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Multiple bursts of pancreatic ribonuclease gene duplication in insect-eating bats

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ABSTRACT

Pancreatic ribonuclease gene (*RNASE1*) was previously shown to have undergone duplication and adaptive evolution related to digestive efficiency in several mammalian groups that have evolved foregut fermentation, including ruminants and some primates. *RNASE1* gene duplications thought to be linked to diet have also been recorded in some carnivores. Of all mammals, bats have evolved the most diverse dietary specializations, mainly including frugivory and insectivory. Here we cloned, sequenced and analyzed *RNASE1* gene sequences from a range of bat species to determine whether their dietary adaptation is mirrored by molecular adaptation. We found that seven insect-eating members of the families Vespertilionidae and Molossidae possessed two or more duplicates, and we also detected three pseudogenes. Reconstructed *RNASE1* gene trees based on both Bayesian and maximum likelihood methods supported independent duplication events in these two families. Selection tests revealed that *RNASE1* gene duplicates have undergone episodes of positive selection indicative of functional modification, and lineage-specific tests revealed strong adaptive evolution in the *Tadarida* β clade. However, unlike the *RNASE1* duplicates that function in digestion in some mammals, the bat *RNASE1* sequences were found to be characterized by relatively high isoelectric points, a feature previously suggested to promote defense against viruses via the breakdown of double-stranded RNA. Taken together, our findings point to an adaptive diversification of *RNASE1* in these two bat families, although we find no clear evidence that this was driven by diet. Future experimental assays are needed to resolve the functions of these enzymes in bats.

1. Introduction

Vertebrate pancreatic ribonuclease (RNase1) is a secretory enzyme that belongs to the ribonuclease A superfamily (Beintema and Kleineidam, 1998). Its biological function in vertebrates is to degrade pathogenic RNA and thus protect organisms as part of the immune system (Sorrentino, 2010; Sorrentino and Libonati, 1994). Previous studies have uncovered high expression levels of RNase in the pancreas of ruminants and Old World colobine monkeys, both of which have evolved foregut fermentation (Barnard, 1969; Beintema, 1990; Beintema et al., 1973). Accordingly it has been proposed that RNase in these taxa might serve an enzymatic function to digest symbiotic bacteria in their foreguts so allowing bacterial derived nitrogen to be utilized efficiently (Barnard, 1969; Beintema, 1990).

Subsequent work has revealed that colobine monkeys possess two or more *RNASE1* genes and that the gene duplicate products are functionally suited to a low pH (Zhang, 2006; Zhang et al., 2002). Specifically, amino acid replacements in pancreatic *RNASE1* have resulted in a greater overall negative charge, and the consequent reduction in isoelectric point (pI) is thought to be an adaptation to the acidified environment of the foregut (Zhang, 2006). The origin of digestive *RNASE1* genes in Asian and African colobines appears to be independent, providing compelling evidence that natural selection has shaped RNase1 evolution for its ability to break down the RNA of cellulolytic bacteria (Yu et al., 2010; Zhang, 2006). Similar *RNASE1* duplications have been reported in ruminants (Breukelman et al., 1998, 2001; Kleineidam et al., 1999), as well as other herbivorous species such as the guinea pig (Van den Berg et al., 1977). Yet *RNASE1* gene duplications are not restricted to herbivores; multiple copies have also been documented in some carnivores, which have also been attributed to dietary efficiency (Yu and Zhang, 2006).

Abbreviations: *RNASE1*, pancreatic ribonuclease gene; RNase1, pancreatic ribonuclease; RNase, ribonuclease; AGT, alanine-glyoxylate aminotransferase 1; GLUT4, glucose transporter 4.

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Bats (order Chiroptera) are divided into two suborders: the Yinpterochiroptera and the Yangochiroptera (Teeling et al., 2005). Both suborders show dietary specializations that include frugivory or nectarivory, insectivory and carnivory (Kunz and Fenton, 2003) and so offer a good opportunity for testing for a link between diet and *RNASE1* duplication events. Recent studies have shown that the enzymes alanine-glyoxylate aminotransferase 1 (AGT) and glucose transporter 4 (GLUT4) have both undergone molecular adaptation in bats related to dietary specialization, probably promoting digestive efficiencies in fruit bats (Liu et al., 2012; Shen et al., 2012). The enzyme AGT was also reported to show adaptive evolution in some insect-eating bats (Liu et al., 2012). An early study reported that the expression level of RNase was very low in bat pancreatic tissue (Beintema et al., 1973), however, to our knowledge no previous study has examined the molecular evolution of ribonucleases in bats. Here we tested for molecular adaptation in the *RNASE1* gene of 24 bat species that collectively exhibit a variety of feeding habits. Based on the results from other mammal groups (Yu and Zhang, 2006), we speculated that bats are good candidates for *RNASE1* duplications, and that *RNASE1* gene evolution in bats would show evidence of different selective regimes associated with divergent trajectories in diet.

2. Materials and methods

2.1. Sample collection and species coverage

Tissue samples of bat wing membrane were collected in the field using biopsy punches, and transferred to ethanol for DNA preservation. Bats were released *in situ* soon after capture. We sequenced *RNASE1* genes from 22 bat species representing a range of dietary specialists. These species covered eight families: Pteropodidae, Hipposideridae, Rhinopomatidae, Emballonuridae, Vespertilionidae, Molossidae, Phyllostomidae and Mormoopidae, five belonging to the suborder Yinpterochiroptera, and the others from the suborder Yangochiroptera (Table S1).

2.2. Experiments and data collection

Genomic DNA was extracted from wing membrane biopsies using DNeasy kits (Qiagen). One primer pair (F1 and R1; see Table S2) was designed based on aligned genome data from *Myotis lucifugus*, *Pteropus vampyrus* and other mammals. We successfully amplified the *RNASE1* gene from 16 bat species using this pair of primers. For amplifying bats from the families Phyllostomidae and Mormoopidae, we designed another pair of primers (F2 and R2) based on the sequence of *Taphozous melanopogon*. To ensure only one *RNASE1* copy was present in these two families, we also designed a set of degenerate primers (F3 and R3) and tested these in the species *Leptonycteris curasoae*, *Phyllostomus latifolius* and *Micronycteris megalotis* (Table S2).

Two independent PCRs were carried out per species using the following protocol: 95 °C for 5 min, 32 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30–40 s, and a final extension of 72 °C for 10 min. At least 44 positive clones from each species were sequenced using an ABI 3730 sequencer. To verify putative duplicates of the *RNASE1* gene detected for the families Vespertilionidae and Molossidae, we conducted additional independent PCRs and sequencing reactions for these groups. Bat sequences that contained at least four amino acid differences, and which were obtained from at least two separate PCRs, were considered to be putative duplicate gene copies (Yu and Zhang, 2006; Yu et al., 2010). *RNASE1* gene sequences of *P. vampyrus* and *M. lucifugus* were retrieved from the Ensembl using BLAT search (www.ensembl.org), and those of *Artibeus jamaicensis* and the douc langur *Pygathrix nemaeus* (AJ535682 and AF449642 respectively) along with an *RNASE1B* sequence from *P. nemaeus* (AF449643) were obtained from NCBI (www.ncbi.nlm.nih.gov). GenBank accession numbers of the bat *RNASE1* genes sequenced in this study are JX624171–JX624211 and JX998072–JX998077 (Table S1).

2.3. Gene tree reconstruction

Bat *RNASE1* sequences (including noncoding regions) were aligned with Clustal W in the MEGA 5 software (Tamura et al., 2011; Thompson et al., 1994) and checked by eye. Based on the Akaike information criterion, TVMef+Γ was the best model chosen by jModelTest 2 (Darriba et al., 2012). Phylogenetic trees were then reconstructed using both Bayesian (Ronquist and Huelsenbeck, 2003) and maximum likelihood (ML) methods (Stamatakis, 2006). Five million generations were set in the Bayesian inference with the first two million discarded as burn-in. We searched 200 tree topologies using the ML method with 1000 bootstrap replicates performed. Cases of duplications were identified from inspecting the tree and duplicates were sorted into groups (suffixed by Greek letters) based on their phylogenetic distinctiveness.

2.4. Molecular evolution analysis

We tested for gene conversion using the software GENECONV under different gscale parameters (Sawyer, 1989), and tested for positive selection using CODEML in PAML 4 (Yang, 2007). For selection tests we used the Bayesian tree topology. To determine the extent to which duplications have led to heterogeneous selection pressures, we first applied two nested branch-wise models: the free-ratio model that assumes independent ω (ratio of the rate of nonsynonymous substitutions [d_N] to the rate of synonymous substitution [d_S]) along each branch and a one-ratio that fixes the same ω across the tree (Yang, 1998). In addition we implemented modified branch-site model A to test for positive selection in each of the detected groups of duplicated genes, in each case setting the ancestral branch to the duplicates as the foreground branch. We conducted test 2 in which ω of the foreground focal branch was fixed at one in the null model (Zhang et al., 2005). Finally, to test further for changes in selection pressure in two groups of duplicates (the *Myotis* γ group and *Tadarida* β group) we applied separate clade models (model C) in which a proportion of sites was allowed to differ in their ω values between the focal clade of duplicates and all of the remaining background sequences (Bielawski and Yang, 2004). Finally, since the parallel evolution of dietary *RNASE1* genes in Asian and African leaf-eating monkeys has been shown to have arisen via parallel amino acid changes (Zhang, 2006), whereas ruminant *RNASE1* genes underwent divergent changes (Zhang, 2003) we also compared the published sequences of leaf-eating monkey to those obtained from bats using the software MEGA 5 (Tamura et al., 2011).

3. Results and discussion

3.1. Gene sequences and duplications of Bat RNASE1

We obtained new sequences from 22 bat species that collectively exhibit a range of dietary habits (listed in Table S1) and supplemented these with published sequences from *P. vampyrus*, *M. lucifugus* and *A. jamaicensis*. In total we identified 52 *RNASE1* gene sequences from 24 bat species, each spanning the entire coding region as well as partial sequences of intron 1 and the 3′ untranslated region. Six of the gene sequences were from six bat species from the suborder Yinpterochiroptera and the other 46 sequences were from 18 species from the suborder Yangochiroptera bats (see full details in Table S1). In most species—including all focal members of the New World family Phyllostomidae—just one *RNASE1* gene copy was detected, despite extensive cloning and sequencing. The absence of any *RNASE1* gene duplicates in the phyllostomids was particularly surprising given that these taxa encompass frugivory, nectarivory and insectivory