Genetic drift does not outweigh natural selection at Toll-like receptor genes in the populations of Galápagos mockingbirds

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Abstract

Population and conservation genetics seek to understand how adaptive diversity is shaped by the interweaving forces of molecular evolution in small and endangered populations. On the one hand, selection shapes variation, on the other hand, genetic drift impedes the selection by stochastic changes of allele frequencies. Drift is hypothesised to prevail if the population size is small. However, in practice empirical estimates of the population size are often challenging. Here we used island size as a proxy to population size to reveal the evolutionary constraints of molecular diversity in Toll-like receptors (*TLR*s) of mockingbirds (genus *Mimus*) inhabiting Galápagos islands. TLRs are crucial for pathogen recognition by host immunity and thus under various selection constraints. We focused on the interaction of drift and selection in *TLR1B*, *TLR4*, and *TLR15* across 12 size-variable insular populations and compared them with the mainland population of the northern mockingbird (*Mimus polyglottos*), aiming to test if population size impacts selection efficiency. Nucleotide diversity positively correlated with the island size indicating an increasing effect of genetic drift in small populations. Despite this pattern, functional TLR properties were largely conserved, presumably due to purifying selection opposing drift independently on the island size. The degree of protein conservatism differed between the loci with TLR15 being the least conserved. Island colonisation did not lead to relaxed selection or to local adaptations. Together with the invariable physicochemical properties of the TLR variants, these observations imply that drift did not outweigh purifying selection despite restricted population size.

Key words: Conservation genetics, Population Genetics - Empirical, Purifying selection, Island birds, Innate immunity, Molecular phenotype

Introduction

Genetic diversity is a voucher for future persistence in ever changing environments (Bijlsma & Loeschcke 2012; Spielman *et al.* 2004). Theory and empirical evidence show that genetic drift reduces this diversity in finite-sized populations (Charlesworth 2009; Kimura 1983), and particularly in insular populations (Frankham 1997). Furthermore, drift also impairs selection efficiency, increasing frequency of potentially suboptimal alleles (Ohta 1992; Galtier 2016). Such distortion of adaptive genetic diversity can lower the fitness of a population and worsen its chance for survival (Spielman *et al.* 2004; Willi *et al.* 2006). Diversity in molecules involved in pathogen detection is particularly important because it determines the ability to adequately respond to infections (DeCandia *et al.* 2018). Several authors reported that even this adaptive diversity is negatively affected by genetic drift in small populations regardless of its functional importance (TLR - Grueber *et al.* 2013; MHC - Miller & Lambert 2004). Here we revise the issue and test to what degree drift and selection shape immuno-genetic diversity in a set of differently sized insular populations.

We focus on single-locus genes that encode Toll-like receptors (TLR), molecules responsible for direct recognition of pathogen-derived ligands. These first-line defence molecules trigger both innate and adaptive immune mechanisms (Iwasaki & Medzhitov 2010; Acevedo-Whitehouse & Cunningham 2006), and their variation is linked with variable resistance to several infectious diseases in humans (Ferwerda *et al.* 2007; Azad *et al.* 2012; Li *et al.* 2016) as well as in birds (Leveque *et al.* 2003). TLRs are an evolutionary conserved family of transmembrane glycoproteins typical for their horseshoe-shaped exodomain structures (Wang *et al.* 2016). Each member of the TLR family recognizes distinct set of ligands (Kumar *et al.* 2011). For example TLR4 detects lipopolysaccharide (LPS) (Nagai *et*

al. 2002), TLR1 lipopeptides (Jin *et al.* 2007) and TLR15 is putatively activated by bacterial and fungal proteases (de Zoete *et al.* 2011). Noncovalent binding of the ligands in the ligand-binding region (LBR) leads to receptor dimerization, which activates downstream signalling pathways (Kumar *et al.* 2011). Therefore, LBR is shaped by natural selection to maintain its proper functionality.

Purifying (i.e. stabilising) selection is the prevalent type of natural selection that shapes overall molecular evolution of TLRs ensuring their conservativeness. This process leaves a distinct pattern in the non-synonymous to synonymous substitutions ratio (dN/dS) that is lower than one across whole vertebrate phylogeny (for TLR4 ~ 0.425; TLR1LB ~ 0.420; TLR15 ~ 0.325) (Wang *et al.* 2016). Prevailing purifying selection in TLR was supported by several studies focusing on analysis of TLR polymorphism at a population level or in closely related taxa (Darfour-Oduro *et al.* 2015; Mukherjee *et al.* 2009; Nelson-Flower *et al.* 2018; Raven *et al.* 2017; Vinkler *et al.* 2015). Signs of site-specific positive (i.e. directional) selection have been detected in multiple taxa (e.g. Alcaide & Edwards 2011; Králová *et al.* 2018; Tschirren *et al.* 2011; Quéméré *et al.* 2015) and association between individual TLR alleles and resistance to pathogens in natural populations provided further support of adaptive importance of the TLR variation (Bateson *et al.* 2016; Gavan *et al.* 2015; Tschirren 2015).

Several authors showed that genetic drift affects different aspects of TLR molecular evolution. Grueber *et al.* (2013) reported that in a small population of Stewart island robin (*Petroica australis rakiura,* Fleming) drift prevails over positive selection in TLR4 because a beneficial allele did not increase in frequency despite its positive effect on survival. Gonzales-Quevedo *et al.* (2015) observed that drift dominated the process of genetic differentiation of TLRs between insular populations of Berthelot's pipit (*Anthus berthelotii,*

Bolle). And finally, Hartmann *et al.* (2014) showed that interaction of drift and purifying selection depleted TLR diversity in a severely bottlenecked population of pale-headed brush-finch (*Atlapetes pallidiceps*, Sharpe). Interestingly, brush-finch's diversity of TLRs was negatively correlated with survival indicating that some deleterious alleles were segregating in the population. Although these studies indicate that interaction of drift with selection is important and drift is often prevalent, it remains unclear how population size affects the adaptive variability maintenance over extended evolutionary timescales. Here we fill this knowledge gap by the analysis of TLR ligand-binding region (LBR) diversity in a set of differently sized populations of mockingbirds inhabiting Galápagos islands.

Four species of mockingbirds occur on the Galápagos islands (hereafter collectively abbreviated GM) - Floreana mockingbird (*M. trifasciatus,* Gould), Hood mockingbird (*M. macdonaldi,* Ridgway), San Cristóbal mockingbird (*M. melanotis,* Gould) and Galápagos mockingbird (*M. parvulus,* Gould). A single ancestor population colonised the archipelago approximately one million years ago and diverged into multiple allopatric populations (Arbogast *et al.* 2006). Crucial feature of the system is that the recent migration rate between major populations is minimal and population size is determined by island size, with populations diverging from each other by the means of genetic drift (Hoeck *et al.* 2010). Floreana mockingbird is a critically endangered species that survives in two tiny populations. Champion census population size fluctuates between 20 - 50 individuals on 9.5Ha island and Gardner population ranges 60 - 180 on 81Ha island (Jiménez-Uzcátegui *et al.* 2011). The Isabela population (Galápagos mockingbird) stands on the other side of the size spectrum with the estimate of effective population size (Ne) above a thousand (Hoeck *et al.* 2010) on 458,812Ha island. The other populations bridge these extremes forming a gradual sequence of Ne roughly from tens to thousands (Hoeck *et al.* 2010). We complement GM populations with a population of Northern mockingbird (*Mimus polyglottos,* Linnaeus)

(hereafter abbreviated NM), a widespread mainland relative of GM (Lovette *et al.* 2012) that diverged from the ancestors of GM around 5.7 million years ago. The selected Californian NM population is the largest population in our study system (Vlček et al. 2016).

In this system we test to what degree population size affects the efficiency of purifying selection in TLRs. We do that by comparison of relationships between population size and different indices of genetic diversity. We hypothesize that “neutral” (synonymous) TLR diversity variance can be attributed to population size and largely explained by the effect of drift. On the contrary, “constrained” (e.g. non-synonymous, protein) diversity variance should be independent of population size if selection dominates. Furthermore, to understand the effect of drift on phenotypic diversity we explore the distribution of protein variants and their physicochemical properties (tertiary structure, surface charge) across the study system. We also compare GM to a larger continental population of NM and test if selection constraints have changed in between these groups and if positive selection was involved.

Materials and Methods

To provide a basis for our investigation, we initially screened diversity of *TLR1A*, *TLR1B*, *TLR2A*, *TLR2B*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15*, *TLR21* in all five species of our study system. This was performed using Sanger amplicon sequencing, which allowed us to compare the patterns of nucleotide polymorphism distribution across loci (Details in Supporting information 1 (SI1), chapter SN.1.1). We used the previously extracted DNA (Vlček *et al.* 2016) originating from blood samples collected between 2003 and 2008 by Hoeck *et al.* (2010). Based on the pattern of polymorphism detected, we selected TLR1B, TLR4 and TLR15 for the population-wide analysis using high-throughput sequencing (details in SI Table S3).

We sequenced the three *TLR* loci in 235 Mockingbird individuals by amplicon sequencing using Illumina MiSeq in the pair-end mode. We used dual-barcoded two-step PCR to prepare the amplicon library. In the first PCR, we amplified *TLRs* by specific primers that were designed based on preliminary sequencing data targeting the LBRs in TLR1B (Jin *et al.* 2007) and TLR4 (Park *et al.* 2009) and the neighbourhood of the putative cleavage site in TLR15 (Wang *et al.* 2016, de Zoete *et al.* 2011); analogous approach to Králová *et al.* (2018). Because the MiSeq sequencing length limit did not allow us to sequence the entire target region of TLR4 and TLR15 in one amplicon, we designed three primer pairs which produced shorter overlapping amplicons (SI1 Table S1-S2). In TLR1B LBR spans only a shorter region and therefore we used only one amplicon. In the second PCR we used primers that complemented the amplicon with sample specific barcodes. The sequencing was performed in the European Molecular Biology Laboratory (EMBL), Heidelberg. Detailed information on library preparation and sequencing is provided in SI1, chapter SN1.2.

To resolve the *TLR* LBR haplotypes and analyse their genetic and phenotypic diversity, we treated the sequence data with the following set of procedures. First, we removed low quality sequences with Trimmomatic (Bolger *et al.* 2014) and amplicons with coverage lower than six. Read pairs were assembled with the program PEAR (http://www.exelixis-lab.org/pear). Then we used the frequencies of unique nucleotide sequences (variants) per amplicon to discern between sequencing errors and genuine alleles and genotypes. If the ratio of the second to the first most frequent variants was equal, we considered the amplicon being heterozygous and we took both of the variants. If the ratio was less than ⅓ we considered amplicon homozygous and only the most frequent variant was used in the next steps (see details and justification in SI1, chapter SN1.3, Figure S1 and Table S4). Homozygous genotypes were assembled automatically by in-house python script while heterozygous

genotypes were assembled manually. Unresolved haplotypes that emerged due to amplification of the three overlapping fragments in TLR4 and TLR15 were inferred by PHASE (Stephens *et al.* 2001). We did not need to phase haplotypes in the case of TLR1B LBR. Analysis of sequencing and phasing accuracy was done by comparison of 20 replicates included in the amplicon sequencing. This analysis confirmed reliability of the approach. The orthology of sequenced loci was confirmed by BLAST. Basic diversity indices (nucleotide, haplotype, synonymous and non-synonymous diversities) and descriptive statistics (Tajima's D & Hardy-Weinberg equilibrium tests) were calculated based on the final dataset per population, per GM and across the whole sampleset (including NM) using dendropy (Sukumaran & Holder 2010), program SNAP (https://www.hiv.lanl.gov/content/sequence/SNAP) and Genepop (http://genepop.curtin.edu.au/, Rousset 1995). The complete bioinformatic pipeline is accessible on Github (https://github.com/vlkofly/TLR-amplicon), for further details see SI1, chapter SN1.3 and supporting data in Vlcek *et al.* (2020).

To assess to what degree drift shaped genetic and phenotypic diversity of *TLR* LBR in GM, we employed linear regression models that fit population diversity indices by island size (Snell et al. 1996). Island size represents a good proxy to effective population size as shown by Hoeck *et al.* (2010). As an explanatory variable, the island size was normalised by natural logarithm. Following population diversity indices were used as response variables in subsequent independent models: nucleotide diversity, haplotype diversity, nucleotide diversity based on synonymous sites, and non-synonymous sites, numbers of nucleotide haplotypes and protein variants. Because the sample size per population was unbalanced we also recalculated linear models with diversity values derived from a sampleset where the number of individuals per population was randomly downsampled to match the population with the least individuals. Linear models were constructed in R 3.5.2 (R core 2016) with

192 package lme4 (Bates *et al.* 2015) and results were plotted using package ggplot (Wickham 2009).

194 Further, we analysed distribution of nucleotide and protein variants across populations. Comparison of haplotype networks among *TLR* loci allowed us to understand a relative role 196 of different evolutionary constraints on each *TLR* locus in Mockingbirds. We hypothesised that under purifying selection protein variants are more likely to be shared and private 198 population haplotypes differ only in synonymous sites, whereas under neutrality, when populations are drifting apart, or under diversifying selection, we expect occurrence of 200 relatively more private haplotypes on both protein and nucleotide levels. We constructed a haplotype network of all nucleotide haplotypes as a minimum spanning network in PopArt 202 (Leigh & Bryant 2015). Then we highlighted the most frequent protein variants by pooling underlying nucleotide haplotypes. In order to inspect how divergence in *TLR* was affected by 204 evolutionary constraints relative to neutral loci, we tested to what degree the pairwise genetic Jost's D distances between populations derived from *TLR* LBR correlate with 206 distances derived from 26 microsatellites adopted from Vlček *et al.* (2016); Details in SI1 (chapter SN1.4.).

208 To understand if observed non-synonymous changes could affect the phenotype of the receptors, we analysed several physicochemical features of the most frequent protein 210 variants. First, we extracted segregating residues and categorized their conservativeness based on a Grantham Scale (such as Rudd *et al.* 2005, Grantham 1974). Second, we 212 characterized protein variants by physicochemical properties of their segregating residues and used principal component analysis (PCA) to explore relative differences between 214 proteins in the composite physicochemical space (Details in SI1 chapter SN1.5). Third, we ascertained the effect of residue substitution on TLR functionality by analysing tertiary 216 protein structures. We modelled the 3-dimensional structures of TLR LBRs using I-TASSER

software (Yang & Zhang 2015). Subsequently, we superimposed the structural models and calculated root-mean-square deviations of atomic positions (RMSD) as the measure of structural differences. Furthermore, we also clustered protein variants based on their surface charge, an important protein feature having impact on the binding of pathogen-derived ligands (Vinkler *et al.* 2014, Walsh *et al.* 2008). The surface charge (electrostatic potential) of TLR LBR haplotypes was analysed by program PIPSA (Richter *et al.* 2008). We used R package pvclust (Suzuki & Shimodaira 2006) to perform hierarchical clustering of proteins based on their surface charge distances and assessed the uncertainty of the clustering by bootstrap resampling. All the statistical analyses were done in program R version 3.5.2 (R Core Team 2016). Further details are available in SI1 chapter SN1.5.

Finally, we used several approaches to evaluate the role of natural selection in molecular evolution of *TLRs* in GM relative to NM. First, we tested a scenario that colonisation of Galápagos led to a significant accumulation of adaptive substitutions in TLR LBRs. We used the webpage platform MKT for McDonald-Kreitman test (Egea *et al.* 2008) to analyse if there is an excess of non-synonymous substitutions between GM and NM relative to polymorphism within GM. We also used common starling (*Sturnus vulgaris,* Linnaeus) as a more diverged outgroup for this test. Second, we tested an alternative scenario that selection pressure got relaxed after the colonization of Galápagos. For that purpose we employed a program RELAX (Wertheim *et al.* 2015) that compares evolutionary rates in a gene between test and reference lineages (alleles), testing whether selection constraints intensified or relaxed in the test lineage relative to the reference branches. We assigned branches leading exclusively to GM alleles as test lineages and branches leading to NM as the reference ones, inferring change in selection constraints in GM relative to NM. Third, we used codon based methods FUBAR (Murrel *et al.* 2013) and FEL (Kosakovsky Pond & Frost 2005) to inspect if particular sites were shaped significantly by a selection constraint. Site

was considered as under selection only if detected by both methods. Further details on selection tests are available in SI1 chapter SN1.6.

Results

We genotyped LBRs of three *TLRs* in 229 individuals of GM and their continental relative (NM), to reveal patterns of functional immunogenetic diversity in the small insular populations. Individual *TLR* LBR loci differed substantially in their levels of diversity, with *TLR1B* showing the lowest values, *TLR4* showing intermediate, and *TLR15* showing the highest values across all estimated parameters (Table 1). All loci showed negative overall Tajima's D and excess of synonymous to non-synonymous diversity. All populations and loci were in Hardy-Weinberg equilibrium except for TLR15 in Fernandina (SI1, Table S5) indicating general reliability of the genotyping approach.

Island size was revealed as an important predictor for several diversity indices in regression tests, however, we found considerable variation among loci (Figure 1, SI1 Figure S2). In TLR15 LBR all diversity indices showed statistically significant positive correlation with island size, while for TLR4 the correlation was significant for all diversity-linked traits but non-synonymous diversity and number of protein variants. In TLR1B none of the diversity index showed significant relationship, because of low diversity in this locus. The regression results remained qualitatively unchanged when based on a downsampled dataset, with only one exception that TLR4 nucleotide diversity relationship became statistically not significant. Results of individual statistical models are available in SI1, Table S6. We further observed that population synonymous diversity was significantly higher than non-synonymous diversity in TLR4 (T-test: t = -4.16, df = 11.37, p-value = 0.0015, SI1, Figure S3) but not in TLR15 and TLR1B supporting thus differences in selection constraints among loci.

The pattern of distribution of the nucleotide and protein variants across populations also indicated that TLR4 and TLR1B are substantially shaped by purifying selection in GM. Although in TLR4 we observed 14 nucleotide haplotypes in GM, after translation, 96% of individuals carried only one of the two most frequent protein variants (P10, P4; Figure 2). TLR1B was nearly monomorphic in GM populations on both nucleotide and protein levels, with protein variant P1 being carried by 99% of individuals. On the other hand, TLR15, was less conservative with 20 nucleotide haplotypes and four major protein variants that were carried by 90% of individuals (Figure 2). We also observed significant positive correlation between pairwise genetic distances of TLRs and microsatellites but this correlation was weaker for TLR4 compared to TLR15 (correlation coefficients 0.573 and 0.797 respectively, Mantel $p < 0.0001$ for both; details available in SI1, Chapter SN1.4, Figure S4 - S5).

Physicochemical and structural differences between the most frequent protein variants in GM were rather insignificant in TLR4 LBR but also in TLR15. In TLR4, the two most frequent haplotypes differed in 3 residues exhibiting conservative (Leu > Val; Thr > Ser) or moderately non-conservative substitutions that differed in charge (Lys > Glu). In TLR15 the most frequent haplotypes showed only one pairwise residue change and they were conservative (Arg > His; Leu > Val; Leu > Phe) (SI1 Table S7). Resulting protein structure differences measured by RMSD were negligible between all protein variants in TLR4 and TLR15 (SI1: Table S11), and, similarly, the clustering based on surface charge did not show any significant differences (Figure 3. Details in SI1 SN1.5, Figure S7-S11).

Comparison of GM with NM indicated that insular populations harbour lower diversity but also that selection constraints in NM were consistent with the patterns observed in GM. Nucleotide and protein diversity was always higher in NM, whether compared to individual populations of GM, or even if diversity was calculated across all GM neglecting population

structure (Table 2, Figure 1). Conversely, the ratio of non-synonymous to synonymous diversity was higher in GM. Interestingly, TLR4 was functionally conserved even in NM where the majority of DNA haplotypes coded for one protein variant P15 and other protein variants were rare (Figure 2). Similar situation was in TLR1B where one major and two rare protein variants occurred. The pattern was different in TLR15 where we observed multiple protein variants segregating at intermediate frequencies. Corresponding to the protein variant network (Figure 2), we also observed that TLR4 haplotypes formed two narrow clusters in physicochemical space specific to GM and NM (SI1 Figure S6). Conversely, TLR15 did not form such narrow clusters, being significantly scattered in NM.

Finally, several methods showed that TLR loci were shaped predominantly by purifying selection in GM and the selection constraints have not become relaxed after colonisation of Galápagos islands. McDonald-Kreitman test, comparing polymorphism in GM with outgroups, was not significant for any locus or divergence level (GM - NM and GM - common starling) (Table 3). Low rate of non-synonymous changes between NM and GM indicates overall conservativeness of the TLR loci in evolution of Mockingbirds. Analysis of reciprocal evolutionary rate by RELAX showed that selection was neither relaxed nor intensified in GM relative to NM (this was marginally non-significant in TLR15, where the values of selection parameter k were lower than one, indicating relaxed selection ($k=0.23$, $p=0.077$). Finally, codon-based tests showed that multiple sites in TLR1B, TLR4 and TLR15 were under purifying selection and one site in TLR15 was under putative positive selection (Details available in SI1 chapter SN.1.6, Table S8-S10).

Discussion

Our study brings a novel perspective on evolutionary processes behind the diversity of pattern recognition receptors in small and endangered populations. We found that purifying selection constrains *TLR* LBRs substantially and it is not completely outweighed by genetic drift in the populations of mockingbirds inhabiting Galápagos islands. Importantly, however, the constraints vary among *TLR* loci affecting the degree to which the diversity is shaped by random drift. Our results suggest that TLR15 is less constrained and thus more affected by genetic drift. On the other hand, predominant purifying selection shaped phenotypic diversity of TLR1B and TLR4, despite different levels of stochasticity in the differently-sized GM populations. Below we discuss our findings in the light of current views on molecular evolution of pathogen recognition receptors.

Purifying selection modulates TLR diversity in GM and NM

Natural selection is one of the four evolutionary forces that shape distribution of genetic and phenotypic diversity (Lynch 2007). With distribution of TLR diversity reliably ascertained across multiple lineages, we investigated to what degree it was shaped by selection and which type of selection was prevalent. We assumed that both positive and purifying selection could be detected in *TLRs*. Positive selection was considered because target regions are crucial for pathogen recognition, which may allow reciprocal co-evolutionary arms races with pathogens (Vinkler *et al.* 2009). Purifying selection was considered because LBRs of individual TLRs have evolved to recognize a specific suit of ligand structures which must be matched by the LBRs (Kumar *et al.* 2011). Moreover, interaction within the molecule and with other molecular components of the pathogen recognition pathway impose further structural constraints (Zeldovich *et al.* 2007).

We found several pieces of evidence indicating that purifying selection was prevalent in the evolutionary history of all the three *TLRs* (15,4,1B) in GM and NM. Tajima's D indicated excess of rare variants, which can be interpreted as the effect of purifying selection, given that we can exclude an alternative scenario of population growth based on available data for GM (Hoeck *et al.* 2010). Furthermore, we found that the majority of non-synonymous mutations were purged, as we observed that pN/pS ratio was lower than one (Table 1). Codon based models showed multiple positions affected by purifying selection, and only one site in *TLR15* under positive selection. McDonald-Kreitman's tests did not support our hypothesis that adaptive molecular changes occurred as a result of the colonisation of Galápagos, and low rate of non-synonymous changes between NM and GM further confirmed purifying selection. Despite potential biases inherent to each method, mainly the effect of population structure and reduced population size on the dN/dS based tests (Kryazhimskiy & Plotkin 2008, Mugal *et al.* 2014), we conclude that LBR of the three *TLRs* were substantially shaped by purifying selection. This result is consistent with some other recent reports showing dominant role of purifying selection on intraspecific level or between closely related taxa in *TLRs* (Nelson-Flower *et al.* 2018, Mukherjee *et al.* 2009, Raven *et al.* 2017). This conservativeness may reflect shared pathogen communities in closely related taxa (Eichler 1948, Clark & Clegg 2017), which seems even more plausible explanation in the case of islands with depauperate pathogen diversity (Wikelski *et al.* 2004).

Different evolutionary constraints in immunogenetic and mitochondrial loci

Our previous research allows us to compare the distribution of genetic diversity of *TLRs* with another immune gene, MHC class II subunit β (*MHCIIβ*), investigated in a mostly identical set of individuals (Vlček *et al.* 2016). It also allows comparison with mitochondrial cytochrome oxidase gene (*COI*) (Štefka *et al.* 2011). Although *TLRs* are functionally more

similar to *MHCIIβ* than to *COI* (given the involvement in pathogen recognition and potential of evolutionary arms race), the *TLR* haplotype networks resembled that of *COI* more than MHC. While in *TLRs* we observed one or two major haplotypes and several rare ones, MHCIIβ network is a mesh of haplotypes with intermediate frequencies widely shared between populations and species (see SI1, Figure S13). On the protein level, TLR haplotypes are shared even between species. However, the sharing involves only one or a few haplotypes that are common across the whole archipelago, whereas in MHC it is the supertype polymorphism that is shared. While TLRs act as direct pattern recognition receptors (Barreiro *et al.* 2009), MHCII presents ligands derived mainly from extracellular pathogens to helper T cells to trigger their responses (Rock *et al.* 2016). MHC in this population follows the well-documented pattern characteristic for loci under pathogen-mediated balancing selection (Doherty & Zinkernagel 1975). Here we show that in the same populations TLRs may be invariantly adapted to specific ligand recognition (Figure 2, Figure 3). We note that comparison between these loci is problematic, due to differences in their length and gene duplication history, nevertheless, our data indicate that the selection constraints are notably dissimilar.

Alleles escaping selection in the smallest GM population

Although purifying selection in all *TLRs* was prevalent, a few private or almost private variants with non-synonymous substitutions reached considerable frequency (TLR4-P4, TLR15-P11, P22). By analysing their physicochemical properties we attempted to estimate whether these variants had any major impact on phenotypic function of the receptor. We hypothesised that in extremely small populations we may see some deleterious variants due to less efficient selection (Ohta 1992). We found that, in general, across all TLRs the residue alterations were rather conservative. However, we observed one haplotype of TLR4 - P4 that has been fixed in the most endangered and smallest population on Champion island and

showed several differences in the surface charge pattern (Figure 3). In contrast, the rest of the populations shared one major protein variant (P10). Assuming that populations share the same pathogens, this P4 haplotype on Champion can be considered as a shift from optimum, a deleterious mutation that got fixed. Moreover, Champion showed substantial reduction in MHCIIβ diversity compared to the other populations (Vlček *et al.* 2016) and impaired health status compared to larger population of the same species on Gardner by Floreana (Deem *et al.* 2011). These observations indicate that in the case of extremely small populations with census size below 50, drift may actually outweigh selection even in the loci involved in pathogen recognition.

Effect of population size differs between TLR loci

Expecting an important role of genetic drift in differently-sized GM populations (Hoeck *et al.* 2010), we hypothesised that island size would be a significant predictor of both neutral and non-neutral diversity represented by synonymous and non-synonymous diversity, respectively. While our regression models supported population-size related effects of genetic drift on TLR diversity, different TLR loci differed in the degree of constraints limiting the effects of drift on non-synonymous diversity. In TLR4 we observed that island size was a statistically significant predictor of synonymous but not non-synonymous diversity (Figure 1), whereas in TLR15 also non-synonymous diversity was positively correlated with island size. Furthermore, only in TLR4 we observed significantly higher synonymous diversity compared to non-synonymous diversity (Figure S3). TLR1B harboured only a low amount of polymorphism and here the island size was not a valid predictor of any of the diversity indices. Scenario of different selection constrains was supported also by haplotype network analysis where in TLR4 we observed one major protein variant (P10) shared by almost all GM populations, but in TLR15 there were 4 major protein variants, two of them species specific (P22 and P11). Moreover, genetic distances between populations based on TLR15

matched the distances based on neutral microsatellites more closely than in the case of TLR4. These patterns imply that TLR15 is shaped more by genetic drift compared to TLR4 and TLR1B, which were more constrained by selection. Selection has not restricted accumulation of non-synonymous mutations in TLR15, while the same class of mutations were purged from TLR4. TLR1B was purged to an even higher degree being virtually devoid of any non-synonymous polymorphism. We did not find any sign of recombination in the loci, so the role of linked selection (Charlesworth 2012), which increased homozygosity especially in the shorter fragment of TLR1B, can be considered. Although differences in sequence lengths could potentially play some role, using per site measurements of population diversities in well diverged populations and well-defined gene region (LBR), we do not consider this factor as an important one.

Differences in diversity between NM and GM further supported the outlined differences in selection constraints between individual TLR loci. We observed a common pattern that TLR genetic diversity was reduced in GM compared to NM. This was most likely caused by a colonisation bottleneck and subsequent evolution in small sized populations. Random drift definitely shaped TLR diversity more in GM compared to NM. This observation is consistent with previously reported microsatellite diversity difference between NM and GM (Vlček *et al.* 2016). Here we argue that some patterns of the loss of TLR diversity point out to the joint effect of natural selection and genetic drift. If no selection was involved in the reduction of genetic diversity, and the mutation rate was equal in each TLR loci, and populations were in drift-mutation equilibrium as indicated by Hoeck *et al.* (2010), then we would expect roughly identical diversities in each TLR locus. On the contrary to this prediction, we observed that nucleotide and haplotype diversities of TLR15 were higher compared to other loci in both GM and NM. In NM nucleotide diversity of TLR15 was ~2 and ~7 times higher compared to TLR4 and TLR1B, respectively (Table 3). Difference between GM and NM was also much

higher in TLR15 (0.004) compared to TLR4 (0.0008) and TLR1B (0.0007) suggesting that TLR15 evolves under less strict selection constraints. Similar patterns showing more dramatic changes in TLR15 diversity compared to other loci were observed also in a study of mainland and insular populations of the song sparrow (Nelson-Flower *et al.* 2018).

The differences in the selection constraints observed between the TLR loci can be explained by the functional differences between the TLRs, where TLR15 appears adapted to a different mechanism of pathogen recognition. Sequenced regions contained 10%, 58% and 53% of sites known to be involved in ligand binding or putative cleavage activation in TLR15, TLR4 and TLR1B, respectively (de Zoete *et al.* 2011 & Wang et al. 2016, Park *et al.* 2009, Jin *et al.* 2007). Despite the lack of knowledge about the exact mechanism leading to TLR15 activation, it seems that its constrained region is either smaller compared to TLR4 and TLR1B or that the selection for maintenance of its general function is weaker. Relative relaxation of selection in TLR15 compared to other TLRs have been reported also in other studies (less sites under positive selection (Velová *et al.* 2018), more non-synonymous polymorphism within single species of the Seychelles warbler (Gilroy *et al.* 2017), increasing diversity after bottleneck in New Zealand robin (Grueber *et al.* 2013)), supporting relaxed selection constraints in TLR15 as a more general feature of this receptor beyond our study system.

Conclusions

Several studies based on free ranging insular populations claimed that in small populations TLRs were predominantly shaped by random genetic drift. Gonzales-Quevedo *et al.* (2015) found that divergence of TLRs between populations was caused by demographic history rather than selection. Grueber *et al.* (2013) observed that even though an allele showed an advantage, its frequency was shaped by drift. Both studies concluded that drift prevails as an

evolutionary process on islands over natural selection. Our results oppose these findings. Using a study system where differential island size serves as a proxy to genetic drift intensity, we show that genetic drift affects neutral variation, but functional sites remain constrained by purifying selection and its strength varies among TLR loci.

Our findings also provide conservation genetics insight into the interplay of evolutionary processes shaping diversity of important pathogen recognition receptors. Although, in general, stochasticity of allele sampling increases with decreasing population size, we show that the detrimental effect of fixation of suboptimal alleles in small populations can be alleviated by purifying selection, at least in some cases. The efficiency of purifying selection is even enhanced in small populations due to the effect of purging, as shown by Robinson *et al.* (2018). On the other hand, we must note that both the increased intensity of purging and drift leads to reduction of diversity (Charlesworth 2009), and thus, although deleterious alleles are purged even in small populations, the future of the small populations is still precarious because of the resulting reduction in genetic diversity (Spielman 2004).

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Data Accessibility

a. DNA haplotypes of TLRs are present on Genebank under accessions MT260894 - MT260972.
b. Genotype data linked with DNA accession numbers, together with amplicon filtering statistics and amplicon frequencies can be found in documented Figshare archive https://doi.org/10.6084/m9.figshare.12180027.v2 (Vlcek *et al.* 2020)
c. Additional data and notes are accessible in the online Supplemental Information file.

Author Contributions

J.V., M.V. and J.S. assembled the study design and provided funding. J.V. performed laboratory analysis. J.V. and M.M. analysed the data. J.V. drafted the manuscript. All authors contributed to the refinement of the drafted manuscript and approved its final version.

Tables and Figures

Table 1. Quantitative summary of each TLR LBR locus across all populations.

Locus	Len. (bp)	№ inds.	№ nuc. alleles	№ prot. var.	π	Hd	πN/πS	TajD
TLR1B	472	212	8	6	0.00096	0.163	0.138	-1.265
TLR4	889	228	34	18	0.00218	0.768	0.200	-1.655
TLR15	970	214	39	22	0.00274	0.832	0.225	-1.495

Len. (bp) - length in nucleotide base pairs (bp);inds. - individuals; nuc. - nucleotide; prot. var. - protein variant; π - nucleotide diversity; Hd - haplotype diversity; pN/pS - ratio of non-synonymous to synonymous nucleotide diversity; TajD - Tajima's D.

Table 2. Comparison of diversity indices between mockingbirds inhabiting Galápagos islands (GM) and northern mockingbird (NM).

Locus	Strata	№ inds.	№ nuc. alleles	№ prot. var.	π	Hd	πN/πS	Charge	RMSD
TLR15	GM tot.	203	20	10	0.0022	0.815	0.25	0.567	2.1
TLR15	GM avg.	16.9	3.1	2.3	0.0005	0.344	0.71†	NA	NA
TLR15	NM	11	19	11	0.0062	0.985	0.19	0.597	2.1
TLR4	GM tot.	211	14	8	0.0019	0.730	0.18	0.633	1.5
TLR4	GM avg.	17.6	2.8	1.8	0.0006	0.386	0.33†	NA	NA
TLR4	NM	17	18	10	0.0027	0.938	0.13	0.615	1.64
TLR1B	GM tot.	196	4	3	0.0001	0.025	1	0.615	2.87
TLR1B	GM avg.	16.3	1.3	1.2	0.0001	0.023	0.92†	NA	NA
TLR1B	NM	16	4	3	0.0008	0.289	0.13	0.7	4.31

GM tot. - diversity indices calculated based on a pool of all GM individuals; GM avg. - diversity indices calculated based on individual GM populations and then averaged; inds. - individuals; nuc. - nucleotide; prot. var. - protein variants; π - Nucleotide diversity ; Hd - haplotype diversity; pN/pS - ratio of non-synonymous to synonymous nucleotide diversity;Charge - average surface charge distance; RMSD - root mean square distance between protein models.† - within population pS was often 0 and then pN/pS was undefined, for that reason we arbitrarily assigned population pN/pS value 1, neglecting potential excess of non-synonymous mutations. The GM average is therefore a conservative estimate, while the real value should be rather higher.

Table 3. Results of McDonald-Kreitman test for two different divergence levels and each TLR locus.

Locus	Outgroup	PN	PS	DN	DS	MKT Chisq	p-value
TLR15	NM	9	8	1	2.01	0.398	0.527
TLR15	Starling	9	8	21.4	13.54	0.324	0.568
TLR4	NM	8	7	1	1	0.008	0.928
TLR4	Starling	9	6	25.74	24.21	0.333	0.563
TLR1B	NM	2	1	1	2	0.68	0.409
TLR1B	Starling	2	1	13.31	15.5	0.456	0.499

PN - Number of non-synonymous polymorphisms in Galapágos mockingbirds (GM), PS - number of synonymous polymorphisms in GM, DN - number of non-synonymous fixed differences between GM and outgroup, DS - number of synonymous fixed differences between GM and outgroup. NM - Northern Mockingbird, MKT Chisq - McDonald-Kreitman Chi Square statistic.

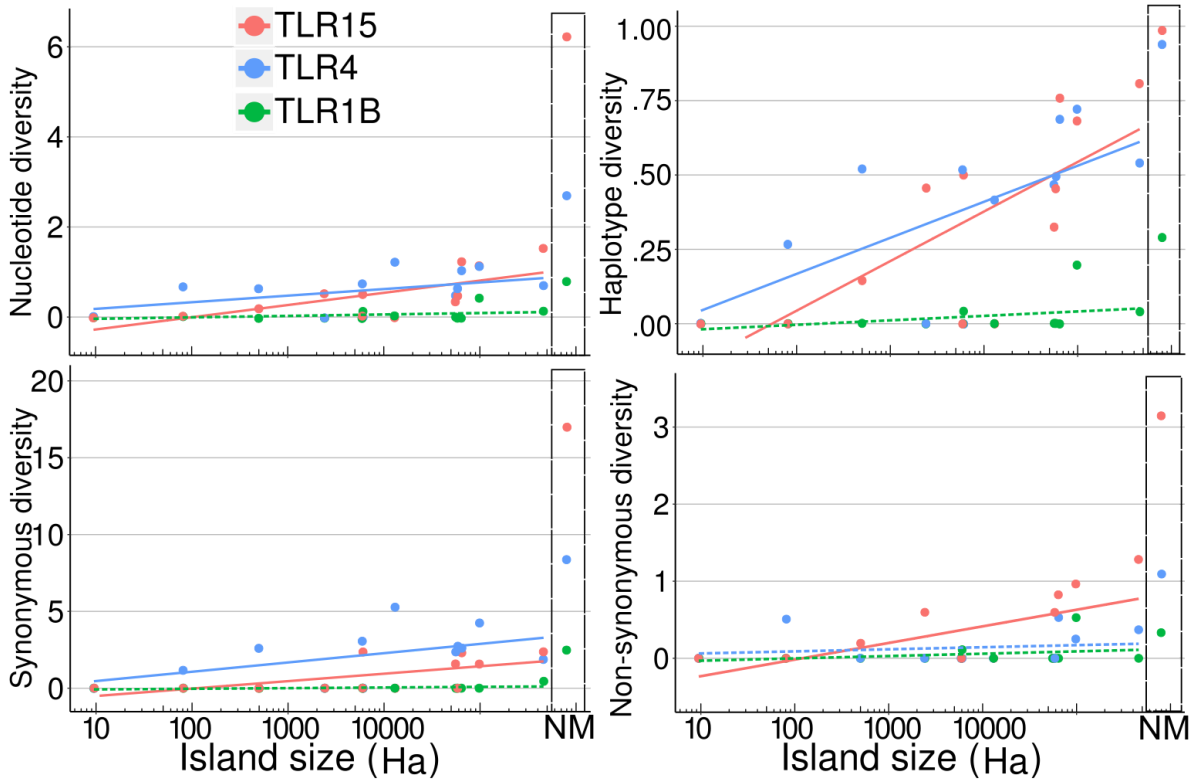

Figure 1: Relationship between population molecular diversity of three TLR loci and island size in hectares (Ha) on logarithmic scale. Majority of relationships are positive and statistically significant (indicated by full line). Statistically non-significant relationships are indicated by dashed lines. The lines represent predictions based on a linear regression model that has been calculated only for Galápagos populations. Values of molecular diversity for northern mockingbird (NM) are shown on the right-hand side of the graph in a dashed rectangle. Nucleotide, synonymous and non-synonymous diversities were multiplied by 1000. Figure with all indices is available in SI1, Figure S2 with test values in Table S6.

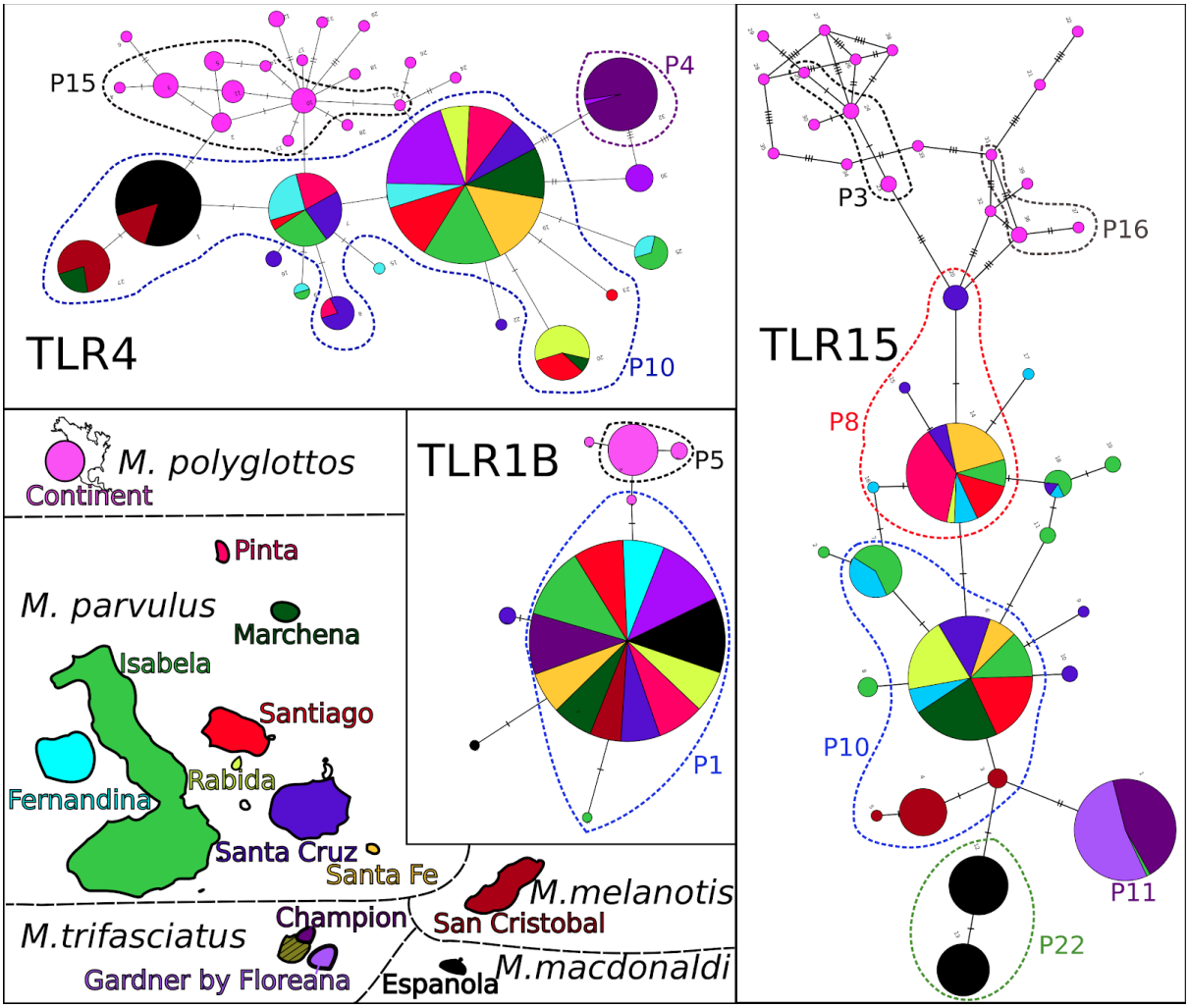

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Figure 2: Haplotype network of all nucleotide haplotypes found in mockingbirds inhabiting
742 Galápagos islands and continental northern mockingbird with schematic distribution of the studied populations. Every pie chart indicates a unique haplotype, with colours denoting
744 population identity and size indicating haplotype frequency. The most frequent protein variants are marked by dashed lines and their identifiers (e.g. P1). Champion and Gardner
746 by Floreana islands are off the scale because of their minute size.

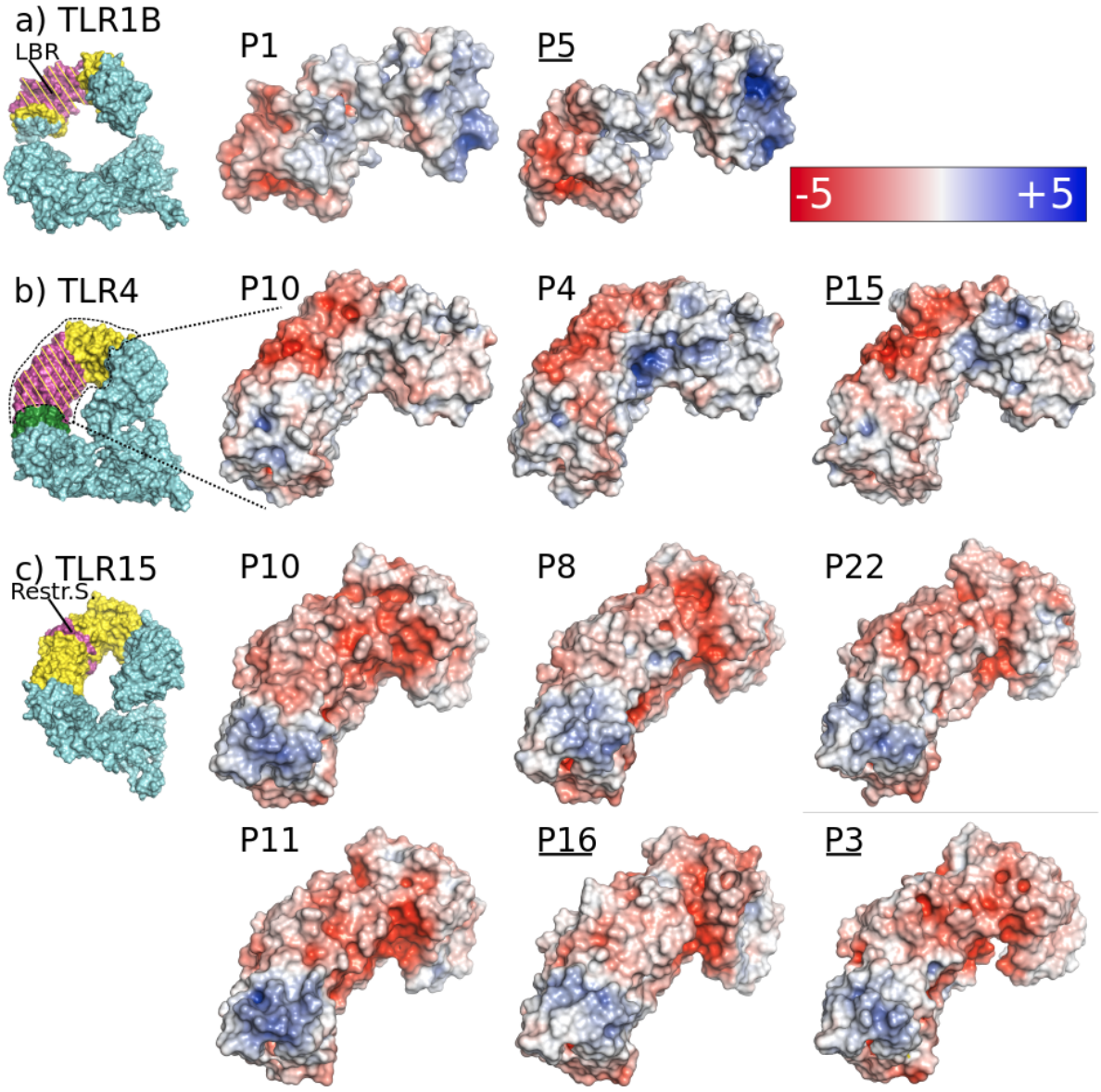

Figure 3: Visualization of surface charge of sequenced segments of the most frequent protein variants. Colour gradient denotes the surface charge: red - negative, blue - positive. Haplotypes found in northern mockingbird are indicated by underlined text. The leftmost column shows complete TLR molecule with ligand binding sites (LBS) (Jin *et al.* 2007, Park *et al.* 2009) or putative cleavage site (Restr. S.) (Wang et al. 2016, de Zoete *et al.* 2011) marked by violet and sequenced region marked by yellow colour (hatched where LBS and sequenced region overlap). Green ribbon in TLR4 denotes an unsequenced part of LBS.