Beyond neutral loci: Examining immune gene variation in tigers *(Panthera tigris)*

B.V. Aditi[1], Uma Ramakrishnan[1, 2]

[1] National Centre for Biological Sciences-TIFR

[2] Senior Fellow, Wellcome Trust DBT India Alliances

Abstract:

Isolated and inbred populations are at a higher risk to extinction due to the loss of genetic diversity. Carnivores are particularly susceptible to isolation due to their ecological requirements. Studies generally assess genetic vulnerability using neutral loci, but these may not accurately reflect a population's adaptive potential. In contrast, diversity at loci putatively involved in fitness and hence survival, such as immune genes, could be a better proxy for future survival. Research on immune genes has traditionally focused on the MHC loci. We extend this investigation to five families of non-MHC immune genes -Tumor Necrosis Factor, Interleukin, Toll-like Receptor, Leukocyte Immunoglobulin Receptors, and Chemokine - involved in adaptive and innate immunity in tigers which exemplifies an endangered carnivore. We compare immune gene diversity to neutral diversity across the genome using whole genome resequencing data from 107 tigers, representing all extant subspecies and populations of different demographic histories.

Our analysis reveals that immune receptor genes (mean nucleotide diversity: 0.0019) show high nucleotide diversity compared to neutral loci (0.0008) and immune signalling genes (0.0004) indicating past positive selection. Heterozygosity at the three classes of loci suggest that most immune genes are evolving neutrally. We confirm that small, isolated populations have lower nucleotide diversity and heterozygosity at both neutral and immune loci compared to large and connected populations. Additionally, genetic differentiation and deleterious mutation load correspond to known signatures from inbreeding and recent bottlenecks. Despite low neutral and immunogenetic diversity in small populations, some loci retain polymorphisms, irrespective of adaptive or innate immune functions. We conclude that drift is the predominant evolutionary force in bottlenecked populations even at adaptive loci.

Introduction:

The recent decline in biodiversity across the globe, resulting in increased extinction risks, is primarily driven by human activities (Barnosky et al. 2011) resulting in habitat loss and fragmentation. Despite significant conservation action, several species continue to be threatened. Carnivores, particularly Felidae, have continued to decline in numbers and rank lower in the IUCN Red List Index (Ripple et al. 2014; Fernández-Sepúlveda and Martín 2022). Carnivores tend to be large bodied, apex predators and need large habitats where they occur at low densities (Cardillo et al. 2008). Habitat loss and fragmentation sequesters such species into small and often isolated populations. Such small and isolated populations are prone to extinction because of environmental and demographic stochasticity as well as genetic effects (Gilpin 1986; Fagan and Holmes 2006).

Population genetic and island biogeography theories predict genetic erosion in small, isolated populations due to random genetic drift and inbreeding (Frankham 1996; Keller and Waller 2002; K. P. Johnson, Adler, and Cherry 2000). The chance effects of genetic drift can outweigh selection, in particular in small populations, and could cause disadvantageous alleles to increase in frequency, (or get fixed) and also could lead to a loss of advantageous alleles. Restricted mating opportunities in such populations could result in inbreeding which rapidly increases homozygosity and manifest in reduced fitness or inbreeding depression. Finally, one or more of these processes could have an impact on individual fitness, which in turn reduces genetic effective population size and population growth rate, eventually contributing to extinction. In the last two decades several case studies provide empirical evidence for this phenomenon (Slate et al. 2000; Liberg et al. 2005; Keller et al. 1994; Saccheri et al. 1998; Khan et al. 2021; Young and Clarke 2000). While the general nature of genetic effects and population decline are well understood, the exact drivers of these processes remain poorly understood, forcing population geneticists to rely on negative and positive genetic correlates of extinction or endangerment.

One such correlate that has been extensively studied is population genetic variation or heterozygosity. Several studies indicate that individuals in populations with low heterozygosity have a lower fitness, and as a result such populations have a higher chance of going extinct (J. A. Johnson and Dunn 2006; Chapman et al. 2009; Liebgold et al. 2018; Annavi et al. 2014; Hansson and Westerberg 2002; Taylor et al. 2010; Velando, Barros, and Moran 2015). Higher heterozygosity and standing genetic variation are thought to be beneficial for species/population survival. However, studies on fitness effects of new mutations indicate that most new mutations are mildly deleterious rather than adaptive (Eyre-Walker and Keightley 2007). Hence, high variation might not indicate adaptive potential. Empirical studies on heterozygosity fitness correlations report only weak correlations, and several endangered populations with very low genetic diversity survive over time (Morin et al. 2021; Robinson et al. 2016; Mathur and DeWoody 2021). Ultimately, it is challenging to determine whether variation is adaptive, neutral or deleterious in nature. As a result, the heterozygosity fitness correlations and extinction vortex continue to be scrutinized.

When attempting to understand fitness effects of low heterozygosity, deleterious mutation load or low genetic variation, it is important to distinguish between adaptive and neutral loci since evolutionary processes differentially shape genetic variation at different loci (Slatkin 1987; Charlesworth and Charlesworth 2017). Loci typically studied in population genetic studies are under weak or no selection and likely do not directly influence fitness. Such neutral loci are shaped by random processes of mutation, recombination and genetic drift and gene flow between populations. Adaptive variation that has a direct effect on fitness is consequently influenced by different types of selection. However, evolution in small populations tends to be dominated by drift, and not selection. As a result, genetic variation at potentially adaptive loci could simply mirror that at neutral loci in small and isolated populations, subject to drift. The study of neutral loci in conservation biology in the context of population viability and fitness is a longstanding and unresolved debate.

Recent studies on adaptive loci spurred by availability of genome assemblies have attempted to understand variation and selection at putatively fitness related loci such as genes related to reproduction and immunity. Immune genes have a direct effect on survival and reproduction, and are an ideal candidate to study adaptive variation, and its connection to population persistence (Netea, Wijmenga, and O'Neill 2012; Hedrick, Lee, and Buchanan 2013; Janeway et al. 2001). Lack of variation at immune genes could lead to increased susceptibility to disease and hence an increased extinction risk. Most of the studies on population genetics of immune genes focus on a single family- the Major Histocompatibility Complex (MHC) which is involved in adaptive immunity. These genes have been examined for diversity in bottlenecked populations, types of selection and associations with diseases (Aguilar et al. 2004; Alcaide 2010; Castro-Prieto, Wachter, and Sommer 2010; Edwards et al. 2000; Edwards, Wakeland, and Potts 1995; Radwan, Biedrzycka, and Babik 2010; Rivero-de Aguilar et al. 2016; Sutton et al. 2011; Wang et al. 2014). MHC genes represent a very small proportion of the immune gene repertoire (Jepson et al. 1997; Acevedo-Whitehouse and Cunningham 2006). With well mapped gene annotations, we can extend study of immune genes to several families of immune genes which could give a broader picture of immunity. Genes for Toll-Like Receptor (TLR) and Chemokine–are characterized in several wild species including those that exist in small populations (Gilroy et al. 2017; Grueber, Wallis, and Jamieson 2014; Grueber et al. 2012; Vlček et al. 2023; Morris et al. 2015).

Evolutionary and population genetic studies have identified contrasting roles of drift and selection in shaping immunogenetic variation. In several species, different immune genes have been known to be under positive selection (Grueber, Wallis, and Jamieson 2014; Shultz and Sackton 2019; Mukherjee et al. 2009). One generalization is that genes that code for receptor molecules that directly interact with proteins such as MHC genes and TLR genes are under positive selection and/or balancing or diversifying selection, whereas genes that code for signalling molecules such as Interleukin and Chemokine are under negative selection (Mukherjee et al. 2009). It has also been observed that higher polymorphisms in genes under balancing selection, prevent erosion of genetic diversity at these loci (Gilroy et al. 2017).

In this paper, we seek to investigate genetic diversity in five families of non-MHC immune genes for tigers. Tigers include six subspecies/populations across several countries in Asia, and present an example of an endangered species that has experienced population decline and habitat fragmentation. Tigers have lost 93 percent of their historic range, and have undergone strong population bottlenecks (Mondol, Karanth, and Ramakrishnan 2009; Armstrong et al. 2021). The Indian subcontinent harbours 60-70% of the global population of wild tigers (Mondol, Karanth, and Ramakrishnan 2009). Indian tiger populations show strong genetic differentiation and exist in different demographic situations: The North West (NW) Indian population is small and isolated while Central Indian (CI) and South Indian (SI) populations are large and connected (Natesh et al. 2017; Armstrong et al. 2021). Genome wide nucleotide diversity is known to be lowest for Amur tigers, and highest for Indochinese tigers (Armstrong et al. 2021). There have been many genomic studies on the different sub species of tigers as well as several genomic assemblies (Armstrong et al. 2021; Shukla et al. 2023) which is rare for an endangered species and allows us to conduct this study.

We attempt to understand adaptive variation in terms of nucleotide diversity and heterozygosity at immune genes by classifying them into two categories based on the possible selective forces acting on them: those under positive selection (genes coding for pathogen recognition molecules) and those under negative selection (genes coding for mediator cytokine molecules). Since tigers have witnessed various population bottlenecks, we expect the effects of selection to be weak, and that immune gene variation will correspond with patterns of variation at putatively neutral regions. However, given that some tiger populations have experienced disease outbreaks recently, it is possible that immune genes might be under selection, and we could see higher diversity at loci under positive selection. We further investigate differentiation in immune gene variation at the subspecies level and population level to investigate whether local, differential histories of disease exposure might have influenced immune gene variation. Finally, we investigate genetic load at immune gene loci to highlight whether immune genes also harbour potentially disadvantageous genetic variation.

2. Methods:

2.1: Identification of immune genes:

Immune gene sequences were extracted from the annotation file of a female wild Indian Tiger genome assembly (Shukla et al. 2023). The associated General Feature Format file was examined to extract rows containing terms related to the immune genes of interest. We searched for genes coding for Toll-Like Receptors (TLR), Leucocyte Immunoglobulin-Like receptors (LILR), Interleukin (IL), Chemokine and Tumor Necrosis Factor (TNF). These genes are well characterized and known to be involved in immunity. The search results were manually reviewed, and sequences with verified gene names and completely assembled Open Reading Frames were selected for further analysis. Genes were chosen for the study if the translated protein were immune receptors or signalling molecules. In the case of numerous entries of the same gene with similar start and end positions, the longest sequence was retained. This allowed us to determine the start and end positions of the entire gene. The annotation also provided information on the start and end positions of exons.

2.3: Whole genome resequencing data

We downloaded resequenced genomes of six sub species of tigers from several studies in the form of raw Fastq files (Armstrong et al. 2021; Khan et al. 2021; Liu et al. 2018; Zhang et al. 2023). In total, 107 genomes were downloaded, trimmed using default parameters of Trimmomatic (Bolger, Lohse, and Usadel 2014) and adapter sequences as mentioned in the published studies and aligned to the Indian tiger reference mentioned above using bwa-mem. We attempted to include as many wild or wild caught individuals as possible. The South China subspecies is only captive with no wild population. The details of samples and accession numbers in Supplementary table 11.

2.2: Creating VCF for intergenic and immune genes:

Since intergenic loci are not expected to be subject to selection, they were found to be a good surrogate for neutral loci. We defined intergenic loci as loci that are 50 kb upstream and downstream of genic regions (Armstrong et al. 2021). We extracted the start and end positions of genic regions from the Indian Tiger annotation mentioned above into a bed file and added a buffer of 50kb upstream and downstream the start and end of the genes. The Variant Call Format files containing both variant and invariant sites were created using the bcftools (Release 1.11 · Samtools/Bcftools). An mpileup was created followed by variant calling along with the relevant depth tags added to the VCF using the --annotate tag. Using bcftools norm, redundant lines were eliminated and variants were left aligned. VCFtools (Variant Call Format and VCFtools | Bioinformatics | Oxford Academic) was used to filter the resulting two VCFs for standard quality filters of minimum base quality of 30 and minimum genotype quality of 30 and to remove alleles occurring three times or less (minor allele count filter of three). We also exclude insertions and deletions since the identification of structural variants is not reliable with the SNP calling method we employ. Further, population genetic filters were applied on the dataset. As recommended in pixy manual, variant and invariant sites were separated and filters were applied separately to these sites. We filtered out SNPs with a Hardy Weinberg p-value less than 0.0001 and retain only biallelic sites. We retained SNPs that were present in at least 70 percentage of the individuals and finally filtered out SNPs in the top and bottom 0.25 quantile of mean depth (Armstrong et al. 2021). Finally, the filtered variant and invariant site VCFs were merged using bcftools concat command.

2.3 Nucleotide diversity, Heterozygosity and F_{ST} estimation

Nucleotide diversity and heterozygosity was estimated in three ways for immune and intergenic loci: First, all samples as a single population; second with assigned sub species and populations and finally as a single population and different genes clubbed together based on gene family. Pairwise Hudson's F_{ST} was estimated for both immune and intergenic datasets with sub species and sub populations assigned in order to account for the unequal sample size for the different populations.

The VCF containing exonic variants and intergenic variants were used as input for pixy to estimate nucleotide diversity pi and F_{ST} for non-overlapping 500 bp windows (average size of exons is 391 bases which we round off to arrive at the window size) (Korunes and Samuk 2021).

Heterozygosity was calculated as proportion of observed heterozygotes for every exonic SNP in immune genes and all SNPs in intergenic loci. Specifically, the –hardy option in vcftools was used to obtain observed genotypes and proportion of observed heterozygotes was calculated by summing up number of observed heterozygotes and dividing by observed genotypes.

2.4 Statistical tests

Differences in nucleotide diversity of immune receptor, signalling and neutral loci between populations was tested with a Zero Inflated Gamma Generalized Linear Model, differences between heterozygosity of immune receptor, signalling and intergenic loci was tested with a Binomial Generalized Linear Model and differences in pairwise Hudson's F_{ST} was estimated with a Gaussian model.

2.5: Identification of deleterious mutation and estimation of genetic load:

Ensembl Variant Effect Prediction (McLaren et al. 2016) was used to predict effect of the variants in immune genes with the custom annotation files, the tag –everything and –pick-allele. Variants were further classified into Untranslated region variants, synonymous variants, missense variants and upstream and Loss of function variants. Missense variants could potentially change the structure of the protein product and this change could be benign or deleterious. Hence, the potential for pathogenicity of identified missense variants was predicted using PolyPhen2 (Adzhubei, Jordan, and Sunyaev 2013) using the protocol HumVar2 which compares known pathogenic amino acid changes in humans to classify query mutations into benign, possibly damaging and probably damaging. Variants that were classified as LOF are splice_region_variant, splice_donor_variant, 3_prime_UTR_variant, downstream_gene_variant, upstream_gene_variant, synonymous_variant, frameshift_variant, intergenic_variant, splice_acceptor_variant, stop_gained, stop_lost, start_lost, transcript_ablation, inframe_insertion and inframe_deletion.

Genetic load was estimated at sub population level in terms of number of alleles using VCFtools –hwe tag. Alleles that were homozygous for alternate allele and heterozygous for missense LOF allele at sub population level. Homozygous missense load helps us to understand realized or inbreeding load whereas heterozygous load is the estimate of masked load in a population.

3. Results*:*

3.1 Summary of Immune genes identified:

Based on the information of the genes obtained from Indian tiger genome annotation, we were able to identify 91 Immune Genes across five gene families related to immunity with complete and contiguous gene sequences. Of these, two gene families code for receptor molecules that are involved in adaptive and innate immunity: Toll-Like Receptor (TLR) code for receptor molecules that recognize pathogens. Leukocyte-like Immunoglobulin Like Receptor (LILR) molecules interact with MHC receptors and regulate the activity of NK Cells. The other three gene families are involved in innate immunity. TNF, Interleukin and Chemokines gene families code for small, secreted molecules called cytokines and their ligands that reach sites of immune reactions and aid in the process by inducing inflammation and signalling

Supplementary Table 1 includes the sizes of genes, and extent of diversity (number of SNPs) in these gene families.

3.2 Patterns of genetic diversity at immune genes in tigers:

To assess patterns of genetic diversity at immune genes and neutral loci, we estimated nucleotide diversity over 500 bp windows and heterozygosity for each SNP. Our results revealed that mean nucleotide diversity at receptor loci (0.0019, 95 % CI: 0.0005-0.0046) is significantly higher than neutral loci (Figure 1 A) whereas signalling loci show lowest mean nucleotide diversity (0.0004, 95 % CI: 0.0003-0.0005). Mean heterozygosity (0.1645, 95 % CI: 0.1370- 0.1975) at receptor loci and signalling loci (0.1437, 95 % CI: 0.1299-0.1576) is lower than neutral loci (0.1973, 95 % CI: 0.1973 - 0.194) (Figure 1 B) but this difference is not significant (GLM results, Supplementary Table 2 and 4).

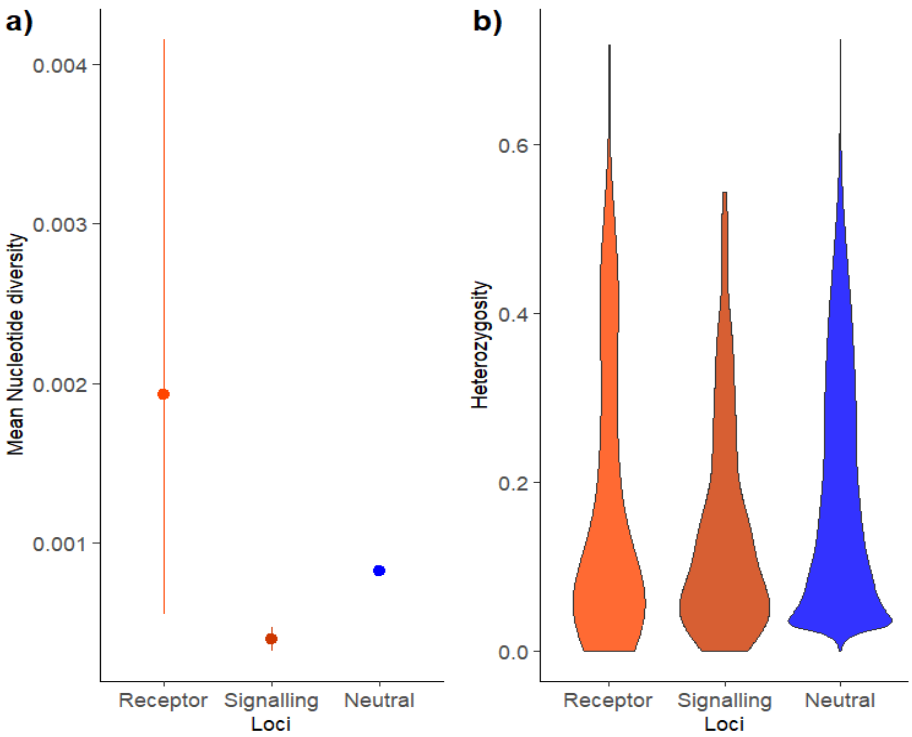

Figure 1: Distribution of a) nucleotide diversity (with 95% confidence intervals around the mean) and b) heterozygosity at receptor and signalling immune genes and neutral loci

We also examined the distribution of nucleotide diversity and heterozygosity values at each family of immune genes. We found significant differences in nucleotide diversity but not heterozygosity of different immune gene families (Figure 2) We observe a higher mean nucleotide diversity at the receptor genes (LILR and TLR) (Figure 2a) and higher heterozygosity values at Toll Like receptor genes, which have the highest mean heterozygosity (0.2442, CI: 0.1828-0.3078) (Figure 2b). (GLM results, Supplementary Table 3 and 5)

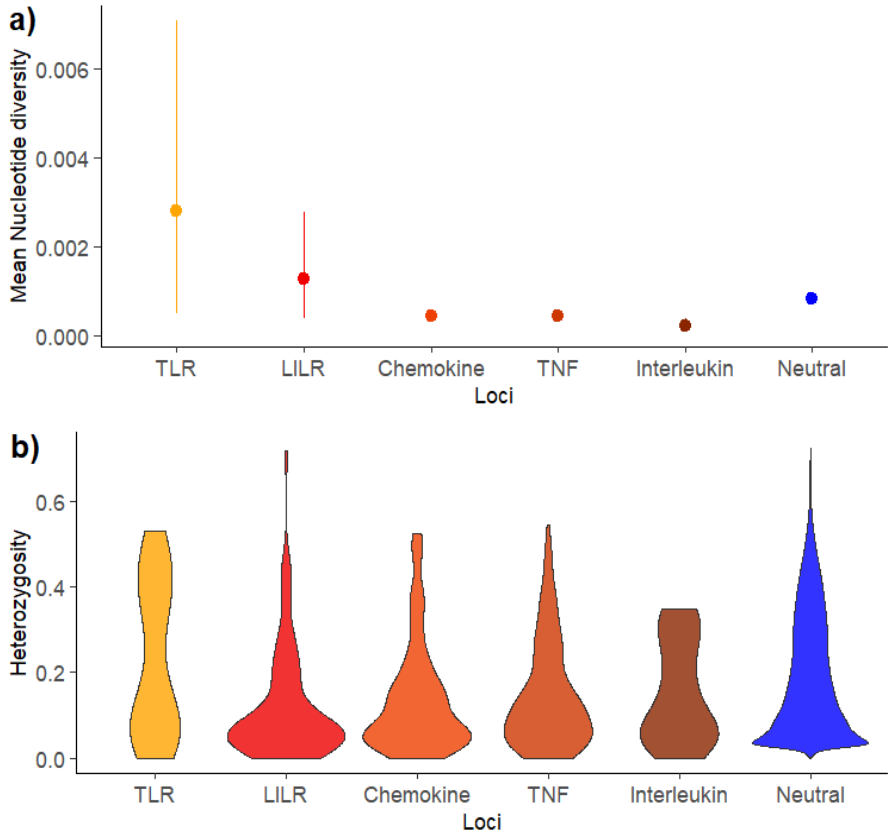

Figure 2: Distribution of a) nucleotide diversity (with 95% confidence intervals) and b) heterozygosity at different families of immune genes

3.3 Differences in immune gene diversity across subspecies and populations of tigers

We investigated the genetic diversity patterns for neutral and immune loci across range wide subspecies and/or populations. Again, we estimated nucleotide diversity and heterozygosity for immune genes and intergenic loci at subspecies/population level. Since we found no significant differences in heterozygosity of different gene families, we combine them to contrast immune genes with neutral loci. Since nucleotide diversity at receptor loci is significantly different from neutral loci, we examine receptor and signalling loci separately and compare with neutral loci.

Our results show that both nucleotide diversity (Figure 3) and heterozygosity (Figure 4) vary similarly across sub species at both immune and neutral loci (GLM results supplementary tables 6,7 and 8). Sumatran tigers have the least nucleotide diversity (Figure 3a) and South China tigers have least heterozygosity at immune and neutral loci (Figure 4a). Indochinese tigers have the highest mean nucleotide diversity and heterozygosity at neutral loci (Figure 3a and 4a). Again, in populations within India, the nucleotide diversity and heterozygosity at immune loci follow a pattern similar to neutral loci (Figure 3b and 4b) . The large, connected populations (CI and SI) have higher nucleotide diversity and heterozygosity when compared to the small, isolated population (NW).

Receptor loci have consistently high nucleotide diversity for subspecies, as well as Indian sub populations. The only exception are Indochinese tigers, where nucleotide diversity fell within inferred nucleotide diversity values. Signalling loci showed lower mean nucleotide diversity when compared to neutral diversity in all populations examined.

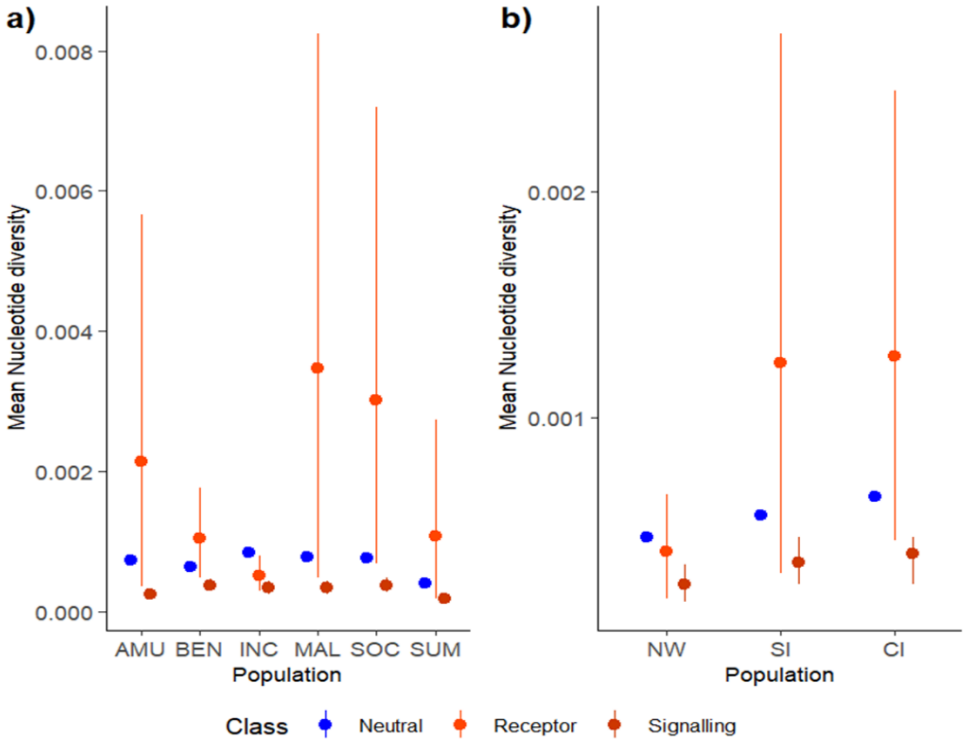

Figure 3: a) Mean and confidence intervals for nucleotide diversity at immune receptor, immune signalling and neutral loci in extant sub-species of tigers including INC (Indochinese), MAL (Malayan), SOC (South China), BEN (Bengal), AMU (Amur) and SUM (Sumatran) B) Mean and confidence intervals for nucleotide diversity at immune receptor, immune signalling and neutral loci in Indian tiger populations NW(Northwest), CI (Central India) and SI (South India)

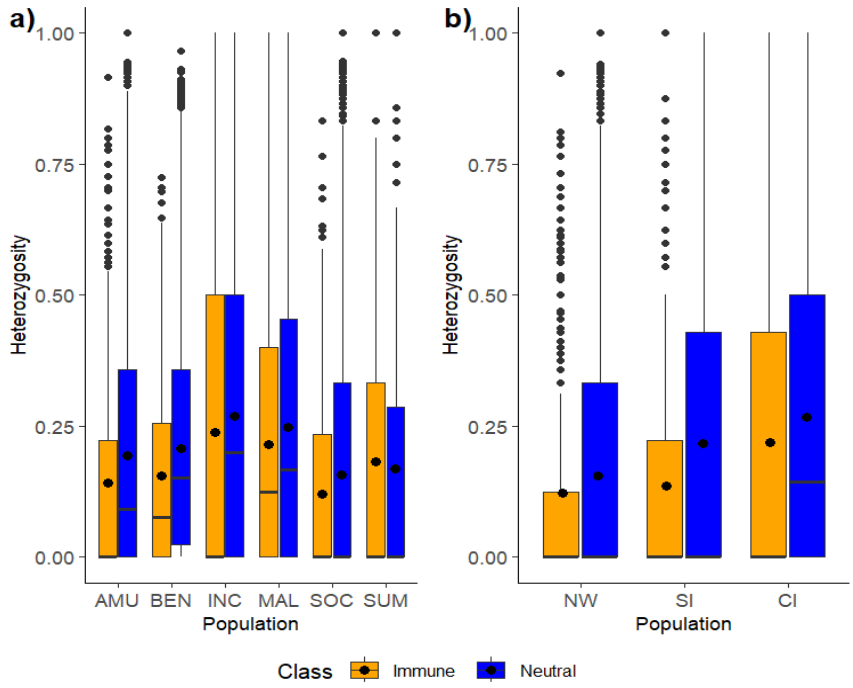

Figure 4: a) Box and whisker plots for heterozygosity at Immune and Neutral loci for ex tant subspecies of tigers including INC (Indochinese), MAL (Malayan), SOC (South Chi na), BEN (Bengal), AMU (Amur) and SUM (Sumatran)
b) Box and whisker plots for heterozygosity at Immune and Neutral loci for different po pulations of Indian tigers NW(Northwest), CI (Central India) and SI (South India)

Although the mean and median for both diversity indices was much lower in the NW population, there are several outliers in this distribution. These outliers are from sites across receptor and signalling genes and not restricted to any one gene family. Interestingly, we found that some of the genes having high diversity were shared among populations (Supplementary table 10).

3.4: Genetic differentiation at immune gene and neutral loci between populations of tigers

Given the differences in habitats of the different subspecies and subpopulations of tigers, it is possible that differential pathogen pressure lead to genetic differentiation at immune loci. We compared differentiation at immune loci to neutral inferences of differentiation using the Hudson's F_{ST} calculation for subspecies (Figure 4a) and subpopulations (Figure 4b). We found no significant difference in F_{ST} distributions of immune receptor, signalling and neutral loci (Supplementary Table 9).

We also examined F_{ST} at immune and neutral loci for populations that have history of disease or drastic demographic change in order to look for loci that might be under selection versus loci whose frequencies have been impacted by drift. We compared F_{ST} for Amur, Sumatran and South China to all other tiger populations and the three Indian sub populations to each other. Sumatran tigers had the highest mean F_{ST} at both neutral and immune loci. Amur tigers

had slightly higher mean F_{ST} at immune loci when compared to neutral loci, but this difference is not significant. Northwestern Indian tigers were the most genetically differentiated amongst the three Indian tiger populations, and similar patterns were reflected in immune genes (Supplementary figure 1 and 2).

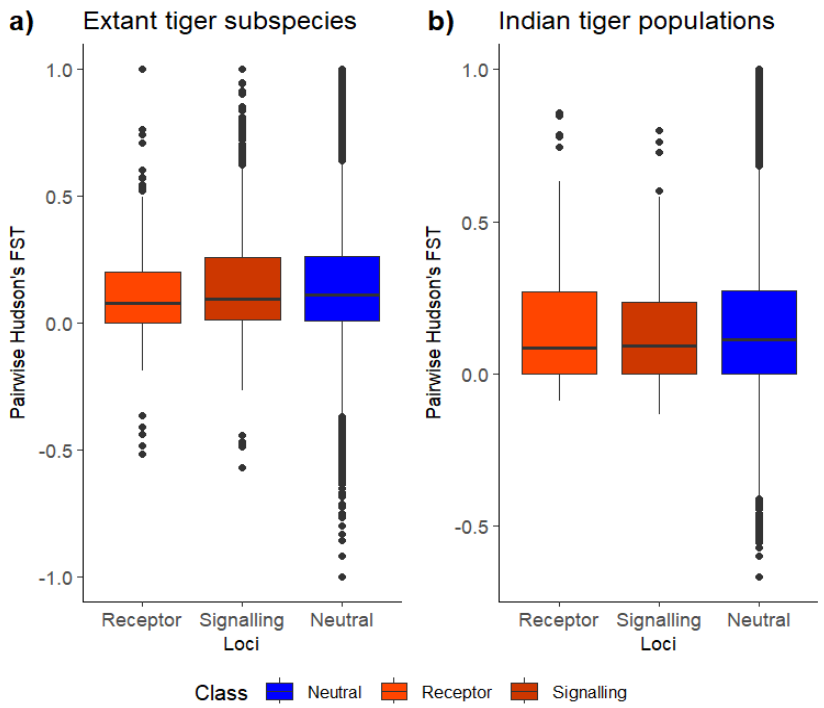

Figure 5: a) Box and whisker plots for Fixation index at immune receptor, immune sign alling and Neutral loci for different subspecies of tigers b) Box and whisker plots for Fix ation index at immune receptor, immune signalling and neutral loci for different popula tions of Indian tigers

3.5: pDeleterious and pLOF mutation load in Indian populations:

Figure 6a reveals the Variant Effect Prediction results categorizing exonic mutations into Loss of Function (pLOF), missense, synonymous and other categories. We found missense and synonymous mutations in all genes examined. Loss of function mutations were only absent in Toll-like Receptor genes but present in all other genes.

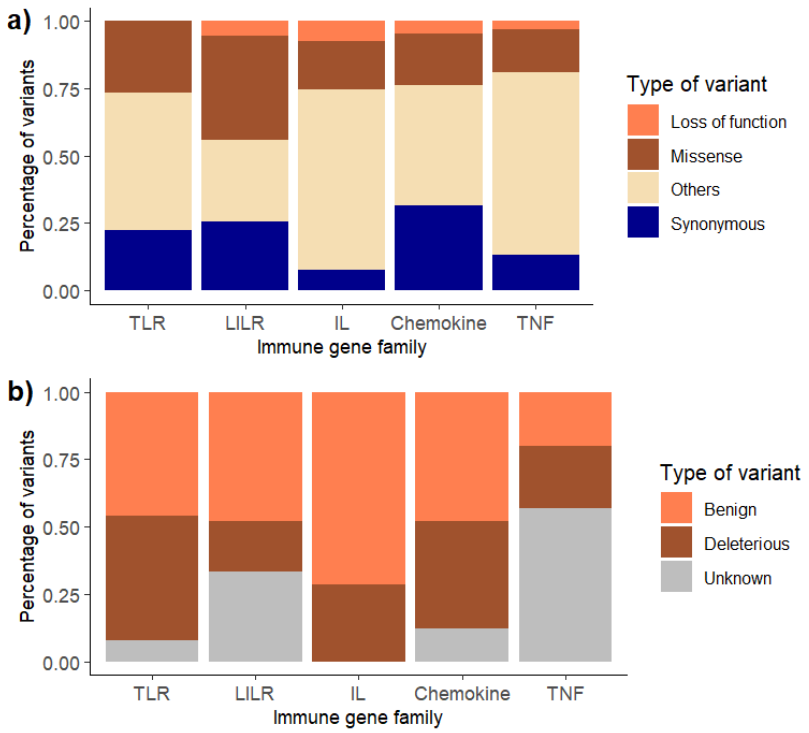

Figure 6: a) Percentage of different variants in exons of immune genes b) Percentage of missense variants predicted as PolyPhen categories of benign, deleterious and unknown effect

The pLOF and pDeleterious mutations identified from VEP and further confirmed through Polyphen were used to estimate genetic load in the Indian subpopulations. Genetic load of damaging mutations in immune genes followed previously established patterns, with large connected populations having high genetic load and small isolated population having low genetic load. Inbreeding load, estimated as homozygous damaging alleles, was much lower in all populations. Most of the genetic load was masked, while the small isolated population had higher pLoF load at immune genes, The large connected central Indian population had the highest masked pDeleterious load (Figure 7).

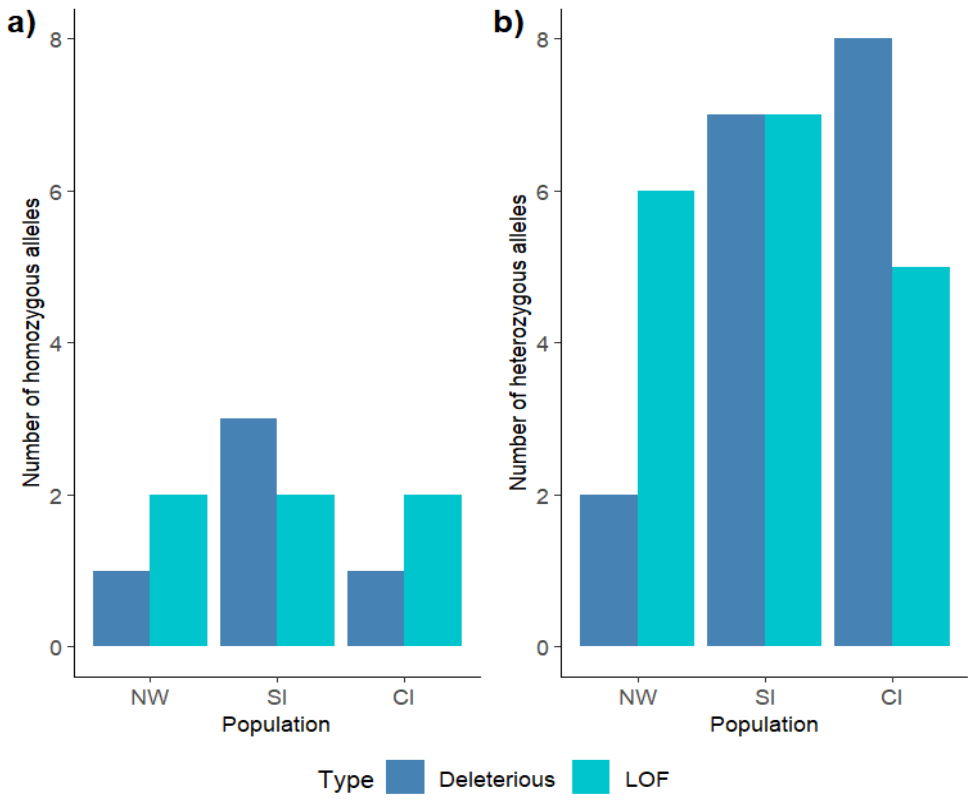

Figure 7: a) Homozygous and b) masked burden of deleterious and loss of function load

We found homozygous pDeleterious mutations in three TLR genes-TLR5, (homozygous in NW and SI), TLR9 and TLR4 (homozygous in SI) as well as TNFRS5 (homozygous in SI and CI). The homozygous pLoF mutations were in CCL5 and CCR5, LILRB3X4 and TNFSFR1, and were fixed in each population.

4. Discussion

4.1: Heterozygosity and nucleotide diversity at different immune gene families show contrasting patterns

We characterized variation at several families of immune genes in tigers spanning adaptive and innate immune functions as well as carrying out signal transduction and pathogen recognition. We found missense and potential loss of function mutations in all families of genes examined; TLR genes are conspicuous due to the absence of potential Loss of function mutations. Using genetic diversity indices of nucleotide diversity and heterozygosity at intergenic regions, we inferred neutral diversity, against which we compare the same diversity indices of immune gene. Immune genes that code for receptors have significantly higher nucleotide diversity but heterozygosity does not differ significantly from neutral loci at both receptor and signalling immune loci. Given that nucleotide diversity does not erode as quickly as heterozygosity, we infer that all TLR and LILR genes may have undergone positive selection in the past. In the recent past, all immune gene families are largely under neutral evolution irrespective of the type of immune function they are involved in. Some of these genes are known to be under positive selection in a previous study with the Indian reference we use here (Shukla et al. 2023).

Interestingly, all of the genes detected to be under positive selection are signalling genes. None of the immune receptor genes have been detected to be under positive selection. This suggests that purifying selection and drift largely shape evolution at immune genes in tigers.

4.2: TLR genes seem to be evolving under purifying selection with some relaxation of selective constraints

Toll-like receptor genes seem to have higher heterozygosity than other gene families, but is not significantly different than neutral loci ruling out balancing selection. After MHC loci, it is TLR loci that are frequently found to be under balancing selection (Kloch et al. 2018). TLR genes are involved in recognition of structurally conserved molecules of microbes such as virus, bacteria and fungus and are an essential part of innate immunity. These functions of TLR genes seem to have led to balancing and directional selection acting on them. However, there are also several studies that find purifying selection acting on TLR genes (Stefanović et al. 2020). The absence of putative Loss of function mutations in TLR genes in tigers indicates the action of purifying selection. Given these contradicting results, we infer a relaxation of selection constraint on TLR genes where most variants are removed by purifying selection but selection might increase diversity at certain parts of the gene (Podlaszczuk et al. 2020).

4.3: Patterns of genetic diversity of immune genes mirror that of neutral loci across populations with differing demographic history

To further ascertain whether drift is the major evolutionary force in tigers, we contrasted immune and neutral variation between the different sub species of tigers and the different populations of Indian tigers which are known to have varying effective sizes and connectivity status. All tiger sub-species have undergone range contractions and population decline (Armstrong et al. 2021; Liu et al. 2018; Harihar et al. 2018). We find that the changes in neutral variation is reflected in immune gene variation as well, suggesting that most immune genes examined are evolving neutrally. Receptor genes have significantly higher nucleotide diversity but this is correlated with neutral diversity across populations. Our results substantiate earlier inferences that most loci in coding regions of the tiger genome are influenced by drift, regardless of which subspecies or population, potentially due to demographic fluctuations and bottlenecks (Armstrong et al. 2021). Most studies examining adaptive variation examine one or few populations which have undergone bottlenecks or exist in both mainland and island populations. To our knowledge, this is the first study examining adaptive variation in range wide populations of a species and one of the few studies with populations of diverse demographic histories.

The Indochinese and Bengal tigers were found to have the highest genetic diversity at both immune and neutral loci. Indochinese tigers are geographically close to the ancestral tiger population (South China tiger) whereas Bengal tigers have the largest population size of extant tigers. Receptor immune genes do not differ significantly from neutral loci in Indochinese tigers, indicating these genes did not undergo positive selection. Sumatran tigers (that live on the island of Sumatra) have the lowest nucleotide diversity at immune and neutral loci whereas the South China tigers that are extinct in the wild have the lowest homozygosity at immune

and neutral loci. The Malayan tigers have genetic diversity values similar to the Indochinese and Bengal populations whereas Amur tigers having undergone strong recent bottlenecks similar to Sumatran tigers, have genetic diversity similar to Sumatran tigers at both immune and neutral loci.

Our results correspond with the findings in several other endangered species where decline in population size and isolation leads to loss of adaptive genetic diversity, along with neutral diversity. Such patterns have been found for immune gene diversity in Tasmanian Devils (Morris et al. 2015), Black grouse (Strand et al. 2012), dunnocks (Lara et al. 2020), snub-nosed monkeys (Luo et al. 2012).

4.4: Several immune genes retain high polymorphisms even in bottlenecked populations

We observe several genes having high heterozygosity values in the Northwestern population of Indian tigers, which is small and isolated and has low genome wide heterozygosity. This is also true in Sumatran and the South China population. These are genes performing diverse immune functions, and not just receptor genes. Some of these genes, like C-X-C chemokine receptor type 4 are known to have been under positive selection. Overall, this suggests that despite a bottleneck and drift, these loci might retain high polymorphisms, potentially maintained by past selection. Such variation has previously been described previously as "ghosts of selection past," and might get eroded with time due to drift debt (Gilroy et al. 2017; Oliver and Piertney 2012; Davies et al. 2021).

4.5: Patterns of genetic differentiation at immune loci in tigers is similar to differentiation at neutral loci

We use fixation index or F_{ST} to contrast differentiation at subspecies level and amongst Indian populations while comparing with differentiation at neutral loci. Our findings further confirm the predominant role of drift in shaping evolution of tigers, including immune loci. F_{ST} values for population pairs, where one (a) had a history of disease (e.g. Amur tigers (Gilbert et al. 2014)), (b) had undergone major bottlenecks (e.g. South China and North western Indian tigers), or (c) had undergone founding events (e.g. Sumatran tigers in the Sunda islands) were not significantly different for immune gene and neutral loci. Amur tigers had a higher mean F_{ST} at immune loci than neutral loci, which could indicate selection for certain alleles that protect from Canine distemper virus but this difference is not significant. Our results align with population genetic theory that suggests that small populations that have been through bottlenecks are expected to be affected more by drift than selection (Whitlock 2002). Additionally, tigers being a solitary, with very little social contact with other tigers and felids, the possibility of infection through horizontal transmission is limited. Oral consumption and nasal exposure could be two possible modes of infection, of which the latter is less likely. This could have prevented a high prevalence of infections and weaker pathogen mediated selection, making it difficult to detect over the strong signatures of demographic history.

We have not used the traditional population genetic indices to distinguish between neutral and non-neutral evolution since these statistics cannot distinguish between selective and

demographic events. Because all subspecies of tigers have undergone drastic bottlenecks throughout the evolutionary history, the use of such statistics is redundant.

4.6: Genetic load and diversity sheds light on populations of conservation importance

We add in an additional layer of assessment of load at immune genes, an aspect that is ignored in most studies focussed on adaptive variation. We carried out an additional step of identifying pathogenicity of missense mutations instead of assuming all missense mutations to be pDeleterious. PolyPhen helps classify the extent of potential impact of damaging mutations. These results indicate that most of the missense variants are benign. We were able to discern potential damaging effect for thirty missense mutations (Figure 7b). There were several missense mutations for which Polyphen assignment was not possible, often the case for non-model organisms like tigers. Polyphen assignments were successful in TLR, Interleukin and Chemokine missense mutations, probably because these genes are well characterized for several species.

Overall, load at immune genes is quite low as damaging mutations were identified at eight loci only. We find damaging mutation load to vary across population sizes of Indian tigers as seen previously (Khan et al. 2021). We find a slight difference in the pattern of the load in the Northwestern population of India where pLoF is higher than pDeleterious mutation load. We carried out an extra step assessing the damaging nature of deleterious mutations due to which we find fewer pDeleterious mutations than the previous study.

Loss of protective polymorphisms in immune genes could result in increased susceptibility to novel infectious diseases, that could cause further population decline (Sommer 2005). Tasmanian devils are particularly notorious for lowered immune function and increased susceptibility to facial tumours that has contributed to population decline. Although specifics mechanisms that result in lowered immune genetic diversity are not explored due to technical challenges, Galapagos sea lions with higher homozygosity are known to have lowered immunity when exposed to invasive species and pollution (Brock et al. 2015). Inbred song sparrows are known to have lowered immunity to parasitic infections varying with sex (Reid et al. 2007). Although many immune genes undergo somatic recombination to increase variety of pathogen recognition motifs, genetic diversity of immune genes is known to aid in production of many protein motifs that could help identify novel pathogens and impart disease resistance (Trowsdale and Parham 2004).

Host immunity appears to be an important facet for survival of tiger and other carnivore populations in the future. Several species of large carnivores have suffered disease epidemics in the recent past leading to population decline(Becker et al. 2013; Mccarthy, Shaw, and Goodman 2007). Canine distemper virus epidemics have affected African wild dogs, African and Asiatic lions and Amur tigers. Inbreeding in lions in South Africa is known to increase susceptibility to bovine tuberculosis whereas the Devil Facial Tumour disease epidemic in Tasmanian devil has led to a population crash (Morris et al. 2015; Trinkel et al. 2013). We suggest that an understanding of immune gene variation in the different subspecies and various populations of tigers is important for understanding adaptability in the context of future disease.

In our study, we find that the Sumatran subspecies and the South China subspecies have low nucleotide diversity and heterozygosity and are most likely to be susceptible to new infections. More worrisome is the Northwestern sub-population of Indian tigers which has low neutral and immune gene variation. A previous study has shown the prevalence of Canine Distemper Virus in the region (Sidhu et al. 2019), and we suggest active disease surveillance in this population to avoid exposure.

In order to understand the adaptive potential of a population, it would be ideal to have a measure of beneficial mutations. This is difficult to obtain without information of fitness-in this case, proved resistance of a mutation to infections. Even the variants identified as pDeleterious are bioinformatic predictions that might not necessarily be damaging. Only experiments establishing susceptibility to disease and increased disease mortality could truly identify pDeleterious load. Unfortunately, it is very difficult to carry out such studies in wild species, particularly elusive carnivores.

Finally, as shown in Kardos et al., 2023, population genetic theory and empirical evidence in several studies show that neutral variation is indicative of extinction risk and genome wide variation is important for population viability (Kardos and Shafer 2018). A comprehensive understanding of functional variation can be obtained only for species with genomic information. Our results suggest that when such information is not available, genome-wide neutral markers would be indicative of adaptive potential. In some cases, adaptive loci might better inform conservation priorities (Oliver and Piertney 2012) and situations where gene targeted approaches are more useful than neutral loci have been previously described (Flanagan et al. 2018).

5: Conclusion:

Our study attempts to answer the longstanding question in conservation genomics, or the correlation between neutral and adaptive variation in genetically depauperate populations with differing demographic histories. We show that immunogenetic variation, if measured as heterozygosity correlates with neutral variation. However, when it is measured as nucleotide diversity, immune genes coding for receptor molecules (that interact with pathogens and other MHC molecules) have higher genetic variation than neutral loci in tigers. Populations that have undergone bottlenecks and founding effects have lower immune gene (as well as neutral loci) diversity, and show slightly higher differentiation, implicating the dominant role of neutral processes in such populations. However, despite drift, some receptor coding immune genes, and signalling loci retain high polymorphisms. Other studies (Shukla et al., 2023) have shown some of these genes to be under positive selection in tigers. We identify populations and subspecies with putatively disadvantageous immune gene variation. Finally, we conclude that in the absence of large-scale genomic data, such as in our study, neutral markers could adequately inform managers about the vulnerability of populations and species to extinction risks from pathogens and infectious diseases.

Acknowledgments:

We are thankful to Megan Aylward, Laura Bertola, Mayuresh Gangal, Sabarinathan Radhakrishnan, Vinay Sagar, Anubhab Khan, Praveen Prakash, Marty Kardos, Harsh Shukla and Kushal Suryamohan for discussion and inputs; Kritagnya Vadar for help with mining annotations and Chakrapani and Prasantha with help with High Performance Computing clusters. BVA was supported by the NCBS/Tata Institute of Fundamental Research (TIFR) (Department of Atomic Energy). The NCBS data cluster used is supported under project 12-R&D-TFR-5.04-0900, Department of Atomic Energy, Government of India. This work was supported by the NCBS/TIFR internal plan fund awarded to UR. (Project Identification RTI 4006, Department of Atomic Energy, Government of India) and DBT Wellcome Trust Senior Fellowship (IA/S/16/2/502714) awarded to UR.

References:

Acevedo-Whitehouse, Karina, and Andrew A. Cunningham. 2006. "Is MHC Enough for Understanding Wildlife Immunogenetics?" *Trends in Ecology and Evolution* 21 (8): 433–38. https://doi.org/10.1016/j.tree.2006.05.010.

Adzhubei, Ivan, Daniel M. Jordan, and Shamil R. Sunyaev. 2013. "Predicting Functional Effect of Human Missense Mutations Using PolyPhen-2." *Current Protocols in Human Genetics* 76 (1): 7.20.1-7.20.41. https://doi.org/10.1002/0471142905.hg0720s76.

Aguilar, Andres, Gary Roemer, Sally Debenham, Matthew Binns, David Garcelon, and Robert K. Wayne. 2004. "High MHC Diversity Maintained by Balancing Selection in an Otherwise Genetically Monomorphic Mammal." *Proceedings of the National Academy of Sciences of the United States of America* 101 (10): 3490–94. https://doi.org/10.1073/PNAS.0306582101.

Alcaide, Miguel. 2010. "On the Relative Roles of Selection and Genetic Drift in Shaping MHC Variation." *Molecular Ecology* 19 (18): 3842–44. https://doi.org/10.1111/J.1365-294X.2010.04772.X.

Annavi, Geetha, Christopher Newman, Christina D. Buesching, David W. Macdonald, Terry Burke, and Hannah L. Dugdale. 2014. "Heterozygosity–Fitness Correlations in a Wild Mammal Population: Accounting for Parental and Environmental Effects." *Ecology and Evolution* 4 (12): 2594–2609. https://doi.org/10.1002/ECE3.1112.

Armstrong, Ellie E, Anubhab Khan, Ryan W Taylor, Alexandre Gouy, Gili Greenbaum, Alexandre Thiéry, Jonathan T Kang, et al. 2021. "Recent Evolutionary History of Tigers Highlights Contrasting Roles of Genetic Drift and Selection." *Molecular Biology and Evolution* 38 (6): 2021. https://doi.org/10.1093/molbev/msab032.

Barnosky, Anthony D., Nicholas Matzke, Susumu Tomiya, Guinevere O. U. Wogan, Brian Swartz, Tiago B. Quental, Charles Marshall, et al. 2011. "Has the Earth's Sixth Mass Extinction Already Arrived?" *Nature* 471 (7336): 51–57. https://doi.org/10.1038/nature09678.

Becker, Matthew S., Nicholas M. Sakuya, Cathleen A. Hanlon, Jassiel M'soka, Mike R. Dunbar, Rachel McRobb, Egil Droge, Are R. Berentsen, and Wigganson Matandiko. 2013. "Rabies, Canine Distemper, and Canine Parvovirus Exposure in Large Carnivore

Communities from Two Zambian Ecosystems." *Vector-Borne and Zoonotic Diseases* 13 (9): 643–49. https://doi.org/10.1089/vbz.2012.1233.

Bolger, Anthony M., Marc Lohse, and Bjoern Usadel. 2014. "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data." *Bioinformatics* 30 (15): 2114. https://doi.org/10.1093/BIOINFORMATICS/BTU170.

Brock, Patrick M, Simon J Goodman, Ailsa J Hall, Marilyn Cruz, and Karina Acevedo-whitehouse. 2015. "Context-Dependent Associations between Heterozygosity and Immune Variation in a Wild Carnivore." *BMC Evolutionary Biology*, 1–10. https://doi.org/10.1186/s12862-015-0519-6.

Cardillo, Marcel, Georgina M Mace, John L Gittleman, Kate E Jones, Jon Bielby, and Andy Purvis. 2008. "The Predictability of Extinction: Biological and External Correlates of Decline in Mammals." *Proceedings of the Royal Society B: Biological Sciences* 275 (1641): 1441–48. https://doi.org/10.1098/rspb.2008.0179.

Castro-Prieto, Aines, Bettina Wachter, and Simone Sommer. 2010. "Cheetah Paradigm Revisited: MHC Diversity in the World's Largest Free-Ranging Population." *Molecular Biology and Evolution* 28 (4): 1455–68. https://doi.org/10.1093/molbev/msq330.

Chapman, J. R., S. Nakagawa, D. W. Coltman, J. Slate, and B. C. Sheldon. 2009. "A Quantitative Review of Heterozygosity-Fitness Correlations in Animal Populations." *Molecular Ecology* 18 (13): 2746–65. https://doi.org/10.1111/J.1365-294X.2009.04247.X.

Charlesworth, B., and D. Charlesworth. 2017. "Population Genetics from 1966 to 2016." *Heredity*. Nature Publishing Group. https://doi.org/10.1038/hdy.2016.55.

Davies, Charli S., Martin I. Taylor, Martijn Hammers, Terry Burke, Jan Komdeur, Hannah L. Dugdale, and David S. Richardson. 2021. "Contemporary Evolution of the Innate Immune Receptor Gene TLR3 in an Isolated Vertebrate Population." *Molecular Ecology* 30 (11): 2528–42. https://doi.org/10.1111/mec.15914.

Edwards, Scott V., Joe Gasper, Daniel Garrigan, Duane Martindale, and Ben F. Koop. 2000. "A 39-Kb Sequence around a Blackbird Mhc Class II Gene: Ghost of Selection Past and Songbird Genome Architecture." *Molecular Biology and Evolution* 17 (9): 1384–95. https://doi.org/10.1093/oxfordjournals.molbev.a026421.

Edwards, Scott V., E. K. Wakeland, and W. K. Potts. 1995. "Contrasting Histories of Avian and Mammalian Mhc Genes Revealed by Class II B Sequences from Songbirds." *Proceedings of the National Academy of Sciences of the United States of America* 92 (26): 12200–204. https://doi.org/10.1073/pnas.92.26.12200.

Eyre-Walker, Adam, and Peter D. Keightley. 2007. "The Distribution of Fitness Effects of New Mutations." *Nature Reviews Genetics* 8 (8): 610–18. https://doi.org/10.1038/nrg2146.

Fagan, William F., and E. E. Holmes. 2006. "Quantifying the Extinction Vortex." *Ecology Letters* 9 (1): 51–60. https://doi.org/10.1111/j.1461-0248.2005.00845.x.

Fernández-Sepúlveda, Jaime, and Carlos A. Martín. 2022. "Conservation Status of the World's Carnivorous Mammals (Order Carnivora)." *Mammalian Biology* 102 (5–6): 1911–25. https://doi.org/10.1007/S42991-022-00305-8/FIGURES/5.

Flanagan, Sarah P., Brenna R. Forester, Emily K. Latch, Sally N. Aitken, and Sean Hoban. 2018. "Guidelines for Planning Genomic Assessment and Monitoring of Locally

Adaptive Variation to Inform Species Conservation." *Evolutionary Applications* 11 (7): 1035–52. https://doi.org/10.1111/eva.12569.

Frankham, Richard. 1996. "Relationship of Genetic Variation to Population Size in Wildlife." *Conservation Biology* 10 (6): 1500–1508. https://doi.org/10.1046/j.1523-1739.1996.10061500.x.

Gilbert, Martin, Dale G. Miquelle, John M. Goodrich, Richard Reeve, Sarah Cleaveland, Louise Matthews, and Damien O. Joly. 2014. "Estimating the Potential Impact of Canine Distemper Virus on the Amur Tiger Population (Panthera Tigris Altaica) in Russia." *PLOS ONE* 9 (10): e110811. https://doi.org/10.1371/JOURNAL.PONE.0110811.

Gilpin, Michael E. 1986. "Minimum Viable Populations: Processes of Species Extinction." *Conservation Biology: The Science of Scarcity and Diversity*, 19–34.

Gilroy, D. L., K. P. Phillips, D. S. Richardson, and C. van Oosterhout. 2017. "Toll-like Receptor Variation in the Bottlenecked Population of the Seychelles Warbler: Computer Simulations See the 'ghost of Selection Past' and Quantify the 'drift Debt'." *Journal of Evolutionary Biology* 30 (7): 1276–87. https://doi.org/10.1111/jeb.13077.

Grueber, Catherine E., Graham P. Wallis, and Ian G. Jamieson. 2014. "Episodic Positive Selection in the Evolution of Avian Toll-like Receptor Innate Immunity Genes." *PLoS ONE* 9 (3). https://doi.org/10.1371/journal.pone.0089632.

Grueber, Catherine E., Graham P. Wallis, Tania M. King, and Ian G. Jamieson. 2012. "Variation at Innate Immunity Toll-Like Receptor Genes in a Bottlenecked Population of a New Zealand Robin." *PLOS ONE* 7 (9): e45011. https://doi.org/10.1371/JOURNAL.PONE.0045011.

Hansson, Bengt, and Lars Westerberg. 2002. "On the Correlation between Heterozygosity and Fitness in Natural Populations." *Molecular Ecology* 11 (12): 2467–74. https://doi.org/10.1046/J.1365-294X.2002.01644.X.

Harihar, Abishek, Pranav Chanchani, Jimmy Borah, Rachel Jane Crouthers, Yury Darman, Thomas N E Gray, Shariff Mohamad, et al. 2018. "Recovery Planning towards Doubling Wild Tiger Panthera Tigris Numbers : Detailing 18 Recovery Sites from across the Range," 1–16.

Hedrick, Philip W., Rhonda N. Lee, and Colleen Buchanan. 2013. "Canine Parvovirus Enteritis, Canine Distemper, and Major Histocompatibility Complex Genetic Variation in Mexican Wolves." *Journal of Wildlife Diseases* 39 (4): 909–13. https://doi.org/10.7589/0090-3558-39.4.909.

Janeway, Charles A., Paul Jr. Travers, Mark Walport, and Mark J. Shlomchik. 2001. "Immunobiology: The Immune System in Health and Disease," 910. https://doi.org/10.1017/CBO9781107415324.004.

Jepson, A, W Banya, F Sisay-Joof, M Hassan-King, C Nunes, S Bennett, and H Whittle. 1997. "Quantification of the Relative Contribution of Major Histocompatibility Complex (MHC) and Non-MHC Genes to Human Immune Responses to Foreign Antigens." *Infection and Immunity* 65 (3): 872.

Johnson, Jeff A., and Peter O. Dunn. 2006. "Low Genetic Variation in the Heath Hen Prior to Extinction and Implications for the Conservation of Prairie-Chicken Populations." *Conservation Genetics* 7 (1): 37–48. https://doi.org/10.1007/s10592-005-7856-8.

Johnson, Kevin P., Frederick R. Adler, and Joshua L. Cherry. 2000. "Genetic and Phylogenetic Consequences of Island Biogeography." *Evolution* 54 (2): 387–96. https://doi.org/10.1111/j.0014-3820.2000.tb00041.x.

Kardos, Marty, and Aaron B. A. Shafer. 2018. "The Peril of Gene-Targeted Conservation." *Trends in Ecology & Evolution* 33 (11): 827–39. https://doi.org/10.1016/j.tree.2018.08.011.

Keller, Lukas F., Peter Arcese, James N. M. Smith, Wesley M. Hochachka, and Stephen C. Stearns. 1994. "Selection against Inbred Song Sparrows during a Natural Population Bottleneck." *Nature* 372 (6504): 356–57. https://doi.org/10.1038/372356a0.

Keller, Lukas F, and Donald M Waller. 2002. "Inbreeding Effects in Wild Populations" 17 (5): 19–23.

Khan, Anubhab, Kaushalkumar Patel, Harsh Shukla, Ashwin Viswanathan, Tom van der Valk, Udayan Borthakur, Parag Nigam, et al. 2021. "Genomic Evidence for Inbreeding Depression and Purging of Deleterious Genetic Variation in Indian Tigers." *Proceedings of the National Academy of Sciences* 118 (49): e2023018118. https://doi.org/10.1073/PNAS.2023018118.

Kloch, Agnieszka, Marius A. Wenzel, Dominik R. Laetsch, Olek Michalski, Renata Welc-Falęciak, and Stuart B. Piertney. 2018. "Signatures of Balancing Selection in Toll-like Receptor (TLRs) Genes - Novel Insights from a Free-Living Rodent." *Scientific Reports* 8 (1): 1–10. https://doi.org/10.1038/s41598-018-26672-2.

Korunes, Katharine L., and Kieran Samuk. 2021. "Pixy: Unbiased Estimation of Nucleotide Diversity and Divergence in the Presence of Missing Data." *Molecular Ecology Resources* 21 (4): 1359–68. https://doi.org/10.1111/1755-0998.13326.

Lara, Carlos Esteban, Catherine E. Grueber, Benedikt Holtmann, Eduardo S.A. Santos, Sheri L. Johnson, Bruce C. Robertson, Gabriel J. Castaño-Villa, Malgorzata Lagisz, and Shinichi Nakagawa. 2020. "Assessment of the Dunnocks' Introduction to New Zealand Using Innate Immune-Gene Diversity." *Evolutionary Ecology* 34 (5): 803–20. https://doi.org/10.1007/s10682-020-10070-0.

Liberg, Olof, Henrik Andrén, Hans Christian Pedersen, Håan Sand, Douglas Sejberg, Retter Wabakken, Mikael Åkesson, and Staffan Bensch. 2005. "Severe Inbreeding Depression in a Wild Wolf (Canis Lupus) Population." *Biology Letters* 1 (1): 17–20. https://doi.org/10.1098/rsbl.2004.0266.

Liebgold, Eric B., Christian F. Kramer, Tamar C. Roomian, Gina M. Dolezar, and Paul R. Cabe. 2018. "Heterozygosity–Behavior and Heterozygosity–Fitness Correlations in a Salamander with Limited Dispersal." *Population Ecology* 60 (3): 251–60. https://doi.org/10.1007/S10144-017-0604-3/FIGURES/4.

Liu, Yue Chen, Xin Sun, Carlos Driscoll, Dale G. Miquelle, Xiao Xu, Paolo Martelli, Olga Uphyrkina, James L.D. Smith, Stephen J. O'Brien, and Shu Jin Luo. 2018. "Genome-Wide Evolutionary Analysis of Natural History and Adaptation in the World's Tigers." *Current Biology* 28 (23): 3840-3849.e6. https://doi.org/10.1016/j.cub.2018.09.019.

Luo, Mao Fang, Hui Juan Pan, Zhi Jin Liu, and Ming Li. 2012. "Balancing Selection and Genetic Drift at Major Histocompatibility Complex Class II Genes in Isolated Populations of Golden Snub-Nosed Monkey (Rhinopithecus Roxellana)." *BMC Evolutionary Biology* 12 (1): 207. https://doi.org/10.1186/1471-2148-12-207.

Mathur, Samarth, and J. Andrew DeWoody. 2021. “Genetic Load Has Potential in Large Populations but Is Realized in Small Inbred Populations.” *Evolutionary Applications* 14 (6): 1540–57. https://doi.org/10.1111/eva.13216.

Mccarthy, Alex J, Marie-anne Shaw, and Simon J Goodman. 2007. “Pathogen Evolution and Disease Emergence in Carnivores,” no. October, 3165–74. https://doi.org/10.1098/rspb.2007.0884.

McLaren, William, Laurent Gil, Sarah E. Hunt, Harpreet Singh Riat, Graham R. S. Ritchie, Anja Thormann, Paul Flicek, and Fiona Cunningham. 2016. “The Ensembl Variant Effect Predictor.” *Genome Biology* 17 (1): 122. https://doi.org/10.1186/s13059-016-0974-4.

Mondol, Samrat, K. Ullas Karanth, and Uma Ramakrishnan. 2009. “Why the Indian Subcontinent Holds the Key to Global Tiger Recovery.” *PLoS Genetics* 5 (8). https://doi.org/10.1371/journal.pgen.1000585.

Morin, Phillip A., Frederick I. Archer, Catherine D. Avila, Jennifer R. Balacco, Yury V. Bukhman, William Chow, Olivier Fedrigo, et al. 2021. “Reference Genome and Demographic History of the Most Endangered Marine Mammal, the Vaquita.” *Molecular Ecology Resources* 21 (4): 1008–20. https://doi.org/10.1111/1755-0998.13284.

Morris, Katrina M., Belinda Wright, Catherine E. Grueber, Carolyn Hogg, and Katherine Belov. 2015. “Lack of Genetic Diversity across Diverse Immune Genes in an Endangered Mammal, the Tasmanian Devil (*Sarcophilus Harrisii*).” *Molecular Ecology* 24 (15): 3860–72. https://doi.org/10.1111/mec.13291.

Mukherjee, Souvik, Neeta Sarkar-Roy, Diane K. Wagener, and Partha P. Majumder. 2009. “Signatures of Natural Selection Are Not Uniform across Genes of Innate Immune System, but Purifying Selection Is the Dominant Signature.” *Proceedings of the National Academy of Sciences of the United States of America* 106 (17): 7073–78. https://doi.org/10.1073/pnas.0811357106.

Natesh, Meghana, Goutham Atla, Parag Nigam, Yadvendradev V. Jhala, Arun Zachariah, Udayan Borthakur, and Uma Ramakrishnan. 2017. “Conservation Priorities for Endangered Indian Tigers through a Genomic Lens.” *Scientific Reports* 7 (1): 1–11. https://doi.org/10.1038/s41598-017-09748-3.

Netea, Mihai G., Cisca Wijmenga, and Luke A. J. O’Neill. 2012. “Genetic Variation in Toll-like Receptors and Disease Susceptibility.” *Nature Immunology* 13 (6): 535–42. https://doi.org/10.1038/ni.2284.

Oliver, Matthew K., and Stuart B. Piertney. 2012. “Selection Maintains MHC Diversity through a Natural Population Bottleneck.” *Molecular Biology and Evolution* 29 (7): 1713–20. https://doi.org/10.1093/molbev/mss063.

Podlaszczuk, Patrycja, Piotr Indykiewicz, Janusz Markowski, and Piotr Minias. 2020. “Relaxation of Selective Constraints Shapes Variation of Toll-like Receptors in a Colonial Waterbird, the Black-Headed Gull.” *Immunogenetics* 72 (4): 251. https://doi.org/10.1007/S00251-020-01156-8.

Radwan, Jacek, Aleksandra Biedrzycka, and Wiesław Babik. 2010. “Does Reduced MHC Diversity Decrease Viability of Vertebrate Populations ?” *Biological Conservation* 143 (3): 537–44. https://doi.org/10.1016/j.biocon.2009.07.026.

Reid, Jane M, Peter Arcese, Lukas F Keller, Kyle H Elliott, Laura Sampson, and Dennis Hasselquist. 2007. "Inbreeding Effects on Immune Response in Free-Living Song Sparrows (Melospiza Melodia),” no. November 2006, 697–706. https://doi.org/10.1098/rspb.2006.0092.

"Release 1.11 · Samtools/Bcftools." n.d. GitHub. Accessed July 22, 2024. https://github.com/samtools/bcftools/releases/tag/1.11.

Ripple, William J., James A. Estes, Robert L. Beschta, Christopher C. Wilmers, Euan G. Ritchie, Mark Hebblewhite, Joel Berger, et al. 2014. "Status and Ecological Effects of the World's Largest Carnivores." *Science* 343 (6167): 1241484. https://doi.org/10.1126/science.1241484.

Rivero-de Aguilar, J., H. Westerdahl, J. Martínez-de la Puente, G. Tomás, J. Martínez, and S. Merino. 2016. "MHC-I Provides Both Quantitative Resistance and Susceptibility to Blood Parasites in Blue Tits in the Wild." *Journal of Avian Biology* 47 (5): 669–77. https://doi.org/10.1111/JAV.00830.

Robinson, Jacqueline A., Diego Ortega-Del Vecchyo, Zhenxin Fan, Bernard Y. Kim, Bridgett M. vonHoldt, Clare D. Marsden, Kirk E. Lohmueller, and Robert K. Wayne. 2016. "Genomic Flatlining in the Endangered Island Fox." *Current Biology* 26 (9): 1183–89. https://doi.org/10.1016/j.cub.2016.02.062.

S, Sommer. 2005. "The Importance of Immune Gene Variability (MHC) in Evolutionary Ecology and Conservation." *Frontiers in Zoology* 2 (October). https://doi.org/10.1186/1742-9994-2-16.

Saccheri, Ilik, Mikko Kuussaari, Maaria Kankare, Pia Vikman, Wilhelm Fortelius, and Ilkka Hanski. 1998. "Inbreeding and Extinction in a Butterfly Metapopulation." *Nature* 392 (6675): 491–94. https://doi.org/10.1038/33136.

Shukla, Harsh, Kushal Suryamohan, Anubhab Khan, Krishna Mohan, Rajadurai C. Perumal, Oommen K. Mathew, Ramesh Menon, et al. 2023. "Near-Chromosomal de Novo Assembly of Bengal Tiger Genome Reveals Genetic Hallmarks of Apex Predation." *GigaScience* 12. https://doi.org/10.1093/gigascience/giac112.

Shultz, Allison J., and Timothy B. Sackton. 2019. "Immune Genes Are Hotspots of Shared Positive Selection across Birds and Mammals." *eLife* 8 (January). https://doi.org/10.7554/eLife.41815.

Sidhu, Nadisha, Jimmy Borah, Sunny Shah, Nidhi Rajput, and Kajal Kumar Jadav. 2019. "Is Canine Distemper Virus (CDV) a Lurking Threat to Large Carnivores? A Case Study from Ranthambhore Landscape in Rajasthan, India." *Journal of Threatened Taxa* 11 (9): 14220–23. https://doi.org/10.11609/jott.4569.11.9.14220-14223.

Slate, J., L. E.B. Kruuk, T. C. Marshall, J. M. Pemberton, and T. H. Clutton-Brock. 2000. "Inbreeding Depression Influences Lifetime Breeding Success in a Wild Population of Red Deer (Cervus Elaphus)." *Proceedings of the Royal Society B: Biological Sciences* 267 (1453): 1657–62. https://doi.org/10.1098/rspb.2000.1192.

Slatkin, Montgomery. 1987. "Gene Flow and the Geographic Structure of Natural Populations." *Science* 236 (4803): 787–92. https://doi.org/10.1126/science.3576198.

Stefanović, Milomir, Mihajla Djan, Nevena Veličković, Yasin Demirbaş, Ladislav Paule, Csongor István Gedeon, Annika Posautz, Christoph Beiglböck, Anna Kübber-Heiss, and Franz Suchentrunk. 2020. "Purifying Selection Shaping the Evolution of the Toll-

like Receptor 2 TIR Domain in Brown Hares (Lepus Europaeus) from Europe and the Middle East." *Molecular Biology Reports* 47 (4): 2975–84. https://doi.org/10.1007/s11033-020-05382-x.

Strand, Tanja M., Gernot Segelbacher, María Quintela, Lingyun Xiao, Tomas Axelsson, and Jacob Höglund. 2012. "Can Balancing Selection on MHC Loci Counteract Genetic Drift in Small Fragmented Populations of Black Grouse?" *Ecology and Evolution* 2 (2): 341–53. https://doi.org/10.1002/ece3.86.

Sutton, Jolene T., Shinichi Nakagawa, Bruce C. Robertson, and Ian G. Jamieson. 2011. "Disentangling the Roles of Natural Selection and Genetic Drift in Shaping Variation at MHC Immunity Genes." *Molecular Ecology* 20 (21): 4408–20. https://doi.org/10.1111/J.1365-294X.2011.05292.X.

Taylor, Sabrina S., Rebecca J. Sardell, Jane M. Reid, Thomas Bucher, Nathan G. Taylor, Peter Arcese, and Lukas F. Keller. 2010. "Inbreeding Coefficient and Heterozygosity–Fitness Correlations in Unhatched and Hatched Song Sparrow Nestmates." *Molecular Ecology* 19 (20): 4454–61. https://doi.org/10.1111/J.1365-294X.2010.04824.X.

Trinkel, Martina, Dave Cooper, Craig Packer, and Rob Slotow. 2013. "Inbreeding Depression Increases Susceptibility To Bovine Tuberculosis in Lions: An Experimental Test Using an Inbred–Outbred Contrast Through Translocation." *Journal of Wildlife Diseases* 47 (3): 494–500. https://doi.org/10.7589/0090-3558-47.3.494.

Trowsdale, John, and Peter Parham. 2004. "Defense Strategies and Immunity-Related Genes." *European Journal of Immunology*. Eur J Immunol. https://doi.org/10.1002/eji.200324693.

"Variant Call Format and VCFtools | Bioinformatics | Oxford Academic." n.d. Accessed July 22, 2024. https://academic.oup.com/bioinformatics/article/27/15/2156/402296?login=false.

Velando, Alberto, Álvaro Barros, and Paloma Moran. 2015. "Heterozygosity–Fitness Correlations in a Declining Seabird Population." *Molecular Ecology* 24 (5): 1007–18. https://doi.org/10.1111/MEC.13092.

Vlček, Jakub, Matěj Miláček, Michal Vinkler, and Jan Štefka. 2023. "Effect of Population Size and Selection on Toll-like Receptor Diversity in Populations of Galápagos Mockingbirds." *Journal of Evolutionary Biology* 36 (1): 109–20. https://doi.org/10.1111/JEB.14121.

Wang, Biao, Robert Ekblom, Ignas Bunikis, Heli Siitari, and Jacob Höglund. 2014. "Whole Genome Sequencing of the Black Grouse (Tetrao Tetrix): Reference Guided Assembly Suggests Faster-Z and MHC Evolution." *BMC Genomics* 15 (1): 180. https://doi.org/10.1186/1471-2164-15-180.

Whitlock, Michael C. 2002. "Selection, Load and Inbreeding Depression in a Large Metapopulation." *Genetics* 160 (3): 1191 LP – 1202.

Young, Andrew Graham, and Geoffrey Maurice Clarke. 2000. *Genetics, Demography and Viability of Fragmented Populations.*

Zhang, Le, Tianming Lan, Chuyu Lin, Wenyuan Fu, Yaohua Yuan, Kaixiong Lin, Haimeng Li, et al. 2023. "Chromosome-Scale Genomes Reveal Genomic Consequences of Inbreeding in the South China Tiger: A Comparative Study with the Amur Tiger."

Molecular Ecology Resources 23 (2): 330–47. https://doi.org/10.1111/1755-0998.13669.

Author contributions:

Conceived and designed the analysis: BVA UR. Performed the analysis: BVA. Wrote the paper: BVA UR.