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Interspecific introgression of MHC genes in *Triturus* newts: Evidence from multiple contact zones

Tomasz S. Gaczorek[1] | **Marzena Marszałek**[1] | **Katarzyna Dudek**[1] | **Jan W. Arntzen**[2,3] | **Ben Wielstra**[2,3] | **Wiesław Babik**[1]

[1]Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland

[2]Naturalis Biodiversity Center, Leiden, The Netherlands

[3]Institute of Biology, Leiden University, Leiden, The Netherlands

Correspondence
Tomasz S. Gaczorek and Wiesław Babik, Institute of Environmental Sciences, Faculty of Biology, Jagiellonian, University, Kraków, Poland.
Email: tomasz.gaczorek@doctoral.uj.edu.pl; wieslaw.babik@uj.edu.pl

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Abstract

The major histocompatibility complex (MHC) genes are central to the adaptive immune response in vertebrates. Selection generally maintains high MHC variation because the spectrum of recognized pathogens depends on MHC polymorphism. Novel alleles favoured by selection originate by interallelic recombination or de novo mutations but may also be acquired by introgression from related species. However, the extent and prevalence of MHC introgression remain an open question. In this study, we tested for MHC introgression in six hybrid zones formed by six *Triturus* newt species. We sequenced and genotyped the polymorphic second exons of the MHC class I and II genes and compared their interspecific similarity at various distances from the centre of the hybrid zone. We found evidence for introgression of both MHC classes in the majority of examined hybrid zones, with support for a more substantial class I introgression. Furthermore, the overall MHC allele sharing outside of hybrid zones was elevated between pairs of *Triturus* species with abutting ranges, regardless of the phylogenetic distance between them. No effect of past hybrid zone movement on MHC allele sharing was found. Finally, using previously published genome-wide data, we demonstrated that MHC introgression was more extensive than genome-wide introgression, supporting its adaptive potential. Our study thus provides evidence for the prevalence of MHC introgression across multiple *Triturus* hybrid zones, indicating that MHC introgression between divergent hybridizing species may be widespread and adaptive.

KEYWORDS
adaptive introgression, hybridization

1 | INTRODUCTION

Interspecific introgression is considered a ubiquitous process (Arnold & Kunte, 2017; Payseur & Rieseberg, 2016), backed up by our recently acquired power to detect introgression with genome-wide data (Li & Akey, 2019; Roux et al., 2016; Taylor & Larson, 2019). However, the evolutionary significance of introgression is less clear. Is it primarily a manifestation of porous species boundaries, akin to intraspecific gene flow, or is it a process of adaptive significance? As reasonably well-documented examples of adaptively introgressed variants accumulate (Hedrick, 2013; Jones et al., 2018), the field moves towards identification of its general properties. The broad view emerges that introgression may be a significant source of variation when novel mutations and standing variation are insufficient for rapid adaptation (Marques et al., 2019; Suarez-Gonzalez et al., 2018). This may be the case during adaptive radiations, enabling the rapid filling of available niches (Edelman et al., 2019; Meier et al., 2017), or when organisms have to cope with an abrupt environmental change, be it pollution (Oziolor et al., 2019), changing climatic conditions (Leroy et al., 2020) or pathogens (Bechsgaard et al., 2017).

Many examples of adaptive introgression would be particular genetic variants, located in various genes, that became available via introgression and confer a selective advantage in the recipient species. Certain classes or categories of genes may regularly be subject to adaptive introgression because selection acting on them generally favours novel variants (Hedrick, 2013; Muirhead, 2001; Schierup et al., 2000). Such genes, evolving under balancing selection, are often involved in arms races of pathogens and hosts. Likely adaptive introgression of such genes has been reported in humans (Enard & Petrov, 2018; Gouy et al., 2020) and other species (Fijarczyk et al., 2018), including parasites (Konczal et al., 2020; Platt et al., 2019). A prime example of long-term balancing selection concerns the major histocompatibility complex (MHC) genes, which encode proteins crucial for the vertebrate adaptive immune response (Radwan et al., 2020). The adaptive advantage of novel MHC alleles, promoted by negative frequency-dependent selection, has been demonstrated experimentally (Phillips et al., 2018). Increased, and probably adaptive, MHC introgression has been reported in a handful of systems, including humans (Abi-Rached et al., 2011), Alpine ibex (Grossen et al., 2014), hares (Pohjoismäki et al., 2021) and small-bodied newts (Dudek et al., 2019; Fijarczyk et al., 2018; Nadachowska-Brzyska et al., 2012). It has been hypothesised that genes under strong balancing selection, such as MHC, may introgress adaptively, even when the overall reproductive isolation between diverging species is strong, placing them among the last genes to stop introgressing at the final stages of speciation (Castric et al., 2008; Dudek et al., 2019). However, a convincing demonstration of increased introgression is challenging because selection leads to long-term retention of ancestral polymorphism, generating patterns that resemble introgression (Fijarczyk & Babik, 2015).

Natural hybrid zones are an excellent tool to overcome this limitation and are especially powerful if multiple transects are studied (Dudek et al., 2019). The elevated interspecific sharing of MHC alleles in proximity to the contact zone, especially if replicated over multiple transects, indicates introgression by ruling out alternative explanations. Furthermore, MHC variation within species is often geographically structured (reviewed by Spurgin & Richardson, 2010), enabling the localisation of introgression events and their detection over extended periods. Groups of distinct but incompletely reproductively isolated species offer yet another level of replication and a prospect of broader generalization in studies of MHC introgression. In such species groups, outcomes of multiple geographically separated and largely evolutionary-independent hybridisation events can be studied. Each hybrid zone involves two (occasionally more) species, and a single species can hybridize with multiple other species, allowing us to separate the effect of various factors, such as species and geographic region, on MHC introgression.

A promising example is the newt genus *Triturus*, which inhabits most of western Eurasia (Figure 1). The genus comprises two main clades, a western (marbled newts) and an eastern (crested newts) one, that diverged c. 18–25 million years ago (Ma) (Arntzen et al., 2015; Kieren et al., 2018; Rancilhac et al., 2021; Steinfartz et al., 2007; Zhang et al., 2008). The western species, *T. marmoratus* and *T. pygmaeus*, split c. 4.1 Ma, whereas the deepest divergence within the eastern clade is dated at c. 7.3 Ma (Figure 1). The most closely related pairs within the eastern clade are *T. anatolicus*-*T. ivanbureschi* and *T. cristatus*-*T. dobrogicus*, diverged c. 4.3 and 4.5 Ma (Wielstra et al., 2019). Hybrid zones have been documented between all *Triturus* species pairs with abutting ranges (Arntzen et al., 2014; Wielstra, Burke, Butlin, Avcı, et al., 2017). These zones differ in the extent of hybridisation—from unimodal, where hybrids are common, to bimodal zones with hardly any hybridisation (Arntzen et al., 2014). Extensive information on the history of contacts between species and range changes is available. In particular, genetic data, current geographic distribution, and historical distribution modelling support large-scale hybrid zone movement and species replacement in some parts of the ranges (Arntzen, López-Delgado, et al., 2021; Arntzen & Wallis, 1991; Themudo & Arntzen, 2007; Wielstra et al., 2013; Wielstra, Burke, Butlin, & Arntzen, 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017). This comprehensive background knowledge and the detailed information on the distribution of all species (Arntzen, 2018; Arntzen, 2022; Wielstra, Burke, Butlin, & Arntzen, 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017; Wielstra, Sillero, et al., 2014) greatly facilitate testing MHC introgression and interpretation of the results.

Here, we study interspecific introgression of MHC class I (MHC-I) and II (MHC-II) in the genus Triturus, focusing on extensively sampled contact zones between six pairs of species: *T. cristatus* x *T. macedonicus* (Figure 1a), *T. anatolicus* x *T. ivanbureschi* (b), *T. cristatus* x *T. ivanbureschi* (c), *T. ivanbureschi* x *T. macedonicus* (d) (Balkan Peninsula), *T. marmoratus* x *T. pygmaeus* (e, Iberia), and *T. cristatus* x *T. marmoratus* (f, France). The three analysed regions are characterized by unique but complementary features (Arntzen et al., 2014). The Balkan contact zones in which hybridisation occurs are narrow but complex, as the ranges of four *Triturus* species meet in the northern part of the peninsula. The hybrid zone in Iberia is also narrow but involves only two species, and it is geographically isolated from other hybrid zones. The contact zone in France, between highly diverged species that hybridize only rarely and experience very limited introgression (Arntzen, 2022; Arntzen, Jehle, & Wielstra, 2021), involves, on the other hand, a broad area of sympatry and syntopy.

Our study has several specific aims. First, we assess the variation of both MHC classes within species and check whether range expansions were associated with a loss of variation, as previously described for *T. cristatus* (Babik et al., 2009). Second, we test whether MHC introgression occurs between species with abutting ranges and whether the extent of introgression differs among the hybrid zones or between the two MHC classes. Third, we test whether MHC introgression exceeds the genome-wide average, as expected under an adaptive introgression scenario. Fourth, we test whether the time of divergence and presence of secondary contact can explain the

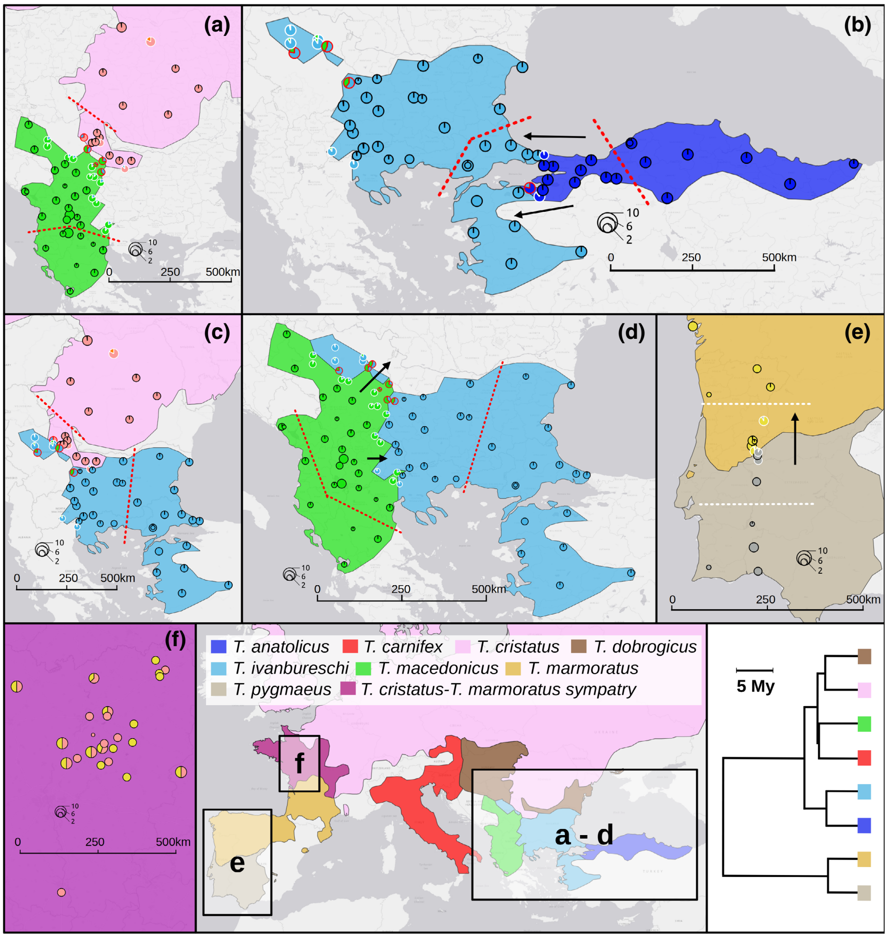

FIGURE 1 The examined *Triturus* hybrid zones together with species ranges and sampling scheme. a) *T. cristatus-T. macedonicus*, b) *T. anatolicus-T. ivanbureschi*, c) *T. cristatus-T. ivanbureschi*, d) *T. ivanbureschi-T. macedonicus*, e) *T. marmoratus-T. pygmaeus*, f) *T. cristatus-T. marmoratus*. Species are represented with different colours, as shown in the legend. Pie charts show the averaged population ancestry in all panels except for (f) where genetic admixture is negligible, and where the pie charts represent ponds where *T. cristatus* and *T. marmoratus* co-occur. The colour outlines of pie charts group them into three categories, depending on the level of admixture; red - >20%, white - >3%, black - <3%. The size of the pie charts corresponds to the number of sampled individuals. Red dotted lines separate allopatric populations into alloC and alloF categories used in the introgression tests, while white dotted lines separate parapatric and allopatric populations. The black arrows show the direction of historical contact zone movements. The map was generated with QGIS version 3.16. The dated *Triturus* phylogeny is from Wielstra et al. (2019).

patterns of MHC similarity between species. MHC introgression is studied by applying complementary approaches, based on interspecific allele sharing and geographic clines, adapted to the characteristics of MHC data. We hypothesise that MHC introgression will be detectable in all contact zones and will consistently exceed the average for genome-wide introgression.

2 | MATERIALS AND METHODS

2.1 | Sampling scheme

To investigate MHC variation and introgression, we used previously collected samples (Arntzen, 2018; Arntzen et al., 2014; Wielstra, Burke, Butlin, & Arntzen, 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017) from six hybrid zones located in three geographical regions: the Balkans (including western Anatolia), the Iberian Peninsula, and France. The analysed localities covered contact zones as well as allopatric parts of the range of each species. In contact zones, we did not analyse localities with substantial genome-wide admixture because these are not informative about MHC introgression. We intended to maximize the number of localities (ponds, which are newt breeding sites) to increase the power of our tests while sampling, whenever possible, at least three individuals to obtain information on within-population variation.

Among the analysed regions, the Balkans are the most complex, with four examined *Triturus* species forming four contact zones (Figure 1a–d). We sampled 20, 17, 40, and 42 localities of *T. anatolicus*, *T. cristatus*, *T. ivanbureschi*, and *T. macedonicus*, respectively. In the Iberian Peninsula, a single hybrid zone is present and was sampled along a transect comprised of nine *T. marmoratus* and eight *T. pygmaeus* localities (Figure 1e). In France, in the broad area of sympatry, eight syntopic and 17 allotopic (nine *T. cristatus* and eight *T. marmoratus*) ponds were examined (Figure 1f). We also genotyped MHC in five populations for which the information about the genome-wide admixture was lacking (three from Iberia and two close to the *T. anatolicus*-*T. ivanbureschi* contact zone). These five populations were used only in the cline analysis (see below). Additionally, to obtain a comprehensive picture of MHC variation in the Balkan peninsula, we sampled three populations of *T. dobrogicus*, even though contact zones with this species were not the focus of attention. The list of all samples used in the study can be found in Table S1.

2.2 | MHC genotyping and its repeatability

The polymorphic second exon of both MHC classes was PCR amplified and sequenced on the Illumina platform following the approach of Fijarczyk et al. (2018). The primers used for amplification can be seen in Table S2. Forward and reverse primers contained 6 bp barcodes that allowed the assignment of sequencing reads to samples. The 10 µl PCR reactions contained 1 µM of each forward and reverse primer (in the case of MHC-I, it was the total concentration of a pool of forward and reverse primers) and 5 µl of Multiplex Master Mix (Qiagen). PCR conditions were as follows: Initial denaturation at 95°C for 15 min, followed by 33 (MHC-I) or 35 (MHC-II) cycles: 94°C for 30 s, 56°C (MHC-I) or 55°C (MHC-II) for 30 s and 72°C for 70 s, with a final elongation at 72°C for 10 min. Amplicons were pooled approximately equimolarly based on gel-band intensity, pools were gel-purified, Illumina adaptors were ligated using the NEBNext Ultra II DNA Library Prep kit (NEB), and sequenced on the Illumina MiSeq (version 3600 cycles kits). The sequencing data were then processed with AmpliSAS (Sebastian et al., 2016). Clustering and filtering parameters are described in Table S3. Sequence variants that deviated by more than six base pairs from the expected length (223 bp - MHC-I, 200 bp - MHC-II) or possessed a stop codon were treated as nonfunctional and were removed. The genotyping repeatability was estimated for six individuals amplified and sequenced in two replicates by dividing the number of alleles present in both replicates by the total number of detected alleles. Finally, the data were transformed into a binary, presence-absence matrix used for subsequent introgression analyses.

2.3 | Assignment of MHC alleles into transcription categories

A consequence of rapid genomic evolution in the MHC region is the presence of classical genes, nonclassical genes of various ages, and MHC pseudogenes. These different categories of genes may have similar sequences but may be affected by different evolutionary forces, for example, whereas classical genes evolve under balancing selection, purifying selection may dominate the evolution of nonclassical genes (Ballingall & McKeever, 2005). MHC pseudogenes, in turn, would be affected mainly by drift and only indirectly by linked selection (Babik et al., 2008). We used the expression level (Wielstra et al., 2019) and sequence similarity to classify MHC alleles into putatively functional (HEX) and putative nonclassical/pseudogene (LEX). This approach followed Fijarczyk et al. (2018) and is described in detail in the Supporting Information (Appendix S1; Figure S1, S2). Although this procedure does not allow an unambiguous distinction of classical MHC alleles from other sequences, it is nevertheless useful because the HEX category should be enriched in functional alleles of classical MHC genes and LEX in nonclassical/nonfunctional alleles. To avoid the potential effect of evolutionary mechanisms that may drive the evolution of nonclassical/nonfunctional genes and counteract balancing selection, we used only the HEX category for testing introgression.

2.4 | Genome-wide admixture

To compare patterns of MHC introgression with genome-wide introgression, we used information about average genome-wide admixture in the form of Q-scores estimated by Structure software (Pritchard et al., 2000). For the contact zone of *T. anatolicus* and *T. ivanbureschi*, the admixture was assessed with 49 SNPs (Wielstra, Burke, Butlin, Avcı, et al., 2017), and for the remaining Balkan species and contact zones, from 52 SNPs (Wielstra, Burke, Butlin, & Arntzen, 2017). The SNPs were located in 3′ untranslated regions of protein-coding genes (Wielstra, Duijm, et al., 2014), which are usually less constrained by selection than the coding regions and because a relatively large number of SNPs was used, they should provide a reliable estimate of genome-wide introgression. In the Iberian *T. marmoratus* x *T. pygmaeus* zone, we used the averaged population Q-score calculated over four enzyme loci (Arntzen, 2018). In a few cases, we considered individuals as nonadmixed based on the distance from the centre of the contact zone and geographic proximity to other nonadmixed individuals. In the *T. cristatus* x *T. marmoratus* zone in France, all individuals were considered nonadmixed because previous long-term studies detected introgression to be near-absent (Arntzen et al., 2014; Arntzen, Jehle, & Wielstra, 2021; Arntzen & Wallis, 1991). Following previous work (Wielstra, Burke, Butlin, & Arntzen, 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017), we considered individuals with a minimum ancestry of 0.97 from a single species as nonadmixed. Those nonadmixed individuals were used to assess MHC variation and to define the reference allopatric groups for the cline analysis (see below).

2.5 | Testing MHC introgression

In the presence of recent hybridisation, gene exchange is expected between species with abutting ranges across the contact zone. The introgressed alleles are initially present only in the populations adjacent to the contact zone but may gradually spread to more distant populations. The rate and strength of introgression depend on the direct and linked selection pressures, with adaptive variants introgressing faster than neutral markers due to the fitness advantage they confer (Edelman & Mallet, 2021; Hedrick, 2013; Pialek & Barton, 1997). Under recent or ongoing adaptive MHC introgression, one can therefore expect the elevated sharing of MHC alleles between populations of two species in the proximity of the contact zone, beyond the point where neutral introgression is detectable.

2.5.1 | Interspecific allele sharing in allopatry

We tested for the signal of MHC-I introgression by comparing the fraction of alleles shared between pairs of *Triturus* species with and without geographically adjacent ranges. As species pairs with non-abutting ranges could not have experienced recent introgression, to make this test comparable for all species pairs, we used only individuals outside the contact zones, without a signal of genome-wide admixture, which we term allopatric. We used the GLM, binomial error model (see Appendix S2; Figure S3) with the phylogenetic relationship between species included to control for various expected levels of ancestral polymorphism. If the MHC introgression was present and prevalent, we would expect an elevated sharing of MHC-I alleles between species with adjacent ranges. Due to limited allele sharing and the presence of, essentially, a single locus, we did not apply the model to MHC-II.

2.5.2 | Interspecific allele sharing in the proximity of contact zones

Within each hybrid zone, we tested for MHC introgression by comparing the extent of allele sharing between parapatric and allopatric populations of hybridizing species. In turn, allele sharing between allopatric populations (i.e., without a signal of genome-wide introgression) closer and further from the contact zone was used to test for adaptive MHC introgression. The significance of all comparisons was assessed with a randomisation test (10,000 replications) in which individuals within species were randomly assigned to population types. Apart from the randomisation tests, principal component analysis (PCA) plots for MHC-I were generated for each contact zone to visualize similarities of MHC genotypes between population types, which may provide independent evidence for introgression. PCA was not performed for MHC-II because, essentially, only a single functional locus was present.

We designated parapatric (up to 20% admixture from other species) and allopatric (less than 3% admixture) populations in most of the examined hybrid zones. Whenever possible, allopatric populations were classified into two groups based on the distance from the contact zone (see below); close (alloC) and far (alloF). This classification was conservative, ensuring that alloF populations were not located in the proximity of historical contact zones when inferred past hybrid zone shifts were considered. The minimum distance of the alloF population to the contact centre was 120 km. There were, however, two exceptions to the rules described above. First, we did not designate parapatric populations in the Balkan *T. cristatus* and in the eastern part of the *T. ivanbureschi* range as restricted genome-wide introgression resulted in nonadmixed populations even in the proximity of the contact zone. Second, the unique characteristics of sympatry observed in the French *T. cristatus* x *T. marmoratus* hybrid zone required a different approach. There, we considered ponds inhabited by both *T. cristatus* and *T. marmoratus* as parapatric, ponds inhabited by only one species as alloC, and distant populations from Iberia (*T. marmoratus*) and Romania (*T. cristatus*) as alloF populations. The classification of populations is visualized in Figure 1.

2.5.3 | Geographic clines

Another approach we used to test whether MHC introgression exceeded the genome-wide introgression average was the comparison of the width and position of geographic MHC and genome-wide clines. We expected rapid introgression of beneficial alleles under balancing selection, even if the hybrid zone poses a strong barrier to neutral introgression, similar to the case of a beneficial allele under directional selection (Pialek & Barton, 1997). This process, however, would not result in a wave of advance and the fixation of the beneficial allele but would rather lead to the widening of the MHC cline. It is because many introgressed alleles would be favoured due to their novelty and rarity, leading to bidirectional introgression, and the selective advantage of the introgressed alleles will decrease as they become common. The wider MHC cline may persist for long periods because, as long as the frequencies of MHC alleles will not be homogenized between species, we can expect a general selective advantage of introgressed alleles, whereas geographic structuring of MHC within species and considerable turnover of alleles within populations should prevent the homogenisation. The expectations regarding centre shift, which indicates asymmetric introgression, are less clear. Such asymmetry is certainly possible, for example, when MHC variation in one of the hybridizing species was depleted prior to secondary contact, making the selective advantage of introgressed alleles higher on average in this species. Another possibility is asymmetry caused by the selective advantage of particular introgressed alleles in one of the species. Such a signature, however, should be transient and not easily detectable from the average MHC clines.

Because the localities did not form one-dimensional transects, to apply the cline analysis, we first calculated the distance of each locality from the centre of the zone and then ordered the localities into a pseudo-transect accordingly. The centre of each hybrid zone was established by drawing an isoline of genome-wide admixture between a given species pair equal to 0.5, using the “akima” package (Akima & Gebhardt, 2021) in R (R Core Team, 2022, version 4.1.3). Then, evenly distributed points (every 1 km) along the isoline were generated using the SAGA plugin (Conrad et al., 2015) in QGIS, and the distance from each locality to the centre of the contact zone was calculated as the distance to the nearest point on the isoline. The distance values for populations of the first species in a pair (ordered alphabetically) were considered negative to create a pseudo-transect with the centre at zero.

For each locality, the average Q-score from Structure was taken as the genome-wide hybrid index. The MHC hybrid index was calculated for each MHC class separately by maximizing, for each individual, the likelihood function from Buerkle (2005). The species-specific MHC allele frequencies were based on individuals without any sign of genome-wide genetic admixture. It allowed us to effectively remove admixed individuals, whose MHC alleles are uninformative in terms of ancestry, and potential false species assignees. Each MHC allele was treated as an independent, biallelic, fully dominant locus, which was necessary because we could not assign the alleles to the actual loci based on their sequence similarity (Huang et al., 2019). This was probably due to frequent duplications, which would make some alleles within the locus more different than some alleles from different loci, or interlocus recombination and gene conversion, which create alleles carrying genetic material from multiple genes. Simple simulations described in the Supporting Information (Appendix S3) validated this approach because the inferred h-index values strongly correlated with those calculated from known haplotypes but might also suggest a slight artefactual widening of clines fitted to h-index data obtained this way (Figure S4). The hybrid index was averaged over all individuals in a given population and the clines were fitted with the "hzar" (Derryberry et al., 2014) R package. For each hybrid zone, the optimal cline model for the genome-wide data was identified from among 15 available models, based on the AIC criterion (Derryberry et al., 2014). The chosen model was then fitted to both genome-wide and MHC data. The parameters of genome-wide and MHC clines were compared based on the two-log-likelihood intervals. Due to the broad sympatry in the *T. cristatus*-*T. marmoratus* hybrid zone, cline analysis was not performed for this species pair.

3 | RESULTS

3.1 | MHC variation and assignment of alleles into transcription categories

MHC variation was studied in 178 localities of seven *Triturus* species inhabiting the Balkans, France, and Iberia. 566 individuals were genotyped in class I and 547 in class II (Table S4), including six additional individuals used only in the expression analysis (see below). Genotyping repeatability estimated using six individuals genotyped in replicates was 87.7% for MHC-I and 100% for MHC-II. We detected a total of 1135 class I and 197 class II alleles (excluding sequences with stop codons and frameshifts), of which 934 (82.3%) and 121 (61.4%) were classified as putatively functional (HEX). All allele sequences are available at the Dryad Digital Repository (doi:10.5061/dryad.pk0p2ngqs).

The per-individual number of alleles ranged from 8 to 28 for MHC-I (2–22 for MHC-I HEX) and 1 to 10 for MHC-II (1–4 for MHC-II HEX, Figure 2). A maximum of two MHC-II HEX alleles were detected in an overwhelming majority of individuals (97.8%). It strongly suggests the presence of a single functional MHC-II locus (*DAB*) as a rule for the genus *Triturus*, in accordance with a previous study of MHC-II variation in *T. cristatus* (Babik et al., 2009). The twelve individuals with more than two alleles can result from either incorrect assignment of some alleles to the HEX category, the presence of rare haplotypes carrying two functional MHC-II genes, or rare spontaneous triploidy (Borkin et al., 1996). The number of MHC-I HEX alleles per individual suggests the presence of 11 or more putative functional genes. The broad range of the number of alleles per individual (Figure 2) may reflect differences in the level of heterozygosity, copy number variation and, possibly, sharing of identical alleles between different genes. The absolute differences among species in the per-individual number of alleles were substantial for MHC-I HEX (medians between 5 and 22) and negligible for MHC-II HEX (medians of 1 or 2). The ANOVA followed by post-hoc Tukey's test showed significant differences in the per-individual number of MHC alleles between all examined species pairs, except for *T. marmoratus*-*T. pygmaeus*, in at least one MHC class: *T. cristatus*-*T. ivanbureschi* and *T. ivanbureschi*-*T. macedonicus* (both classes), *T. cristatus*-*T. macedonicus* and *T. cristatus*-*T. marmoratus* (MHC-I), and *T. anatolicus*-*T. ivanbureschi* (MHC-II) (Table S5). Based on allelic richness, the marbled newts inhabiting western Europe have lower MHC-I variation than the eastern crested newts, while the apparent loss of MHC-I variation in French populations of *T. cristatus* is possibly the effect of colonization from a distant refugium (Figure 2, Wielstra et al., 2015).

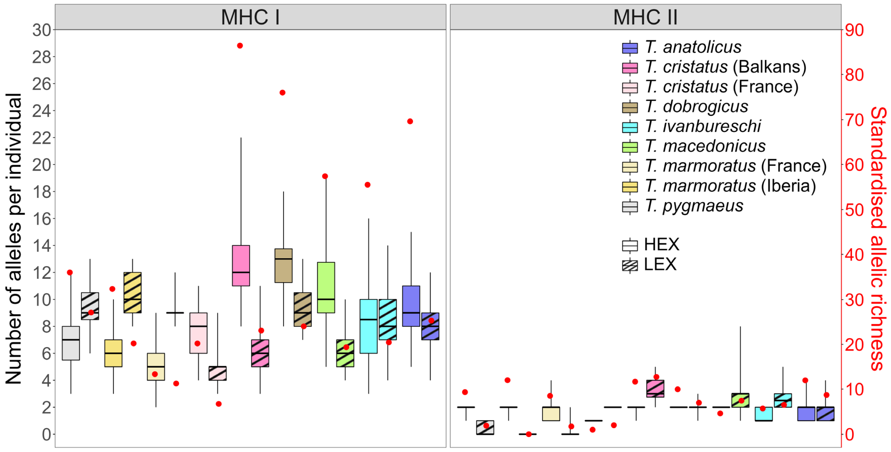

FIGURE 2 MHC polymorphism. The number of MHC alleles per individual (boxplots) and allelic richness standardized to the per-species sample size of 10 individuals (red dots). In boxplots, the thick horizontal line, box, and whiskers correspond to the median, values between the first and third quartile, and the range, respectively. HEX stands for highly expressed alleles and LEX for nonfunctional or nonclassical, lowly expressed alleles.

3.2 | MHC allele sharing and introgression

Allele sharing was detected between allopatric populations of several species, but the degree differed between the MHC classes (Figure 3). In MHC-I HEX, significantly elevated allele sharing was observed between species pairs with abutting ranges (GLM, $p = .02$), with no significant effect of the time of species divergence ($p = .44$). In the Balkan region, all species pairs shared some MHC-I HEX alleles (mean = 4.64%), and the highest values were observed in *T. cristatus-T. dobrogicus* (12.34%), *T. anatolicus-T. ivanbureschi* (8.29%), and *T. cristatus-T. ivanbureschi* (6.91%). In contrast, allele sharing in MHC-II HEX was lower (mean = 2.86%) with *T. anatolicus-T. ivanbureschi* (10.53%), *T. anatolicus-T. macedonicus* (6.25%), and *T. cristatus-T. ivanbureschi* (6.25%) standing out. In France, allele sharing between *T. cristatus* and *T. marmoratus* was observed in both classes, whereas the Iberian *T. marmoratus* and *T. pygmaeus* shared only MHC-I alleles (15.17%) (Table S6).

An excess of MHC alleles shared between species in the proximity of a contact zone, as inferred from the randomisation tests, indicated ongoing or recent MHC introgression (Figure 4; Figure 5; Figure S6). Allele sharing between parapatric populations of two species was significantly elevated compared to the sharing between allopatric populations in all tested hybrid zones and for both MHC classes. In MHC-I, parapatric populations shared 25.8% (32/124, *T. macedonicus-T. ivanbureschi*) to 50% (15/30, *T. cristatus-T. marmoratus*) of detected alleles while sharing among alloF populations did not exceed 4.3% in any hybrid zone (maximum 9 alleles in *T. anatolicus—T. ivanbureschi*). In MHC II, 5.9% (1/17, *T. cristatus-T. marmoratus*) to 20% (1/5, *T. ivanbureschi-T. macedonicus*) of alleles were shared close to the contact zone while no alleles were shared between alloF populations. For most hybrid zones, these findings also surfaced in the PCA ordination of MHC-I genotypes, where parapatric populations tended to overlap more than allopatric along PC2 or PC3 (Figure S7). Geographic clines showed the same pattern as the PCA, as some parapatric populations, for example, from the *T. ivanbureschi-T. macedonicus* hybrid zone showed intermediate h-index values (Figure S8).

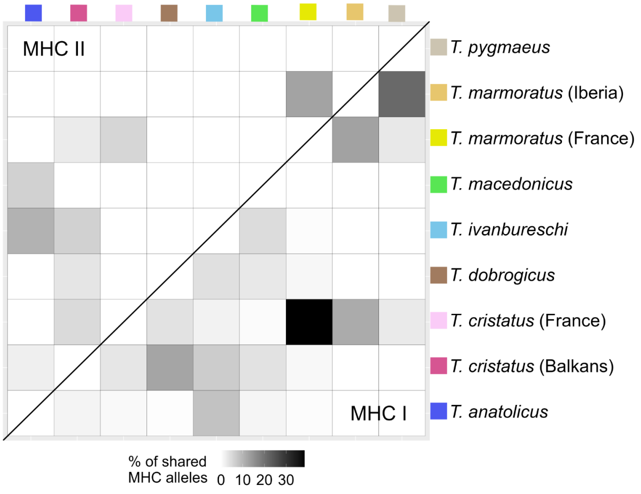

FIGURE 3 Percent of MHC HEX alleles shared between allopatric individuals of each species pair.

3.3 | MHC vs. genome-wide introgression

The hypothesised selective advantage of novel MHC alleles is expected to elevate the rate of their introgression compared to neutral markers. We confront the two rates in two independent analyses: tests for an excess of shared alleles and the comparison of geographic clines. The patterns of allele sharing between allopatric populations closer (alloC) and further (alloF) from the contact differed between species pairs (Figure 4). In MHC-I, a significant excess of allele sharing between alloC groups, indicative of increased MHC-I introgression compared to the genome-wide introgression average, was detected in all three hybrid zones involving *T. ivanbureschi*. It resulted mainly from limited allele sharing between *T. ivanbureschi* alloF populations and those of *T. anatolicus*, *T. cristatus*, and *T. macedonicus* (1.0%–4.3% alleles shared between alloF and 9.5%–11.5% between alloC). A striking difference was also found for the *T. cristatus-T. marmoratus* hybrid zone where 26.5% of alleles were shared between alloC and none between alloF groups. In MHC-II, allele sharing between allopatric populations of different species was more limited than in MHC-I, with no alleles shared between any alloF populations. Moreover, in only two out of six examined hybrid zones, allele sharing between alloC populations was detected (*T. anatolicus-T. ivanbureschi* - 15.4% and *T. cristatus-T. ivanbureschi* - 16.7%). In both cases, the excessive allele sharing between the alloC populations was statistically significant ($p < .01$). Interestingly, *T. marmoratus* and *T. cristatus* populations occupying different ponds (alloC) shared no MHC-II alleles despite extensive sharing of MHC-I alleles.

The comparison of MHC and genome-wide cline parameters provides insights into the extent and directionality of introgression. We expected wider MHC clines due to elevated introgression rate, while we did not have clear expectations regarding shifts of cline centre, which would indicate asymmetry of MHC introgression. The MHC clines were indeed significantly wider than genome-wide clines in *T. cristatus-T. macedonicus* (MHC-I), *T. marmoratus-T. pygmaeus* (MHC-I), *T. anatolicus-T. ivanbureschi* (MHC-II), and *T. cristatus-T. ivanbureschi* (both MHC classes) hybrid zones (Figure 5, Figure S9; Table S7). The MHC-I cline for *T. marmoratus-T. pygmaeus* was the only one with a shifted centre (towards *T. marmoratus*).

4 | DISCUSSION

We tested for MHC introgression and compared its extent to the average genome-wide introgression in multiple hybrid zones between *Triturus* species. We found a widespread signal of MHC introgression, but its extent differed between hybrid zones and MHC classes. The elevated sharing of MHC alleles that we detected in the proximity of the contact zones is best explained by introgression. Two alternative explanations, namely similarity due to the retention

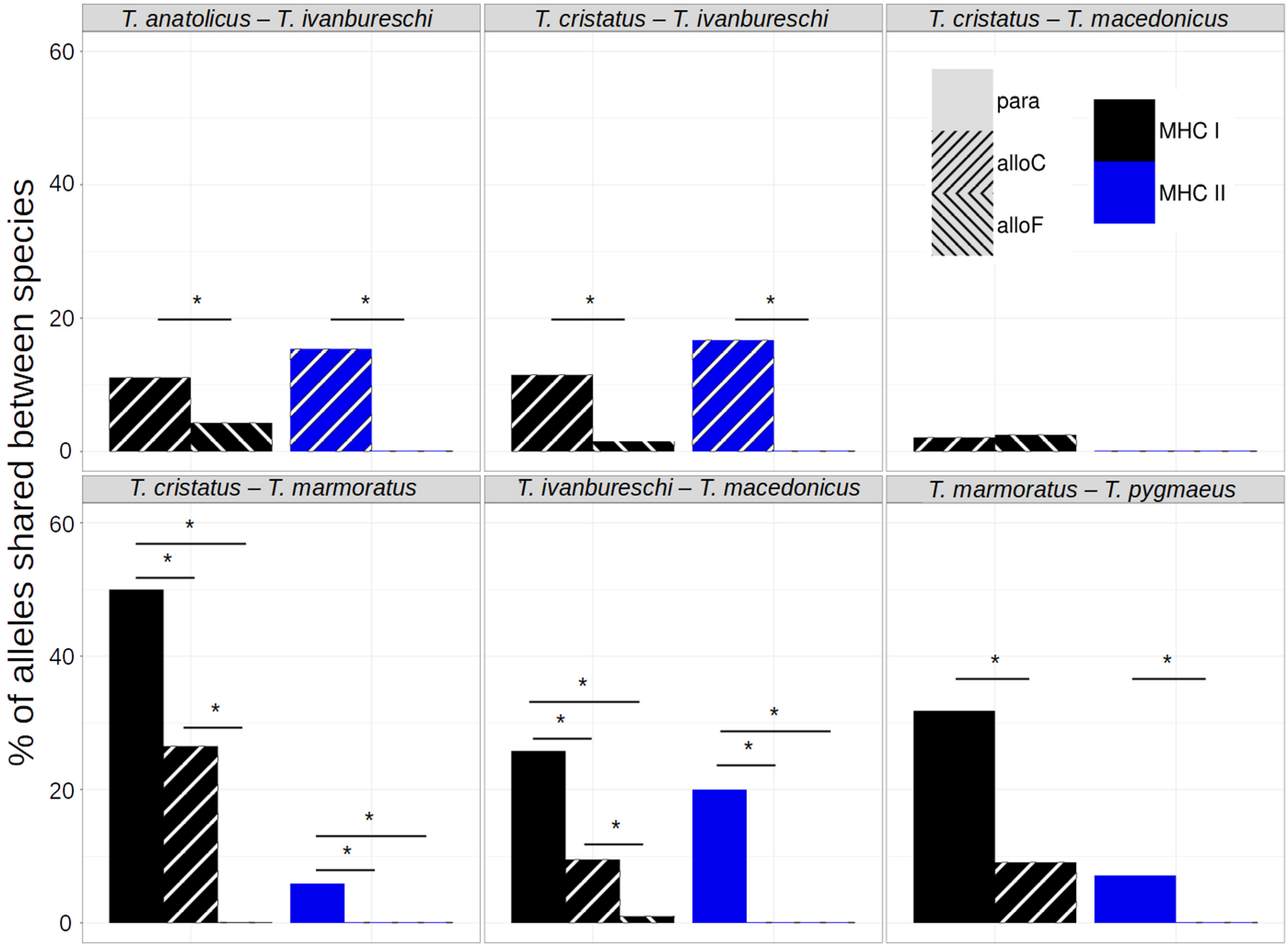

FIGURE 4 The results of randomisation tests comparing the extent of interspecific allele sharing for various population classes in each hybrid zone. Asterisks denote statistically significant differences ($p < .01$). Note that for some species pairs, certain population classes are lacking. Para - populations with 3%–20% genome-wide admixture located close to the contact zone; alloC - populations with less than 3% admixture located in the proximity of the contact zone; alloF - populations with less than 3% admixture located far from the contact zone. In the *T. cristatus* x *T. marmoratus* hybrid zone, a unique approach was applied due to sympatry and near-absent genome-wide introgression. Coinhabited ponds were considered parapatric, ponds inhabited by only one species - alloC, and distant populations from Iberia (*T. marmoratus*) and Romania (*T. cristatus*) - alloF populations.

of ancestral polymorphism and parallel changes in allele frequencies (Kaesler et al., 2017; Klein et al., 2007; Lenz et al., 2013) are both unlikely. The retention of ancestral polymorphism should result in a uniform distribution of shared alleles within species, so no elevated allele sharing close to the contact zone is expected under this scenario. The parallel increase in the frequency of the same MHC alleles, driven by common pathogens in the contact area, would require that the same ancestral alleles were maintained in both species at low frequency. The consistent pattern of long-term retention of such alleles and then parallel increase in frequency across multiple distant hybrid zones is unlikely. The comparison of the extent of MHC-I allele sharing between species (considering only individuals without detectable genome-wide admixture) further supports the presence of introgression as we found elevated allele sharing between species with abutting ranges while controlling for the time of species divergence.

4.1 | Adaptive MHC introgression

To test for the adaptive MHC introgression, we checked whether it was more extensive than the genome-wide introgression average. In agreement with theoretical predictions (Schierup et al., 2000), we found evidence for increased, likely adaptive, MHC introgression in most hybrid zones. Nowhere, however, was MHC introgression as extensive as reported between *Lissotriton montandoni* and *L. vulgaris* newts in the Carpathian Basin (Dudek et al., 2019). An elevated interspecific allele sharing in at least one MHC class was observed in the proximity of the contact zone but outside the area of detectable genome-wide introgression in all examined species pairs except for *T. cristatus*-*T. macedonicus*. A limited genome-wide introgression characterizes this pair. Nevertheless, they still share some MHC-I alleles, relatively evenly distributed throughout the species ranges. These might be instances of ancestral polymorphism or the result

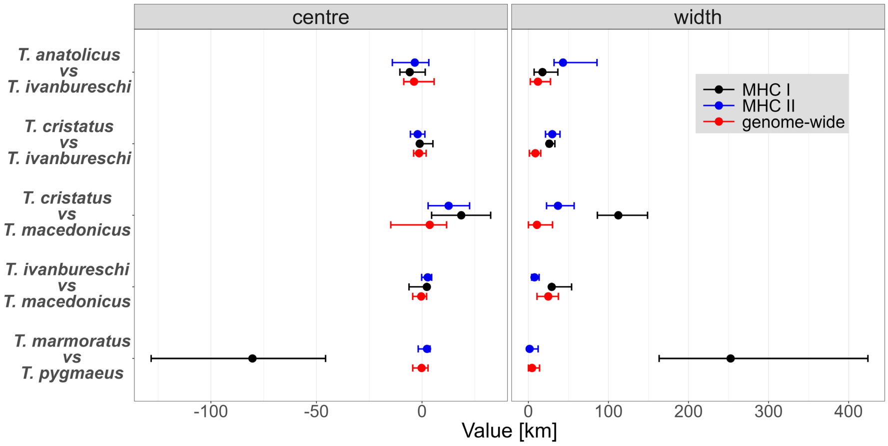

FIGURE 5 A comparison of Centre and width parameters of MHC and genome-wide geographic clines with the error bars corresponding to the 2 log-likelihood intervals. Due to broad sympatry, the *T. cristatus-T. marmoratus* zone was not suitable for cline analysis. Within each zone, the same cline model was fitted for the genome-wide, MHC-I, and MHC-II data.

of independent introgression from the adjacent *T. ivanbureschi* into each species. As multiple introgression events were observed in other systems, such as *Heliconius* butterflies (Pardo-Diaz et al., 2012) and oaks (Eaton et al., 2015), the latter explanation seems likely.

The analysis of clines also supports the adaptive introgression hypothesis. If introgression is prevalent and reaches allopatric populations, one can expect MHC clines to be wider than the genome-wide cline. We indeed detected broader clines for at least one MHC class in all hybrid zones except for *T. ivanbureschi-T. macedonicus*. This exception comes as a surprise, considering the extensive sharing of MHC-I alleles between the alloC populations of these two species. The *T. ivanbureschi-T. macedonicus* contact zone is the longest of the zones analysed here and is geographically complex (Wielstra, Burke, Butlin, & Arntzen, 2017). Some populations (e.g., in the northern part of the *T. macedonicus* range) may even experience introgression from two directions. It may be that the projection of such a complex 2D setting onto a 1D transect, as required for cline fitting, distorts the pattern. If the relative rate of MHC introgression differs in various parts of the zone, such a projection may place some non-introgressed populations close to the centre, therewith artificially narrowing the cline (Macholán et al., 2011). On the other hand, the MHC clines may be slightly widened as a consequence of using the hybrid index estimated from presence/absence data, although this effect should decrease with an increase in the number of analysed loci (Buerkle, 2005). Still, this effect might have contributed to the much wider MHC-I cline for *T. marmoratus* and *T. pygmaeus*, which are characterized by lower MHC-I polymorphism than Balkan species.

4.2 | Comparison between MHC classes

MHC-I introgression appears more extensive than MHC-II introgression. Allele sharing between allopatric populations from different species was commonly observed in MHC-I, while it was rare in MHC-II. No MHC-II alleles were shared between alloF populations, and sharing between alloC populations occurred in only two out of six hybrid zones. For instance, in the *T. cristatus-T. marmoratus* hybrid zone in France, no MHC-II alleles were shared between species in alloC populations (the ponds occupied by a single species) despite an extensive interspecific sharing of MHC-I alleles. The signal of interspecific MHC-I allele sharing in alloC populations was derived from many individuals and populations of both species, effectively ruling out the possibility of "contamination" caused by the incorrect assignment of a few individuals into species.

The elevated rate of MHC-I introgression may be related to its function. MHC-I is responsible for binding and displaying antigenic peptides from rapidly evolving intracellular pathogens, such as viruses (Murphy & Weaver, 2017). An intense selective pressure following secondary contact, for example, resulting from the exposure of the host species to new viruses, may facilitate introgression (Komar et al., 2003; Parrish et al., 2008; but see Streicker et al., 2010), as inferred for several antiviral genes that introgressed from Neanderthals to modern humans (Enard & Petrov, 2018). As *Ranavirus*, a major viral threat for newts and amphibians in general (Price et al., 2017), was shown to be a generalist that lacks host-specific genetic structuring, interspecific transfer of viruses following secondary contact seems likely (Epstein & Storfer, 2016).

However, the apparently stronger MHC-I introgression might also have a non-adaptive explanation. First, MHC-II variation is generally lower than MHC-I variation, which is well visible in the *T. cristatus-T. marmoratus* zone in France, where genetic variation was strongly reduced in the process of postglacial colonization from distant refugia (Wielstra et al., 2013, 2015). Consequently, MHC-II alleles in both species might be able to present a restricted and similar repertoire of antigens (for constrained MHC, see de Groot et al., 2017). Under these conditions, the introgressed MHC-II alleles would not confer a selective advantage, and increased introgression would not be expected. This hypothesis contrasts with the results of recent simulations of self-incompatibility genes in plants, which are also evolving under balancing selection. Pickup et al. (2019) showed that, irrespective of the barrier strength, the introgression is elevated if there are fewer S alleles. The contrast may, however, be only apparent. The hypothetical MHC scenario would correspond to the situation when the reduced variation in S genes does not result in sets of alleles of distinct specificities in each species but rather, in sets of distinct alleles with similar specificities in both species. Then, elevated introgression would not be expected as introgressed alleles would confer little fitness advantage. Second, while mostly a single functional MHC-II gene occurs in all *Triturus* species, multiple putatively functional MHC-I genes are present, so our MHC-I data simply represents a longer genomic region than the MHC-II data. Thus, the pure chance of maintenance and detection of some introgressed MHC-I alleles would be higher, even if introgression was neutral or equally beneficial in both MHC classes (Baird et al., 2003; Sachdeva & Barton, 2018).

4.3 | MHC introgression and hybrid zones history

Because the amount of ancestral polymorphism shared between species generally decreases over time, though the rate of the decrease may be slowed down by balancing selection, we expected that nonadmixed individuals of closely related species would share more MHC alleles (Dufresnes et al., 2021). Surprisingly, we did not detect the expected negative relationship between the fraction of shared MHC-I alleles and time of species divergence, when the para- vs allopatry of species ranges was taken into account. We did observe a tendency though, especially for the most closely related *T. anatolicus*-*T. ivanbureschi* species pair, which shared more alleles in both MHC classes than any other contact zone involving *T. ivanbureschi*. On the other hand, the distantly related *T. cristatus* and *T. marmoratus* showed the highest fraction of MHC allele sharing among all examined hybrid zones. It, therefore, seems that introgression determines the fraction of alleles shared, even between distantly related species, as long as the abutting or overlapping geographic ranges create an opportunity for introgression.

Two of the Balkan hybrid zones, *T. anatolicus*-*T. ivanbureschi* and *T. ivanbureschi*-*T. macedonicus*, experienced a considerable shift in their position (Wielstra, Burke, Butlin, & Arntzen, 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017). Theory predicts increased rates of neutral introgression from receding into advancing species (Barton & Hewitt, 1985; Currat et al., 2008; Petit & Excoffier, 2009; Rohwer et al., 2001; Secondi et al., 2006; Wielstra, 2019). *Triturus ivanbureschi*, which in both cases was a receding species, also forms a hybrid zone with *T. cristatus*; yet the location of this zone has been relatively stable (Wielstra, Burke, Butlin, & Arntzen, 2017). Surprisingly, we did not observe notable differences in allele sharing between these three hybrid zones, suggesting a similar rate of introgression. The past movement of the contact centre was also detected for the *T. marmoratus*-*T. pygmaeus* hybrid zone, where *T. pygmaeus* advanced by up to 200 km (Arntzen, López-Delgado, et al., 2021; López-Delgado et al., 2021). It is the only hybrid zone for which we observed a shift of the centre of the MHC cline (MHC-I) in comparison to the genome-wide cline. However, the direction of the shift is contrary to neutral expectations, as elevated introgression into advancing species should result in a cline shift towards *T. pygmaeus* rather than *T. marmoratus* territory. The expectations may differ in the case of adaptive introgression, which deserves further research. In principle, the asymmetry could be driven by the selective advantage of introgression into MHC variation-depleted species. However, as the differences in variation between species were negligible, asymmetry could perhaps be due to beneficial alleles that originated in *T. pygmaeus* before secondary contact.

4.4 | Comparison with other studies and limitations of the study

The evidence for MHC introgression in multiple *Triturus* hybrid zones provided here adds to a small set of recently published studies indicating that MHC introgression may be a widespread phenomenon. MHC introgression was reported in humans (Abi-Rached et al., 2011, MHC-I), Alpine ibex (Grossen et al., 2014, MHC-II), *Lacerta* lizards (Sagonas et al., 2019, MHC-I), *Lissotriton* newts (Fijarczyk et al., 2018; Dudek et al., 2019, both MHC classes) and hares (Pohjoismäki et al., 2021, MHC-II). MHC introgression, with various strengths of supporting evidence, was also suggested for penguins (Hibbets et al., 2020, MHC-II), fire-bellied toads (de Cahsan et al., 2021, MHC-II), chickens (Manjula et al., 2021, MHC region), and trouts (Talarico et al., 2021, MHC-II). Yet, a direct comparison of MHC introgression with genome-wide markers or the analysis of replicated transects has rarely been applied. Thus, despite the likely signatures of adaptive introgression, for example, rapid expansion of introgressed MHC alleles, alternative explanations have only infrequently been explicitly tested and ruled out. To the best of our knowledge, our study is the first one to indicate differences in the extent of introgression between MHC classes.

Our study has several limitations, mainly stemming from the nature of MHC polymorphism and the available molecular tools. First, our tests were based on the assumption of recent or ongoing introgression that has not permeated the entire species' ranges. This assumption has been partially relaxed by using models that tested the effect of para−/allopatry of species ranges and the time of species divergence on interspecific allele sharing outside the hybrid zones. Second, the genomic complexity of newt MHC, class I in particular, necessitates simultaneous amplification of multiple genes. The alleles could not be assigned to loci and were necessarily analysed as binary presence/absence data, which may have led to less accurate estimates of the hybrid index compared to estimates from single codominant loci. The simple approach based on binary encoded alleles may be considered unsatisfactory; however, viable alternatives in non-model organisms are currently lacking. The ideal data for testing MHC introgression and its adaptive nature would consist of haplotype-resolved MHC regions from multiple individuals, densely sampled for each species involved. Fortunately, the rapid advances in long-read sequencing or methods such as haplo-tagging bring some promise (Amarasinghe et al., 2020; de Coster et al., 2021; Meier et al., 2021). However, it is unlikely that we will be able to obtain the relevant population-scale data for nonmodel systems soon. Therefore, a medium-term goal would be to validate and explore the limits of simpler methods, similar to the one applied here, to detect MHC introgression and test its adaptive character based on MHC data from various species that may differ considerably in the genomic complexity of the MHC region. Finally, for introgression testing, classifying populations into parapatric and allopatric groups might be considered subjective. To overcome this limitation, we used a conservative approach based on a threshold of genome-wide admixture.

4.5 | Conclusions

Our analysis of the *Triturus* system sheds light on several aspects of MHC introgression. A widespread signal of MHC introgression was detected in hybrid zones between several *Triturus* species, including the deeply divergent crested and marbled newts. We obtained evidence that MHC introgression is more extensive than genome-wide introgression, suggesting its adaptive character. Our work, together with a small number of previous studies, suggests that MHC introgression may be a widespread phenomenon, providing adaptively relevant variation even at the final stages of speciation (Barton, 2020). We notice an urgent need for broader comparative studies that would bring a quantitative view of the role of MHC introgression in generating new variation, including possible differences between MHC classes in the potential for adaptive introgression.

AUTHOR CONTRIBUTIONS

Wiesław Babik, Tomasz S. Gaczorek and Ben Wielstra designed the study. Laboratory work was performed by Katarzyna Dudek and Marzena Marszałek. Analyses were performed by Tomasz S. Gaczorek. Tomasz S. Gaczorek and Wiesław Babik wrote the manuscript with help from Ben Wielstra and Jan W. Arntzen. All authors revised the manuscript and approved the final version.

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DATA AVAILABILITY STATEMENT

Due to the short length of MHC-II alleles (≤ 200 bp), we were unable to upload the detected MHC sequences to GenBank. However, they have been made available at the Dryad Digital Repository (doi:10.5061/dryad.pk0p2ngqs).

ORCID

Tomasz S. Gaczorek https://orcid.org/0000-0001-6370-5161
Katarzyna Dudek https://orcid.org/0000-0002-9810-2536
Jan W. Arntzen https://orcid.org/0000-0003-3229-5993
Ben Wielstra https://orcid.org/0000-0002-7112-5965
Wiesław Babik https://orcid.org/0000-0002-1698-6615

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