we characterized their taxonomic assignment and extent of admixture, which will be important to inform future management strategies.

Methods

Sampling and DNA extraction

Crested newts were captured from 29 localities (n = 203 individuals) scattered across the Lake Geneva region, from the Geneva area to the upper Rhone Valley, which correspond to the remaining stations where it is known to occur. To characterize their nature and origin, reference populations of *T. cristatus* (n = 97 individuals from 11 localities) and *T. carnifex* (n = 33 individuals from 3 localities) were also sampled from different parts of their natural ranges. Details on sample origins are available in Additional File S1. DNA was obtained from buccal swabs (study area; Broquet et al. 2007) or tail tips (preserved in absolute ethanol; reference populations), and extracted using the Qiagen Biosprint Robotic workstation.

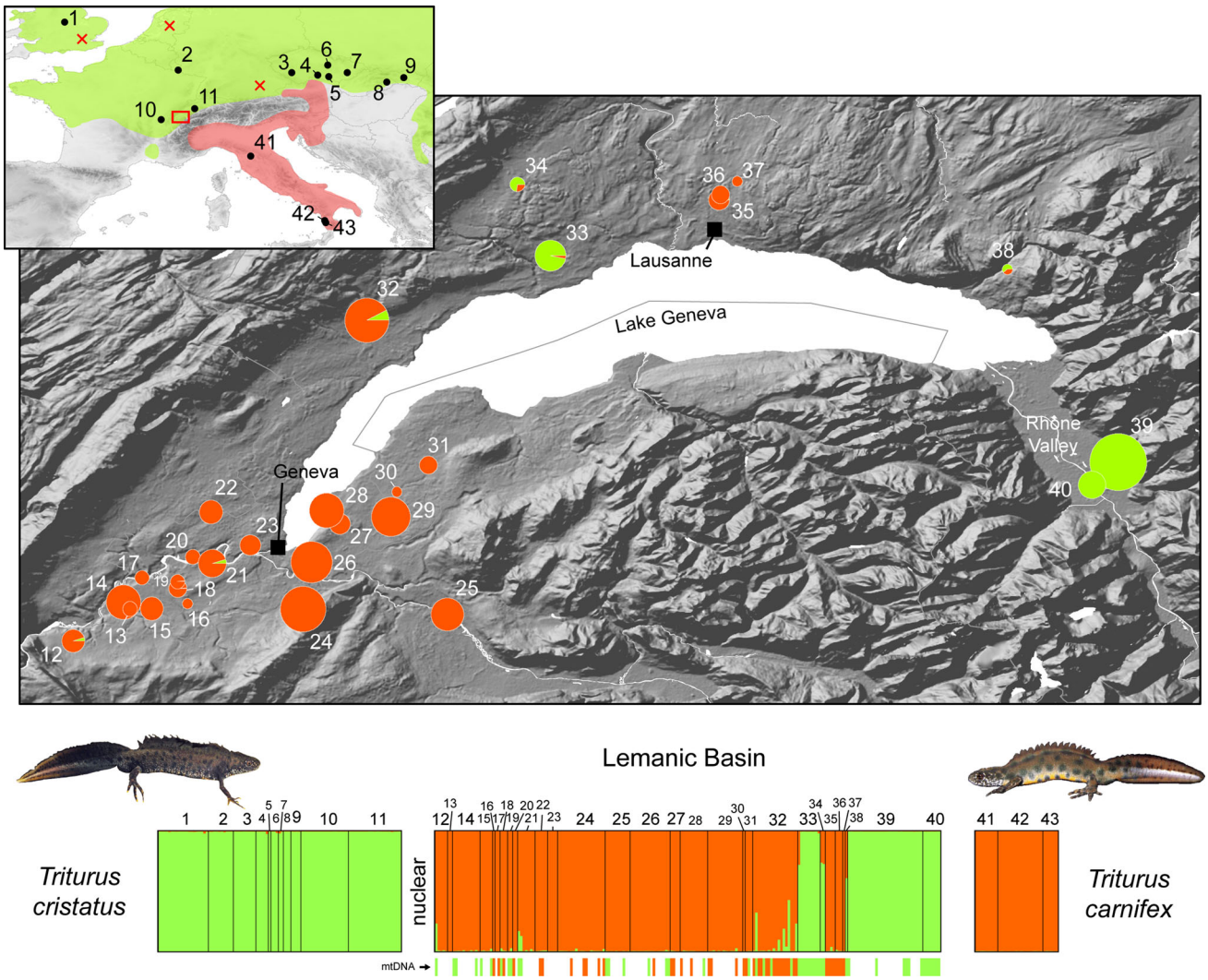

Fig. 1 Bayesian clustering of microsatellite genotypes with STRUCTURE (nuclear) and mitotypes of crested newts from Western Switzerland (*green*: *T. cristatus*, *red*: *T. carnifex*). The map displays the average STRUCTURE assignments of each population. Natural distributions of the two species, known other introductions of *T. carnifex* (*red crosses*), location of the study area (*red rectangle*) and the reference populations are shown on the top-left frame. Photo credits: Radek Sejkora (*T. carnifex*) and Andréas Meyer (*T. cristatus*)

Microsatellite genotyping and mtDNA sequencing

To infer their nuclear background, individuals were genotyped for 11 microsatellite loci, cross-amplifying in both species and featuring interspecific polymorphism. Five loci were optimized from Krupa et al. (2002) and six loci were developed for this study. The latter were isolated from a library of *T. carnifex* DNA enriched with microsatellite repeat motifs (GIS, Chatsworth, CA), tested and chosen for cross-amplification in *T. cristatus*, and for species-specific polymorphisms. Markers and primer sequences are listed in Table 1.

Markers were amplified separately in 10µL PCRs with 2µL of DNA template (10–100 ng), 1 × Qiagen PCR buffer, 0.5 mM of each primer, 0.25 mM of dNTPs, 0.25 units of Qiagen Taq, and either 0.19 mM (for *Tcri*13, *Tcri*35, *Tcri*29, *Tcri*46), 0.33 mM (for *Tcri*36) or 0.21 mM (for *A*7, *A*126, *D*1, *D*5, *A*8, *D*127) of $MgCl_2$. PCRs were run as follows: (a) for *Tcri*13, *Tcri*35, *Tcri*29, *Tcri*46, *Tcri*36: 2′ at 94 °C, 39× (1′ at 94 °C, 1′ at annealing temperature, 1′ at 72 °C), and 5′ at 72 °C; (b) for A7, A126, D1, D5, A8, D127: 2′ at 94 °C, 35× (30″ at 94 °C, 30″ at annealing temperature, 1′ at 72 °C) and 5′ at 72 °C. PCR products were pooled for genotyping as followed. (a) Mix 1:*Tcri*13 (diluted 4.5×), *Tcri*35 (diluted 1.8×) and *Tcri*46 (diluted 4.5×) with respective ratios of 1:2.5:1; (b) Mix 2: *Tcri*29 (diluted 7×) and *Tcri*36 ((diluted 7×) with a ratio 1:1; (c) Mix 3: *A*7 and *A*126 with a ratio of 1:1; (d) Mix 4: *D*1 and *D*5 with a ratio of 1:1; (e) Mix 5: *A*8 (diluted 2×) and *D*127 (diluted 4×) with ratio 1:1. Pooled

Table 1 Details on the markers used in this study

Marker	Ref.	Primers	Dye		*T. cristatus*		*T. carnifex*	
				T_a	Range	N_a	Range	N_a
Microsatellites								
*Tcri*13	1	F: GTGATGGTTGCCAAGC	FAM	53	111–129	9	95–107	6
		R: GATCCAAGACACAGAATATTTAG						
*Tcri*35	1	F: CCAACTGGTATGGCATTG	HEX	55	203–297	11	225–315	18
		R: GATCACAGAAACTCTGAATATAAGC						
*Tcri*46	1	F: CAAGTTTCCTCTGAAGCCAG	FAM	53	234–337	16	222–284	13
		R: GTTTCTTGCCTGACAAAGTAATGCTTC						
*Tcri*29	1	F: CGAGTTGCCCAGACAAG	ATTO	53	288–334	10	246–250	2
		R: GATCACATGCCCATGGA						
*Tcri*36	1	F: GATCATCTGAATCCCTCTG	FAM	53	231–311	16	179–221	14
		R: ATACATTCATGACGTTTGG						
A7	2	F: GCTTTCTTTCATTTCACTCTTC	FAM	56	145–293	23	133–209	17
		R: AGACCATTCCATTGGTGAG						
*A*126	2	F: CCAACAAGATCCCAGATGC	HEX	58	282–374	23	210–330	20
		R: GCTTGCTTCACAATTTCTCG						
*D*1	2	F: GCATTTGTTTGCCTTTTCT	HEX	57	173–318	17	188–190	2
		R: GCGCTACCCTATATTAGGTGT						
*D*5	2	F: GAAATATCCACCCAGCAGAAC	ATTO	56	277–283	2	283–299	7
		R: CACAGCGTGAGTGTGTTATCA						
*A*8	2	F: ATGTGCTTGATTTGGACAGG	HEX	58	258–499	43	239–489	25
		R: ATACGGACCACAACTTCGTG						
*D*127	2	F: CGATAATATGCACGATCATTC	FAM	57	183–221	2	217–229	6
		R: ATTTGGCTGATATGACTTATGG						
Mitochondrial control region								
Amplicon 1	2	L-Uro: TGGCACCCAAGGCCAAAATTC						
		H-Uro: CAAGACTTTATGGGGCATTGA						
Amplicon 2	2	L-CR-Uro*: TCAATGCCCCATAAAGTCTTG						
		H-tRNAPhe-Uro: CTGTCTTAACATTTTCAGTGC						

Ref references ([1]Krupa et al. 2002, [2]this study); T_a annealing temperature (°C); N_a number of alleles; ranges are in base pairs. N_a and *Ranges* were calculated from reference populations

* Reverse complement of H-Uro

amplicons were then run on an ABI Prism 3100 genetic analyzer; peaks were scored with GeneMapper 4.0 (Applied Biosystem).

To infer the mitochondrial background of Western–Swiss crested newts, we analyzed 644 bp of the mitochondrial control region (CR) in a total of 100 individuals (91 from the study area, plus 9 from reference populations). Two adjacent parts of this marker were amplified by two separate PCRs using newly developed primers conserved across urodeles: L-Uro/H- Uro (375–376 bp amplicon) and L-CR-Uro/H-tRNAPhe-Uro (385 bp amplicon). Primer sequences are available in Table 1. Both amplicons were amplified in 25μL PCRs with 2μL of DNA template (10–100 ng), 1 × Qiagen PCR buffer, 0.15 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.5 mM of each primer, and 0.625 units of Qiagen Taq. PCRs were run as follows: 3′ at 95 °C, 35 × (30″ at 95 °C, 30″ at 55 °C, 30″ at 72 °C) and 5′ at 72 °C. Amplicons were purified using the Promega purification kit and sequenced in both directions on an ABI3730 genetic analyzer (Applied Biosystems). Quality-checked sequences obtained from each amplicon were concatenated and aligned for subsequent analyses.

Population genetic analyses

For reference populations with large sample sizes ($n \geq 9$; *T. cristatus*: loc. 1–3, 10–11; *T. carnifex*: loc. 41–42), we checked for the presence of genotyping errors and null

alleles with Microchecker (Oosterhout et al. 2004), and used Arlequin (Excoffier et al. 2005) to calculate Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium between loci.

Several analyses were conducted to characterize the genetic structure of Western–Swiss crested newts. First, we performed Bayesian clustering assignment of individual microsatellite genotypes using STRUCTURE (Pritchard et al. 2000). We tested from 1 to 11 groups (K), with 10 replicate runs per K, each run consisting of 100,000 iterations (after a burnin' of 10,000). The ΔK test was applied to determine the most likely number of groups (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and VonHoldt 2012). Replicates of the most likely solution were combined using CLUMPP (Jakobsson and Rosenberg 2007) and graphical displays of individual ancestry coefficients (barplots) were built using DISTRUCT (Rosenberg 2003). Second, we conducted a principal component analysis on microsatellite genotypes (*adegenet* R package, Jombart 2008).

Relationships between mitochondrial haplotypes were reconstructed by a maximum-likelihood phylogenetic inference with PhyML (Guindon and Gascuel 2003), using a HKY model of sequence evolution (MrAIC, Nylander 2004) and 1,000 bootstrap replicates. A published sequence of *T. marmoratus* (Wielstra and Arntzen 2011, GenBank HQ697279) was used as outgroup.

Results

Data quality

Potential null alleles were detected in a few instances for *A*126 (loc. 1, 11 and 42), *D*1 (loc. 1) and *A*8 (loc. 3). Accordingly, *A*126 and *D*1 were the only markers significantly departing from HWE in some *T. cristatus* reference populations (*A*126: loc. 1 and 11; *D*1: loc.1) after Bonferroni corrections. We did not detect significant linkage disequilibrium for any pair of loci in any reference population.

Clustering of microsatellite genotypes

Bayesian clustering assignment using STRUCTURE unambiguously recovered two groups ($\Delta K = 2452.1$; Additional File S2), corresponding to the gene pools of *T. cristatus* and *T. carnifex*, based on our reference populations (Fig. 1). In Western Switzerland, all individuals from the Geneva area (loc. 12–31) were assigned by nuclear loci to *T. carnifex*, with evidence for admixture in only three individuals (i.e. with ancestry coefficients below 0.9; e.g. loc. 21). Populations distributed further east along the lake coast displayed a mixed pattern: some are deeply introgressed by *T. carnifex* (loc. 32, 35–37, 38), while others retained most (loc. 33–34) if not all the local *T. cristatus* nuclear genome (loc. 39–40).

The Principal Component Analysis (PCA) on microsatellite genotypes confirmed this result: the first axis discriminated between the two species (Additional File S3), while the second contrasted southern (loc. 42–43) versus northern *T. carnifex* native populations (loc. 41). Most Western–Swiss newts clearly group with the latter, and only a few populations remained similar to *T. cristatus*. Some individuals had intermediate scores on the first axis, indicative of genetic admixture.

Distribution of mitochondrial lineages

Phylogenetic reconstruction of CR haplotypes allowed perfect discrimination between the two species (3 % of divergence, Fig. 2; GenBank KU743871-KU743879). Reference *T. cristatus* ($n = 6$ sequences) displayed three different haplotypes (CRI1-3), with only one haplotype (CRI1) occurring in Western Switzerland (out of $n = 33$ sequences). Four *T. carnifex* haplotypes (CAR1-3, CAR6) were recovered in our study area (out of $n = 44$ sequences), with the most abundant (CAR1) also present in Northern Italy (loc. 41). In contrast, reference individuals from Southern Italy (loc. 42–43) possessed specific haplotypes, not found elsewhere (CAR4-5).

Interestingly, distributions of the two mtDNA lineages across our study area contrast with the nuclear pattern: 40 % of the newts sampled on the west end of the lake retained *T. cristatus* mtDNA despite being assigned by microsatellites to *T. carnifex* (25 out of 58 sequenced individuals; Fig. 2). Furthermore, admixed (loc. 33–34, 38) and pure (based on microsatellites, loc. 39–40) *T. cristatus* populations only host the native *T. cristatus* mtDNA.

Discussion

Our genetic survey revealed that the introduction of exotic *T. carnifex* in the Geneva area has been followed by massive introgressive hybridization and genetic replacement of the native crested newt *T. cristatus* across Western Switzerland. This pattern parallels the situation in Dutch crested newt populations, where genetic pollution of local *T. cristatus* by introduced *T. carnifex* resulted in the formation of an artificial, geographically continuous hybrid zone (Meilink et al. 2015).

Two lines of evidence support replacement by introgression rather than ecological exclusion only. First, several newts harbored direct signs of nuclear admixture, suggesting incomplete backcrossing (loc. 12, 21, 32–34

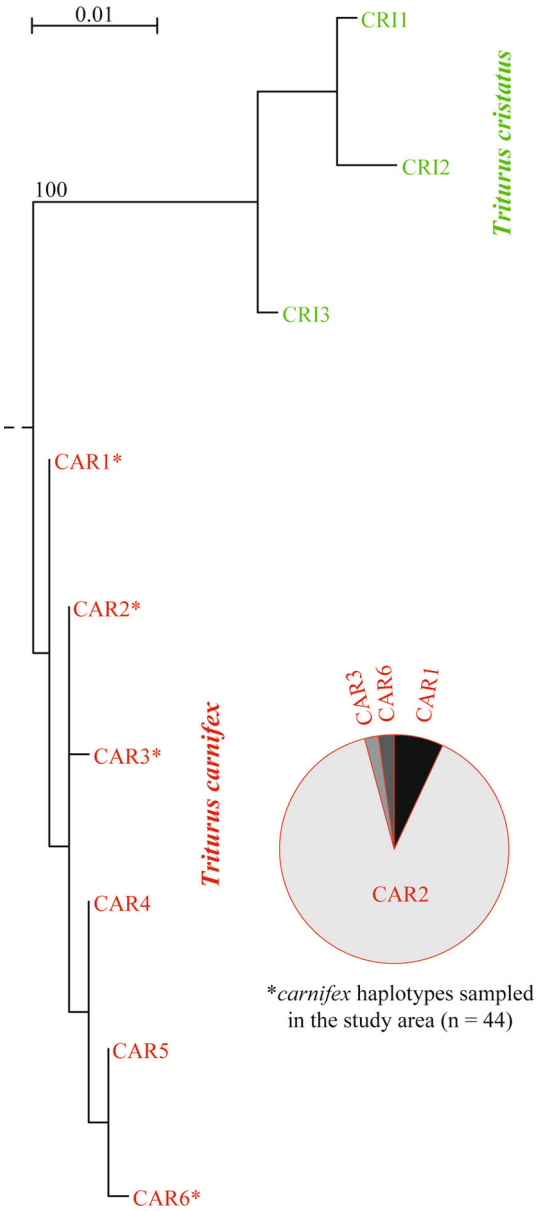

Fig. 2 Maximum-likelihood phylogenetic reconstruction of mitochondrial CR haplotypes (*green*: *T. cristatus*, *red*: *T. carnifex*). For *T. carnifex*, haplotypes sampled in Western Switzerland are indicated by an *asterisk*, and their relative frequencies are displayed (pie chart)

and 38). Second, cyto-nuclear discordance was detected in as many as 25 specimens. Interestingly, the discordance was strongly asymmetric: about half of nuclearly pure *T. carnifex* retained the mtDNA of *T. cristatus* but not the reverse, i.e. we found no pure (or admixed) *T. cristatus* carrying *T. carnifex* mtDNA. Given that mtDNA is only maternally-transmitted, this pattern may reflect male-biased dispersal of *T. carnifex*, coupled with allele surfing in expanding populations (Klopfstein et al. 2006). It has been shown that during the first phases of expansions, introgression can be an asymmetric process going from the local to the invading species, irrespective of relative densities, with local alleles surfing at the edge of the expansion wave, thus reaching high frequencies in the invading species. This pattern is predicted to be stronger for organelle markers with low effective size such as mtDNA (Currat et al. 2008; Petit and Excoffier 2009).

Alternatively, or in addition, this pattern could stem from reproductive isolation between the two species, for instance due to asymmetric incompatibilities between reciprocal hybrid crosses, i.e. lower fitness of offspring from ♂ *T. cristatus* × ♀ *T.carnifex* crosses compared to ♂ *T. carnifex* × ♀ *T.cristatus* crosses. So far, laboratory experiments did not find evidence for differential performance between *T. cristatus* and *T. cristatus* × *T. carnifex* hybrid larvae (Wyssmüller 2007). Yet, in the Netherlands, hybrids between invasive *T. carnifex* and local *T. cristatus* may face negative selection across their artificial hybrid zone (i.e. the transition appears bimodal, suggesting selection against hybrids, Meilink et al. 2015). Asymmetric viability was detected directly in hybrid crosses between *T. cristatus* and another European relative, the marbled newt *T. marmoratus* (Arntzen et al. 2009), although it is more distantly related than *T. carnifex* (Wielstra and Arntzen 2011). Here, *T. cristatus* and *T. carnifex* diverged as early as ~9 Mya (Wielstra and Arntzen 2011) and have thus likely accumulated reproductive isolation. In amphibians, complete isolation is often observed after shorter divergences in the wild (Pleiocene, <5 Mya; Dufresnes et al. 2015a), and is likely maintained by pre-zygotic barriers (e.g. assortative mating). Accordingly, *T. carnifex* and *T. cristatus* present sharp transitions and hardly hybridize across their natural contact zone in Eastern Europe (Maletzky et al. 2008). In introduced ranges, as observed in Swiss and Dutch crested newts, hybridization could occur more frequently due to the lack of opportunity for assortative mate choice (i.e. the introduced species is initially present at low density). Even distantly-related species may thus pose a threat to the genetic integrity of native taxa (Dufresnes et al. 2015b).

Our data indicate a probable North Italian origin of *T. carnifex* in Western Switzerland, as previously suggested (Arntzen 2001). Microsatellites clearly distinguished between the southern and northern lineages of *T. carnifex* (Canestrelli et al. 2012), and all Swiss genotypes unambiguously clustered with the latter (Additional File S3). The low number of available mitochondrial sequences from native *T. carnifex* population (n = 3, only 1 from North-Italian individuals), however, prevents to confirm this origin. Yet, the presence of four different *T. carnifex* mtDNA haplotypes in the Geneva area suggests multiple releases of exotic specimens. Historical records suspected at least one introduction of *T. carnifex* in Geneva, possibly close to population 26 (Grossenbacher et al. 2012 and ref.

therein). Furthermore, unlike for the Dutch introduction (Meilink et al. 2015), here the expansion of *T. carnifex* eastward along Lake Geneva's shore (loc. 32, 35–37, 38) was discontinuous and more likely resulted from additional illegal translocations rather than natural dispersal. Two arguments support this hypothesis. First, in Western Switzerland, crested newts greatly suffer from the lack of suitable breeding sites and the last remaining populations are disconnected, strongly limiting natural dispersal (Fig. 1). Second, individuals have been presumably translocated in the past decades in several additional places (Geneva area and loc. 35–37, 38; S. Dubey and JT pers. com.), where the *T. carnifex* genome is now present.

Our results provide insights on the future management of these populations. First, frequent monitoring should inform on the progress of invasive *T. carnifex* across Western Switzerland and adjacent regions. Second, conservation efforts should be prioritized on the last indigenous *T. cristatus* populations (loc. 39–40, but also loc. 33–34 which are relatively free of introgression), both to protect their breeding sites and prevent future introductions. Furthermore, if hybrids face asymmetric incompatibilities, massive regular translocations of female *T. cristatus* may help restoring the native gene pool. Exhaustive experimental studies, involving all types of possible crosses and at different stages (larval and adults of F1 s and F2 s), are obviously required to characterize potential incompatibilities beforehand. Future surveys should also focus on the ecology of *T. carnifex* in its introduced range, in comparison to *T. cristatus*, in order to better understand the ecological causes and consequences of the invasion (e.g. competitive exclusion). In contrast to *T. cristatus*, introduced *T. carnifex* is associated to anthropogenically-disturbed ponds in Western Switzerland, which may partly explain its success in this heavily-impacted region (Ferrantin 2007).

The case of crested newts in Western Switzerland emphasizes the invasive potential of introduced taxa through genetic introgression ("genetic pollution"). This aspect of invasions is often neglected (Laikre et al. 2010), particularly in amphibians where morphological similarities between closely-related species and their hybrids complicate population monitoring (e.g. Dubey et al. 2014, this study). Rates of illegal amphibian translocations may be underestimated (Fisher and Garner 2007; Smith and Sutherland 2014), and our study contributes to understanding the extent and genetic consequences of anthropogenic introductions on indigenous related taxa.

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