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Widespread introgression of MHC genes in Iberian *Podarcis* lizards

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Abstract

Major histocompatibility complex (MHC) genes are crucial for the adaptive immune response of jawed vertebrates. Their variation, reaching extreme levels, is driven mainly by an arms race between hosts and pathogens. One hypothesised mechanism contributing to MHC polymorphism is adaptive introgression, the exchange of genetic variants between hybridising species favoured by selection, yet its effect on MHC variation is poorly understood. Detection of adaptive MHC introgression, though challenging, may be facilitated by the analysis of species complexes forming multiple hybrid zones. Here, we investigated MHC introgression in six hybrid zones formed by seven species of *Podarcis* lizards inhabiting the Iberian Peninsula. To differentiate adaptive introgression from neutral introgression, we compared the patterns of gene exchange in MHC and genome-wide markers. We found elevated sharing of MHC alleles in the proximity of contact beyond the areas of detectable genome-wide admixture in most hybrid zones and, in half of them, asymmetric MHC exchange. In general, the elevated MHC allele sharing between species pairs with abutting ranges compared to geographically isolated species pairs also supports the prevalence of introgression. Collectively, our results demonstrate widespread MHC introgression in the Iberian *Podarcis* complex and suggest its adaptiveness. Contrary to previous results from *Triturus* newts, we did not observe differences in the rate of introgression between MHC classes. Our work adds support to the emerging view of adaptive introgression as a key mechanism shaping MHC diversity. It also raises questions about the effect of elevated MHC variation and factors leading to the asymmetry of adaptive introgression.

KEYWORDS
adaptive introgression, hybridisation

1 | INTRODUCTION

Introgression is a widespread phenomenon, detected and described in many animal and plant systems (Abbott et al., 2013; Anderson, 1953; Edelman & Mallet, 2021; Mallet, 2005). It is the process of gene exchange between differentiated populations, including species that are not fully reproductively isolated, through hybridisation and repeated backcrossing of hybrid offspring into the parental populations (Aguillon et al., 2022; Anderson, 1953). Introgression is often limited by reduced fitness of hybrids stemming

from genetic, behavioural or ecological factors (Araripe et al., 2010; Barton, 2001; Barton & Hewitt, 1985; Forsdyke, 2019; Ishikawa & Kinoshita, 2009; Johnson, 2010; Vaid & Laitinen, 2019). Other factors may, however, facilitate introgression.

One such mechanism is recombination, which reduces the fitness cost of introgression by separating beneficial or neutral loci from the genome segments that are deleterious on the foreign genomic background (Barton, 1983; Barton & Bengtssont, 1986). The other factor facilitating introgression is positive selection—a strong fitness advantage of some introgressing variants may directly outweigh the fitness costs of linked deleterious variants (Barton, 1979; Pialek & Barton, 1997). If introgression of a foreign allele results in an immediate fitness increase of individuals carrying it, it is referred to as adaptive introgression. Both factors interact—the fitness advantage may outweigh fitness costs more easily if recombination reduces the linked deleterious load (Fitzpatrick et al., 2010; Uecker et al., 2015).

Well-known examples of adaptive introgression include newly acquired traits conferring clear fitness benefits and rapidly rising to high frequencies under directional selection, for example, the *EPAS1* gene of Denisovan origin (Huerta-Sánchez et al., 2014), contributing to the adaptation of Tibetans to high altitude, pesticide resistance genes in mice (Song et al., 2011), or genes responsible for the environmental adaptation in oaks (Leroy et al., 2020) and canids (Wang et al., 2020). Apart from directional selection, balancing selection that favours novel or rare alleles may also enhance adaptive introgression (Castric et al., 2008). Interestingly, the adaptive introgression driven by balancing selection has some unique characteristics resulting from how selection shapes allele frequencies. First, the introgressed gene variants are usually not driven to fixation, as in the directional selection scenario, but are maintained as polymorphisms. Consequently, genetic variation may increase instead of being lost. Second, introgression driven by balancing selection is not a single event but might be recurrent, unless the system becomes saturated or reproductive isolation between species becomes complete. Hence, genes under balancing selection should be among the last that stop introgressing as speciation becomes complete. Although the potential of adaptive introgression under balancing selection is well recognised (Castric et al., 2008; Fijarczyk et al., 2018), its rate and effect on genetic diversity are poorly understood.

Evidence for adaptive introgression has recently started to accumulate in several genetic systems evolving under balancing selection, such as the self-incompatibility complex in plants (Castric et al., 2008; Glémin et al., 2005), wing pattern genes in *Heliconius* butterflies (Pardo-Diaz et al., 2012) and vertebrate MHC genes (Abi-Rached et al., 2011; Dudek et al., 2019; Gaczorek et al., 2023; Grossen et al., 2014; Hedrick, 1999; Schierup et al., 2001; Sommer, 2005). The MHC is a particularly interesting example because of its extraordinary polymorphism, its role in the adaptive immune response and its importance for conservation biology (Sommer, 2005).

Major histocompatibility complex genes encode protein receptors that bind antigenic peptides and activate T lymphocytes. Two classes are generally distinguished, class I (MHC-I), responsible for the detection of intracellular pathogens (e.g. viruses), and class II (MHC-II), which recognises extracellular threats (e.g. bacteria or parasites) (Murphy & Weaver, 2017). The extreme diversity of MHC genes is seen at both the individual and population levels and is manifested as numerous alleles per locus and numerous gene copies per genome, their number often differing between haplotypes. In isolation, the novel MHC diversity is generated mostly by gene conversion events but point mutations, following frequent duplication events, and reciprocal recombination are also involved (Klitz et al., 2012). After secondary contact and hybridisation between related species, the pool of MHC alleles may be expanded via adaptive introgression. While the probability of de novo beneficial mutation is low, adaptive introgression is a source of variants already selected at another genomic background and might be an efficient way to obtain the well-adapted alleles in the constant arms race between hosts and pathogens (Hedrick, 2013).

The assessment of the effect of adaptive introgression on MHC variation is challenging for several reasons. First, the detection of introgression might be difficult. The reason is that ancestral polymorphism (trans-species polymorphism sensu stricto), parallel evolution and introgression can generate similar patterns of allele sharing between species (Fijarczyk & Babik, 2015). These mechanisms can, however, be differentiated when a broader picture is considered. Ancestral polymorphism alone should result in a geographically uniform distribution of alleles shared between species, while multiple independent cases of parallel evolution are unlikely. Second, adaptive introgression must be distinguished from neutral introgression. Such distinction requires a direct comparison of introgression rate between a presumably adaptive locus and the genome-wide variation of neutral introgression levels. Third, frequent duplications of MHC genes and interlocus allele sharing make it hard to reconstruct the structure of MHC haplotypes, limiting the use of standard methods for introgression testing. Following Dudek et al. (2019), our general solution is to compare MHC and genome-wide markers in a framework of multiple hybrid zones. An elevated rate of MHC allele sharing in the proximity of the contact and repeatability of pattern among independent hybrid zones demonstrates recent introgression by ruling out alternative explanations. If such a pattern is observed for populations without signs of genome-wide introgression, adaptive introgression is strongly suggested.

Here, we test for adaptive MHC introgression in lizard species from *Podarcis hispanicus* complex inhabiting the Iberian Peninsula. The spatial complexity of this system and the available dense sampling make it an excellent model for such analyses. Eight *Podarcis* species belonging to *P. hispanicus* complex occur in Iberian Peninsula (*P. bocagei*, *P. carbonelli*, *P. guadarramae*, *P. hispanicus*, *P. liolepis*, *P. lusitanicus*, *P. vaucheri* and *P. virescens*). They diverged approximately 8–13 million years ago (Mya; Kaliontzopoulou et al., 2011; Yang et al., 2021). Although their relationships are still disputed, with several conflicting phylogenies available in the literature (Caeiro-Dias, Rocha, et al., 2021; Salvi et al., 2021; Yang et al., 2021), there is an agreement that the *P. hispanicus* complex, including the Iberian and North African species, form a monophyletic group. Recent studies suggest that some speciation events overlapped the Messinian

Salinity Crisis (5.96–5.33 Mya), which might have contributed to speciation due to the reduction of ecological niche availability (Yang et al., 2021). Following, most likely, allopatric speciation (Caeiro-Dias et al., 2018), Iberian taxa from that species complex expanded their ranges and established secondary contact zones. Until now, multiple hybrid zones have been reported, though in most of them, the overall genome-wide introgression appears highly limited (Caeiro-Dias, Brelsford, et al., 2021; Caeiro-Dias, Rocha, et al., 2021; Pinho et al., 2009). The hybrid zone between *P. carbonelli* and *P. bocagei* on the northwest coast of Portugal is the most well-studied and provided solid evidence for introgression (Caeiro-Dias, Brelsford, et al., 2021; Caeiro-Dias et al., in review; Pinho et al., 2009). The evidence for introgression was also found in areas where *P. carbonelli* is sympatric with *P. lusitanicus*, *P. vaucheri* and *P. virescens* (Caeiro-Dias, Brelsford, et al., 2021; Caeiro-Dias et al., in review). They all experience variable but limited genome-wide patterns of introgression (Pinho et al., 2008; Yang et al., 2021). Currently, evidence of substantial introgression in other contact zones is lacking.

We start with describing MHC variation and signal of selection in MHC sequences. Then, we compare levels of admixture for MHC genes with the genomic background of admixture estimated with SNPs genotyped by RAD sequencing; a repeated pattern of elevated admixture for MHC genes relative to the genomic average would suggest adaptive introgression. We also compare the levels of introgression between the two MHC classes. Finally, we take advantage of the *P. hispanicus* complex in Iberian Peninsula, with multiple species exhibiting complex geographic distributions, to address two further questions. First, we check whether an opportunity for MHC introgression is sufficient for it to occur. To test it, we compare the overall MHC allele sharing between species with and without abutting distributions. Second, we test whether MHC introgression is symmetrical, focussing on the trio of species that have the potential to hybridise with each other: *P. guadarramae*, *P. liolepis* and *P. virescens*.

2 | MATERIALS AND METHODS

2.1 | Samples and genome-wide data

2.1.1 | Samples

Major histocompatibility complex variation in the Iberian *Podarcis* was studied using available, previously collected samples (Caeiro-Dias, Brelsford, et al., 2021; Caeiro-Dias, Rocha, et al., 2021; Caeiro-Dias et al., in review; Kaliontzopoulou et al., 2011; Table S1). The sampling attempted to maximise the coverage of species ranges and the number of localities, preferably with the known level of genome-wide admixture. In total, we sampled 178 unique localities (666 individuals) of seven *Podarcis* species (*P. bocagei*—17 (39 individuals), *P. carbonelli*—29 (30), *P. guadarramae*—12 (67), *P. hispanicus*—13 (24), *P. liolepis*—32 (111), *P. vaucheri*—12 (80), *P. virescens*—39 (158) and undefined or consisting of multiple species—24 (157), Figure 1a). Among chosen samples, 50.9% had direct information about genome-wide ancestry, while for 29.6%, the ancestry was inferred based on species distributions (see below). Due to the lack of DNA samples, our study does not include hybrid zones of *P. lusitanicus*.

2.1.2 | Species distributions

To map the distributions of our focal Iberian *Podarcis* species, we used the database maintained in CEFE (Montpellier, France) and

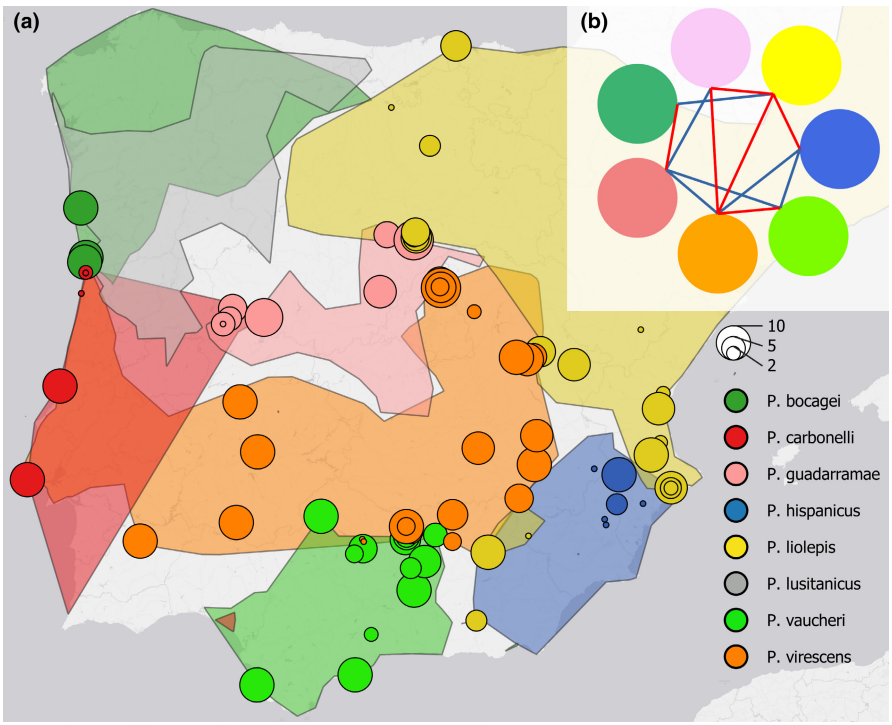

FIGURE 1 (a) Map of genotyped *Podarcis* samples, together with the species distributions inferred from the databases of georeferenced localities (see *Species distributions* section). (b) The net of analysed *Podarcis* species. The lines connect species with abutting ranges. The hybrid zones with available dense sampling close to the contact zones, which are explored in this article, are denoted with red lines.

in CIBIO-InBio (Vairão, Porto, Portugal), containing georeferenced locations of *Podarcis* individuals identified by genetics or morphology on the basis of voucher specimens or photographs or live individuals (date of access, 24 February 2023). The distribution range of each species was defined as the minimum area overlapping the corresponding records. To generate the distribution ranges in QGIS (v3.26.0), we used the 'concave hull' algorithm with the *k* parameter equal to 40 and a prior assumption of few enclaves; *P. carbonelli* within *P. vaucheri* distribution (near Doñana National Park, Spain), *P. guadarramae* within *P. virescens* (near Trujillo, Spain), *P. vaucheri* adjacent to *P. hispanicus* (near Almeria, Spain), and *P. liolepis* within *P. hispanicus* territory (eastward of Cazorla; Sierra Espuña Regional Reserve; Sierra de Baza Nature Reserve; Sierra Nevada National Park, Spain; Pinho et al., 2007a, 2007b, used database).

2.1.3 | Genome-wide ancestry

We used the available STRUCTURE v2.3.4 (Pritchard et al., 2000) Q-scores based on genomic SNPs as estimates of the average genome-wide ancestry. For *P. bocagei-P. carbonelli* hybrid zone Q-scores were obtained from Caeiro-Dias et al. (in review). For individuals from the other five species, Q-scores were estimated in the context of another ongoing comparative study of hybrid zones. Briefly, sequencing data were acquired from two libraries prepared following a double digestion restriction site associated DNA (ddRAD) protocol modified from Peterson et al. (2012), Parchman et al. (2012) and Purcell et al. (2014). The complete protocol is described by Brelsford et al. (2016). One library containing a total of 665 individuals was previously described by Caeiro-Dias, Brelsford, et al. (2021), and another containing 749 individuals was reported by Caeiro-Dias, Rocha, et al. (2021). Raw sequences were demultiplexed, SNPs called and filtered, and STRUCTURE analysis was conducted as described in Caeiro-Dias, Brelsford, et al. (2021). All Q-scores and the number of SNPs used are reported in Table S2. Note that due to the overrepresentation of RADseq data in contact proximity and low level of overall genome-wide admixture, STRUCTURE analyses were performed only for pairs of neighbouring species. For introgression analyses, the individual's ancestry estimates (Q-scores) of the remaining species were set to 0. We compiled/collected the ancestry estimates for 339 individuals (135 populations) representing all seven analysed *Podarcis* species. We defined an individual as nonadmixed if its ancestry from any species was ≥99%. Such a strict threshold was chosen to limit possible misassignments stemming from uneven sampling causing the underestimation of low-level admixture (Toyama et al., 2020). Despite the strict threshold, only 21 individuals (6.2%) showed any sign of admixture, confirming that the current genome-wide admixture between the Iberian *Podarcis* species is extremely limited. Additionally, we treated 197 individuals which lacked genetic information as nonadmixed if they were located at least 25 km from the margin of other species' ranges or the locality they belonged to included only genetically identified nonadmixed individuals.

2.2 | MHC genotyping

We genotyped the variable second exon of MHC-I and MHC-IIB genes. Below we describe in detail the design of primers and genotyping procedure.

2.2.1 | Primer design

We used available predictions of protein-coding DNA sequences (CDS) based on the genome assembly of one *P. muralis* individual (GCA_004329235, Andrade et al., 2019) to identify sequences of second exon of MHC-I and MHC-IIB genes. We found MHC genes by comparing *P. muralis* CDSs with human MHC proteins HLA-B and HLA-DRB1 using tblastn implemented in BLAST+ v2.9.0 (Camacho et al., 2009). To increase the sensitivity of our searches for even highly differentiated MHC sequences, we extracted second exon of genes identified in the previous step and used them as queries in the final blastn (BLAST+) search of *P. muralis* CDS and three de novo transcriptome assemblies: *P. cretensis* (SRR3201591, SRR3201796), *P. liolepis* (SRR9090247) and *P. siculus* (SRR3479613-24). Then, to enrich for functional classical MHC sequences, we filtered out those showing signs of pseudogenisation or nonclassical nature. To this end, we removed sequences at the end of very long branches on the neighbour-joining tree, as well as those covering genomic regions shorter than 1000 bp or encoding proteins which lack amino acids conserved throughout vertebrate classical MHC proteins (Kaufman et al., 1994). Finally, we designed primers based on 51 different MHC-I (6—*P. cretensis*, 11—*P. muralis*, 3—*P. liolepis*, 31—*P. siculus*) and 26 MHC-II (2—*P. cretensis*, 9—*P. muralis*, 4—*P. liolepis* and 11—*P. siculus*) sequences. We ended up with two primers for MHC-I (1F, 1R) and six for MHC-II (3F, 3R). Their sequences are shown in Table S3.

2.2.2 | Genotyping and its repeatability

The second exon of MHC genes was PCR amplified and sequenced (pair-end) on the Illumina® platform following Fijarczyk et al. (2018). We amplified 233 bp and 235 bp (excluding primers) fragments of the second exon of MHC-I and MHC-IIB, respectively, in 10 μL PCR reactions containing: 50–100 ng of genomic DNA (gDNA) and 5 μL of Multiplex PCR kit (Qiagen). All MHC-I primers were used at 1 μM, while the MHC-II primers were used at 0.25 (F2, F3, R3) or 0.5 μM (F1, R1, R2). PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 33 (MHC-I) or 35 (MHC-II) cycles: 95°C for 30 s, 56°C (MHC-I) or 58°C (MHC-II) for 30 s and 72°C for 70 s and the final elongation at 72°C for 10 min. Individual amplicons were barcoded with a combination of 6 bp indexes at the 5′ end of forward and reverse primers. Amplicons were pooled approximately equimolarly based on gel-band intensity, pools were gel-purified, Illumina adaptors were ligated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs), and libraries were quantified

with Qubit fluorometer (Invitrogen®, Thermo Fisher Scientific) and sequenced on Illumina® MiSeq (v3 600 cycles kits).

The sequencing data were then processed with AmpliSAS software which identified MHC alleles occurring in each individual (Sebastian et al., 2016). Clustering and filtering parameters are described in Appendix S1 (Table S4). Sequence variants that deviated by more than six base pairs from the expected length (233 bp—MHC-I and 235 bp—MHC-II) were treated as nonfunctional and were filtered out. The genotyping repeatability was estimated for 30 individuals, amplified and sequenced in two replicates. Precisely, we divided the number of variants present in both replicates by the total number of detected variants. Finally, the data were transformed into a binary, presence-absence matrix, with MHC sequence variants in columns and individuals in rows, which was used for subsequent introgression analyses. Throughout the text, we use for simplicity the term 'allele' for MHC sequence variants, although we are aware that the 'alleles' are derived from multiple MHC genes. Consequently, most individuals have more than two alleles in the genotype matrix (see results).

2.2.3 | Identification of putatively nonfunctional alleles

Apart from classical MHC genes central to the adaptive immune response, nonclassical MHC genes and MHC pseudogenes can occur in the MHC region. They may show sequence similarity to and co-amplify with classical MHC genes (Chattopadhyay et al., 2014). The forces shaping their evolution are, however, strikingly different. While variation in the classical MHC genes is maintained mainly by balancing selection, the evolution of nonclassical and nonfunctional genes is shaped mostly by negative selection and neutral processes (Ballingall & McKeever, 2005; Tsukamoto et al., 2013). Mixing genes evolving under such contrasting evolutionary mechanisms may reduce the power of tests of adaptive introgression, which is expected mostly for classical MHC genes. Therefore, we decided to exclude putatively nonfunctional and nonclassical sequences from the set of amplified alleles. Our approach was similar to the one used by Fijarczyk et al. (2018) and Gaczorek et al. (2023) and is described in Appendix S1 (Figures S1 and S2). Note that this procedure should not be mistaken for filtering out putatively nonfunctional genomic or transcript sequences performed during primer design. After filtering, we excluded 14 (0.6%) MHC-I and 78 (4.6%) MHC-II alleles from our final dataset. The remaining 2152 MHC-I and 1632 MHC-II unique alleles were used to describe MHC diversity in the Iberian *Podarcis* species from the *P. hispanicus* complex and to test introgression. The final dataset should be enriched for functional classical MHC variants but might still contain some nonfunctional or nonclassical sequences.

2.3 | Selection analysis

Selection analysis of individual allele sequences was conducted in MEGA v11 (Kumar et al., 2012) and HyPhy (Pond & Muse, 2005) software. In MEGA, we calculated mean pairwise nucleotide distances (Tamura-Nei), amino acid distances (Poisson correction) and synonymous/nonsynonymous substitutions (Nei-Gojobori). We then performed an overall average codon-based *Z*-test of selection, which compares the rates of synonymous and nonsynonymous substitutions and calculates the probability of selection acting on the sequences. For all analyses, the standard error was estimated with 100 bootstrap replicates. These results were obtained first for all alleles (excluding those removed beforehand, e.g. putative nonfunctional alleles) and for all codons in the sequence. Next, the same analysis was conducted, but we differentiated between peptide-binding (PBS) and non-PBS sites. PBS sites were identified based on the alignment with human MHC sequences: HLA-A, HLA-B and HLA-C for class I; and HLA-DP, HLA-DQ and HLA-DR for class II (Reche & Reinherz, 2003).

In HyPhy v2.2.4, sites under positive and negative selection were identified for both MHC classes with the FUBAR model with default settings (Murrell et al., 2013), using all sequences regardless of individual ancestry. Before analysis, we checked the sequences for signatures of recombination using RDP4 v5.23 software (Martin et al., 2021). At a *p*-value of .05 following Bonferroni correction, RDP and GENECOV methods detected no recombinant sequences, while MaxChi identified <20 sequences which were removed. The phylogenetic trees used in the FUBAR analysis were constructed in MEGA v11 with the neighbour-joining method using maximum likelihood nucleotide distances. An agreement between the positively selected sites and PBS was assessed with the chi-square test. A consensus sequence (frequency threshold = 0.4) and a sequence logo representation were generated using EMBOSS (Rice et al., 2000) and WebLogo v2.8.2 (Crooks et al., 2004).

2.4 | The number of MHC genes in *P. muralis* genome

The available *P. muralis* genome assembly (GCA_004329235, Andrade et al., 2019) is chromosome level and includes a relatively well-assembled MHC region. We used it to estimate the number of MHC genes in *P. muralis* and to explore the possibility of assigning MHC alleles detected in our focal Iberian *Podarcis* to loci. We extracted sequences of the second exon of MHC genes from *P. muralis* CDSs (see *Primer design*, 43—MHC-I and 26—MHC-II) and compared their annotated chromosomal coordinates. Based on the nonoverlapping coordinates, we identified 18 MHC-I and 10 MHC-II putative loci, of which 6 MHC-I and 1 MHC-II loci were classified as nonfunctional or nonclassical according to the criteria described above. Thus, we expect that *P. muralis* has a minimum of 12 and 9 putatively functional MHC-I and MHC-II genes, respectively (Figure S3).

Unfortunately, we were not able to assign MHC alleles from other *Podarcis* species to the genes identified in the *P. muralis* genome. The poorly supported relationships between alleles ruled out an assignment of the alleles to loci based on sequence clustering. Moreover, some transcripts presumably corresponding to

different loci, based on chromosomal coordinates, were almost identical in sequence. It might result from errors in the genome assembly or specific MHC characteristics in *Podarcis*, such as recent gene duplications or MHC allele sharing between genes. In MHC-II, some putative loci were also present on unassembled contigs. They might be the assembly artefacts, causing an overestimation of the number of MHC-II loci.

2.5 | MHC allele sharing among *Podarcis* species

If MHC introgression commonly affects large parts of species ranges, then the geographic opportunity for hybridisation should predict the extent of allele sharing between species. To test this prediction, we modelled, using GLM, the fraction of alleles shared between species pairs as a function of three explanatory variables: type of contact (the pair has abutting ranges or species are geographically isolated, Figure 1b), time of divergence (Yang et al., 2021) and MHC class. The model and the permutation procedure (Dryad Repository, doi: 10.5061/dryad.hqbzkh1m7) addressing the problem of non-independence among pairwise observations followed Gaczorek et al. (2023) with two meaningful differences. First, by including information about MHC classes, we directly tested for differences between them while controlling for the time of species divergence and type of contact. Second, we added the observation-level random effect (OLRE) to deal with overdispersion in the fitted binomial model (DHARMa nonparametric dispersion test; Hartig, 2022). We excluded all interaction terms from the model as their effect was not significant, and the meaning of the interactions was unclear (e.g. regression lines were clearly separated at the analysed timescale). We admit, however, that their removal significantly increased the residual deviance (Chi-square $p = .01$).

2.6 | Patterns of MHC admixture

To assess the extent of interspecific admixture in the Iberian *P. hispanicus* system, we estimated, separately for each hybrid zone and MHC class, the per-individual hybrid index (h-index). The obtained values, ranging from 0 to 1, can be seen as the probability of belonging to a given species based on the analysed genetic marker, with 0 and 1 referring to nonadmixed individuals. To estimate the h-index, we considered each MHC allele as a dominant biallelic locus and implemented the method of Buerkle (2005). This approach was previously tested with computer simulations and shown to perform satisfactorily (Gaczorek et al., 2023).

Unfortunately, due to the characteristics of the Iberian *P. hispanicus* complex, we could not apply the standard introgression testing methods utilising the hybrid index, that is, geographic and genomic clines (Gompert et al., 2017; Szymura & Barton, 1986). The method of geographic clines could not be used because we were not able to meaningfully reduce the spatially complex sampling to one-dimensional transects in the majority of contact zones. The fitting of genomic clines was problematic because of the extreme bimodality of the genome-wide ancestry. Thus, we only visualised the MHC admixture by plotting the hybrid index against genome-wide ancestry.

2.7 | Testing adaptive introgression

Assuming the system has not been saturated and introgression is still ongoing, we should observe differences in the fraction of exchanged MHC alleles across the species distribution. The farther from the contact zone, the fewer alleles of foreign origin are expected. The adaptive and neutral introgression differ, however, in their geographic signatures of introgression, with adaptive introgression being more extensive. The adaptive variants introgressing faster than other genomic fragments (Martin & Jiggins, 2017) are supposed to reach populations with no signs of genome-wide introgression. This, in turn, generates a stratification among nonadmixed populations with an excess of introgressed genes in the contact proximity compared to populations located farther. It is because the allele transfer into parental species, though driven by selection, is still limited by individual dispersal rates (Barton & Hewitt, 1985). Thus, considering only populations without detectable genome-wide admixture, an elevated interspecific MHC similarity in parapatry would suggest ongoing adaptive introgression of MHC genes in the *Podarcis* system.

We tested for the signal of adaptive MHC introgression using a randomisation test by comparing MHC allele sharing between parapatric and allopatric populations of two hybridising species. Both categories of populations included only individuals without detectable genome-wide admixture. Note that exclusively for this test, to include the *P. bocagei*–*P. carbonelli* hybrid zone, we treated all individuals from the Subportela (northmost locality of *P. bocagei*) as nonadmixed. They served as the representation for *P. bocagei* allopatry. We lack SNP data for this population, and initially, it was not classified as nonadmixed due to the overlapping distribution with *P. lusitanicus*. *Podarcis bocagei* and *P. lusitanicus* live in broad sympatry and are known for their strong reproductive isolation with only occasional admixture (Caeiro-Dias, Rocha, et al., 2021). The sympatry, however, spans over or is adjacent to all sampled populations of *P. bocagei*. Thus, potential admixture from *P. lusitanicus* should equally affect para- and allopatry and should not affect our test results for the *P. bocagei*–*P. carbonelli* hybrid zone.

Para- and allopatry were defined based on the shortest distance to the inferred distribution of the other species—the populations at the distance of minimum 25 km were considered allopatric, and the remaining were considered parapatric. Importantly, our classification was independent for each hybrid zone so that a particular population could fall into the parapatric or allopatric category depending on the analysed species pair. The detailed population assignment is shown in Table S5.

Following the assignment of localities into allo- and parapatric, we calculated and compared the percentage of alleles shared

between species for the two population categories and each hybrid zone. We tested for significance of difference with 10,000 permutations randomly assigning individuals within species to allo- and parapatry. The *p*-value was calculated as the fraction of permutations with a larger difference in allele sharing between both categories than in the observed data.

3 | RESULTS

3.1 | MHC variation

We successfully genotyped 665 individuals for MHC-I and 657 individuals for MHC-II (Table S6), detecting 2152 MHC-I and 1632 MHC-II putatively functional alleles. The repeatability of genotyping was 98% for MHC-I and 96% for MHC-II. On average, a nonadmixed *Podarcis* individual possessed 16.3 MHC-I and 10.5 MHC-II alleles. The number of alleles per individual in *P. liolepis* MHC-II was significantly larger than in all other species ($p < .001$), except for *P. hispanicus* ($p = .051$, Tukey post hoc test, Figure 2). Significant differences were also detected between *P. guadarramae* and *P. vaucheri* (both MHC classes), *P. carbonelli*–*P. vaucheri* (MHC-I), and *P. bocagei*–*P. guadarramae* (MHC-II, Table S7). Singletons constituted 40.3% of MHC-I and 46.5% of MHC-II alleles. Most detected alleles were also private to a single species (80.0%—MHC-I and 90.6%—MHC-II). Within species, however, the percentage of private alleles varied from 45.1% (*P. guadarramae*) to 76.7% (*P. vaucheri*) for MHC-I and 61.2% (*P. hispanicus*) to 91.4% (*P. vaucheri*) for MHC-II (Table S8). *Podarcis liolepis* and *P. virescens* showed the highest values of allelic richness standardised to the sample size of 10 individuals for both MHC classes. This might reflect extensive sampling covering the broad ranges of these two species.

3.2 | Selection analysis

The sequence divergence between alleles is presented in Table S9. Overall, both nucleotide and amino acid distances were substantial. Regarding all codons, the mean nucleotide distance between alleles for MHC-I equalled 0.16 ± (SE) 0.02 and for MHC-II 0.20 ± 0.02. The amino acid distance equalled 0.29 ± 0.04 for MHC-I and 0.38 ± 0.04 for MHC-II. For MHC-I, the dN/dS value for the whole sequence was 0.76. The *Z*-test of selection suggested negative selection for whole sequences and non-PBS and positive selection for PBS, though the results were significant only for the non-PBS sites ($p = .01$). For MHC-II, dN/dS ratio for the whole sequence was 2.05, and the *Z*-test indicated positive selection in every category, with significant ($p < .05$) values for the whole sequence and the PBS.

FUBAR identified 18 codons under positive selection in MHC-I and 21 in MHC-II at posterior probabilities higher than 95%. FUBAR also identified 24 and 23 codons under negative selection in MHC-I and II, respectively. Positively selected sites corresponded somewhat to PBS inferred from a comparison with human PBS, with more PBS under diversifying than under purifying selection. The chi-square test for an overlap between positively selected sites and PBS showed a significant ($p = .03$) result for MHC-I and nearly significant ($p = .054$) for MHC-II. The results of the analyses are presented in Figure S4.

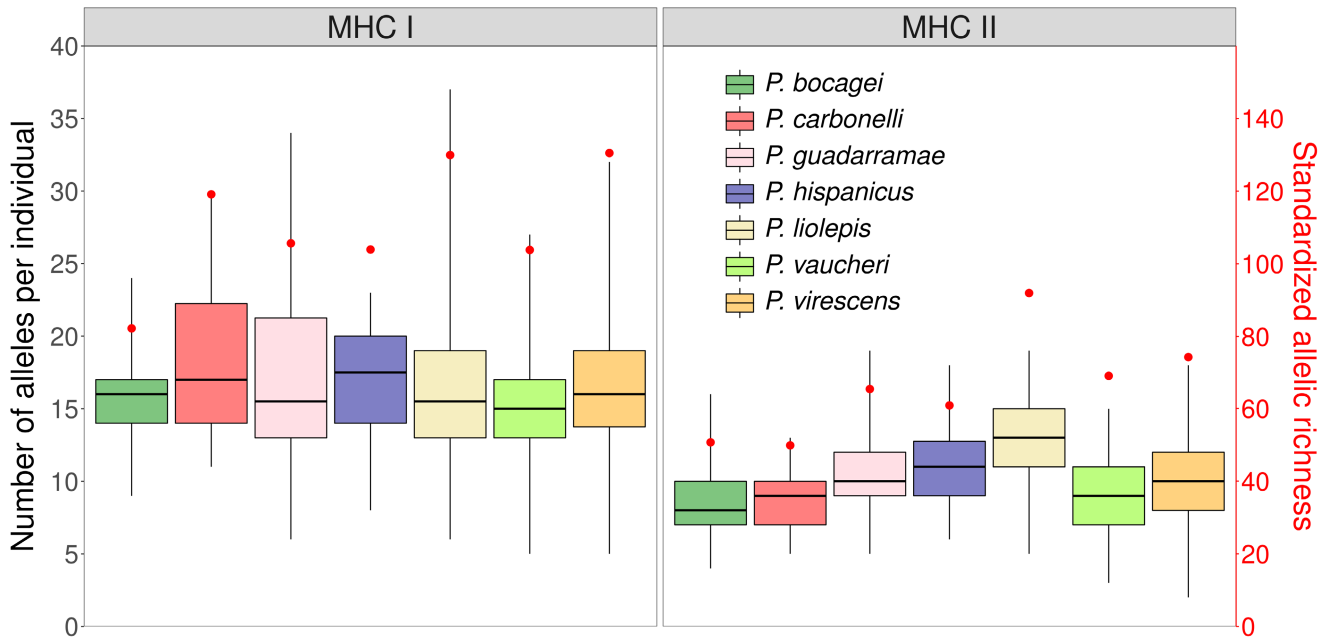

FIGURE 2 Number of alleles per individual (boxplots) and allelic richness standardised to the sample size of 30 individuals (red dots). Note separate axes for both measures. The thick horizontal line, box and whiskers in boxplots correspond to the median, values between the first and third quartile, and the range, respectively.

3.3 | MHC allele sharing among Podarcis species

The sharing of MHC alleles between pairs of *Podarcis* species, considering only nonadmixed individuals, calculated by diving the number of shared alleles by the total number of alleles within a focal pair, was limited, with a mean value of 5.33% for MHC-I and 2.20% for MHC-II (Figure 3). The most extensive sharing in MHC-I was detected for the *P. guadarramae–P. virescens* pair (12.81%) and in MHC-II for *P. bocagei–P. carbonelli* (8.41%). The GLM showed an elevated allele sharing between species with abutting ranges ($p = .007$) but no significant differences between MHC classes ($p = .97$). Sharing between isolated species was not significantly higher than zero ($p = .07$), but it might result from adding the OLRE factor, reducing detection power and, thus, widening confidence intervals. The effect of divergence time was not significant ($p = .80$, Figure S5).

3.4 | Testing for asymmetry and signals of adaptive introgression

A comparison of genome-wide ancestry and the MHC h-index revealed asymmetry in MHC introgression within three out of six analysed hybrid zones (Figure 4, Figure S6, Table S10). Almost unidirectional flow of MHC alleles was detected from *P. bocagei* to *P. carbonelli*, from *P. guadarramae* to *P. virescens* and from *P. hispanicus* to *P. liolepis*. We did not observe any substantial differences between the MHC classes.

We observed an elevated MHC allele sharing between parapatric populations of hybridising species compared with allopatric populations in most of the analysed hybrid zones (Figure 5, Figure S7). The only exceptions were *P. vaucheri–P. virescens*, where the difference was not significant ($p = .12$—MHC-I, $p = .09$—MHC-II), and *P. guadarramae–P. liolepis*, where no allele sharing in parapatry was detected in MHC-II. The sharing of alleles in parapatry ranged from 6.1% to 35.8% for MHC-I and from 0.0% to 17.2% for MHC-II, while in allopatry, from 3.8% to 8.9% for MHC-I and from 1.2% to 4.7% for MHC-II. The repeatably higher allele sharing in parapatry may suggest an ongoing adaptive introgression of MHC alleles between multiple pairs of the Iberian *Podarcis* species.

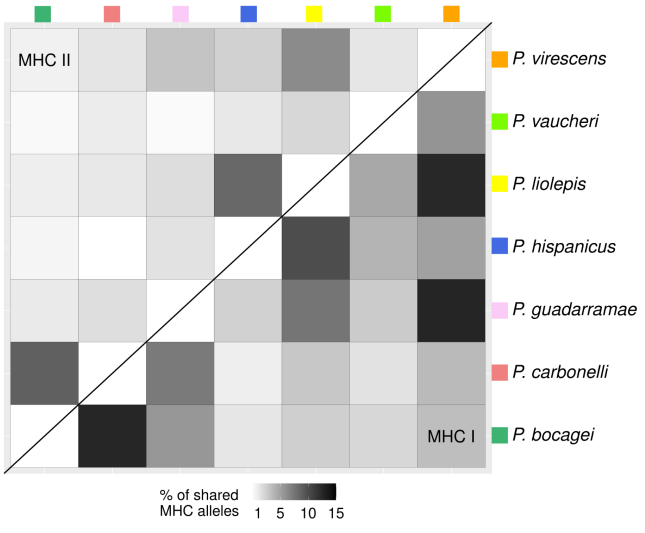

FIGURE 3 Percentage of shared alleles between nonadmixed individuals of analysed *Podarcis* species.

4 | DISCUSSION

In almost all analysed hybrid zones, we observed an elevated sharing of MHC alleles in the proximity of the contact zone compared with the sharing between allopatric populations. The only exception is the *P. vaucheri–P. virescens* hybrid zone, where the sharing in parapatry, though not significant, was still elevated for both MHC classes. This indicates widespread introgression between several species from *P. hispanicus* complex. The other plausible explanations, such as the retention of ancestral MHC polymorphism facilitated by balancing selection or parallel adaptation on both sides of each contact zone, are unlikely. The first would be expected to increase the similarity between species across the species ranges, not only close to the contact zone (Schierup et al., 2000). The second would require similar pathogen pressure on both sides of all examined contact zones (Lenz et al., 2013) and the availability of identical alleles that would increase in frequency in parallel in both species (Dudek et al., 2019). Furthermore, because this result was obtained considering only individuals without detectable genome-wide admixture, it suggests that this pattern was at least partly driven by adaptive introgression. We admit that for a single hybrid zone, the observed pattern could also be explained by the stochastic interlocus variation in the level of neutral introgression, with MHC at the tail of the distribution (Bonnet et al., 2017). However, such stochasticity is highly unlikely to generate the observed, repeatable pattern of enhanced MHC introgression in almost all analysed hybrid zones.

The results of selection analysis are generally consistent with previous studies on MHC in finding high nucleotide and amino acid divergence (Bernatchez & Landry, 2003) and signs of positive selection acting on PBS (Aguilar et al., 2004; Hedrick et al., 2002; Zhao et al., 2013). The observed interplay between diversifying and purifying selection in MHC has previously been reported for the parts of protein directly involved in antigen binding (Fijarczyk et al., 2018; Kamath & Getz, 2011). While diversifying selection frequently occurs at sites responsible for pathogen recognition, other sites, such as those maintaining the proper shape of the peptide-binding groove, tend to be under purifying selection (He et al., 2020) and are conserved even in distantly related vertebrates (Kaufman et al., 1994). Though not evidence in itself, the unambiguous signal of long-term positive selection suggests the plausibility of adaptive introgression. It is, however, essential to note that these tests reflect mostly more distant evolutionary history and are poorly suited for the short-term dynamics expected under strong balancing selection (Kryazhimskiy & Plotkin, 2008; Mugal et al., 2014).

Observed patterns suggesting adaptive introgression align well with previous studies examining MHC introgression. It is the third

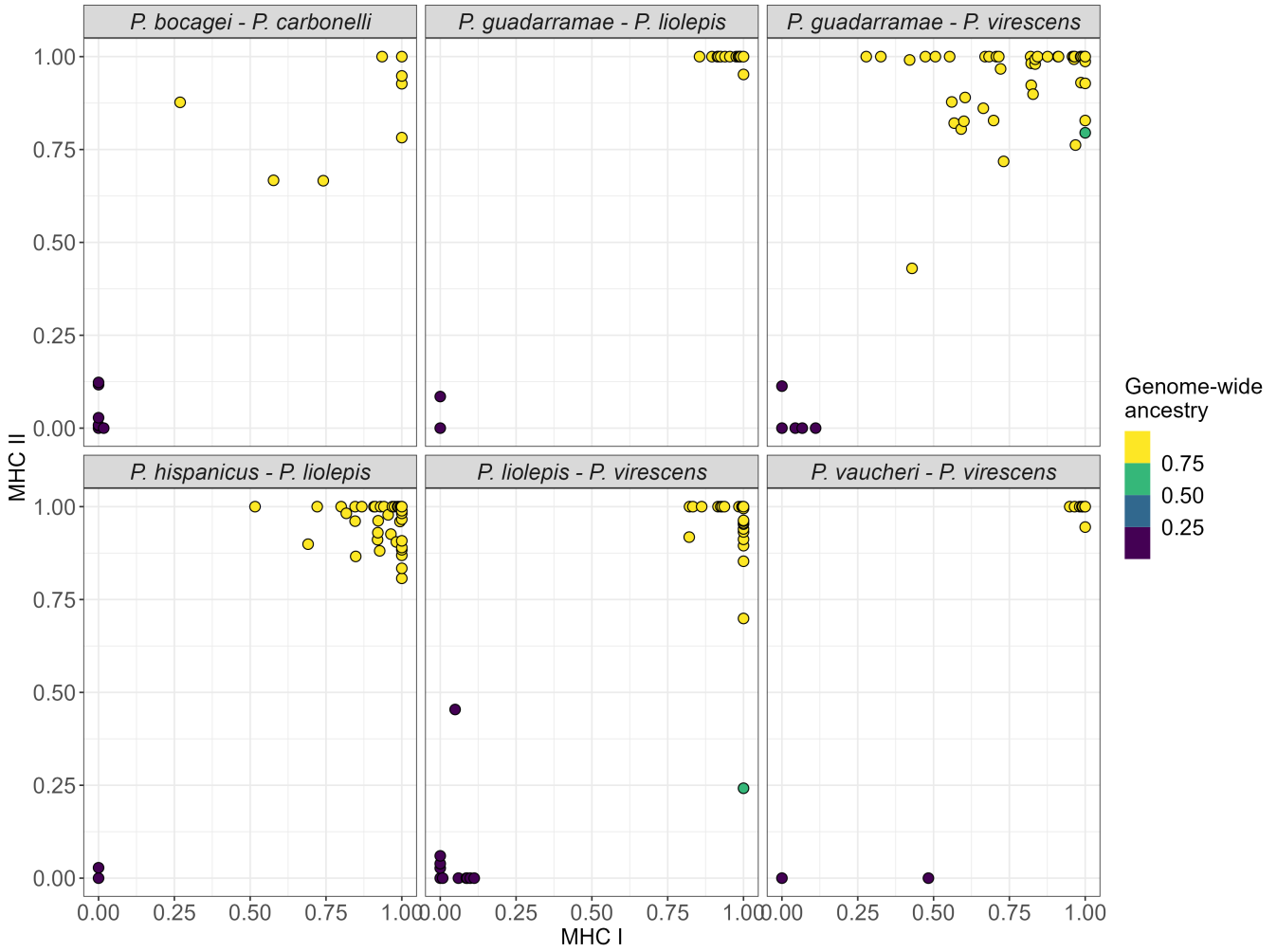

FIGURE 4 Comparison between Q-scores from STRUCTURE analyses used as a proxy for genome-wide ancestry and hybrid indexes estimated based on MHC-I and MHC-II alleles. For all parameters, 0 corresponds to the first species in the pair. If all values are equal, we expect all points to lie on the diagonal and gradually change colour. Note that the number of observations is ignored, and the colour is averaged for points with the same h-index for MHC-I and MHC-II.

vertebrate system, after *Lissotriton* (Dudek et al., 2019) and *Triturus* (Gaczorek et al., 2023) newts, for which the introgression in MHC was directly compared with genome-wide markers across multiple hybrid zones. As in the current work, introgression in MHC genes was prevalent in both previous studies, suggesting its universality and adaptive character. Presumably adaptive MHC introgression was also reported in humans (Abi-Rached et al., 2011), Alpine ibex (Grossen et al., 2014), hares (Pohjoismäki et al., 2021), and the only other reptile system, *Lacerta* lizards (Sagonas et al., 2019). Although the introgression tests used in those studies were mostly robust, the scarcity of explicit comparisons of MHC with genome-wide markers makes it difficult to exclude alternative explanations, for example, neutral introgression event followed by adaptation from standing genetic variation.

Although MHC introgression in all examined *Podarcis* hybrid zones exceeds the genome-wide introgression, its extent differs between zones. It is well visible for *P. guadarramae*, *P. liolepis* and *P. virescens*, which can potentially hybridise with each other—MHC introgression between *P. guadarramae* and *P. liolepis* is more limited than in the other two pairs. As there is limited evidence for female choosiness in *Podarcis* lizards (Yang et al., 2020 but see Runemark et al., 2011; Gabirot et al., 2012), we expect the differences in the extent of introgression to be related to differences in the selective advantage of introgressed MHC or a stronger genetic or environmentally mediated barrier. We also detected asymmetric MHC introgression in three of the hybrid zones examined. Interestingly, those species pairs showed the highest (though not necessarily consistent between MHC classes) deviation of MHC h-index values from the predictions based on the genome-wide admixture. This suggests asymmetry in the selective advantage of MHC introgression caused by, for example, local interspecific differences in the levels of MHC variation or in the strength of pathogen pressure. The asymmetry might also result from the combination of introgression and contact zone movement (Barton & Hewitt, 1985; Wielstra et al., 2017). In the case of neutral introgression, a species extending its range and replacing the other species is expected to absorb more alleles

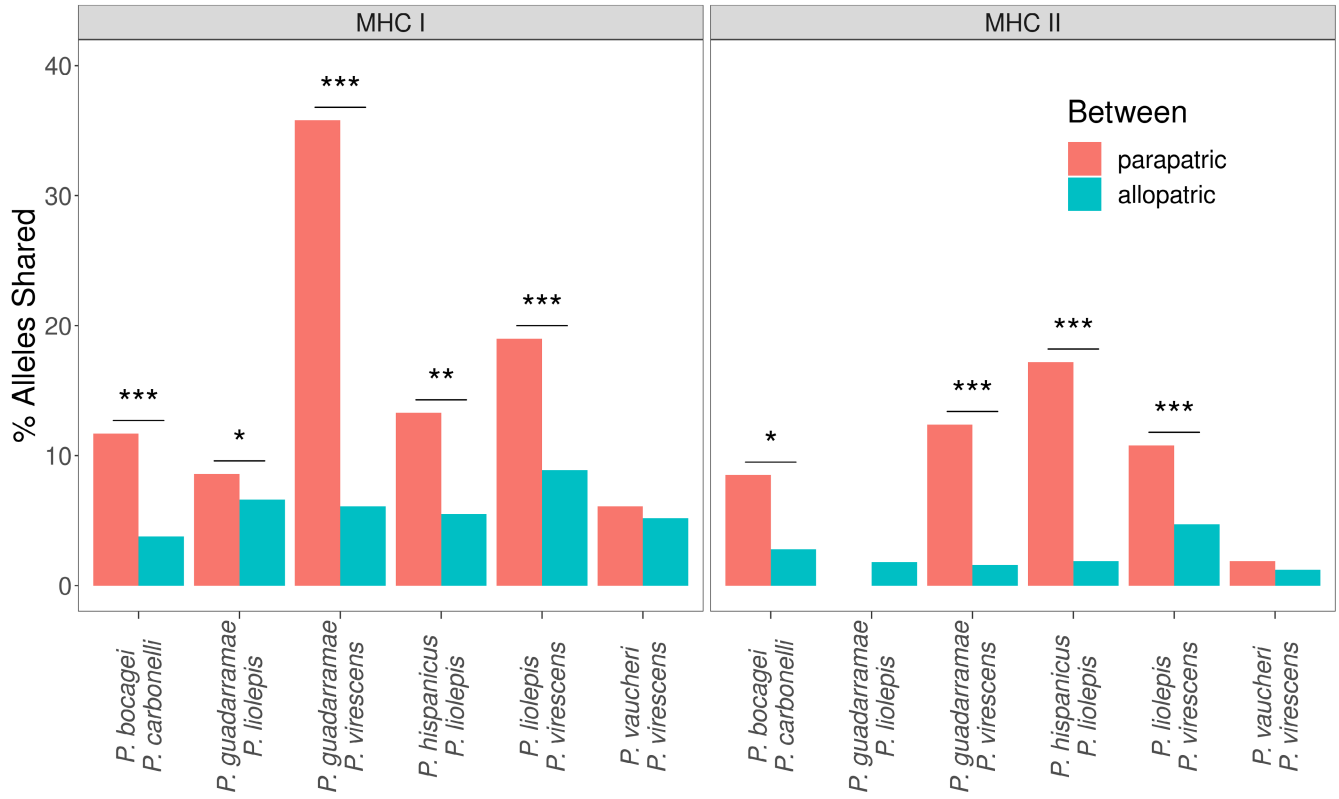

FIGURE 5 Percentage of MHC alleles shared between parapatric and allopatric populations of hybridising *Podarcis* species. The asterisks denote a significant difference based on the permutation tests (*$p \leq .05$, **$p \leq .01$ and ***$p \leq .001$).

(Buggs, 2007; Currat et al., 2008). The pattern agrees with studies in *P. carbonelli*, suggesting hybrid zone movement towards *P. bocagei* territory (Caeiro-Dias et al., in review). We hypothesise that the effect of contact zone movement might be similar or even stronger for adaptively introgressed variants. Unfortunately, no other contact zones were studied from this perspective.

Unlike our previous work on *Triturus* newts (Gaczorek et al., 2023), we did not find clear evidence for the varying rate of introgression between MHC classes. No significant differences were detected for allele sharing or h-index deviations from the genome-wide predictions. At the same time, the selection analysis brings a stronger signal of positive selection for MHC-II, which is consistent with the literature (Minias et al., 2016, 2019). It is worth noting that selection signal for MHC-II alleles might be simply easier to detect. Ejsmond and Radwan (2015) and Ejsmond et al. (in review) showed that selection pressure from slowly evolving pathogens leads to high-amplitude fluctuations in allele frequency and incomplete sweeps that may cause stronger dN/dS signal. On the contrary, the chances of detecting selection signal might be reduced for MHC-I. Assuming their fast evolution in response to quickly evolving intracellular pathogens, for example, viruses (Kelley et al., 2005), manifested by frequent duplication and deletions, one might expect a quick loss of gene orthology resulting in periodical erosion of the selection signal. Overall, the results are inconclusive, and we hypothesise that the lack of substantial difference between MHC classes in *Podarcis* may result from their similar polymorphism, unlike in *Triturus*, which possesses only one functional MHC-II locus. This might suggest differences in either dynamics of introgression or detection power depending on the level of MHC polymorphism. The latter complies with Baird et al. (2003) and Sachdeva and Barton (2018), who showed that introgression of longer genome fragments is easier to be detected.

The MHC introgression signal does not follow the pattern of genome-wide introgression. As mentioned above, genome-wide introgression is highly limited. Still, recent work (Caeiro-Dias, Brelsford, et al., 2021) and our results show that its extent differs between contact zones. In some hybrid zones, for example, *P. bocagei–P. carbonelli* or *P. liolepis–P. virescens*, hybrids of various generations were detected, while the others, like *P. guadarramae–P. liolepis* included solely parental species. Regarding that, the lack of corresponding differences in the MHC introgression pattern between hybrid zones is interesting, as it may suggest similar permeability of different zones for MHC. It agrees with theoretical predictions that the introgression of beneficial alleles is only slightly delayed in contact zones with stronger but still incomplete genetic barriers (Barton, 1979; Barton & Hewitt, 1985). This phenomenon, however, might also be explained by nearly complete system saturation, where introgression is restricted to a few beneficial alleles originating by de novo mutations. Although this hypothesis is backed by the evidence of pervasive ancient hybridisation among the Iberian *Podarcis* species (Yang et al., 2021), potentially homogenising the MHC allele pool, long-term maintenance of polymorphism in multiple species is unlikely. First, the simulation work, examining the host-pathogen arms

race, points to extreme dynamics of MHC allele frequencies and turnover at short time scales (Ejsmond & Radwan, 2015). Second, and more importantly, we still observe substantial MHC differentiation between species. Potentially, the differences in genome-wide introgression between hybrid zones might also be explained by fitness advantage of hybrids in the narrow contact area, maintaining hybrid populations. So far, however, no reports suggested hybrid fitness advantage in the proximity of *Podarcis* contact zones.

We admit that the outcomes of our analyses might have been affected by the complex nature of MHC variation in the *P. hispanicus* complex. The genotyped individuals possess fewer alleles than expected based on the number of loci inferred from *P. muralis* genome assembly. It might suggest that we did not genotype the whole MHC variation. If the genotyped subset was nonrandom, for example, it covered only a specific part of the antigen recognition spectrum, it might restrain our inference. The obtained difference, however, can also be an artefact of reduced allele diversity caused by the presence of monomorphic loci, overly conservative filtering, genome assembly errors or gene duplications. Although we cannot exclude the possibility that the analysed species could have experienced gene duplications, such duplications would have probably occurred prior to the divergence of the Iberian species from the *P. hispanicus* complex, as all of them show a similar number of alleles per individual. Such potential duplications shared between species are unlikely to bias our results. A similar caveat concerns the presence of monomorphic loci. Our previous work (Gaczorek et al., 2023) showed that the presence of low-polymorphic genes, among others, high-polymorphic genes, might artificially widen confidence intervals for hybrid index estimation in gene complexes with unresolved haplotypes, such as MHC. However, due to the similar per-individual number of alleles among species, we also expect the haplotype structure to be similar. Thus, if the bias is indeed present, it should equally affect all compared hybrid zones, altering the extent of the signal but not its presence.

In conclusion, this study found MHC introgression extending beyond the areas of detectable genome-wide introgression across most examined contact zones of the Iberian *Podarcis* lizards, suggesting the potential adaptive nature of MHC introgression. The MHC introgression was detected even between otherwise strongly reproductively isolated species. Thus, our work adds to the growing evidence indicating that MHC introgression persists even at late stages of speciation and may be a major source of adaptive variation. Additionally, we observed asymmetry in introgression in half of the analysed hybrid zones but, contrary to our previous report from *Triturus* newts, we did not find evidence for a difference in introgression rate between the MHC classes. Our findings raise further questions that should be addressed in future studies. Does depleted MHC variation in one species typically lead to asymmetric introgression? What are the fitness consequences of asymmetric MHC introgression? Does lower MHC polymorphism result in a lower rate of adaptive introgression, or it only affects our detection power? Finally, does the adaptive introgression pattern for contact zone movement reflect the pattern expected under neutral introgression? We believe that research answering those questions should include an analysis of introgression between closely related species with varying levels of polymorphism, for example, depleted by the severe bottleneck, and known fitness effects of introgressing alleles. We consider the latter prerequisite crucial as it may help to estimate the fraction of introgressed alleles that is indeed favoured by selection.

AUTHOR CONTRIBUTIONS

Wieslaw Babik and Tomasz Sebastian Gaczorek designed the study. MHC laboratory work was performed by Katarzyna Dudek, and MHC analyses were performed by Tomasz Sebastian Gaczorek and Mateusz Chechetkin. Catarina Pinho and Guilherme Caeiro-Dias performed genome-wide ancestry analyses. Pierre-Andre Crochet and Philippe Geniez provided a database for species distribution inference. Tomasz Sebastian Gaczorek, Mateusz Chechetkin and Wieslaw Babik wrote the manuscript with help from Catarina Pinho, Pierre-Andre Crochet, Guilherme Caeiro-Dias and Philippe Geniez. All authors revised the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Sequences of MHC alleles were uploaded to GenBank under accession nos. OQ852955–OQ856738 and OQ856780–OQ856871. They have also been made available, together with an R script for the GLM model, at the Dryad Digital Repository (doi: 10.5061/dryad.hqbzkh1m7). Raw sequence reads, used for Q-scores estimation, are deposited in NCBI Sequence Read Archive (SRA) under the BioProject accession no. PRJNA665746.

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