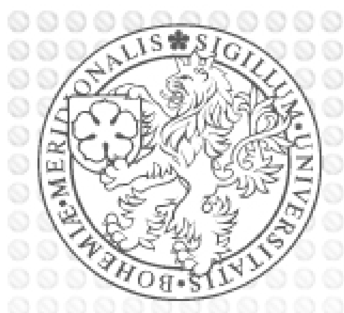

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Genetic structure of the *Triturus cristatus* complex in central Europe

(Ph.D. Thesis)

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Annotation:

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Abstract:

We have explored patterns of genetic variability in *Triturus cristatus* complex in two case studies. In the first study, we have surveyed genetic variability in central European populations of *T*. *carnifex*, *T*. *cristatus* and *T*. *dobrogicus* using partial mitochondrial cytochrome b sequences. Only one *T*. *carnifex* haplotype carried by single specimen was found in central Europe. All *T*. *cristatus* specimens belonged to the one clade of seven, highly similar haplotypes without any correlation to geography. In *T*. *dobrogicus*, three rather diversified clades with significant geographical associations were found.

Using both nuclear and mitochondrial molecular markers and morphology, we have described also contact zone between *T*. *cristatus* and *T*. *carnifex* in Salzburg region, Austria. We propose the secondary hybrid zone is of unimodal type with possible impact of landscape pattern. In contrast to microsatellite data, mitochondrial cytochrome b sequences show considerable uniformity suggesting this region was colonised only limited number of times.

Hereby I declare I've written this thesis all by myself with help of literature cited beyond. My contribution to presented manuscripts is specified further.

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1 Introduction

1.1 *Triturus cristatus* superspecies

1.1.1 Origin and phylogeny

Newts of genus *Triturus* were traditionally, according to their body size, placed into the three groups: the large bodied (crested and marbled newts), the intermediate sized (alpine and banded newt) and the small bodied (*T. vulgaris* species group). Recent molecular phylogenies based on the mtDNA more or less confirmed a monophyly of the respective groups, however questioned the monophyly of the whole genus (Caccone et al., 1994, 1997; Titus and Larson, 1995; Litvinchuk et al., 2005; Weisrock et al., 2006; Steinfartz et al, 2007). Litvinchuk et al. (2005) suggest only the large bodied species to retain generic name *Triturus*, which was refrained also in extensive study of Steinfartz et al. (2007).

The fossil evidence places the origin of the large bodied *Triturus* newts to the Lower Miocene. Böhme (2003) dates the fossil *Triturus* cf. *marmoratus* to 24.2–23.8 mya. The genus *Triturus* s.s. (sensu Litvinchuk et al., 2005) can be divided into the two monophyletic clades with a strong geographic pattern of distribution. The “western” clade contains the so called “marbled“ species (*T. pygmaeus* and *T. marmoratus*) distributed on the Iberian Peninsula and most of the France. The eastern clade, also known as the *Triturus cristatus* complex (or superspecies), encompasses the “crested” species *T. cristatus* (Laurenti 1768), *T. carnifex* (Laurenti 1768), *T. dobrogicus* (Kiritzescu 1903) and *T. karelinii* (Strauch 1870), whose distribution ranges from the France and the British Isles on the west, to the Ural mountains, the Caspian sea and the Turkey on the east (Arntzen, 2003). The evolutionary centre of marbled newts is though to be on Iberian Peninsula, where as in crested newts it is the Balkans and the Black sea (Crnobrnja-Isailović *et al.* 1997; Arntzen 2003).

The “crested” clade comprises of four species *T. cristatus*, *T. carnifex*, *T. dobrogicus* and *T. karelini*. Its origin was, based on the *T. cristatus*-like fossil found in Poland and Slovakia (Sanchíz & Mlynarski 1979; Hodrová 1985), traditionally dated to cca 8 mya.

The mitochondrial RFLP and allozyme data point to the divergence cca 5-7 MYA and 2.5-3 MYA respectively (Wallis and Arntzen, 1989, MacGregor et al., 1990). Based on complete cytochrome b (*cytb*) mtDNA sequences, Steinfartz et al. (2007) estimated the origin of the oldest *T. karelini* lineage between 11-17 mya and the rest of species to appear between 3-6 mya. While monophyly of the group and respective species have never been questioned, its phylogeny is still poorly understood. The basal position of *T. karelini* is accepted by most of authors, however mutual relationships between other three species remains unclear. Morphology and ecological preferences point to some similarities between *T. cristatus* and *T. carnifex,* where as allozyme (Horák, 2000) and microsatellite (Mikulíček, 2005) data put *T. cristatus* as a sister group to the *T. carnifex*-*T. dobrogicus* clade. Recent studies employing mtDNA sequences reveal very fast radiation, though slightly favouring (karelini(carnifex(cristatus,dobrogicus))) topology (Weisrock et al. 2006; Steinfartz et al., 2007; Figure 2).

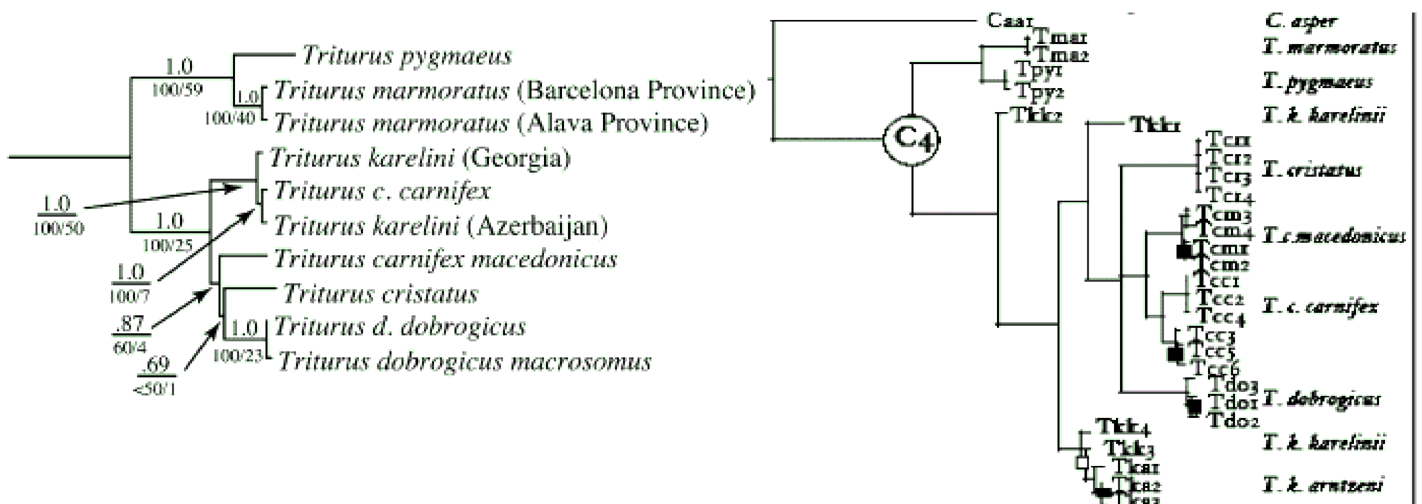

Fig 2 Phylogeny of large bodied *Triturus* newts, based on a) 2700 bp fragment of mtDNA (from Weisrock et al, 2006), b) complete *cytb* mtDNA (from Steinfartz et al., 2007) sequences

The reasons behind the rapid radiation within complex are still unknown. Although all published time estimates significantly differ, they all undoubtedly point to pre-Pleistocene vicariation. Quarternary glaciations thus did not cause speciation in crested newts, however, they might strongly affect the genetic variability and strucure of respective species, especially those with northern distribution (*T. cristatus*; Wallis and Arntzen, 1989). Crnobrnja-Isailović *et al.* (1997) proposed scenario, where a movements of the Miocene sea-level created a physical barrier to a gene flow. Separated populations accumulated mutations that restricted further genetic exchange and started the process of speciation. Short internal branches found in mitochondrial based trees published so far (Weisrock, 2006; Steinfartz, 2007) seem to support this hypothesis.

1.1.2 Present distribution and genetic differentiation

There is only limited amount of data about pre-Pleistocene distribution of crested newt species (Sanchíz & Mlynarski, 1979; Hodrová, 1985), so one can not provide any solid comparison with the present situation. However, Miocene fossils from Včeláre locality reveals presence of *T. cristatus* and *T.* cf. *marmoratus* from central Europe (Hodrová, 1985; Hodrová , unpublished data). Nowadays, the species distribution is parapatric (Fig. 3) and genetic interaction on sites where the areals of respective species meet, is described from numerous studies (Wallis and Arntzen, 1989; Crnobrnja et al., 1989; Klepsch, 1994, Kalezic et al., 1997; Horák, 2000; Mikulíček et al. 2004). Of all crested newts, the most widespread and northernmost distribution belongs to *T. cristatus*. This species can be found from British Isles, central Sweden and Norway in the north, central Russia and the Ural mountains on the east, to Atlantic coast of France on the west and western Romania on the south. Distribution of *T. dobrogicus* is limited to the lowland environment of the basins of the Middle and Lower Danube River and its large tributaries It spans from northern Bulgaria in the south to south-eastern part of Czech republic on the north. *Triturus carnifex* inhabits Apennine and Balkan Peninsulas with extension to the central

Europe (up to the southern part of Czech Republic), and *T. karelinii* is distributed from eastern Balkans to the Caspian Sea (Arntzen & Borkin, 1997; Arntzen, 2003).

Based on the allozyme and mtDNA data, the crested newts are thought to posses the lowest genetic variability compared to other newt groups (Kalezic and Hedgehog, 1980; Crnobrnja et al., 1989; Steinfartz et al. 2007). This applies especially for *T. cristatus*, species with the widest and northernmost distribution, that is extremely homogenous as far as mtDNA RFLP and sequential data (Walis and Arntzen, 1989, Steifartz 2007) reveals. There is no morphological variation (sensu subspecies or morphs) described.

The areal of *T. dobrogicus* is disrupted into two parts (Fig3). Litvinchuk and Borkin, (2000), based on the morpholocial differences, suggested the Panonnian basin to be inhabited by *T. d. macrosomus*, while the Dobrogean basin by *T. d. dobrogicus*. However, both the allozyme (Litvinchuk et al. 1994) and mtDNA data (Arntzen and Wallis, 1989) does not support this separation and show a great similarity between both regions.

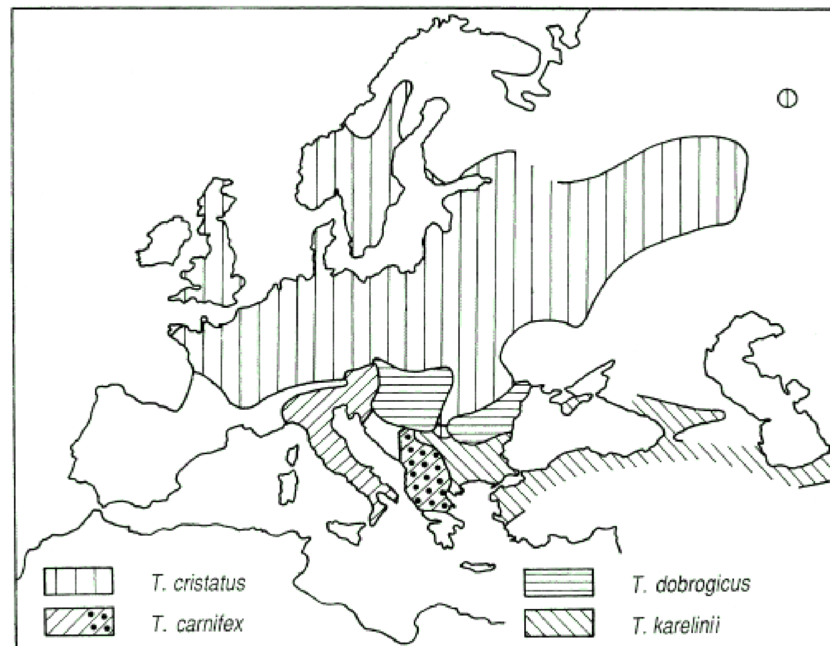

Fig. 3 Distribution of *Triturus cristatus* complex (from Arntzen, 1995)

On the other hand, remaining two species of the complex are genetically much more variable. In *T*. *carnifex*, two subspecies are being recognized based on morphology and genetics (Crnobrnja *et al.* 1989; Kalezić *et al.* 1997; Arntzen & Wallis 1999; Arntzen 2001). *T*. *c*. *carnifex* inhabits Apennines and north-eastern part of Balkans while *T*. *c*. *macedonicus* is found from west Albania and Greece to central Serbia and Macedonia on the east. Scillitani and Picarelio (2000) and Arntzen (2001) found some genetic structuring also within nominal subspecies (allozyme data). Only little is known about variability of *T*. *c*. *macedonicus*.

Although the distribution of *T. karelini* is continuous, this species is recently divided into two subspecies. Triturus. *k.* arntzeni is described from eastern Balkans and possibly western Turkey, where as the rest of its range is inhabited by *T. k. karelinii*. Contrary to the morphological similarity, the subspecies level seems to be supported by the allozyme and genome size differences (Litvinchuk et al., 1999). However, the mtDNA-based phylogeny from Steinfartz et al. (2007) with a rather extensive taxon sampling reveals that this species is formed by a paraphyletic assemblage of mitochondrial lineages on the base of the crested newt's tree. *Triturus karelinii* thus represents an ancestral form, from which the rest of crested newts evolved.

The above stated implies that the species with southern distribution (*T. carnifex* and *T. karelinii*) are genetically and morphologically more variable than those with more northern distribution (*T. cristatus* and *T. dobrogicus*). Similar pattern can be followed in many groups of organisms (i.e. Hewitt, 2004; but see Stewart & Lister, 2001) and is mostly explained by the bottleneck and founder effect phenomena during, between and after glaciation(s), more pronounced in northern areas with the stronger climatic oscillations.

1.1.3 Adaptation, biology and eco-morphological differences

If we accept ancestral position of *T*. *karelinii* and a derived status of *T*. *cristatus* and *T*. *dobrogicus*, as suggested by phylogenetic analyses of mtDNA (Weisrock et al., 2006; Seinfartz et al., 2007), we can also reconstruct a direction in evolution of the

complex. Though it is hard to recognize, whether the morpho-ecological adaptations were the driving force or rather the consequence of the cladogenesis, one can follow a clear pattern. Species with southernmost distribution, *T. karelinii* has bulky body shape with long legs and thick skin; going north and (probably) climbing up the evolutionary tree, the body-plan changes. This is expressed by decreasing values of Wolterstorff index (forelimb lenght to interlimb distance ratio; Wolterstorff, 1923) and/or increasing number of rib-bearing vertebrae. The ancestral state in latter statistic is 13 and 14 in *T. marmoratus* and *T. karelinii* respectively, whereas *T. carnifex* is described to have 15, *T. cristatus* 16 and *T. dobrogicus* 16 – 18 (Crnobrnja-Isailović *et al.* 1997; Arntzen & Wallis 1999; Litvinchuk & Borkin 2000). Both indices describe the same phenomenon: the body loses bulkiness, the legs get shorther. Traditionally, this is being explained as an adaptation to a more aquatic life style as can be seen in *T. cristatus* and especially *T. dobrogicus*. The latter one is most aquatic of all crested newts as can be seen from an average length of aquatic phase (6 months compared to 4-5 months of other species; Jehle et al., 1997; Arntzen & Wallis, 1999) and microhabitat preferences (contrary to other crested newts, *T. dobrogicus* inhabits also slowly-flowing water). Unfortunatelly, very little is known about habitat preferences in amphibians as a whole and the same applies to the crested newts too.

Having an elongate and slender body, a longer tail, and shorter limbs should facilitate undulatory swimming, but reduce speed of movement on land. However, Gvoždík & VanDamme (2007) found no evidence for “running-swimming” trade off in females of *Triturus* newts. Moreover, the average individual dispersal in *T. marmoratus* and *T. cristatus* is described to be comparable and, in both cases, very low (Jehle & Arntzen, 2000). So, although the evolution of body plan within crested newts probably can be explained as an adaptation to different life-style and ecological requirements, its evolutionary importance and effect on movement performance is, at present state of knowledge, unclear.

Results A

Horák A, Piálek J.

Genetic differentiation and post-Pleistocene history of central European crested newts (*Triturus cristatus complex*) as revealed by mitochondrial cytochrome b sequences.

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I have performed all lab work and analyses presented in this manuscript, written with the help of coauthor. Contribution of other people is given in Acknowledgements.

Genetic differentiation and post-Pleistocene history of central European crested newts (*Triturus cristatus* complex) as revealed by mitochondrial cytochrome b sequences.

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Running title: genetic differentiation of crested newts

Abstract:

Large bodied newts of the *Triturus cristatus* complex were considered to be the least differentiated from european salamandrids with both intra- and interspecific distances considerably lower than those of other newt species. This lack of genetic variability was mostly assigned to the impact of quarternary glaciation events connected with the genetic bottleneck, founder effect phenomena and biology of respective species. We have attempted to describe the genetic structure and variability within central European populations previously assigned to *T. carnifex*, *T. cristatus* and *T. dobrogicus* using partial sequences of mitochondrial cytochrome b and to estimate their postglacial history. Analysis of at total 113 specimens from 48 localities revealed intraspecific sequential variability aproximately one magnitude higher than previously described. As expected, *T. cristatus* was the most uniform of the three species, the geographic pattern behind distribution of haplotypes was least pronounced. The sequence of the only central European specimen possessing the *T. carnifex* haplotype was more similar to the Appenines samples than specimens from Balkans. *T. dobrogicus* was found to be the most variable and structured. Three rather separated clades found on a relatively small geographic scale suggest several colonization events probably from different refugial stocks. Possible demographic processes affecting present distribution of *T. cristatus* and *T. dobrogicus* are also discussed.

Introduction

It is generally accepted that Pleistocene glaciations played a key role in structuring of, especially, the European and North American biota (Avise, Walker & Johns , 1998; Hewitt, 2000; Hewitt, 2004). Populations expanding from refugia between glaciation peaks had been gradually loosing a considerable amount of genetic variability due to the repeated genetic bottleneck and founder effect phenomena. This can be followed in almost any group of animals and plants, even those with high dispersal power, such as migratory birds (Mila, Smith & Wayne, 2006) or marine fishes (Bremer et al., 2005). However, the most pronounced, and most often described is effect on organisms such as small mammals (Seddon et al., 2001; Jaarola & Searle, 2002), invertebrates (Lunt, Ibrahim & Hewitt, 1998; Schmitt Giessl & Seitz, 2002, Weider & Hobaek, 2003) or freshwater fishes (Kotlik & Berrebi, 2001; Slechtova et al., 2004; Culling et al., 2006), where limitation of long-range dispersal and/or sort of specific site/environment dependence can be found. Some aspects of amphibian biology (e.g. dependence on water at least at the larval stage and limited ability of dispersal) suggest that this group of vertebrates may be a suitable model for explaining the postglacial history of temperate regions. This fact is reflected by the growing number of studies on genetic structure and phylogeography of amphibian fauna (Steinfartz et al., 2000; Babik et al., 2005; Pabijan & Babik, 2006; but see also Schneider, Cunningham & Moritz., 1998).

The large bodied crested newts of the *T. cristatus* complex, formally known as the *T. cristatus* superspecies , form a monophyletic clade comprised of four presently recognized species: *T. cristatus* (*Laurenti, 1768*), *T. karelinii* (Strauch, 1870) , *T. dobrogicus* (Kiritzescu, 1903) and *T. carnifex* (Laurenti, 1768). Allozyme and mtDNA RFLP data point to the divergence at about 5-7 MYA and 2.5-3 MYA, respectively (Wallis & Arntzen, 1989, MacGregor Sessions & Arntzen, 1990). Based on cytochrome b (*cytb*) mtDNA fragment, Steinfartz et al. (2007) estimated the origin of the *T. karelinii* lineage between 11-17 MYA and the rest of species to appear between 3-6 MYA. The present

distribution of species is mostly parapatric with relatively wide areas, where some sort of genetic interaction was described (Wallis & Arntzen, 1989; Crnobrnja et al., 1989; Klepsch, 1994). *T. cristatus* is found in most of Europe from the British Isles and from to southern Romania to western part of the Ural mountains; *T. karelinii* from the Balkans to the Caspic sea region; *T. dobrogicus* is distributed in the Danube River Basins and around its large tributaries and *T. carnifex* inhabits hilly areas on the Apennines and the Balkans with extension to central Europe (Arntzen, 2003). While *T. cristatus* and *T. carnifex* occupy mainly hilly regions, *T. dobrogicus* is restricted to lowlands (Arntzen & Borkin, 1997; Arntzen, 2003; Mikulíček *et al.*, 2004). Steinfartz et al. (2007) describes the inter- and intra-specific mitochondrial variability within the *T. cristatus* complex as markedly lowest from all *Triturus* species, which corresponds to the mtDNA RFLP data of Wallis & Arntzen (1989) and Arntzen (2001). This low level of genetic differentiation could be the consequence of genetic bottlenecks associated with expansion towards northern areas after the Pleistocene glaciations (particularly in the case of *T. cristatus* as a species with northernmost distribution), and/or high connectedness between populations in lowlands facilitating gene flow (particularly in the case of *T. dobrogicus*; Wallis & Arntzen, 1989).

Historically, only *T. cristatus* and T. *dobrogicus* were reported from central Europe north of Danube valley, though the presence of *T. carnifex* was suspected (Sochurek, 1978). However, morphological (Piálek, Zavadil & Valíčková, 2000) and allozyme data (Klepsch, 1994; Horák, 2000, Arntzen, 2001) have recently revealed the presence of *T. carnifex* in the northern Austria and the southern part of the Czech Republic. In the present study we used a partial sequence of mitochondrial cytochrome b to describe the genetic structure of Czech and Slovak crested newt populations with special emphasis on regions of possible contacts zones between species as suggested by Horák (2000) and Mikulíček et al. (2004). We also attempted to characterize intraspecific structure and to infer demographic processes behind the present distribution. This study is the first large-scale intrapopulation survey on the sequential variability of the crested newts.

Material and Methods

Sampling and sequencing

In total, 106 newt specimens were collected from 41 sampling sites listed in Table 1. All animals were, prior tissue sampling, anaesthetised using Menocain (Infusia,) and after recovering released back to their locality. Tissue samples (toes and/or tail tips) were stored in 96% ethanol until DNA extraction, which was carried out using Qiaquick Tissue DNA kit (Qiagen) following the manufacturers instruction. Part of the mitochondrial cytochrome b (cytb) was amplified using TRIcytb-f (5'- CCT CAC AGG CCT ATT CCT AGC -3' and TRIcytb-r (5'- TAG AAG AGA TAC CTG TTG GGT -3') oligonucleotides in 25 ul PCR reaction of composition: 1x polymerase buffer containing 2.5 mM $MgCl_2$, 0.2 U Taq DNA polymerase (Top-Bio), 8 pmoles of each primer, 200 uM of DNTP’s, and 1-100 ng of template DNA. The thermal profile of PCR consisted of 5 min initial denaturation at 94°C followed by 30 cycles of 94°C/45sec initial denaturation, 50°C/30sec annealing step sec and 30 sec. extension at 72°C. Amplicons of expected size were gel-purified from ethidium bromide or SYBR green stained 1% agarose gel with Qiaquick Gel extraction kit (Qiagen). Alternatively 2 ul of PCR reaction were checked for the presence of a PCR product. The rest was purified using Qiaquick PCR purification kit (Qiagen) and then directly sequenced on Beckman Coulter or ABI-PRISM 3700 automatic sequencer (Applied Biosystems) using manufacturers’s chemistry and instructions. Both strands were sequenced so as at least two independent reads were available for each specimen. Contigs were completed using Editseq and Seqman of DNAStar software package. Sequences were aligned in amino acid open reading frame with the Clustal algorithm and then back translated to nucleotides with Bioedit (Hall, 1999). The sequences were deposited at GeneBank under accession Numbers: EU030807 - EU030937.

Phylogenetic analyses

The bayesian phylogenetic tree was constructed in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR+I+G model of evolution with the Markov chain was set to 2 x 10^6 generations, every 100th tree was sampled and the first 5 x 10^5 generations were omitted from phylogeny reconstruction. The Maximum parsimony (MP) bootstrap support was calculated using PAUP* 4.0b10 (Swofford, 2003). Maximum likelihood bootstraping was performed under the HKY+I+G (second model according to the Akaike Information Criterion as implemented at Modeltest [Posada & Crandall, 1998]) using Phyml 2.4.4 (Guindon & Gascuel 2003). All bootstraping was carried out for 1000 iterations. The median joining haplotype network (Bandelt, Forster & Röhl, 1999) with consequent Steiner MP analysis (Polzin & Daneschmand, 2003) was constructed using Network 4.2.0.1 (http://www.fluxus-ingeneering.com). Statistical parsimony network constructed with TCS 1.21 (Clement, Posada & Crandall, 2000) was used to check for alternative network topology.

Genetic diversity analyses

Molecular diversity indices, analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro. 1992), pairwise F_{ST} tests (Slatkin, 1991; significance on P=0.05 was tested with 10000 permutation), exact test for population differentitation (Raymond & Rousset, 1995; 100000 steps in Markov chain), and tests for selection (Tajima's *D*-test, Tajima 1989) were calculated using Arlequin version 3.1 (Excoffier, Laval & Schneider , 2005) software.

Demographic analyses

We attempted to analyse demographic processes acting in history of studied populations (*T. cristatus* and *T. dobrogicus* only) using two complementary approaches. Nested clade analysis (NCA; Templeton 1998, 2004) was performed using AneCa v1.1 software (Panchal & Beaumont, 2007). This software incorporates TCS and Geodis programs (Posada, Crandall & Templeton, 2000) and automatizes NCA procedure and inference of patterns of dispersal standing behind the distribution of

the nested haplotype clades according to the most recent version of the inference key as suggested by Templeton (http://inbio.byu.edu/faculty/kac/crandall_lab/computer.html; Templeton 2004). Histograms of mismatch distributions show the pattern of nucleotide site differences among individuals within each population and can be used to test for population size change. Slowly declining or long-term stable populations will generate multimodal mismatch distribution, while recent population expansions or past bottlenecks will generate a unimodal distribution (Slatkin & Hudson1991; Rogers & Harpending 1992). Mismatch distribution analysis was performed using Arlequin 3.1. We have also tested goodness-of-fit to the sudden demographic expansion model (Excoffier, 2004). Ten thousands parametric bootstrap replicates were used to generate expected distribution according to the above mentioned model. Proportion of simulations producing larger sum-of-squared deviation (SSD) than observed SSD were then considered as a significance level of statistic. Raggedness index (Harpending 1994) was also calculated using Arlequin 3.1 to quantify smoothness of observed mismatch distribution. Small values are considered to be sign of population expansions, whereas higher values are typical in populations in demographic equilibrium or those who undergone bottleneck (Harpending et al., 1993; Harpending 1994). Significance of observed values was tested as in case of SSD.

Results

We have characterised 379 bp of *cytb* gene for 105 individuals belonging to the *T. cristatus* complex and one T. *marmoratus* specimen. With the exclusion of the outgroup (i. e. *T. marmoratus*, *T. k. karelinii* and *T. k. arntzeni*) used for rooting the phylogenetic trees, we have identified 42 haplotypes, defined by 63 polymorphic sites (49 transitions, 13 transversions, no indels). The reference sequences of *T. dobrogicus* (Bulgaria, Hungary and Romania), *T. carnifex* (Slovenia and Italy) and *T. cristatus* (Romania, UK) originating from this study, and work of Steinfartz (2007) were used to identify the mitochondrial *cytb* haplotype of central European samples.

The Bayesian based phylogeny separated the central European specimens into three clades. According to the presence of reference sequences, the most basal was recognized as *T. carnifex* represented by one Czech (18BOS), four Slovenian, three Italian and one Serbian sample. The Serbian *T. c. macedonicus* significantly differs from the rest of the *carnifex* clade, confirming thus its supspecific status. *T. cristatus* and *T. dobrogicus* specimens (Fig. 2) formed the crown group of the tree. The most abundant was the "*dobrogicus*" clade with 61 sequences; the "*cristatus*" contained 34 sequences. The bootstrap support for basal groups was rather low in any of the tree reconstructing methods employed (ML, MP, BI), however, they all gave consistent topology, differing only by the mutual position of *T. k. karelinii* and *T. k. arntzeni* (irrelevant for purpose of this study).
Molecular variation indices for the respective haplotype groups are listed in Table 2. The nucleotide (pi) diversity was highest within *T. carnifex* (0.01824) and lowest in *T. cristatus* (0.00365). Due to the low taxon sampling from most of the localities and overall low degree of variability within the sequenced region, we have grouped the representatives of respective populations and species to the larger groups according to their geography (see Table 1 and Figure. 3 for details). We also omitted *T. carnifex* from further analyses as only one specimen found in the central Europe. The "mitochondrial *dobrogicus*", specimens from the vicinity of Znojmo were grouped separately due to their presumable "*carnifex*-like" allozyme and morphology structure (see Discussion). The F_{ST} statistic indicates stronger differentiation within the *T. dobrogicus* samples, where most of the values were also significant. The distribution of genetic variation within and among populations was checked with use of the analysis of molecular variance (AMOVA, Excoffier, Smouse & Quattro, 1992)). Results for both *T. cristatus* and *T. dobrogicus* indicated that the largest part of variation is shared among populations within groups (Table 2).
The median joining network corrected with the maximum parsimony Steiner algorithm, enriched also by the relevant sequences originating from study of Steinfartz (2007) resulted in three, well defined, clades with good congruence to the convenient phylogeny methods (Fig. 3). The *T. dobrogicus* was found to be 16 substitutions from the nearest haplotype of both *T. cristatus* and *T. carnifex*. Six

highly diversified haplotypes were observed in the *T. carnifex* part of the network, with the sole Czech sample being more similar to both the Italian sequences (six and seven substitutions difference) rather then samples from the Balkans (ten and more subs.). The *T.* c*ristatus* clade comprises of seven haplotypes of which the central and most widespread covers representatives of four geographic groups ranging from England to Romania. The largest distance between haplotypes was three substitutions. The *dobrogicus* clade was found to be most diverse with 13 haplotypes observed. The largest and the most widespread group contained 13 sequences from 5 geographical subunits ranging from the Danube Delta to eastern Slovakia and the Czech Republic. Samples from the vicinity of the town Znojmo (in southern Moravia near the Austrian borders), with prevailing *T. carnifex* morphology and allozyme sructure as well as the *dobrogicus* samples from south eastern Moravia and western Slovakia, belonged to the "central" haplotype group. All eastern Slovakian samples except one (Svätá Mária) formed a separate "eastern" clade. The samples from westernmost localities were found in rather differentiated clade, here called "western".

Differences between *T. cristatus* and *T. dobrogicus* clades exceeded 95% confidence interval for creating parsimony network, we therefore performed NCA for both species separately. In T. cristatus, only two nesting levels were required for connecting all haplotypes (Figure 4). No statistically significant geographical associations were found. *T. dobrogicus* haplotypes were nested into four levels (Figure 4). Two of six second level nested clades and two of three third-level clades were found to be significantly associated with geography (Figure 4, Table 5). One inference outcome was inconclusive (clade 3-3), in three clades (2-2, 2-4 and 3-1) software suggest rapid expansion or isolation by distance. Analysis of total *T. dobrogicus* part of network (fourth level) yielded continuous range expansion inference. The nesting pattern was in good agreement with our division of clades (Figure 3) with exception of haplotype D21 (assigned to the "central" clade in NCA). In *T. cristatus*, with low sequential variability, unimodal distribution of mismatch distribution (Figure 5) corresponds to the sudden population expansion/recent bottleneck model (Rogers 1995; Rogers et al. 1996); however, none of the neutrality test indices with nearly neutral and non significant values

seem to support this statement. In *T. dobrogicus* mismatch distribution follows a rather bimodal pattern advocating stable or slowly declining populations, when all sequences analyzed (Figure 5). Analysis of the "central" clade yielded similar pattern as in the case of *T. cristatus*.

Discussion

Mitochondrial DNA has proven to be "gold standard" for studying low-level phylogeny, phylogeography or effects of post-glacial expansions (Avise, 2000, 2004). Though there is growing controversy on its use as the sole genetic marker (Arntzen & Wallis, 1999; Babik, Szymura & Rafinski., 2003), it is still widely used, and this is reflected in many recent studies on amphibians or reptiles (e.g. Starkey et al., 2003, Babik et al., 2004; Chiari et al., 2005; Noonan & Gaucher, 2006; Veith et al. 2006).

The conventional phylogenetic methodology is, in combination with an appropriate marker, very useful for reconstructing relationships on as low levels as species to order. However, it may fall short when intra-specific or generally low-taxonomic level relationships are examined and/or variability within sample is insufficient. Here, the coalescent-based methods for reconstructing gene genealogies are more appropriate (Posada & Crandall, 2001). This also proved to be true in the case of crested newts reported here. All tree-building methods employed provided similar topology and the composition of the respective clusters was identical (Fig. 2). The bootstrap support was, with the exception of BI, nonetheless low even for the basal nodes and there was no resolution below species level, caused by the low sequential variability.

The basic evolutionary scenario proposed here is, however, in good congruence with other mitochondrial phylogenies published so far (Weisrock et al., 2006; Steinfartz et al., 2007). The tree rooted with *T. marmoratus* reveals species with present southern distribution (*T. karelinii* and *T. carnifex*) to be ancestral. This suggests south-to-north evolution and is in agreement with placing of the evolutionary centre of the complex in the Balkans (Crnobrnja-Isailovic et al., 1997).

The close relationship between *T. cristatus* and *T. dobrogicus* is thus supported with a mtDNA fragment of total length cca 3 kbp. On the other hand, morphology and ecological preferences point to some similarities between *T. cristatus* and *T. carnifex* and allozyme and microsatellite data (Horák, 2000; Mikulíček, 2005) put *T. cristatus* as a sister group to the *T. carnifex*-*T. dobrogicus* clade. However, their usage as phylogenetic markers has some limitations and has been questioned (Takezaki & Nei, 1996, Peatkau et al., 1997). The commonly used Rag1, Rag2, SSU and LSU rRNA genes carry almost no phylogenetic signal in this respect (Horák et al., unpublished data), so other, more variable nuclear markers are to be sequenced in order to solve the question, which is crucial for understanding the forces driving evolution of the complex.

Nevertheless, for the purpose of this study, the main reason behind performing the phylogenetic analysis was its authority to assign haplotype to respective species. To get further insight into the intra-specific haplotype structure of central European populations, we constructed a median-joining network with consequent maximum parsimony analysis of the looped regions. The distribution of haplotypes into clades representing respective species was identical to the phylogenetic analysis, as well as resulting network topology was identical to those constructed with use of TCS 1.21 (Clement Posada & Crandall, 2000). .

The most obvious pattern of distribution of sequential variability could be seen in *T. cristatus*. The haplotype network (Fig. 2) shows only one group, containing seven closely related haplotypes, This species is considered to be the most genetically homogenous of all crested newts (Wallis & Arntzen, 1989; Arntzen & Wallis, 1999). In this study, we found the most frequent haplotype distributed from England to Romania. Only this sequence was also represented among the *T. cristatus* specimen scored in south-western Austria by A. Maletzky et al. (unpublished data). The analysis of molecular variance (Table3) reveals the largest portion of sequential variability to be shared within populations and the lowest between geographical groups. Also, in comparison with *T. dobrogicus*, the F_{ST} statistics and exact population differentiation test show significantly weaker structuring (Table 4).

The lack of mitochondrial DNA variability indicates that a significant part of modern *T. cristatus* populations originates from very few or even one refugial stock, situated probably northernmost of all crested newt species. Also, the histogram of mismatch distribution, Raggednes index value (Figure 5) and the star-like pattern of haplotype network (Figure 3), suggests recent expansion from, possibly, northern refugium, where populations might undergone massive bottleneck, with no apparent geographic associations.. Importance of northern refugia was recently highlighted and is reflected in the growing number of studies on several groups of organisms (Stewart & Lister 2001; Jaarola & Searle 2002; Kotlík et al. 2006). Several haplotypes found within central European samples point to foothills of the Carpathians or adjacent lowlands as a probable refugium in *T. cristatus* during glaciation(s). The same was recently suggested also for Alpine newts (Pabijan & Babik, 2006). However, more representative taxon sampling, especially from Western Europe and Russia is necessary before drawing any robust conclusions.

A somewhat similar situation was expected in *T. dobrogicus* (Walis & Arntzen, 1989). The occurrence of this species is restricted to basins of the Danube River and its large tributaries (Arntzen & Borkin, 1997). Of all crested newts, *T. dobrogicus* is also the most adapted to the aquatic life (relatively shorter limbs, hydrodynamic body-shape, thinner skin, longer aquatic phase etc.; Arntzen, 2003; but see Gvoždík & Van Damme, 2006). Those aspects of biology facilitate its active (i.e. upstream) and especially its passive (downstream) spread and limit it on the other hand mostly to water bodies. While typical annual dispersal of *T. cristatus* and *T. marmoratus* (species with a more terrestrial life style) was described to range from tenths meters to approximately one kilometre (Arntzen & Wallis, 1991; Haley Oldham & Arntzen, 1996, Jehle & Arntzen, 2000), one can easily imagine the passive dispersal of *T. dobrogicus* can reach several kilometres within few days during annual floods. The relative connectedness between populations that results from their dispersal ability, was argued to be a possible cause of genetic homogeneity in *T. dobrogicus* as reported by Wallis & Arntzen (1989). This is in agreement with the fact that the most represented group of highly

related haplotypes, here called the " central", was found in individuals from southern part of the Czech Republic and eastern Slovakia to the Danube river Delta in Romania and northern Bulgaria (i.e. from northernmost to southernmost part of its range). Also histogram of the mismatch distribution for the "central" clade and star-like shape of respective part of haplotype network (Figure 3) corresponds to the recent expansion model (Rogers, 1995; Rogers et al. 1996). However, two other clades, or haplotype groups, with significant associations to the geography (Figure 3, 4, Table 5), can be recognized in *T. dobrogicus*. Although there is growing controversy on biological interpretation of the NCA and its performance under some conditions (Knowles & Maddison, 2002; Paulo et al. 2002; Masta et al. 2003; Smith & Farrel, 2005; Johnson, Dunn & Bouzat, 2007; but see Templeton, 2004), it is still probably the most widely used method for assessing biogeographic associations and population history from molecular data. In case of *T. dobrogicus*, the NCA suggested, in our opinion, artificial nesting of neighbouring haplotypes from the "western" and "central" clades (clade 2-4, Figure 4). Other than that, the results are in good agreement with our conclusions about the geographic structuring with *T. dobrogicus* (and lack of thereof in *T. cristatus*). The "eastern" clade was mostly represented among specimens from southern and south-eastern Slovakia, though it included also two Romanian samples. Nuclear markers (Horák, 2000; Mikulíček, 2005) confirm their position within *T. dobrogicus* (in some cases with admixture of *T. cristatus*). On the other hand, the "western" clade of rather diversified sequences found in south-western part of the Czech Republic, is formed by specimens with mostly unclear taxonomical status. Allozyme (Horák, 2000) and microsatellite (Mikulíček, 2005) data reveal different levels of admixture of *T. carnifex* into the *T. cristatus* genome or *vice versa* (also discussed further). While nuclear data suggest that specimens from the "eastern" and "western" clades probably belong to different taxa, geographicaly, in comparison with localities represented in the "central" clade, they both (with exception of the DOM population in south Slovakia) share considerable distance from the Danube river and its large tributaries. They may well represent remnants of previous/minor colonisation waves, possibly from different refugia, in "accesible" part of distribution range recently covered with latest massive spread

of the “central” haplotype group.

Triturus c. carnifex populations could be separated to the Balkan and Apennines part suggesting different history of this (sub)species in respective areas, resulting from probable different glacial refugia or postglacial colonisation routes. The sampling was poor so more populations have to be scored before any strict statement in this respect could be made, however, this separation is, at least partially, supported also by allozyme data (Arntzen, 2001). The only Czech *T. carnifex* haplotype was found to be more relative to those from Apennines rather than Balkans. However, the assumption about “Apennine” origin of this specimen is complicated by the clearly Balkan-like mtDNAof *T. carnifex* populations from the south-western Austria and adjacent part of Germany (Maletzky et al., unpublished data). This suggests that this particular part of Austria, logical stepping stone between Italy and the Czech Republic, had to be colonised later, with no genetic traces of presumably original “Apennine” sequence type. Alternatively, the Czech Republic could be colonised via some alternative, though, from modern perspective and strictly human insight, peculiar way. Even allochtonous origin, in *T. carnifex* reported also from Bavaria (Franzen, Gruber & Heckes, 2002) and Switzerland (Arntzen & Thorpe, 1999), cannot be fully discarded, although there are no data for its support

Locality BOS, where the only Czech mitochondrial *T. carnifex* specimen was found, is about 100 km far from where the most *carnifex*-like newts were described based on their morphology (Piálek, Zavadil & Valíčková, 2000), allozyme and microsatellite profiles (Horák, 2000; Mikulíček et al.,unpublished data). The same data sets suggest that the western populations show varying level of admixture of *T. carnifex* and *T. cristatus* . However, their mtDNA was in some cases replaced by mtDNA of *T. dobrogicus* of the central and western type. This pattern was especially pronounced in the Znojmo region, where 18 out of 19 specimens scored carried *T. dobrogicus* mtDNA of the central type. Present distribution of crested newts in southern Moravia revealed cca 40 km gap of abused

agriculture land between *T. carnifex* and *T. dobrogicus*. However, local environment, favouring presence of both species makes the past genetic contact probable. The spread of *T. dobrogicus* further to the west, though, would be limited due to preferred association of this species to basins of the large rivers and the lowlands (Arntzen & Borkin, 1997; Arntzen, 2003). Hence, we can follow a unidirectional introgression of mtDNA as far as 100 km from the expected contact zone. Alternatively, this "switch" of mtDNA can be explained by ancestral polymorphism or incomplete lineage sorting phenomena (Moran & Kornfield, 1993; Muir & Schlotterer, 2005; Spaulding et al., 2006). However, at least in BOS locality, the introgression hypothesis is supported also with allozyme and microsatellite data (Horák, 2000; Mikulíček 2005; Mikulíček, personal comm.). Discordant mobility of nuclear and mitochondrial markers through contact zone is described from numerous studies (e.g. Garcia-Paris et al., 2003; Leach & Cole, 2007) and has been recently, though to the much lesser extent, reported also in *T. cristatus* complex from Austria (A. Maletzky et al., unpublished data. One can only speculate about selective forces standing behind such a massive and deep spread.

Also, the origin of *carnifex*-like populations carrying *T. dobrogicus* mtDNA remains unclear. Are they extension of Balkan populations, or, together with the only *T. carnifex* Czech specimen, do they represent Apennine branch and have been recently introgarded by *T. dobrogicus* and *T. cristatus*? Unfortunately, very little is known about status of Austrian crested newts north of the Danube River. Arntzen & Thorpe (1999) suggest existence of contact zone with all three species included stretching from Vienna to the south-eastern Austria and adjacent part of Germany. Arntzen (2001) surveyed genetic variability of *T.* c. *carnifex* using more than 40 allozyme loci and found specimens from north-eastern Austria in clade with Slovenian sample leaving the Italian samples as a sister group. Klepsch (1994), based on allozyme profiles, reported mixed population from north-eastern Austria, mostly with all three genotypes presented. Low allelic variability of used markers disables any conclusions to be made in this respect. With the present state of knowledge we have to leave this question open to the further research.

This work present first large-scale survey into the intraspecific sequential variability of crested newts. Overall level of intraspecific variability was found to be ranging from 1% in T. cristatus to 6% in *T. dobrogicus*. Those values are of one magnitude higher than data published so far (Wallis & Arntzen 1989; Steinfartz et al., 2007) and are comparable to the interspecific distances within complex and to the intraspecific distances of other newts (Babik et al. 2004; Pabijan & Babik, 2006; Steinfartz, 2007). In fact, the variability within central European specimens of *T. dobrogicus* exceeded its distance both to *T. cristatus* and *T. carnifex*. On relatively small geographic scale covering central Europe, we have uncovered considerable amount of, previously unknown variability and genetic structuring. Although the evolutionary centre of complex is thought to be on Balkans, our data suggest that central Europe, especially in case of *T. cristatus,* might have played important role in colonisation of northern parts of areals of respective species. They also indicate recent genetic contact, although more proper genetic markers have to be employed to fully uncover its pattern and range (Mikulíček et al., unpublished data)

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Table 1

Locality (country)	abreviation	coordinates (N/E)	N	haplotype	region
Pastviny (CZE)	PAS	50º04'/16º33'	3	CR4,CR6	WB
Cep (CZE)	CEP	48°55'/14°50'	3	CR1,CR61	SB
Bor (CZE)	BOR	48°53'/14°48'	2	CR3	SB
Bosna (CZE)	BOS	48°53'/14°56'	5	CA5,D21	SB
Stradov (CZE)	STR	48°51/14°33'	1	CR4	SB
Nové Hrady (CZE)	NH	48°45'/14°47'	5	CR1	SB
Nová Bystřice (CZE)	NB	49°2'/15°5'	3	CR6	WM
Brandlín (CZE)	BRA		1	CR6	WM
Horní Slatina (CZE)	HS	49°6'/15°33'	5	CR1,CR6,D2	WM
Řečice (CZE)	REC	49º08'/15º21'	4	CR1,D23	WM
Třebětice (CZE)	TRE	49º03'/15º30'	4	CR1,CR61,D2,D22	WM
Zblovice (CZE)	ZBL	48°57'/15°42'	3	D2	ZN
Olbramkostel (CZE)	OLB	49°6'/15°33'	1	D1	ZN
Citonice (CZE)	CIT	48°52'/15°57'	3	D2	ZN
Žerůtky (CZE)	ZER	48°54'/15°57'	3	D2	ZN
Podmolí (CZE)	POD	48°51'/15°56'	3	D1,D3,D6	ZN
Únanov (CZE)	UNA	48°53'/16°3'	1	CR1	ZN
Tasovice (CZE)	TAS	48°50'/16°8'	3	D1,D3	ZN
Mašovice (CZE)	MAS	48°51'/15°58'	3	D1,D3	ZN
Nové Mlýny (CZE)	NM	48°51'/16°42'	1	D5	EMWS
Bulhary (CZE)	BUL	48°50'/16°45'	1	D3	EMWS
Moravský Písek (CZE)	MP	48°58'/17°19'	3	D1,D3	EMWS
Kurovice (CZE)	KUR	49°17'/17°31'	3	CR2	EMWS
Rusovce (SK)	RUS	48º03'/17º09'	3	D1	EMWS
Devínske jazero (SK)	DJ	48º17'/16º57'	4	D5,D51	EMWS
Domica (SK)	DOM	48º28'/20º28'	2	D1,D42	ES
Silica (SK)	SIL	48º34'/20º32'	1	CR1	ES
Jovsa (SK)	JOV	48º48'/22º04'	3	CR5,D4	ES
Teplý Vrch (SK)	TV	48º28'/20°08'	2	D4,D42	ES
Veľký Blh (SK)	VB	48º26'/20º06'	3	D4	ES

Veškovce (SK)	VES	48º33'/22º 06'	3	D43	ES
Svätá Mária (SK)	SM	48º27'/21º49'	1	D1	ES
Fülöpháza (HU)	FUL	46º53'/19º24'	3	D3	OUT
Delta Dunarea (ROM)	DEL	unknown	2	D1,D4	OUT
Matena (SLO)	MAT	45°58'/14°31'	5	CA1	OUT
Tuscany (IT)	MM	43°01'/10°53'	1	CA2	OUT
Bujanovac (SER)	BUJ	unknown	2	*T. k. arntzeni*	outgroup
Ljuberadja (SER)	LJU	unknown	2	*T. k. arntzeni*	outgroup
Vrtovac (SER)	VRT	unknown	1	*T. k. arntzeni*	outgroup
Toledo (SPA)	TOL	unknown	1	*T. marmoratus*	outgroup
Abant (TUR)	ABA	unknown	3	*T. k. karelinii*	outgroup
Peterborough (UK)*	PET	unknown	1	*T. cristatus*	outgroup
Sinaia (ROM)*	SIN	unknown	1	*T. cristatus*	outgroup
Zimnicea (ROM)*	ZIM	unknown	1	*T.dobrogicus*	outgroup
Svishtov (BUL)*	SVI	unknown	1	*T.dobrogicus*	outgroup
Alap (HUN)*	ALA	unknown	1	*T.dobrogicus*	outgroup
Ano Kaliniki (GRE)*	ANO	unknown	1	*T. c. macedonicus*	outgroup

Table 2

Source of variation	d.f.	Percentage of variation
T. dobrogicus		
among groups	4	30.42
among population within groups	20	53.61
within populations	59	15.97
T. cristatus		
among groups	5	21.44
among population within groups	9	42.12
within populations	19	36.44

Table 3

	n/npop	h	Hd	subst./PIS	S/NS	Pi	k
dobrogicus	**60/27**	**13**	**0.819**	**23/18**	**19/4**	**0.00684**	**2,591**
ES	12/7	4	0.712	8/7	6/2	0,00772	2,924
EMWS	12/5	4	0,803	3/3	3/0	0,00328	1,242
ZN	18/6	4	0,652	3/3	2/1	0,00214	0,810
WM	6/3	4	0.800	14/7	13/1	0,01601	6,067
SB	4/1	1	0	0/0	0/0	0	0
OUT	8/5	4	0,786	5/2	5/0	0,00452	1,714
carnifex	**10/6**	**6**	**0.778**	**19/7**	**21/5**	**0.01824**	**6,911**
cristatus	**34/15**	**7**	**0.783**	**6/5**	**3/3**	**0.00365**	**1,381**
ES	2/2	2	1.000	1/0	1/0	0,00264	1,000
EMWS	3/1	1	0	0/0	0/0	0	0
WM	12/5	3	0,682	2/2	0/2	0,00220	0,833
SB	11/4	4	0,691	4/3	2/2	0,00355	1,345
WB	4/1	2	0,500	2/0	1/1	0,00264	1.000
OUT	2/2	1	0	0/0	0/0	0	0

Table 4

	ES	EMWS	ZN	WM	SB	OUT
ES		**0.34276***	**0.48694***	**0.26327***	**0.74636***	**0.17642***
EMWS	0.86364		**0.29388***	**0.30830***	**0.87915***	0.11090
ZN	n/a	n/a		**0.36346***	**0.89103***	**0.24756***
WM	0.30451	**0.72806***			**0.45631***	**0.21352***
SB	0.04946	**0.52546***	n/a	**0.12043**		**0.85410***
WB	0.33333	0.72806*	n/a	**0.39040***	**0.23489**	
OUT	0	1	n/a	0.15977	-0.14487	0.31429

Table 5

Group	X^2 statistic	P	Chain of inference	Inference
2-2	44.00	0.000	1-2-3-4 NO	RGF w/IBD
2-4	25.00	0.000	1-19-20-2-3-4 NO	RGF w/IBD
3-1	12.00	0.033	1-2-3-4 NO	RGF w/IBD
3-3	69.00	0.000	1-2 IO	undetermined
total cladogram	140.39	0.000	1-2-11-12 NO	CRE

Legends

Table 1

List of localities together with country of their origin. Abbreviation used further in text and figures are: GPS coordinates, number of specimens sequenced, haplotype assignment according to the Fig 2 and region denomination (WB - West Bohemia, SB - South Bohemia, WM – West Moravia, ZN – Znojmo region, EMWS East Moravia/South-West Slovakia, ES – East Slovakia, OUT – outgroup populations). Asterisks denote sequences adopted from Steinfartz et al. (2007)

Table 2

Results of analysis of molecular variation

Table 3

Molecular diversity indices computed for respective species and regions using Alrequin 3.01 software (n/npop – number of individuals/population in analysis, *h* - number of haplotypes observed, *Hd* – haplotype diversity, *subst./PIS* - number of substituion/parsimony informative sites observed, *S/NS* – synonymous vs. nonsynonymous substitutions ratio, *Pi* – nucleotide diversity, *k* – average number of nucleotide differences)

Table 4

Parwise F_{ST} comparison and results of exact population differentiation test within *T. dobrogicus* (upper diagonal) and *T. cristatus* (lower diagonal) localities Asterisks denote values significant on 0.05 level as tested with 10000 permutations, bold numbers indicate significant results of exact population differentiation test)

Table 5

Biogeographical history inferred using NCA in nested clades with significant geographic association. RGF w/IBD = restricted gene flow with isolation by distance, CRE = continuous range expansion

Figure 1

Map showing geographical position of localities/specimens sequenced within the frame of this study. (for abbreviations of regions see the legend in Table 1). Asterisks denote sequences from Steinfartz et al. (2007).

Figure 2

Phylogeny of T. cristatus complex as revealed by Bayesian inferrence under the GTR+I+G model of evolution using MrBayes 3.1.2 software (see text for details). The topology within the *T. cristatus* and *T. dobrogicus* clades are not shown due to the low resolution and graphical constraints (see Fig. 3 and text for further details). Numbers above branches represent bootstrap support (ML/MP, 1000 iterations), numbers in box denote bayesian posterior probability (see text for details)

Figure 3

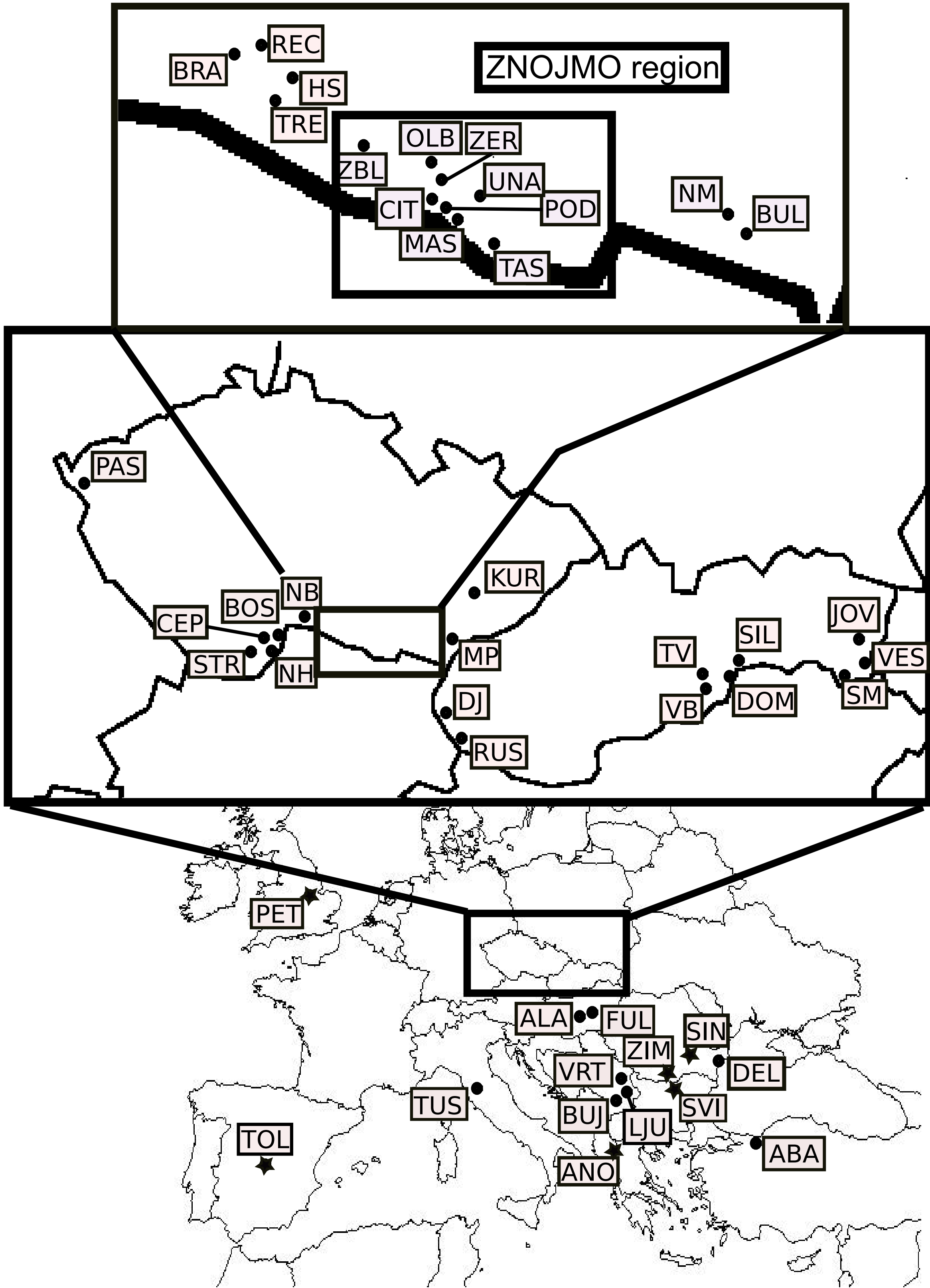
Median joining haplotype network with subsequent maximum parsimony Steiner tree analysis (Polzin et al., 2003), performed with Network 4.2 software (Bandelt et al, 1999) Pie charts represent haplotypes, with size proportionate to the number of identical sequences forming the haplotype. Different shading denotes geographic origin (WB - West Bohemia, SB - South Bohemia, WM – West Moravia, ZN – Znojmo region, EMWS East Moravia/South-West Slovakia, ES – East Slovakia, OUT – outgroup populations). Numbers beside lines reveals number of mutationals steps between respective haplotypes, Text in boxes shows the haplotype code and number of sequences contained. Asterisks denote sequences from Steinfartz et al. (2007).

Figure 4

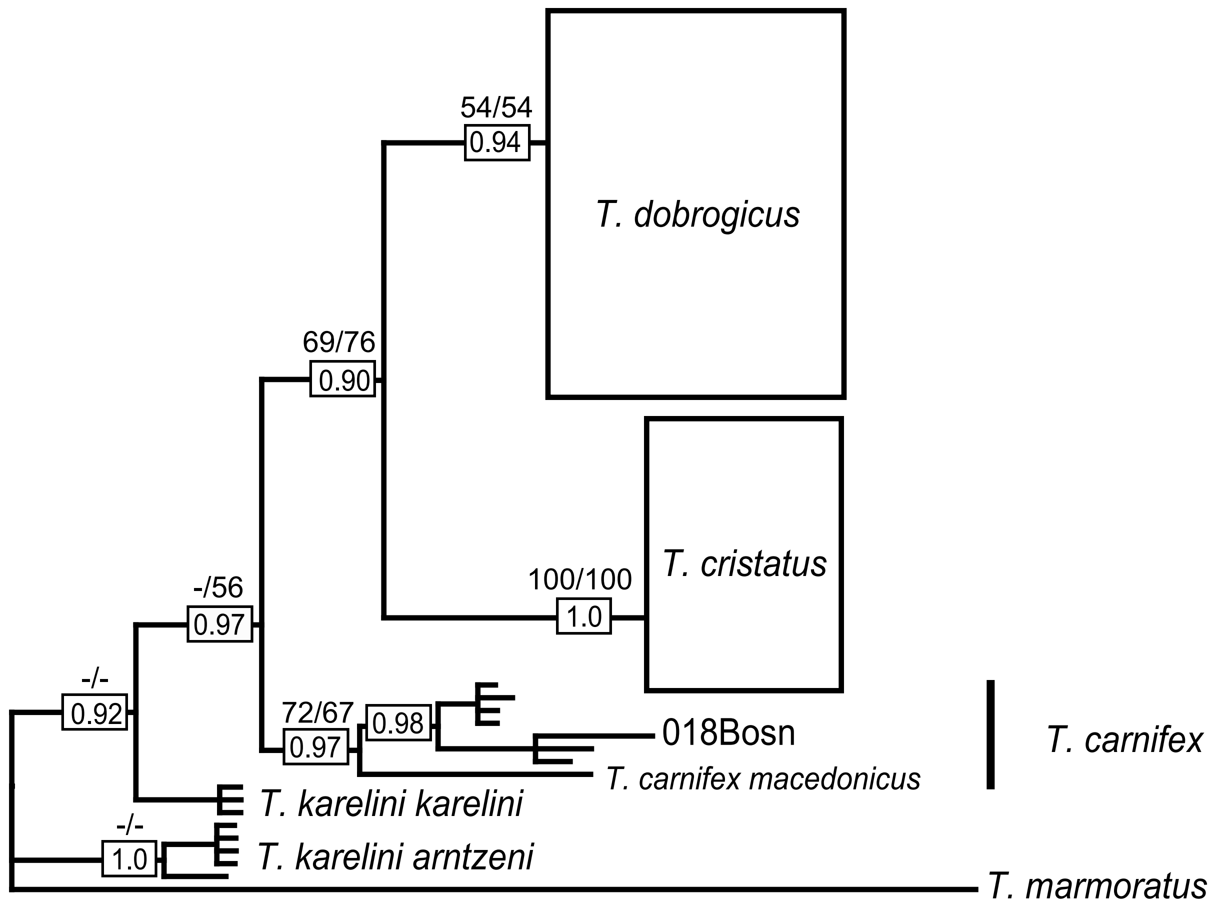
Nested clade design of *T. cristatus* and *T. dobrogicus cyt b* haplotype network constructed using TCS 1.18 software. Large ovals represents haplotypes (corresponding to the haplotypes on Fig. 3), small circles stand for missing haplotypes.

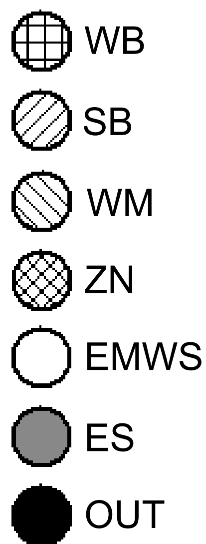
Figure 5

Histograms of mismatch distribution of central-european crested newts. Black curves show shape of theoretical distribution according to the sudden expansion model. SSD, sum-of-square deviation; P_{SSD}, P-value of SSD observation; RI, raggedness index (Harpending, 1994); P_{RI}, P-value of raggedness index measure.

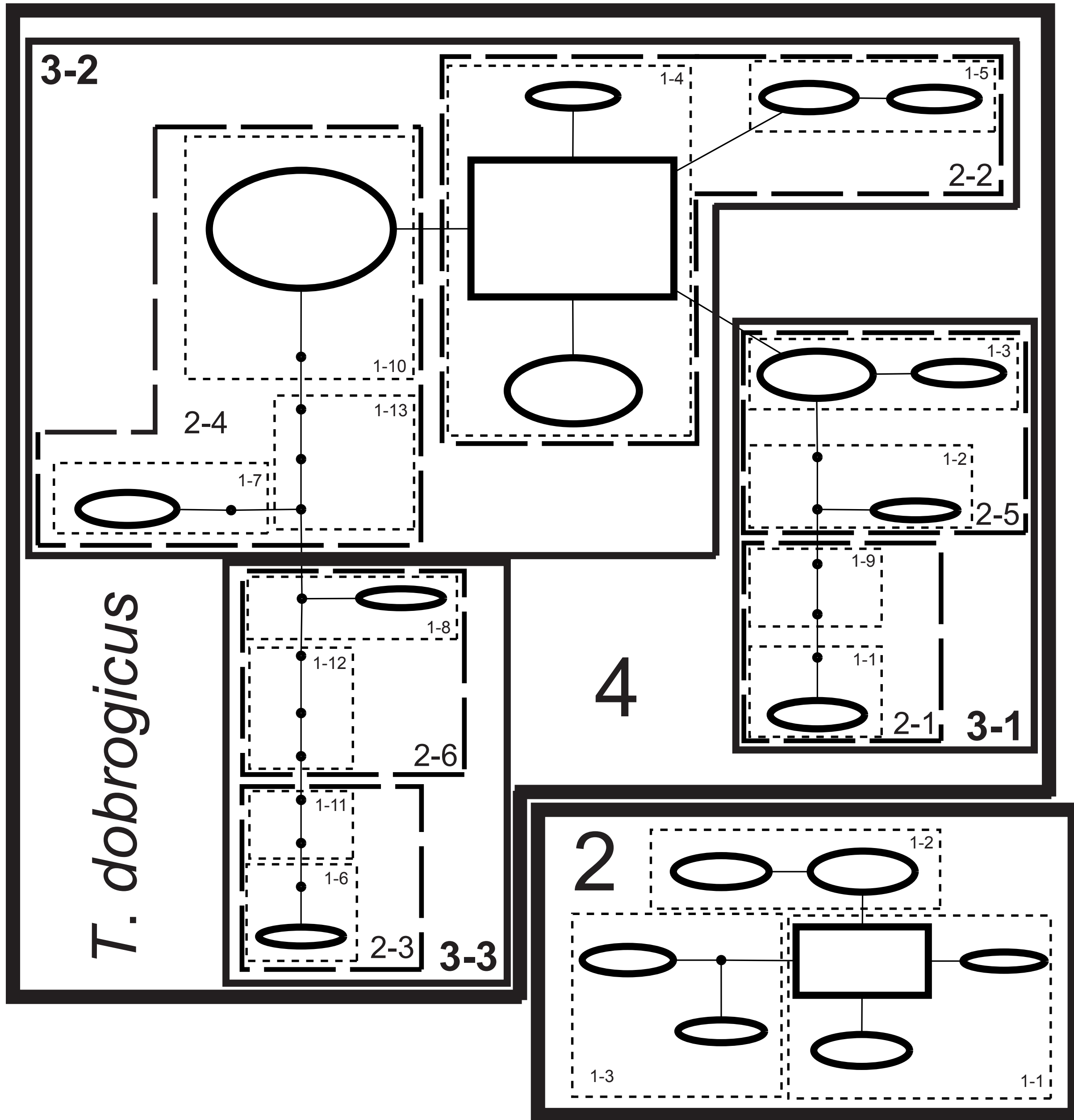


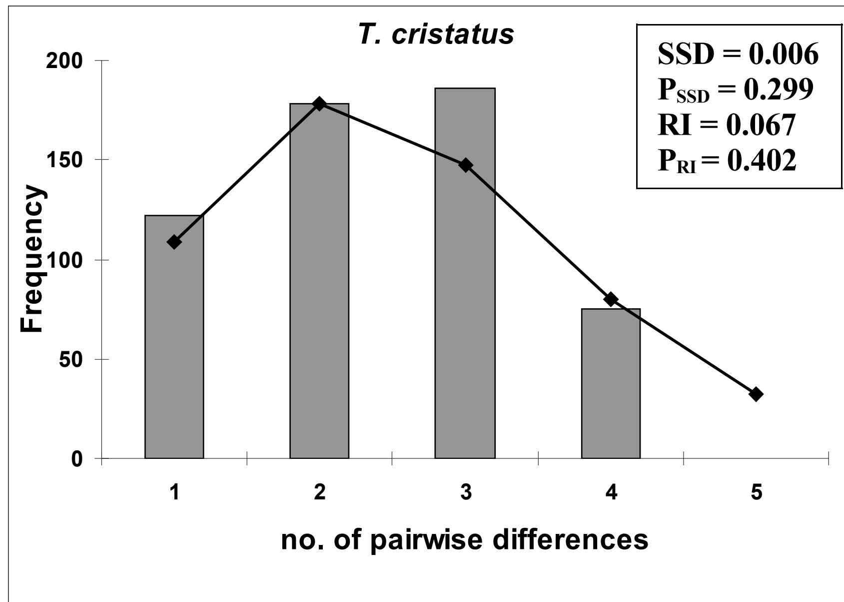
ZNOJMO region
REC
BRA
HS
TRE
OLB
ZER
ZBL
UNA
CIT
POD
MAS
TAS
NM
BUL
PAS
KUR
NB
BOS
CEP
STR
NH
MP
DJ
RUS
TV
SIL
JOV
VES
SM
VB
DOM
PET
ALA
FUL
SIN
ZIM
VRT
DEL
TUS
SVI
BUJ
LJU
TOL
ABA
ANO

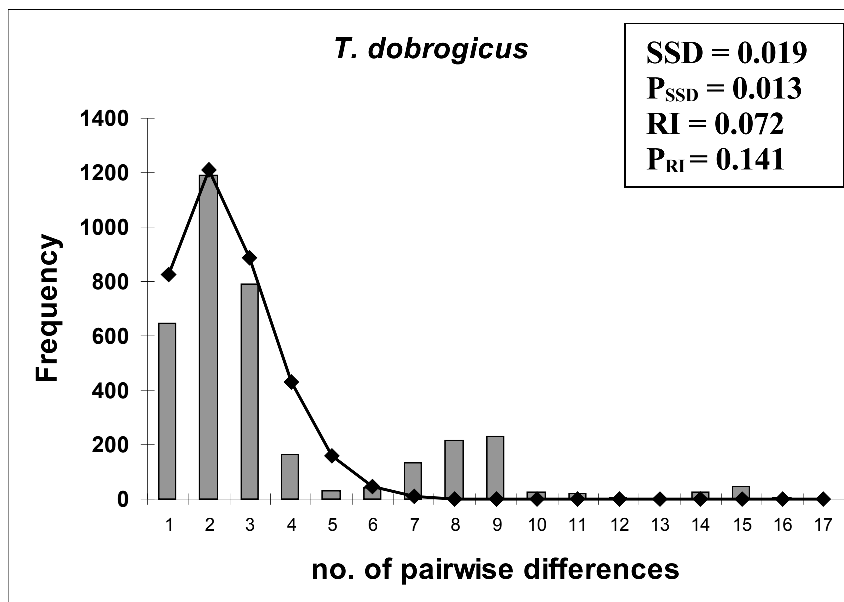

54/54
0.94
T. dobrogicus
69/76
0.90
T. cristatus
100/100
1.0
-/56
0.97
-/-
0.92
72/67
0.97
0.98
018Bosn
T. carnifex
T. carnifex macedonicus
T. karelini karelini
-/-
1.0
T. karelini arntzeni
T. marmoratus
0.1

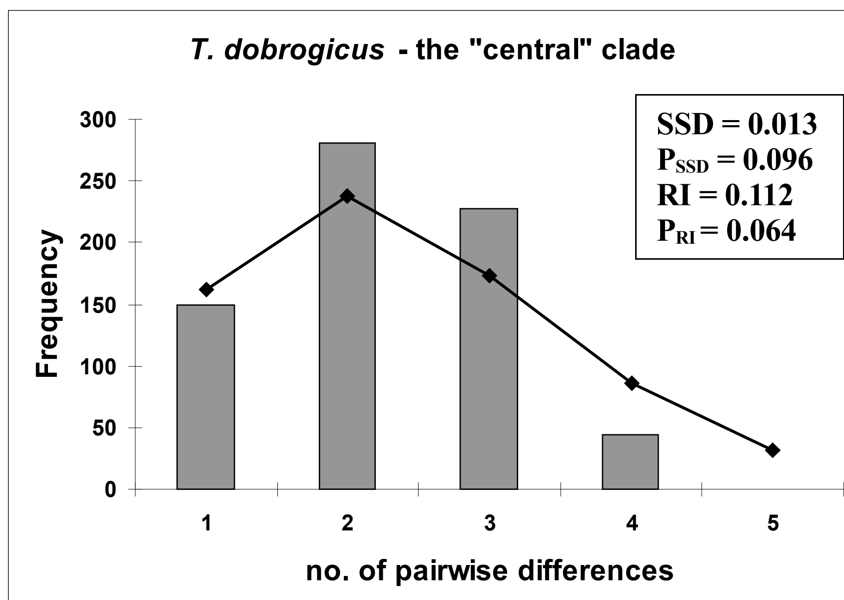


WB
SB
WM
ZN
EMWS
ES
OUT
western
central
D21
(4)
D22
(1)
D23
(1)
D2
(12)
D6
(1)
D51
(2)
D5
(3)
D1
(13)
D3
(11)
central
eastern
D4
(7)
D41
(3)
D42
(1)
D43
(3)
T. dobrogicus
T. cristatus
T. carnifex
T. c. macedonicus
CR61
(4)
CR6
(6)
CR1
(14)
CR2
(2)
CR3
(3)
CR4
(4)
CR5
(1)
Balkans
Appeninnes
018BOS
Czech Rep.


3-2
1-4
1-5
2-2
1-10
2-4
1-13
1-7
1-3
1-2
2-5
1-9
1-1
2-1
3-1
4
1-8
1-12
2-6
1-11
1-6
2-3
3-3
T. dobrogicus
2
1-2
1-3
1-1
T. cristatus


T. cristatus
SSD = 0.006
P_{SSD} = 0.299
RI = 0.067
P_{RI} = 0.402
Frequency
200
150
100
50
0
1
2
3
4
5
no. of pairwise differences


T. dobrogicus
SSD = 0.019
P_{SSD} = 0.013
RI = 0.072
P_{RI} = 0.141
Frequency
1400
1200
1000
800
600
400
200
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
no. of pairwise differences


T. dobrogicus - the "central" clade
SSD = 0.013
P_{SSD} = 0.096
RI = 0.112
P_{RI} = 0.064
Frequency
300
250
200
150
100
50
0
1
2
3
4
5
no. of pairwise differences

Results B

Maletzky A, Mikulíček P, Franzen M, Goldschmid A, Gruber HJ, **Horák A**, Kyek M.

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.

Journal of Herpetology, submitted

I have sequenced cytochrome b, performed analyses of sequential variability and written relevant parts of manuscript.

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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Running title: Hybridization between crested newts

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ABSTRACT

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 due to morphological data. In our study we compared distribution patterns and hybridization on the basis of one morphological (Wolterstorff-Index) and two (*cyt b*, microsatellites) molecular markers. A total of 35 autochthonous populations in this region were surveyed. 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) already were present 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 three regions where admixed populations and individuals were present in Salzburg and Upper Austria. No autochthonous population of *T. carnifex* could be detected in Bavaria. The hybrid zone is probably unimodal, with hybrid individuals predominate in the centre. As the present day distribution ranges of both species in the surveyed area are fragmented and population are heavily reduced in numbers we only can observe the remains of former hybrid zones.

The analysis of molecular markers revealed considerable genetic uniformity. The studied area seems to be colonised of a limited number of individuals and probably less times, then areas with slightly higher diversity like in the Czech and Slovak Republic. Hybrid zones in the study region were most probably formed by one *T. carnifex* genotype and two different *T. cristatus* genotypes.

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: *Triturus cristatus* superspecies, Salamandridae, microsatellites, mtDNA, Wolterstorff-Index, Bayesian analysis, admixture, hybrid zone.

INTRODUCTION

Hybrid zones (abbreviated as HZ in the following) 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 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 paleoclimatic changes during the Pleistocene (Hewitt, 2000; 2001). Many animal and plant taxa had glacial refugia in the 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 HZ (e.g. Butlin & Hewitt, 1985; Arntzen & Wallis, 1991).

Several models have been proposed to describe the structure and dynamics of HZ (for review see Arnold, 1997). Among them 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 HZ as clines of various characters maintained by 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 HZ 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 the one or other parental genotype. A continuum from unimodal to bimodal genotypic distributions in HZ 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 (e.g. Rieseberg *et al.*, 1998). Postzygotic incompatibilities are similar in bimodal and unimodal zones (Jiggins & Mallet, 2000).

HZ go along with common genetic features like heterozygote deficit and linkage disequilibria. A shortage of heterozygotes compared with Hardy-Weinberg (H-W) 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 HZ 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 recognised 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; Bucci-Innocenti *et al*., 1983; Wallis & Arntzen, 1989). They are considered to be relatively young species “in statu nascendi”, among which genetic isolation is not fully developed. 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 HZ (e.g. Freytag, 1978; Klepsch, 1994; Wallis & Arntzen, 1989; Arntzen & Wallis, 1999). Crested newts differ by morphological (Wolterstorff, 1923; Arntzen & Wallis, 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 HZ are documented for several regions, concerning 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 due to morphological data (Wolterstorff, 1929; Schüller, 1963; Schmidtler, 1976; Freytag, 1978), rendering its extension and the distribution ranges of the two species unknown (e.g. Arntzen & Thorpe, 1999; Thiesmeier & Kupfer, 2000; Arntzen, 2003). According to Arntzen & Thorpe (1999) a 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 up to now and is obscured.

In this study we combined morphological markers (Wolterstorff-Index) and two types of molecular markers (mtDNA and microsatellites) to describe the structure of HZ 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 HZ with respect to geographic distribution of genotypes, 2) 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 Tab. 1 and Fig. 1. Most samples from Bavaria were collected and preserved in

the year 2001 during a study on populations in this region (Franzen *et al*., 2001). The *T. carnifex* population Isen (Ise) was introduced 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 the years 2004 and 2005. Newts were taken into the laboratory and anaesthetised 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 X 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*., 2001). 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 (*cytb*) 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* cytochrome 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 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 94ºC followed by 39 cycles of 1 min at 94ºC, annealing for 1 min at 53ºC and elongation at 72ºC for 2 min. An additional 5 min period for elongation at 72ºC followed the last cycle. For routine genotyping, primers were commercially labelled with fluorochromes HEX, FAM, TET and NED and 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), 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).

Microsatellite markers

We calculated gene diversity (H_O) and allelic richness (A) 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, 1969; 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 equilibrium in a population. If the basic model is expanded to the model with admixture, the admixture proportion of each individual *q* (i.e. 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 100.000 iterations. In a first approach the population structure was assessed assuming an unknown number of K (range K = 2 to K = 10) and posterior 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). The value of K = 4 showed the highest ΔK value with two putative *T. cristatus*-like clusters (C1 and C2) and two *T. carnifex*-like clusters (C3 and C4), and was used for further analyses. 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 genotype should belong to more than one of four inferred clusters. We followed a similar approach as described by Pierpaoli *et al*. (2003). In an initial procedure all individuals were assigned to the four inferred clusters without any prior population information. In a second procedure, prior population information (Clusters 1 to 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 nine populations we found average WI-

values corresponding to *T. cristatus* in both sexes, in two further populations only females were measured, also showing *T. cristatus*-like characteristics. In seventeen populations only *T. carnifex*-like WI-values were found, in five of them only males were sampled, while in one population only information on females was available. The six remaining populations (Irl, Fue, Unt, Som, and Ann from Salzburg as well as Bad from Upper Austria) showed intermediate index values in males and females. 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 (Tab. 2, Fig. 2).

Mitochondrial DNA

We have analysed a 520 bp long fragment of *cyt b* for 117 specimens collected from 32 sites. The vast majority of samples 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 the population Ise possessed haplotype CAR2, differing from CAR haplotype by two substitutions (A to G at positions 280 and 345) and one specimen from the population Har possessed haplotype CAR1 differing by only one substitution (G to A at position 361). No insertion/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), populations Fue, Ach, Bue, Rie and Neu (all from Salzburg) showed occurrence of both CAR and CRI haplotypes (Tab. 5, Fig. 3). From the perspective of mtDNA these are possible candidates for detecting a contact zone. The populations Ann and Fre from Salzburg are the southernmost to show pure CAR haplotypes. In Upper Austria the only individual tested from the population Gun was assigned to CRI haplotypes, despite being located within *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 number 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 (Tab. 3). 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 significant deficit of heterozygotes in the populations Bab and Sur from Bavaria, Stg and Gun from Upper Austria, Bue, Rie, Fre, Zec and Ach from Salzburg as well as the allochthonous *T. carnifex* population Ise (Tab. 3). 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 entire sample (n = 291) consisted of four distinct clusters (ΔK for $K = 4$ was 192.21, Ln P (D) = -4946.97; Tab. 4). Two clusters could each 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 river Salzach and 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). Considerable amount of membership for C3 was also found in the Danube area (Hai and Baz) where proportion of C1 was slightly higher than 0.8. Admixture between C3 and C2 was detected in population Rie from Salzburg (Tab. 5, Fig. 4).

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 (Tab. 6). No autochthonous population of *T. carnifex* was found in surveyed populations from Bavaria. Only individuals from population Nie showed a considerable amount of the *T. carnifex* genotype.

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 Wolterstorff-Index or number of rip-bearing vertebrae might lead to a high level of false determinations. Mitochondrial DNA markers have provided powerful tools to gain accuracy. However, some instructive cases showed 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 into another species 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. That is why both types of genetic markers (nuclear and mtDNA) together with morphological markers should be used in species determination and hybrid detection.

Due to the high legal protection status and rareness in the study area in Austria we decided to use adult individuals and non-destructive sampling methods. 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 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 again reported in the north and northeast of the province of Salzburg (Schüller, 1963; Freytag, 1978), as well as from the 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*.

According to our data there are no present autochthonous populations of *T. carnifex* in Bavaria. We analysed individuals from 3 out of 5 populations – the remaining two have most probably vanished – presented by Schmidtler (1976). Of these, the population in Sillersdorf (Sil), which possessed morphological characteristics of *T. carnifex* (see also Franzen *et al*., 2001), was clearly *T. cristatus* according to molecular markers. All further populations could be assigned to *T. cristatus*, with only Niederau (Nie) containing one hybrid individual. As already assumed by Thiesmeier & Kupfer (2000), to the best of our knowledge no pure *T. carnifex*-populations occur in Bavaria.

The results of 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 (1978), 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). The number of crested newt localities north of the Danube in particular is scarce. However it would be interesting to study some further populations to gain a more precise characterisation of the contact zone there. 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 population Bad is proved to have its seeds on anthropogenic interference by introduction of newts from different populations in one garden pond (R. Mysliwietz, pers. comm.).

The former extension of HZ in the studied area is hard to determine. There is a clear NW-SE direction at a large geographic scale, but this pattern is not so clear on a smaller geographic 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, this pattern is caused by stochastic processes associated with recolonization. In Salzburg and Bavaria we found admixed individuals in a region of about 30 kilometres width, with *cristatus*-like and *carnifex*-like populations converging to 2 (Som and Unt) and 3.5 kilometres (Ame and Ann). Crested newts have suffered from severe declines in population numbers in all studied regions (e.g. Franzen *et al*., 2001, Kyek & Maletzky, 2006). Thus it is evident that only the remains of former HZ can be found today.

Variability in genetic markers & 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, in previous studies *T. cristatus* was found to be genetically homogenous over most of its distribution range (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. This haplotype has a sequence identical to Slovenian samples from Matena. The same applies for *T. cristatus*, where only one haplotype was found which has the same sequence as the most *T. cristatus* specimens in the Czech Republic (Horák *et al*., unpublished). Moreover, the same haplotype is also described from England and Romania, showing extreme uniformity of *T. cristatus* mtDNA (Steinfartz *et al*., 2007). Salzburg area is more isolated and was more likely colonised with limited number of individuals and probably less times, then areas with slightly higher diversity like in the Czech and Slovak Republic.

Although intrapopulation variability at microsatellite loci was high in most studied populations, sub-structuring of the whole sample was relatively low, revealing only two distinct clusters for each *T. cristatus* and *T. carnifex* (one exclusively found in Ise). In fact, our study shows that HZ in the surveyed region were most probably formed by one *T. carnifex* genotype and two different *T. cristatus* genotypes. These findings correlate well with mtDNA results. Crested newts, and here particularly the two *T. cristatus* clusters clearly are associated with landscape structure and river valleys that might constitute constraints in the postglacial re-colonization 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, HZ are like a cline where the frequency of genotypes is changing from pure *T. cristatus* to pure *T. carnifex* in 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 kilometres 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 predominate in the centre and parental genotypes at the edges of the zone (Jiggins & Mallet, 2000). Similar results were gained in HZ of these species in the southern part of Czech

Republic (Mikulíček, 2005). Typical for unimodal HZ (Jiggins & Mallet, 2000), assortative mating might be absent in our studied populations from the contact zone and reproductive isolation mechanisms between the species are not fully developed. 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).

Although a significant deficit of heterozygotes was also observed in hybrid populations, 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 H-W 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 *T. cristatus* x *T. carnifex* hybrid zone and heterozygote in analysis, the deficit 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, 2002). *Triturus carnifex* shows the widest ecological amplitude of all crested newt species and is considered to be fairly adaptive and competitive (e.g. Arntzen, 2003). Therefore the introduced populations are a threat to native crested newts by hybridization or displacement (e.g. Arntzen & Thorpe, 1999). In this context, the Ise population requires special attention. This population is based on offspring (100-200 larvae) from one pair, which was captured near Rovinj, Croatia (Franzen *et al*., 2002). Thus, maximally four different alleles should be detected in one heterozygote individual at a given microsatellite locus. However, we detected between 3 and 9 alleles per locus, rendering this information highly questionable. The presence of two *T. carnifex*-haplotypes may either signalize their presence in the donor locality, or alternatively one came from Balkans with main colonisation wave. However, there

are no data about occurrence of crested newts in this particular locality and surrounding area is inhabited by *T. cristatus*. Therefore, we prefer the former explanation. 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 records of autochthonous *T. cristatus* from that locality (or nearby areas) in the Bavarian amphibian mapping programme prior to 1990 (Franzen *et al*., 2002). Nearest current *T. cristatus* localities are at distances of 7 and 8 km and are separated from the sampled locality by large corridors of mostly unsuitable habitats (i.e. few ponds, large stretches of agricultural areas, many roads). The assumed dispersal distance for crested newts is 1km/year (Arntzen & Wallis, 1991; Halley *et al*., 1996). Hence a 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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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).

Locality Name	Province	N	Latitude (°)	Longitude (°)	Locality Name	Province	N	Latitude (°)	Longitude (°)
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′

TABLE 2: Wolterstorff-Index (mean ± std. dev. 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*

TABLE 3: Average gene diversity (H_O), Allelic richness (A) and inbreeding coefficient (F_{IS}) for each sampled population. F_{IS}-values with single asterisks (*) indicating significant and double asterisks (**) highly significant results; n.e. = not evaluated.

Population	H_O	A	F_{IS}	Population	H_O	A	F_{IS}
Kochel (Koc)	0,46	2,15	0.009	Irlach (Irl)	0,54	2,29	-0.060
Niederau (Nie)	0,48	2,27	0.152	Bürmoos (Bue)	0,51	2,56	0.061*
Lengthal (Len)	0,57	n.e.	0.000	Fürwag (Fue)	0,64	2,45	0.007
Roibach (Roi)	0,38	n.e.	0.135	Ried (Rie)	0,42	2,61	0.122**
Sillersdorf (Sil)	0,37	1,81	0.028	Freimoos (Fre)	0,57	2,79	0.178*
Babensham (Bab)	0,24	1,69	0.204*	Guggenthal (Gug)	0,55	2,46	-0.188
Bischofwiesen (Bis)	0,57	n.e.	0.000	Unterkoppl (Unt)	0,52	2,57	-0.006
Surheim (Sur)	0,49	2,69	0.228**	Sommeregg (Som)	0,77	2,57	0.106
Reichersberg (Rei)	0,54	2,67	0.071	Neuhofen (Neu)	0,69	2,57	-0.013
Haibach (Hai)	0,48	n.e.	0.000	Koppl (Kop)	0,17	n.e.	-0.200
Bad Zell (Baz)	0,66	2,72	-0.040	Zecherl (Zec)	0,20	2,66	0.225**
Bad Ischl (Bad)	0,49	2,30	-0.064	Achleiten (Ach)	0,43	2,95	0.255**
St. Georgen (Stg)	0,55	1,70	0.211**	Strobl (Str)	0,46	2,10	-0.172
Seewalchen (See)	0,58	n.e.	0.180	Ameisensee (Ame)	0,36	2,36	-0.011
Stadl-Paura (Sta)	0,50	n.e.	0.071	Annaberg (Ann)	0,36	n.e.	-0.106
Gunskirchen (Gun)	0,51	2,29	0.296**	Kainisch (Kai)	0,29	1,70	0.064
Micheldorf (Mic)	0,65	n.e.	0.167	Hartberg (Har)	n.e.	n.e.	n. e.
Ternberg (Ter)	0,50	n.e.	0.273	Isen (Ise)	0,50	2,90	0.172*

TABLE 4: Population genetic clustering results based on the Bayesian-modelling method in STRUCTURE. The best fit model K = 4 (bold characters) 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

TABLE 5: 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).

Population	Cluster 1 (C1)	Cluster 2 (C2)	Cluster 3 (C3)	Cluster 4 (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

TABLE 6: Population assignment and inferred ancestry of admixed individuals without (first value) and with (second value) using prior population information.

	Cluster 1 (CRI 1)	Cluster 2 (CRI 2)	Cluster 3 (CAR 1)	Cluster 4 (CAR 2)
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	0.165 - 0.155	**0.626 - 0.599**	**0.202 - 0.240**	0.006 - 0.006
Nie_4	**0.216 - 0.227**	**0.640 - 0.601**	0.136 - 0.164	0.008 - 0.007
Nie_5	**0.538 - 0.529**	**0.392 - 0.347**	0.062 - 0.116	0.007 - 0.008
Len_2	**0.336 - 0.492**	**0.656 - 0.500**	0.004 - 0.004	0.005 - 0.005
Bab_2	**0.688 - 0.774**	**0.304 - 0.218**	0.004 - 0.004	0.004 - 0.004
Sur_3	**0.409 - 0.445**	**0.571 - 0.534**	0.013 - 0.014	0.007 - 0.007
Hai_2	**0.538 - 0.450**	0.007 - 0.007	**0.451 - 0.538**	0.004 - 0.004
Baz_3	**0.770 - 0.726**	0.006 - 0.006	**0.220 - 0.264**	0.004 - 0.004
Baz_5	**0.605 - 0.492**	0.008 - 0.010	**0.383 - 0.494**	0.004 - 0.004
Baz_8	**0.680 - 0.640**	0.054 - 0.052	**0.261 - 0.304**	0.005 - 0.005
Baz_10	**0.734 - 0.662**	0.008 - 0.009	**0.253 - 0.324**	0.004 - 0.004
Irl_2	**0.781 - 0.788**	0.033 - 0.032	**0.182 - 0.176**	0.004 - 0.004
Rie_2	0.031 - 0.037	**0.427 - 0.378**	**0.537 - 0.578**	0.006 - 0.006
Rie_3	0.034 - 0.034	**0.284 - 0.287**	**0.678 - 0.674**	0.004 - 0.004
Rie_5	0.005 - 0.172	**0.659 - 0.021**	**0.331 - 0.802**	0.004 - 0.005
Rie_6	0.014 - 0.005	**0.595 - 0.657**	**0.383 - 0.334**	0.007 - 0.004
Rie_7	0.140 - 0.014	**0.739 - 0.598**	**0.111 - 0.381**	0.011 - 0.007
Rie_12	**0.193 - 0.143**	**0.020 - 0.738**	**0.781 - 0.108**	0.005 - 0.011
Fre_2	**0.667 - 0.648**	0.021 - 0.021	**0.300 - 0.320**	0.012 - 0.012
Fre_4	**0.323 - 0.331**	0.054 - 0.052	**0.618 - 0.613**	0.005 - 0.004
Fre_8	**0.656 - 0.644**	0.043 - 0.043	**0.297 - 0.309**	0.004 - 0.004
Som_3	0.004 - 0.004	**0.428 - 0.462**	**0.560 - 0.526**	0.008 - 0.008
Som_18	0.006 - 0.006	**0.588 - 0.589**	**0.397 - 0.396**	0.009 - 0.009
Kop_1	0.009 - 0.009	**0.263 - 0.271**	**0.722 - 0.714**	0.006 - 0.006
Zec_1	0.005 - 0.005	**0.706 - 0.710**	**0.281 - 0.277**	0.008 - 0.008
Zec_7	0.010 - 0.011	**0.193 - 0.141**	**0.782 - 0.834**	0.014 - 0.015
Zec_11	0.101 - 0.106	**0.137 - 0.131**	**0.757 - 0.757**	0.006 - 0.006
Ame_17	**0.192 - 0.189**	**0.599 - 0.603**	0.008 - 0.008	**0.201 - 0.200**
Ann_2	**0.404 - 0.342**	0.068 - 0.064	**0.516 - 0.583**	0.012 - 0.011
Bad_4	**0.759 - 0.681**	0.036 - 0.044	**0.199 - 0.269**	0.005 - 0.006
Bad_5	**0.282 - 0.263**	0.012 - 0.013	**0.702 - 0.720**	0.004 - 0.004
Bad_6	0.011 - 0.013	**0.268 - 0.250**	**0.716 - 0.732**	0.005 - 0.005
Gun_1	0.009 - 0.009	**0.377 - 0.381**	**0.608 - 0.603**	0.006 - 0.006

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

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 Tab. 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 Tab. 1; BAV = Bavaria, UA = Upper Austria, SBG = Salzburg.

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 of the sample sites are given in Tab. 1; BAV = Bavaria, UA = Upper Austria, SBG = Salzburg.

FIG. 1.

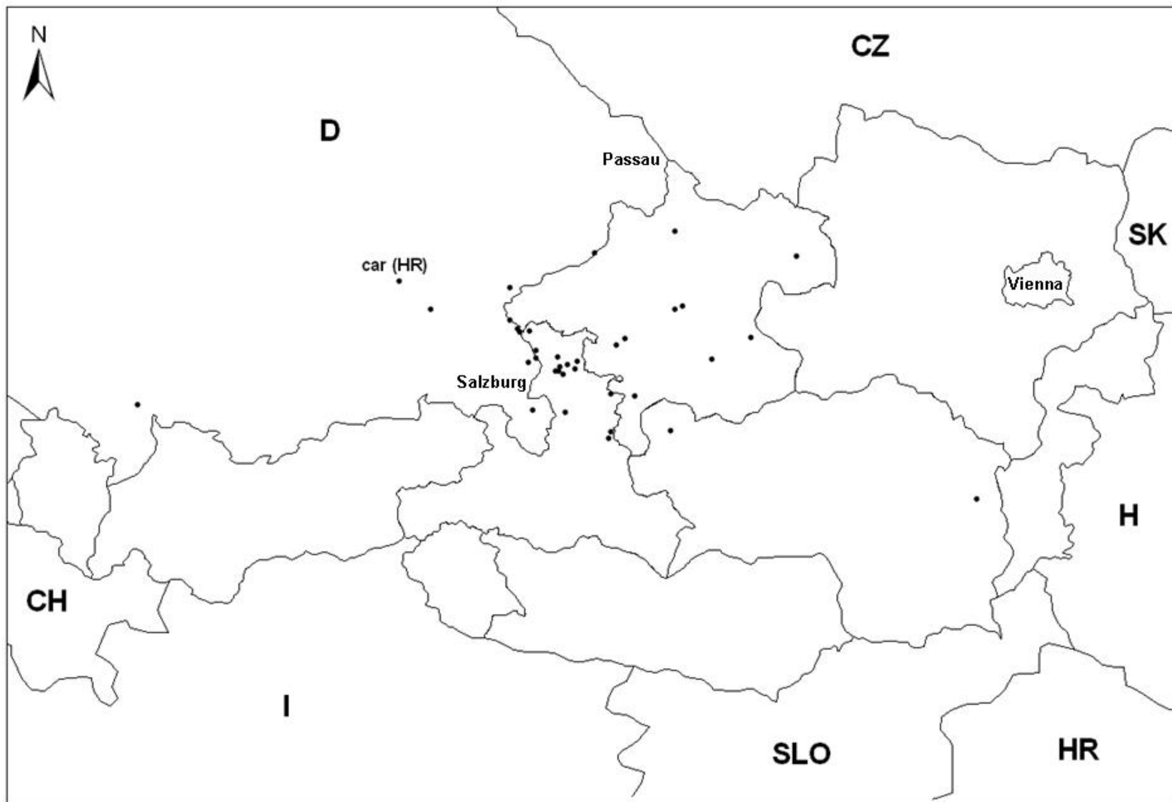


FIG. 2

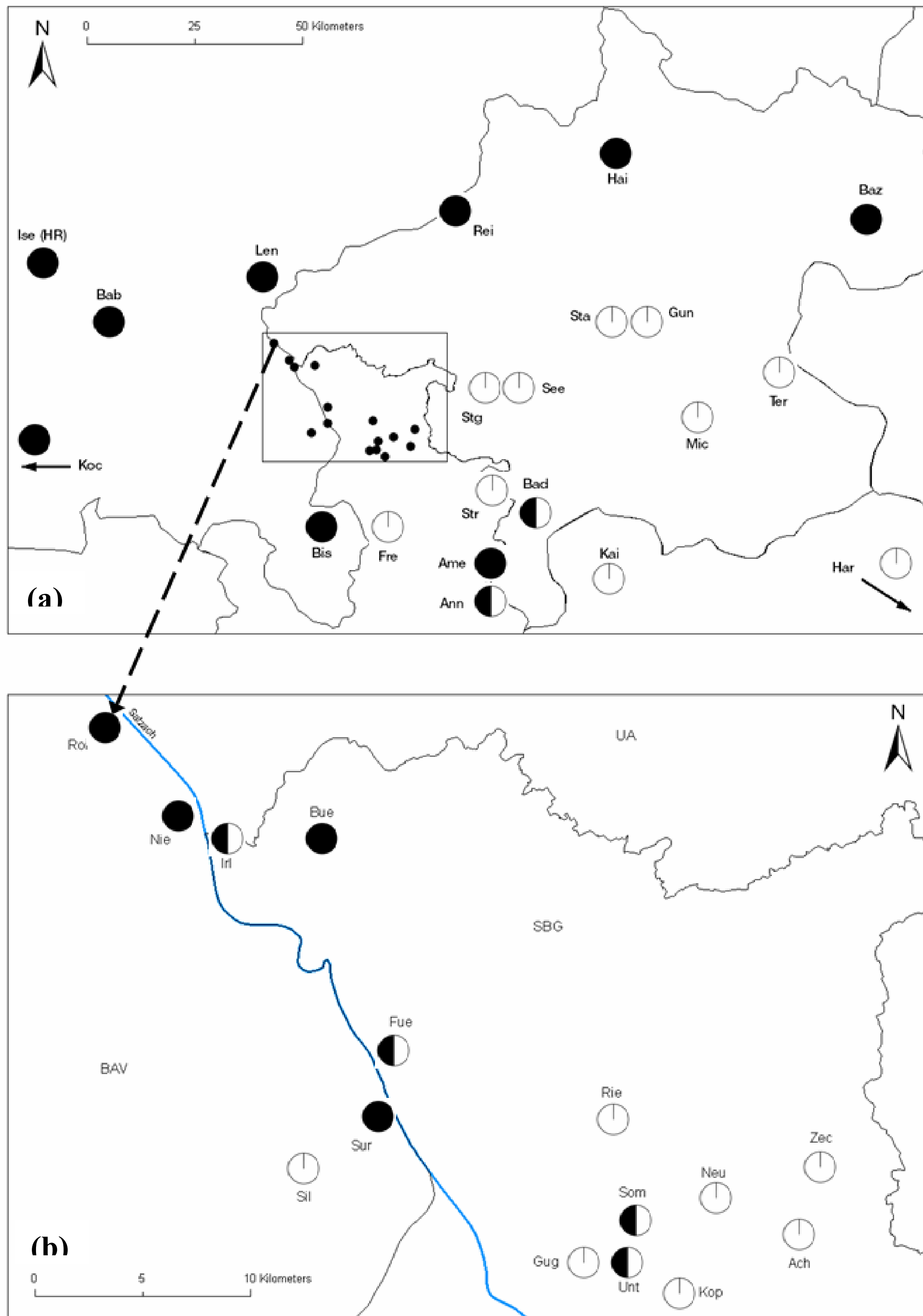


FIG. 3.

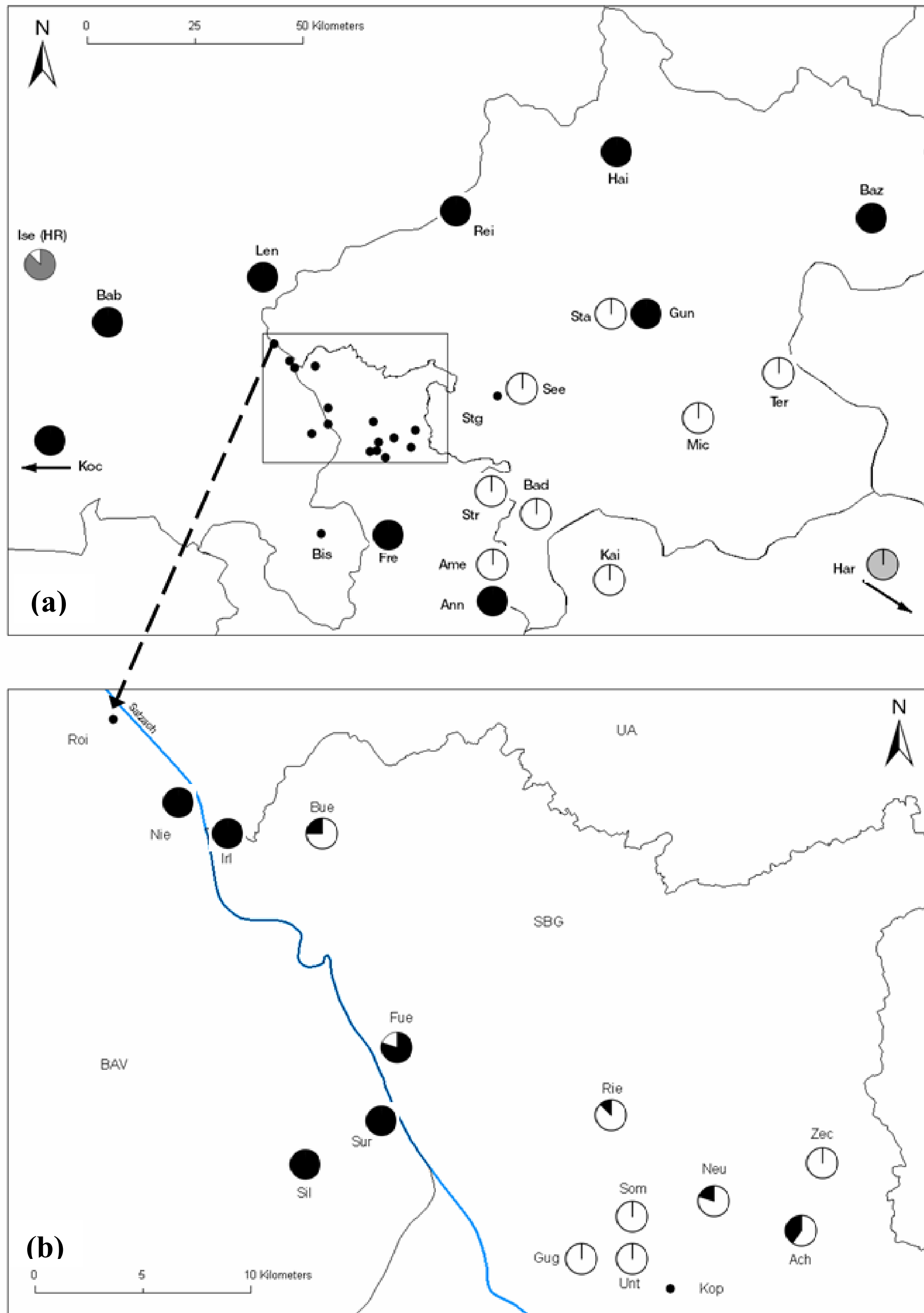
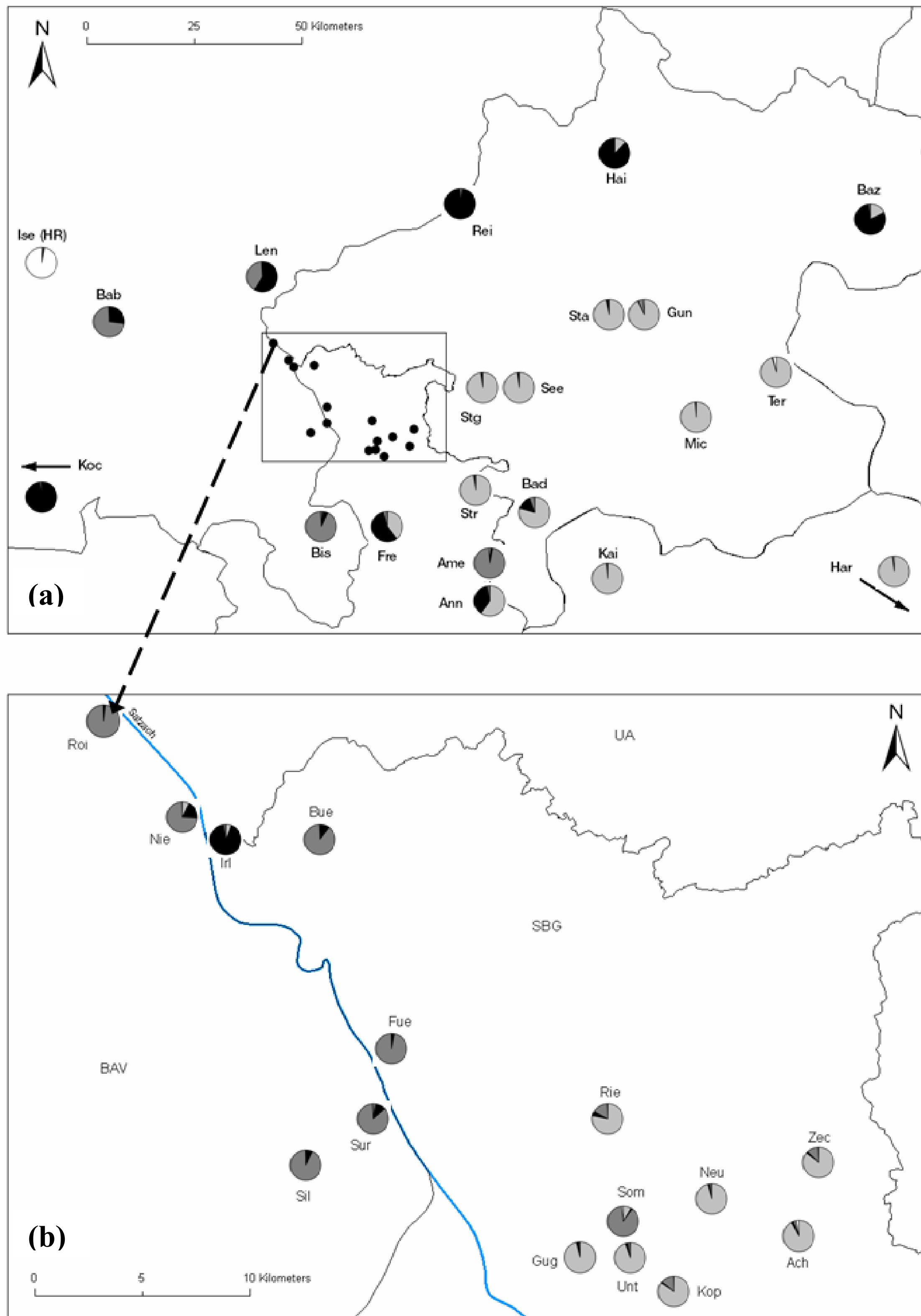


FIG.4.



3 Conclusions

1. Analysis of partial cytochrome b sequences of *T. cristatus* complex populations from the Czech and Slovak Republic revealed considerably higher amount of genetic variability than those from Austria. Six haplotypes of *T. cristatus* unique to central Europe were found to be highly similar to the most prevalent one (known from Austria, Czech Republic, England, Romania and Slovakia so far) without clear geographical pattern. On the other hand, *T. dobrogicus* haplotypes were grouped to three clades of which two of them could be assigned to particular region, whereas the central clade was distributed from southernmost to northernmost part of *T. dobrogicus* areal.

2. Contrary to morphological and nuclear data, only one *T. c. carnifex* haplotype carried by the sole specimen was found among the Czech and Slovak crested newts. Phylogenetic analyses suggested its Italian origin. The rest of the *carnifex*-like newts possessed the *dobrogicus*-like mtDNA of central and western type.

3. Based on partial mitochondrial cytochrome b sequences, microsatellites and morphology, we have uncovered contact zone between *T. c. carnifex* and *T. cristatus* in the Salzburg region, Austria. The patterns of introgression in both molecular nuclear and mitochondrial markers suggests that the hybrid zone (HZ) is of unimodal type. However, assortative mating typically linked with unimodal HZ, resulting in deficit of heterozygotes, was found only in some of the localities with admixed individuals. Selection against hybrids is thus not the prevalent mechanisms forming the shape and width HZ (the possible role of environmental factors is also discussed).

4. In contrast to previous reports, molecular markers revealed no autochtonous localities of *T.* c. *carnifex* in Bavaria. We have found no signs of genetic interaction between *T.* c. *carnifex* specimens from allochtonous locality Isendorf (of Croatian origin) and surrounding *T. cristatus* population

5. Analysis of mtDNA further reveals local *T. cristatus* populations posses only the most prevalent haplotype found throughout the Europe. In case of *T. c. carnifex*, the Salzburg region was colonised from the northern Balkans. Both species show haplotype uniformity, which suggests this rather isolated and geographically highly indent region was colonised only limited number of times.

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Zvláštní poděkování nakonec patří ovšem mé báječné ženě a dětem, bez nichž by to ostatní tak nějak postrádalo smysl.

!Díky!

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1994 – 1997 BA level in Zoology, title of the BA thesis: Hybridization between the fire-bellied toads, Bombina bombina and B. variegata in South Bohemia

1997 – 2000 MSc level in Ecology and Etology of Vertebrates, title of the MSc thesis: Genetic study of hybridization within the newt Triturus cristatus superspecies

2000 – present PhD student at Department of Zoology, topic of the PhD thesis: Genetic study of the hybridization of Triturus cristatus superspecies newts. Expected termination: October 2007

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Supervisor of 3 BA and 3 MSc theses on ecology of hybridization in fire-bellied toads, all theses defended at FBS/FS.

Leader of the laboratory and field seminars in Zoology of Vertebrates, Zoology of Lower Vertebrates and Herpetology courses at FBS/FS

Since February 2002 research assistant in Laboratory of Molecular Taxonomy, Biology Centre of Academy of Sciences of the Czech Republic, Institute of Parasitology (head ing. M. Obornik, PhD), where I am responsible for keeping the lab going, teaching students molecular methods and helping them with data analysis.

Current Research Projects I am involved in besides of my PhD:

Molecular phylogeny of East African agamas using mitochondrial and nuclear markers.

Evolution of the *Leishmania* with special emphasize to the *L. donovani* complex

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International presentations

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