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The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data

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The shape of hybrid zones provides insight into genetic isolation between the evolutionary lineages involved: the greater the limitation to gene flow, the farther along the unimodal to bimodal continuum. We study hybrid zone modality in a group of closely related species at a variety of levels of genetic divergence. We explore the degree and the geography of hybridization in 12 transects of *Triturus* newts, for nine of the ten secondary contact zones in Europe, using nuclear and mitochondrial encoded genetic data and morphological characteristics. Species status is verified by examining the correlation between the three marker systems in a geographical context. At 40 allozyme loci, two transects classify as unimodal, three as bimodal and two as intermediate, while the signal for hybridization is weak or absent in five transects. One zone studied in duplicate was classified as intermediate in one region and as bimodal in another region. mtDNA introgression is frequent and extends beyond nuclear introgression in two transects. Morphology provides additional evidence for hybridity, including one transect for which the signal of nuclear gene flow is weak. Compared to simulations allowing panmixia, the observed allozyme transitions at contact zones show a deficit of backcrossing to various degrees. Over all transects, there is a weak negative relationship between the level of hybridization and allozyme genetic distance for species pairs, consistent with Bateson–Dobzhansky–Muller effects. This observation, based upon highly comparable data for a single genus, supports conclusions derived from analyses over a wide variety of other taxa. *Biological Journal of the Linnean Society*, 2014, **113**, 604–622.

ADDITIONAL KEYWORDS: allozymes – Balkan Peninsula – cline – hybridization – introgression – *Triturus cristatus* superspecies – *Triturus marmoratus*.

INTRODUCTION

Hybrid zones are regions where genetically distinct populations meet, mate and produce offspring of mixed ancestry. Hybrid offspring usually possess genetically less compatible gene combinations (intrinsic selection), and later generation hybrids possess introgressed alleles that tend to reduce fitness in a novel ecological and behavioural setting (extrinsic selection). The hybrid zone is often maintained because of the continuous removal of unfit genotypes, balanced by gene flow into the zone from the parental populations (Barton & Hewitt, 1985). There may also be some instances of positive selection, where alien alleles actually raise fitness (Macholán *et al.*, 2011). Hybrid zones are of particular interest as natural laboratories, where genetic interactions of related or incipient species can be examined (Kocher & Sage, 1986; Hewitt, 1988; Harrison, 1993).

The shape of hybrid zones depends on the degree of genetic isolation that has evolved between the participating lineages (Harrison & Bogdanowicz, 1997; Jiggins & Mallet, 2000). If genetic isolation is limited,

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hybrid zones with a unimodal shape are formed, where individuals in the hybrid zone have mainly recombinant genotypes. If genetic isolation is more pronounced, hybrid zones with a bimodal shape arise, where individuals are mostly parental type and recombinants are rare. As independently evolving lineages accumulate genetic differences causing cumulative post-zygotic isolation over time (Bateson, 1909; Coyne & Orr, 2004; Bolnick & Near, 2005; Arntzen *et al*., 2009), one might expect that the degree of hybridization and introgression would be inversely proportional to genetic distance between the hybridizing units. Following from that, unimodal hybrid zones would be expected to occur among closely related species, whereas more distantly related species would engage in bimodal hybrid zones. In a review paper, Jiggins & Mallet (2000) show bimodality to be strongly associated with assortative mating or fertilization and only weakly with overall levels of genetic divergence or intrinsic genomic incompatibility. The data in this review are, however, gathered over a wide array of taxa and may not be directly comparable. Since Jiggins & Mallet (2000), several studies have been carried out with replication over different geographical and ecological settings, either involving different races, lineages or ecotypes of one species (Morgan-Richards & Wallis, 2003; Panova, Hollander & Johannesson, 2006; Berner, Grandchamp & Hendry, 2009; Singhal & Moritz, 2012) or two species (Aboim *et al*., 2010; Culumber *et al*., 2011; Schaefer, Duvernell & Kreiser, 2011; Bailey *et al*., 2012). No replicate studies are as yet available that deal with a wide range of related species. We here explore nine hybrid zones involving taxa in a single genus (*Triturus* newts) with widely different degrees of genetic and ecological divergence, bringing together genetic and morphological data collected over 30 years. The system offers a near-unique possibility for the direct comparison of interspecies characteristics, such as the test for association between genetic incompatibility and genetic divergence. While sample size from specific ponds is often rather low, this usually reflects biological reality; the strength lies in the number of transects we have made, at the expense of local sampling intensity.

Triturus newts are widely distributed across western Eurasia, showing an intricate biogeographical pattern (Fig. 1) reflecting the general picture of Pleistocene refugia and subsequent post-glacial expansion (Taberlet *et al*., 1998; Ruiz-González *et al*., 2013). Eight species are currently recognized: two marbled newts (*T. marmoratus*, *T. pygmaeus*) and six crested newts (*T. carnifex*, *T. cristatus*, *T. dobrogicus*, *T. ivanbureschi*, *T. karelinii*, and *T. macedonicus*; together known as the *T. cristatus* superspecies). The two species of marbled newt meet on the Iberian Peninsula, and five species of crested newt meet in central Europe and the Balkan Peninsula; the sixth crested species (*T. karelinii*, from which *T. ivanbureschi* was recently split) is allopatric in the Caucasus region (Wielstra *et al*., 2013c). One marbled and one crested newt species have broader contact with each other in western and central France (Lescure & de Massary, 2012). Local studies within the area of range overlap revealed a pattern of ecological separation of species following forestation and altitude, with low numbers (ca. 4% on average) of F_1 hybrids and very limited nuclear introgression (< 0.5%) (Schoorl & Zuiderwijk, 1981; Arntzen & Wallis, 1991). Data for the marbled newt contact (*T. marmoratus* and *T. pygmaeus*) on the Iberian Peninsula are incomplete, but give a similar picture (Espregueira Themudo & Arntzen, 2007a; Espregueira Themudo, Nieman & Arntzen, 2012). Among the five European crested newt species, eight out of ten theoretically possible contact zones exist in nature (Fig. 1). Our knowledge of the contact zones predominantly comes from an early phylogeographic study (49 population samples) using mtDNA (Wallis & Arntzen, 1989), and a subsequent study (142 population samples, including marbled newts) that incorporated morphometrics (Arntzen & Wallis, 1999). These data suggest that widespread blending of gene pools is absent, but hybridization between species clearly does take place. Table 1 summarizes the current knowledge by contact zone. In short, we have a good picture of the *T. marmoratus* – *T. cristatus* contact zone and an emerging picture for the *T. marmoratus* – *T. pygmaeus* contact zone. In contrast, published information for the crested newt contacts is limited.

Although morphology and mtDNA generally provide reliable criteria for species identification, mingling of these features occurs at contact zones between crested newt species, where intermediate phenotypes and mitochondrial introgression are observed (Wallis & Arntzen, 1989; Arntzen & Wallis, 1999; Arntzen, 2003; Wielstra & Arntzen, 2012). Nuclear markers are required to explore these contact zones in more detail. We have accumulated genotypic data across a large array of allozyme loci and increased sampling intensity in nearly all areas of contact. Our sampling includes populations well away from contact zones, as well as several transects of the zones themselves. Improved analytical tools have recently become available that permit detailed analysis of hybridity. With the newly constructed allozyme background, we are in a position to ask: What is the extent of nuclear genetic introgression among *Triturus* species? Subsequently, we put mitochondrial and morphological variation, and especially their mismatch with the allozyme background, into a spatial context. Finally, we test whether the interspecific

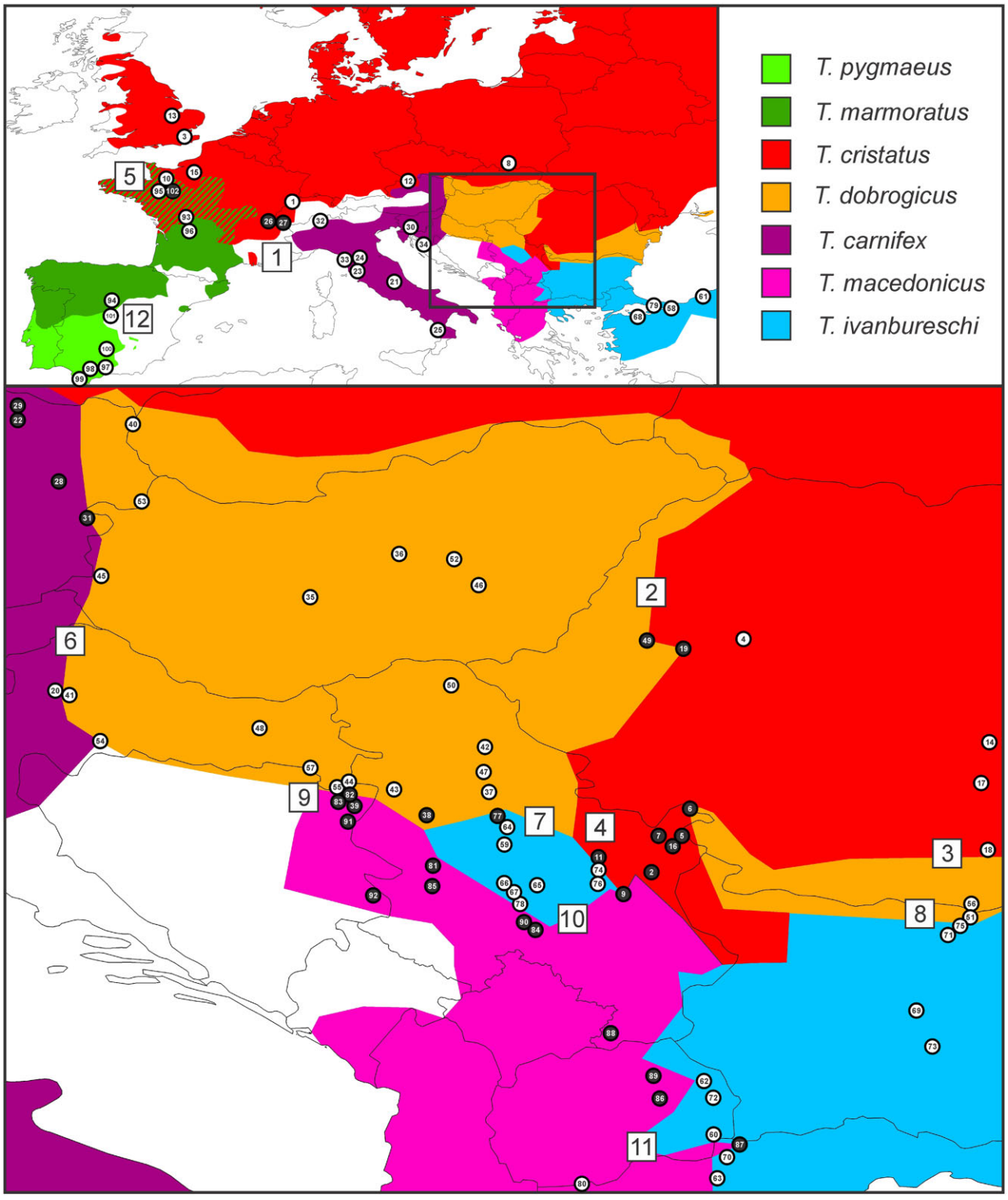

Figure 1. The distribution of seven species of the genus *Triturus* and sampling design. The inset shows the sampled populations in Central Europe and the Balkan Peninsula. Details on the numbered populations are provided in Appendix S1. Populations with introgressed mtDNA are presented as black spots with white numbering (instead of the reverse). The approximate position of the 12 transects is shown by boxed numbers. The exact partitioning of populations over transects is shown in Table 2.

Table 1. Features of contact regions among seven species of the newt genus *Triturus*

Species pair	Region	Differential ecology	History	mtDNA introgression	Chromosome differences	Fitness	References	Transect
T. cristatus–*T. carnifex*	Austria, Czech Republic; artificially in Geneva basin	Largely separated by Alps	Fragmenting	Some	55% chiasmata in F_1 males, inversion bridges	High mortality in F_2	Spurway & Callan, 1950; Callan & Spurway, 1951; Wallis & Arntzen, 1989; Arntzen & Thorpe, 1999; Brede *et al*., 2000; Arntzen, 2001; Maletzky *et al*., 2008	1
T. cristatus–*T. dobrogicus*	Slovakia, Romania	Hilly/Montane vs. Lowland	Stable	No	?	?	Wallis & Arntzen, 1989; Mikulícek *et al*., 2004	2, 3
T. cristatus–*T. ivanbureschi*	Serbia, Bulgaria	Not studied	Moving S at expense of *T. ivanbureschi*	?	54% chiasmata in F_1 males, inversion bridges, ≥ 2 translocations	High mortality in F_2	Spurway & Callan, 1950; Callan & Spurway, 1951; Wallis & Arntzen, 1989; Arntzen & Wallis, 1999	4
T. cristatus–*T. macedonicus*	Serbia	Not studied	Lengthening due to *T. macedonicus* expanding N	?	?	?	Arntzen & Wallis, 1999	Unresolved
T. cristatus–*T. marmoratus*	France	Open vs. Forest; flat vs. Hilly areas	Moving at expense of *T. marmoratus*	Very slight	Extensive rearrangements (based on *T. ivanbureschi* only)	Reduced; male F_1 sterile	Lantz & Callan, 1954; Arntzen & Hedlund, 1990; Arntzen & Wallis, 1991; Arntzen *et al*., 2009	5
T. carnifex–*T. dobrogicus*	NW Balkans, Austria, Hungary	Hilly/Montane vs. Lowland	Stable	?	?	?		6
T. carnifex–*T. ivanbureschi*	Balkan Peninsula	Separated by Dinaric karst and congeneric species		No	50% chiasmata in F_1 males, inversion bridges, ≥ 2 translocations	High mortality in F_2	Spurway & Callan, 1950; Callan & Spurway, 1951	NA
T. carnifex–*T. macedonicus*	Balkan Peninsula	Separated by Dinaric karst		No	?	?		NA
T. dobrogicus–*T. ivanbureschi*	Serbia, Bulgaria	Lowland vs. Hilly/Montane	Stable	Some	?	High	Wallis & Arntzen, 1989; Arntzen & Wallis, 1999	7, 8
T. dobrogicus–*T. macedonicus*	Croatia, Bosnia-Herzegovina, Serbia	Lowland vs. Hilly/Montane	Stable	?	?	?	Wallis & Arntzen, 1989	9
T. ivanbureschi–*T. macedonicus*	Serbia, Macedonia, Greece	None	Moving N at expense of *T. ivanbureschi*	High	?	High	Wallis & Arntzen, 1989; Arntzen & Wallis, 1999	10, 11
T. marmoratus–*T. pygmaeus*	Portugal, Spain	Relief, July temperature, land use	Moving N at expense of *T. marmoratus*	High	?	?	Arntzen & Espregueira Themudo, 2008; Espregueira Themudo & Arntzen, 2007a, b; Espregueira Themudo *et al*., 2012	12

NA, not available.

genetic distances based on both allozymes and mtDNA are correlated with the amount of hybridization and the shape of the hybrid zone.

MATERIAL AND METHODS

Sampling strategy

We sampled *Triturus* newts from 102 populations (nine marbled, 92 crested, one hybrid marbled/crested) across Europe, with denser sampling in areas where species transitions were expected as judged from documented geographical ranges (Fig. 1). In their area of overlap, marbled (*T. marmoratus*) and crested (*T. cristatus*) newts show practically no backcrossing (Arntzen & Wallis, 1991), but we included F_1 hybrids between the two (which are easily identified by their intermediate morphologies (Vallée, 1959; Muratet, 2008). We also included the contact between the two marbled newts *T. marmoratus–T. pygmaeus*. Among European crested newts, neither *T. carnifex–T. ivanbureschi* nor *T. carnifex–T. macedonicus* contacts exist in nature. No transect was made of the short *T. cristatus–T. macedonicus* contact because it had not been resolved at the time of collecting. The *T. carnifex–T. cristatus* contact in the Vienna area presented two disadvantages: it is close to a three-way meeting (with *T. dobrogicus*) and the local distribution of crested newts is in severe decline (Klepsch, 1994). Instead we studied the Geneva basin, where *T. carnifex* was introduced amid autochthonous *T. cristatus* a century ago (Arntzen & Thorpe, 1999). Three zones (*T. cristatus–T. dobrogicus*, *T. dobrogicus–T. ivanbureschi*, *T. ivanbureschi–T. macedonicus*) were studied in duplicate.

Laboratory methods

Genetic profiles for 40 nuclear gene loci were established by allozyme electrophoresis for 749 newts (Appendix S1). For 729 of these, we sequenced a 658-bp segment of subunit 4 of the NADH dehydrogenase (ND4) mitochondrial gene complex, omitting the ten *T. cristatus–T. marmoratus* F_1 hybrids, for which RFLP data were available (missing data 1.3%). In addition, 736 newts were X-rayed to determine the number of rib-bearing pre-sacral vertebrae (NRBV) on a Faxitron 43855C/D with an exposure of 20–40 s at 3 mA and 70 kV (missing data 1.7%). Laboratory protocols and some of the data (ca. 62% allozymes, 19% mtDNA) are published elsewhere (Arntzen, 2001; Arntzen, Espregueira Themudo & Wielstra, 2007; Arntzen & Wallis, 1991; Arntzen & Wallis, 1999; Arntzen & Wielstra, 2010; Vörös & Arntzen, 2010; Wielstra & Arntzen, 2012; Wielstra *et al.*, 2010).

Species identity and hybridization based on allozymes

First, we used a Bayesian analysis of population structure with the program BAPS v.5.3 (Corander *et al.*, 2008) to: (1) determine the most probable number of distinct gene pools (k); and (2) assign individuals to the inferred gene pools probabilistically, based upon allele frequencies across loci. BAPS makes no a priori assumptions about k and can be used to apportion individuals for any value up to the number of individuals present in the input dataset. Here, k was evaluated over the $2 \leq k \leq 50$ range, under BAPS default settings.

Second, we arranged a subset of populations into 12 transects across nine parapatric contact zones in central Europe and the Balkans, central Iberia, western France, and the Geneva basin in Switzerland and France (Fig. 1 and Table 2). To minimize the effect of intraspecific geographical variation, transects were as far as possible defined within BAPS groups and anchored by populations located away from the considered range edge, preferably from a locality central to the range (Table 2). We used the admixture method based on pre-defined populations in order to estimate the degree of hybridity for each individual in each transect, based upon these anchor populations. Gene flow estimates from transects involving pairs of species were derived from BAPS in two steps, involving the clustering of all individuals from the transect in two groups, followed by admixture analysis.

Third, we used NewHybrids 1.1b3 (Anderson & Thompson, 2002) to infer membership of genetically admixed individuals to four different hybrid classes (F_1, F_2, backcross to A = F_2A and backcross to B = F_2B) against two parental anchor classes (A, B). NewHybrids was run with burn-in and post burn-in runs of 10 000 iterations each. For two transects, NewHybrids and BAPS analyses were also run on larger samples with fewer loci, for which data were taken from the literature (Arntzen & Wallis, 1991; Arntzen & Thorpe, 1999).

Fourth, we simulated bivariate BAPS versus NewHybrids plots, using anchor population genotypes under the assumption of panmixia, with the software HybridLab v.1.0 (Nielsen, Bach & Kotlicki, 2006). Anchor populations were crossed with themselves *in silico* to obtain source populations A and B with sample sizes of 200. F_1 were produced by crossing A and B, F_2 by crossing F_1 with F_1, and backcrosses in each direction by crossing F_1 with A and B (F_2A and F_2B, respectively). The population of 1200 individuals (200 in each class) was then analyzed with BAPS and NewHybrids and plotted as before. These simulations are to tell us what the BAPS vs. NewHybrids plots should look like with no pre-zygotic or post-zygotic

Table 2. Details of 12 transects among seven species of the newt genus *Triturus*

Species pair	Transect number – region	Anchor sites (n = pooled sample size)	Sites inside contact zone (n = pooled sample size)	D (Nei, 1978); K2P mtDNA (Kimura, 1980)	Gene flow estimate BAPS (%); average NewHybrid score inside contact zone (%)
T. cristatus–T. carnifex	1 – Geneva basin Switzerland, France (introduced)	13, 3, 15, 10, 1 (n = 65) vs. 32, 33, 24, 23, 21 (n = 25)	26, 27 (n = 27)	0.157; 0.061	0.46; 0.04
Idem, data from Arntzen & Thorpe (1999)	1a – idem, data for three diagnostic loci	Idem	Seven localities in the Geneva basin and adjacent France (n = 182)	Idem	0; 0.12
T. cristatus–T. dobrogicus	2 – Hungary, Romania	4 (n = 10) vs. 46, 52, 36 (n = 9)	19, 49 (n = 16)	0.152; 0.063	1.39; 9.86
T. cristatus–T. dobrogicus	3 – Romania, Bulgaria, Serbia, Hungary	14, 17 (n = 20) vs. 50 (n = 14)	18, 6, 56, 51 (n = 17)	0.092; 0.063	4.35; 11.98
T. cristatus–T. ivanbureschi	4 – Serbia	5, 16, 7 (n = 15) vs. 59 (n = 9)	2, 11, 9, 74, 76 (n = 19)	0.175; 0.069	2.30; 24.84
T. cristatus–T. macedonicus	Serbia	4, 14, 17 (n = 30) vs. 92, 85, 81, 84, 88, 86, 80 (n = 64)	Not sampled	0.131; 0.064	NA
T. cristatus–T. marmoratus	5 – W France	13, 3, 15, 1 (n = 16) vs. 93, 96, 94 (n = 12)	10, 95 (n = 88)	0.724; 0.135	0; 0.011
Idem, data from Arntzen & Wallis (1991)	5a – idem, data for nine diagnostic enzyme loci for large samples outside and inside the area of range overlap	Eight localities in the UK and the French depts. Pas-de-Calais and Manche (n = 132) vs. two localities in dept. Charente (n = 109)	Fourteen localities in dept. Mayenne (n = 582), excluding 10 pre-selected F_1 hybrids	Idem	0; 0.007
T. carnifex–T. dobrogicus	6 – Slovenia, Croatia, E Austria, W Hungary	30, 34 (n = 9) vs. 35 (n = 4)	20, 31, 28, 22, 29, 54, 41, 45, 53, 40 (n = 34)	0.037; 0.060	0; 7.57
T. carnifex–T. ivanbureschi	Apennine peninsula vs. SE Europe	32, 33, 24, 23, 30 (n = 29) vs. 69, 73, 72, 60 (n = 21)	NA	0.173; 0.065	NA
T. carnifex–T. macedonicus	Apennine vs. C Balkan peninsula	32, 33, 24, 23, 30 (n = 29) vs. 92, 85, 81 (n = 32)	NA	0.126; 0.039	NA
T. dobrogicus–T. ivanbureschi	7 – Hungary, Serbia	50, 42 (n = 28) vs. 59, 65, 66, 67 (n = 25)	47, 37, 77, 64 (n = 26)	0.222; 0.066	0; 0.14
T. dobrogicus–T. ivanbureschi	8 – Hungary, Romania, Bulgaria	50 (n = 14) vs. 69, 73 (n = 8)	56, 51, 75, 71 (n = 15)	0.221; 0.066	0; 0.05
T. dobrogicus–T. macedonicus	9 – Croatia, Bosnia-Herzegovina, W Serbia	48, 57, 44 (n = 19) vs. 92, 85, 81 (n = 32)	43, 55, 39, 38, 82, 83, 91 (n = 75)	0.136; 0.064	1.40; 19.70
T. ivanbureschi–T. macedonicus	10 – Serbia	59, 65, 66 (n = 19) vs. 84 (n = 8)	67, 78, 90 (n = 15)	0.158; 0.067	0; 0.26
T. ivanbureschi–T. macedonicus	11 – Bulgaria, Macedonia, Greece	69, 73 (n = 8) vs. 80 (n = 11)	62, 72, 60, 70, 63, 87, 86, 89, 88 (n = 53)	0.248; 0.067	1.90; 4.09
T. marmoratus–T. pygmaeus	12 – C Spain	95, 93, 96 (n = 44) vs. 100, 97, 98, 99 (n = 53)	94, 101 (n = 18)	0.211; 0.039	0; 0.03

Nei D (Nei, 1978) is calculated over anchor sites with BIOSYS1 (Swofford & Selander, 1981).

Full mtDNA pairwise distances (K2P; Kimura, 1980) are based on the data presented in Wielstra & Arntzen (2011) and calculated in MEGA 5.05 (Tamura *et al.*, 2011).

Gene flow estimates with BAPS (Corander *et al.*, 2008) and NewHybrids (Anderson & Thompson, 2002) hybridity are described in the text.

NA, not available.

isolation and provide a null expectation against which to compare the empirical bivariate plots.

Hybridity versus genetic distance for species pairs

To test for a relationship between the amount of hybridization and relatedness as captured by genetic distance, we correlate the BAPS estimate of gene flow and the average score at NewHybrids (P_{NH}) across each transect with: (1) Nei's D (Nei, 1978) (allozymes); and (2) Kimura's K2P (Kimura, 1980) (mtDNA) between pairs of anchor populations, using Spearman's r_s, in which tied values were randomly permuted in 100 iterations (Sokal & Rohlf, 1981).

Clustering mtDNA to source species

The ND4 mtDNA sequences were aligned and merged into unique haplotypes in MacClade 4.08 (Maddison & Maddison, 2005). In order to have all species represented we added three *T. karelinii* sequences, and *Calotriton asper* was used as an outgroup (GenBank accession numbers GU982391, GU982399, GU982406 and GU982378) (Wielstra *et al*., 2010). To assort individuals to a particular mtDNA lineage, we used a maximum likelihood based phylogenetic analysis in PAUP* (Swofford, 2003) under a model of nucleotide substitution selected by jModelTest under the AIC criterion (Darriba *et al*., 2012). This was the TIM1 + I + G model. The support for monophyly of the haplotype groups was assessed with 2000 bootstrap replicates. A haplotype network was constructed for all sequences with HaploViewer (available at http://www.cibiv.at/~greg/haploviewer). Each *Triturus* species represents a distinct mtDNA lineage (Wielstra & Arntzen, 2011), so identifying whether an individual possesses homospecific or introgressed mtDNA, is straightforward.

Morphology

Wolterstorff (1923) originally identified the crested newt species on the basis of forelimb to interlimb ratio and arranged them in a morphological series from stocky (*T. karelinii*) to slender (*T. dobrogicus*). We have since determined that this 'Wolterstorff Index' is a crude reflection of the number of pre-sacral rib-bearing vertebrae (NRBV) (Arntzen & Wallis, 1999; Arntzen, 2003). NRBV ranges from 11–13 in marbled newts and from 12–17 in crested newts. There is some intraspecific variation, but modal values of NRBV reliably discriminate the five *Triturus* morphotypes: *T. marmoratus* and *T. pygmaeus* (12), *T. ivanbureschi* and *T. karelinii* (13), *T. carnifex* and *T. macedonicus* (14), *T. cristatus* (15) and *T. dobrogicus* (16 or 17). Although other features such as ventral patterning are also informative for discriminating *Triturus* species to a certain extent, NRBV is more easily quantifiable.

RESULTS

Allozyme data with BAPS and NewHybrids

Allozyme data are presented in Appendix S2. As markers of hybridity, allozymes have enduring power. Net charge encompasses a huge number of possible amino-acid substitution permutations over the entire length of the protein, ranging from interior to exterior, making both convergence and incidental co-migration on a gel highly unlikely. In contrast, microsatellite loci (though possessing many alleles) will often converge in copy number through time, exacerbated by high mutation rate. Bayesian analysis of population structure revealed 16 distinct gene pools: four within *T. carnifex*, two in *T. cristatus*, two in *T. dobrogicus*, four in *T. ivanbureschi*, one in *T. macedonicus*, one in *T. marmoratus*, one in *T. pygmaeus* and one for F_1 hybrids between *T. cristatus* and *T. marmoratus* (Appendix S1). BAPS allocation to species is mostly concordant with *a priori* expectations based on phenotype and geographical range. The exceptions occur in two transects and concern three individuals from Donja Čadjavica and Gornja Čadjavica in the *T. dobrogicus* – *T. macedonicus* transect and three individuals from Virfuri and Sebis in the western *T. cristatus* – *T. dobrogicus* transect. Another exception is the classification of two individuals from Sebis (716, 718) in the group of north-eastern *T. carnifex* (Appendix S1), which is more likely explained by retention of ancestral polymorphism or rare alleles derived from hybridization [hybrizymes; sensu Woodruff (1989)] than by genuine contemporary affiliation to that species.

BAPS and NewHybrids (NH) analyses of transect material tell us two closely related, but subtly different things. BAPS infers an assignment probability for each multilocus genotype to each of the two alternative parental types (P_{DC} – using anchor population samples); NH gives the probability (P_{NH}) of hybridity to four different hybrid categories, pooled for our purposes here (Fitzpatrick, 2012). We express these results using bivariate plots for each of the 12 transects, with P_{DC} on the horizontal axis and P_{NH} on the vertical axis (Fig. 2). Individuals at the bottom left and bottom right ($P_{DC} < 0.15$ or > 0.85 and $P_{NH} < 0.1$, i.e. falling inside the boxed areas illustrated in Fig. 2A) are classified similar to anchor populations. Those in the centre top are F_1, or are in any later hybrid class with approximately equal proportions of A and B alleles. Individuals intermediate to these categories are backcrosses of various types. Note that many

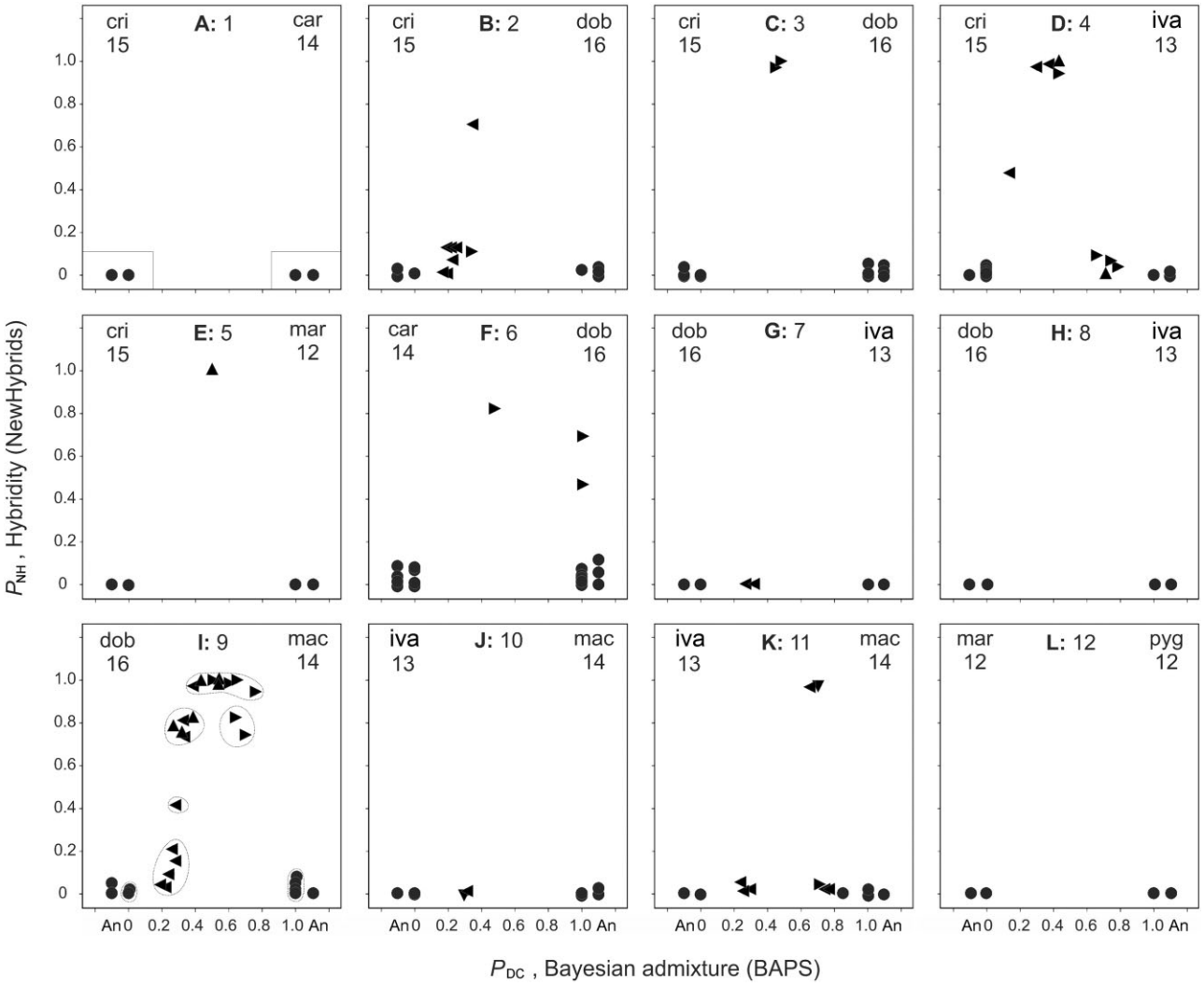

Figure 2. Bivariate plots of nuclear genotypes for 12 transects across nine *Triturus* species pairs (cri = *T. cristatus*, car = *T. carnifex*, dob = *T. dobrogicus*, iva = *T. ivanbureschi*, mac = *T. macedonicus*, mar = *T. marmoratus* and pyg = *T. pygmaeus*). Values on the horizontal axis present the scores obtained with Bayesian admixture (P_{DC}, with BAPS) and those on the vertical axis present hybridity (P_{NH}, with NewHybrids). Values shown underneath the species code represent the modal number of rib-bearing pre-sacral vertebrae (NRBV). Individuals within the boxed area shown in Figure 2A (round symbols, $P_{DC} < 0.15$ or > 0.85 and $P_{NH} < 0.1$) have the parental or near-parental genotypes. At either side, anchor populations (An) are offset along the horizontal axis, for clarity of presentation. Genetically admixed individuals are shown by triangular symbols. The direction the triangle points refers to the individuals morphology as follows: left and right – NRBV as indicative for the species listed in the top-left and top-right of each diagram respectively, upwards – intermediate NRBV score and downward pointing – NRBV unknown. For constituent data see Appendices S1, S2 and S4.

points, particularly parental types and F_1 hybrids, overlie each other almost exactly. All individuals with $0.15 < P_{DC} < 0.85$ or $P_{NH} > 0.1$ are listed in Appendix S4.

With these facts in mind, we make some broad comparative observations of the different zones. Transects 1, 8 and 12 give no signal of hybridity at all (Fig. 2A, H, L), while transects 7 and 10 give minimal signal, with $P_{NH} << 0.1$ (Fig. 2G, J). Analysis with three diagnostic loci and large samples for transect 2, re-analysed from Arntzen & Thorpe (1999), also reveals no hybrids and shows introgressive hybridization at low frequency (transect 1a in Table 2). The other seven transects are considered in turn below. By diagnostic allele, we mean unambiguously diagnostic based on anchor samples; by novel allele we mean an allele never seen in either anchor sample, possibly a hybrizyme.

Transect 2 (Fig. 2B) shows evidence of admixture in the two central populations (Sebis, Virfuri) and is a sharp cline with species syntopy at Virfuri (Appendix S1). Only one genotype (Virfuri 715) gets classified with a high degree of hybrid likelihood (P_{NH} = 0.69). Five more or less intermediate genotypes cluster at P_{NH} = 0.07–0.10 (Sebis 366, 368, 716, 717; Virfuri 356). A feature of this group is their low P_{NH} values, despite having at least three diagnostic alleles from each species. Although the distribution of alleles across these five newts favours *T. cristatus*, 20/24 of the *T. cristatus* alleles occur in homozygotes in contrast to 8/17 *T. dobrogicus* alleles. BAPS scores them all as about 70–80% *T. cristatus*. Two individuals are adjacent to this group (Virfuri 357, 359) but with even lower P_{NH} values. Three potentially hybrid genotypes (Sebis 367, 718; Virfuri 711) are classified as pure *T. dobrogicus* by BAPS. The first has 11 *T. dobrogicus* alleles, one *T. cristatus* allele and one novel allele (P_{NH} = 0.22); the second has 11 *T. dobrogicus* alleles and one novel allele (P_{NH} = 0.02); the third has ten *T. dobrogicus* alleles, one (rare) *T. cristatus* allele and one novel allele (P_{NH} = 0.02). Five individuals from Virfuri (355, 358, 712–714) classify as pure *T. cristatus*, with concomitant very low NH scores (P_{NH} < 0.005).

For transect 3 (Fig. 2C), both newts from Kladovo (462, 463), each with at least two alleles from each parental type, are strongly admixed. They appear to be later generation hybrids (not backcrosses), either F_2 or part of a hybrid swarm. With just two individuals sampled and Kladovo being geographically displaced in the transect (and thus possibly genetically distinct), it is uncertain. All other genotypes are effectively parental type, so curve shape is not resolved. As with transect 2, involving the same species pair, the cline is quite sharp, but there is no syntopy in this case.

Transect 4 (Fig. 2D) concerns a broad region of hybridity and is anchored with Arandjelovac to the west as there appears to be no parental *T. ivanbureschi* in the immediate vicinity. Four individuals (Resavaci Pecina 825, 826; Sisevac 827, 875) have 2–4 *T. cristatus* alleles and 9–12 *T. ivanbureschi* alleles, and lie in the 65–78% *T. ivanbureschi* range, but with low hybridity values (P_{NH} < 0.10). This is probably because evidence of *T. cristatus* is mainly restricted to the locus *Ndh*-2, which is polymorphic in *T. cristatus*. One individual from Milanovac (791) has nine *T. cristatus* and two *T. ivanbureschi* alleles, and P_{NH} = 0.48. The newts making up the cluster near the peak (Milanovac 789, 883, 884; Lukovo 933) all have at least three alleles from each species, and at least one novel allele (totals: 29 *T. cristatus*, 20 *T. ivanbureschi* and seven novel).

For transect 5 (Fig. 2E) no introgressed genotypes were found. The analysis with eight diagnostic loci (see Appendix S2) shows ten F_1 hybrids, all at P_{DC} = 0.5 and P_{NH} = 1. Analysis with nine diagnostic loci and large samples, as in Arntzen & Wallis (1991), reveals no F_1 hybrids and shows introgressive hybridization at very low frequency (transect 5a in Table 2).

Transect 6 (Fig. 2F) has several individuals with low NH scores and just three individuals with high P_{NH}. This result is related to a lack of diagnostic loci, but several diagnostic alleles, which leads to some spread in anchor populations too. All genotypes except Vendropolje (861) are classified as pure *T. carnifex* or *T. dobrogicus* by BAPS. Tadten (310) has four *T. dobrogicus*, one *T. carnifex*, and two novel alleles (P_{NH} = 0.48); Dugo Selo (880) has five *T. dobrogicus*, two *T. carnifex*, and four novel alleles (P_{NH} = 0.70). Both of these are classified as *T. dobrogicus* by BAPS. Vendropolje (861) is an almost exactly intermediate genotype.

Transect 9 (Fig. 2I) shows the highest admixture, with a curve peaking at unity on the NH axis. To help interpret this graph we have split newts into seven arbitrary categories as they appear on the graph. The first two categories are parental or near parental (*T. dobrogicus*: 24 Debrc, two Donja Čadjavica, 11 Glusci and five Vrsani; *T. macedonicus*: one Donja Čadjavica, three Gornja Čadjavica and eight Tavna Monastire). A third category, towards the bottom left, are mainly *T. dobrogicus*, with 1–2 *T. macedonicus* alleles and no novel alleles (four from Debrc and one from Vrsani). A single individual (centre left, Gornja Čadjavica), has four *T. dobrogicus*, seven *T. macedonicus* and two novel alleles, which appear to lower its P_{NH} value (0.42). A cluster of five individuals (four Donja Čadjavica, one Vrsani) show only 1–2 *T. macedonicus* alleles, a single novel allele and have a P_{NH} of about 0.80. An equivalent pair of newts (one from Tavna Monastire and one from Donja Čadjavica) at P_{NH} = 0.80 towards the right have single *T. dobrogicus* alleles, 3–4 diagnostic *T. macedonicus* alleles and a single novel allele. A final cluster of eight individuals (one Donja Čadjavica, one Donja-Gornja Čadjavica, five Gornja Čadjavica and one Vrsani) lie around the peak and have at least two (usually three) diagnostic alleles from each species and a maximum of one novel allele.

Transect 11 (Fig. 2K) shows three newts (Probistip 862, 863, 866) with 4–5 *T. ivanbureschi*, 11–15 *T. macedonicus* and three novel alleles each, positioned towards the bottom right of the graph. Mirroring these are three newts (Bigla 892, Dafnochori 815, Kentriko 814) with 2–4 *T. macedonicus*, 11–12 *T. ivanbureschi* and 0–4 novel alleles, positioned towards the bottom left. Once again, these newts have remarkably low P_{NH} values (P_{NH} < 0.06) and we note a skewed distribution of genotypes for the completely diagnostic loci. The three Probistip admixed newts

Table 3. Summary of 12 *Triturus* contact zone characteristics in terms of shape, range overlap and hybridity

Transect – species pair	Zone characteristics and hybridity	Range overlap, hybrid zone modality (*)
1 – *T. cristatus–T. carnifex*	Hybridizing, incomplete mixing (Arntzen & Thorpe, 1999; Maletzky *et al*., 2008)	None, hybrids not observed
2 – *T. cristatus–T. dobrogicus*	Occasional hybrids (Wallis & Arntzen, 1989; *)	Medium (20 km), intermediate
3 – *T. cristatus–T. dobrogicus*	Local hybridization at Iron Gate (*)	None, bimodal
4 – *T. cristatus–T. ivanbureschi*	mtDNA introgression (Wallis & Arntzen, 1989), local mixing (*)	None, unimodal
5 – *T. cristatus–T. marmoratus*	Low nuclear introgression; the ca. 4% F1 hybrids that are near sterile (Arntzen & Wallis, 1991; Arntzen *et al*., 2009)	Wide (300 km), bimodal
6 – *T. carnifex–T. dobrogicus*	Occasional hybrids and limited mixing in two populations (*)	None, bimodal
7 – *T. dobrogicus–T. ivanbureschi*	Introgressed mtDNA, morphological intermediates at Tresjna (Wallis & Arntzen, 1989)	None, ambiguous
8 – *T. dobrogicus–T. ivanbureschi*	No mixing or overlap (Wallis & Arntzen, 1989; *)	None, hybrids not observed
9 – *T. dobrogicus–T. macedonicus*	Near complete mixing in narrow zone (*)	None, unimodal
10 – *T. ivanbureschi–T. macedonicus*	No mixing or overlap (Wallis & Arntzen, 1989; *)	None, ambiguous
11 – *T. ivanbureschi–T. macedonicus*	Mixing, mtDNA broadly introgressed into *T. macedonicus* (Wallis & Arntzen, 1989; Wielstra & Arntzen, 2012; *)	Local, intermediate
12 – *T. marmoratus–T. pygmaeus*	No hybrids known, relict part of the zone (García-París *et al*., 1989; *)	None, hybrids not observed

*present paper.

include: 17 *T. macedonicus* homozygotes, five *T. ivanbureschi* homozygotes and one heterozygote. The other three newt hybrids have: three *T. macedonicus* homozygotes, 15 *T. ivanbureschi* homozygotes, and one heterozygote. Finally, two individuals at the peak from Livadia (646) and Probistip (864) between them have 20 *T. macedonicus* and ten *T. ivanbureschi* diagnostic alleles (a minimum of four of each), and P_{NH} values approaching unity. The distribution of genotypes for fully diagnostic loci is: seven *T. macedonicus* homozygotes, three *T. ivanbureschi* homozygotes and four heterozygotes.

In summary, two zones classify as unimodal (transects 4 and 9), two as intermediate (2, 11) and three as bimodal (3, 5, 6). The signal for hybridization is absent in three transects (1, 8, 12). A weak nuclear hybridity signal is observed in transects 7 and 10 where, however, mtDNA and morphology (transect 7) support the hypothesis of interspecific gene flow (see below). The zones studied in duplicate classify as intermediate and bimodal in *T. cristatus–T. dobrogicus* (2, 3), as providing weak and no signal in *T. dobrogicus–T. ivanbureschi* (7, 8) and as weak and intermediate in *T. ivanbureschi–T. macedonicus* (10, 11) (Table 3).

Hybrid Laboratory simulations

In Figure 3 we show the results of HybridLab simulations as a series of 12 graphs, plotting BAPS allocation (P_{DC}) against NH allocation (P_{NH}) as in Figure 2. Scatter is generally more attributable to NH classification rather than BAPS (although see Fig. 3F, transect 6). Lower scatter is seen in zones between parentals with higher Nei's *D* (e.g. Fig. 3D, E, G, H, K, L; cf. Table 2) and vice versa (e.g. Fig. 3C). For example, in transect 3 (Fig. 3C), only *Me* is completely diagnostic between anchor populations, so crosses involving hybrids and backcrosses generate many parental genotypes.

Many plots show asymmetry (Fig. 3A–D, F, G), particularly transects 1–3, reflecting differences between anchor populations in the number of diagnostic alleles. In transect 2 (Fig. 3B), for example, many backcrosses to *T. dobrogicus* (open red), and some F_2 (+), get classified with anchor *T. dobrogicus* (solid red symbols), but fewer backcrosses to *T. cristatus* (open green) get classified with anchor *T. cristatus* (solid green). This asymmetry in misclassification can be attributed to the higher gene diversity in *T. dobrogicus*. In addition to three completely diag-

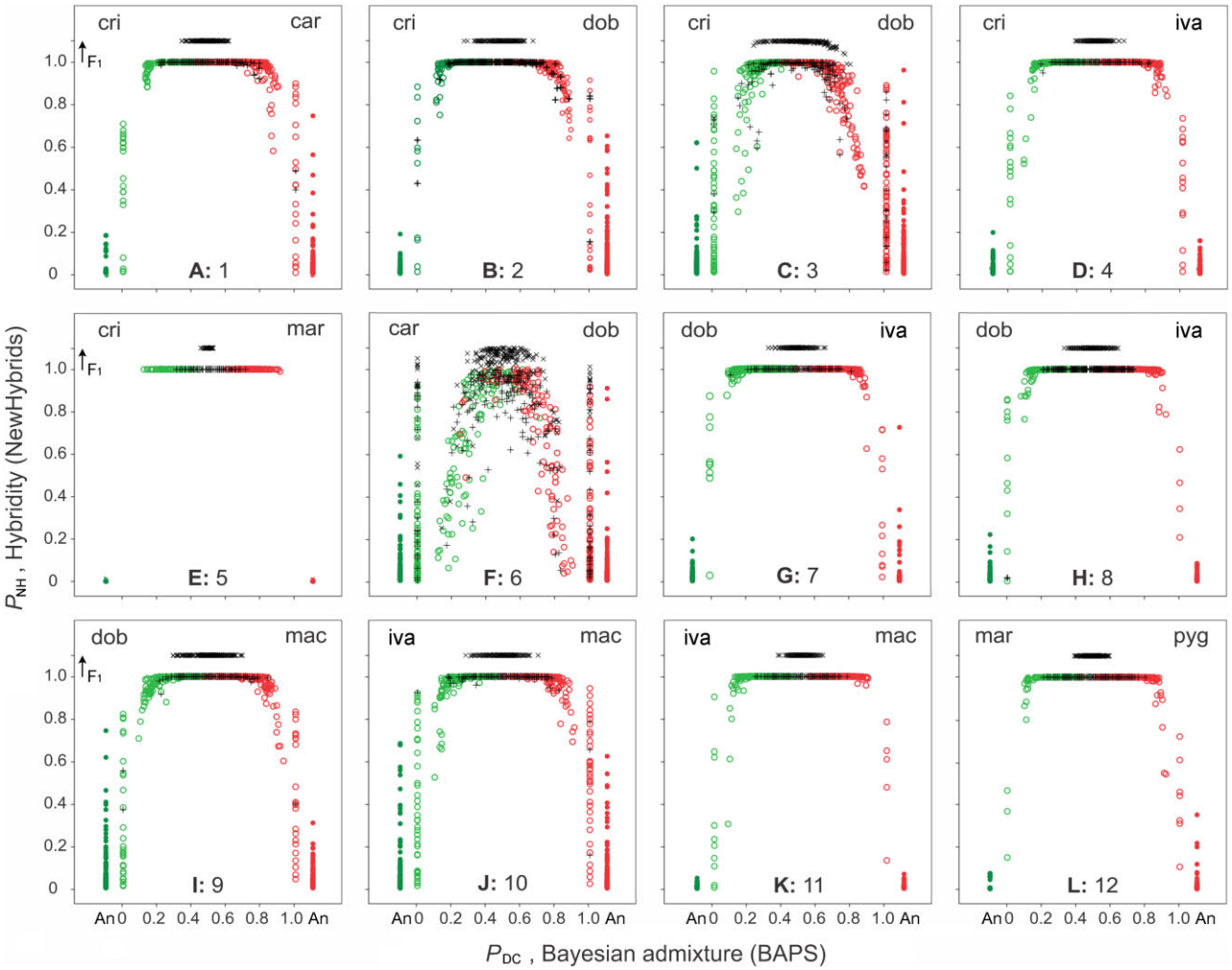

Figure 3. Bivariate plots of nuclear genotypes as modelled under the assumption of two generations of panmixia in 12 *Triturus* species pairs. Species codes are as in Figure 2. Data were generated from anchor populations (An) with HybridLab (see text for details) and analyzed as in Figure 2. Individuals from the parental populations ('A' and 'B') are shown by green and red solid symbols; backcrosses F_2A and F_2B are shown by open green and red symbols, respectively while F_1 and F_2 hybrids are shown by cross (×) and plus (+) symbols, respectively. For clarity of presentation, anchor populations are offset along the horizontal axis (An) on either side, as are F_1 populations along the vertical axis. For the anchor populations see Table 2, with Appendix S1 for locality information and Appendix S2 for genotypic data.

nostic loci, anchor *T. dobrogicus* has 12 alleles unique to that species (across ten polymorphic loci), in contrast with only two such alleles (at two polymorphic loci) in *T. cristatus*. Thus, although one-eighth of all backcross hybrids, in either direction, will be homozygous at all three completely diagnostic loci (i.e. they inherit three matching co-segregating anchor alleles from their F_1 parent), *T. cristatus* backcrosses are much more likely to possess other *dobrogicus*-specific alleles at the other (ten) loci, than *T. dobrogicus* backcrosses are to possess *cristatus* alleles from the two polymorphic loci with *T. cristatus*-specific alleles. Having a larger number of species-specific alleles (in addition to the strictly diagnostic loci) makes it harder to lose the signature of that genome (i.e. *T. dobrogicus*), so later hybrid generations retain evidence of *T. dobrogicus* parentage for longer. Indeed, many F_2 and *T. dobrogicus* backcrosses segregate out to be like anchor *T. dobrogicus*, because of a dearth of *T. cristatus*-specific alleles.

A more surprising result is the degree of scatter in NH classification of anchor populations themselves in most simulations (Fig. 3A–C, F–G, I–J). The cause of this pattern relates to the argument above, since the scatter of anchor populations shows strictly parallel asymmetry. So in transect 2 (Fig. 3B), for example, the scatter is much higher (0.0–0.69) for anchor

T. dobrogicus than for anchor *T. cristatus* (0.0–0.2). Once again, a dearth of polymorphic loci in *T. cristatus*, and therefore few *T. cristatus*-specific alleles outside of the three diagnostic loci, leads to high homogeneity within simulated anchor populations. In contrast, simulated *T. dobrogicus* anchors produce a vast array of genotypes, which in theory could possess anything from 0–20 *T. dobrogicus*-specific alleles at non-diagnostic polymorphic loci. This result highlights an important difference between the two analyses. Whereas BAPS is forced to consider anchor populations as two discrete parental classes (evidenced by three totally diagnostic loci), NH takes the other ten loci polymorphic into account, and considers the chance of hybridity higher as the number of *T. dobrogicus*-specific alleles at these ten loci reduces.

Another general observation can be made about the way the two analyses contrast in their results. With the possible exception of transects 3 (Fig. 3C, F; highest mixing) and 5 (Fig. 3E; lowest mixing), NH tends to scatter allocation of anchor and backcross types while maintaining tight allocation of F_1 and F_2, whereas BAPS tends to scatter allocation of F_1 and F_2 while maintaining relatively tight allocation of backcrosses. This makes sense, because BAPS is allocating genotypes with respect to one or the other anchor population (i.e. what is the probability of an individual being A or B), whereas NH is allocating genotypes with respect to four classes of hybrids. So NH shows little or no scatter for F_1 or F_2, because it has the option to categorize 50:50 genotypes as F_1 if they are heterozygous for all diagnostic loci, or F_2 if showing a mix of parental and heterozygous genotypes for these loci. BAPS simply scales genotypes based on the proportion of the genome that looks like species A/B, which has the effect of smearing out the F_1 slightly, and F_2 substantially, due to other partially diagnostic loci (F_1 and F_2) and segregation (F_2 only). For transect 5 (Fig. 3E), there is zero scatter in NH results because of the large number (16, of which only eight were studied in population 102) of completely diagnostic loci studied: presumably all 2 × 200 backcrosses maintain at least one diagnostic allele from each parent (5/20 expected) and there are no other diagnostic alleles at polymorphic loci to scatter anchors.

mtDNA sequences

The mtDNA dataset includes 87 *Triturus* haplotypes; 47 are new and the other 40 were previously identified (Wielstra *et al*., 2010; Wielstra & Arntzen, 2012). Sequences are available from the GenBank database (see Appendix S3 for accession numbers). Haplotypes cluster into eight (including *T. karelinii*) well supported genetic lineages, corresponding to species (Fig. 4). In general, mtDNA introgression is observed only at contact zone sites, but not all contact zone sites (transects 8 and 12) and not all individuals in contact zone sites contain introgressed mtDNA (Fig. 1; Table 2; Appendix S1). There are two exceptions: (1) eight out of 15 newts (53%) from the three *T. cristatus* anchor sites in the *T. cristatus–T. ivanbureschi* transect contain *T. ivanbureschi* mtDNA; and (2) all but one of the sampled *T. macedonicus* populations contain only *T. ivanbureschi* mtDNA (Fig. 1).

Morphology

NRBV data are given in Appendix S1. Non-modal values may represent intraspecific variation or result from hybridization. In *T. marmoratus–T. pygmaeus*, NRBV is not discriminatory and in *T. cristatus–T. marmoratus*, intermediate NRBV is well documented for their F_1 hybrids (Vallée, 1959). In the remaining ten transects, non-modal values were found in 36 individuals out of 286 (12.6%) in anchor populations and for 71 individuals out of 283 (25.1%) in central populations (*G*-test of independence, $G = 14.8$, $P < 0.001$). Similarly, a significant result was obtained in three individual transects ($P < 0.05$ in transects 4, 6 and 9) and the result was marginally significant in transects 7 ($0.05 < P < 0.10$). In BAPS versus NH plots, 65 individuals have $0.15 < P_{DC} < 0.85$ or $P_{NH} > 0.1$ (i.e. falling outside of the boxed areas illustrated in Fig. 2A). Non-modal NRBV scores are more frequently observed in transects (23.3%) than in anchor populations (15.9%). For transects this is significantly more than one would expect from the documented (cf. Arntzen, 2003) background variation at 12.7% (*G*-test for goodness of fit, $G = 34.5$, $P < 0.0001$). At anchor populations the number of non-modal NRBV scores is also higher than expected, but not significantly so ($G = 3.52$, $0.05 < P < 0.10$). The left and right pointing triangles in Figure 2, representing specimens with a non-modal NRBV, have on average significantly different P_{DC} and P_{NH} values, indicating an association between genetic composition and morphology (Student's *t*-test, P_{DC}: $t = 5.86$, $P < 0.0001$; P_{NH}: $t = 2.94$, $P < 0.01$).

Relationship between gene flow and hybridity with genetic distance

Of the 14 entries in Table 2 we consider the three spatial replicates to yield independent results, whereas the results for the two temporal replicates in transects 1 and 5 are averaged. With 12 observations, we find a significant correlation between the level of hybridity (NH) and Nei's *D* ($r_s = -0.62$, $P < 0.05$). Log-transformed data analysed for Pearson's correlation coefficient also provide a significant result ($r = -0.65$,

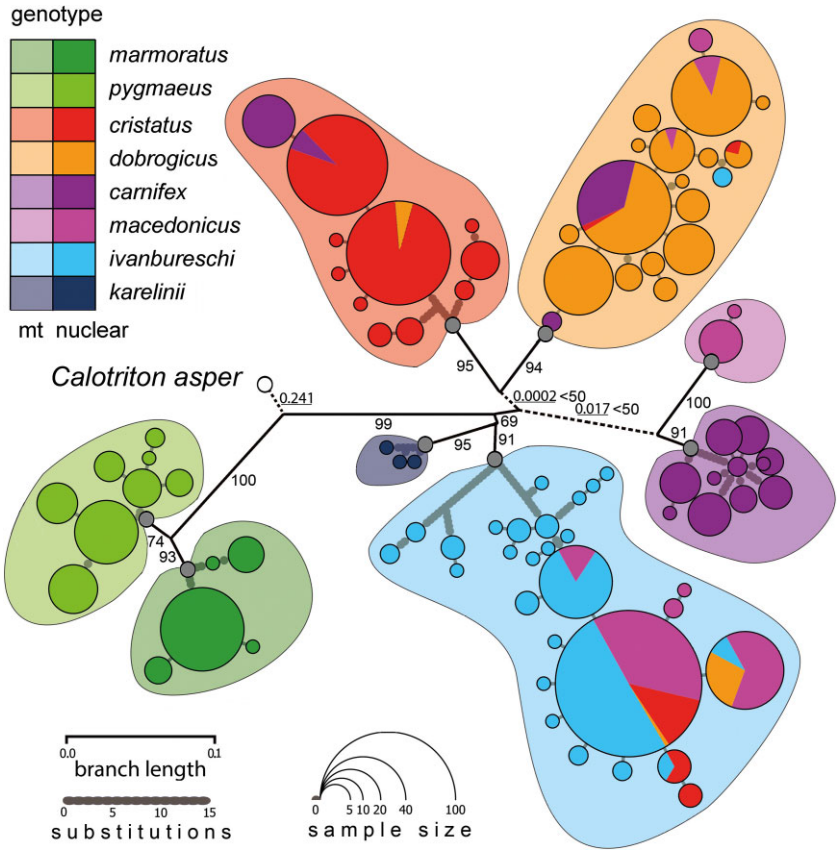

Figure 4. Network of 729 ND4 mtDNA haplotypes in the genus *Triturus* plus outgroup *Calotriton asper*. Colour codes are as in Figure 1, with light shades applied to the species-specific mtDNA haplotype groups and heavy shades applied to the inferred species' nuclear genetic background. Note that, e.g. *T. macedonicus* is heavily introgressed with *T. ivanbureschi* mtDNA. At the species level the tree topology is determined by maximum likelihood phylogenetic inference. Branches shown by interrupted lines are out of proportion and have actual lengths indicated (underlined, see legend for comparison). The support for monophyly of the haplotypes for the species is shown by percent bootstrap values. Note that the branching order is identical to that obtained with full mtDNA which tree has high statistical support, except for the branch leading to *T. cristatus* and *T. dobrogicus* (see Wielstra & Arntzen, 2011: Table 2). The within-species network configuration, external to the large grey dots, is determined with HaploViewer on the basis of the ML tree. Details on the distribution of haplotypes are presented in Appendix S1 and GenBank numbers are in Appendix S3.

$P < 0.05$; Fig. 5). Otherwise no significant correlations are found between genetic differentiation and either gene flow or hybridity ($r_s > -0.30$ and $P > 0.05$ in all cases). The four transects in which the morphological signal for hybridity is near-significant do not have significantly lower Nei's D than the other transects (Student's t-test, $t = 0.421$, $P > 0.05$).

DISCUSSION

Since early attempts to look at hybrid zones of *Triturus* newts using mtDNA and morphology (Wallis & Arntzen, 1989; Arntzen & Wallis, 1999), we have taken a fresh look incorporating nuclear-encoded allozymes. The motivation behind this is that allozymes are not subject to some of the limitations of mtDNA (Ballard & Whitlock, 2004) (single clonal haploid marker) and morphology (Hillis, 1987) (environmental effect, polytypism, convergence, dominance, limited phenotypes, number of loci unknown). Thus, allozymes are more suitable to allocate individuals to parental species or admixed classes, and so accurately map transition zones among the taxa.

UNIMODALITY AND BIMODALITY IN *TRITURUS* HYBRID ZONES, AND HETEROGENEOUS HYBRID ZONES

Our transect graphs show a dearth of hybrid classes for most species contacts. Where hybrid classes are present, they are rarer than parental types, and much rarer than in our simulations that mix parental genotypes over two generations. This deficit strongly

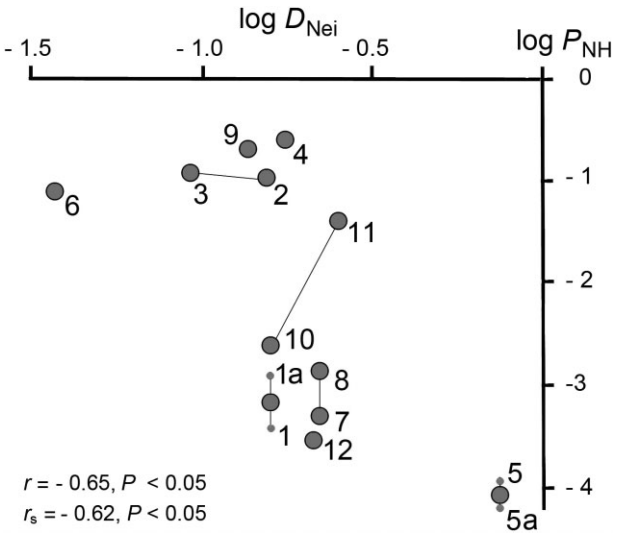

Figure 5. Bivariate plot of hybridity (log P_{NH}) versus allozyme genetic distance (log D_{Nei}) across European *Triturus* contact zones. Numbers refer to transects as in Table 2. Replicated species combinations are connected in which spatial replicates are considered independent while temporal replicates are averaged (transects 1 and 5). Pearson's and Spearman's correlation coefficients are represented by r and r_s, respectively.

suggests pre- and/or post-zygotic isolation, since migration among ponds is low and swamping by parentals from allotopic ponds is not plausible; typically 90–95% of *Triturus* newts derive from local recruits (Jehle *et al*., 2005). In keeping with this idea, each pond has its own characteristic allele frequencies. Another feature of our simulated hybridizations that differs from real life is the intensity of hybridization. That is, our observed hybrid plots are narrower (leptokurtic) than the simulated plots; the 'shoulders' are largely missing. Part of the explanation for this difference is the sheer number of offspring in the simulations, which tend to populate the graph more broadly. Additionally, we see a tendency for later hybrid generation classes to show heterozygote deficiency. That is, there is a tendency for them to be homozygous AA or BB for different loci, representing alternative parental genotypes. This correlation of alleles within individuals is best explained by genetic structure among populations. That is, drift in hybrid populations tends to drive parental frequencies away from a 50:50 mix (Excoffier & Ray, 2008) [possibly helped by selection for alien alleles or hybrizymes, (Woodruff, 1989; Arntzen, 2001)], tending to produce new combinations of homozygous genotypes. Heterozygote disadvantage, epistatic selection (Bateson-Dobzhansky-Muller (BDM) effects) and null alleles could be added factors. A caveat here is our sampling intensity: although our sampling was at least two-phased (i.e. involved return to each location after initial position had been located), it is possible that more intensive sampling at a scale of, say, 100s or 1000s of metres, may have found hybrids for zones in which they appear to be absent, such as at transects 8 and 12.

For transect 10, all Geneva genotypes are *T. carnifex* with no sign of *T. cristatus* whatsoever, but denser sampling here and elsewhere (with fewer loci) has revealed hybridization (Arntzen & Thorpe, 1999; Maletzky *et al*., 2008). Similarly, though transect 5 shows no introgression in *T. cristatus* or *T. marmoratus* in these particular samples from the Mayenne region in western France, we have detected very low levels with fewer loci but much larger sample sizes in the same location (Arntzen & Wallis, 1991; see Table 2). Although transect 12 shows no introgression between *T. marmoratus* and *T. pygmaeus* near Madrid, elsewhere, we have detected low levels (Espregueira Themudo & Arntzen, 2007a). We here sampled the eastern part of the zone where population numbers are dwindling (García-París *et al*., 1989) and locally the contact might be characterized as residual [*sensu* Szymura (1993)].

The sharp contact zones revealed by the allozyme data reassert that the *Triturus* newts manage to retain species identity, despite occasional interspecific mating. We show that hybrid zone shape varies widely, even among different regions in the same contact zone [coined heterogeneous hybrid zones by Espregueira Themudo *et al*. (2012)]. Although comparisons involving *T. dobrogicus*, *T. macedonicus* and *T. pygmaeus* are unknown, the accumulating examples of post-zygotic isolation (White, 1946; Spurway & Callan, 1950; Callan & Spurway, 1951; Arntzen *et al*., 2009) (see Table 1) and the evenness of effect across the other species (Callan & Spurway, 1951) make it likely that all combinations experience hybrid breakdown. We imagine that much of the observed pattern is governed by reduced fitness of hybrids, but pre-zygotic isolation probably plays a role too. Not much is known yet about pre-zygotic reproductive isolating mechanisms in *Triturus* newts in the field, other than *T. cristatus* and *T. marmoratus* (Zuiderwijk & Sparreboom, 1986; Zuiderwijk, 1990). Experimental studies and observations from breeders are reviewed elsewhere (Macgregor, Sessions & Arntzen, 1990).

mtDNA introgression and morphological signatures of hybridization

The allozyme data provide a reliable background against which to interpret the mtDNA and morphological data. *Triturus* newts have deeply differentiated mitochondrial genomes [Table 1; Wallis &

Arntzen (1989); Wielstra (2011)]. However, we observe mismatches between mtDNA type and species identity at most contact zones (Fig. 1). mtDNA introgression might simply reflect neutral diffusion, though introgression involves a particularly extensive area for *T. macedonicus–T. ivanbureschi*. The likely explanation for this pattern of asymmetrical mtDNA introgression is displacement of *T. ivanbureschi* by *T. macedonicus* accompanied by low-frequency hybridization (Currat *et al*., 2008; Wielstra & Arntzen, 2012). Consequently, attempting to assign species identity on the basis of mtDNA alone is unreliable at *Triturus* contact zones over a broad swathe of southern Europe, from the Iberian Peninsula (Espregueira Themudo *et al*., 2012), via the Alps (Maletzky *et al*., 2008), through the Balkans (Wallis & Arntzen, 1989; Wielstra & Arntzen, 2012; Wielstra *et al*., 2013b) into Asia minor (Wielstra, Baird & Arntzen, 2013a).

The mode of inheritance of morphological characters is complex and far from understood in general, let alone in *Triturus* (Ivanović & Arntzen, 2014). Furthermore, variation could reflect both hybridization and intraspecific variance (due to natural polymorphism and environmental influences). Placing the NRBV in an allozyme framework reveals that hybridization accounts for most of the intraspecific variation: most deviation from the norm occurs at contact zones, and is often intermediate between the species in contact. Such individuals are more often picked out by BAPS than by NH as genetically admixed. Significantly more newts at the centre of contact zones show a deviation from modal NRBV than in anchor populations. Furthermore, a few individuals in populations distantly located from the contact zone also show aberrant NRBV. These data underline that although gene flow accounts for intraspecific NRBV variation, there is also some natural variation in NRBV within *Triturus* species.

Hybridity and genetic distance

We find no statistically significant relationship between mtDNA genetic distances across contact zones and the degree of interbreeding of the species pairs, either expressed in the (nuclear) gene flow measure (BAPS software), or as the extent of hybridization (NewHybrids software). For nuclear genetic distance, as measured by Nei's *D*, the relationship is significant for hybridity alone. The signal diminishes if F_1 hybrids in transect 5 are included in the analyses, but these are rare (ca 4%), and the hybrid breakdown that they exhibit (Arntzen *et al*., 2009) is itself evidence for BDM effects interrupting the regular decline of hybridization potential. These results, overall, are consistent with a higher number of BDM effects (or possibly more aberrant gene expression in hybrids) between pairs of species with greater genetic differentiation (Coyne & Orr, 2004), as is being found for viability of hybrids in many vertebrate species (Sasa, Chippindale & Johnson, 1998; Arrieta, Lijtmaer & Tubaro, 2013). This weak relationship could be disrupted by reproductive character displacement through reinforcement, or other forms of selection, where ecological and behavioural factors add to or override isolation resulting from genetic divergence alone. The weak correlation between the ability to hybridize and phylogenetic distinctiveness is in line with the general conclusions of Jiggins & Mallet (2000) from a survey over a wide variety of organisms. For *Triturus* newts, a weak correlation was to be expected, as the group radiated in a narrow time interval of *c.* 8.7–10.4 Mya (three crested newt lineages) and at *c.* 5.3 Mya (*T. carnifex* – *T. macedonicus* and marbled newts) (Arntzen, Espregueira Themudo & Wielstra, 2007; Espregueira Themudo, Wielstra & Arntzen, 2009; Wielstra & Arntzen, 2011). Recent multi-marker (Macholán *et al*., 2011; Larson *et al*., 2014) and genomic studies (Feder *et al*., 2013; Larson *et al*., 2014; Seehausen *et al*., 2014) are showing highly heterogeneous degrees of introgression among markers. One might expect that neutral diffusion, positive selection and hitch-hiking (exacerbated by chromosomal rearrangements) of genes in the blocks marked by our isozymes could overwhelm detection of BDMs through the correlations we make, but we have nonetheless demonstrated one relationship of significance. Our case is possibly helped by the isolated nature of habitat (ponds), which reduces gene flow and permits genetic differentiation over more loci to accrue (Feder *et al*., 2013), and perhaps also by strong selection (Larson *et al*., 2014).

Conclusion and scope for further research

The modality of hybrid zones reflects the evolutionary independence of the taxa involved. The system here described is excellent for researching reproductive character displacement, using multiple contact zones, involving similarly distinct newt species, showing a degree of post-zygotic isolation. Future research could focus on analysing pre-zygotic isolation and its reinforcement under natural conditions. Additionally, ecological parameters, and differential adaptation to those parameters, may play an important role in constraining hybrid zones. We expect this might be the case in particular for *T. dobrogicus* versus the other species, restricted as it is to lowland river valleys (Arntzen *et al*., 1997; Arntzen, 2003).

Denser sampling is now required in each contact zone. Allozymes are potentially subject to selection, and although this possibility is interesting in itself,

further work should employ multiple neutral nuclear markers (Wielstra *et al*., 2014) as a background for looking at selective movement of alleles. Microsatellite markers are more problematic to work with (homoplasy, large numbers of alleles, excessive population structure in low gene flow species), but can in principle be employed at a local level. However, for old, well-separated species such as *Triturus*, we expect that microsatellites are less likely to be useful (Mikulícek, Crnobrnja-Isailović & Piálek, 2007). Future analyses of the recently discovered contact between *T. cristatus* and *T. macedonicus* is heightened by *T. macedonicus* having a less definable morphology, overlapping considerably with *T. cristatus* in colour and patterning (Freytag, 1988). Nuclear genetic data are therefore especially required for this contact.

The types of genetic analyses outlined above will provide sharper evidence for differences in ability to hybridize (e.g. cline width and shape). Additionally, phylogenomics can provide a better estimate of branching pattern. Combining these two approaches with analysis of fitness of hybrids in the wild, as we have started for *T. cristatus* × *T. marmoratus* (Arntzen *et al*., 2009), should make for clearer resolution of this issue. Alternatively, it may be that ecological parameters, and differential adaptation to those parameters, play a dominant role in constraining hybrid zones.

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Data accessibility

The data on which this paper is based are presented in the Electronic Appendices S1–3: Appendix S1 – sampled individuals with locality information, Appendix S2 – allozyme data in individual genotype format and Appendix S3 – GenBank accession numbers.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Sampled individuals with locality information (population numbers matching with Fig. 1), mtDNA haplotype, number of rib-bearing vertebrae and BAPS groupings.
Appendix S2. Allozyme data in individual genotype format.
Appendix S3. GenBank accession numbers for the ND4 mtDNA haplotypes.
Appendix S4. Individuals identified in the transect analyses as genetically admixed by BAPS ($0.15 < P_{DC} < 0.85$) and/or New Hybrids ($P_{NH} > 0.10$).

