Hybridization and introgression between two species of crested newts (*Triturus cristatus* and *T. carnifex*) along contact zones in Germany and Austria: morphological and molecular data

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In the area between south-eastern Bavaria (Germany) and Upper Austria the distribution ranges of northern (*Triturus cristatus*) and Italian crested newts (*T. carnifex*) are narrowly sympatric and a hybrid zone has been suggested on the basis of morphological data. In our study of 35 autochthonous populations in this region, we compared distribution patterns and hybridization on the basis of one morphological (Wolterstorff index, WI) and two molecular (cytochrome b, microsatellites) markers. Furthermore, we studied the status of an introduced *T. carnifex* population, originating from Croatia and thriving in a locality near Munich for more than 15 years. Tissue samples from Bavarian populations (preserved phalanges) were already available from a previous study. Austrian samples were gained non-destructively, by collecting buccal cells with sterile cotton buds. Results showed good concordance for all markers in most populations. Average WI values per population were within the range of the species *T. cristatus* and *T. carnifex*. Six populations from Salzburg and Upper Austria showed intermediate index values in males and females. Applying standard measures of genetic diversity within populations as well as Bayesian analysis of population structure, we detected admixed populations and individuals in three regions of Salzburg and Upper Austria. No autochthonous population of *T. carnifex* could be detected in Bavaria. The hybrid zone is probably unimodal, with hybrid individuals predominating in the centre. As the present-day distribution ranges of both species in the surveyed area are fragmented and populations are heavily reduced in numbers, we only can observe their remains. The analysis of molecular markers revealed considerable genetic uniformity. The studied area has been colonized by a limited number of individuals and probably less often than areas with slightly higher diversity. Hybrid zones in the study region were most probably formed by one genetically different *T. carnifex* population and two different *T. cristatus* populations. The allochthonous *T. carnifex* population in Isen (Bavaria) showed no signs of interbreeding with native *T. cristatus*. The assumption that this population was based on offspring from one pair is highly questionable according to our data.

Key words: admixture, Bayesian analysis, hybrid zone, microsatellites, mtDNA, Salamandridae, *Triturus cristatus* superspecies, Wolterstorff index

INTRODUCTION

Hybrid zones (HZs) are narrow regions where two distinct genetic forms meet, mate and produce offspring of mixed ancestry (Barton & Hewitt, 1985; Hewitt, 1988). Such zones may vary in width, length, patchiness and fitness of various hybrid genotypes (Arnold, 1997). Natural hybridization may influence evolution in a variety of ways, contributing either as a barrier to gene flow between distinct populations, or as a driving force for the development of new evolutionary lineages (e.g. Barton, 2001). Nevertheless, increased anthropogenic hybridization (by introduction of allochthonous taxa) is harmful and can lead to the extinction of many populations or even taxa by both replacement and genetic admixture (e.g. Arntzen & Thorpe, 1999; Allendorf et al., 2001; Pierpaoli et al., 2003).

Two types of HZ – primary and secondary – are recognized on the basis of their origin (Barton & Hewitt, 1985). Primary zones are those where the differences between populations evolved in a continuous distribution, owing to selection for different alleles towards two sides of an environmental gradient. In the case of secondary zones, the differences evolved while the two populations were geographically isolated. In temperate latitudes, biodiversity was greatly affected by palaeoclimatic changes during the Pleistocene (Hewitt, 2000, 2001). Many animal and plant taxa had glacial refugia in the

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Table 1. List of 36 sampled localities with abbreviations in parentheses, number of individuals (*n*) used for microsatellite and *cyt b* (in parentheses) analyses, as well as geographical coordinates; Bav = Bavaria (Germany), UA = Upper Austria, Sbg = Salzburg (Austria), Sty = Styria (Austria), Isen (*) = introduced population from Rovinj (Croatia). Lat. = latitude; long. = longitude.

Locality	Province	*n*	Lat.	Long.	Locality	Province	*n*	Lat.	Long.
Kochel (Koc)	Bav	16 (2)	47°39′	11°23′	Irlach (Irl)	Sbg	7 (4)	47°59′	12°52′
Niederau (Nie)	Bav	6 (4)	48°00′	12°51′	Bürmoos (Bue)	Sbg	16 (4)	47°59′	12°56′
Lengthal (Len)	Bav	2 (2)	48°11′	12°48′	Fürwag (Fue)	Sbg	17 (4)	47°54′	12°58′
Roibach (Roi)	Bav	3 (0)	48°2′	12°48′	Ried (Rie)	Sbg	20 (8)	47°52′	13°6′
Sillersdorf (Sil)	Bav	5 (4)	47°51′	12°55′	Freimoos (Fre)	Sbg	8 (7)	47°38′	13°10′
Babensham (Bab)	Bav	7 (3)	48°5′	12°17′	Guggenthal (Gug)	Sbg	7 (4)	47°49′	13°6′
Bischofwiesen (Bis)	Bav	2 (0)	47°38′	12°57′	Unterkoppl (Unt)	Sbg	7 (3)	47°49′	13°7′
Surheim (Sur)	Bav	7 (4)	47°52′	12°58′	Sommeregg (Som)	Sbg	18 (3)	47°50′	13°7′
Reichersberg (Rei)	UA	5 (4)	48°20′	13°21′	Neuhofen (Neu)	Sbg	16 (5)	47°50′	13°10′
Haibach (Hai)	UA	4 (4)	48°25′	13°53′	Koppl (Kop)	Sbg	2 (0)	47°48′	13°8′
Bad Zell (Baz)	UA	10 (3)	48°19′	14°42′	Zecherl (Zec)	Sbg	11 (4)	47°58′	13°14′
Bad Ischl (Bad)	UA	9 (2)	47°42′	13°37′	Achleiten (Ach)	Sbg	7 (5)	47°49′	13°13′
St. Georgen (Stg)	UA	5 (0)	47°56′	13°30′	Strobl (Str)	Sbg	8 (2)	47°43′	13°27′
Seewalchen (See)	UA	3 (1)	47°57′	13°33′	Ameisensee (Ame)	Sbg	19 (5)	47°33′	13°27′
Stadl-Paura (Sta)	UA	4 (3)	48°5′	13°52′	Annaberg (Ann)	Sbg	4 (3)	47°31′	13°27′
Gunskirchen (Gun)	UA	8 (1)	48°6′	13°55′	Kainisch (Kai)	Sty	8 (4)	47°33′	13°51′
Micheldorf (Mic)	UA	2 (1)	47°52′	14°7′	Hartberg (Har)	Sty	1 (1)	47°15′	16°02′
Ternberg (Ter)	UA	2 (2)	47°57′	14°22′	Isen (Ise)*	Bav	16 (8)	48°12′	12°04′

southern regions of Europe and North America where they persisted and diverged during glacial periods. After the glaciations, species ranges expanded, came into contact with each other again and (when hybridized) formed secondary HZs (e.g. Butlin & Hewitt, 1985; Arntzen & Wallis, 1991).

Several models have been proposed to describe the structure and dynamics of HZs (for a review see Arnold, 1997). Among these, mosaic and tension zone models are most important. These models incorporate processes like dispersal, selection and fitness of parental and hybrid genotypes in certain environments, but differ in the relative importance of these components. While the mosaic model assumes exogenous selection and the lower fitness of hybrids in comparison to the parental forms (Howard, 1986; Harrison & Rand, 1989), the tension zone model sees HZs as clines of various characters maintained by a balance between dispersal and selection against hybrids (Key, 1968; Barton & Hewitt, 1985; Szymura & Barton, 1986). The fitness of hybrids in this case can be reduced, independent of environmental traits due to incompatibilities of parental genomes (endogenous selection).

A further useful classification of HZs is to discriminate between unimodal, flat and bimodal zones (Harrison & Bogdanowicz, 1997; Jiggins & Mallet, 2000). While in unimodal zones intermediate hybrid genotypes predominate, bimodal zones generally consist of individuals genetically similar to one or other parental genotype. A continuum from unimodal to bimodal genotypic distributions in HZs is predicted, with the flat HZ as an intermediate form with a more even mixture of parental and hybrid genotypes. Bimodality within a local population indicates that speciation is almost complete. Bimodal zones are invariably coupled with strong assortative mating or assortative fertilization within hybrid populations and generally show strong prezygotic isolation between hybridizing species (Rieseberg et al., 1998). Postzygotic incompatibilities are similar in bimodal and unimodal zones (Jiggins & Mallet, 2000).

HZs go along with common genetic features like heterozygote deficit and linkage disequilibria. A shortage of heterozygotes compared with Hardy–Weinberg (HW) expectations is usually measured by the inbreeding coefficient F_{IS} and can be caused by selection against heterozygotes, assortative mating and migration between divergent populations as well as inbreeding. Linkage disequilibria, stable associations between loci, are more often found in HZs and are commonly generated by migration of individuals between genetically divergent populations or, within populations, by assortative mating and/or epistatic selection (e.g. Jiggins & Mallet, 2000).

The currently recognized species of the crested newt complex from Europe and western Asia, originally described as subspecies based on morphological features (Wolterstorff, 1923), are *Triturus cristatus* (Laurenti, 1768), *T. dobrogicus* (Kiritzescu, 1903), *T. carnifex* (Laurenti, 1768) and *T. karelinii* (Strauch, 1870). They are considered to be relatively young species *in statu nascendi*, among which genetic isolation is not fully developed (Bucci-Innocenti et al., 1983; Wallis & Arntzen, 1989). Divergence time among species was estimated at 5–7 million years based on allozyme data (Kalezić & Hedgecock, 1980; Macgregor et al., 1990) and at 2.5–3 million years based on mtDNA RFLP data (Wallis & Arntzen, 1989). These closely related taxa show parapatric distributions, whose contacts are often characterized by narrow HZs (Freytag, 1978; Klepsch, 1994; Wallis & Arntzen, 1989; Arntzen & Wallis, 1999). Crested newts differ in morphological (Wolterstorff, 1923; Arntzen & Wallis,

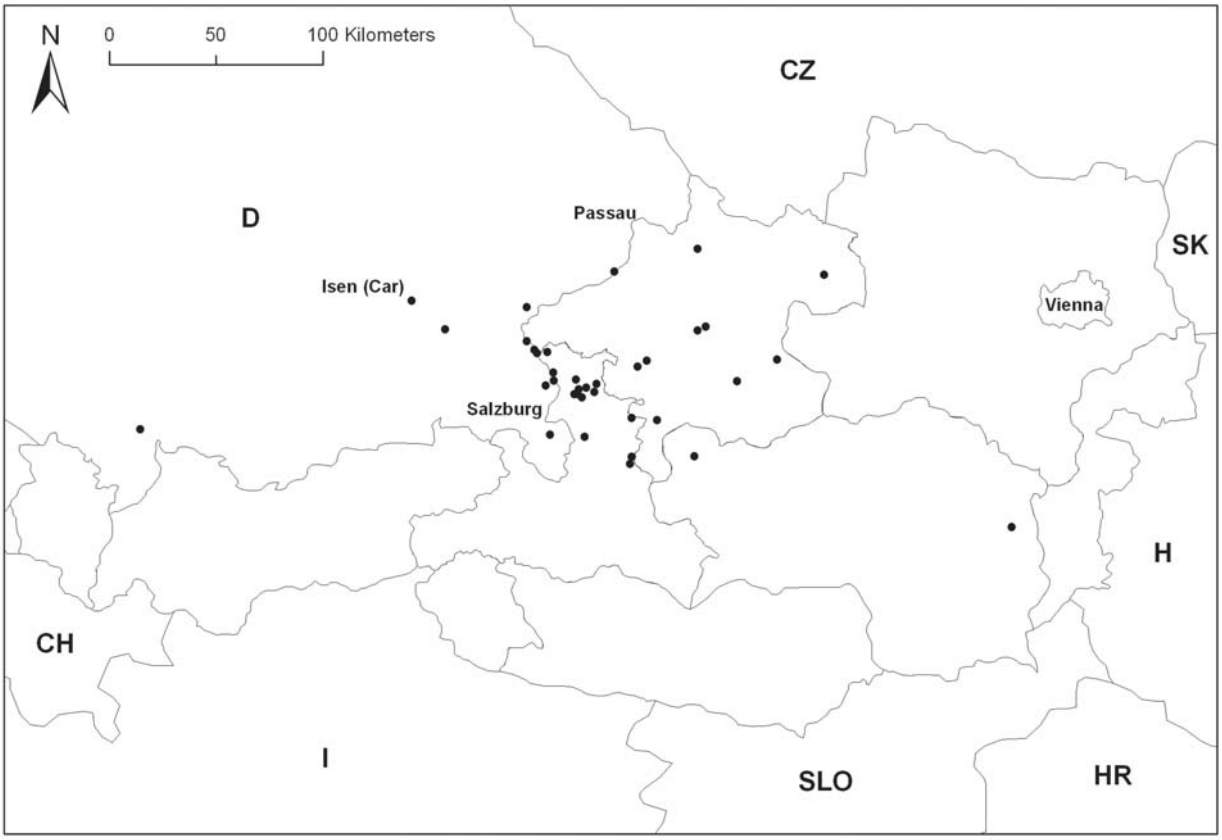

Fig. 1. Map of Austria and the surrounding regions with full circles indicating the sampled localities; Isen (Car) indicates the allochthonous *T. carnifex* population introduced from Rovinj, Croatia. D = Germany, CZ = Czech Republic, SK = Slovak Republic, H = Hungary, HR = Croatia, SLO = Slovenia, I = Italy, CH = Switzerland.

1994) and genetic (Bucci-Innocenti et al., 1983; Arntzen & Thorpe, 1999; Krupa et al., 2002; Mikulíček & Piálek, 2003) characters but their ecological differentiation and habitat preferences are poorly understood.

In Austria such natural HZs are documented in several regions and all three occurring taxa, i.e. *T. cristatus*, *T. carnifex* and *T. dobrogicus* (Mayer, 2001). In north-eastern Austria and adjacent Moravia (a part of the Czech Republic), hybrid populations with introgression between all three species can be found (Klepsch, 1994; Arntzen & Wallis, 1999; Mikulíček, 2005). In eastern and south-eastern Austria introgression between *T. carnifex* and *T. dobrogicus* has been documented (Wallis & Arntzen, 1989; Mayer, 2001). A third area of narrowly sympatric distribution ranges (*T. cristatus*, *T. carnifex*) is located between south-eastern Bavaria (Germany) and the provinces of Salzburg and Upper Austria (Austria). The presence of hybrid populations in this area has been suggested based on morphological data (Wolterstorff, 1929; Schüller, 1963; Schmidtler, 1976; Freytag, 1978), but its extension and the distribution ranges of the two species are unknown (Arntzen & Thorpe, 1999; Thiesmeier & Kupfer, 2000; Arntzen, 2003). According to Arntzen & Thorpe (1999), the collection of data from this region is desirable to gain further insight into the genetic compatibility of these taxa.

Furthermore, a population of *T. carnifex* originating from Rovinj (Croatia) was established in Bavaria as a result of human introduction in 1990 or 1991 (Franzen et al., 2002). Hybridization of introduced *T. carnifex* with local *T. cristatus* was not documented and has been obscure up to now.

In this study we combined morphological markers (Wolterstorff Index) and two types of molecular markers (mtDNA and microsatellites) to describe the structure of HZs and the extent of introgression between *T. cristatus* and *T. carnifex* in the area between south-eastern Bavaria and Upper Austria. Our aims were to find out: 1) the structure of HZs with respect to geographic distribution of genotypes, 2) the direction of introgression of mtDNA and nuclear microsatellites and 3) whether hybridization between the introduced *T. carnifex* from Croatia and the original *T. cristatus* occurred.

MATERIALS AND METHODS

Study area and sampling

In total we examined 36 localities situated in the German province of Bavaria (Bav, 9), and the Austrian provinces of Salzburg (Sbg, 15), Upper Austria (UA, 10) and Styria (Sty, 2). Their names and geographical locations, as well as the number of studied individuals per site, are shown in Table 1 and Figure 1. Most samples from Bavaria were collected and preserved in 2001 during a study on populations in this region (Franzen et al., unpublished data). The *T. carnifex* population Isen (Ise) was intro-

duced in 1990 or 1991 and originates from Rovinj, Croatia (Franzen et al., 2002). Newts from Austrian populations and population Sillersdorf (Sil) in Bavaria were captured during the breeding season in 2004 and 2005. Newts were taken into the laboratory and anaesthetized with MS 222 (Sigma-Aldrich, Vienna) for measuring and tissue sampling.

Morphological markers

For morphological studies we rejected individuals that were immature and/or smaller than 100 mm total length. We placed the individuals on a moistened white towel and measured their morphometric characters to the nearest 0.1 mm with a vernier calliper. Wolterstorff's morphometric index (WI = 100 × forelimb length/interlimb distance) was calculated (Wolterstorff, 1923). WI values are typically lower than 64 (males) or 54 (females) in *T. cristatus* and higher than this in *T. carnifex* (Arntzen & Wallis, 1994). The newts were returned to the sites of original sampling within 24 hours. Results from Bavarian populations were obtained in the course of an earlier study (Franzen et al., unpublished data). In total we measured 305 individuals (162 males, 143 females) belonging to 36 populations.

Molecular markers

Tissue for DNA extraction was obtained by two different methods. For localities in Bavaria it originated from phalanges preserved in ethanol during an earlier study in 2001, while in all other cases tissue was gained non-destructively by collecting buccal cells with sterile cotton buds which were stored in 96% ethanol (Pidancier et al., 2003). Total genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel). A part of mitochondrial cytochrome b (*cyt b*) and seven highly polymorphic microsatellite loci were used in this study.

Mitochondrial DNA. The amplification of a 520 bp long cytochrome b (*cyt b*) fragment was carried out using *Tri_cyb2F* (5' ACAGCAGACACACAATCGGCAT 3') and *Tri_cyb2R* (5' GGTAACTAAGGAGTTTGCTGGG 3') primers designed according to the partial *T. carnifex cyt b* sequences, available at sequence databases. We used the following reaction conditions: 10–100 ng of template DNA, 0.2U Taq DNA polymerase (Top-Bio), 500μM dNTPs (Top-Bio), 200nM of each primer and 5μl of reaction buffer containing 25 nM of $MgCl_2$ filled in to the 50 μl reaction volume by bi-distilled water. PCR was performed under the following conditions: 94 °C for 5 min as initial denaturation, followed by 30 cycles of 94 °C for 45 sec, 60 °C for 45 sec and 72 °C for 45 sec. PCR products were then purified with Qiaquick PCR purification kit (Qiagen) and directly sequenced using Big Dye Terminator Cycle Sequencing v3.1 on the ABI-Prism 377 automatic sequencer. Both strands were sequenced, so that each region was available for at least two independent reads.

Microsatellite markers. We used seven microsatellite loci (*Tcri13*, *Tcri29*, *Tcri32*, *Tcri35*, *Tcri36*, *Tcri43* and *Tcri46*), originally designed from a *T. cristatus* library (Krupa et al., 2002). For amplification of microsatellite loci, total reaction volumes of 10 μl were used with a final concentration of 0.1 mM dNTPs, 2–3 mM $MgCl_2$ (depending on the locus), 0.1 mM of each primer, 0.25 U DNA polymerase (Fermentas) and DNA sample in the manufacture's buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20). Thermal profiles for all microsatellite loci consisted of 2 min initial denaturation at 94 ºC followed by 39 cycles of 1 min at 94 ºC, annealing for 1 min at 53 ºC and elongation at 72 ºC for 2 min. An additional 5 min period for elongation at 72 ºC followed the last cycle. For routine genotyping, primers were commercially labelled with fluorochromes HEX, FAM, TET and NED. PCR products were run on an automated ABI 310 DNA sequencer. Allele sizes were surveyed using ABI Genescan software and Tamra 500 size standard (Applied Biosystems).

Data analysis

Mitochondrial DNA. For nucleotide sequence analysis in mitochondrial DNA, chromatograms were assembled and contigs were produced using Editseq and Seqman (DNAStar software package). Sequences were then aligned and alignment was edited with Bioedit (Hall, 1999); the DNA polymorphism pattern was analysed with DnaSP 4.01 (Rozas et al., 2003). Samples from the Slovenian locality Matena for *T. carnifex* and Peterborough, United Kingdom for *T. cristatus* provided reference sequences (Steinfartz et al., 2007; Horák et al., unpublished data).

Microsatellite markers. We calculated mean observed (H_O) and expected (H_E) heterozygosity as well as allelic richness (A) per population, using the program FSTAT 2.9.3.2 (Goudet, 1995). Measures of allelic richness (mean number of alleles per locus) were corrected for variation in sample size by using the rarefaction method implemented in the program. Populations with $n<5$ were omitted. Hardy–Weinberg (HW) equilibrium and linkage disequilibria (LD) were computed using GENEPOP 3.3 (Raymond & Rousset, 1995). Deviations from HW equilibrium were measured by F_{IS} coefficient (Wright, 1978; Weir & Cockerham, 1984). Negative F_{IS} values indicate heterozygote excess, positive values indicate heterozygote deficiency. Estimation of exact *P* values of F_{IS} was performed using the Markov chain algorithm based on 1000 iterations.

We assessed population structure using the model-based Bayesian approach implemented in the program STRUCTURE 2.1 (Pritchard et al., 2000; Falush et al., 2003). This model assumes that there is an unknown number of K populations (or genetic clusters), each of which is characterized by a set of allele frequencies at each locus. STRUCTURE assumes Hardy–Weinberg and linkage equilibria in a population. If the basic model is expanded to the model with admixture, the admixture proportion of each individual *q* (i.e. the proportion of individual's genome that originates from population K) can be determined. Individuals are probabilistically assigned to one or more clusters. For each value of K we performed three runs to evaluate the consistency of the results using the entire sample set (n=291). All STRUCTURE analyses were based on runs of 10^6 iterations, following a burn-in period of 10^5 iterations. In a first approach the population structure was assessed assuming an unknown number of K (range K=2 to K=10) and posterior

Table 2. Wolterstorff index (mean ± SD and range) for males and females in each locality with assignment to the species *T. cristatus* (*Tcri*) and *T. carnifex* (*Tcar*).

Locality	N (m, f)	WI (males)	Species	WI (females)	Species
Koc	16 (8,8)	58.3±2.6 [53.8–61.9]	*Tcri*	46.4±2.5 [44.0–52.0]	*Tcri*
Nie	6 (2,4)	54.6±4.6 [51.3–57.9]	*Tcri*	52.0±4.0 [48.7–57.1]	*Tcri*
Len	2 (0,2)	–	–	52.1±1.0 [51.4–52.8]	*Tcri*
Roi	3 (0,3)	–	–	57.3±0.7 [56.7–58.1]	*Tcar*
Sil	10 (2,8)	66.3±5.3 [62.5–70.0]	*Tcar*	55.8±3.4 [50.0–59.6]	*Tcar*
Bab	7 (1,6)	57.1	*Tcri*	52.6±3.5 [47.6–55.3]	*Tcri*
Bis	4 (2,2)	61.1±6.7 [56.3–65.8]	*Tcri*	49.1±1.3 [48.1–50.0]	*Tcri*
Sur	7 (1,6)	59.2	*Tcri*	53.2±6.4 [48.6–65.9]	*Tcri*
Rei	5 (0,5)	–		53.2±4.7 [45.8–58.8]	*Tcri*
Hai	4 (3,1)	62.9±0.8 [62.1–63.6]	*Tcri*	51.1	*Tcri*
Baz	10 (6,4)	63.4±2.4 [60.0–66.7]	*Tcri*	51.2±3.3 [47.2–54.2]	*Tcri*
Bad	7 (2,5)	64.4±1.4 [62.5–65.6]	*Tcar*	53.5±4.9 [47.9–62.5]	*Tcri*
Stg	5 (2,3)	65.3±0.7 [64.9–65.8]	*Tcar*	57.8±0.9 [56.8–58.5]	*Tcar*
See	3 (1,2)	66.7	*Tcar*	57.1±2.6 [55.3–59.0]	*Tcar*
Sta	4 (2,2)	65.8±1.3 [64.9–66.7]	*Tcar*	54.5±0.7 [54.1–55.0]	*Tcar*
Gun	8 (4,4)	66.9±0.5 [64.7–67.7]	*Tcar*	58.3±0.3 [57.9–58.5]	*Tcar*
Mic	2 (2,0)	66.7	*Tcar*	–	–
Ter	2 (2,0)	66.2±0.6 [65.8–66.7]	*Tcar*	–	–
Irl	7 (5,2)	63.1±2.6 [58.8–65.7]	*Tcri*	56. 1±2.1 [54.6–57.6]	*Tcar*
Bue	22 (15,7)	62.8±2.7 [58.8–66.7]	*Tcri*	52.0±4.1 [46.7–57.1]	*Tcri*
Fue	17 (13,4)	63.1±3.8 [54.8–66.7]	*Tcri*	56.4±2.4 [53.3–58.3]	*Tcar*
Rie	21 (11,10)	64.3±2.4 [58.8–66.7]	*Tcar*	56.0±2.9 [50.0–58.3]	*Tcar*
Fre	8 (6,2)	64.0±2.5 [61.0–66.7]	*Tcar*	58.2±0.2 [58.1–58.3]	*Tcar*
Gug	7 (7,0)	65.7±0.8 [64.7–66.7]	*Tcar*	–	–
Unt	7 (4,3)	62.4±3.5 [58.1–65.8]	*Tcri*	56.9±2.2 [54.4–58.1]	*Tcar*
Som	18 (8,10)	62.4±2.6 [59.0–65.7]	*Tcri*	54.0±4.3 [46.7–59.0]	*Tcar*
Neu	16 (9,7)	66.2±0.7 [64.7–66.7]	*Tcar*	56.8±1.9 [53.7–58.8]	*Tcar*
Kop	2 (0,2)	–	–	55.2±4.4 [52.1–58.3]	*Tcar*
Zec	11 (5,6)	65.7±0.7 [65.0–66.7]	*Tcar*	56.3±3.2 [50.0–58.7]	*Tcar*
Ach	7 (7,0)	65.3±1.7 [61.5–66.7]	*Tcar*	–	–
Str	8 (4,4)	65.7±0.1 [65.6–65.7]	*Tcar*	58.6±0.3 [57.9–59.5]	*Tcar*
Ame	20 (13,7)	60.7±3.5 [54.6–66.7]	*Tcri*	53.4±3.0 [50.0–57.1]	*Tcri*
Ann	4 (2,2)	62.2	*Tcri*	55.6±1.8 [54.4–56.8]	*Tcar*
Kai	8 (4,4)	65.4±1.3 [63.6–66.7]	*Tcar*	58.0±1.5 [55.8–59.0]	*Tcar*
Har	1 (1,0)	65.8	*Tcar*	–	–
Ise	16 (8,8)	60.7±3.7 [54.8–66.7]	*Tcri*	53.7±2.9 [50.0–59.5]	*Tcri*

probability log likelihood values (Ln P (D)) were evaluated. To estimate the correct number of clusters we calculated ΔK following the approach by Evanno et al. (2005). All populations were assigned to the cluster in which the average proportion of individuals was ≥0.80. If the average proportion of membership for one cluster was less, populations were assigned to more clusters.

We also used these results to search for admixed individuals in our sample set. Therefore we investigated the ancestry of newts, assuming that each hybrid genotype should belong to more than one inferred cluster. We followed a similar approach to that described by Pierpaoli et al. (2003). In an initial procedure all individuals were assigned to the inferred clusters without any prior population information. In a second procedure, prior population information (clusters 1–4) was used for all individuals except newts which were identified as admixed putative hybrids after the first procedure. These individuals were again assigned without prior information.

RESULTS

Morphological markers

Average WI values per population ranged between 57.1 and 66.9 and were thus within the range of the species *T. cristatus* and *T. carnifex*. In 13 populations we found average WI values corresponding to *T. cristatus*. In 17 populations only *T. carnifex*-like WI values were found. The six remaining populations (Irl, Fue, Unt, Som and Ann from Salzburg as well as Bad from Upper Austria) showed intermediate index values. All populations from Bavaria except Sil showed WI values corresponding to *T. cristatus*. The introduced Bavarian *T. carnifex* population Ise interestingly showed *cristatus*-like characteristics in both sexes (Table 2, Fig. 2).

Mitochondrial DNA

We analysed a 520 bp long fragment of *cyt b* for 117 specimens collected from 32 sites. The vast majority of samples

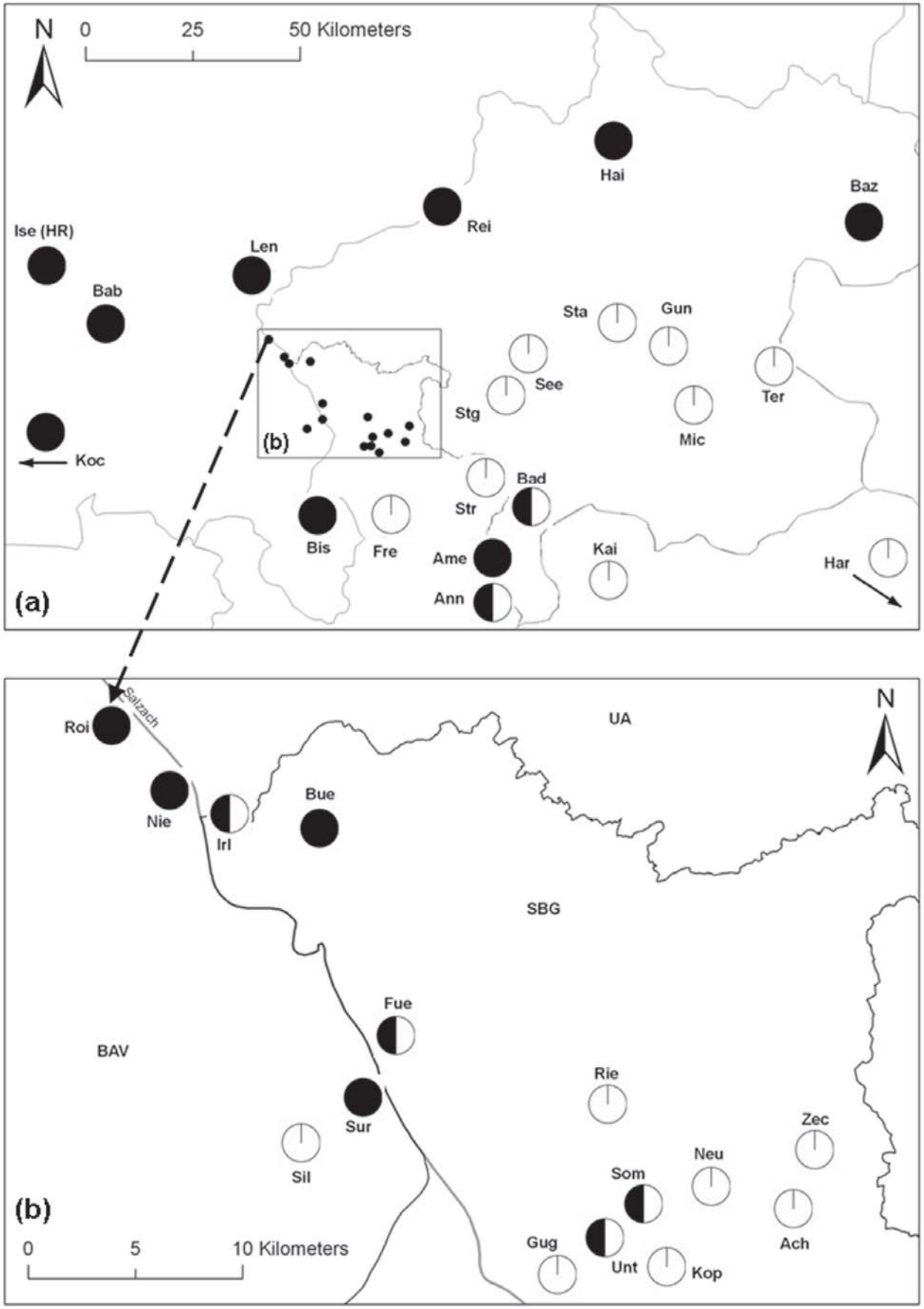

Fig. 2. Pie charts showing the assignment of studied populations to the species *T. cristatus* (black) and/or *T. carnifex* (white) according to average WI values for females and males. Abbreviations of the sample sites are given in Table 1; BAV = Bavaria, UA = Upper Austria, SBG = Salzburg.

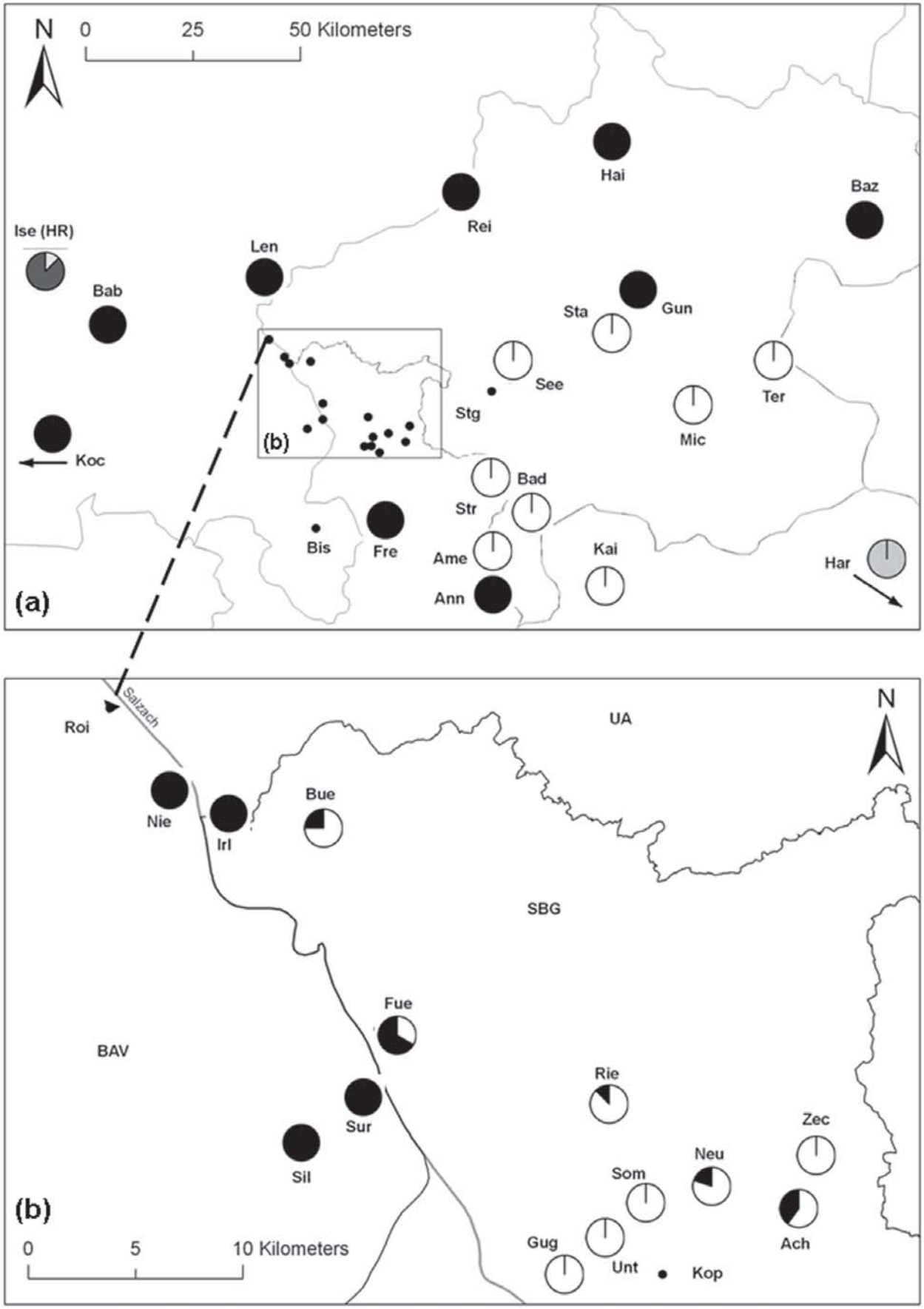

Fig. 3. Pie charts showing the distribution and proportion of four detected *cyt b* haplotypes among studied populations of *T. cristatus* and *T. carnifex*; CRI 1 = black sectors, CAR = white sectors, CAR 1 = light grey sectors, CAR 2 = dark grey sectors. Abbreviations of the sample sites are given in Table 1; BAV = Bavaria, UA = Upper Austria, SBG = Salzburg.

Table 3. The average proportion of membership (*q*) for 36 studied populations (*n*=291) to the inferred clusters (C1, C2, C3, C4) generated by STRUCTURE (Pritchard et al., 2000), and corresponding *cyt b* haplotypes (CRI, CAR, CAR1, CAR2). Clusters to which the different populations are assigned are in italics.

Population	C1	C2	C3	C4	*cyt b*
Koc	*0.976*	0.016	0.004	0.004	CRI
Nie	*0.188*	*0.735*	0.071	0.006	CRI
Len	*0.584*	*0.405*	0.003	0.008	CRI
Roi	0.016	*0.977*	0.003	0.004	–
Sil	0.063	*0.927*	0.006	0.004	CRI
Bab	*0.264*	*0.724*	0.006	0.006	CRI
Bis	0.054	*0.924*	0.014	0.008	–
Sur	0.113	*0.859*	0.020	0.008	CRI
Rei	*0.965*	0.009	0.017	0.009	CRI
Hai	*0.871*	0.006	0.119	0.004	CRI
Baz	*0.804*	0.017	0.174	0.005	CRI
Irl	*0.917*	0.020	0.057	0.006	CRI
Bue	0.092	*0.894*	0.008	0.006	CRI/CAR
Fue	0.022	*0.967*	0.007	0.004	CRI/CAR
Rie	0.047	*0.163*	*0.783*	0.007	CRI/CAR
Fre	*0.560*	0.038	*0.396*	0.006	CRI
Gug	0.024	0.012	*0.960*	0.004	CAR
Unt	0.026	0.022	*0.946*	0.006	CAR
Som	0.015	*0.884*	*0.084*	0.017	CAR
Neu	0.018	0.019	*0.958*	0.005	CRI/CAR
Kop	0.013	0.137	*0.845*	0.005	–
Zec	0.019	0.118	*0.856*	0.007	CAR
Ach	0.022	0.030	*0.918*	0.030	CRI/CAR
Str	0.008	0.012	*0.975*	0.005	CAR
Ame	0.018	*0.957*	0.011	0.014	CAR
Ann	*0.371*	0.024	*0.598*	0.007	CRI
Bad	*0.151*	0.049	*0.795*	0.005	CAR
Kai	0.005	0.005	*0.986*	0.004	CAR
Stg	0.011	0.007	*0.977*	0.005	–
See	0.01	0.008	*0.978*	0.004	CAR
Sta	0.018	0.009	*0.968*	0.005	CAR
Gun	0.006	0.055	*0.926*	0.013	CRI
Mic	0.005	0.004	*0.987*	0.004	CAR
Ter	0.004	0.004	*0.955*	0.037	CAR
Har	0.004	0.005	*0.977*	0.014	CAR1
Ise	0.005	0.009	0.012	*0.974*	CAR/CAR2

belonged to the two most widespread haplotypes, CAR (represented by 54 specimens) and CRI (55 specimens), assigned to *T. carnifex* and *T. cristatus* respectively. Haplotypes differed by 38 substitutions, of which 10 were non-synonymous. Furthermore, seven newts from population Ise possessed haplotype CAR2, differing from the CAR haplotype by two substitutions (A to G at positions 280 and 345), and one specimen from population Har possessed haplotype CAR1, differing by only one substitution (G to A at position 361). No insertions/deletions were observed among sequenced samples. Of the 34 localities scored, six proved to contain two different haplotypes. While at Ise two different *T. carnifex* haplotypes were found (CAR, CAR2), in populations Fue, Ach, Bue, Rie and Neu (all from Salzburg), both CAR and CRI haplotypes occurred (Table 3, Fig. 3). From the perspective of mtDNA these are possible candidates for detecting a contact zone. Populations Ann and Fre from Salzburg are the southernmost localities to show pure CRI haplotypes. In Upper Austria the only individual tested from population Gun was assigned to CRI haplotypes, despite being located within the *T. carnifex* area. No autochthonous individual from Bavaria could be assigned to *T. carnifex* haplotypes.

Microsatellite markers

The seven surveyed microsatellite loci were all highly polymorphic. We detected 7–18 different alleles per locus and a total of 97 alleles (average per locus = 13.9, SD=4.5). Allelic richness (A) per population ranged between 1.69 and 2.95. The values for observed heterozygosity per population showed a wide range with a minimum of 0.17 for population Kop and a maximum of 0.77 for population Som. Expected heterozygosity values were mainly higher than H_O, ranging from 0.28 to 0.75 (Table 4). Results by locus revealed a significant deficit of heterozygotes in loci *Tcri-32* ($P<0.01$), *Tcri-35* ($P<0.001$) and *Tcri-46* ($P<0.01$). F_{IS} values calculated over all loci indicated a significant deficit of heterozygotes in populations Bab and Sur from Bavaria, Stg and Gun from Upper Austria, Bue, Rie, Fre, Zec and Ach from Salzburg and the allochthonous *T. carnifex* population Ise (Table 4). No significant linkage disequilibria were observed when testing each locus pair across all populations. Thus free recombination between loci can be assumed.

According to Bayesian analyses performed with STRUCTURE, the value of K=4 showed the highest ΔK (ΔK = 192.21, Ln P (D) = –4946.97; Table 5). The entire sample (*n*=291) consisted of four distinct clusters. Two clusters each could be attributed to *T. cristatus* (C1, C2) and *T. carnifex* (C3, C4), with admixed populations (membership *q* to one cluster ≤0.8) detected between all clusters except C4, which was exclusively assigned to the allochthonous population Ise. Populations which were assigned to C1 are distributed mainly in the north (rivers Danube and Inn) and far west (population Koc) of the surveyed area. Irl from Salzburg is the southernmost C1 population, but admixed populations with C3 can also be found further south along the river Salzach and its tributaries (Fre and Ann) and in Bab. C2 is found mainly along the putative border of distribution between *T. carnifex* and *T. cristatus* in south-eastern Bavaria and Salzburg. Furthermore, populations Som and Ame were assigned to this cluster in contrast to *cyt b* results that only showed CAR haplotypes. C3 was found in the largest area in the east and south-east of the study area. Both populations from Styria, as well as all southern populations from Upper Austria and the eastern part of Salzburg, could be assigned to this cluster. Admixed populations between C3 and C1 were detected in the eastern border area between Salzburg and Upper Austria (Fre, Ann and Bad). A considerable degree of membership for C3 was also found in the Danube area (Hai and Baz), where the proportion of C1 was slightly higher than 0.8. Admixture between C3 and C2 was detected in population Rie from Salzburg (Table 3, Fig. 4).

Table 4. Average observed (H_O) and expected (H_E) heterozygosity, allelic richness (A) and inbreeding coefficient (F_{IS}) for each sampled population. F_{IS} values with single asterisks (*) indicate significant and double asterisks (**) highly significant results; n.e. = not evaluated.

Population	H_O	H_E	A	F_{IS}	Population	H_O	H_E	A	F_{IS}
Kochel (Koc)	0.46	0.46	2.15	0.009	Irlach (Irl)	0.54	0.59	2.29	–0.060
Niederau (Nie)	0.48	0.53	2.27	0.152	Bürmoos (Bue)	0.51	0.59	2.56	0.061*
Lengthal (Len)	0.57	0.50	n.e.	0.000	Fürwag (Fue)	0.64	0.64	2.45	0.007
Roibach (Roi)	0.38	0.44	n.e.	0.135	Ried (Rie)	0.42	0.69	2.61	0.122**
Sillersdorf (Sil)	0.37	0.39	1.81	0.028	Freimoos (Fre)	0.57	0.67	2.79	0.178*
Babensham (Bab)	0.24	0.31	1.69	0.204*	Guggenthal (Gug)	0.55	0.65	2.46	–0.188
Bischofwiesen (Bis)	0.57	0.57	n.e.	0.000	Unterkoppl (Unt)	0.52	0.58	2.57	–0.006
Surheim (Sur)	0.49	0.65	2.69	0.228**	Sommeregg (Som)	0.77	0.62	2.57	0.106
Reichersberg (Rei)	0.54	0.54	2.67	0.071	Neuhofen (Neu)	0.69	0.62	2.57	–0.013
Haibach (Hai)	0.48	0.52	n.e.	0.000	Koppl (Kop)	0.17	0.75	n.e.	–0.200
Bad Zell (Baz)	0.66	0.64	2.72	–0.040	Zecherl (Zec)	0.20	0.64	2.66	0.225**
Bad Ischl (Bad)	0.49	0.60	2.30	–0.064	Achleiten (Ach)	0.43	0.73	2.95	0.255**
St. Georgen (Stg)	0.55	0.33	1.70	0.211**	Strobl (Str)	0.46	0.44	2.10	–0.172
Seewalchen (See)	0.58	0.61	n.e.	0.180	Ameisensee (Ame)	0.36	0.54	2.36	–0.011
Stadl-Paura (Sta)	0.50	0.58	n.e.	0.071	Annaberg (Ann)	0.36	0.62	n.e.	–0.106
Gunskirchen (Gun)	0.51	0.57	2.29	0.296**	Kainisch (Kai)	0.29	0.28	1.70	0.064
Micheldorf (Mic)	0.65	0.50	n.e.	0.167	Hartberg (Har)	n.e.	n.e.	n.e.	n.e.
Ternberg (Ter)	0.50	0.46	n.e.	0.273	Isen (Ise)	0.50	0.66	2.90	0.172*

Admixed individuals between three clusters (C1, C2 and C3) were found in populations Rie and Zec from Salzburg. Individual Ame_17 showed admixture between clusters C1 (*T. cristatus*), C2 (*T. cristatus*) and C4 (*T. carnifex*). Admixture between the two *cristatus* clusters was observed in populations Len, Bab and Sur. Putative hybrid individuals between C1 (*T. cristatus*) and C3 (*T. carnifex*) were found in the populations Hai, Baz and Bad from Upper Austria, as well as Irl and Ann from Salzburg. Putative hybrids between C2 (*T. cristatus*) and C3 (*T. carnifex*) were detected from populations Rie, Som, Kop and Zec in Salzburg, as well as Bad and Gun from Upper Austria (Table 6). No autochthonous population of *T. carnifex* was found in surveyed populations from Bavaria. Only one individual from population Nie showed a considerable amount of the *T. carnifex* genotype. Linking the data from the Wolterstorff index with putative hybrid individuals that were identified in STRUCTURE analysis, no clear correlation could be found. Eleven out of 28 putative hybrids showed divergent WI values compared to the other analysed individuals. A further seven individuals showed intermediate WI values (Table 6).

Table 5. Population genetic clustering results based on the Bayesian modelling method in STRUCTURE. The best fit model K=4 (italics) was determined following recommendations in Evanno et al. (2005); K = number of assumed subpopulations, Ln P (D) and Var Ln P (D) are posterior probabilities and variance for each model, ΔK = value based on the rate of change in the log probability of data between successive K values.

K	Ln P (D)	Var Ln P (D)	dK
2	–5631.77	177.03	9.24
3	–5244.03	260.17	24.34
4	*–4946.97*	*309.50*	*192.21*
5	–4779.53	342.27	71.30
6	–4702.93	407.80	38.72
7	–4618.10	454.80	10.02
8	–4569.87	526.43	1.67
9	–4511.03	544.47	18.23
10	–4500.27	616.43	6.71

DISCUSSION

Comparison of markers and non-destructive sampling

In our study we compared distribution patterns and hybridization on the basis of three types of markers in southern Germany and northern Austria. Crested newts (*T. cristatus* superspecies) are characterized by an exceptionally high level of phenotypic variation, particularly in contact zones (Arntzen & Wallis, 1994, 1999). Thus analysis of morphological characters like the Wolterstorff index or number of rib-bearing vertebrae might lead to a high level of false determinations. Mitochondrial DNA markers have provided powerful tools to increase accuracy. However, some instructive cases have shown that only using mitochondrial markers could lead to incomplete conclusions (e.g. Arntzen & Wallis, 1999; Babik et al., 2003). Mitochondrial DNA is maternally inherited and introgression from one species into another over comparatively large distances is well known in contact zones (Babik et al., 2005). When only mtDNA markers are used, misdeterminations due to introgression should be taken into account, and so both types of genetic markers (nuclear and mtDNA), together with morphological markers, should be used in species determination and hybrid detection.

Because of the newts' high level of legal protection and rarity in the study area in Austria, we decided to use adult individuals and non-destructive sampling methods.

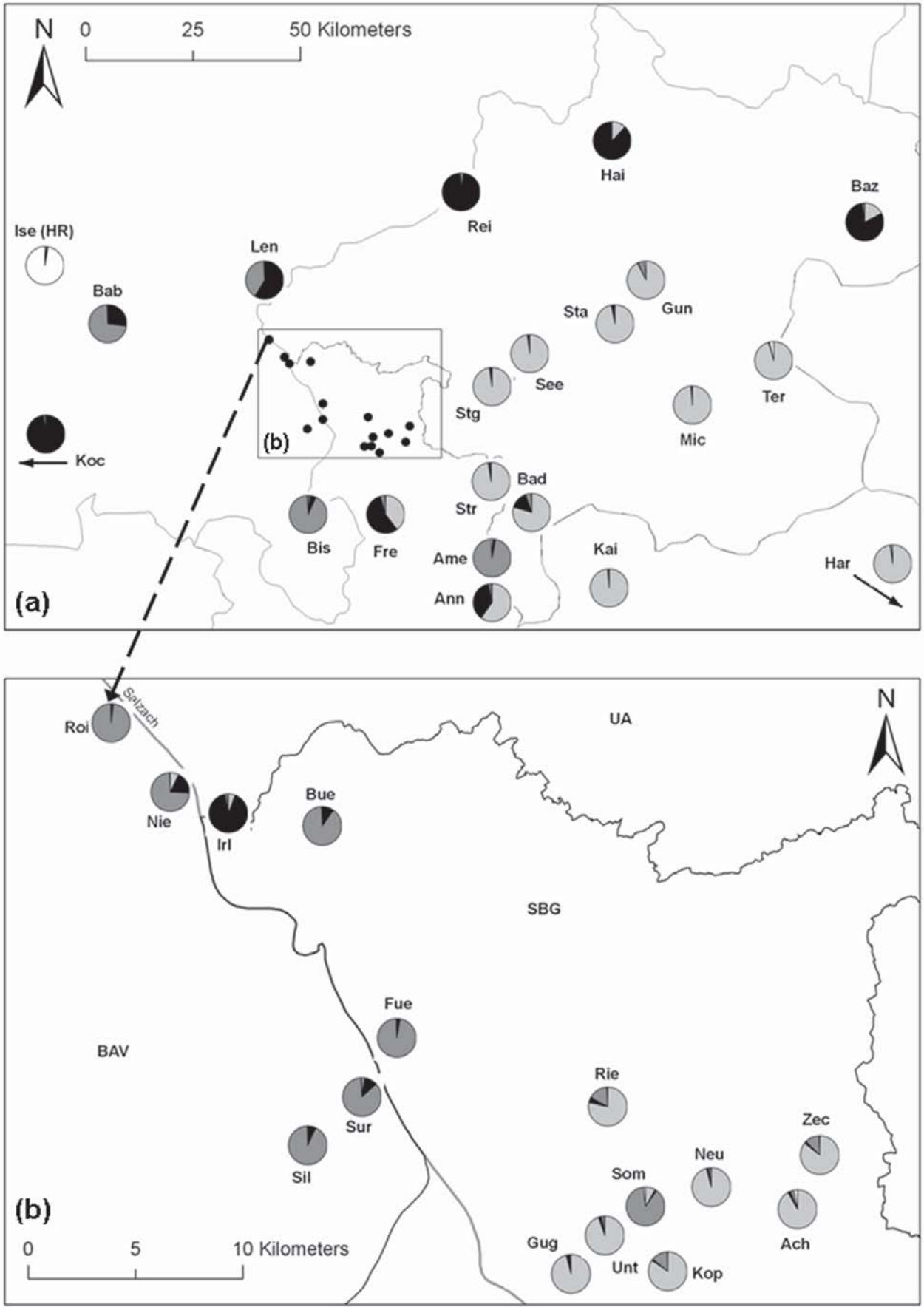

Fig. 4. Pie charts showing the proportion of individual genotypes (*q*) from microsatellite loci, averaged per population, computed using STRUCTURE 2.1 (Pritchard et al., 2001) with K=4. C1 = black sectors, C2 = dark grey sectors, C3 = light grey sectors, C4 = white sectors. Abbreviations for sample sites are given in Table 1; BAV = Bavaria, UA = Upper Austria, SBG = Salzburg.

Table 6. Population assignment and inferred ancestry of admixed individuals without (first value) and with (second value) using prior population information, as well as WI values. Clusters to which the different admixed individuals are assigned are in italics.

	Cluster 1 (CRI 1)	Cluster 2 (CRI 2)	Cluster 3 (CAR 1)	Cluster 4 (CAR 2)	WI
C1	*0.947–0.934*	0.017–0.016	0.030–0.045	0.006–0.005	
C2	0.040–0.062	*0.939–0.918*	0.014–0.013	0.007–0.007	
C3	0.020–0.020	0.019–0.019	*0.953–0.953*	0.008–0.008	
C4 (Isen)	0.005–0.005	0.009–0.009	0.012–0.012	*0.974–0.974*	
Nie_3 (female)	0.165–0.155	*0.626–0.599*	*0.202–0.240*	0.006–0.006	57.1
Nie_4 (female)	*0.216–0.227*	*0.640–0.601*	0.136–0.164	0.008–0.007	53.3
Nie_5 (female)	*0.538–0.529*	*0.392–0.347*	0.062–0.116	0.007–0.008	48.7
Len_2 (female)	*0.336–0.492*	*0.656–0.500*	0.004–0.004	0.005–0.005	52.8
Bab_2 (female)	*0.688–0.774*	*0.304–0.218*	0.004–0.004	0.004–0.004	n.e.
Sur_3 (female)	*0.409–0.445*	*0.571–0.534*	0.013–0.014	0.007–0.007	n.e.
Hai_2 (male)	*0.538–0.450*	0.007–0.007	*0.451–0.538*	0.004–0.004	62.9
Baz_3 (male)	*0.770–0.726*	0.006–0.006	*0.220–0.264*	0.004–0.004	66.7
Baz_5 (male)	*0.605–0.492*	0.008–0.010	*0.383–0.494*	0.004–0.004	65.7
Baz_8 (male)	*0.680–0.640*	0.054–0.052	*0.261–0.304*	0.005–0.005	62.9
Baz_10 (female)	*0.734–0.662*	0.008–0.009	*0.253–0.324*	0.004–0.004	47.2
Irl_2 (female)	*0.781–0.788*	0.033–0.032	*0.182–0.176*	0.004–0.004	54.6
Rie_2 (female)	0.031–0.037	*0.427–0.378*	*0.537–0.578*	0.006–0.006	58.3
Rie_3 (male)	0.034–0.034	*0.284–0.287*	*0.678–0.674*	0.004–0.004	65.6
Rie_5 (male)	0.005–0.172	*0.659–0.021*	*0.331–0.802*	0.004–0.005	62.5
Rie_6 (female)	0.014–0.005	*0.595–0.657*	*0.383–0.334*	0.007–0.004	55.0
Rie_7 (female)	0.140–0.014	*0.739–0.598*	*0.111–0.381*	0.011–0.007	54.3
Rie_12 (male)	*0.193–0.143*	*0.020–0.738*	*0.781–0.108*	0.005–0.011	65.7
Fre_2 (female)	*0.667–0.648*	0.021–0.021	*0.300–0.320*	0.012–0.012	58.3
Fre_4 (male)	*0.323–0.331*	0.054–0.052	*0.618–0.613*	0.005–0.004	65.8
Fre_8 (female)	*0.656–0.644*	0.043–0.043	*0.297–0.309*	0.004–0.004	58.1
Som_3 (female)	0.004–0.004	*0.428–0.462*	*0.560–0.526*	0.008–0.008	53.9
Som_18 (male)	0.006–0.006	*0.588–0.589*	*0.397–0.396*	0.009–0.009	64.7
Kop_1 (female)	0.009–0.009	*0.263–0.271*	*0.722–0.714*	0.006–0.006	52.1
Zec_1 (female)	0.005–0.005	*0.706–0.710*	*0.281–0.277*	0.008–0.008	50.0
Zec_7 (male)	0.010–0.011	*0.193–0.141*	*0.782–0.834*	0.014–0.015	65.9
Zec_11 (female)	0.101–0.106	*0.137–0.131*	*0.757–0.757*	0.006–0.006	57.1
Ame_17 (male)	*0.192–0.189*	*0.599–0.603*	0.008–0.008	*0.201–0.200*	63.2
Ann_2 (male)	*0.404–0.342*	0.068–0.064	*0.516–0.583*	0.012–0.011	62.2
Bad_4 (female)	*0.759–0.681*	0.036–0.044	*0.199–0.269*	0.005–0.006	56.0
Bad_5 (male)	*0.282–0.263*	0.012–0.013	*0.702–0.720*	0.004–0.004	65.6
Bad_6 (female)	0.011–0.013	*0.268–0.250*	*0.716–0.732*	0.005–0.005	47.9
Gun_1 (female)	0.009–0.009	*0.377–0.381*	*0.608–0.603*	0.006–0.006	58.5

Buccal swabs have been successfully used for extracting mtDNA and microsatellite markers in a range of amphibian species including crested newts (Pidancier et al., 2003) and allow efficient and reliable microsatellite genotyping according to a study by Broquet et al. (2007). As the Bavarian part of our samples was gained using toe clips, we can state that we obtained high quality DNA and PCR products in both non-destructively gained tissue and toe clips. The use of adult individuals is obviously reflected by limited sample sizes, rendering some tests (e.g. linkage disequilibria in populations) impossible.

HZ extension

We detected three regions where admixed populations and individuals were present in the area between Bavaria and Upper Austria. Wolterstorff (1929) was the first to describe the presence of putative admixed individuals in the surroundings of Salzburg in connection with individuals he obtained from F. Mahler. The existence of some admixed individuals or even populations was later reported again in the north and north-east of the province of Salzburg (Schüller, 1963; Freytag, 1978), as well as from neighbouring south-eastern Bavaria (Berchtesgadener Land), where *T. carnifex* individuals with introgression of *T. cristatus* features and even pure *T. carnifex* populations were suspected, according to their morphology (Schmidtler, 1976). Sochurek (1957, 1978) predicted the contact zone between the two species ran along the 100 km line from Salzburg in the south to Passau in the north, suspecting no occurrence of *T. carnifex* north of the river Danube in Austria. Arntzen & Thorpe (1999) claimed that this contact zone even reached as far as Vienna, where the two species meet the western distribution border of *T. dobrogicus*.

As already assumed by Thiesmeier & Kupfer (2000), to the best of our knowledge no pure *T. carnifex*

populations presently occur in Bavaria. We analysed individuals from three out of five populations – the remaining two have most probably vanished – presented by Schmidtler (1976). Of these, individuals from the population in Sillersdorf (Sil), which possessed morphological characteristics of *T. carnifex* (Franzen et al., unpublished data), could clearly be assigned to *T. cristatus* according to molecular markers. All further populations could also be assigned to *T. cristatus*, with only Niederau (Nie) containing one hybrid individual.

The results from Austrian populations showed good concordance for morphological and molecular markers, but WI values must be treated cautiously in admixed populations. In addition to the predicted hybrid zone north and northeast of the city of Salzburg, we also detected admixed *cristatus*-like individuals on both sides of the river Danube in Upper Austria, where introgression of *T. carnifex* can be found. Previous studies from the north of Lower Austria have already disproved Sochurek's (1978) prediction, locating *T. carnifex* populations north of the Danube and even as far as Moravia and South Bohemia, Czech Republic (Piálek et al., 2000; Horák et al., unpublished data). A further contact zone with admixed populations is located south of the city of Salzburg, where introgression from *T. carnifex* could be detected in the populations Fre and Ann, while the large high-altitude population at lake Ameisensee (Ame; Maletzky et al., 2004) appears to be isolated to a high degree. Its relatedness to the Ise population might be caused by discrete convergent developments. The hybrid character of the Bad population is based on anthropogenic interference, with several introductions of newts from at least two different populations into one private garden pond (R. Mysliwietz, pers. comm.).

The former extension of HZs in the study area is hard to determine. There is a clear NW–SE cline at a large geographic scale, but this pattern is not so clear on a smaller scale, where geographically close populations are genetically different (see populations Som and Unt or Ame and Ann; Maletzky, 2007). This pattern could on the one hand be caused by a mosaic distribution of genotypes, associated with specific habitat. More likely, however, it is caused by stochastic processes associated with occasional long-distance migration, recolonization and founder events, i.e. processes, which were also observed in other hybrid zones (Babik et al., 2003; Macholán et al., 2007). In Salzburg and Bavaria we found admixed individuals in a region about 30 kilometres wide, with *T. cristatus*-like and *T. carnifex*-like populations converging to 2 km (Som and Unt) and 3.5 km (Ame and Ann). Crested newts have suffered from severe declines in population numbers in all regions studied (Franzen et al., unpublished data; Kyek & Maletzky, 2006). Thus it is evident that only the remains of former HZs can be found today.

Variability in genetic markers and HZ type

The analysis of one mitochondrial DNA marker revealed considerable genetic uniformity. As a consequence of the Pleistocene glaciations in Europe, populations in southern or eastern refugia could accumulate high genetic richness, while rapidly expanding northern populations are genetically pure and uniform (Bilton et al., 1998; Hewitt, 2001). As a more northerly distributed species, *T. cristatus* was found to be genetically homogenous over most of its distribution range in previous studies (Wallis & Arntzen, 1989; Arntzen & Wallis, 1999). These developments are presumably a result of bottleneck effects associated with expansion towards northern areas after Pleistocene glaciations and can also be detected in our studied populations. Apart from the introduced population Ise and one sampled specimen from Hartberg (Har) close to the Hungarian and Slovenian border, we only found one *cyt b* haplotype for *T. carnifex* throughout the surveyed region. The same haplotype was found in Slovenia. In *T. cristatus* the only detected haplotype was also found in the Czech Republic (Horák et al., unpublished data), England and Romania (Steinfartz et al., 2007), showing extreme uniformity of *T. cristatus* mtDNA. Salzburg area is more isolated and was more likely colonized with a limited number of individuals, and probably fewer times, than areas with slightly higher diversity such as in the Czech and Slovak Republics.

Although intrapopulation variability at microsatellite loci was high in most populations studied, sub-structuring of the whole sample was relatively low, revealing only two distinct clusters each for *T. cristatus* and *T. carnifex* (one found exclusively in Ise). In fact, HZs in the surveyed region were most probably formed by one genetically different *T. carnifex* population and two different *T. cristatus* populations. These findings correlate well with mtDNA results. Crested newts, and here particularly the two *T. cristatus* clusters are clearly associated with landscape structure and river valleys that might constitute constraints in the postglacial recolonization of this region.

According to Barton & Hewitt (1985), most hybrid zones may be seen as clines of different characters maintained by the interaction between dispersal and selection against hybrids. It seems that in our case, at a given geographical scale, HZs are like a cline where the frequency of genotypes is changing from pure *T. cristatus* to pure *T. carnifex* in a NW–SE direction. We did not observe syntopic occurrence of parental genotypes, although in two cases *T. cristatus*-like and *T. carnifex*-like populations are coexisting less than 4 km apart. Bi-directional introgression, i.e. from *T. cristatus* to *T. carnifex* and vice versa, was observed in both molecular markers. Therefore this zone is probably unimodal, with hybrid individuals predominating in the centre and parental genotypes at the edges of the zone (Jiggins & Mallet, 2000). Similar results were gained in HZs of these species in the southern part of the Czech Republic (Mikulíèek, 2005). Typical for unimodal HZs, assortative mating might be absent in our study populations from the contact zone and reproductive isolation mechanisms between the species are not fully developed (Jiggins & Mallet, 2000). We can only speculate about habitat–species association. *T. carnifex* is assumed to be more ecologically adaptive and use a larger number of habitats (e.g. Arntzen, 2003).

When selection against heterozygotes operates in hybrid zones, heterozygote deficit should be detected in

populations of mixed origin. Although a significant deficit of heterozygotes was also observed in some hybrid populations under study, the cause of this pattern remains unknown. Out of ten populations showing a significant heterozygote deficit, only four were admixed (Gun, Rie, Fre, Zec). Other admixed populations showed either HW equilibrium (Nie, Hai, Som) or heterozygote excess (Baz, Bad, Kop, Ann). According to these results, it seems that selection against heterozygotes (hybrids) is not the prevalent mechanism acting in the *T. cristatus* × *T. carnifex* hybrid zone and the deficit of heterozygotes in analysis may also be caused by the presence of null alleles.

The allochthonous population Isen

In the past decades several allochthonous populations of *T. carnifex* have been reported in western and central Europe (e.g. Arntzen & Thorpe, 1999; Bogaerts, 2002; Franzen et al., 2002). *T. carnifex* shows the widest ecological amplitude of all crested newt species and is considered to be fairly adaptive and competitive (Arntzen, 2003). Therefore the introduced populations pose a threat to native crested newts from hybridization or displacement (Arntzen & Thorpe, 1999). In this context, the Ise population requires special attention.

This population was reported to be based on offspring (100–200 larvae) from one pair, which was captured near Rovinj, Croatia (Franzen et al., 2002). Thus, a maximum of four different alleles should be detected in one heterozygote individual at a given microsatellite locus. However, we detected between three and nine alleles per locus, rendering this information highly questionable. The presence of two *T. carnifex* haplotypes may either signal their presence in the donor locality, or alternatively one came from Balkans with the main colonization wave. However, there are no data about the occurrence of crested newts in this particular locality and the surrounding area is inhabited by *T. cristatus*. Therefore, we prefer the former explanation.

Franzen et al. (2002) have already discussed the low WI values within the Ise sample and stated that in contrast all individuals were morphologically rather "typical" *T. carnifex* with respect to their low number of rib-bearing vertebrae (14 vs 15 in most other Bavarian populations), their comparably smooth skin, the almost complete lack of small light markings on the flanks and the rather constant presence of a yellow vertebral line. We can also not exclude the possibility that the low WI values may reflect different measurement techniques, rather than biological variation (Arntzen & Wallis, 1994).

The analysis of nuclear markers revealed a very uniform structure (data not shown). Sampled individuals from all Austrian *T. carnifex* samples were strongly differentiated, resembling a distinct cluster. According to molecular data, newts from Ise did not interbreed with newts from the nearest autochthonous populations, at least until 2001 when the samples were collected. However, there is no information about the status of the nearest *T. cristatus* populations. There are no records of autochthonous *T. cristatus* from that locality in the Bavarian amphibian mapping programme prior to 1990. Nearest current *T. cristatus* localities are at distances of 7 and 8 km and separated from the sample locality by large corridors of mostly unsuitable habitats (i.e. few ponds, large stretches of agricultural land, many roads). The assumed dispersal distance for crested newts is 1 km per year (Arntzen & Wallis, 1991; Halley et al., 1996). Monitoring of the populations in this region is important to minimize the danger of displacement developments as described for the area around Geneva (Arntzen & Thorpe, 1999).

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