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Patterns of genetic diversity in five species of Passeriformes co-distributed in an environmental gradient

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ABSTRACT

Understanding the evolutionary processes that influence the distribution of genetic diversity in natural populations is a key issue in evolutionary biology. Both species' distribution ranges and environmental gradients can influence this diversity through mechanisms such as gene flow, selection, and genetic drift. To explore how these forces interact, we assessed neutral and adaptive genetic variation in three widely distributed and two narrowly distributed bird species co-occurring along the Cauca River canyon in Antioquia, Colombia—a region of pronounced environmental heterogeneity. We sampled individuals across eight sites spanning the canyon's gradient and analyzed genetic diversity and structure using microsatellites and toll-like receptors (TLRs), a gene family involved in innate immunity. Widely distributed species consistently exhibited higher genetic diversity at both marker types compared to their narrowly distributed counterparts. Although we did not find a significant relationship between microsatellite heterozygosity and TLR heterozygosity, we evidenced a negative trend for widely distributed species and a positive trend for narrowly distributed species. This result suggests that there is a stronger effect of genetic drift in narrowly distributed species. Our results highlight the role of distribution range in maintaining genetic diversity and suggest that environmental gradients, by interacting with gene flow and selection, may influence patterns of adaptive variation.

1. Introduction

Genetic diversity, consisting of allele and genotype variation within the genome, provides the raw material for evolution and is a fundamental source of biodiversity (Fisher, 1930; Frankham et al., 2002). Genetic diversity allows populations to adapt to environmental changes (Reed and Frankham, 2003) and is therefore important for the maintenance of populations and species over time. The loss of genetic diversity in some cases drives to inbreeding depression and reduced biological fitness (Reed and Frankham, 2003). Determining the relative role of evolutionary forces on patterns of genetic diversity in natural populations has been therefore a central goal of evolutionary biology and has implications for evolutionary ecology and conservation biology (DeWoody et al., 2021).

Genetic diversity can be classified as neutral or adaptive, depending on which evolutionary forces act upon it. Neutral genetic diversity is influenced by mutation, genetic drift and gene flow. Genetic drift tends to have a more pronounced impact on small populations, which often exhibit lower levels of neutral genetic diversity (Frankham, 1996; Hoeck et al., 2010; Zapata et al., 2020). On the other hand, adaptive genetic diversity is influenced by these evolutionary forces as well as by natural selection (Holderegger et al., 2006). Natural selection leads to the adaptation of populations to local conditions (Linhart and Grant, 1996; Vellend and Geber, 2005). Depending on the context, natural selection can favor a decrease of genetic diversity, or its retention through balancing selection (Spurgin and Richardson, 2010; Kirk and Freeland, 2011). Since both neutral and adaptive genetic diversity play a fundamental role in the genetic diversity of a population or species, it is necessary to assess both to determine the relative contribution of the evolutionary forces in shaping genetic diversity.

Adaptive molecular markers are directly related to the response of populations to environmental changes (Hoffmann and Willi, 2008). One

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of the most commonly used adaptive molecular markers in genetic diversity studies are genes associated with the immune response, as these loci are subject to various selective pressures related to susceptibility and resistance to pathogenic diseases (Hedrick, 1999; Piertney and Oliver, 2006; Dalton et al., 2016; Grueber et al., 2012). Toll-like receptors (TLRs) are excellent candidates for assessing adaptive genetic diversity because they are an important component of the innate immune response (Roach et al., 2005). These receptors recognize pathogen-associated molecular patterns (PAMPs) and initiate an immune response through an intracellular signaling cascade (Medzhitov, 2001; Kawai and Akira, 2006). Vertebrate TLRs can be classified into six major families, each distinct in their recognition of specific PAMPs (Roach et al., 2005). For example, the TLR3 family recognizes dsRNA, the TLR4 family binds lipopolysaccharides, and the TLR5 family binds flagellin (Roach et al., 2005). TLR families vary in the length of their extracellular leucine-rich domains (Roach et al., 2005). It has been suggested that the observed variation in genes encoding TLRs is driven by positive and/or balancing selection (Grueber et al., 2014). This variation in TLRs is associated with resistance to parasite infection. Thus, greater variation in TLRs reflects a greater ability to respond to a variety of pathogens and a greater potential for populations to adapt to changing environments (Grueber et al., 2012; Grueber and Jamieson, 2013; Dalton et al., 2016). Several studies have evaluated TLR variation in wild populations to assess evolutionary potential (Tschirren et al., 2013; Gonzalez-Quevedo et al., 2015; Kloch et al., 2018; Davies et al., 2021; Quéméré et al., 2021), for its importance in pathogen recognition and activation of an immune response (Knafler et al., 2017; Minias and Vinkler, 2022).

When assessing the importance of evolutionary forces, it is paramount to differentiate the role of adaptive forces (natural selection) and neutral forces (i.e., genetic drift, gene flow). For this purpose, variation at adaptive markers should be compared to that found at neutral markers. Microsatellites have been one of the most widely used molecular markers to study neutral genetic diversity because they provide information on demographic history and population genetic structure (Jarne and Lagoda, 1996; Ellegren, 2004; Corrêa et al., 2010). Due to their multiallelic nature and high mutation rates (Zhivotovsky and Feldman, 1995; Brumfield et al., 2003; Ellegren, 2004), they are usually one of the best markers for assessing population differentiation (Foster et al., 2010). Despite being gradually replaced by the more informative genome-wide SNPs, microsatellites are still widely used to assess neutral genetic diversity in wild populations and are an important tool in conservation genetics (Tokarska et al., 2009; Camacho-Sanchez et al., 2020; Zimmerman et al., 2020).

The ecological processes of species influence the maintenance and structure of genetic diversity in populations and have an impact on how evolutionary forces operate. Among the ecological processes that influence genetic diversity of wild populations is the geographic distribution of species. Widely distributed species are characterized by having locally abundant populations and tend to occupy a large number of sites, with a wide variety of habitats, while restricted species are confined to limited geographic regions and are found in very small areas and few habitat types (Brown, 1984; Gaston, 1996; Blackburn et al., 1997; Krabbe and Rahbek, 2010; Verberk, 2011). Differences in genetic diversity between widespread and restricted species may have implications for their evolution. Population genetics theory predicts that species with restricted ranges will generally have small population sizes that are more susceptible to the effects of genetic drift, which erodes levels of genetic diversity (Lande, 1988; Levy et al., 2016). In these populations, balancing selection on loci important for local adaptation is not sufficient to counteract the effect of drift. As a result, small populations have low levels of genetic diversity, which impairs their ability to respond to environmental changes and increases their risk of extinction (Spielman et al., 2004; Frankham, 2005; Evans and Sheldon, 2008). On the other hand, widely distributed species may have higher genetic diversity because they are generally composed of larger populations in which natural selection and gene flow play a fundamental role in keeping the frequencies of deleterious alleles low (Frankham, 2005). Widely distributed species are also expected to have greater genetic diversity than narrowly distributed species because the former are composed of subpopulations throughout their distribution range where genetic differences can be maintained due to local adaptation processes (Mayr, 1942; Wang et al., 2022).

When studying how evolutionary forces affect genetic diversity in wild species, it is important to have a study model that consists of species that differ in the ecological processes under study. Birds are a good model for studying these processes because they are a very diverse group in which we find a wide variety of ecological traits. Furthermore, some bird species have wide distributions inhabiting areas with diverse habitats, while others have very restricted distributions. Although several studies have focused on determining neutral and adaptive genetic diversity in the species of interest, few have assessed neutral and adaptive genetic diversity in co-distributed species (subject to similar environments) that differ in their distribution patterns (see Hartmann et al., 2014 for an exception).

In the present study, we investigated the effect of distribution range on neutral (microsatellites) and adaptive (TLRs) genetic diversity in bird species co-distributed in an environmental gradient with two types of habitats: a tropical dry forest and a habitat in the transition between tropical dry forest and tropical humid forest (Rivera-Gutiérrez et al., 2018; Idárraga-Piedrahíta et al., 2022). The aims of this study were: (1) to analyze the neutral genetic diversity of widely distributed species and compare it to that found in species with restricted distributions; (2) to analyze the adaptive genetic diversity of widely distributed species and compare it to that found in species with restricted distributions; (3) to compare patterns of genetic structure between widespread and restricted bird species; and (4) to analyze the role of natural selection in maintaining genetic diversity in adaptive markers.

2. Materials and methods

2.1. Study area and species

Our study was carried out in the Cauca River canyon, (Antioquia, Colombia) in the area of direct influence of the Ituango Hydroelectric Project (Hidroituango), which includes eight municipalities, from north to south: Briceño, Toledo, Ituango, Peque, Sabanalarga, Buriticá, Liborina and Santafé (Fig. 1). This canyon, located in the northwest of the department of Antioquia, presents an environmental gradient with a temperature range from 24 °C to 38 °C and annual precipitation from 1000 mm to 5000 mm. This environmental gradient has different biomes, the tropical dry forest, transition dry-rainforest and rain forest (Fig. 1) (Rivera-Gutiérrez et al., 2018; Idárraga-Piedrahíta et al., 2022). In addition, the environmental gradient influences the distribution of some pathogens between forest types. For example, Martínez-Alvarado et al. (2019) reported a higher prevalence of avian Hemosporidia infections in the transition zone compared to the tropical dry forest. We selected two populations of each species, one in the tropical dry forest and one in the transition dry-rainforest. The tropical dry forest includes the municipalities of Santafé, Buriticá, Liborina, Sabanalarga and Peque. The transition dry-rainforest includes the municipalities of Briceño, Ituango and Toledo (Fig. 1).

Among the species present in the study area, we selected three bird species with wide distribution: the Black-striped Sparrow (*Arremonops conirostris*), the White-breasted Wood-wren (*Henicorhina leucosticta*) and the Dusky-capped Flycatcher (*Myiarchus tuberculifer*). Additionally, we selected two species with restricted distribution: the Apical Flycatcher (*M. apicalis*) and the Antioquia Wren (*Thryophilus sernai*). We selected pairs of phylogenetically related species (within the same family) with different distribution ranges to reduce potential heterogeneity arising from differences in their evolutionary histories. Comparisons included *H. leucosticta–T. sernai* and *M. tuberculifer–M. apicalis*. We also included

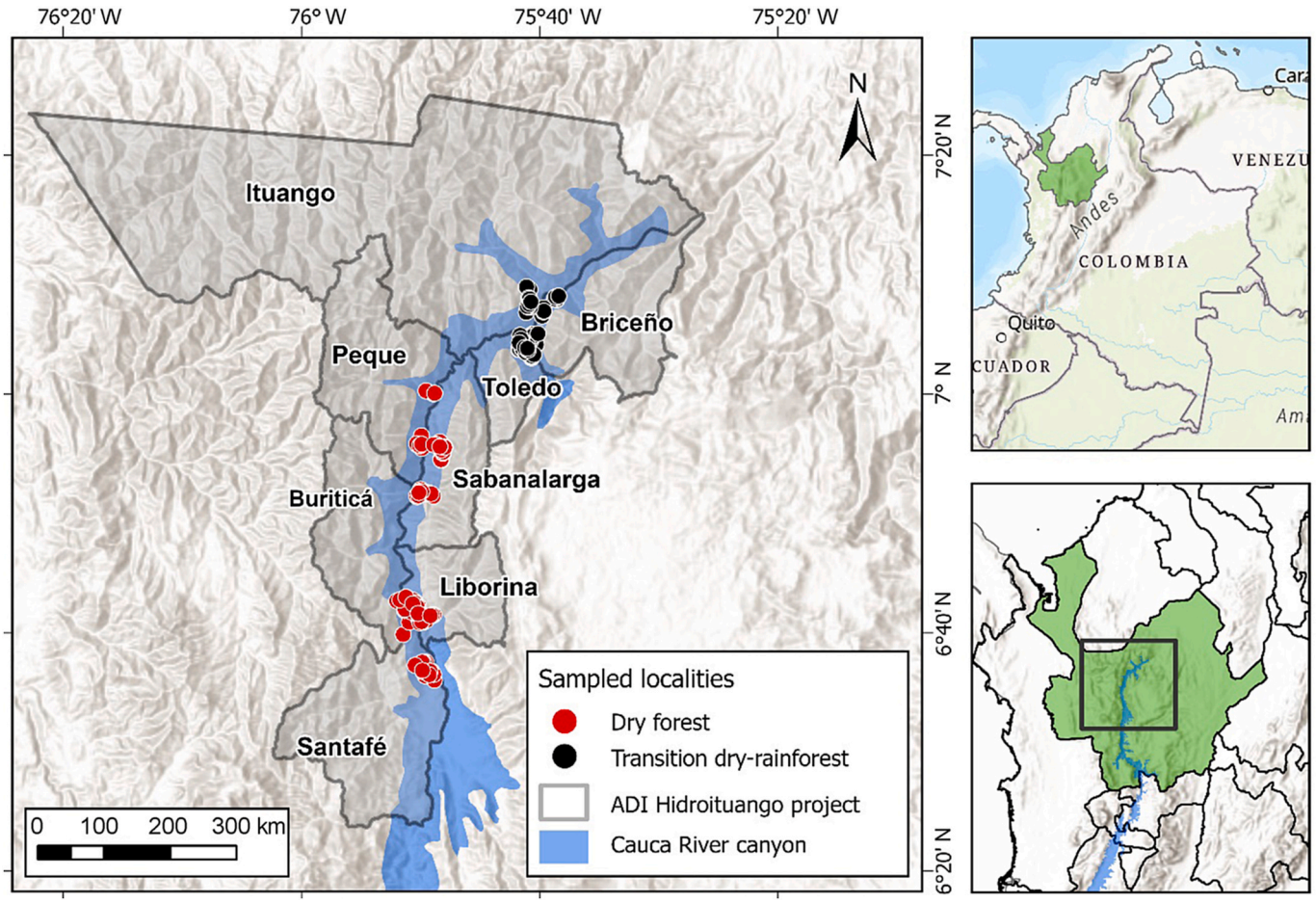

Fig. 1. Map of the study area. Sampling sites in the area of influence of the Hidroituango project. Map created using ArcGIS Pro v.2.7 (ESRI, 2022).

A. conirostris as it is the most abundant, widely distributed species in the study area and has shown high levels of genetic diversity in preliminary analyses. We classified species based on their global geographic range. Species considered widely distributed (*A. conirostris*, *H. leucosticta*, *M. tuberculifer*) occur broadly across the Neotropics, while narrowly distributed species (*T. sernai*, *M. apicalis*) are endemic to Colombia and have small global range sizes. Detailed description of the study species can be found in Appendix A and Appendix Figs. S1–S5.

2.2. Sampling

Birds were captured between 2015 and 2021 using mist nets (Ecotone, Poland). We used playback to increase the capture rate in the area where the species of interest were detected. We collected blood samples of approximately 20 μL by brachial venipuncture using 0.4 mm diameter hypodermic needles and capillaries. We used a hypodermic needle for each individual, cleaning the area with alcohol before and after blood sampling. Samples were stored in Queen lysis buffer (Seutin et al., 1991). Finally, we performed genomic DNA extraction using the Salting Out protocol (Miller et al., 1988). The DNA samples were quantified in a nanodrop.

2.3. TLR genotyping

To assess adaptive genetic diversity, we genotyped birds at nine TLR loci: TLR1LA, TLR1LB, TLR2A, TLR2B, TLR3, TLR4, TLR5, TLR15, and TLR21 using the primers published by Alcaide and Edwards (2011), and by Grueber et al. (2012). These primers amplify fragments of the extracellular domains of TLRs, regions associated with pathogen recognition and where the greatest variability is expected. Details on the PCR amplifications of TLRs can be found in the Appendix text and Table S1. Purified PCR products were forward sequenced using the Sanger sequencing method. Sequences were edited, aligned, and translated in Geneious 11.0.4 (Biomatters Ltd.) (Kearse et al., 2012). Single nucleotide polymorphisms (SNPs) were identified by visual examination of chromatograms. The International Union of Pure and Applied Chemistry (IUPAC) code for degenerate nucleotides was used for heterozygous positions. SNPs that were detected in only one sample were confirmed by repeating the amplification and sequencing procedures.

2.4. Estimation of the genetic diversity and population structure in TLR

The PHASE algorithm (Stephens and Donnelly, 2003) implemented in DnaSP 5.10 (Librado and Rozas, 2009) was used to identify individual haplotypes when more than one heterozygous position was found in the sequence. A threshold of genotype assignment probability >0.9 was used. In the same program, haplotype richness (h), nucleotide diversity (π), Watterson's estimator (θ_W) and a Tajima's D neutrality test (Tajima, 1989) were calculated. Observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). Hardy–Weinberg equilibrium (HWE) tests were performed using the web version of GENEPOP (http://genepop.curtin.edu.au/) (Raymond and Rousset, 1995). To account for differences in sample size between populations, allelic richness (A) was calculated in FSTAT 2.9.4 by implementing the rarefaction algorithm (Goudet, 1995). To compare between species, allelic richness adjusted for the smallest sample size of each species (A') per locus was estimated using FSTAT.

F_{ST} values for population differentiation between dry forest and

transition dry-rainforest were estimated for two data sets: each TLR locus per species, and for the set of TLR loci per species. Pairwise F_{ST} values were calculated using Arlequin (Excoffier and Lischer, 2010). To infer relationships between TLR haplotypes, haplotype networks were generated using the median-joining method with PopArt (Leigh and Bryant, 2015).

2.5. Selection tests

To quantify natural selection acting on TLRs, the rate (ω) of non-synonymous to synonymous nucleotide substitutions per site (dN/dS) was calculated in MEGA 10 (Kumar et al., 2018) for each locus using the haplotype sequences identified across species, taking into account that not all loci were successfully genotyped in all five species. The significance of the relationships between dN and dS (dN<dS, dN>dS) was assessed using 10,000 bootstrap replicates and the Kimura 2-parameter model. Under negative or purifying selection, the proportion of non-synonymous substitutions is lower than that of synonymous substitutions (dN < dS), whereas under positive or diversifying selection, the proportion of nonsynonymous substitutions is higher than that of synonymous substitutions (dN > dS) (Yang and Nielsent, 2002; Rocha et al., 2006). To identify codons under positive selection, two codon-based selection detection models were run using the HyPhy package (Pond et al., 2005) implemented on the Datamonkey web server (Weaver et al., 2018): (1) the mixed effects evolution model (MEME), which uses a maximum likelihood approach to detect episodic selection (Murrell et al., 2012); and (2) the fast unconstrained Bayesian approach (FUBAR) to detect generalized selection (Murrell et al., 2013). Analyses were performed with the default settings of a significance threshold of 0.1 for MEME and a posterior probability of 0.9 for FUBAR. For codon selection tests, we used the alignments of each TLR including all alleles identified in all species where the marker was successfully amplified. Finally, we aligned the amino acid sequences of the TLRs with those of *Gallus gallus* for reference (NCBI accession numbers: FJ915290 [TLR1LA], FJ915334 [TLR1LB], AB046119 [TLR2], FJ915472 [TLR3], FJ915520 [TLR4], FJ915552 [TLR5]).

2.6. Microsatellite genotyping

To assess neutral genetic diversity, we genotyped 15 microsatellites for *A. conirostris* (Nietlisbach et al., 2015), 10 microsatellites for *H. leucosticta*, 15 microsatellites for *T. sernai* (Cabe and Marshall, 2001; Brar et al., 2007; Bowie et al., 2012) and, 10 microsatellites for *M. tuberculifer* and *M. apicalis* (McDonald and Potts, 1994; Piertney et al., 2002; Bardeleben and Gray, 2005; Duval and Nutt, 2005; Beheler et al., 2007). We used microsatellites described in closely related species and standardized with different PCR conditions (Appendix Tables S2–S5). Details of the PCR amplification of microsatellites can be found in the Appendix text. Each locus was amplified separately, and capillary electrophoresis was performed on an ABI 3500 HD Gene Analyzer with the GeneScan LIZ 600 size standard (Applied Biosystems), combining 3 or 4 loci in each run. Allele sizes were determined using Geneious (Biomatters Ltd).

2.7. Estimation of the genetic diversity and population structure of microsatellites

Hardy–Weinberg equilibrium (HWE) for each locus and linkage disequilibrium for each pair of loci were tested using Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). For each microsatellite, evidence of null alleles or genotyping errors due to stuttering and dropout of large alleles was assessed using Microchecker 2.2.3 (Van Oosterhout et al., 2004). Genetic diversity was assessed by calculating expected heterozygosity (H_e), observed heterozygosity (H_o) and number of alleles for each locus using Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). To correct for differences in sample size between populations, allelic richness (A) was calculated in FSTAT by implementing the rarefaction algorithm (Goudet, 1995). We calculated the multilocus heterozygosity metric internal relatedness (IR) from the microsatellite genotypes with the Rhh package in R (Alho et al., 2010).

To study the patterns of genetic diversity distribution throughout the study area and to compare these patterns among species groups, genetic structure was analyzed using several approaches. Bayesian assignment analyses implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) were used to estimate the most probable number of genetic clusters (K). The analysis was performed in two ways. The first assumed two hypothetical populations: dry forest and transition dry-rainforest. The second assumed eight hypothetical populations, corresponding to the eight municipalities. Although very similar results were obtained with both approaches, only the STRUCTURE results for the eight populations are shown in order to detect possible genetic substructure corresponding to the discrete sampling sites. For *H. leucosticta*, individuals were assigned to five populations, as this species was only captured in the municipalities of Briceño, Ituango, Toledo, Buriticá and Sabanalarga. Genetic substructure can occur due to intrinsic species characteristics (e.g., low dispersal ability (Moore et al., 2008), reproductive system (Lee et al., 2009; Morinha et al., 2017)), or environmental heterogeneity (Wang and Bradburd, 2014) driving differentiation patterns. The program was run using the admixture model with the correlated allele frequency model configuration. Each run consisted of an MCMC run-in of 100,000 followed by 500,000 steps of sampling, 10 iterations were performed for each K. Estimation of ΔK was performed as recommended by Garnatje et al. (2013), Puechmaille (2016), and Arnelas et al. (2022) with $n + 2$, where n is the number of hypothetical populations. Following this methodology, the value of K was estimated from 1 to 10 when run with all 8 populations (except for *H. leucosticta*, where K was estimated from 1 to 7 because only 5 populations were present), and K was estimated from 1 to 4 when run with the 2 hypothetical populations (dry forest and transition dry-rainforest). The most probable number of clusters, K, was determined using Evanno's method (Evanno et al., 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). Genetic differentiation between populations was measured by calculating the pairwise F_{ST} fixation index between dry forest and transition forest in Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). The F_{ST} was also estimated between populations separated by the Cauca River, as a river can be a barrier to gene flow in understory bird species in the Neotropics (Mayr, 1969; Brawn et al., 1996; Burney and Brumfield, 2009) due to low dispersal capacity (Moore et al., 2008). Population structure has even been reported in bird species isolated by smaller rivers (Musher et al., 2022), following the hypothesis of fluvial barriers (Voelker et al., 2013). The municipalities of Ituango, Peque, Buriticá, and Santafé are located on the left bank of the Cauca River, and the municipalities of Briceño, Toledo, Sabanalarga, and Liborina are located on the right bank (Fig. 1). In addition, to further explore the genetic structure, we performed a Principal Component Analysis (PCA) using the ADEGENET package in R (Jombart, 2008).

2.8. Association of TLR and neutral genetic variation

We examined the ability of microsatellite multilocus heterozygosity (MLH) to predict TLR heterozygosity of individuals of each species using a generalized linear mixed model (GLMM) implemented by the glmer function of the R package lme4 (Bates et al., 2014). Individual MLH was calculated from microsatellite data using the Rhh package (Alho et al., 2010) implemented in R. We used the internal ratio (IR) metric, which is a measure of individual genetic diversity (Amos et al., 2001). Given that our response variable is a proportion, the model was estimated with a logit link function. In the GLMM, the response variable was the proportion of TLR loci genotyped that were heterozygous for each individual and microsatellite IR was our fixed predictor variable.

2.9. Relationship of microsatellite and TLR heterozygosities with the species geographic range

We quantified phylogenetic signal of microsatellite heterozygosities, TLR heterozygosities and species geographic range using Pagel's λ (Pagel, 1999) and Blomberg's K (Blomberg et al., 2003) in the R package phytools (Revell et al., 2008). Both λ and K use a Brownian motion (BM) model of trait evolution. For both metrics, a value close to zero indicates phylogenetic independence and a value of one indicates trait similarity between related species as expected under BM (Münkemüller et al., 2012). For the phylogenetic signal test we used the phylogenetic tree of Jetz et al. (2012). To assess whether range was associated with patterns of genetic diversity, Pearson's correlation coefficients between the range of species distribution and mean heterozygosity were calculated for microsatellite and TLR data. To estimate the range of each species within the study area, we calculated the intersection between the species' total geographic range and the boundaries of our sampling region using ArcGIS Pro v.2.7 (ESRI, 2022). This regional range size was used as a proxy for distribution extent in the correlation analyses. Importantly, the species classified as widely distributed also had a larger geographic extent within the study area than the narrowly distributed species, supporting the validity of this proxy (see Results).

It is important to note that our genetic data were collected exclusively from populations within the Cauca River canyon. Therefore, the genetic diversity estimates reflect regional, not species-wide, patterns. For this reason, we used regional range size rather than global range in our analyses, in order to maintain consistency between the spatial scales of genetic and geographic data. For *T. sernai*, *H. leucosticta* and *M. tuberculifer* we used the distribution maps available in BirdLife (BirdLife International, 2023). For *A. conirostris* and *M. apicalis* we calculated the potential distribution using the Maxent algorithm (Phillips et al., 2006), as the distributions were not available in BirdLife. Details of the methodology for the MaxEnt algorithm can be found in Appendix A. Correlation analyses were performed in R version 4.3.1.

3. Results

3.1. Adaptive genetic diversity

A total of 374 individuals were captured in the sampling and used for genetic analyses (*A. conirostris*, $n = 93$; *H. leucosticta*, $n = 35$; *M. tuberculifer*, $n = 36$; *M. apicalis*, $n = 36$; *T. sernai*, $n = 174$). Not all TLR loci were successfully amplified in all species. All TLR alleles reported in this study have been deposited in GenBank (accession numbers: PV904822–PV904863, PV904864–PV90492, PV904925–PV904969, PV904970–PV905011, PV905012–PV905032, PV905033–PV905095). Summary variation in successfully amplified loci for each species is shown in Table 1. TLR1LA and TLR4 were only successfully amplified for two widely distributed species, *A. conirostris* and *H. leucosticta*. In a previous study in *T. sernai*, TLR1LA, TLR3, TLR5 were also amplified, being monomorphic for a total of 35 genotyped individuals (Zapata et al., 2020). The monomorphism of these markers was further confirmed by genotyping two samples per municipality distributed throughout the study area from the new sampling in this study. There were no frame-shift mutations or stop codons for any TLR locus, except for TLR5 for *A. conirostris*, where stop codons were found. This may be because a pseudogene was amplified in this species as reported in a study for passerines (Bainová et al., 2014) or because the locus is not functional. With the data we have it is not possible to determine this. For this reason, we excluded this marker in this species from further analyses.

We detected 200 SNPs in total over all loci and species; there were more nonsynonymous substitutions (113) than synonymous substitutions (87) (Table 1). Overall, a pattern is shown with the highest levels of genetic diversity in widely distributed species compared to restricted distribution species. This pattern is consistent with that found in allelic richness (A) and allelic richness between species (A'), where independent of sample size, greater diversity was found in the widely distributed species (Table 1).

We found significant deviations from the Hardy–Weinberg

Table 1
Measures of genetic diversity in TLR for widely distributed species (*Arremonops conirostris*, *Henicorhina leucosticta* and *Myiarchus tuberculifer*) and for species with restricted distribution (*Myiarchus apicalis* and *Thryophilus sernai*).

Loci	Distribution	Species	N[b]	SNPs (s:n)[c]	H[d]	Π[e]	θ_W[f]	Tajima's D[g]	A[h]	A'[i]	H_o[j]	H_e[k]	HWE[l]
TLR1LA	Wide	*A. conirostris*	79	14 (7:7)	25	0.0031	0.0026	0.554	24.54	15.45	0.37	0.79	0[a]
		H. leucosticta	29	18 (11:7)	17	0.0037	0.0046	−0.634	17.00	17.00	0.86	0.85	0.0329[a]
TLR1LB	Wide	*M. tuberculifer*	35	38 (22:16)	39	0.0059	0.0082	−0.917	31.76	35.66	0.97	0.98	0.0014[a]
	Narrow	*M. apicalis*	30	15 (6:9)	18	0.0054	0.0040	0.842	15.89	18.00	0.57	0.91	0[a]
		T. sernai	107	3 (0:3)	4	0.0011	0.0006	1.398	4.00	3.86	0.56	0.55	0[a]
TLR2	Wide	*A. conirostris*	76	7 (3:4)	17	0.0025	0.0014	1.835	17.00	12.91	0.76	0.83	0.0152[a]
		H. leucosticta	32	15 (5:10)	25	0.0037	0.0036	0.097	23.92	25.00	0.88	0.95	0[a]
	Narrow	*T. sernai*	99	2 (0:2)	3	0.0006	0.0004	0.724	3.00	2.79	0.44	0.50	0.5116
TLR3	Wide	*A. conirostris*	85	7 (3:4)	13	0.0009	0.0012	−0.004	12.35	8.09	0.34	0.57	0[a]
		H. leucosticta	34	9 (0:9)	13	0.0014	0.0019	−0.708	12.14	11.46	0.76	0.73	0.7676
		M. tuberculifer	30	11 (5:6)	11	0.0021	0.0024	−0.281	10.00	10.32	0.62	0.64	0.4347
	Narrow	*M. apicalis*	26	3 (0:3)	5	0.0004	0.0008	−0.941	4.52	5.00	0.31	0.31	0.1081
TLR4	Wide	*A. conirostris*	82	9 (3:6)	14	0.0021	0.0025	−0.406	13.56	9.63	0.77	0.74	0.1447
		H. leucosticta	32	4 (1:3)	7	0.0016	0.0013	0.394	6.81	7.00	0.71	0.73	0.4611
TLR5	Wide	*H. leucosticta*	31	7 (2:5)	9	0.0032	0.0019	1.730	8.71	8.19	0.81	0.81	0.5126
		M. tuberculifer	33	35 (19:16)	48	0.0070	0.0091	−0.879	37.10	37.19	0.97	0.99	1
	Narrow	*M. apicalis*	26	3 (0:3)	6	0.0010	0.0006	1.257	5.00	6.00	0.75	0.75	0.0002[a]

[a] Significant deviations from Hardy–Weinberg equilibrium.
[b] Number of individuals analyzed per locus.
[c] Number of synonymous (s) and nonsynonymous (n) SNPs.
[d] Number of inferred haplotypes.
[e] Nucleotide diversity.
[f] Watterson's estimator of the population mutation rate.
[g] Tajima's D neutrality test.
[h] Allelic richness corrected for sample size difference.
[i] Allelic richness corrected for sample size difference between species.
[j] Observed heterozygosity.
[k] Expected heterozygosity.
[l] P-value of the exact test of Hardy–Weinberg equilibrium.

equilibrium in TLR1LA, TLR1LB, TLR2, TLR3, and TLR5 (Table 1). A deficit of heterozygotes was found at all these loci, except for TLR1LA in *H. leucosticta* where an excess of heterozygotes was observed (Table 1). F_{ST} values for each TLR locus were very low between the two populations (dry forest and transition dry-rainforest) in all species and was only significant for TLR1LA in *A. conirostris* and *H. leucosticta*, and for TLR2 in *A. conirostris* (Appendix Table S6). Total F_{ST} values for population differentiation in the species was also very low and not significant: *A. conirostris* ($F_{ST} = 0$, $P = 0.9$), *H. leucosticta* ($F_{ST} = -0.007$, $P = 0.5$), *M. tuberculifer* ($F_{ST} = 0$, $P = 0.9$), *M. apicalis* ($F_{ST} = 0$, $P = 0.9$) and, *T. sernai* ($F_{ST} = 0$, $P = 0.9$). The haplotype networks (Appendix Figs. S6–S10) are consistent with what was found in the F_{ST} values, where no population structure was detected. In the TLR1LA haplotype network for *A. conirostris* and *H. leucosticta*, we found private haplotypes in the transition dry-rainforest and private haplotypes in dry forest, which explains the F_{ST} value obtained with low structuring.

Codon-level selection tests detected evidence of positive selection at all loci when comparing between species. The MEME model identified between 1 and 11 sites at all TLRs. The FUBAR model identified between 3 and 9 sites in all TLRs. Sites identified under positive selection by both methods were between 1 and 5. The dN/dS ratio was <1 for all loci, being slightly higher for TLR4 and TLR5 (Appendix Table S7).

3.2. Neutral genetic diversity

In none of the species studied we found a significant F_{ST} when comparing populations on both banks of the Cauca River; therefore, in the following paragraphs only the F_{ST} estimates by habitat type will be mentioned. Microsatellite genotypes for all species can be found in Appendix B. We present the results by species pairs, comparing closely-related species (same family), with different geographic distribution ranges. In the two Troglodytidae species (*H. leucosticta* and *T. sernai*), 15 microsatellites were tested. In *H. leucosticta*, nine microsatellites were successfully amplified, of which seven were polymorphic. In *T. sernai*, only four of the 15 markers were polymorphic. Two microsatellite loci amplified in common in both species, Hle284 and ThPl30. The summary variation of polymorphic loci for *H. leucosticta* and *T. sernai* is shown in Appendix Table S8. At the Hle284 locus, seven alleles were found for *T. sernai*, while for *H. leucosticta* this was the most polymorphic locus with a total number of 17 alleles. On the other hand, the ThPl30 locus showed a very similar number of alleles in *T. sernai* and *H. leucosticta*. However, it is important to note that in *T. sernai* this marker showed a significant deviation from Hardy–Weinberg equilibrium, presenting an excess of heterozygotes, as found in a previous study in this species (Zapata et al., 2020). It was also evidenced that the TA-C3-B (2) locus in *H. leucosticta* was out of Hardy–Weinberg equilibrium, showing an excess of homozygotes. Neither of these two loci showed evidence of null alleles; therefore, they were retained within the analyses, because removing them did not yield different results.

In the STRUCTURE analysis for *H. leucosticta* (Appendix Fig. S11) the ΔK of $K = 2$ was found to be the most probable. No pattern of genetic structuring between groups was found; on the contrary, individuals are observed to be equally likely to belong to one or the other population. This result is consistent with the F_{ST} value detected (transition dry-rainforest–dry forest: $F_{ST} = 0.03842$, $P < 0.05$), where there is a low population differentiation. In contrast, a pattern of group differentiation is observed in the PCA (Appendix Fig. S12), in which the locality of Buriticá is separated from the rest of the clusters. It is important to note that this result in the PCA may be influenced by the low representativeness of samples in this locality.

Similar results were found in the genetic structure for *T. sernai*, in which for the STRUCTURE analysis (Appendix Fig. S13) the highest ΔK value indicates that the most probable number of populations is two, but no population differentiation is evident. The F_{ST} value was low but significant (transition dry-rainforest–dry forest: $F_{ST} = 0.0097$, $P < 0.05$), indicating that there is very low genetic structuring. In the PCA (Appendix Fig. S14), the individuals are mainly grouped in one genetic group, although it is evident that the Santafé locality is separated from the others.

In the two Tyrannidae *M. apicalis* and *M. tuberculifer* 10 microsatellites were amplified. In *M. apicalis* eight loci of the 10 microsatellite loci successfully amplified were polymorphic, whereas in *M. tuberculifer* all 10 microsatellite loci were found to be polymorphic (Appendix Table S9). The number of alleles ranged from two to 10 and 2 to 21 for *M. apicalis* and *M. tuberculifer*, respectively. A higher allelic richness was also evident in *M. tuberculifer*. Two markers in common: AAAG-33 and LTMR8 were found to show significant deviation from Hardy–Weinberg equilibrium for both species, due to an excess of homozygotes. Two other loci, SAP50 and AAGG-209 in *M. apicalis* and *M. tuberculifer*, respectively, were not in Hardy–Weinberg equilibrium. These loci were retained in the analyses, since testing to remove them did not influence the result in genetic diversity and population differentiation metrics.

In the STRUCTURE analysis for *M. tuberculifer* (Appendix Fig. S15), no pattern of genetic structuring between groups was found, as it is observed that individuals have the same probability of belonging to any group. The ΔK indicates that the most probable number of populations is two. The F_{ST} value (transition dry-rainforest–dry forest: $F_{ST} = 0.0133$, $P = 0.2072$), is consistent with the evidence that there is no genetic structure shown by the STRUCTURE analysis. However, in the PCA (Appendix Fig. S16) a clustering of three or four genetic groups is observed.

The STRUCTURE analysis in *M. apicalis* (Appendix Fig. S17) shows four genetic clusters; however, this clustering is evenly distributed among the populations studied. The F_{ST} value (transition dry-rainforest–dry forest: $F_{ST} = 0.06513$, $P < 0.05$) shows that there is population differentiation although it is low, and the PCA (Appendix Fig. S18) shows that the genetic groups are more separated.

In *A. conirostris*, 13 of the 15 microsatellite loci genotyped were polymorphic (Appendix Table S10). At the polymorphic loci the number of alleles ranged from three to 12, and the highest allelic richness at these loci was 11.95. Four markers were found to be outside the Hardy–Weinberg equilibrium: Sosp-ng146, Sosp-ng149, Sosp-ng197, Sosp-ng269, resulting from the excess of homozygotes as these markers were found to have null alleles. However, in the estimation of the F_{ST} value, a similar value was found when excluding or including them in the analyses; therefore, they remained within the results.

The results of the ΔK of Evanno's method show three genetic groups ($K = 3$) for *A. conirostris* (Appendix Fig. S19). These three genetic groups are distributed among the populations studied but the assignment probabilities are not high in any of the groups, indicating that the genetic structure does not fit the geographic distribution. On the other hand, the F_{ST} values obtained among the populations were low but significant (transition dry-rainforest–dry forest: $F_{ST} = 0.02264$, $P < 0.05$). Likewise, the PCA (Appendix Fig. S20) showed a separation of the Buriticá locality with respect to the other localities, which may be in line with the low structuring found in the F_{ST} values.

3.3. Association of TLR and neutral genetic variation

For widely distributed species, MLH showed no relationship with TLR heterozygosity; the very weak negative effect was statistically nonsignificant: the 95% credible interval included zero (Correlation coefficient: −0.1222, *P*: 0.1228) (Fig. 2A). For narrowly distributed species, MLH showed no relationship with TLR heterozygosity; the very weak positive effect was statistically nonsignificant: the 95% credible interval included zero (Correlation coefficient: 0.1259, *P*: 0.1222) (Fig. 2B). The GLMM model suggested that there were some species-specific effects (Fig. 3). *A. conirostris* and *M. apicalis* species do not have random effects significantly different from zero. On the other hand, *H. leucosticta*, *M. tuberculifer* and *T. sernai* species have random effects that are significantly different from zero.

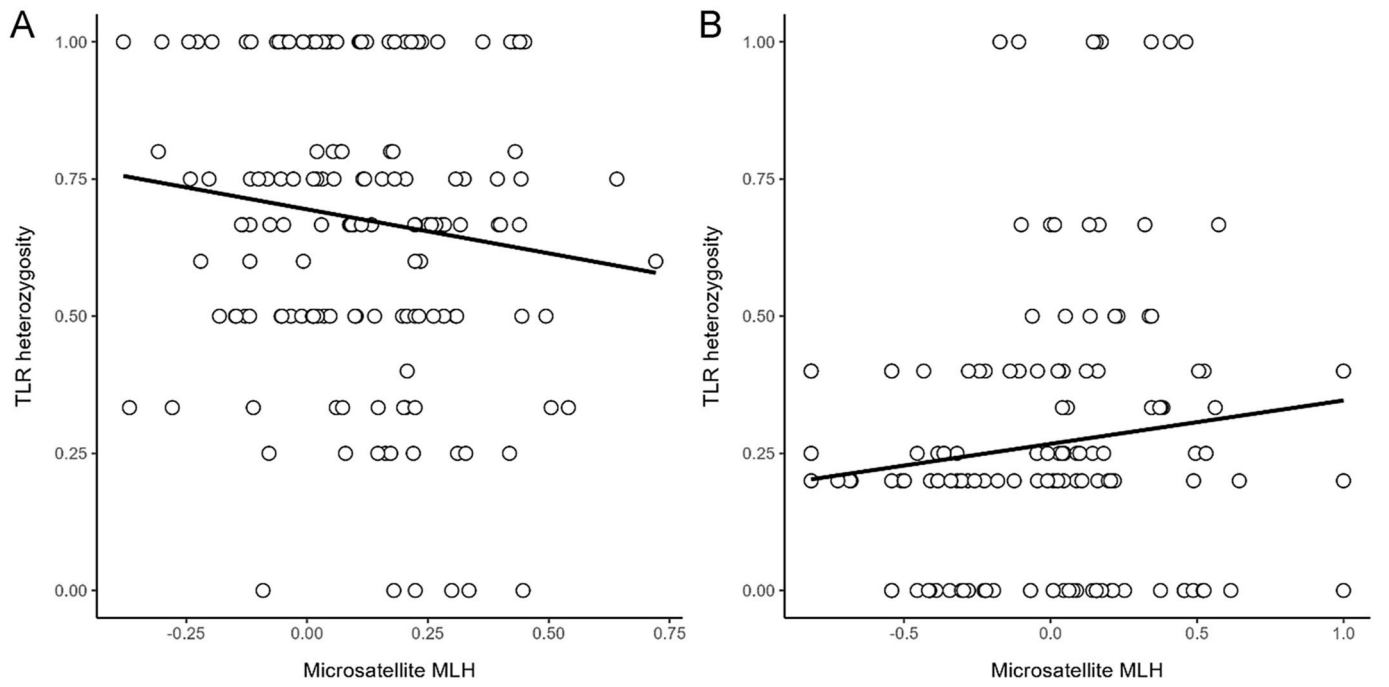

Fig. 2. Relationship between IR, a microsatellite-based measure of individual multilocus heterozygosity (MLH), and TLR heterozygosity in (A) widely and (B) narrowly distributed species.

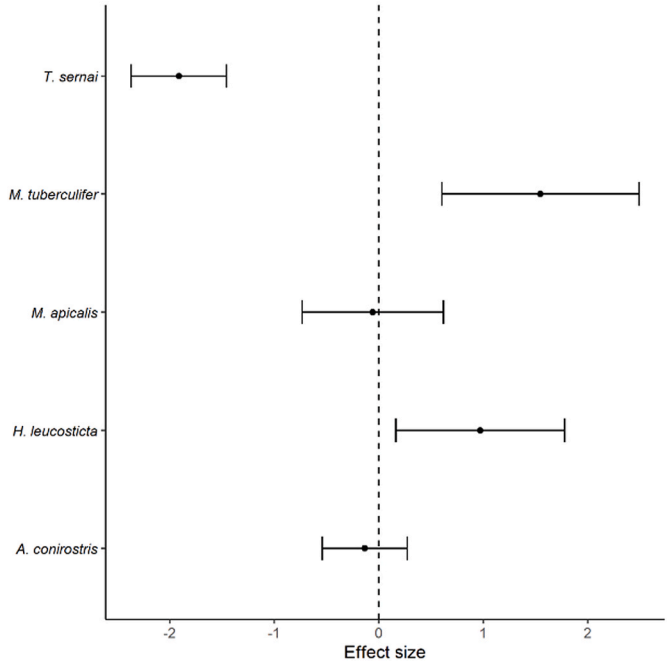

Fig. 3. GLMM model showing variation in genetic effect sizes of widely distributed species and narrowly distributed species based on neutral and adaptive genetic diversity.

3.4. Relationship of microsatellite and TLR heterozygosities with the species geographic range

Geographic range and heterozygosity at microsatellites and TLRs exhibited a non-significant phylogenetic signal according to the two metrics (λ and K, Appendix Table S11). Therefore, it is not possible to conclude that phylogeny influences these traits. Mean heterozygosity at TLRs ranged from 0.209 in *T. sernai* to 0.868 in *M. tuberculifer*, while heterozygosity at microsatellites ranged from 0.145 in *T. sernai* to 0.591 in *A. conirostris* (Table 2). The total distribution area of the species and the distribution area in the study area are reported in Table 2. No significant correlation was found between average heterozygosity at microsatellites and species range ($r = 0.674$, $P = 0.2121$). On the other hand, a significant correlation was found between average heterozygosity at TLRs and species range ($r = 0.896$, $P = 0.0394$).

4. Discussion

4.1. Patterns of genetic diversity in TLRs

Toll-like receptors are key for the innate immune response; therefore, variation at these loci is thought to play a pivotal role in adaptive processes. However, genetic drift and gene flow also influence the patterns of genetic diversity at adaptive loci; therefore, we expected to find signatures of selection at TLRs in all species, and a higher TLR diversity in widely distributed species, compared to those with a restricted distribution. At the species level, *M. tuberculifer* showed the highest genetic diversity among widely distributed species, with the highest polymorphism in TLR1LB and TLR5 (Table 1), which exhibited a substantial number of nonsynonymous SNPs (Table 1). The high levels of polymorphism found may be driven by balancing selection, where several alleles are maintained in the population. This is reasonable because TLR1LB recognizes bacterial lipoproteins (Takeda et al., 2002), microorganisms that may be commonly found in the environments these birds

Table 2
Measurements of mean heterozygosity in TLRs and microsatellites and total distribution areas (IUCN, 2025) and of the distribution area of the species in the Hidroituango hydroelectric project study area.

Species	Total area (km^2)	Study area (km^2)	H_e (TLRs)	H_e (MSATS)
A. conirostris	3,990,000	3502	0.734	0.591
H. leucosticta	8,050,000	3175	0.814	0.438
M. tuberculifer	21,100,000	4538	0.868	0.543
M. apicalis	117,000	848	0.656	0.480
T. sernai	6360	866	0.209	0.145

occupy. It is possible that these alleles confer an advantage in responding to pathogens, although this remains to be explored. On the other hand, in the haplotype network, private haplotypes were evident in the dry forest (Appendix Fig. S8), revealing the presence of many low-frequency allelic variants in this forest type. This is consistent with the Tajima's *D*-neutrality test (Table 1), where the value was negative which is concordant with excess of polymorphism associated with a population expansion process. However, the finding of such high levels of polymorphism should be interpreted with caution due to such a small sample size and uneven sampling across populations of this species ($n_{\text{dry forest}} = 28$, $n_{\text{transition dry-rainforest}} = 5$). As a result, more haplotypes were found in the dry forest than in the transition dry-rainforest ($h_{\text{dry forest}} = 45$, $h_{\text{transition dry-rainforest}} = 8$). It is possible that *M. tuberculifer* has preferences for a drier habitat type as reported in a previous study, where the characteristics of a tropical dry forest seem suitable for the species (Maglianesi, 2009).

The results for *A. conirostris* suggest the existence of genetic differentiation between the two forest types (Appendix Table S6). In addition, the presence of private alleles in the dry forest and in the transition dry-rainforest (Appendix Fig. S6) could be related to local adaptation events, possibly driven by differences in selective pressures exerted by pathogens. This species shows a high prevalence of a *Plasmodium* parasite in the study area (Martínez-Alvarado et al., 2019), but no analyses have been done to assess whether this prevalence varies between the two habitat types. Further exploration of the possible association of habitat type, infection status, and individual TLR genotypes is warranted in this species.

The genetic variation observed at TLR loci in *H. leucosticta* suggests that balancing selection is the main driving force behind these patterns, probably in response to pathogen-mediated pressures. Furthermore, in TLR1LA, significant F_{ST} values and the presence of private alleles in the dry forest and transition dry-rainforest indicate a possible local adaptation process. These findings reinforce the idea that environmental heterogeneity contributes to the maintenance of genetic diversity in genes involved in the immune response.

Species with restricted distribution exhibited low diversity in TLR. In *M. apicalis*, levels of haplotype diversity were low (5–18 haplotypes per locus for three loci) compared to levels observed for *M. tuberculifer* (11–48 per locus for three loci) with a similar sample size. However, most substitutions were non-synonymous which may correspond to a balancing selection scenario.

In *T. sernai*, the species exhibiting the least diversity in TLR, only two loci of the five analyzed were polymorphic. TLR1LB and TLR2, presented few SNPs, but all resulted in nonsynonymous substitutions (Table 1). In addition, TLR1LB showed deviations from Hardy–Weinberg equilibrium (HWE) with excess of heterozygotes (Table 1). Additionally, a positive Tajima's *D* (Table 1) at both loci could be an indication of balancing selection acting in the population. Despite the low TLR diversity in this species, the amino acid variants may persist due to functional changes in the protein, potentially enabling the species to withstand environmental selective pressures from pathogens (Zapata et al., 2020). This finding in TLR diversity for *T. sernai* is consistent with results reported in the Stewart Island Robin (*Petroica australis rakiura*), a threatened species that exhibited few haplotypes at the loci analyzed but presented nonsynonymous variation (Grueber et al., 2012).

We found that the values of allelic richness per locus and allelic richness adjusted for sample size per locus among species were higher for widely distributed species than for narrowly distributed species (Table 1). Although it is important to note that differences in genetic diversity among species may be due to evolutionary processes unique to each lineage, a pattern can be recognized in which allelic richness is more sensitive to declines in population sizes (Bichet et al., 2015) and restricted distribution species exhibit a small population size, as reported for *T. sernai* (Zapata et al., 2020). Despite the difference between diversity metrics in TLRs for species according to their distribution range, we found no significant genetic differences between the two habitat types evaluated. Here we suggest two hypotheses to explain this result. First, selection pressures in both habitat types are similar or genetic variation in TLRs is not high enough to detect significant differences. A second possibility is that there is high gene flow between localities and that this same evolutionary force maintains the high diversity patterns in the widely distributed species. In addition, widely distributed species have the ability to colonize new habitats, even when these are fluctuating, unlike restricted distribution species that only use stable habitats (Glazier, 1986). The low genetic diversity found in TLRs in restricted distribution species may be attributed to the low capacity of the species to adapt to new conditions. It is likely that this low diversity allows them to thrive only in their range as reported for *T. sernai* (Zapata et al., 2020), and may be sufficient to confer some advantage if there is a specific type of selection acting (Hartmann et al., 2014), since as reported in the present study, we found that most substitutions found in *T. sernai* and *M. apicalis* corresponded to nonsynonymous substitutions.

Codon-specific selection tests suggest evidence of positive selection operating at TLR loci (Appendix Table S7). On the other hand, we found that the rate of nonsynonymous to synonymous nucleotide substitutions per site (dN/dS) was <1 indicating that most nonsynonymous substitutions are removed from populations by negative selection. This pattern can be explained because the dN/dS estimator is calculated for the entire sequence that is primarily conserved, whereas per-codon selection tests can detect specific codons that are being subjected to the effect of positive selection (Appendix Table S7). However, it is noteworthy that a good proportion of nonsynonymous substitutions was found even in restricted-distribution species, suggesting that positive selection can drive TLR evolution in birds (Velová et al., 2018; Włodarczyk et al., 2023).

Our findings on the diversity of TLRs suggest that these immune genes may evolve under positive selection or balancing selection where certain allelic variants may confer resistance to diverse pathogens. Our study area is located in a region with a high prevalence and diversity of avian Hemosporidia (Martínez-Alvarado et al., 2019) and it is likely that these parasites constitute a selective pressure driving diversity in TLRs. A recent study demonstrated how balancing selection driven by avian Hemosporidia shapes diversity at TLR loci (Antonides et al., 2019). While we hypothesize that environmental differences between dry forest and transition dry-rainforest may impose distinct selective pressures—potentially via variation in pathogen communities—we did not directly evaluate pathogen presence or infection status in this study. This limits our ability to establish a direct link between TLR variation and pathogen-mediated selection. To explore potential differences in adaptive genetic diversity between habitats, we compared allelic richness and nucleotide diversity for each TLR locus across forest types (see Appendix Figs. S21–S22). However, no statistical comparisons were performed due to limited species-level data for most loci. Future studies should assess pathogen prevalence and diversity in the study area—particularly for Hemosporidia and other parasites—as this information is essential for evaluating the role of pathogen-mediated selection in shaping TLR variation.

4.2. Patterns of genetic diversity in microsatellites

In contrast to TLRs, microsatellite markers could not be compared in general terms among all species because they were specific for each family. However, it was possible to observe a pattern in which the widely distributed species showed greater genetic diversity than the restricted distribution species. In the Troglodytidae family, in *H. leucosticta*, 78% of the loci analyzed were polymorphic, while in *T. sernai* only 27% were polymorphic. Allelic richness and heterozygosity values were lower for *T. sernai* than for *H. leucosticta* (Appendix Table S8). These results can be explained because a species such as *T. sernai* with a decreased population size (BirdLife International, 2023), may suffer mostly from decreases in the number of alleles and expected heterozygosity per locus (Nei et al., 1975). No population structuring

was found in microsatellites for *T. sernai* or *H. leucosticta*, although the F_{ST} values were significant, they were very low, indicating the high gene flow for the species in the sampled localities. It is noteworthy that *H. leucosticta* was only sampled in five municipalities, three of which were part of the transition dry-rainforest. Although the sampling effort was greater for the dry forest population because it included five municipalities, it is possible that the species was not captured mostly in this type of forest because it may prefer more humid habitats. According to this, finding that in the PCA (Appendix Fig. S12) Buriticá was separated as an individual cluster may be associated with the low representativeness of samples compared to the rest of the municipalities. On the other hand, the PCA for *T. sernai* (Appendix Fig. S14) does not correspond with the STRUCTURE plot (Appendix Fig. S13), since two genetic clusters are evident, where Santafé is separated from the rest of the municipalities. As in *H. leucosticta*, there is a separation mainly from one municipality with very few individuals. Although wrens are characterized by their low dispersal capacity associated with their ecology and morphology, it seems that the environmental gradient and geographic characteristics in the study area do not represent a limit to dispersal. In general terms, no population structure is evident for wren species, which may imply that gene flow is an important evolutionary force shaping neutral genetic diversity in these species.

In the two flycatcher species (Tyrannydae family), a decrease in allelic richness but not in heterozygosity was identified for *M. apicalis* (Appendix Table S9). Moderate to high heterozygosity was observed at all loci for both species. In small populations such as *M. apicalis*, a species of restricted distribution and according to predictions about geographic range and population size, loss in allelic diversity may occur faster than loss of heterozygosity (Maruyama and Fuerst, 1985). In contrast to what was reported for the wren species, *H. leucosticta* and *T. sernai*, a different pattern in genetic structuring was found in *M. tuberculifer* and *M. apicalis*. The STRUCTURE analysis for *M. tuberculifer* (Appendix Fig. S15) showed a pattern of admixture with equal probability of individuals belonging to either genetic group. This was consistent with the F_{ST} value, where no significant differences were found between the two populations, dry forest and transition dry-rainforest. Although in the PCA analysis (Appendix Fig. S16) four genetic groups were recovered, we rescue the notorious difference of the low sampling in the two localities of Peque and Ituango, which suggests that it influences the separation of the clusters. On the other hand, the analysis of genetic structure in *M. apicalis* revealed four genetic groups that are homogeneously distributed (Appendix Fig. S17). This is consistent with what was found in the PCA (Appendix Fig. S18) and the low population differentiation for the species. These results may evidence a higher structure for *M. apicalis*, which may be attributed to a lower gene flow in a species with a restricted distribution range where population densities are lower (Brown, 1984) and, therefore, the movement of individuals between localities is more limited.

In *A. conirostris*, although we could not compare diversity metrics with a species of restricted distribution, we found interesting patterns to highlight. This widely distributed species showed high levels of polymorphism, and moderate to high values of allelic richness and heterozygosity (Appendix Table S10). Most interestingly, unlike the other widely distributed and restricted distribution species, we found a higher population structure where individuals are assigned to three genetic groups (Appendix Fig. S19). However, low population differentiation was found and there was no definite grouping between clusters in the PCA (Appendix Fig. S20). These results may be an indication of the high genetic diversity in the species and although there is a low pattern of differentiation it is likely that there is still a lot of gene flow between populations and constant movement of individuals between localities.

4.3. Association of TLR and neutral genetic variation

Although we did not find a significant relationship between mean microsatellite and TLR diversity in widely distributed species and restricted distribution species, we observed a slight negative and positive correlation, respectively (Fig. 2A and B). In widely distributed species, the negative correlation may be due to the fact that neutral genetic diversity not necessarily reflects adaptive genetic diversity (Holderegger et al., 2006; Barton, 2010; Whitlock, 2014; Chung et al., 2023), and in these populations, selection processes can be evidenced by the high rates of nonsynonymous substitution as an indication of balancing selection. On the other hand, in the restricted distribution species we observed a positive correlation. Although we found signatures of selection resulting from several nonsynonymous substitutions, it is possible that selection is not strong enough to counteract the effect of genetic drift in restricted distribution species (Gonzalez-Quevedo et al., 2015). In the GLMM, the effect size (Fig. 3) is probably explained by the effect of *H. leucosticta* and *M. tuberculifer* which had a high genetic diversity. In *A. conirostris* and *M. apicalis* the diversity in microsatellites does not explain the diversity in TLRs as was reported by Grueber et al. (2015) for ten threatened species. In *T. sernai* the negative effect can be explained because there was very low genetic diversity in TLRs where only two out of five markers were polymorphic. However, it is important to note that the lack of significance in assessment could be due to two key factors. First, species microsatellite data mainly comprise loci characterized by family (Tyrannidae, Troglodytidae and Passerellidae), implying that each microsatellite marker has a different evolutionary history and rate of substitution. Therefore, caution is needed when comparing microsatellite data between species. Second, it was not possible to successfully amplify the same TLR loci across all species, which could affect the assessment of TLR diversity and hinder interspecies comparisons.

Patterns of diversity in microsatellite loci and TLRs were consistently higher in widely distributed species, as expected for species with broad distribution ranges and higher abundance. (Brown, 1984; Gaston, 1996; Blackburn et al., 1997; Krabbe and Rahbek, 2010; Verberk, 2011). Widely distributed species have higher population densities per unit area but, at the same time, because of their wide geographic range, can colonize a diversity of heterogeneous landscapes (Wang et al., 2022), where although there are variable selective pressures, populations may be sufficiently close or abundant in an area for genetic exchange to continue to occur. Correlation analyses between average heterozygosity and the range of distribution were high and significant for TLR, but not for microsatellites, which may correspond with a scenario where widely distributed species exhibit greater variation possibly driven by positive and/or balancing selection. This could result from exposure to a greater diversity of pathogens encountered in the wide variety of habitats associated with a large distribution. In addition, it is important to mention that the correlation results may show a pattern of the relationship of species range and genetic diversity but should be taken with caution given the unique nature of each molecular marker and the comparison of different microsatellite markers defined in this study by bird family. When we performed the phylogenetic signal tests to evaluate phylogenetic independence in microsatellite heterozygosity (MLH), TLR heterozygosity and species range, we found that it was not significant with K and λ. This supports our results and suggests that our conclusion of higher genetic diversity in species with a wide distribution compared to those of restricted distribution is not due to those species being closely related. However, the low number of lineages included in the analysis limits the robustness of the phylogenetic signal tests (Blomberg et al., 2003; Revell et al., 2008; Münkemüller et al., 2012), and therefore, these results should be interpreted with caution.

The patterns of increased diversity in TLRs for widely distributed species are comparable with other studies in wide-ranging bird species, the Lesser Kestrel (*Falco naumanni*) and House Finch (*Haemorhous mexicanus*) (Alcaide and Edwards, 2011), and a recent study in 11 bird species (Włodarczyk et al., 2023) that showed how patterns of increased variation at TLRs are maintained by diversifying selection. Other studies are consistent with patterns of diversity in TLRs found in our restricted distribution species, such as those reported for the Stewart Island Robin

(*Petroica australis rakiura*), a New Zealand subspecies in threatened status that showed low variation in TLRs, but where much of it corresponded to nonsynonymous variation associated with the action of natural selection (Grueber et al., 2012). These patterns are consistent with theoretical expectations that wider distribution ranges may maintain greater genetic diversity due to larger effective population sizes and increased gene flow. However, we caution that our findings are based on five species sampled regionally, and genetic diversity estimates were derived only from populations within the Cauca River canyon. Therefore, while the observed trends are informative, they should be interpreted with caution. Additional studies incorporating a wider range of taxa and geographic contexts will be necessary to assess the generality of these patterns.

Overall, F_{ST} values between the two populations (dry forest and transition dry-rainforest) were significant for microsatellite markers, but not for TLRs. Our findings are comparable to what was found by Bichet et al. (2015) when evaluating the genetic structure of island and continental populations from microsatellite and major histocompatibility complex (MHC) gene data. Our results showed that neutral processes as well as adaptive processes are very important in shaping patterns of variation at these loci. Low structure was found with both types of genetic markers but was lower in adaptive loci. The fact that there is less differentiation in adaptive relative to neutral loci may be associated with the fact that allele frequencies of populations can be homogenized by gene flow, allowing a homogeneous distribution of alleles to respond to the pressures exerted by pathogens in all environments. In addition, genetic diversity in TLRs is being driven by natural selection, promoting the maintenance of several nonsynonymous substitutions and allelic variants in the different species as could be recognized in both widely distributed and restricted distribution species. High gene flow may prevent local adaptation, but we do not know whether the parasite communities to which TLR alleles respond are differential across sites. This clearly needs to be further explored.

This study adds to the growing evidence that larger population sizes and wider range can result in the maintenance of higher levels of genetic diversity. This is important because maintaining high genetic diversity is crucial for maintaining the evolutionary potential of populations to respond to environmental changes. On the other hand, populations with restricted geographic ranges are susceptible to environmental changes due to their limited range, small population sizes and lower genetic diversity, which hinders their adaptation and makes them more prone to local extinctions.

CRediT authorship contribution statement

Marcela Restrepo-Arias: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Héctor F. Rivera-Gutiérrez:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition. **Iván Darío Soto-Calderón:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Ernesto Pérez-Collazos:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Catalina González-Quevedo:** Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics statement

The samples used in this study were collected under the Framework Permit for the collection of specimens of wild species of the biological diversity for non-commercial scientific research purposes, granted by the ANLA (Autoridad Nacional de Licencias Ambientales) to the Universidad de Antioquia by resolution 524 of 2014.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hector Fabio Rivera-Gutierrez reports financial support was provided by Empresas Públicas de Medellín. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.avrs.2025.100279.

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