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Hybridization and introgression in deeply differentiated salamander species – molecular genetics and a reappraisal of Dr. Louis Vallée's osteological data

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Abstract

Two morphologically and genetically distinct salamander species (the crested newt, *Triturus cristatus* and the marbled newt, *T. marmoratus*) engage over a large area in the west of France where they hybridize at ca. 4%. The species interaction is characterized by ecological differentiation and limited gene flow beyond the F_1 hybrid generation. Incompletely isolated species like these allow to investigate the genetic mechanisms and evolutionary forces that maintain their identity in the face of ongoing gene flow, to which a large mosaic hybrid zone provides excellent opportunities. A reanalysis of published morphological data supports the partial breakdown of the species barrier whereas extensive genetic data show that introgression is low yet asymmetric, in line with the dynamics of species replacement inferred earlier. The current work, with seven diagnostic nuclear markers studied for a large sample, revises the estimates of introgression in both *T. cristatus* (to 0.24%) and *T. marmoratus* (to 0.11%). Difficulties remain in the recognition of potential triploid hybrids versus backcross hybrids. Haldane's rule is partially supported, but deeper analyses require the use of a molecular marker for sex that is not yet available.

Keywords

asymmetric species interactions – genetic footprints – Haldane's rule – morphological anomalies – morphometrics – osteology – single nucleotide polymorphisms – *Triturus* newts

Introduction

Speciation is the process by which groups of populations evolve to become distinct species. Tempo and modo may be fast or even instantaneous (Soltis et al., 2003; Lamichhaney et al., 2018; Vos et al., 2020), but more often acquiring reproductive isolation takes a long time over which barriers to gene exchange accumulate, to the point that the merger of differentiated gene pools is no longer feasible (Kulmini et al., 2020). Diverging populations will initially not be able to stably coexist, because they interbreed too much, or due to ecological competition for resources (Weber & Strauss, 2016; Irwin & Schluter, 2022). However, at some advanced stage in the speciation process habitat differentiation will contribute more to divergence than genomic incompatibility (Jiggins & Mallet, 2000; Germain et al., 2021) and a parapatric contact zone may by then 'collapse into broad sympatry' (Barton & Hewitt, 1985: 121).

Naturally, most speciation research is carried out on taxon pairs although, to increase power and depth, research programmes would ideally involve a hierarchy of life forms (Coyne & Orr, 1989; Rabosky & Matute, 2013). One group showing great research potential under natural conditions is the Eurasian genus of aquatic salamanders (newts) *Triturus*. The ten species and several subspecies it harbours engage in a fortuitous geographical arrangement

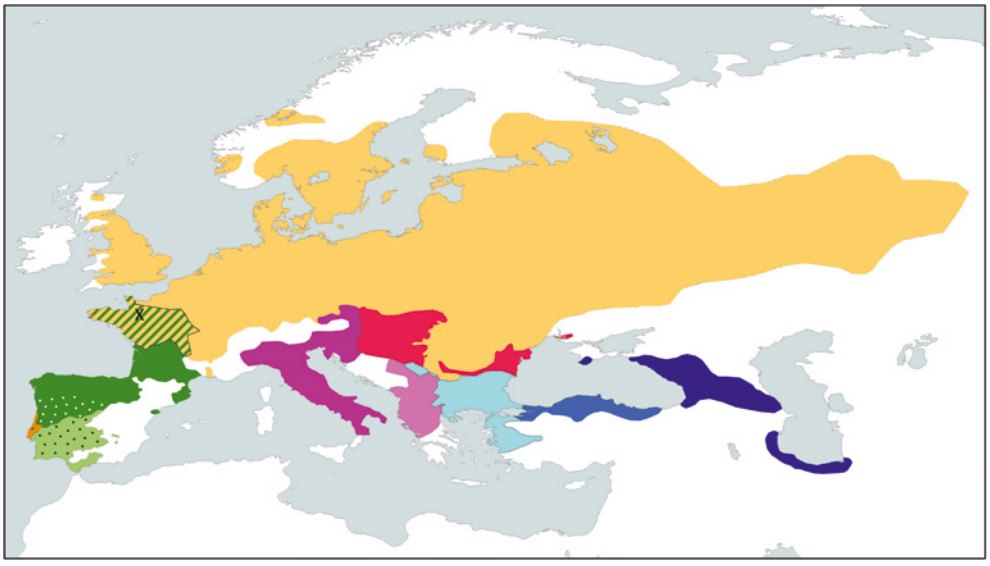

FIGURE 1 Distribution of ten species in the genus *Triturus* (after Wielstra et al., 2014 and Arntzen, 2023b, 2024a,b,c). Colour codes from west to east are: brown – *T. rudolfi* Arntzen, 2024, light green – *T. pygmaeus* (Wolterstorff, 1905), dark green – *T. marmoratus* (Latreille, 1800), ochre – *T. cristatus* (Laurenti, 1768), purple – *T. carnifex* (Laurenti, 1768), red – *T. dobrogicus* (Kiritzescu, 1903), pink – *T. macedonicus* (Karaman, 1922), light blue – *T. ivanbureschi* Arntzen & Wielstra, 2013, medium blue – *T. anatolicus* Wielstra & Arntzen, 2016 and dark blue – *T. karelinii* (Strauch, 1870). Stippled areas are the approximate ranges of the subspecies *T. m. harmannis* Arntzen, 2024 (light stipples) and *T. p. lusitanicus* Arntzen, 2023 (dark stipples). The study area of dept. Mayenne is shown by a cross.

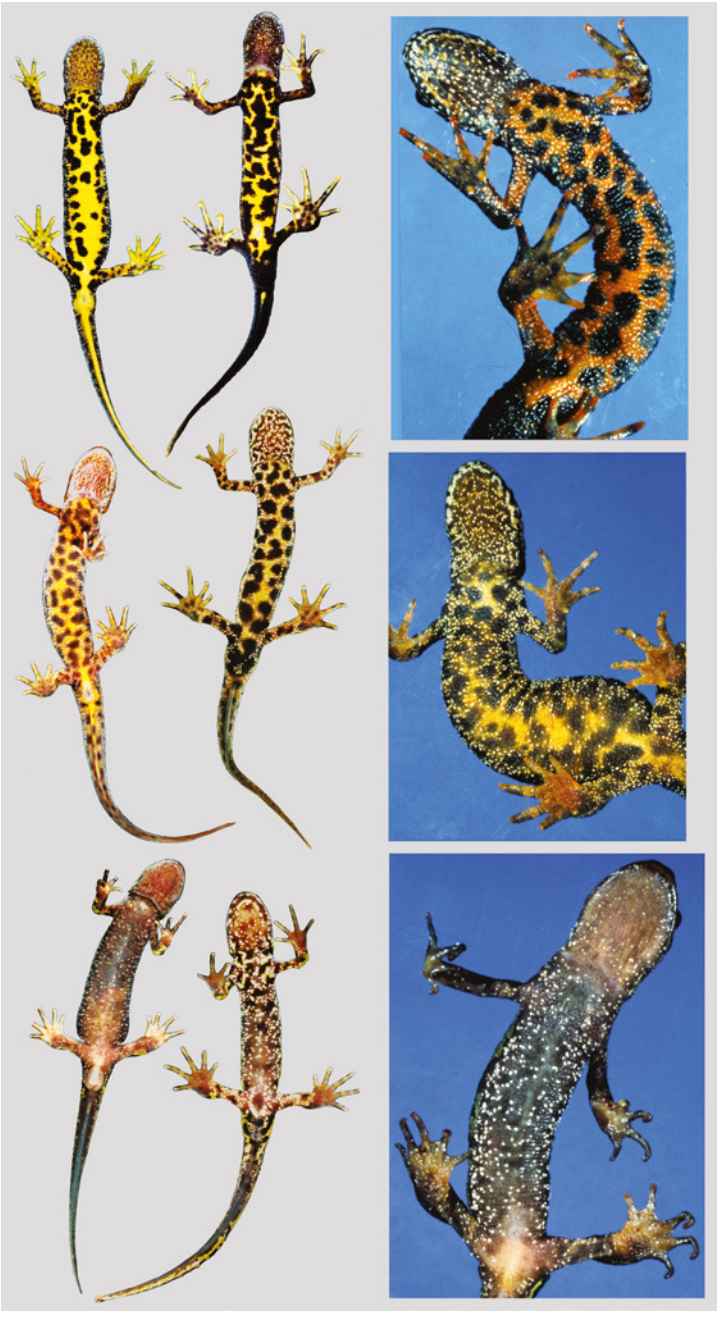

FIGURE 2 Ventral sides of *Triturus cristatus* (top), F_1 hybrid (middle) and *T. marmoratus* (bottom) from dept. Mayenne, France. Left column are females and right column are males. Inserts at a blue background: close-up of the snout-vent region of three different individuals, from top to bottom: *Triturus cristatus* male, F_1 hybrid female and *T. marmoratus* female.
PHOTOGRAPHY BY L.A. VAN DER LAAN

that provides a multitude of contact zones for taxa at varying stages of genetic incompatibility (Arntzen et al., 2014). Species ranges and full names are shown in fig. 1. The groups' phylogeny has been resolved (Wielstra et al., 2019) and the fossil record places the most recent common ancestor of *Triturus* species at ca. 18 Ma or more (Estes & Hoffstetter, 1976; Estes, 1981; Marjanović & Laurin, 2014, see also Steinfartz et al., 2007). Some of the contact zones are short, but many are long or very long, which offers the research prospect of spatial replication. The oldest representatives of the genus that are in contact are the crested newt, *T. cristatus* and the marbled newt, *T. marmoratus* that engage in a wide area of range overlap in the west of France (Arntzen, 2023a,b). The substantial but incomplete reproductive isolation among them is reflected in a trimodal frequency distribution of forms that include F_1 hybrids (fig. 2). The observed high levels of genetic and ecological differentiation place this species pair firmly at the far end of the speciation continuum (Arntzen et al., 2021). Unlike the other species pairs in the genus that show more or less narrow contact zones, it appears that *T. cristatus* and *T. marmoratus* have indeed 'collapsed into broad sympatry' and therewith represent a research field at the very interface (or 'nexus') of ecology and evolution (Weber & Strauss, 2016).

I here address the question to what extent *T. cristatus* and *T. marmoratus* are reproductively isolated. In principle this is not a difficult issue to address because the species are morphologically different and diagnostic genetic markers are numerous, as shown by early work with allozymes and mitochondrial (mt) DNA fragment analysis (Wallis & Arntzen, 1989; Arntzen & Wallis, 1991). However, a drawback at this level of differentiation is that gene flow may be low, so that testing for introgression requires large samples, as achieved in the present study (N > 6,000). Other issues addressed are the level of support for asymmetries in the strength of reproductive isolation depending on the direction of the cross (known as 'Haldane's rule') and the question if intermediate phenotypes could not actually be triploid F_1 hybrids. I also present a reanalysis of a data set on the osteology of species and hybrids that sheds light on the matter from a morphological perspective.

Materials and methods

The area of research is the département (dept.) Mayenne in the west of France, where *T. cristatus* and *T. marmoratus* both occur. Morphological differentiation of both species and their hybrids has been studied by Dr. Louis Vallée (1908–2001) on local populations (three groups, N = 216) and on material from northern (*T. cristatus*, N = 76) and southern (*T. marmoratus*, N = 49) reference populations (Vallée, 1959). Characters studied were the following osteological features: length of the humerus (1) and the femur (2), total skull length (3), skull length at the level of the frontal process of the maxilla (4), the trapezoid shape of the ceratobrachial as measured by its relative width at the proximal and distal ends (5), the depth of the indentation at the ceratohyal (6), the number of teeth at the upper (7) and lower jaw (8),

and the number of vertebrae (9) (Vallée, 1959). The counts of rib-bearing vertebra (NRBV) here presented exclude the cervical vertebra (atlas) and the sacrum (Lanza et al., 2010; Arntzen et al., 2015). Six missing data points (0.20%) were filled in with the modal value for the group. The original data are here made available as scans from Dr. Louis Vallée's thesis and as Excel files (supplementary fig. S1 and supplementary table S1). Univariate statistical analyses were carried out for both sexes separately on untransformed data by non-parametric methods. In particular, the prediction was tested that character states for sympatric populations are different from allopatric populations of the same species, in direction of the counterpart species. Sexual dimorphism in *T. cristatus*, *T. marmoratus* and hybrids was studied in a similar way. A multivariate analysis by principal component analysis (PCA) was carried out on normalized and (if so preferred) log-transformed data. Statistical analyses were done with SPSS 26 (IBM SPSS, 2019). Chi^2-tests are tests for independence, unless indicated otherwise.

Tissue samples for molecular genetic analyses were collected starting in 1979 for a variety of projects, with a large total sample size over 99 *Triturus* populations from dept. Mayenne and consisted of larval or adult tail tips stored on 95% ethanol. Alternatively, recently deposited eggs (embryos) were raised in 5l buckets until hatching and harvested for future DNA analysis. Occasionally samples were a toe, tail tip or a larval gill from ethanol preserved material. Individuals studied for seven nuclear loci with no missing data were 4,277 adults, 222 hatchlings and 742 larvae, and 4,990 (95%) of these were analysed for mtDNA also.

Genotyping was conducted on stored DNA extracts at the single nucleotide polymorphism (SNP) genotyping facility of the Institute of Biology, Leiden University, using the Kompetitive Allele-Specific PCR (KASP) genotyping system (LGC Genomics). The KASP technology encompasses fluorescence-based genotyping (Semagn et al., 2014). The SNP variant present in each individual (both variants in the case of a heterozygote) was determined in uniplex assays, based on two allele-specific primers with a final base complementary to one of the two potential SNP variants that also possess a unique tail sequence. Different fluorescently labelled primers present in the KASP master mix correspond to each tail sequence and are activated when incorporated during subsequent PCR cycles, with further cycling causing signal intensity to increase. Species diagnostic loci were available from a panel of markers used earlier (Arntzen et al., 2021). Of the eight loci studied, seven were nuclear markers (abl, dlgap, edc4, eif4ebp2, ngef, phf and slc5) and one was mitochondrial (ND4). Considering the vast genome of *Triturus* newts (Gregory, 2005), and because the loci were chosen at random, the markers were assumed to be unlinked.

Data points 'uncalled' by the KASP software mostly reflect a poor signal due to poor quality DNA, but may also represent a strong but difficult-to-classify signal, on account of a position in between clouds of homozygous and heterozygous genotypes (fig. 3). Although such ambiguous signals may be genuine outliers in the

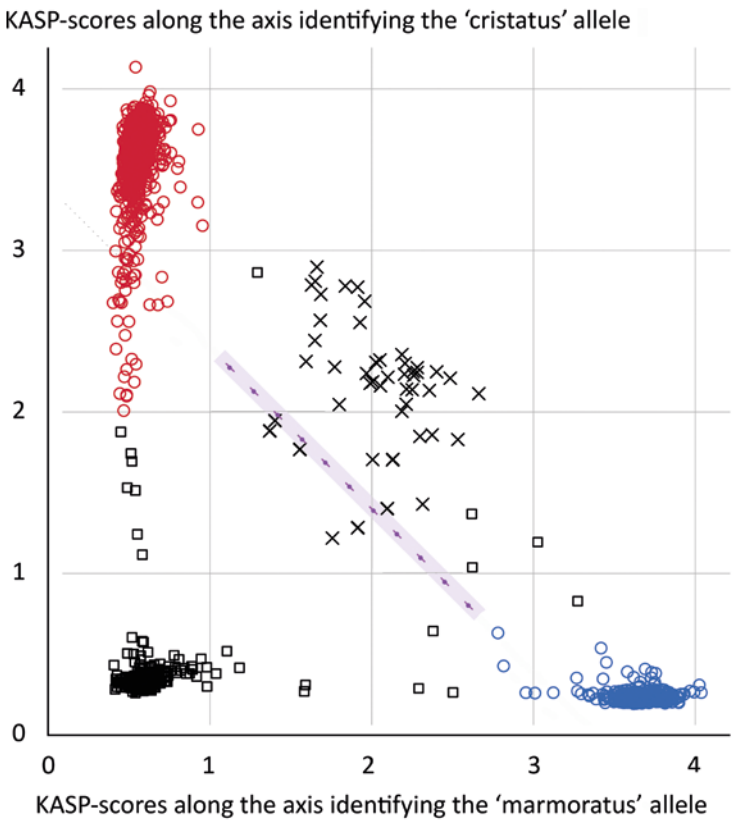

FIGURE 3 Bivariate plot of KASP-scores for the locus *abl*, the first out of seven markers, in the first run (N = 1,535). Individuals top left are classified as homozygous for abl^C allele that is typical for *Triturus cristatus* (CC, open round symbols in red), individuals bottom right are homozygous for the abl^M allele that is typical for *T. marmoratus* (MM, open round symbols in blue) and those in the centre are considered heterozygous (CM, crossed symbols). Open square symbols denote individuals for which no genotype call was made, either on account of a strong but intermediate signal (five data points in between the CC, CM and MM clusters) or low signal strength. It is here considered that the former group might represent F_1 hybrids with a duplicated *abl* gene (one CCM, four CMM), as might arise from e.g., trisomy or triploidy. The interrupted line indicates a signal strength of 3.4.

clouds of points for the homozygous or the heterozygous condition, they could also represent a triplicated chromosome or chromosome region, as in trisomic or triploid individuals. For data analysis, I followed two complementary approaches, that are based on (i) the genotype calls as provided by the KASP software and (ii) the raw (not interpreted) KASP-scores.

In the first approach individuals were considered for which all seven nuclear markers were called (N = 5,241 out of

6,295, 83.3%). Per individual a species index (SI) was calculated as the number of *T. marmoratus*-alleles observed over seven loci. Accordingly, SI ranges from zero for pure *T. cristatus* to 14 for pure *T. marmoratus* while F_1 hybrids would be expected to have SI = 7. In the second approach, all KASP-scores (and not the called genotypes) were analysed by metric multidimensional scaling (mMDS) with Primer v7 software following the manual (Clarke & Gorley, 2015). A 'resemblance matrix' of Euclidian distances was constructed from normalized data and the program was run with 500 random starts and a minimum stress value of 0.001. To allow comparison of results across runs, the scores over the first axis (Dim1) were linearly scaled, with the average value for phenotypically identified *T. cristatus* and *T. marmoratus* set at zero and unity, respectively. The results over the second axis (Dim2) were not straightforward to interpret, yet strongly correlated with signal strength (supplementary fig. S2). Decisions to *a posteriori* exclude individuals from the analysis were therefore not determined from Dim2, but based upon signal strength averaged over the seven nuclear markers. The threshold value chosen was a Manhattan distance of 3.4, with N = 5,557 (88.3%) above and N = 738 (11.7%) below the threshold. I also carried out a simple simulation with HybridLab software (Nielsen et al., 2006) to gain an impression of where diploid (CM) and triploid hybrids (CCM and CMM) might be expected to show up along Dim1, based on gene dosages and on the assumption that the results along the first MDS axis scale linearly. Thereto the data for the first axis obtained for reference *T. cristatus* (C) and *T. marmoratus* (M) were randomly combined as CC, CCM, CM, CMM and MM. Normal distributions were fitted to 1,000 combinations per class, for comparison with the profiles observed from the original data.

Results

The analysis of Vallée's morphometric data with PCA yielded a wide scatter of data points. Of the total variance in the data, 66.9% is explained along the first axis and 11.8% along the second axis (fig. 4). Species morphological differentiation is most apparent along the first axis, with hybrid individuals taking the anticipated intermediate position. Loadings (L) on the first axis were high for all nine characters (|L| > 0.73). Character state values were lower in *T. cristatus* than in *T. marmoratus*, except for NRBV. Populations of both parental species from dept. Mayenne are somewhat more similar to one another (and to the hybrid group) than are the northern and southern reference populations. The second PC-axis shows almost no species differentiation, but slightly different values for the hybrids than for the parental species, possibly reflecting transgression for characters 1, 2, 5 and 6 that have relatively high loadings (|L| > 0.43). Sexual dimorphism is near-absent in *T. cristatus*, moderate in hybrids and pronounced in *T. marmoratus*. In univariate analyses, the hypothesized morphological shift for sympatric parental populations in direction of the counterpart species was observed for single characters for a total of 31 out of 36 comparisons (table 1). When restricted to characters for which the morphological difference is nominally significant, the

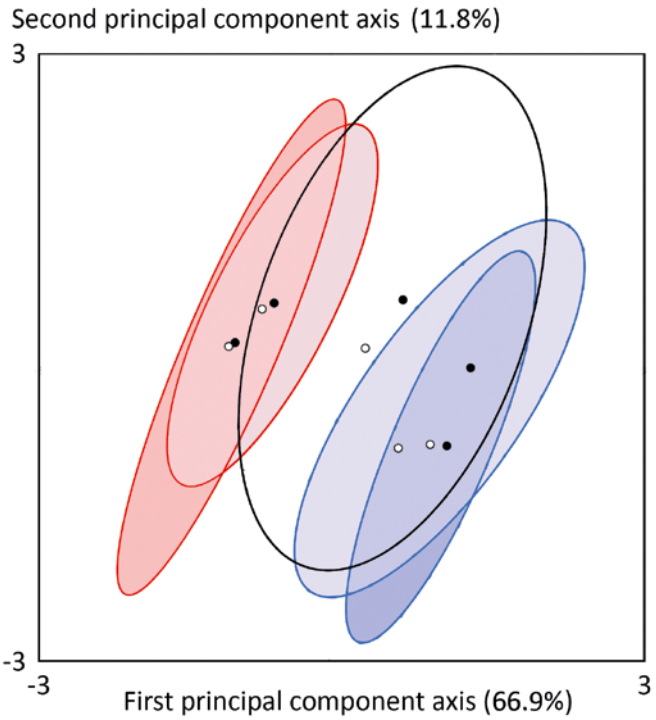

FIGURE 4 Results of a principal component analysis of morphometric data on *Triturus cristatus* (in red), *T. marmoratus* (in blue) and interspecific hybrids (in white), with allopatric and sympatric parental populations shown by bright and shaded colours, respectively. Ellipses represent the 95% confidence interval of the mean. The proportion of the total variance explained is shown along either axis (%). Averages for males and females are separated out and shown by solid versus open round symbols. Note that sexual dimorphism is pronounced in *T. marmoratus* and the hybrids and not in *T. cristatus*. The constituent data are from Vallée (1959).

numbers at that arbitrary threshold are 9/9 for *T. cristatus* and 5/5 for *T. marmoratus*. Under a Bonferroni adjustment that accounts for the testing of multiple cases, the remaining score of 6/6 is still statistically significant (binomial test, $P < 0.05$). A substantial sexual dimorphism in the studied osteological characters is found for *T. cristatus* in one character, for three characters in hybrids and for five out of nine characters in *T. marmoratus* (table 2).

Among 4,277 individuals with no missing KASP calls and phenotype known (i.e., adults), 2,653 were *T. cristatus* (62.0%), 1,516 were *T. marmoratus* (35.4%) and 108 were hybrids (2.5%). As expected from the use of diagnostic markers, these groups show a distinctly trimodal species

TABLE 1 Univariate analysis of morphometric data for *Triturus cristatus* and *T. marmoratus* from the area of sympatry (dept. Mayenne, western France) versus northern and southern allopatric reference populations. Sample sizes are in parentheses. Data are from Vallée (1959). The prediction tested is that character states are more similar to the counterpart species in sympatry than in allopatry. Two-sided statistical testing is by the Kruskal-Wallis procedure for characters 1–8, and for character number 9 by a chi-square test for independence under one degree of freedom.

Character	Measurement	*T. cristatus*				*T. marmoratus*			
		Males (41, 34)		Females (46, 42)		Males (30, 20)		Females (34, 29)	
		Significance	Prediction supported	Significance	Prediction supported	Significance	Prediction supported	Significance	Prediction supported
1 – Humerus	Length	*	Yes	**	Yes	NS	Yes	NS	Yes
2 – Femur	Length	*	Yes	NS	Yes	NS	Yes	NS	Yes
3 – Skull 1	Length (log)	***	Yes	***	Yes	NS	Yes	*	Yes
4 – Skull 2	Length (log)	NS	No	NS	Yes	*	Yes	NS	Yes
5 – Trapezoid	Shape	NS	Yes	NS	Yes	NS	Yes	NS	Yes
6 – Ceratohyal indentation	Depth	NS	No	NS	Yes	NS	No	NS	No
7 – Teeth upper jaw	Number	***	Yes	***	Yes	NS	Yes	*	Yes
8 – Teeth lower jaw	Number	***	Yes	***	Yes	*	Yes	NS	Yes
9 – Rib-bearing vertebrae	Number	NS	No	NS	No	NS	No	*	Yes

Significance levels are: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and NS $P > 0.05$.

TABLE 2 Sexual dimorphism in nine osteological characters in *Triturus cristatus*, *T. marmoratus* and interspecific hybrids from France in data from Vallée (1959), with sample sizes in parentheses. The values shown are averages. Statistical testing is by the Kruskal-Wallis procedure for characters 1–8, and for character number 9 by a chi-square test for independence under one degree of freedom.

Character	*T. cristatus*			Hybrids			*T. marmoratus*		
	Males (75)	Females (88)	Significance	Males (22)	Females (43)	Significance	Males (50)	Females (63)	Significance
1 – Humerus length	8.14	8.19	NS	9.33	9.52	NS	8.98	9.77	***
2 – Femur length	7.37	7.36	NS	8.41	8.91	NS	8.43	9.10	***
3 – Skull length 1 (log)	1.080	1.090	NS	1.117	1.150	*	1.128	1.154	**
4 – Skull length 2 (log)	0.595	0.611	*	0.699	0.730	*	0.719	0.755	***
5 – Trapezoid shape	1.981	1.968	NS	2.536	2.428	NS	2.802	2.871	NS
6 – Ceratohyal indentation depth	0.076	0.124	NS	0.767	0.537	NS	1.074	1.114	NS
7 – Teeth number upper jaw	19.95	20.25	NS	24.05	28.63	**	27.06	30.06	***
8 – Teeth number lower jaw	34.07	34.01	NS	43.91	46.40	NS	45.30	46.67	NS
9 – Number of rib-bearing vertebrae	15.04	15.02	NS	13.59	13.81	NS	12.22	12.19	NS

Significance levels are: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and NS $P > 0.05$.

index distribution, with SI ≤ 3 for *T. cristatus*, SI ≥ 11 for *T. marmoratus* and intermediate values (4 < SI < 10) for hybrids (table 3). Hatchlings and larvae were classified accordingly and the results suggest a discrepancy among frequencies of the three life stages (Chi² test, df = 4, chi = 54.63, P < 0.0001), with more hybrids among larvae (5.0%) than in adults (2.5%) and hatchlings (2.3%). Frequencies of alleles typical for the counterpart species ('alien alleles') were not dissimilar for the three life stages (Chi² test, df = 2; *T. cristatus* – chi = 4.77, P > 0.05; *T. marmoratus* – chi = 2.61, P > 0.05). Although overall levels are low, *T. cristatus* appears significantly more introgressed than *T. marmoratus*, at the level of the gene (0.24% versus 0.11%; Chi² test, df = 1, chi = 16.75, P <0 .0001), as well as at the level of the individual (2.0% versus 0.9%; chi = 9.90, P < 0.01). Frequencies of alien alleles across the seven marker loci are not dissimilar among species (Chi² test, df = 6, chi = 5.51, P > 0.05).

Metric multidimensional scaling of KASP-values (rather than called genotypes) yielded substantial separation of individuals with positions over the first dimension related to the species index and on the second axis related to signal strength (supplementary fig. S2). Stress values ranged from 0.05–0.06. For all individuals with an average signal strength >3.4 the distribution along the first dimension is trimodal, with few individuals positioned in between the peaks (fig. 5). A simulation based on the KASP-scores obtained for both parental species suggest that backcrosses and triploids would fall in between the three peaks, but also that the various groups may be difficult to disentangle and in practice no clearly separated classes were observed. For signal strengths <3.4 and >3.4, frequencies of *T. cristatus*, F_1 hybrids and *T. marmoratus* (for convenience data

TABLE 3 Cross tabulation of life stages and species index (SI) for numbers of *Triturus cristatus, T. marmoratus* and hybrids from dept. Mayenne, western France. The species index corresponds to the number of *T. marmoratus*-alleles observed over seven diagnostic markers. Adults were grouped on basis of their phenotype whereas hatchlings and larvae were classified on basis of genetic criteria as established for adults (SI ≤ 3 – *Triturus cristatus*, 4 < SI < 10 – hybrids and SI ≥ 11 – *T. marmoratus*).

Group	Species index	Life stage		
		Adults	Hatchlings	Larvae
Triturus cristatus				
	0	2596	130	357
	1	31	2	0
	2	13	1	0
	3	13	1	2
Hybrids				
	4	6	0	0
	5	3	0	1
	6	6	0	2
	7	82	5	33
	8	5	0	1
	9	4	0	0
	10	2	0	0
Triturus marmoratus				
	11	5	0	0
	12	3	0	0
	13	5	2	2
	14	1503	81	344

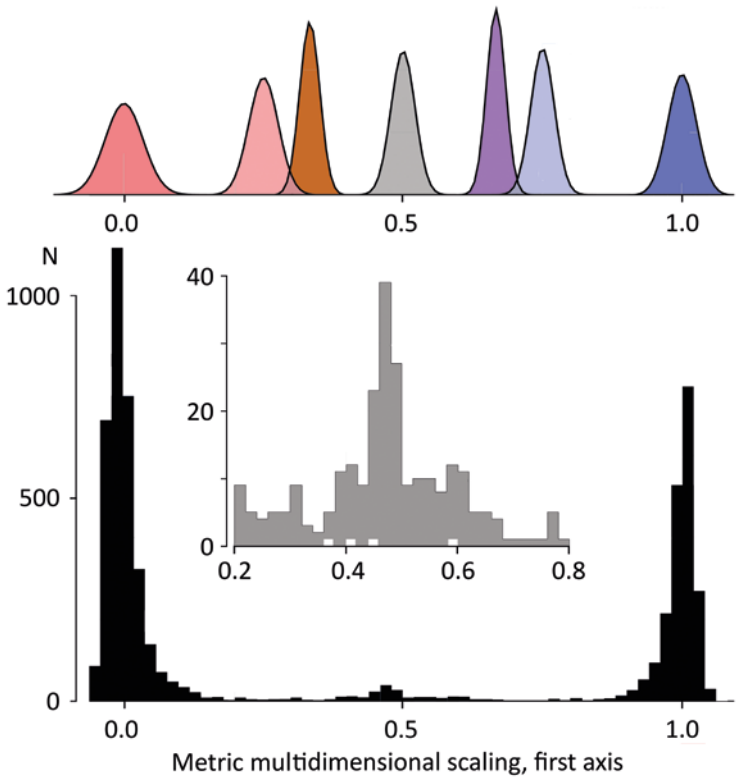

FIGURE 5 Histogram of values over the first dimension of a metric multi-dimensional scaling analysis of KASP-scores for 5557 individuals with signal strength >3.4 (see fig. 3). Values for the first dimension are scaled between zero and unity for the averages of phenotypic *Triturus cristatus* and *T. marmoratus*, respectively, to allow pooling of results over five KASP runs. The insert with the histogram in grey shows the results for intermediate genotypes (0.2 < Dim1 < 0.8) in detail. Curves in the top panel are normalised distributions for the parental species in red (*T. cristatus* – CC) and blue (*T. marmoratus* – MM), along with those for simulated hybrid combinations with, from left to right: diploid backcrosses in direction of *T. cristatus* (C(CM)) in light red, triploid hybrids (CCM) in brown, diploid hybrids (CM) in grey, triploid hybrids (CMM) in purple and diploid backcrosses in direction of *T. marmoratus* (M(CM)) in light blue. Observations over the 0.2 ~ 0.4 and 0.6 ~ 0.8 ranges suggest that *T. cristatus* × *T. marmoratus* hybrids are not just F_1's, but also backcrosses and/or triploids that from the observed data will, however, be difficult to disentangle. Four hatchlings scored as heteroplasmic for mtDNA are shown in white, and although they have a SI = 7 (see table 5), three out of four appear to be outliers to the that class, as resolved with mMDS, suggesting that they may be nuclear triploids.

pooled over the Dim1 <0.2, 0.4–0.6 and >0.8 ranges) versus potential backcrosses and triploids (data pooled over the Dim1 0.2–0.4 and 0.6–0.8 ranges) are dissimilar (Chi^2 test, df = 1, chi = 949.1, P < 0.0001). When frequencies are compared for signal strengths in the 3.4–3.6 range versus yet higher values, no significant differences were found among the classes (Chi = 0.59, P > 0.05) (table 4), suggesting that setting the threshold at 3.4 is an adequate choice, at which classification results are not significantly affected by low signal strengths. The KASP-scores can be found in supplementary table S2.

TABLE 4 Classification of individuals from KASP genotypic data by metric multidimensional scaling (mMDS), partitioned for signal strength categories. Groups are defined by their position at the first mMDS dimension as <0.2, 0.4–0.6 and >0.8 for parental species and F_1 hybrids (group 1), and 0.2–0.4 and 0.6–0.8 for potential backcrosses and triploids (group 2) (see fig. 5). The test applied is Chi^2-square test for independence.

	Signal strength comparisons			
	Low versus high		**High versus higher**	
Manhattan distance	3.0–3.4	> 3.4	3.4–3.6	> 3.6
Group 1	538	5464	2718	2746
Group 2	200	93	50	43
	Chi = 949.1 ***		Chi = 0.592 NS	

Significance levels are: *** P < 0.0001 and NS P > 0.05.

The mtDNA haplotype was determined for 4,990 individuals with nuclear genotypes called (missing data 4.8%). The haplotypes observed were 'cristatus' and 'marmoratus', and mostly corresponded with the nuclear genotype (table 5). Levels of mtDNA introgression are not significantly different for adults versus hatchlings and larvae in either species (Chi^2 test, *T. cristatus* – chi = 0.00, P > 0.05; *T. marmoratus* – chi = 0.12, P > 0.05). Also, the overall levels of mtDNA introgression at 0.6% in *T. cristatus* and 1.0% in *T. marmoratus* are roughly at par with the levels measured for nuclear data (see above). The marmoratus haplotype is about equally frequent in *T. cristatus* (19 times, 0.62%) as in the reverse combination (18 times, 1.02%) (Chi^2 test, chi = 2.47, df = 1, P > 0.05). However, mtDNA introgression is higher in introgressed individuals than in 'pure' individuals, in both species (Chi^2 test, *T. cristatus* – chi = 656.8, P < 0.0001, *T. marmoratus* – chi = 359.2, P < 0.0001). In 142 hybrid individuals ($4 \leq SI \leq 10$) the cristatus haplotype was found 116 times and the marmoratus haplotype 22 times, which frequency difference is significant (Chi^2 test for equal proportions, df = 1, chi = 64.03, P < 0.0001). Four hatchlings were classified as heteroplasmic (i.e., with both mtDNA haplotypes). Although these individuals have an SI = 7 and are thus classified as F_1-hybrids, positions at the first mMDS dimension are somewhat off-centre, suggesting that three of them may be nuclear triploids (fig. 5).

Of adult hybrids with the cristatus haplotype 25 were males and 54 were females whereas among those with the marmoratus haplotype 16 were males and two were

TABLE 5 Classification results across life stages for *Triturus cristatus, T. marmoratus* and hybrids from dept. Mayenne, western France, partitioned for the mitochondrial haplotype observed (cri – 'cristatus' and mar – 'marmoratus'). Groups are established as in table 3.

Group	Species index	Life stage					
		Adults		Hatchlings		Larvae	
		cri	mar	cri	mar	cri	mar
Triturus cristatus							
	0	2547	3	128	0	349	0
	1	27	3	1	1	0	0
	2	8	5	0	1	0	0
	3	8	5	0	1	2	0
Hybrids							
	4	2	3	0	0	0	0
	5	1	2	0	0	1	0
	6	3	2	0	0	2	0
	7 #	64	9	2	3	31	1
	8	5	0	0	0	1	0
	9	3	1	0	0	0	0
	10	1	1	0	0	0	0
Triturus marmoratus							
	11	5	0	0	0	0	0
	12	2	1	0	0	0	0
	13	1	4	0	2	0	2
	14	6	1406	0	67	4	259

Four more hatchlings in this category were classified as heteroplasmic.

females. The sex-ratio is significantly different from 50/50 in both, be it opposite, directions of the cross (Chi² test for equal proportions, df = 1, chi = 10.65, P < 0.01 and chi = 10.89, P < 0.001). On aggregate the numbers observed are: *T. cristatus* mothered hybrids – 78 males and 156 females, and *T. marmoratus* mothered hybrids – 40 males and five females (Arntzen & Wallis, 1991; Arntzen et al., 2009, 2021, present paper) (Chi-test, chi = 47.7, P < 0.0001).

Discussion

Among the many hybrid zones that the genus *Triturus* has to offer that of *T. cristatus* and *T. marmoratus* stands out on account of its size and patchy structure. The area of range overlap encompasses ca. 150,000 km², which amounts to almost a quarter of the surface of continental France. This spatial configuration sustains the replication of research

and independent assessment of results. Conversely, small hybrid zones, such as found in *Triturus pygmaeus* (fig. 1) and other systems (e.g., Caeiro-Dias et al., 2023; Kaleantzis et al., 2023), are limited in the replication they allow.

The two-species' mosaic distribution is set by habitat preferences for flat and open terrain for *T. cristatus* versus hilly and forested terrain for *T. marmoratus*, yet many of the aquatic breeding sites accommodate both species (Arntzen, 2023a,b). Adult hybrids between the species are clearly identifiable as such (fig. 2) and in extensive surveys, their frequency has been estimated at around 4% (Vallée, 1959; Schoorl & Zuiderwijk; 1981; present paper). The constituent data are, however, erratic for which the two main reasons are: (i) uneven species frequencies that makes the classification of breeding localities as allotopic (with a single species and no hybrids) versus syntopic (with two species and/or hybrids) strongly dependent on sample size, and (ii) occasionally high hybrid numbers, suggesting that (negative) selection is not uniform across admixed genotypes. It transpires that those hybrids that make it into adulthood represent the fortuitous combination of co-adapted genotypes. However, selection against F_1-hybrids is mostly strong, as evidenced by a nine times lower hatching rate for embryos from heterospecific versus homospecific crosses, followed by a five times lower survival rate over the hatchling to adulthood trajectory (Vallée, 1959: Table 4). In the present study, the observed difference in hybrid frequencies for larvae (5.0%) versus adults and hatchlings (2.3–2.5%) is probably best explained by the samples originating from different sets of ponds (cf. Arntzen et al., 2009).

Hybridization beyond the first generation

Support for hybridization and introgression comes from morphological and genetic data. Whereas a previous genetic study showed a low, but significant frequency of alien alleles (0.51%) with no difference among the species (Arntzen & Wallis, 1991), the five times larger sample size with SNPs yielded a lower overall frequency of alien alleles, more so in *T. marmoratus* (0.11%) than in *T. cristatus* (0.24%) (table 3). Support for gene flow beyond the first hybrid generation is supported by Vallée's (1959) osteological data because *T. cristatus* and *T. marmoratus* from dept. Mayenne (i.e., within the area of range overlap) are more similar to one another than are the species in allopatry (table 1, fig. 4), as would be expected from ongoing interspecific gene flow. A shortcoming in the present work is that SNP data gathering was restricted to within the area of *T. cristatus* – *T. marmoratus* range overlap so that, without data from allopatric reference populations, it cannot be excluded that the observed 'alien' alleles represent ancient polymorphisms. A shortcoming in the morphometric (re)analysis is that several of the osteological characters (nos 1–4, see above) represent organismal size that, more than the others, may be affected by environmental conditions, reflect age, and be subject to (clinal) geographical variation (Arntzen, 2000). More decisive results might have been obtained if individual size would have been known, but these details were no longer available (L. Vallée, pers. comm., 1986).

An independent line of argument supporting introgressive hybridisation comes from morphological anomalies. These are mostly digital malformations such as fused, split, or curved fingers and toes, but

supernumerary limbs and tail-tip aberrations have also been observed. The crucial observation is that malformations are frequent in hybrids (16.9%), moderately frequent in sympatric parental populations (4.6% in *T. cristatus*, 6.3% in *T. marmoratus*) and absent or rare in allopatry (Vallée, 1959; Arntzen & Wallis, 1991; Arntzen, 2018), pointing to some incompatibility of the parental genomes. While a wide variety of malformations has been reported for amphibians with an array of potential causes (Henle et al., 2017), these are not expected to show specifically in hybrid zones. Vallée (1959) also observed two cases of transitional vertebrae in sympatric populations (0.9%) which frequency is substantially lower than the 5.1% observed for the genus, so that homeotic transformations cannot be considered typical for introgressive hybridization (Arntzen et al., 2015; Slijepčević et al., 2015). With three independent lines of evidence, the case for *T. cristatus* – *T. marmoratus* hybridization to proceed beyond the first generation is strong. The morphometric analyses by Vallée (1959) already pointed to this conclusion, but it is unfortunate that he refrained from statistical analysis of his data.

A confounding factor in establishing the (low) amount of backcrossing in the *T. cristatus* – *T. marmoratus* system with KASP is the false positive signal potentially provided by triploid F_1-hybrids. None are reported for the *T. cristatus* – *T. marmoratus* system, but triploids have been documented at low frequency in laboratory backcrosses of *T. marmoratus* and *T. karelinii* (Lantz & Callan, 1954), and in (or close to) the natural hybrid zone of *T. cristatus* and *T. dobrogicus* (Borkin et al., 1996). To identify heterozygous triploids with the KASP line, a consistently strong signal is required across loci, but such was not always obtained in the present study, presumably on account of the age and condition of the DNA extracts available for analysis.

Asymmetries of hybridisation and introgression

Asymmetrical gene flow has frequently been documented in naturally occurring hybrid zones (e.g., Johnson et al., 2015; Kenney & Sweigart, 2016; Sardell & Uy, 2016; Purcell et al., 2016) and can result from various genetic, behavioural, morphological and demographic factors, including the genetic architecture of reproductive incompatibilities, patterns of mate choice and demographic characteristics (Burke & Arnold, 2001; Tiffin et al., 2001; Svensson et al., 2007; Abbot et al., 2013). While multiple processes may be at play, the higher level of introgression towards *T. cristatus* than towards *T. marmoratus* is probably best explained by the process in which the former species replaces the latter species, in a phenomenon known as 'allele surfing' (Currat et al., 2008; Quilodrán et al., 2020). The alien alleles then represent the 'genetic footprint' of the species that was displaced, in this system yielding marmoratus-alleles carried by *T. cristatus*. Species replacement in the corresponding direction has indeed been documented over the southern part of the dept. Mayenne (Arntzen & Wallis, 1991). Recent change is presumably associated with agrarian reform, in particular hedgerow removal and the turn from pasture to arable farming practices in the decades following the second World War (Schoorl

& Zuiderwijk, 1981). This affected the more aquatic *T. cristatus* less than the more terrestrially operating *T. marmoratus*. Wider surveys indicate that the process operates over the entire area of range overlap, in which *T. cristatus*, with a post-glacial origin from the Balkan peninsula (Wielstra et al., 2013), made it to the Atlantic coast and in which the *T. marmoratus* range shrank and became increasingly fragmented (Arntzen, 2023a,b). Results from a recent survey in the dept. Mayenne suggest that species replacement may have stalled, possibly because the distances between remaining populations are becoming too large to sustain a healthy metapopulation (Visser et al., 2017).

Haldane's rule which states that 'when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous (i.e., heterogametic) sex' (Haldane, 1922; see also Orr, 1997; Schilthuizen et al. 2011; Delph & Demuth, 2016; Cowell, 2023) is weakly supported in the *T. cristatus* × *T. marmoratus* system. The observed asymmetry in the direction of the cross, with one class (*T. cristatus*-mothered) making up the majority of F_1 hybrids (Arntzen et al., 2019) is here confirmed with larger sample sizes. However, the two classes of reciprocal-cross hybrids (*T. cristatus* mothered versus *T. marmoratus* mothered) behave differently. The observed low frequency of females in the latter category of adult F_1 hybrids (contradicting Haldane's rule) is best explained by an incompatibility between the *T. cristatus* X chromosome and *T. marmoratus* cytoplasm (Arntzen et al., 2009). It is thus important to distinguish the two classes of reciprocal-cross hybrids before making general statements about whether Haldane's rule is observed. Currently, research is hampered by the unavailability of molecular sex markers for *Triturus*. Once these are available, analyses can be extended to include the pre-adult life stages.

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Supplementary material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.26124259

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