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The Transcriptomes of Tent-making Bats (*Uroderma*): Testing for Adaptive Divergence in Recently Diverged Species

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Abstract

Molecular evidence of adaptation is of interest to evolutionary biologists trying to understand the role of adaptive divergence in the speciation process. In bats (Order Chiroptera), evidence of directional selection has been associated with drastic changes in lifestyle, especially those related to the evolution of flight and echolocation. Here, the transcriptomes of submandibular glands of two closely related species of tent-making bats (*Uroderma davisi* and *U. convexum*) were reported, annotated, and described. Also, episodic directional selection was tested in 21 genes reported to be involved in lipid lysis, transport, and usage for energetics in a phylogenetic framework centered on bats (Chiroptera), using novel genomic and transcriptomic data. Transcriptomes of the two *Uroderma* species were highly similar. Ectopic expression (in the submandibular salivary glands) of genes of interest (*C3*, *Lcn2*, *Psap*, and *Clu*) in tent-making bats was found, expanding an observation made earlier in *Myotis*. Eight out of the twenty-one genes screened had at least one codon under positive selection as detected by aBSREL. Four (*Apoe*, *Atgl*, *Hadh*, and *Lcn2*) were exclusive to some branches of the tree and four (*C3*, *Lsr, Plin1*, and *Psap*) appeared in more than one branch. These results indicate that these selective processes affecting the target loci are scattered along several branches, including those leading to species of *Uroderma*, rather than being restricted to the early divergence of bats from other mammals, where flight evolved. Results also indicate that the strength of selection over the energy generation systems in diverse lineages of bats involve both recruitment of new genes for ectopic expression and adaptive divergence of coding sequences.

Key words: Chiroptera, ectopic expression, episodic positive selection, free fatty acids, lipolysis, Phyllostomidae, submandibular salivary glands

Supplementary material related to this manuscript is available online at https://drive.google.com/open?id=1ZKXiSYfhbgIgPMsSkcAak3YNcDVjlEGx.

Introduction

With at least 59 genera and more than 200 species (Koopman 1993; Simmons 1998; Wetterer et al. 2000; Baker et al. 2003; Baker et al. 2016; Cirranello et al. 2016), the New World leaf-nosed bat family Phyllostomidae is the second largest of the extant bat families. It also exhibits more variation in morphological features than any other family-level group of mammals, including modifications associated with feeding habits that are also unusually diverse (Wetterer et al. 2000; Baker et al. 2003). Dietary specializations include sanguivory (blood-feeding), insectivory, carnivory, omnivory, nectarivory, palynivory (pollen-feeding), and frugivory. These dietary specializations occur in addition to those required by flight (an energetically demanding lifestyle [Speakman and Thomas 2003]).

Evolutionary adaptations can be manifested through changes in gene sequence and/or gene expression that contribute to locally adaptive phenotypes (Feder and Mitchell-Olds 2003; Coyne and Orr 2004). Transition from local divergence within species to incipient species is the first step of ecological specia-

tion, but early divergence is often subtle and difficult to characterize (Andrew and Riesberg 2013). In recent years, the advent of massively paralleled sequencing technologies (see, Metzker 2010 for a review) has permitted the generation of data providing new insights into chiropteran adaptations, using both genomic (Parker et al. 2013; Zhang et al. 2013) and transcriptomic (Shaw et al. 2012; Phillips et al. 2014) data analyses. Zhang et al. (2013) found a concentration of positively selected genes among those possibly associated with the evolution of flight. Parker et al. (2013) highlighted the convergence of directional selection signals throughout mammalian evolution in genes related to echolocation and vision. Further, Shaw et al. (2012) detected a smaller set of genes associated with structural development and highlighted a list of interesting genes for the study of defense against viral infections. Finally, Phillips et al. (2014) detected the ectopic expression of seven genes related to lipid metabolism and insulin resistance in the submandibular salivary gland in the insectivorous *M. lucifugus* and pointed to a relationship between diet and flight in gene expression. The results of Phillips et al. (2014) were consistent with those of Voigt et al. (2010), who reported that rapid combustion of recently ingested lipids was used to cover flight energetic demands by the insectivorous bat *Noctilio albiventris*. Undoubtedly, lipid energetics has been of central importance in the dietary specialization and adaptation to flight in bats. Examination of recently diverged bats that are not strictly insectivorous might help understand whether natural selection on genes related to lipid metabolism is continuing to operate at this level.

The *Uroderma bilobatum* (Peters' tent-making bat) species complex (Chiroptera: Phyllostomidae: Stenodermatini) offers an opportunity to identify genomic targets of selection among recently diverged taxa. This complex is distributed widely in the New World tropics. Five species of Peters' tent-making bat currently are recognized (*U. bilobatum*, *U. convexum*, *U. davisi*, *U. magnirostrum*, and *U. bakeri*; Mantilla-Meluk 2014 and references therein), supported by chromosomal differences (Baker et al. 1972; 1975 and Baker 1979; 1987), mitochondrial DNA sequences (Cuadrado-Ríos and Mantilla-Meluk 2016), and morphology (Mantilla-Meluk 2014). Among these, *U. davisi* (2n = 44) is found along the Pacific versant of El Salvador, Guatemala, Honduras, and Mexico, and, *U. convexum* (2n = 38) can be found in the remainder of Central America and along the Pacific versant of Colombia and northern Ecuador. These two species come into contact and hybridize at only one locality (Honduras, Departamento Valle, 17 km SSW of Nacaome) in Central America. These are not sister species, as *U. convexum* is sister to (*U. davisi* + *U. bakeri*) (Cuadrado-Ríos and Mantilla-Meluk 2016). However, average divergence between *U. davisi* and *U. convexum* is only 2.5% in the mitochondrial cytochrome-*b* gene (Hoffmann et al. 2003), and the most recent common ancestor was estimated at 3.8 MYA (Cuadrado-Ríos and Mantilla-Meluk 2016). Studies of genetic markers have shown limited hybridization and suggested diversifying selection might be at work in limiting introgression (proposed by Baker 1981; Greenbaum 1981; Barton 1982; see also Lessa 1990; and Hoffmann et al. 2003).

The recent divergence among species and the plausible role of diversifying selection in this process offers a suitable scenario to identify genetic targets of selection acting in early stages of speciation and to compare them with those found in other cases such as the chiropteran basal lineage or the major chiropteran clades, namely Yangochiroptera, and Yinpterochiroptera (Springer et al. 2001; Teeling et al. 2005). The study herein focused on two sets of candidate genes related to lipid catalysis and fatty acid energetics: a) a group of twenty-two loci posited to be important in fatty acid energetics in mammals (*e.g.* Schoiswohl et al. 2014); and b) a group of seven genes related to the regulation and processing of lipids that were found to express ectopically in salivary glands of the little brown bat (*M. lucifugus*, Phillips et al. 2014) (supp Table S1). The aims of this study were twofold. First, the genetic divergence between the hybridizing species of *Uroderma* using transcriptomes of submandibular salivary glands was characterized. Second, the role of directional selection on the aforementioned genes in the divergence of bats in a broader phylogenetic framework was examined to gain insights into the phylogenetic localization and functional significance of adaptive change at the molecular level.

MATERIALS AND METHODS

Sample collection, RNA extraction, and sequencing.—The submandibular gland (SMG) of one specimen each of *U. davisi* (TK169381) and *U. convexum* (TK165187) were collected and preserved in liquid nitrogen for posterior total RNA isolation with Trizol Reagent (Invitrogen, Carisbad, California, US). TK numbers correspond to samples archived in the Genetic Resources Collection of the Natural Science Research Laboratory, Museum of Texas Tech University, Lubbock, Texas. RNA and library preparation quality, purity and integrity were evaluated using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, United States) (see supp. Appendix S1 for detailed estimates of quality, purity and integrity of samples, RIN, rRNA ratio, and 260/280 rate minimum values required). Poly-A based mRNA enrichment and paired-ends library preparation were conducted using Illumina TruSeqTM RNA sample preparation kits. Sequencing was performed on a Hiseq2000 and a Genome Analyzer IIx (*U. convexum* and *U. davisi*, respectively), using a complete lane for each sample.

Assembly and annotation.—Sequence quality descriptors were generated using FastQC (Andrews 2010), version 0.10.1, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) which were used to guide read trimming with Fastx-toolkit (Gordon and Hannon 2010), version 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit). Post-processed reads were assembled *de novo* using Trinity (Grabherr et al. 2011), separately for each sample. This is a method for efficient and robust *de novo* reconstruction of transcriptomes from RNA-seq data (Grabherr et al. 2011; Henschel et al. 2012); the algorithm partitions the sequence data into many individual de Bruijn graphs (de Bruijn 1946; Good 1946), each representing the transcriptional complexity at a given gene or locus, and then processes each graph independently to extract full-length splicing isoforms and to identify transcripts derived from paralogous genes.

Contigs from each assembly were annotated using a BLASTx search (*e-value* $\leq 1.00\times10^{-10}$) against *M. lucifugus* CDSs extracted from the OMA Browser (Altenhoff et al. 2018). For annotated contigs, the gene ontology distribution was assigned using the Blast2GO software function (Conesa et al. 2005; Götz et al. 2008) against the Swissprot database with stringency conditions similar to those used for blastx search. Through Blast2GO, contigs were classified into the three main GO (Gene Ontology) categories: molecular functions, biological processes, and cellular components. Each category also contains detailed inner GO terms assignments, allowing examination of function, processes and components.

Positively selected genes.—An initial set of 29 candidate genes (potential targets of positive selection) related to lipid catalysis and fatty acid energetics was defined from two sources: a) twenty-two loci previously identified as functionally important by Schoiswohl et al. (2014) in lipid catalysis or linked to the same GO terms (functions); and b) a set of seven genes that were found to express ectopically in the submandibular salivary glands of the little brown bat (*M. lucifugus)* by Phillips et al. (2014) (supp. Table S1). To identify orthologs for subsequent analysis of molecular adaptation, coding sequences from species of Chiroptera available on public databases were surveyed. Some were available as CDSs already annotated, whereas others were obtained from raw reads in transcriptome sequencing data (in these cases, filtering, assembly, and annotation was performed as previously described for the two species of *Uroderma*). These data were combined with those for major Laurasiatheria representatives and the assemblages of *U. davisi* and *U. convexum* (see Table 1). Only 1 to 1 orthology class, as classified by OMA-Browser v.24 (Altenhoff et al. 2018) was used for subsequent analyses. Nucleotide sequence alignments were combined with the corresponding aligned protein sequences using Pal2nal (Suyama et al 2006) and indels were retained. After excluding loci that were poorly represented in the targeted taxa, analyses were narrowed to 21 loci (see Table 2). Alignments for these genes were inspected visually and adjusted manually.

Phylogenetic relationships within Chiroptera were specified as in Agnarsson et al. (2011) and Feijoo and Parada (2017); the former defines all major clades, and the latter adds all additional taxa used in the study reported herein (Fig. 1). aBSREL (adaptive Branch-Site Random Effects Likelihood) (Smith et al. 2015)

was used to detect individual branches and sites subject to positive selection within Chiroptera, which was set as the foreground group. Computations were performed using the Datamonkey online suite (Weaver et al. 2018) using default validation parameters.

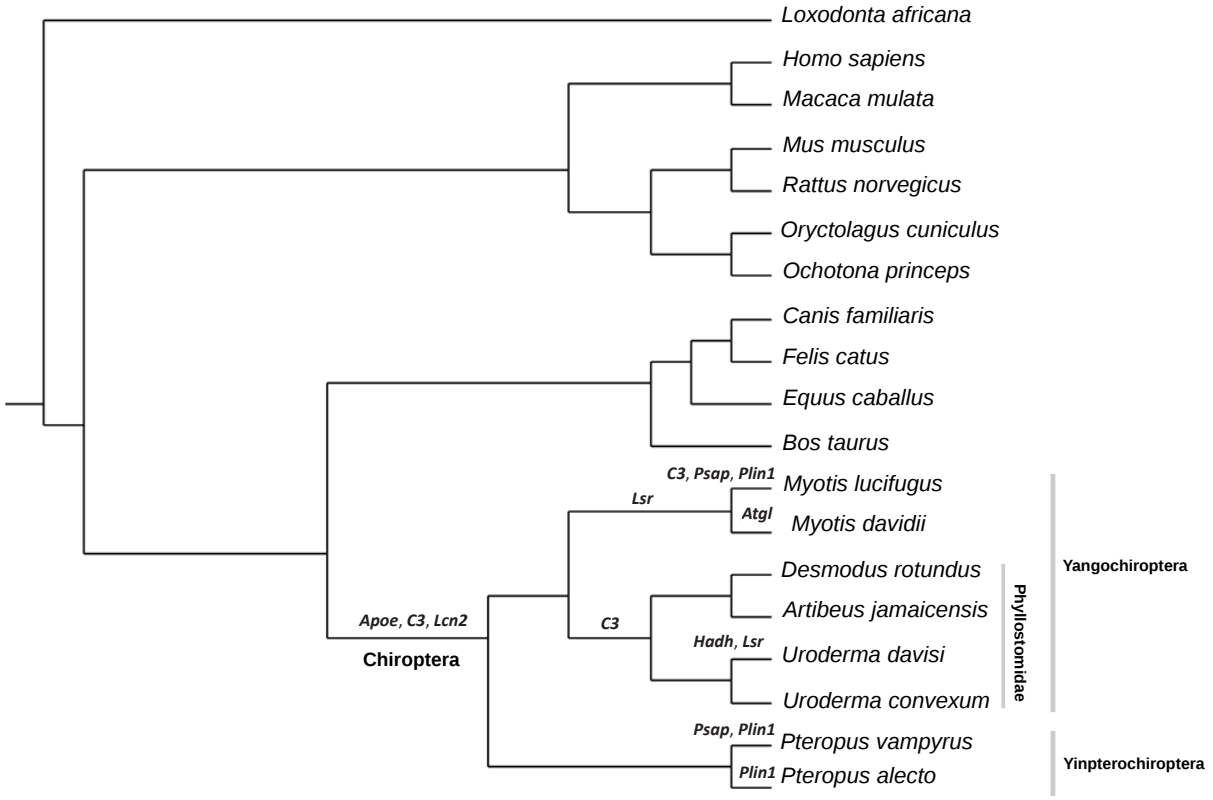

Figure 1. Phylogenetic relationships of taxa used in this study based on Bayesian analysis of multilocus data (following Agnarsson et al. 2011, Song et al. 2012, and Feijoo and Parada 2017). Some branches (Chiroptera, Yangochiroptera, and Yinpterochiroptera) are named as the corresponding crown groups. Name of loci that were recovered to be under positive selection are located along specific branches of the tree (see Table 2 for details on these loci).

RESULTS

Data generation and assembly.—One transcriptome was sequenced from the submandibular salivary gland of each of the hybridizing species, *U. convexum* and *U. davisi* with Illumina-SOLEXA (Bentley et al. 2008) technology, using adult specimens. Raw sequences generated herein also were used for a phylogenomic study (Feijoo and Parada 2017) and deposited in NCBI-SRA database (Table 1). After removal and trimming of reads, sequencing provided a total of 138.5 million high-quality (> 30 phred, each base) paired-end reads with average read-length of 74 bp. *De novo* assembly using Trinity (Grabherr et al. 2011) resulted in an average number of contigs assembled and average contig lengths of ~121,000 and 1,093 bp, respectively. By way of size comparison to a reference transcriptome of a related species (*M. lucifugus* 10.4 million bp), and assuming a similar transcriptome size for *U. bilobatum*, an average depth of 175x of bases sequenced was obtained for *U. davisi* and *U. convexum*. Completness of assembly assessed using BUSCO (Simao et al. 2015) with default parameters in gVolante (Nishimura and Kuraku 2017) with mammal references was similar, and sufficient for *de novo transcriptome* in both samples (63% and 75%, *U*

Table 1. Source of data for the 19 taxa used for analyses. For each species, the database for annotated sequences or accession number for raw sequences and the referenced article are provided.

Taxa	Database	Observations	Reference
Ingroup			
Major Lausrasiatheria representatives			
Bos taurus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Canis familiaris	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Eqqus caballus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Felis catus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Homo sapiens	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Macaca mulata	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Mus musculus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Ochotona princeps	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Oryctolagus cuniculus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Rattus norvegicus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Chiroptera			
Artibeus jamaicensis	SRA-NCBI SRR539297	de novo assembly-anotation for this article	Shaw et al. 2012
Desmodus rotundus	SRA-NCBI SRR606911	de novo assembly-anotation for this article	Francischetti et al. 2013
Myotis davidii	SRA-NCBI SRR628072	de novo assembly-anotation for this article	Zhang et al. 2013
Myotis lucifugus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Pteropus alecto	SRA-NCBI SRR628071	de novo assembly-anotation for this article	Zhang et al. 2013
Pteropus vampyrus	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018
Uroderma convexum (2n = 38 cytotype)	SRX768594	de novo assembly-anotation for this article	Feijoo and Parada 2017; This article
Uroderma davisi (2n = 44 cytotype)	SRX768593	de novo assembly-anotation for this article	Feijoo and Parada 2017; This article
Outgroup			
Afrotheria			
Loxodonta africana	OMA Browser 1:1 orthology	OMA Release v24	Altenhoff et al. 2018

davisi and *U convexum,* respectively). Also, mapping reads to assembled transcripts indicated high-quality assemblies for which mapping rates were 73% and 75% for *U. davisi,* and *U. convexum,* respectively.

For phylogenetic analysis of positive selection at the 21 genes set along major lineages, a sequence data set of 19 taxa (Table 1) was constructed. This data set included the two *Uroderma* species, the *de novo* assembly and annotation of transcriptome raw data available on NCBI-SRA database of four additional bat species, and the OMA-Browser (Altenhoff et al. 2018) 1:1 orthologous genes of 13 species (*Myotis lucifugus, Pteropus vampyrus,* and ten additional species selected to represent major clades of Laurasiatheria, and one Proboscidea as outgroup).

Functional annotation of Uroderma *transcriptomes.*—Annotation of contigs against *M. lucifugus* CDSs from OMA database through Blastn (Altschul et al. 1990) analysis, resulted in 9,691 unique IDs for *U. davisi* and 11,783 for *U. convexum*. From these, 9,379 CDSs were shared between species, whereas 312 and 2,404 were restricted to *U. davisi* and *U. convexum*, respectively. For functional annotation, the international standardized gene functional classification system (Gene Ontology–GO–, Ashburner et al. 2000), using Blast2GO (Conesa et al. 2005) against the SwissProt (Uniprot Consortium 2018) database. This system offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe the properties of genes and their products in any organism using three main categories: Biological process, Molecular function and Cellular component. Then, the graphical data for comparisons of functional categories between species was done. In total, for both species, out of the 88% and 86% of the SwissProt/GO sequences with positive hits for *U. davisi* and *U. convexum*, respectively, 32% were classified for biological process, 32% for molecular function, and 36% for cellular component categories.

Positive selection.—From the initial list of 29 candidate genes, 21 were recovered for analyses (Table 2). Of these, 8 genes (38%) were found to be significantly selected for at least one branch. These included: a) four genes positive selected at individual lineages and b) four genes positive selected in multiple lineages. Three genes were positively selected along the branch leading to Chiroptera (*Apoe*, *C3*, and *Lcn2*), three genes were exclusively selected within Yangochiroptera (*Atgl*, *Lsr,* and *Hadh*) and two were positively selected in both Yango- and Yinpterochiroptera (*Plin1* and *Psap*). No genes were positive selected solely within Yinpterochiroptera. The genes *Lsr* and *Hadh* were positively selected in *U. davisi*, and *C3* was positively selected in *U. convexum.*

DISCUSSION

Comparison of the two Uroderma *transcriptomes.*—With 19,862 sequences of protein-coding DNA available for *M. lucifugus* in the OMA-Browser database, and assuming similar gene contents for the two *Uroderma* species, the submandibular gland transcriptome included ~50% (*U. davisi*) and ~60% (*U. convexum*) of genes currently identified in *M. lucifugus*. Their functional annotation showed minimal differences in biological functional categories assignments against the GO database (Fig. 2), as expected for two closely related species that have experienced recent divergence (Hoffmann et al. 2003). It was found, as expected, that the majority of sequences expressed in the submandibular gland were associated with housekeeping and that expressed loci tied to specific tissue functions were mostly shared between species (supp. Appendix S2). Nucleotide divergence between species calculated for genes of interest was, as expected, very low, 1 every 3650 bp (0,027%). Some genes were uncovered uniquely in one of these species, but transcriptomes of additional individuals are needed to understand whether such apparent differences in expression are characteristic of species or represent individual or temporal differences.

For *M. lucifugus*, a strictly insectivorous bat, Phillips et al. (2014) identified a set of proteins recruited for ectopic expression in the submandibular glands associated with lipids hydrolysis (CEL), insulin resistance to avoid lipid storage (C3, LCN2, and RETNLB) and lipid transport and receptor-mediated endocytosis (PSAP, APOE, and CLU). The analyses also uncov-

Table 2. Genes examined for positive selection in 19 taxa. The genes are identified as in the Ensembl database for *M. lucifugus* (* indicates *P. vampyrus* id where *M. lucifugus* ortholog was not found) and named following Uniprot. Lineages under positive selection are those for which analyses using aBSREL (Smith et al. 2015) uncovered at least some codons under positive selection. The first five loci are those selected from Phillips et al. (2014) and last 16 are taken from Schoiswohl et al. (2014) and from GO related term search.

Ensmbl gene ID (*M. lucifugus*)	Gene name	Lineages under positive selection (aBSREL)
ENSMLUG00000006546	*Apoe*	Chiroptera basal branch
ENSMLUG00000006721	*Clu*	None
ENSMLUG00000011254	*C3*	Chiroptera and Phyllostomidae basal branches, and *M. lucifugus*
ENSMLUG00000015746	*Psap*	*P. vampyrus* and *M. lucifugus*
ENSMLUG00000016210	*Lcn2*	Chiroptera basal branch
ENSMLUG00000006040	*Acsa*	none
ENSMLUG00000007408	*Appl2*	none
ENSPVAG00000014851*	*Atgl*	*M. davidii*
ENSMLUG00000024511	*Bscl2*	none
ENSMLUG00000001134	*Elmo3*	none
ENSMLUG00000008844	*Hadh*	*U. davisi*
ENSMLUG00000016739	*Hsl*	none
ENSMLUG00000000120	*Kdsr*	none
ENSMLUG00000029908	*Lsr*	*Myotis* basal branch and *U. davis*i
ENSMLUG00000017152	*Mgll*	none
ENSMLUG00000015655	*Pip4k2b*	none
ENSMLUG00000012035	*Plin1*	*P. alecto, P. vampyrus* and *M. lucifugus*
ENSMLUG00000010899	*Plin2*	none
ENSMLUG00000011457	*Plin3*	none
ENSMLUG00000008255	*Prkaca*	none
ENSMLUG00000012359	*Unv119*	none

ered, through reads mapping counts using RSEM (Li and Dewey 2011), consistent expression of *C3*, *Lcn2*, *Psap*, and *Clu* in the transcriptome of submandibular glands of both *Uroderma* specimens. *Uroderma* is known to be primarily frugivorous, although its diet includes insects as well (Fleming et al. 1972). It thus seems that ectopic expression of at least some of the genes discussed by Phillips et al. (2014) is not limited to insectivorous bats, such as the little brown bat (*Myotis*). The phylogenetic distribution of ectopic expression of these genes in the submandibular glands of bats warrants additional studies.

Phyllostomidae is the most diverse extant chiropteran family in terms of ecological and morphological features (Wetterer et al. 2000; Baker et al. 2003). Among Phyllostomidae, the *Uroderma bilobatum* complex is a particularly interesting group to study ongoing speciation and divergent selection processes. Two chromosomally distinct species (*U. davisi* and *U. convexum*) of this complex are known to hybridize in a contact zone, reflecting their phylogenetic affinity and some level of genetic compatibility. *U. davisi* occupies a somewhat more arid habitat than *U. convexum* (Baker et al. 1975). However, if the entire range of the

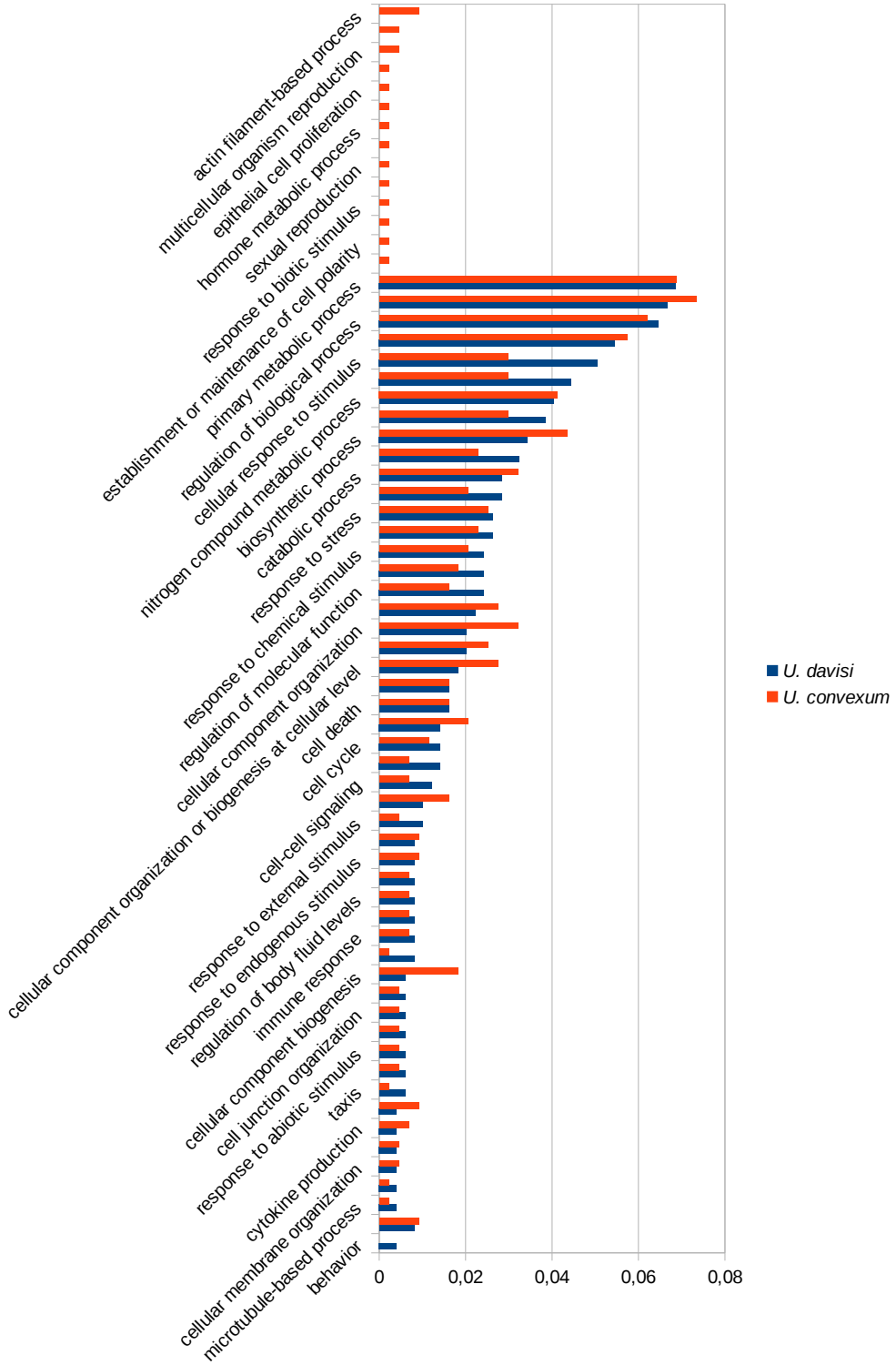

Figure 2. Comparison of the proportion of genes assigned to each biological category with Blast2GO (level 3, biological processes) in the transcriptomes of *U. davisi* and *U . convexum*. The first 14 and last 1 terms are uniquely represented in *U . convexum* and *U. davisi*, respectively.

two respective species is considered, there are no clear ecological factors to readily distinguish the habitat of the two chromosomal races (Baker et al. 1975). No differences have been reported, for example, in diet, roost characteristics or reproduction and life history. These general similarities do not rule out yet to be discovered ecological differences, but nonetheless suggest ecological divergence is limited. The hybrid zone formed by these two species has been extensively studied, and several studies suggest some level of selection against introgression (Baker 1981; Greenbaum 1981; Barton 1982; Lessa 1990; Owen and Baker 2001; Hoffmann et al. 2003). However, the role of diversifying selective forces in the generation and maintenance of divergence has been difficult to assess.

This study of a small set of 21 loci related to lipid processing and metabolism uncovered three loci under positive selection for phyllostomids. Of these, LSR (*lipolysis stimulated lipoprotein receptor*) was positively selected for *U. davisi* and also was uncovered as positively selected in the branch leading to *Myotis*. *C3* (*Complement component 3*) was positively selected in several bat lineages (including the branch leading to Phyllostomidae), and *Hadh* was positively selected in *U. davisi*. *Hadh* (Hydroxyacyl-CoA Dehydrogenase) is a member of the 3-hydroxyacyl-CoA dehydrogenase gene family. The encoded protein functions in the mitochondrial matrix to catalyze the oxidation of straight-chain 3-hydroxyacyl-CoA as part of the beta-oxidation pathway, where fatty acid molecules are broken down to generate acetyl-CoA. Then, acetyl-CoA enters the citric acid cycle and NADH and FADH2 co-enzymes, which are used in the electron transport chain (Yang et al. 2005; Houten et al. 2010). The lipolysis-stimulated lipoprotein receptor, LSR, is a multimeric protein complex in the liver that undergoes conformational changes upon binding of free fatty acids, thereby revealing a binding site (s) that recognizes both apoB and apoE. Its central role, described for rat and human, is the clearance of triglyceride-rich lipoprotein from blood (Yen et al. 2008). With regard to expression of a *C3* gene, for *M. lucifugus* it was reported that salivary gland uses the anaphylatoxin processing pathway (C3a–C3b) typically observed in hepatocytes. This is the enzymatic cleavage within the N-terminal of the nascent C3 protein that produces a 77 amino acid anaphylatoxin peptide (C3a) with immunological functions (Caporale et al. 1980; De Brujin and Frey 1985). The C3 protein that remains after cleavage is termed C3b. Consequently, hepatocytes secrete two proteins -the anaphylatoxin peptide (C3a) and a large C3b protein- both of which are processed from a single precursor. Besides the inmunological function of C3a, the C3b protein is associated with insulin resistance and free fatty acids trapping. Over-production of the C3b protein has been linked to hyperlipidemia in humans (Verseyden et al. 2003). In this sense, the hyperlipidemia resulting from the abundant secretion of the C3b protein would be advantageous in processing and using insect lipids while foraging, being used not only by insectivorous bats but also by omnivorous taxa.

Three of the 21 loci (14%) analyzed were determined to be under positive selection in *Uroderma*, and a total of eight of 21 loci (38%) were positively selected somewhere in the bat radiation. However, focus was set on a group of candidate genes associated with lipid processing and metabolism, which are known to be of particular physiological importance in bats. Most likely, therefore, the fraction of genes under positive selection across the bat genome in general is much smaller. On the other hand, phylogenetic methods for detecting selection aim at reducing type I error and will fail to detect selection in many instances (Anisimova and Yang 2007; Kosiol et al. 2008). Therefore, it seems that selective pressures on genes related to lipid metabolism have been very significant in the evolutionary diversification of bats. Additionally, the results show that these selective processes are scattered along several branches, including those leading to species of *Uroderma*, rather than being restricted to the early divergence of bats from other mammals, where flight evolved.

Positively selected genes.—Although identifying signatures of positive selection is only the first step in defining the genetic basis of species differentiation, such analyses are able to identify potential candidate genes of ecological, behavioral, morphological, physiological, or other functional significance. The aBSREL method allows w (=*dn*/*ds*) to change among lineages, as well as among codons. This is a more realistic assumption on how selective pressure acts and results in greater power for identifying selected sites when compared with other molecular selection algorithms (Murrell et al. 2012).

In addition to the genes positively selected in Phyllostomidae discussed above, five genes were positively selected somewhere in the bat radiation: *Atgl* (in *M. davidii*), *Plin1* (in *Pteropus vampyrus*, *Pteropus alecto*, and *M. lucifugus*), *Apoe* and *Lcn2* (in the Chiropteran basal lineage), and *Psap* (in *P. vampyrus* and *M. lucifugus*). The sequential hydrolysis of triacylglycerols in adipocytes producing free fatty acids is catalyzed by a cascade of lipolytic enzymes, with different substrate preferences (Watt 2008). The committed enzyme catalyzing the first step of triacylglycerol hydrolysis is ATGL (*Adipose triglyceride lipase*). ATGL is the major triacylglycerol lipase in adipose tissue and expression in other tissues is rather low (Morak et al. 2012). PLIN1 (*Perilipin 1*) is the major lipid droplet coat protein in mature adipocytes and plays a critical role in the regulation of lipolysis, the process via which fatty acids and glycerol are liberated from triglyceride in the lipid droplet (Girousse and Langin 2012; Zechner et al. 2012). The majority of perilipin is associated with the lipid droplet, although a small but significant proportion has been reported to be bound to the endoplasmic reticulum membrane, where it may be involved in the function of lipids droplets that bud from this organelle (Rochford 2014). PLIN1 controls the access to the adipocyte triglyceride stores and thus plays an important role in energy homeostasis. Depending on the energy state of the organism, PLIN1 either limits lipase access to stored triglyceride (in the fed state) or facilitates hormonally stimulated lipolysis (in the fasted state) (Ordovas 2017).

The secreted form of APOE (Apolipoprotein E) consists of two domains—the NH2-terminal domain that binds to the low-density lipoprotein (LDL) cell surface receptor, and the COOH-terminal that binds to LDLs (Mahley 1998). In humans, three *Apoe* alleles encode proteins that are associated with differing lipoprotein plasma concentrations (Elosua et al. 2003). Consequently, *Apoe* manly participates in the distribution or redstribution of lipids among various tissues and cells of the body (Huang and Mahley 2014). *Lcn2* (Lipocalin 2) is a cytokine with a role in regulating lipid metabolism and increasing insulin resistance (Guo et al. 2010; Jin et al. 2012). The protein has the b-barrel motif similar to other lipocalins, including an array of secretory and intracellular lipid-binding proteins (Flower 1996; Flower et al. 2000). In the extracellular milieu, the LCN2 protein can bind to fatty acids or iron and has been investigated from in connection with obesity, insulin resistance, and inflammation (Wang et al. 2007; Zhang et al. 2008). The LCN2 protein also modulates the peroxisome proliferator-activated receptor-c, which in turn regulates energy expenditure and lipid homeostasis in adipocytes (Spiegelman 1998). The PSAP (Prosaposin) protein is thought to be secreted before its final processing and the secreted form serves as a lipid transporter that delivers bound sphingolipids to cell plasma membranes and into an endocytotic pathway (Hiraiwa et al 1992). The intracellular prosaposin peptides—referred to as saposins (termed A, B, C, and D)—enhance lysosomal hydrolytic activity (Yuan et al. 2007).

Bat specializations related to lipid metabolism could be explained as related to hibernation and/or daily torpor mechanisms presented in major chiropteran lineages (Yuan et al. 2011), where efficient usage of adipose tissue for heat production and control is required. Another interesting mechanism is energetic performance during active flight. During flight, an elevated metabolic rate must be balanced by a great nutrient combustion for energy (Speakman and Thomas 2003). In this context, Voigt et al. (2010) proposed, in a study of metabolism in *Noctilio albiventris*, that bats might quickly mobilize and combust just-ingested nutrients instead of utilizing endogenous lipids. This may have enabled bats to conquer the nocturnal niche of aerial insectivory. Given the diversity of feeding habits that exist, these authors suggest that all bats might be using predominantly newly ingested nutrients to meet the high energy requirements of flapping flight. Therefore, genes associated with these processes might be expected to be under positive selection. In contrast to *M. lucifugus,* the diet of *U. bilobatum* consists of fruits and a small fraction of insects (e.g. Fleming et al. 1972). These findings are in line with the views of Voigt et al. (2010) in that selection acting on energy generation systems may be widespread in diverse lineages of bats.

De novo assembly of sequences of non-model species is a rapidly growing area of research and of methodological development (reviewed in Martin and Wang 2011) and evaluation (Misner et al. 2013; O'Neil and Emrich 2013). With increased taxonomic density and better annotation, the number of orthologous genes that can be reliably identified is expected to

increase rapidly. The present work limited analyses to candidate genes with previously published evidence of functional importance. The representation of lineages of bats is significantly increased relative to that of Shen et al. (2010); in relation to Parker et al. (2013), bat taxa sampled were increased and a more recent and sensitive method for testing positive selection was used. As illustrated by this work, taxonomic density may be greatly increased by generating orthologous sets of genes via transcriptomics and combining them with genomic data (see also Feijoo and Parada 2017).

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