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Article type : Special Issue

LARGE-SCALE GENOME SAMPLING REVEALS UNIQUE IMMUNITY AND METABOLIC ADAPTATIONS IN BATS

GENE FAMILY EVOLUTION IN BATS

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Abstract

Comprising more than 1,400 species, bats possess adaptations unique among mammals including powered flight, unexpected longevity, and extraordinary immunity. Some of the molecular mechanisms underlying these unique adaptations includes DNA repair, metabolism and immunity. However, analyses have been limited to a few divergent lineages, reducing the scope of inferences on gene family evolution across the order Chiroptera. We conducted an exhaustive comparative genomic study of 37 bat species, one generated in this study, encompassing a large number of lineages, with a particular emphasis on multi-gene family evolution across immune and metabolic genes. In agreement with previous analyses, we found lineage-specific expansions of the APOBEC3 and MHC-I gene families, and loss of the proinflammatory PYHIN gene family. We inferred more than 1,000 gene losses unique to bats, including genes involved in the regulation of inflammasome pathways such as epithelial defense receptors, the natural killer gene complex and the interferon-gamma induced pathway. Gene set enrichment analyses revealed genes lost in bats are involved in defense response against pathogen-associated molecular patterns and damage-associated molecular patterns. Gene family evolution and selection analyses indicate bats have evolved fundamental functional differences compared to other mammals in both innate and adaptive immune system, with the potential to enhance anti-viral immune response while dampening inflammatory signaling. In addition, metabolic genes have experienced repeated expansions related to convergent shifts to plant-based diets. Our analyses support the hypothesis that, in tandem with flight, ancestral bats had evolved a unique set of immune adaptations whose functional implications remain to be explored.

Key Words

daptive immunity, gene family evolution, innate immunity, inflammatory pathway, metabolism, viral tolerance

Introduction

omparative genomics provides a framework for identifying the molecular mechanisms underlying unique rganismal adaptations, in their endless forms. To date, comparative genomic approaches have revealed the mechanisms underlying terrestrial adaptations in mudskipper fish (You et al., 2014), heat tolerance in coral (Bay, Rose, Logan, & Palumbi, 2017), cold stress tolerance in *Draba* (Nowak et al., 2020), and extreme longevity in naked mole rats (X. Zhou et al., 2020). In most cases the search for molecular adaptations has focused on orthologous single-copy genes, but gene loss and duplication can also be adaptive and are critical o understanding of how phenotypic adaptations evolve. Analyses based on highly contiguous genome assemblies have uncovered gene expansions likely associated with production of urushiol and anthocyanins in mango (P. Wang et al., 2020), the earliest events of gene duplication in cytoskeletal and membrane-trafficking families in eukaryotic cellular evolution (Vosseberg et al., 2020), pseudogenization in genes associated with esticular descent in afrotherian mammals (Sharma, Lehmann, Stuckas, Funke, & Hiller, 2018), gene losses ssociated with diving–related adaptations in cetaceans (Huelsmann et al., 2019), and losses associated with physiological and metabolic adaptations in fruit bats (Sharma, Hecker, Roscito, Foerster, Langer & Hiller, 018). Given the importance of gene family evolution, multiple large-scale genome sequencing consortia such as the Earth BioGenome Project (Lewin et al., 2018), the Vertebrate Genomes Project (Rhie et al., 2020), and Bat1K (Teeling et al., 2018) aim to generate high-quality genome assemblies for species spanning entire lades and even the entire phylogenetic 'Tree of Life', thereby enabling greater confidence in analyses of gene nd gene family evolution.

Gene family expansions and contractions are influenced by selection, including from biological factors uch as pathogens. Host-pathogen interactions are shaped by reciprocal selection, an evolutionary arms race which has forced hosts to evolve complex immune defense mechanisms (Papkou et al., 2019; Sironi, Cagliani, orni, & Clerici, 2015). Vertebrates have two types of immune response: innate immunity, which is non-specific and acts as a first line of defense; and adaptive immunity, which is highly specific and generates mmune memory (Delves, Martin, Burton, & Roitt, 2017; Janeway & Travers 2001.). Several immune-related ene families that have experienced substantial evolutionary changes during mammal evolution. While many important facets of the immune system are conserved, immune gene families have high rates of evolution

whether measured via substitution rate ratios or birth–death turnover (Bernatchez & Landry, 2003; Goebel et al., 2017; Minias, Pikus, Whittingham, & Dunn, 2019; Santos et al., 2016; Shultz & Sackton, 2019; Van Oosterhout, 2009). This is especially true of the Major Histocompatibility Complex (MHC), which is responsible for generating cell surface proteins that play essential functions in the adaptive immune system (Janeway & Travers 2001).

This combination of highly conserved, and highly variable components of the immune system, is particularly intriguing among bats. Among mammals, bat diversity is second only to that of rodents, and encompasses over 1,400 species that occupy a broad diversity of ecological niches on six continents (Fenton & Simmons, 2015; Nogueira et al., 2018). The success of bats is likely related to a suite of adaptations unique both to the clade as a whole and to various subclades within the Order Chiroptera. The most obvious of these is powered flight, allowing bats to occupy a unique aerial niche not utilized by any other mammal. While this unique niche limits body size, within that constraint bats have been exceptionally successful and have diversified in ways unparalleled among other mammals. For example, bats evolved virtually every mammalian dietary strategy (e.g., frugivory, carnivory, nectarivory, piscivory) and have done so in a relatively short evolutionary time frame (Dumont et al., 2012). Another less obvious but likely more interesting adaptation is the exceptional longevity and increased health span (the period of life during which an organism is in generally good health) exhibited by many bat species given their body size. Many species such as the Bechstein's bat (*Myotis bechstein)* the little brown bat, Brandt's bat (*Myotis brandtii*), greater mouse-eared bat (*Myotis myotis*) and greater horseshoe bat (*Rhinolophus ferrumequinum*) have unexpectedly long health spans, living 30 - 40 years (Fleischer, Gampe, Scheuerlein & Kerth, 2017; Foley et al., 2018; Podlustsky, Khritankov, Ovodov & Austad, 2005; Seim et al., 2013; Wilkinson & Adams, 2019). Such longevity defies the expectation that large species are longer-lived than small species; despite constrained body size, bats live longer than other mammals of similar size (Austad & Fischer, 1991; Healy et al., 2014). Bat longevity and health span may be influenced by their exposure to extrinsic mortality factors. Powered, mostly nocturnal flight may lower bats' exposure to some sources of extrinsic mortality, including predation (Healy et al., 2014). Yet, the risk of exposure to another extrinsic source of mortality, contagious infection, increases among bat species that roost in large colonies (Brook & Dobson, 2015; H. Han et al., 2015). Thus, to achieve such longevity and decreased senescence, long–lived bat populations must overcome the burden of infectious diseases.

The uniqueness of bats extends to the immune repertoire. Early in the age of whole-genome analyses, it was clear that inflammation-related gene families had expanded or contracted, and certain single–copy genes associated with immunity and cell repair had experienced selection in bats (G. Zhang et al., 2013). There is still debate as to whether bats harbor a disproportionately large number of viruses, or whether viral load is simply a function of species richness (Moratelli & Calisher, 2015; Olival et al., 2017; Mollentze & Streicker, 2020). Howeverthere is no doubt that several recent viral intrusions into our own species ultimately originated from bat hosts (Drexler et al., 2012; Goldstein et al., 2018; Hu et al., 2017; Memish, Perlman, Van Kerkhove, & Zumla, 2020; Towner et al., 2007). This likely includes the current SARS-CoV-2 pandemic (Boni et al., 2020; Lau et al., 2020). Bats appear to have the ability to tolerate these viruses with few health impacts, hence recent studies have focused on bat comparative genomics (Jebb et al., 2020) and its emphasis on viral response (reviewed in: Gorbunova, Seluanov, & Kennedy, 2020; Hayman, 2019). Although little is known from this perspective, there is a growing body of functional analyses showing that bats are unusual among mammals in how they deal with viruses (Ahn et al., 2019; A. Banerjee et al., 2020; Miller et al., 2016; Schountz, Baker, Butler, & Munster, 2017; Xie et al., 2018).

The 'inflammosome' is typically highly conserved across mammals, but bats exhibit a reduced inflammatory response that may be tied to their ability to cope with viral infection while experiencing minimal impact (Pavlovich et al., 2018). For example, the PYHIN gene family, namely, appears to have been almost completely lost in bats (Ahn, Cui, Irving, & Wang, 2016; G. Zhang et al., 2013) while at least one PYHIN gene can be found in all other eutherians examined. Similarly, in bats, the inflammatory function of interferons (G. Zhang et al., 2013) appears distinct among bat species, where IFN contractions and constitutive expression of IFN-α has been observed in some bats (P. Zhou et al., 2016), and the APOBEC3 repertoire, which is associated with anti-viral response, is expanded (Jebb et al., 2020; Hayward et al., 2018). These functional patterns suggest an overall dampened inflammatory reaction despite a robust immune response to viruses whose origins may lie in the gene repertoires available to bats (A. Banerjee, Rapin, Bollinger, & Misra, 2017; A. Banerjee et al., 2020).

Gene family evolution also likely plays a role in the unique dietary ecology of bats. Several studies have found a variety of mechanisms influencing dietary adaptation. For example, convergent amino acid substitutions in several lineages of frugivorous bats have occurred independently (Gutiérrez-Guerrero et al., 2020; Shen, Han, Zhang, Rossiter, & Zhang, 2012; Teeling et al., 2018; K. Wang et al., 2020), and are associated with the shift to a high-sugar diet. Another strategy has been to repurpose a given gene to

accommodate such dietary shifts (Shen, Han, Jones, Rossiter, & Zhang, 2013). With the exception of olfactory receptors (Hayden et al., 2014; Hughes et al., 2018; Tsagkogeorga, Müller, Dessimoz, & Rossiter, 2017), the roles of gene loss and gain in shaping dietary evolution of bats have not been comprehensively explored.

Here we investigate bat gene family evolution related to immunity, metabolism, and dietary adaptations, using the most extensive genomic sampling within bats to date. Despite variability in quality of assemblies, the ecological diversity of lineages for which assemblies are available allows, for the first time, an investigation of gene family evolution across 10 families, two suborders, and a complete coverage of the entire range of diets. We find two major patterns. First, system-wide gene losses related to inflammatory response and selection on genes associated with antiviral immunity appear to have influenced bat lineages. This suggests that bats— compared to other mammals such as cow, dog, horse, pig, mouse and human— have evolved complex, complementary adaptations across multiple functional pathways to simultaneously reduce inflammatory response while maintaining strong antiviral defenses, potentially underlying their suspected tolerance of viruses. Second, the move from the ancestral arthropod diet to high-sugar nectar and fruit-based diets is associated with lineage-specific gene family expansions in metabolic gene families.

Materials and Methods

Whole genome sequencing

We generated a whole genome assembly for a male *Phyllostomus hastatus*, PE091, collected in Jenaro Herrera, Peru. Field-collected tissues from *Phyllostomus hastatus* specimen PE091 were lawfully collected under permit #0122–2015–SERFOR–DGGSPFFS, exported under SERFOR permit #0002287, and imported under USFW 3-177 2015MI1694291.

Samples were preserved in RNAlater for one week before flash–freezing in a liquid nitrogen dry shipper, following previously published protocols (Yohe et al., 2019). High molecular weight genomic DNA was extracted from flash-frozen liver using the Qiamp DNA Micro Kit (Germantown, MD, USA) and sequenced on a PromethION instrument (Oxford Nanopore Technologies, New York, NY, USA) at Cold Spring Harbor Laboratory. Additionally, short-read Illumina whole genome sequencing was performed at Novogene, Inc (California, USA). Genomic DNA from lung was randomly fragmented to 350bp, end-repaired, adenylated, ligated with Illumina sequencing adapters, and further PCR–enriched. The final libraries were purified (AMPure XP system) and library quality and size verification were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Molar concentration was assessed using real-time PCR.

De novo genome assembly was performed using Flye v.2.7.1 (Kolmogorov, Yuan, Lin, & Pevzner, 2019) using default *--nano-raw* parameterization. The obtained pre-assembly was polished using Illumina short-reads with POLCA tool built-in MaSuRCA genome assembly and analysis toolkit (Zimin et al., 2013).

Genome database construction

Publicly-available genome assemblies for an additional 36 bat species (Supplementary Table 1) were downloaded from open-source databases to maximize bat taxonomic sampling (D. Dong et al., 2017; Eckalbar et al., 2016; Gutiérrez-Guerrero et al., 2020; Jebb et al., 2020; Parker et al., 2013; Seim et al., 2013; K. Wang et al., 2020; Zepeda Mendoza et al., 2018; G. Zhang et al., 2013). Assemblies were masked with RepeatMasker v.4.1.0 (Smit, Hubley, & Green, n.d.) using a custom library combining known mammalian transposable elements (TE) from Repbase (v20181026), a *de novo* mammalian TE library generated using assemblies from the Zoonomia Project (Genereux et al., 2020) and the Dfam database, and a custom bat–specific TE library generated by manual curation (Jebb et al., 2020).

All assemblies were annotated or re-annotated with the MAKER annotation pipeline v.2.31.10 (Holt & Yandell, 2011) to avoid bias in downstream analyses caused by differences in genome assembly annotation quality. Two iterations of MAKER were performed for each species. During the first run we provided expressed sequence tags (ESTs) and transcriptomic data as inputs (Davies et al., 2020; Potter et al., n.d.) (Supplementary Table 2). If species-specific transcriptomic data were unavailable, we used information from a related species of the same genus. We used two databases for protein homology the Uniprot/Swiss-Prot protein sequence database (Bateman, 2019) and a bat–specific protein database obtained from high-quality genome annotations for six bat species (Jebb et al., 2020). Repeat evidence was provided using the repeat annotation GFF3 file generated by RepeatMasker. Gene models generated on the first run were used for gene predictions with two gene software packages, SNAP (Korf, 2004) and Augustus (Stanke & Waack, 2003). Only gene models with an AED score < 0.25 and with more than 50 amino acids were retained. For the second run, focusing on re-annotation, the MAKER control file was edited to include the GFF3 output file from the first run gene predictions generated by SNAP and the Augustus gene prediction species model as inputs. Functional annotation was performed with BlastP (Camacho et al., 2009) using the Uniprot/Swiss-Prot database and protein domain annotation with InterProScan (Jones et al., 2014).

Homology inference

Protein homology was inferred among the proteins of 43 mammals: Including *Homo sapiens* and *Mus musculus,* two well-studied model organisms, and more closely related species from the superorder Laurasiatheria: *Sus scrofa, Bos taurus, Equus caballus, Canis lupus familiaris,* and the 37 bat species (Supplementary Table 1). Orthologous groups (orthogroups) were assigned with Orthofinder v.2.4.0 (Emms & Kelly, 2019). When no orthologs were inferred for the Chiroptera in a given orthogroup, we independently analyzed the genome data to confirm gene losses in bats (Supplementary Fig. 1). To this end, we performed a BLAST search against the 37 bat genomes using the following criteria: an e-value of 1e-6 and an identity and protein coverage greater than 80%. Then, genomic regions with a BLAST hit were extracted along with 200bp upstream and downstream. Sequences were aligned with the MAFFT aligner tool v.7.402 (Katoh & Standley, 2013) and visualized using Geneious version 11.1.3 (Kearse et al., 2012) to discriminate annotation errors. Additionally, BLAST searches were also performed against transcriptomic data from 22 bat species (Supplementary Table 2) (Potter et al., n.d.). For these searches, potential matches were filtered more strictly, and those with identity and protein coverage ≥ 90% were retained. Subsequent blast hit extraction, alignment and visualization were as for the genome searches.

Enrichment in chiropteran gene losses

We conducted pathway enrichment analyses with the final list of genes missing from all bat species using two databases: BioPlanet (R. Huang et al., 2019) and DICE GOnet (Pomaznoy, Ha, & Peters, 2018). In each case, we used the list of gene symbols as input with a cutoff value of 0.05 (BioPlanet) and a similar p–value in the DICE GOnet biological process classification for the mouse model. In both cases, all genes found to be missing were used as input and compared to a reference set of genes annotated in the corresponding database.

Inferring bat phylogeny

To infer gene family evolution, we first inferred an ultrametric phylogenomic tree based on 350 single copy orthologous genes (207,551 amino acid sites). All the orthologs were concatenated into a single 207,551–amino acid "contig" and sequence alignment was performed using the MAFFT aligner tool v.7.402 (Katoh & Standley, 2013). We evaluated the best-fit models of protein evolution with ProtTest v.3 (Darriba, Taboada, Doallo, & Posada, 2011) using two criteria: the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) (distribution JTT, +G +I +I +G and 80% consensus threshold). A maximum likelihood tree was inferred for the concatenated data set with RAxML v.8 (Stamatakis, 2014). Estimation of species

divergence times was performed with Bayesian phylogenetic methods using the MCMCtree tool in the PAML 4.9 package (Yang, 2007). We calibrated divergence dates using six points based on fossil records: 1) *Icaronycteris*, considered as one of the oldest echolocating fossil bats, dated at 52 Mya (Gunnell & Simmons, 2005; Simmons, Seymour, Habersetzer, & Gunnell, 2008); *Tachypteron,* the oldest known emballonurid fossil from the early Middle Eocene, with an age range of 48.6 to 40 Mya (Storch, Sigé, & Habersetzer, 2002); *Hipposideros africanum*, the oldest fossil record of the family Hipposideridae, its records date at 41.3 Mya (Ravel et al., 2016); Vespertillionidae indet. (41.3 Mya) (Eiting & Gunnell, 2009); Phyllostomidae indet. (30 Mya) (Nicholas J Czaplewski, 2010), and *Palynephyllum* (11.8 Mya) (Nicolas J Czaplewski, Takai, Naeher, & Setoguchi, 2003; Dávalos, Velazco, Warsi, Smits, & Simmons, 2014). Additionally, we included and corroborated the molecular dates for the base of the ingroup root estimated by Teeling et al. (2005).

Gene family evolution

While previous analyses that included bat species have analyzed signals of positive selection across bats (e.g. Parker et al., 2013), fewer have explicitly centered on gene family evolution (Jebb et al., 2020; Tsagkogeorga et al., 2017). To analyze our comprehensive bat-focused sample, we modeled gene family expansions and contractions using CAFE (Computational Analysis of Gene Family Evolution) v.4.2.1 (M. V. Han, Thomas, Lugo-Martinez, & Hahn, 2013). CAFE fits a birth and death parameter (λ) to estimate the probability of gene gains or losses across a specified phylogeny (Hahn, De Bie, Stajich, Nguyen, & Cristianini, 2005), and we used the newly inferred phylogeny to this end.

When we included all species in the CAFE analysis, we observed a systematic bias in gene family contractions among fragmented genomes. This effect of genome quality on downstream gene predictions is well documented and leads to an overestimation of gene gains and losses (Denton et al., 2014; Tsagkogeorga et al., 2017). To mitigate the bias, only genome assemblies with BUSCO completeness scores over 80%, totaling 34 species (28 bat species and 6 outgroup mammals) were used for CAFE. This smaller subset of protein sequences was filtered, retaining only the longest isoform. Homology clustering was performed with Orthofinder v.2.4.0 (Emms & Kelly, 2019).

We filtered the final input for CAFE to reduce systematic bias in inferring gene family evolution. First, we retained only gene families present at the most recent common ancestor of the phylogeny, with at least one gene present in each of the four clades assigned: a) Euarchontoglires (*Homo sapiens* and *Mus musculus*), b) non-Chiroptera Laurasiatheria (*Bos taurus, Canis familiaris, Equus caballus, Sus scrofa*), c) Yangochiroptera and d) Yinpterochiroptera. Second, gene families missing in more than 50% of bat species were excluded.

Finally, families with large gene copy number variance (≥100 gene copies) were excluded for the global birth and death (λ) rate inference.

To analyze families with at least one gene copy across the taxa sampled, we first estimated a global λ for all branches. The global model was compared against a three multi-λ model that fits each lineage with its own gene family evolution rate. To test which model fits better with our dataset, we performed a likelihood ratio test for 100 gene family evolution simulations. We ran CAFE in error correction mode to account for genome assembly and annotation errors and estimate the global distribution of error with the assumption that all branches share a unique λ rate (λ=0.0033734) as described in Han et al. (2013). Finally, we used complementary tools; the Protein Analysis Through Evolutionary Relationships (PANTHER v.15) (Mi, Muruganujan, Ebert, Huang, & Thomas, 2019) and Gene Ontology Analysis (GOnet) to annotate genes with gene ontology (GO) terms (Ashburner et al., 2000; Carbon et al., 2019) and assign them to gene families, pathways, and biological process categories.

Selection tests

We identified genes under positive selection by evaluating 268 single–copy genes involved in immune response, based on a curated database of 1,793 genes downloaded from the IMMPORTDB repository (Bhattacharya et al., 2014) available at https://www.immport.org/home. Gene alignments were built with MAFFT v.7.402 (Katoh & Standley, 2013) and manually filtered to remove sequences with less than 70% of protein coverage based on the homologous human protein. Only alignments represented by at least 30% of the species were used for downstream analysis. For each gene in the codeml analyses, we built a phylogeny with RAxML (Stamatakis, 2014) and a codon alignment for each gene with PAL2NAL (Suyama, Torrents, & Bork, 2006).

We tested for evidence of positive selection among sites along bat lineages using the strict branch–site model (Yang, Wong, & Nielsen, 2005; J. Zhang, Nielsen, & Yang, 2005) with maximum-likelihood estimations implemented in codeml in PAML v.4.9 (Yang, 2007). We implemented model 2 as this allows the dN/dS ratio (ω) to vary across branches and sites and to detect if selection differs in a few amino acid residues in specific lineages (foreground branches). We compared two hypotheses, assigning the 37 bat species as foreground branches: 1) the null hypothesis with a fixed ω (ω=1) for all branches does not allow for positive selection, and 2) an alternative hypothesis assuming that the foreground branches have a greater proportion of sites under positive selection (ω > 1) than the background branches. The null hypothesis was tested against the alternative model with the likelihood-ratio test (LRT); the p-value was calculated under a chi-square

distribution with 1 degree of freedom, additionally we adjusted the p-value using the false discovery rate (FDR) correction. To detect sites under positive selection, we used the Bayes Empirical Bayes (BEB) (Yang et al., 2005) approach to calculate posterior probabilities that a site has a significant value of ω >1. The residues with a high posterior probability (P > 95%) were considered.

To determine how robust the signals of positive selection detected were, we used the adaptive Branch-Site Random Effects Likelihood (aBSREL) (Smith et al., 2015) model, as implemented in HyPhy (Kosakovsky Pond, Frost, & Muse, 2005). The aBSREL model explores whether a proportion of sites have evolved under positive selection in each branch of the phylogeny, and was applied to all alignments using their respective gene trees. The false discovery rate method of multiple testing correction was applied to all p-values generated for each branch and gene.

Results

Genome sequencing

The final assembly for *P. hastatus* comprised 2.1 Gb and has a N50 contig length >39 Mb. Assembly quality completeness was estimated at 95.4%. These values are similar to those observed for bat assemblies inferred using similar methods (Jebb et al., 2020).

Homology inference

BUSCO analysis results indicated that the bat genome assemblies contained between 68.5 and 96.5% of the single–copy orthologs present among mammals (Figure 1). Orthologs were grouped into 42,441 groups, of which 1,193 were single copy. In total, 5,528 orthogroups had at least one representative in each of the entire set of 43 species that were analyzed. In contrast, 1,055 orthogroups were represented in at least 50% of bat species but missing from the six outgroup taxa (Supplementary table 3). To annotate diets, we used the semi–quantitative database compiled by Rojas, Ramos, Fonseca and Dávalos (2018), which focuses on neotropical noctilionoids (Yangochiroptera), supplemented with summaries from Animal Diversity Web (https://animaldiversity.org/).

Enrichment in chiropteran gene losses

We inferred the first densely sampled chiropteran phylogeny based on hundreds of loci (Figure 1). Our results confirmed the monophyly of the suborders Yinpterochiroptera and Yangochiroptera but the phylogeny of the neotropical leaf-nosed bats (family Phyllostomidae) differed from previous phylogenies (Davalos, Velazco, & Rojas, 2020), in the paraphyly of plant-eating lineages. As the obtained phylogeny is the best supported by all

genome-scale analyses available thus far (S.J. Rossiter and M. Hiller pers. obs.), we used this phylogeny for gene family evolution analyses.

A total of 1,115 genes (Supplementary Table 4) were identified as missing in bats, even after filtering BLAST searches against the genomes and transcriptomes. Based on this list, we identified eight over-represented pathways in BioPlanet (Supplementary Table 5) and 63 GO terms in GOnet (Supplementary Table 6). While the former included 104 genes, of which 49 were unique, the latter included 339 unique missing genes. As expected, over-represented categories included chemosensory gene losses in the categories of olfactory transduction, G-protein–coupled receptors (GPCR), and signal transduction. BioPlanet pathways were also enriched for less common categories including immune system pathways that include alpha and beta defensins, antigen process and presentation, and graft-versus-host disease (Supplementary Table 5). GOnet analyses also identified the expected enrichments in chemosensory gene losses and general response to stimuli categories, but also included many more immune categories. Of the latter, the categories comprising the most genes were defense response (58 genes), defense response to other organism (54), response to bacterium (53), innate immune response (46), defense response to bacterium (44), humoral immune response (34), adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (23), lymphocyte mediated immunity (23), and leukocyte mediated immunity (23). Although these categories share many genes across them, a preponderance of immune system losses is evident in Supplementary Table 6. We used BioRender to summarize the immune gene ontology categories and connections, highlighted in Figure 2.

Gene family evolution

To determine branches and gene families with significant gene family expansions and contractions, we analyzed 14,171 orthogroups under two models: a global rate of gene family evolution, and a three multi–λ model. The three–rate model best fit the data ($p < 0.01$), this analysis estimated a higher rate of gene family turnover ($\lambda_{Yangochiroptera} = 0.0048$) in the ancestral Yangochiroptera lineage than in the Yinpterochiroptera ancestral lineage ($\lambda_{Yinpterochiroptera} = 0.0024$), with the lowest turnover rate for outgroup lineages ($\lambda_{Outgroups} = 0.0017$).

With an estimated error distribution of 0.049 (i.e., 4.9% of gene families showed an error in gene size), we identified 2,555 orthogroups with significant expansions or contractions along at least one of the branches in the species tree (Supplementary Table 7). Given our focus on immune system and metabolic evolution, we extracted PANTHER annotations for the most frequent (900 orthogroups) biological process categories:

immune response, metabolic process, and cellular process. All GOnet annotations were used and binned into immune, metabolic, and two additional processes: response to stress (271 orthogroups) and autophagy (19). PANTHER and GOnet annotations were mostly complementary; orthogroups were often annotated in one database but not the other (1,268 orthogroups). When annotations were available from both databases, these tended to agree on both immune and metabolic categories (594 orthogroups), or to agree on one or the other (404), with only 48 orthogroups disagreeing completely in immune and metabolic annotations between the databases. The remaining 241 were not annotated in either database. Categories, locations, and size of significant gene family changes were summarized using tools in the R package ggtree (Yu, Smith, Zhu, Guan, & Lam, 2017) and are shown in Figure 3. Although several pairs of sister species showed apparently large differences along corresponding tips (e.g., *Rhinolophus*, *Miniopterus*), such variation is common in analyses that include genome assemblies of varying quality (Denton et al., 2014; Tsagkogeorga et al., 2017). Therefore, we focus our discussion on the more robust inference of gene family expansions and contractions for non-sister lineages in immunity and metabolism genes.

Selection tests

Branch–site selection tests identified 37 of 268 single–copy genes with evidence for positive selection, of which 27 remained after false discovery rate correction (Table 1). This subset included genes involved in interferon-gamma (IFNG) signaling, inflammatory response, as well as cytokines, chemokines, and interleukins. A total of 16,979 branches across 268 genes were analysed using the aBSREL model in HyPhy. After FDR correction, 683 branches from 191 gene trees were found to be significant, 25 of which were consistent with CODEML results (Supplementary table 8).

Discussion

losses in inflammation–related gene families and positive selection in single–copy genes associated with immune and cell repair functions in mammalian models have been evident since the very first bat genome assemblies were published (G. Zhang et al., 2013). Although subsequent studies have confirmed those initial results (Ahn et al., 2016; Seim et al., 2013), confidence in assessing both gene losses and gene family expansions has strengthened only recently, with the publication of highly contiguous assemblies for a few bat species (Jebb et al., 2020; Scheben et al., 2020). Examining a comprehensive sample of bat lineages while checking against high quality genome assemblies and multi organ RNA Seq, our analyses reveal system wide gene losses with the potential to modify the sensitivity, targets, and magnitude of immune responses across all bats. These inferred losses are particularly concentrated along inflammasome activation pathways, which

are triggered by the innate immune recognition of pathogenic signals through both pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). In contrast with more pathogen–driven PAMPs, DAMPs result from host cellular distress signals such as mitochondrial stress and reactive oxygen species (ROS) (Zheng, Liwinski, & Elinav, 2020), which bats produce during active flight (Costantini, Lindecke, Petersons, & Voigt, 2019). Bat cells, in turn, display exceptional mechanisms of repair (Pickering, Lehr, Kohler, Han, & Miller, 2014) and resist damage (Harper, Salmon, Leiser, Galecki, & Miller, 2007), connecting molecular signaling and cell processes to extreme longevity (Salmon et al., 2009; Wilkinson & Adams, 2019).

Based on our genomic surveys, immune-related losses can be divided into three categories: the epithelial defense receptors (defensins), the Natural Killer gene complex (NKC) and the interferon-induced pathway (IFI; HIN; PYHIN) (Figure 2). This particular combination of losses in crucial components of immune activation seems contradictory, as it would imply that these losses could lead to an ineffective immune response in bats. This contradiction notwithstanding, these results complement previous findings indicating that bats have evolved efficient mechanisms of regulation that allow them to mount a low intensity immune response to primarily intracellular pathogens. Integrating these genomic findings with published functional data suggests complex, systemic adaptation, in line with both previous analyses of bat immune system responses (A. Banerjee et al., 2020; Basler, 2020; P. Zhou, 2020) and the growing body of evidence for cellular mechanisms underlying longevity (Z. Huang, Whelan, Dechmann, & Teeling, 2020; Z. Huang et al., 2019, Kacprzyk et al., 2017). We review these losses in a stratigraphic order, from the outer cellular matrix to the inner cellular pathways, starting with the defensins.

While defensins are the primary barrier of the immune system, with broad antimicrobial activity that covers bacteria, fungi, and viruses (Semple & Dorin, 2012; Xu & Lu, 2020), bat defensin losses consist mainly of orthologs of genes localized to epithelial cells. Our results indicate that both α and β defensin genes have undergone a rapid evolutionary change through either loss or positive selection (Table 1, Figure 2a, Supplementary Table 4). Rapid evolution and diversification of defensins, driven by the microbiome, varies considerably among species, even in closely related species (Tu et al., 2015). Among vertebrates, an expansion of β defensins occurred in mammals, with bovines having the largest number of copies (Tu et al., 2015), while α defensins, exclusive from mammals (Xiao et al., 2004), are lost in bovines (Fjell et al., 2008).

Defensins can function as modulators of the host's cell surface receptors, and α and β defensins genes have pleiotropic effects on the regulation of carcinogenesis and inflammation (Xu & Lu, 2020). By

acting as chemokines to alter the adaptive immune response, defensins also serve as a bridge between innate and adaptive immunity (Grigat, Soruri, Forssmann, Riggert, & Zwirner, 2007). In humans, defensins can elicit proinflammatory cytokine production (Niyonsaba et al., 2010; Wiens, Wilson, Lucero, & Smith, 2014), but overexpression of certain defensins can actually enhance viral infection (Rapista et al., 2011). We hypothesize that specific defensin losses in bats (Figure 2a) complement several other mechanisms (Ahn et al., 2019; A. Banerjee et al., 2017; Xie et al., 2018) contributing to a dampened inflammatory response, reduced host–driven damage from viral infections, and enhanced longevity (Baker & Schountz, 2018; Brook & Dobson, 2015; Gorbunova et al., 2020). For example, modifying defensin repertoires on epithelial cells would result in fewer instances of both immune cell recruitment and initiation of inflammatory pathways known to damage healthy tissue (e.g., focal necrosis in lungs, spleen and lymph nodes during the inflammatory response during SARS-Cov2 infection (Merad & Martin, 2020)). In humans, loss of β-defensins prevents the inhibition of neutrophil apoptosis and thus averts the production of proinflammatory cytokines and chemokines (Nagaoka, Niyonsaba, Tsutsumi-Ishii, Tamura, & Hirata, 2008), avoiding the amplification of the immune response, and may have a similar effect in bats. Losses of some epithelial surface defensins would thus reduce inflammation without compromising responses to intracellular pathogens.

Another result with inferred implications for reducing proinflammatory reactions involves losses of Natural Killer (NK) receptors that play an important role in the recognition of MHC-I molecules and regulation of cytotoxic activity against virus–infected cells. While killer-cell immunoglobulin like receptors (KIR) and killer cell lectin-like receptors (KLR) receptor losses has been previously reported for *Pteropus alecto* and *Myotis davidii* (Papenfuss et al., 2012; G. Zhang et al., 2013), our analyses confirm these losses across Chiroptera (Supplementary Table 4). Although the *Killer Cell Lectin Like Receptor K1* (KLRK1 or NKG2D) gene is present in bats, its ligands, gene subfamilies *RAET1* and *H60* responsible for binding and activating NKG2D receptors, activating natural killer cells, and stimulating them to secrete Interferon gamma (IFN-γ) (Zhi et al., 2010), were absent in all bat species (Figure 2b).

We hypothesize that these losses lead to low recruitment of proinflammatory NK cells and reduce B-cell signaling (Arapović et al., 2009; Stolberg et al., 2014; Takada et al., 2008; Wortham et al., 2012), as they do in mice and humans. Loss of this particular mechanism of activation of the MHC-I pathway prevents proliferation of immune cells, which can be cytotoxic, proinflammatory, and targets of viral infections (Djelloul, Popa, Pelletier, Raguénez, & Boucraut, 2016; Wortham et al., 2012). For example, NKG2D–deficient mice infected with influenza viruses exhibit less airway damage and reduced inflammation without

compromising viral clearance; similarly, knockout of NKG2D in mice and humans during cytomegalovirus infection helps to avoid the destruction of non-infected cells by NK (Muntasell et al. 2010; Slavuljica, Krmpotić, & Jonjić, 2011). NKG2D stimulation is a central pathway to tumor, stress and viral-mediated NK cell hyper responsiveness (Wortham et al. 2012) and has been shown to be involved in autoimmune disorders, such as rheumatoid arthritis, type I diabetes, and celiac disease (reviewed in Caillat-Zucman, 2006; Guerra et al. 2013), and inflammatory diseases such as Crohn's disease (Vadstrup et al. 2017), chronic respiratory diseases (Wortham et al. 2012; Guerra et al. 2013) and more recently with age-dependent COVID–19 severity (Akbar & Gilroy, 2020). During viral exposure, rarer activation of NKG2D function would therefore lead to less inflammatory exacerbation. Reducing instances of NKG2D activation might also reduce B cell signaling, as it occurs in NKG2D–deficient mice (Lenartić et al., 2017; Zafirova et al., 2009), and complements losses of immunoglobulin heavy chain variable regions IGHV1, IGVH3, and IGHV14 genes that modify the B cell receptor signaling pathway, and thus B lymphocyte differentiation (M. Banerjee, Mehr, Belelovsky, Spencer, & Dunn-Walters, 2002; McHeyzer-Williams, Okitsu, Wang, & McHeyzer-Williams, 2012; Reddy et al., 2010). Based on the roles of both NKG2D and B cell activation in promoting inflammation in viral infection, and since some viral proteins have been shown to specifically target the NKG2D receptor via the RAET1 and H60 loci (Arapović et al., 2009), we propose that these losses resulted from selection during viral infections early in the evolutionary history of bats. While the functional implications for bats need to be tested, in humans, lack of specificity of the T and B cells in children results in a broader immune response to novel viruses (Pierce et al., 2020), and it may confer analogous advantages in bats.

Complementing losses in defensins and NK signaling, the third large group of gene losses involves the IFN-γ pathway (Figure 2c). While representatives of the PYRIN and HIN domain (PYHIN) gene family, immune sensors of cytosolic DNA activating the inflammasome and IFN-γ, are present in all mammals, they have not found in any of the bat genomes analyzed thus far examined (Ahn et al., 2016; G. Zhang et al., 2013; Zebb et al., 2020). Previous genomic analyses linked losses in this inflammasome pathway not only to immune implications, but also to the unique demands of bat flight and in response to increased ROS production (G. Zhang et al., 2013). In other mammals, the presence of dsDNA, DAMPs and PAMPs, or, especially, bacteria and DNA viruses, induces the (PYHIN) AIM2 inflammasome, while the IFI16 inflammasome (Interferon-inducible protein 16, also missing in bats) recognizes viruses replicating in the nucleus (Zheng et al., 2020). Hence, these bat gene losses could undermine innate defense against viruses. We hypothesize that bats have evolved mechanisms to overcome this potential disadvantage in rapid recognition and response against

viruses through expansion of MHC-I class genes (Supplementary Table 7). These genes are involved in the recognition and binding of intra cellular peptides, and previous studies have described a unique 5–amino acid insertion at the exon 2 peptide binding region (PBR) on bats which may allow the host to recognize longer peptides (Ng et al., 2016; Papenfuss et al., 2012). Besides implications for immunity, IFN-γ pathway gene losses also point to changes in autophagy. In mice, loss of the IFN-γ inducible immunity related GTPase gene (IRGM1 and IRGM2) results in an IFN-γ induced autophagic death program in lymphocytes (Feng et al., 2008). Along with the loss of other IFN-γ related genes (IGTO, IIGP, TGTP2), these losses may help achieve apoptosis of infected cells without runaway inflammation.

While some mechanisms of activation of IFN-λ are lost in bats, IFN-γ itself is under positive selection within branches (Table 1, Supplementary Table 7). IFN-γ is a crucial part for the first line of defense against viruses, helps shape adaptive immune memory (Schroder, Hertzog, Ravasi, & Hume, 2004), and its deficiency increases inflammation (Loo et al., 2017). Thus, evolutionary adaptation may have shaped bats' unique ability to induce a rapid antiviral response without triggering runaway inflammation. This fine-tuned response may be achieved by expressing high levels of IFN-γ early on, which recruits broad-spectrum immune cells to the site of injury, while negatively regulating the IFN-γ pathway receptors that trigger inflammation (Ahn et al., 2019; Ferber et al., 1996).

By generating a controlled induction of immune response, bats' unique regulatory mechanisms, have sparked an extraordinary immune tolerance against viruses, a key factor in bats as natural viral reservoirs. Evidence of this viral tolerance has been observed in bats with high viral load (reviewed in; Subudhi, Rapin, & Misra, 2019; Irving et al., 2021). In addition, *in silico* experiments have shown that a trade-off of this viral tolerance in bats is the rapid spread of viruses within the host; thus, favoring viruses to evolve adaptations that increase their replication rates (Brook et al. 2020). While this rapid transmission may not have a significant harmful effect in bats, it could be detrimental for other species, as recent spillovers have shown.

In contrast to a pattern of proinflammatory signal losses common to all bats, most other variation in gene families within Chiroptera corresponded to cell processes and metabolic functions with the notable exceptions of APOBEC3 and MHC-I. Besides confirming the previously reported APOBEC3 expansion in *Pteropus vampyrus* (Hayward et al. 2018), we also inferred expansions in the common ancestors of *Desmodus* and *Artibeus*, of Vespertilionids, *Myotis*, and of *M. brandtii* and *lucifugus*, including species-specific expansions in the latter. With this denser sampling, expansions formerly traced to *Myotis myotis* and *Pipistrellus kuhlii* (Jebb et al. 2020), are instead part of broader vespertilionid dynamics especially within

Myotis. Other species-specific expansions were inferred in the phyllostomids *Tonatia saurophila* and *Desmodus rotundus*, both of which shift from an ancestral bat insectivorous diet to one including vertebrates, exclusively so for *Desmodus*. While MHC-I expansions have been highlighted in *Pteropus alecto* (Ng et al. 2016) and *Rousettus aegyptiacus* (Pavlovich et al. 2018), here we find much greater expansions in neotropical noctilionoids including *Noctilio*, *Mormoops*, and especially within Phyllostomidae including *Artibeus*, *Sturnira*, *Tonatia*, *Leptonycteris*, *Musonycteris*, *Anoura*, *Desmodus*, and *Macrotus*. As with APOBEC3, MHC-I evolution in vespertilionids was found to be dynamic, with significant expansions inferred for every *Myotis* species, as well as *Pipistrellus* and *Eptesicus*. While APOBEC3 function has been examined in *Pteropus alecto* (Hayward et al. 2018), our analyses highlight the need for characterization in vespertilionids. With greater potential for ligand binding, rich MHC-I repertoires may provide both better self recognition for NK tuning and finer resolution of MHC-I pathogen mimics (Parham & Moffett, 2013), suggesting further research avenues in phyllostomids, vespertilionids, and *Miniopterus*. Our analyses overlooked both the potential for unique MHC-I features that alter antigen presentation, as in *Pteropus alecto*, and population variation, already found in the phyllostomids *Carollia perspicillata* (Qurkhuli et al. 2019), suggesting these as potential research avenues.

Expansions and contractions in metabolic genes were common throughout the bat phylogeny (Figure 4), but many ecological differences across species (e.g., biogeography, hibernation, life history) could be driving these changes (Seim et al. 2013; Y. Han et al. 2015). Taking advantage of our relatively dense taxon sampling within bats (Figure 1), we focus on parallel adaptation to plant–rich diets across suborders Pteropochiroptera and Yangochiroptera, a set of traits of known metabolic implications (Voigt & Speakman, 2007). Shifts from the ancestral bat insectivorous diet to including nectar and fruit and the resulting mutualistic relationships between bats and plants appear to have led to elevated rates of diversification and the evolution of new morphological traits (Dumont et al., 2012; Jones, Bininda-Emonds, & Gittleman, 2005), but gene family evolution has remained underexplored. Regarding significant expansions (Supplementary Table 7), we identified few —only nine— sets of duplications independently replicated across all pteropodids and phyllostomids with convergent, plant–based diets (Figure 1). In addition to a trace amine associated receptor (TAAR) of unknown chemosensory function (Liberles & Buck, 2006) and a putative homolog of the yeast protein transport protein YIP1, two genes stand out as candidates for diet–linked adaptive gene family evolution: those encoding homologs of *inositol monophosphatase 1* (IMPA1) and *integrin alpha-D/beta-2* (ITAD). Glycolysis, the metabolic pathway that breaks down glucose to ultimately phosphorylate more ADP into ATP than the reverse, begins with the phosphorylation of glucose into D-glucose 6-phosphate (Berg,

Tymoczko, & Stryer, 2002). This metabolite, however, cannot diffuse through the membrane and is thus highly osmotic; its accumulation would cause cells to swell. Through the synthesis of *myo*-inositol from D-glucose 6-phosphate, IMPA1 provides one avenue to protect cells, particularly in the brain (Parthasarathy, Parthasarathy, & Vadnal, 1997), from the osmotic stress of this glucose metabolite (Rafikov et al., 2019). We found independent IMPA1 duplications in the pteropodid ancestor, *A. jamaicensis*, *A. caudifer*, *P. discolor*, and the common ancestor of phyllostomids and *Mormoops*. Except for the aerial insectivore *Mormoops*, all the lineages with IMPA1 duplications include nectar and fruit in their diet (Figure 1), are expected to at least occasionally experience high blood glucose levels (Amitai et al., 2010; Ayala & Schondube, 2011; Kelm, Simon, Kuhlow, Voigh & Ristow, 2011; Welch, Herrera & Suarez, 2008; Meng, Zhu, Huang, Irwin, & Zhang, 2016), and therefore require options for processing metabolites from glycolysis. Although beta integrins, including ITAD, are regulators of leukocyte function and therefore not annotated as directly involved in metabolism, leukocyte adhesion has been found to modulate glucose homeostasis via lipid metabolism (Meakin et al., 2015). Specifically, mice deficient in a paralogous beta-2 integrin become spontaneously obese in old age despite a normal diet (Z. Dong, Gutierrez-Ramos, Coxon, Mayadas, & Wagner, 1997), and when fed a fat rich diet show obesity, inflammation, high neutrophil activity and insulin resistance in skeletal muscle (Meakin et al., 2015). Likewise, mice deficient in this same integrin are unable to respond to fasting by increasing fat uptake and reduce insulin levels slowly compared to normal mice (Babic et al., 2004). We found single ITAD duplications in lineages that include sugar rich foods in their diet: ancestral pteropodids and phyllostomids, as well as *Leptonycteris yerbabuenae*, two each in *Macroglossus*, *Anoura*, and *Tonatia*, and three in *Artibeus jamaicensis*. While the function of these lineage–specific bat paralogs remain unknown, their phylogenetic distribution warrants future exploration and functional analysis.

In summary, our results, grounded on the most comprehensive survey of bat genomes to date, suggest bats have evolved complex mechanisms of inflammasome regulation. These may have evolved to prevent uncontrolled inflammatory response against DAMPs byproducts of the high metabolic rate required for powered flight (Banerjee et al., 2017; Banerjee et al., 2020; Subudhi, Rapin & Misra, 2019; Xie et al., 2018), to better respond against intra-cellular pathogens such as viruses, or some combination of both. Regardless of the ecological origin of selection, compared to mammals such as humans or mice, bat genomes reveal systemwide immune evolution that prevents or dampens aggressive inflammatory responses. In contrast with these gene losses, we found significant expansions in gene families involved with glucose degradation,

coinciding with the transition from a diet based mainly on insects to a high-glucose content diet that includes fruit and nectar.

By undertaking large-scale comparative genomic analyses encompassing many ecologically divergent lineages, the present study demonstrates the impact of genomics in non-model organisms. Such analyses allow elucidating the broad evolutionary mechanisms in a given clade, with potential for functional implications. Yet, heterogeneity in assembly quality continues to limit the scope of inference. Hence, the need to generate high quality genomes for future studies endures.

Acknowledgments

For support in long-read Oxford Nanopore sequencing, we thank Dr. Sara Goodwin from Cold Spring Harbor Laboratories. L.M.D. was supported, in part, by NSF-DEB 1838273, NSF-DGE 1633299, with S.J.R by NSF-DEB 1442142, and with A.M.B. by NSF-IOS 2032063 and 2031906. D.A.R. and D.D.M.S. were supported, in part, by NSF-DEB 1838283, and NSF-IOS 2032006. A.P.C was supported, in part, by NSF-IOS 2032011 and 2031926. T.M.L was supported by NSF-PRFB 2010853. L.R.Y. was supported by NSF-IOS 2032073 and NSF-DBI 1812035. E.C.T. was supported in part by an Irish Research Council Laureate Award IRCLA/2017/58. SCV was supported by a Max Planck Research Group awarded by the Max Planck Gesellschaft, a Human Frontiers Science Program Grant (RGP0058/2016) and a UKRI Future Leaders Fellowship (MR/T021985/1). The authors would like to thank Stony Brook Research Computing and Cyberinfrastructure, and the Institute for Advanced Computational Science at Stony Brook University for access to the high-performance SeaWulf computing system, which was made possible by a $1.4M National Science Foundation grant (#1531492). The High-Performance Computing Center at Texas Tech University and The Scientific Computing Department at the Instituto de Ecología, Universidad Nacional Autónoma de México provided computational infrastructure and technical support throughout the work.

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Declarations

The authors claim no conflicts of interest.

Data Accessibility

Final genome assembly is desposited at Genbank under BioProjectID: PRJNA733208 and accession number JAHKBD000000000.

Scripts for genome assembly, ultrametric tree construction, gene family, and selection test are deposited in Dryad repository https://doi.org/10.5061/dryad.59zw3r265.

Author Contributions

LMD, DAR, DDMS conceived of the study; DAR, JHP, LRY, SJR, and HZ collected samples; DAR, DDMS, ECT, GM, JHP, KTJD, LMD, LRY, PD, SJR, SV, YTGG, and ZH generated data; AMB, APC, DDMS, GH, LMD, TML, YTGG, and ZH analyzed data-guided, in part, by DAR and FGH; APC, DAR, DDMS, LMD, and TML wrote the manuscript. All authors reviewed the manuscript prior to submission.

Tables

Table 1. Branch–site codeml results for all species on single–copy immune system genes. FDR, false discovery rate; LR, likelihood ratio; *P*, nominal *P*-value.

Symbol	Name	Category	Alt	Null	LRT	P-val	FDR
Bbc3	BCL2 Binding Component 3	Inflammatory response	-4704.07	-4724.64	41.15	0.00	0.000
BPIFB5	BPI fold containing family B member 4	Antimicrobials	-5438.63	-5448.49	19.73	0.00	0.000
CCL1	C-C motif chemokine 1	Chemokines/Cytokines/Antimicrobials	-2449.96	-2454.54	9.16	0.00	0.023
CD3E	CD3e molecule	TCR signaling Pathway	-4463.25	-4485.65	44.80	0.00	0.000
CD79B	CD79b molecule	BCR Signaling Pathway	-4298.73	-4303.68	9.91	0.00	0.017
CD86	CD86 molecule	Antimicrobials	-5668.52	-5673.13	9.22	0.00	0.023
CSF2	colony stimulating factor 2	Cytokines	-1895.79	-1901.28	10.98	0.00	0.012
CXCL13	C-X-C motif chemokine 13	Chemokines/Cytokines/Antimicrobials	-2446.76	-2474.82	56.11	0.00	0.000
DEFB129 †	Beta-defensin 129	Antimicrobials	-4093.98	-4100.00	12.05	0.00	0.008
DEFB133	defensin beta 133	Antimicrobials	-935.69	-944.53	17.67	0.00	0.001
F2RL1	F2R like trypsin receptor 1	Antimicrobials	-10695.69	-10741.51	91.64	0.00	0.000
HRK†	Harakiri, BCL2 Interacting Protein	Inflammatory response	-1232.08	-1248.01	31.86	0.00	0.000
IFNG	interferon gamma	Antigen Processing and Presentation	-5525.65	-5538.95	26.60	0.00	0.000
IL17A	Interleukin-17A	Cytokines/Interleukins	-4495.35	-4500.65	10.60	0.00	0.014

IL17RC	interleukin 17 receptor C	Cytokines	-3585.03	-3623.10	76.14	0.00	0.000
IL1A	interleukin 1 alpha	Cytokines	-6876.43	-6880.12	7.39	0.01	0.052
IL20RA	interleukin 20 receptor subunit alpha	Cytokine Receptors	-12518.47	-12522.21	7.49	0.01	0.051
INHBE	Inhibin beta E chain	Cytokines/TGFb family	-8225.60	-8257.30	63.40	0.00	0.000
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	BCR Signaling Pathway	-4109.81	-4141.82	64.03	0.00	0.000
MAPKBP1	Mitogen-Activated Protein Kinase Binding Protein 1	Antimicrobials/Inflammatory response	-17784.73	-17791.00	12.54	0.00	0.006
NPFF	neuropeptide FF-amide peptide precursor	Cytokines	-2619.77	-2623.89	8.23	0.00	0.037
NRG1	neuregulin 1	Cytokines	-1737.10	-1741.12	8.05	0.01	0.038
TRDC	T cell receptor delta constant	TCR signaling Pathway	-4159.64	-4192.39	65.50	0.00	0.000
TRDV3	T cell receptor delta variable 3	TCR signaling Pathway	-2903.04	-2908.09	10.09	0.00	0.016
TRH	Pro-thyrotropin-releasing hormone	Cytokines	-6601.02	-6606.58	11.12	0.00	0.012
TRIML1	Tripartite Motif Family Like 1	Antimicrobials	-10302.78	-10307.91	10.27	0.00	0.015
TYROBP	TYRO protein tyrosine kinase-binding protein	NaturalKiller Cell Cytotoxicity	-1824.09	-1829.22	10.27	0.00	0.015

†Genes non significant in aBSREL

Figures

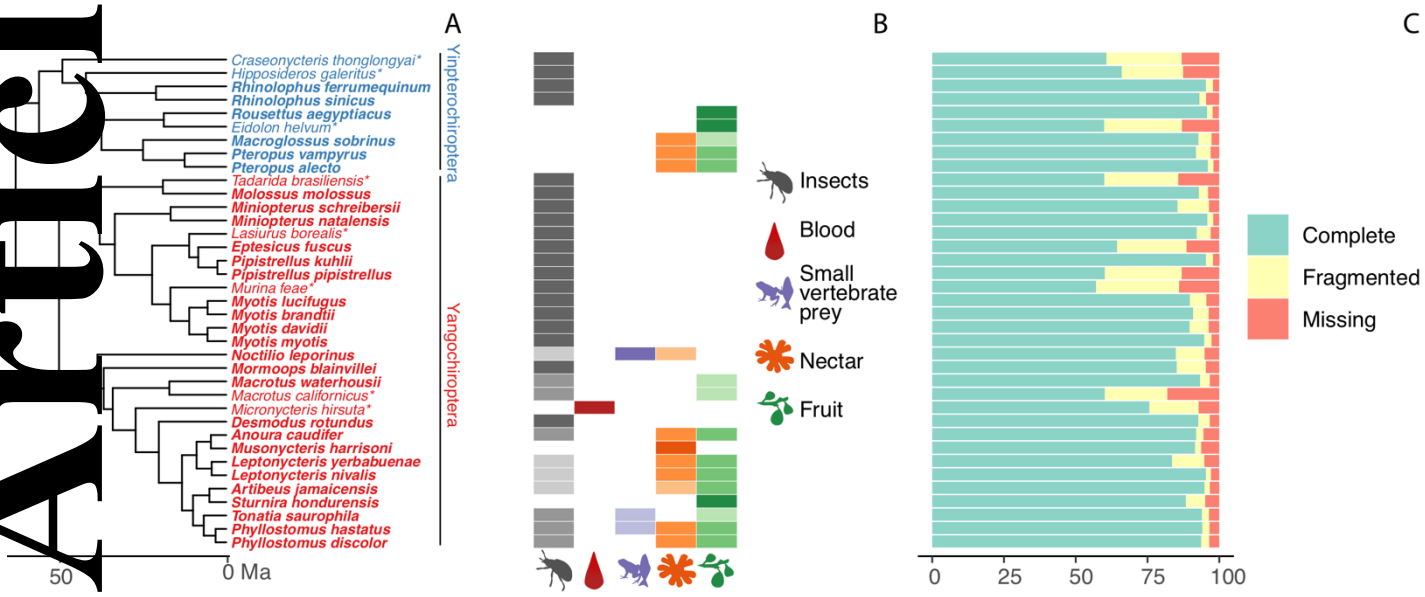

Figure 1. Phylogeny, dietary diversity, and BUSCO completeness across bat genomes. A) Species tree based on 300 genome –wide loci dated using penalized likelihood smoothing. *Genomes excluded from CAFE analyses. B) Diet composition and relative reliance indicated by color intensity (Rojas et al., 2018). C) BUSCO completeness for the corresponding genome.

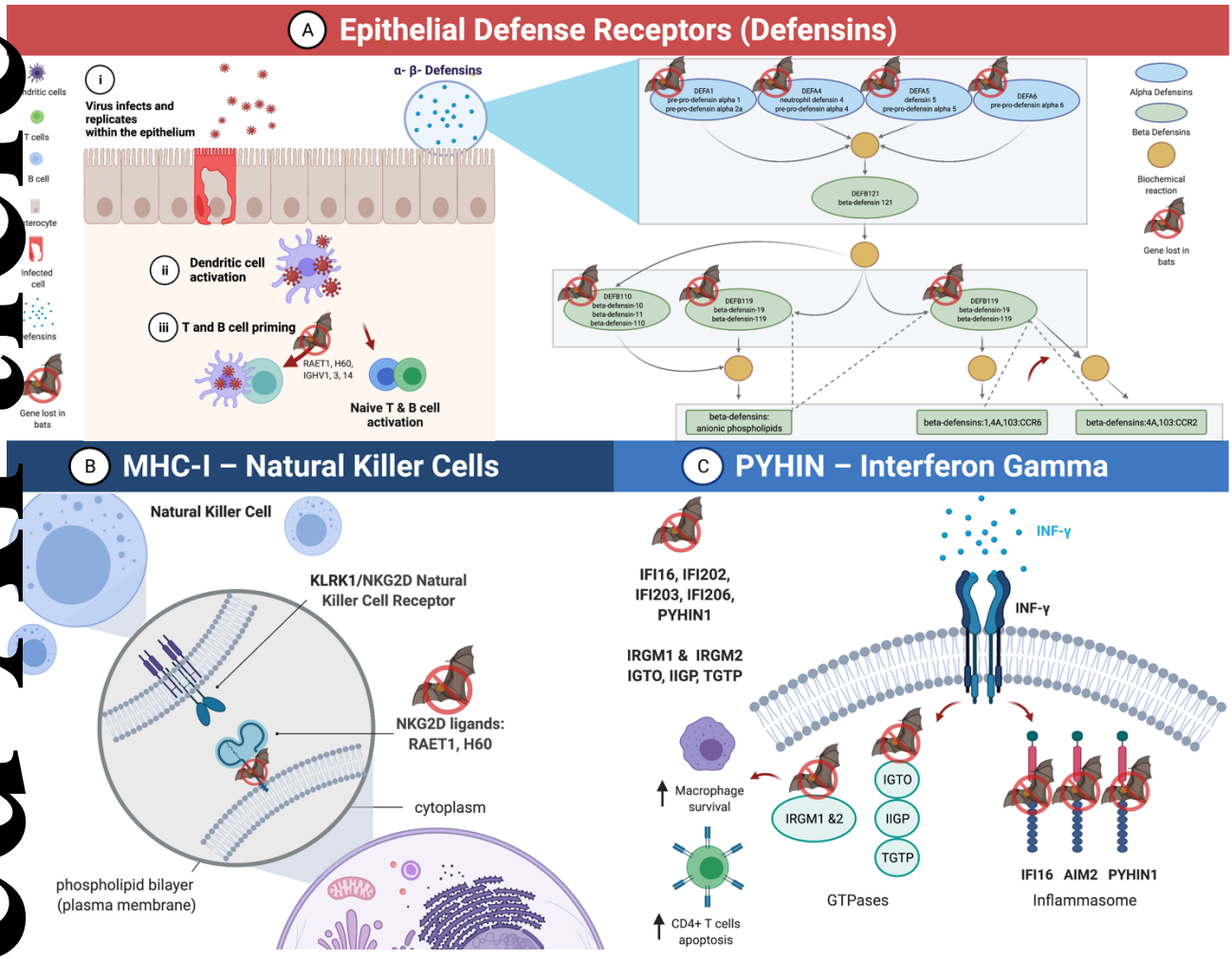

Figure 2. Graphical summary of the cellular location and biological process categorization for genes involved the inflammasome activation pathway found to be missing across all bats. A) Gene loss ofspecific epithelial β defensins. B) Gene losses of NKG2D ligands RAET1 and H60, involved in recruiting NK cells and IFN-γ timulation. C) Losses in IFN-γ activating PYRIN and HIN domain (PYHIN) gene family (AIM2, IFI16, PYHIN1), long with the IFN-γ inducible related GTPase genes (IRGM1, IRGM2, IGTO, IIGP, TGTP2); loss of IRGM1 and 2 esults in increase macrophage survival and CD4+ T cells apoptosis.

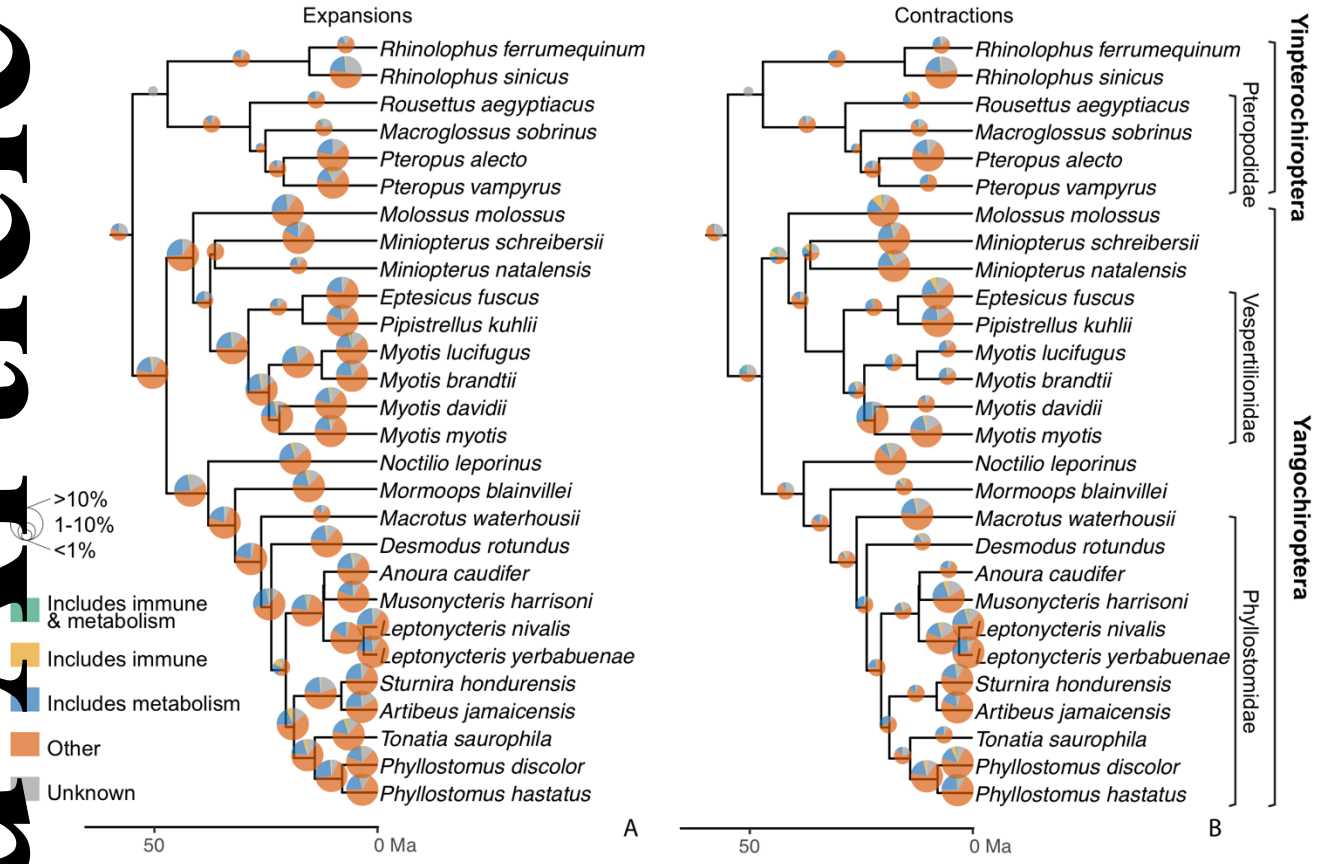

Figure 3. Gene ontology categories, phylogenetic locations, and relative size of significant gene family expansions (A) and contractions (B) inferred using CAFE. "Other" category comprises mostly Panther cellular processes, and GOnet response to stress and autophagy. Pie sizes are relative to a maximum of 594 expansions and 579 contractions.