WWJMRD 2017; 3(12): 15-21
www.wwjmrd.com
International Journal
Peer Reviewed Journal
Refereed Journal
Indexed Journal
UGC Approved Journal
Impact Factor MJIF: 4.25
e-ISSN: 2454-6615

Vishal Kadu
Department of Zoology,
Sathaye College, Mumbai,
India

Ashok P. Manekar
Department of Zoology,
Institute of Science, Mumbai,
India

Correspondence:
Ashok P. Manekar
Department of Zoology,
Institute of Science, Mumbai,
India

Homogentisate 1, 2 Dioxygenase (*HGD*) Gene Characterization from Insectivorous and Frugivorous Bats

Vishal Kadu, Ashok P. Manekar

Abstract
The only true flying mammal bat (Order: Chiroptera) has number of food habits. Depending on the food sources bats are grouped into frugivorous (eat fruit, seed and pollen), insectivorous (eat mosquitoes, moth and beetle), piscivorous (eat fish), nectarivorous (feed on nector), carnivorous (eat frog and lizard), and sanguivorous vampire bats (feed on blood). These differences in feeding habit alter their intake of nutrients like protein and carbohydrates. Diet of frugivorous bats is deficient in proteins but rich in carbohydrates, whereas the insectivorous bats feed on protein rich diet. Bats species are thought to evolve to feed on varied food resources and therefore considered to have undergone the physiological, behavioral, metabolic, and genetic adaptations to cope up with this change. In present research work, we studied Homogentisate 1, 2 dioxygenase (*HGD*) gene in frugivorous bat (*Cynopterus sphinx*) and insectivorous bats (*Hipposideros fulvus* and *Scotophilus heathi).* The HGD enzyme plays role in catabolism of tyrosine amino acid. The nucleotide sequencing analysis across these bat species suggests presence of 90% or above conserved regions in the *HGD* gene. The variable sites in the gene are the result of synonymous as well as non-synonymous substitution occurred during evolution of bats. Most of the nucleotide substitutions are transition type. In comparison to *C. sphinx,* the gene expression of *HGD* for *H. fulvus* showed 26 fold incease while *S. heathi* showed 17 fold increase. Phylogenetic analysis of *HGD* shows divergence of frugivorous bats from insectivorous bats earlier in the evolution.

Keywords: Homogentisate 1, 2 dioxygenase (*HGD*), *C. sphinx, H. fulvus, S. heathi,* qPCR, frugivorous bat, insectivorous bat, alkaptonuria

Introduction
Catabolism of tyrosine takes place in five step process, involving equal number of enzymes encoded by genes *Tat, HPD, HGD, MAAI/GSTZ1* and *FAH* genes [1]. The complete phenylalanine/tyrosine degradation pathway is expressed in hepatocytes and renal proximal tubular cells [2]. The catabolism of tyrosine occurs mainly in liver when it is no longer required or present in excess [3]. In the third reaction of catabolism, homogentisic acid (HGA) is converted to maleylacetoacetate (MAA) by enzyme homogentisate 1, 2 dioxidgenase (HGD) which is encoded by gene *HGD* [4]. In humans alkaptonuria (AKU) or ochronosis a rare autosomal recessive disorder caused by deficiency of HGD, which leads to the accumulation of homogentisic acid (HGA), since it cannot be converted to maleylacetoacetic acid in the tyrosine catabolic pathway [5]. Darkening of the urine upon standing is usually the first sign of AKU, characteristic of homogentisic acid excretion [3]. The human gene *HGD* contains 14 exons and covers 60 kb of genomic DNA [6].
The study of *HGD* in bats is largely confined to hibernating and non-hibernating bats. In hibernating animals commonly number of changes observed, which involve a controlled reversible reduction of body temperature, metabolic rate, and many other physiological processes [7–10]. During hibernation, many hibernators do not feed and depend largely on fat reserves for energy by switching from glucose utilization to lipid consumption [7, 9]. As a preparation for hibernation, the weight and fat mass of some hibernators (e.g., squirrels, bats, and black bears) are greatly increased in summer and autumn [7]. Several mechanisms that

may prevent muscle atrophy or loss of liver proteins have been proposed, including the production of proteolytic inhibitors [11, 12], continual protein synthesis [14, 15], maintenance of proper protein folding [13], and preservation of essential amino acids such as phenylalanine (Phe) and tyrosine (Tyr) [14–16]. These two amino acids are both glucogenic and ketogenic in energy production. They are also precursors of thyroid hormones and catecholamines (e.g., adrenaline, noradrenaline, and dopamine) that can act on adipose tissue to modulate thermal and energy balance during hibernation [17, 18]. In another case, the feeding habits of the bat shows difference in intake of food and its constituents. Bats can feed on diverse food sources and can be grouped into frugivorous (eat fruit, seed and pollen), insectivorous (eat mosquitoes, moth and beetle), piscivorous (eat fish), nectarivorous (feed on nector) carnivorous (eat frog and lizard), and sanguivorous vampire bats (feed on blood) [19]. These differences in feeding habit alter their intake of nutrients like protein and carbohydrates [20, 21, 22]. Diet of frugivorous bats is deficient in proteins but rich in carbohydrates, whereas the insectivorous bats feed on protein rich diet [22, 23]. Bats species are thought to evolve to feed on varied food resources, and therefore considered to have undergone the physiological, behavioral, metabolic, and genetic adaptation to cope up with this change [21, 24]. Gene expression analysis requires sensitive and accurate techniques capable of measuring the levels of messenger RNA (mRNA) expressed in a cell at a specific moment. Until recently, gene expression levels were generally determined through the northern blotting technique, which is slow, requires microgram amounts of mRNA [25] and poorly detects low expression levels. Currently, RT-qPCR is the most sensitive and specific method for detection of gene expression at both low and high levels. Thus, RT-qPCR technique was used for gene expression analysis in liver tissues of bats. A housekeeping gene eukaryotic elongation factor 2 (*EEF2*) was used as intrinsic control.

Material and methods

Animal collection and tissue isolation:

Before collecting the animals from the wild, permission was taken from Maharashtra State Biodiversity Board (MSBB) and animals were handled as per the guidelines of CPCSEA. All three bats species (*C. sphinx, H. fulvus, and S. heathi*) were collected from Elephanta island region (18.96^0N, 72.93^0E), Mumbai, India. During the period from December 2015 to February 2016, by using mesh nets. The *C. sphinx* bats roost in canopy and were collected at the time of feeding on fruits at night, whereas insectivorous species *H. fulvus* and *S. heathi* were collected from the caves and wall crevices during day time. Three specimens of each species were collected and transported to the laboratory in a cage within four to five hours time. The animals identification was done by taking external measurements like ear pinna length, fore arm length, head body length, hind foot length, tail length, third metacarpal length, length of the first phalanx of the third metacarpal, maxillary tooth row, mandibular tooth row [26]. The animals were sacrificed by cervical dislocation method. The liver tissues were isolated and stored in RNAlater solution (Sigma-Aldrich) at 4^0C.

Table 1: PCR primers used for *HGD* gene

Bat species	Set	Primer sequence (5'→3')	Annealing temp (^{0}C)
C. sphinx	CS1	Forward 1: CAGCTCTCAGGATCAGCTTTCA	60.00
		Reverse 1: GGCACTTGGCGATCTTCATA	
	CS2	Forward 2: GGAATGCGGTTCAGCATAGA	58.50
		Reverse 2: CCTGGAATTGGAGGTGAAGT	
H. fulvus	HF1	Forward 1: CAGCTCTCAGGATCAGCTTTCA	60.00
		Reverse 1: GGCACTTGGCGATCTTCATA	
	HF2	Forward 2: GGAATGCGGTTCAGCATAGA	58.50
		Reverse 2: CCTGGAATTGGAGGTGAAGT	
S. heathi	SH1	Forward 1: CAGCTCTCAGGATCAGCTTTCA	60.00
		Reverse 1: GGCACTTGGCGATCTTCATA	
	SH2	Forward 2: GGAATGCGGTTCAGCATAGA	58.50
		Reverse 2: CCTGGAATTGGAGGTGAAGT	

RNA isolation and cDNA preparation:

RNA was extracted using the RNAiso Plus (TaKaRa^{TM}) according to the manufacturer's protocol. For making cDNA, RNA was quantified using Thermo Scientific nanodrop 1000 spectrophotometer.

Gene specific pre sequencing PCR:

In order to amplify the *HGD* gene, the gene specific primers were designed for each bat species. For primer designing, the reference sequences were used from NCBI database. Software like Primer3 and primer-BLAST were used to pick the primers. In order to get overlapping sequences, two sets of PCR primers were used (Table 1).

Polymerase Chain Reaction (PCR) was set up using *emerald* DNA Taq polymerase Master Mix. Initial denaturation for 10 seconds at 94°C. In each cycle, denature 30 seconds at 98°C, primer annealing for 30 seconds at variable temperature as mentioned in table 1. Extend DNA for 1 minute at 72°C. Step denaturation, annealing and extension repeated for 35 cycles, final extension for 10 minutes at 72°C

DNA Sequencing:

For DNA sequencing, 60-100 ng concentration of PCR product was used. The sequencing was done by using Sanger's dideoxy sequencing method, or chain termination sequencing.

A. The specifications for sequencing used

Instrument: 3130xl Genetic Analyzer, Applied Biosystem
Capillary: 16 Capillaries of 50 cm length.

Table 2: Primers used for Real-time PCR

Species name	Gene	Primer	Primer sequence	Annealing temp. (°C)
C. sphinx	*HGD*	F	CTACGGCGTCCACTTTGAG	58.3
		R	GATCTTCATACCAGGCGACAG	
	EEF2	F	GCCGATCCAGAGAACAATCC	60.3
		R	GTGGTGATAGTGCCAGTCTTC	
H. fulvus	*HGD*	F	GAGATCTGCGTCATTCAGAGAG	58.6
		R	CCCAGATCAGGCAACTCAAA	
	EEF2	F	TGAGAAGTACGAGTGGGATGT	56.8
		R	CCACACTGTCCTTGATCTCATT	
S. heathi	*HGD*	F	AGATGTGTTCGAGGAGACTAGA	58.6
		R	TCAGGAAATCACGAGGATTGG	
	EEF2	F	TACCTGGCTGAGAAGTACGA	56.3
		R	GCTGTCCTTGATCTCGTTGA	

B. Sequencing using control DNA:
A control DNA template was used as one of the templates in a set of sequencing reactions. The results from the control help to determine whether failed reactions are the result of poor template quality or sequencing reaction failure. Control DNA sequence used in the reaction pGEM®-3Zf (+) as a double-stranded control

C. 16-capillary 3130xl Genetic Analyzer:
With 16-capillary throughput and advanced automation capabilities, the 3130xl system was used.

D. Detection method:
A sensitive detection system is designed in the 3130xl Genetic Analyzer instrument was used. The emitted fluorescent light is collected, separated by wavelength, and focused onto a charge coupled device (CCD).

E. Spectral array detection:
The fluorescent light collected and dispersed across the CCD was transferred to the computer where it was transformed by chemometric algorithmic processing into 4- or 5-dye electropherograms.

Quantitative PCR (qPCR or Real-time PCR):
In order to know the expression levels of the genes involved in tyrosine catabolism, Real-time PCR was performed using gene specific primers and SYBR green I. The housekeeping gene *EEF2* was used as internal control (Table 2).
The kit used for qPCR was SYBR Premix Ex Taq (Tli RNaseH Plus). The machines used for quantitative PCR was Applied Biosystems 7500 Real-Time PCR System, StepOne PCR SYSTEM.

Analysis and interpretation of the data:
The gene sequenced chromatograms were read and analyzed by BioEdit v7.2.6, MEGA 6, NCBI BLAST and seqin. The BioEdit software was used to trim and isolate gene nucleotide sequences. For alignment purpose, MEGA 6, Clustal W, Clustal Omega and NCBI BLAST were used. Gene sequence submission in NCBI was done using seqin.

Results
Real-Time PCR
After making cDNA from the RNA, relative expression pattern of the genes involved in tyrosine catabolism was studied. On comparison of relative gene expression by quantitative PCR, it was found that the genes involved in tyrosine catabolism are up regulated in the insectivores bats (*H. fulvus* and *S. heathi*) as compared to that in frugivorous bat (*C. sphinx*). The relative expression of these genes was observed to be down regulated in *S. heathi* as compared to that in *H. fulvus* (Table 3 and 4).

Table 3: Quantitative PCR data for frugivorous and insectivorous bats

Species	Gene	Mean Ct and S.D	ΔCt
C. sphinx	*EEF2* (Housekeeping)	23.12±0.34	Control gene
	HGD	26.03±0.66	2.91
H. fulvus	*EEF2* (Housekeeping)	26.30±0.23	Control gene
	HGD	24.48±0.2	-1.81
S. heathi	*EEF2* (Housekeeping)	23.69±0.07	Control gene
	HGD	22.51±0.2	-1.18

The Ct values are inversely proportional to the amount of target DNA in a sample tube. The qPCR performed for *HGD* gene showed variable amount of gene expression across the species. Housekeeping gene Eukaryotic Elongation Factor 2 (*EEF2)* was used as internal control. The ΔCt values in table 3 show low expression of *HGD* as compared to *EEF2* in *C. sphinx.* The ΔCt values for *H. fulvus* and *S. heathi* show similar level of transcripts presence.

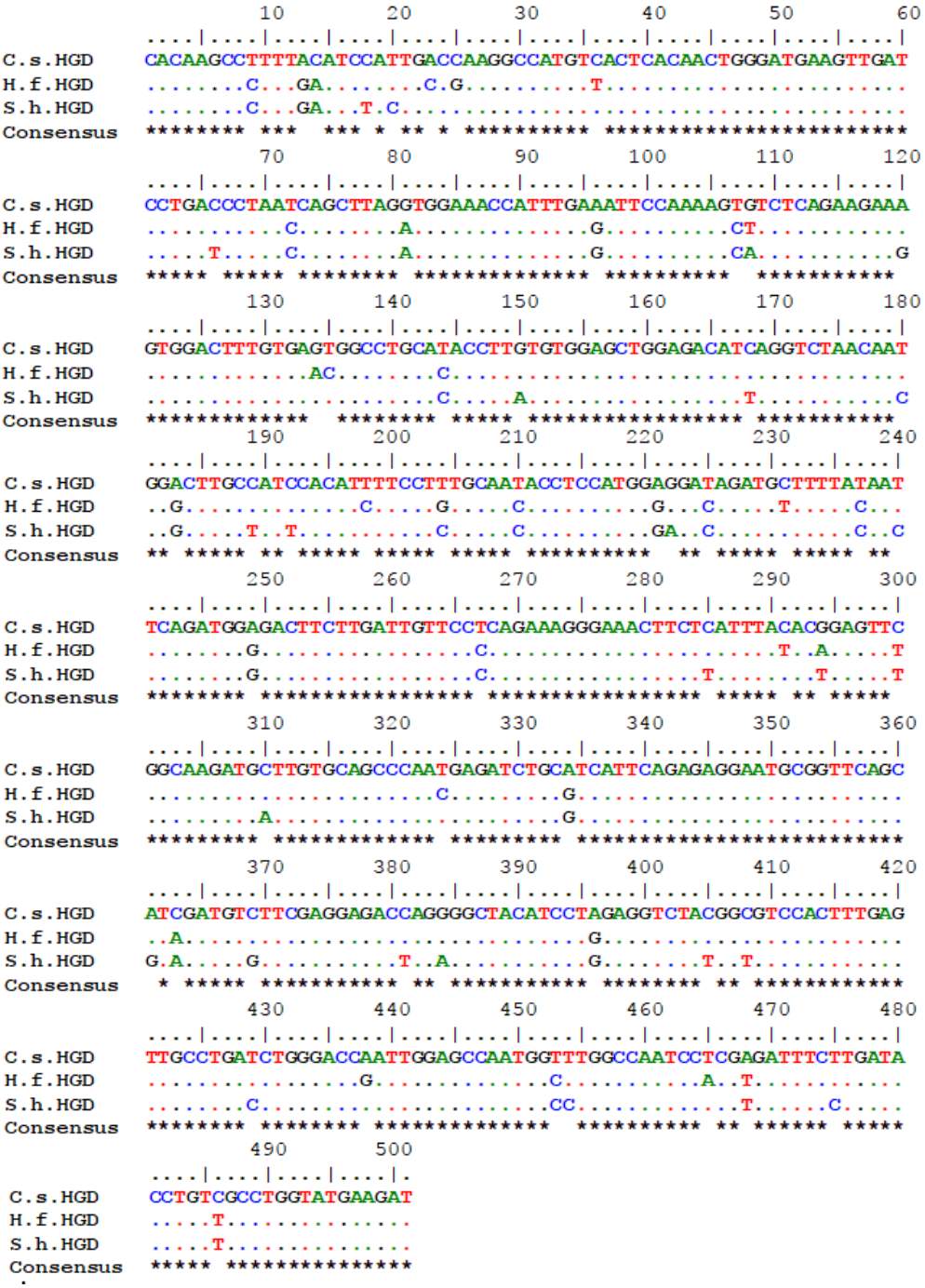
Table 4: Quantitative PCR data analysis, comparison of gene expression between selected species. Gene *EEF2* was used as internal housekeeping control.

Control species	Experimental species	Genes	ΔΔCt	$2^{-\Delta\Delta Ct}$	Analysis
Cynopterus sphinx	*Hipposideros fulvus*	*HGD*	-4.73	26.53	Up regulated
Cynopterus sphinx	*Scotophilus heathi*	*HGD*	-4.10	17.14	Up regulated
Hipposideros fulvus	*Scotophilus heathi*	*HGD*	0.69	0.64	Similar

Further analysis of the *HGD* gene shows, up regulation in insectivorous bats as compared to frugivorous bat species. The expression levels of *HGD* gene are similar for two insectivorous bat species.

Gene nucleotide sequence analysis

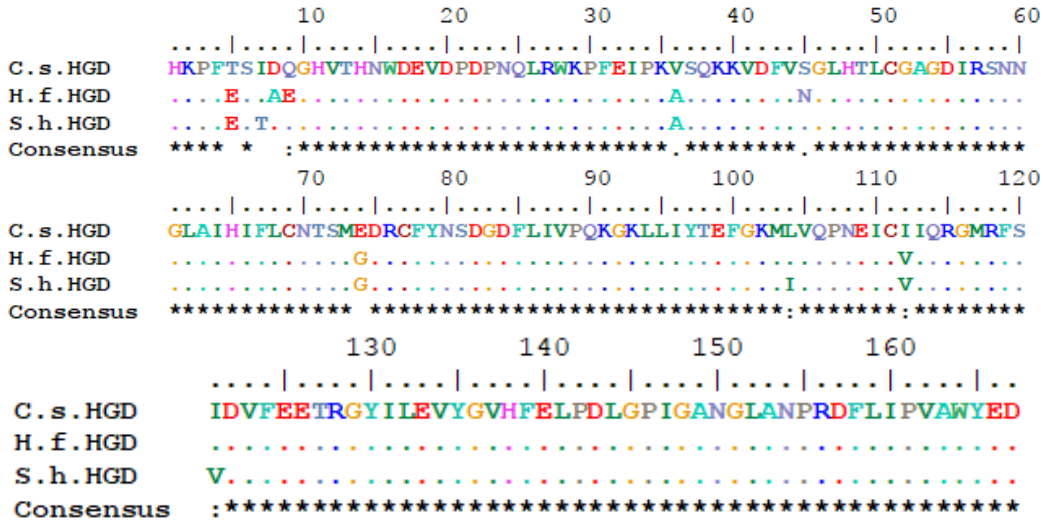
The nucleic acid sequences of genes under study were aligned using ClustalW. The *HGD* gene sequence is deposited in the NCBI GenBank database (accession number MF497589, MF497594, MF497599) for *C. sphinx*, *H. fulvus*, and *S. heathi* respectively. In summary *HGD*, gene sequences show high sequence similarities for bat species under study. In addition the gene sequences for insectivorous bats (*Hipposideros fulvus* and *Scotophilus heathi*) when compared with frugivorous bats (*Cynopterus sphinx*), showed single nucleotide differences at many sites.



Summary of sequence alignment

Sequence 1: C.s.HGD 501 bp
Sequence 2: H.f.HGD 501 bp
Sequence 3: S.h.HGD 501 bp
Sequences (1:2) Aligned. Score: 92%
Sequences (1:3) Aligned. Score: 90%
Sequences (2:3) Aligned. Score: 92%

Multiple sequence alignment for HGD amino acid was performed using ClustalW.



Summary of amino acid sequence alignment
Sequence 1: CSHGD 167 aa
Sequence 2: HFHGD 167 aa
Sequence 3: SHHGD 167 aa

Sequences (1:2) Aligned. Score: 95%
Sequences (1:3) Aligned. Score: 95%
Sequences (2:3) Aligned. Score: 96%

In order to find out the evolution of the *HGD* gene, the deposited nucleotide sequences were taken from NCBI and BLAST was performed. Total eight gene sequences from bats were showed by BLAST result, which were used for constructing phylogenetic tree. The mouse *(M. musculus)* and rat (*R. norvegicus) HGD* gene were used as outgroup.

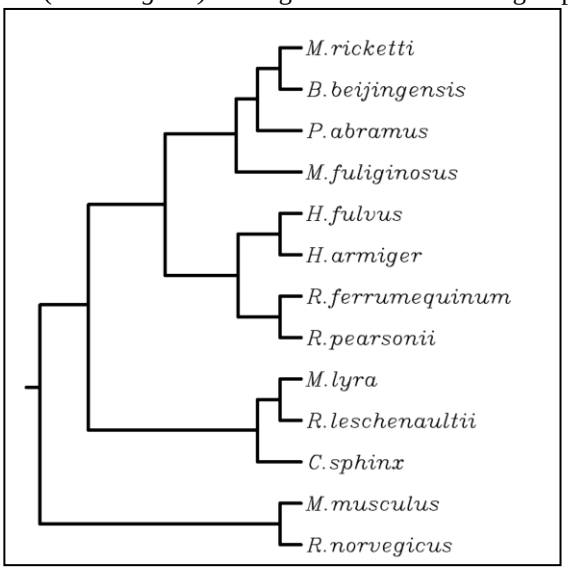


Fig 1: Phylogenetic tree constructed from *HGD* nucleotide BLAST, while constructing the tree, *M. musculus* and *R. norvegicus* was used as outgroup.

In order to study the amino acid sequences of *HGD* and its conserved sites, multiple alignments was performed using BLAST. While constructing the phylogenetic tree, *HGD* sequences of twelve bat species were used from NCBI database. In addition four other sequences were used as outgroup (*M. musculus, C. elegans, D. melanogaster* and *D. rerio*).

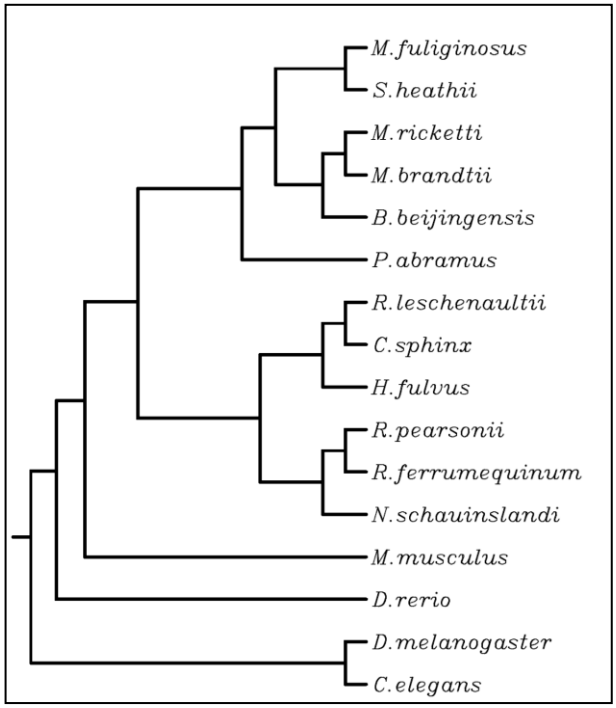


Fig 2: Phylogenetic tree constructed from amino acid sequence of *HGD* gene. Total twelve bat species and four non-bat species were used.

Discussion

Third enzyme in the tyrosine catabolic pathway Homogentisate 1,2-dioxygenase (HGD) mRNA expression levels were studied by RT-qPCR. In comparison to *C. sphinx*, the gene expression of *HGD* for *H. fulvus* showed 26 fold incease while *S. heathi* showed 17 fold increase. The mRNA levels between insectivorous bats showed similar levels of gene expression. These results are in agreement with current knowledge of tyrosine catabolic pathway. The nucleotide sequence for *HGD* between *C. sphinx* and *H. fulvus* shows 92%; whereas that of *C. sphinx* and *S. heathi* shows 90% sequence similarity. The nucleotide sequence comparison between insectivorous bat *H. fulvus* and *S. heathi* is 92%. This shows sequence similarity amongst insectivorous and frugivorous bat species. The amino acid sequence comparison by Clustal Omega shows 95% similarity for *C. sphinx* and *H. fulvus;* and *C. sphinx* and *S. heathi.* The amino acid sequence

comparison *HGD* gene between insectivorous bats *H. fulvus* and *S. heathi* shows 96% identity score. Thus the amino acid sequence and nucleotide sequence comparison shows the conserved regions. Wherever nucleotide differences for *HGD* across the frugivorous and insectivorous species is due to evolutionary different lineages or may be due to synonymous or non-synonymous substitutions over the long evolutionary period.

In human, it is demonstrated that AKU patients are homozygous or compound heterozygous for loss of function mutations in the *HGD* gene. The first two mutations in the *HGD* gene have been described in two Spanish families in 1963. These are two missense mutations: P230S (Pro230Ser) in exon 10 and V300G (Val300Gly) in exon 12. To date, more than 100 different mutations of the *HGD* gene have been identified in patients from many different countries and are described in the new online HGD mutation database [27]. Partial nucleotide sequences of *HGD* gene having size 501bp were considered from three bat species. Out of these 501 nucleotides, there are 25 nucleotide sites which showed single nucleotide substitutions in *C. sphinx* compared to insectivorous bats under study. Amongst these 25 substitution sites, 18 sites (synonymous substitutions) do not affect encoding amino acids, whereas rest 7 substitutions (non- synonymous substitutions) cause alteration in 5 amino acids. In peculiar non-synonymous substitutions are located on following nucleotide positions; 13 and 14 (T5E change in amino acid), 107 and 108 (V36A change), 221 (E74G), 334 (I112V), 363 (I121V). While synonymous substitutions are located at following nucleotide positions 9, 72, 81, 96, 144, 183, 204, 210, 225, 237, 249, 267, 294, 300, 396, 448, 468, 486. Amongst these synonymous substitutios except 204, 294, and 468 positions other shows transition substitution. In non-synonymous substitutions, nucleotide substitutions at 13, 107, 221, and 334 shows transition substitutions. No insertion or deletion has contributed in the variations of nucleotide.

The recent establishment of the crystal structure of the human HGD protein provides a framework for understanding the pathogenic effect of AKU mutations [1]. The HGD is a complex structure, which assembles as a functional hexamer arranged as a dimer of trimers [28]. The active site contains a Fe^{2+} atom, close to the interface between the two trimers. This biologically active structure requires many noncovalent bonds between amino acid residues to maintain the spatial structure of the monomer, but also of the dimer and the hexamer [3]. This complex structure can be easily disrupted by mutations of the *HGD* gene [4]. The effects of some mutations on the HGD enzyme's activity have been studied using mutant HGD proteins in *E. coli*. For other mutations, especially for the missense mutations which are reported to be around 65%, when no functional studies are available, bioinformatics tools (SIFT, POLYPHEN, PANTHER, PNUT, SNAP and FASTSNP) have been generated in order to predict with a 50-80% accuracy the pathogenic effect of these mutations [3].

Phylogenetic studies from figure 1 and 2 shows the possible evolutionary pattern of *HGD* in number of bat species at nucleotide and amino acid levels respectively. Nucleotide phylogenetic tree of *HGD* shows divergent or splitting of *HGD* clade into frugivorous and non-frugivorous bats. Further non-frugivorous bats have evolved and diverged two times to form *HGD* with different nucleotides substitutions. The phylogenetic tree from amino acid sequence shows formation of two clades during evolution and has mixed groups of frugivorous and insectivorous bats. This shows the effect of nucleotide substitutions at different sites.

Conclusion

The nucleotide sequence study of *HGD* in frugivorous and insectivorous bats shows majority of the gene is well conserved across the species. The nucleotide differences at variable sites could be result of synonymous and non-synonymous substitutions. The expression of *HGD* gene varies across bat species depending on the food intake. Frugivorous bat species (*C. sphinx*) has low levels of *HGD* transcripts than insectivorous bat species (*H. fulvus* and *S. heathi*). Despite this the *HGD* gene seems to be well conserved across bats species. Nucleotide phylogenetic data suggest early split of frugivorous bats from insectivorous bat species.

References

1. Titus G P, Mueller H A, Rodriguez de Cordoba S, Penalva M A & Timm D E. Crystal structure of human homogentisate dioxygenase. *Nat Struct Biol* (2000) 7, 542-546
2. Mitchell, G. A., M. Grompe, M. Lambert, and R. M. Tanguay. Hypertyrosinemia, p. 1777–1805. *In* D. Valle (ed.), the metabolic and molecular basis of inherited disease, (2001). 9th ed., vol. 2. *McGraw-Hill, New York*, N.Y.
3. Vilboux T, Kayser M, Introne W, Suwannarat P, Bernardini I. Mutation spectrum of homogentisic acid oxidase (HGD) in alkaptonuria. *Hum Mutat* (2009) 30: 1611–1619.
4. Rodriguez J. M, Timm D. E, Titus G. P, Beltran-Valero de Bernabé D, Criado O, Mueller H. A, Rodriguez de Cordoba S & Penalva M A. Structural and functional analysis of mutations in alkaptonuria. *Hum Mol Genet* 9, (2000) 2341-2350.
5. Phornphutkul C, Introne W. J, Perry M. B, Bernardini I, Murphey M.. D, Fitzpatrick D. L, Anderson P. D, Huizing M, Anikster Y, Gerber L. H, Gahl W. A. Natural history of alkaptonuria. *N Engl J Med.*; (2002) 347:2111–2121.
6. Fernandez-Canon J M, Granadino B, Beltran Valero de Barnabe D, Renedo M, Fernandez-Ruiz E, Penalva M A & Rodriguez de Cordoba S. The molecular basis of alkaptonuria. *Nat Genet* (1996) 14, 19-24
7. Carey HV, Andrews MT, Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev* (2003) 83: 1153–1181.
8. Geiser F. Hibernation. *Current Biology* (2013) 23 (5): R188–R193.
9. Morin P Jr, Storey KB. Mammalian hibernation: differential gene expression and novel application of epigenetic controls. *Int J Dev Biol* (2009) 53: 433–442.
10. Kondo N, Sekijima T, Kondo J, Takamatsu N, Tohya K, et al. Circannual control of hibernation by HP complex in the brain. *Cell* (2006) 125: 161–172.
11. Fedorov VB, Goropashnaya AV, Toien O, Stewart NC, Gracey AY, et al. Elevated expression of protein biosynthesis genes in liver and muscle of hibernating

black bears (Ursus americanus). *Physiol Genomics* (2009) 37: 108–118.
12. Fuster G, Busquets S, Almendro V, Lopez-Soriano FJ, Argiles JM. Antiproteolytic effects of plasma from hibernating bears: a new approach for muscle wasting therapy? *Clin Nutr* (2007) 26: 658–661.
13. Lee K, Park JY, Yoo W, Gwag T, Lee JW, Overcoming muscle atrophy in a hibernating mammal despite prolonged disuse in dormancy: proteomic and molecular assessment. *J Cell Biochem* (2008) 104: 642–656.
14. Epperson LE, Rose JC, Carey HV, Martin SL. Seasonal proteomic changes reveal molecular adaptations to preserve and replenish liver proteins during ground squirrel hibernation. *Am J Physiol Regul Integr Comp Physiol* (2010) 298: R329–R340.
15. Epperson LE, Dahl TA, Martin SL. Quantitative analysis of liver protein expression during hibernation in the golden-mantled ground squirrel. *Mol Cell Proteomics* (2004) 3: 920–933.
16. Nelson CJ, Otis JP, Martin SL, Carey HV. Analysis of the hibernation cycle using LC-MS-based metabolomics in ground squirrel liver. *Physiol Genomics* (2009) 37: 43–51.
17. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* (2004) 84: 277–359.
18. Dark J, Miller DR, Lewis DA, Fried SK, Bunkin D. Noradrenalineinduced lipolysis in adipose tissue is suppressed at hibernation temperatures in ground squirrels. *J Neuroendocrinol* (2003) 15:451–458.
19. Teeling EC, Springer MS, Madsen O, Bates P, O'brien SJ, et al. A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science* (2005) 307: 580–584.
20. Neuweiler G. The biology of bats. Oxford University Press. New York. Gottsberger G, Schrauwen J, Linskens HF (1984) Amino acids and sugars in nectar, and their putative evolutionary significance. *Plant Syst Evol* (2000) 145: 55–77.
21. Kelm DH, Simon R, Kuhlow D, Voigt CC, Ristow M. High activity enables life on a high-sugar diet: blood glucose regulation in nectar-feeding bats. *Proc Biol Sci* (2011) 278: 3490–496.
22. Protzek AOP, Rafacho A, Viscelli BA, Bosqueiro JR, Cappelli AP, et al. Insulin and glucose sensitivity, insulin secretion and beta-cell distribution in endocrine pancreas of the fruit bat Artibeus lituratus. *Comp Biochem Physiol AMol Integr Physiol* (2010) 157: 142–148.
23. Caviedes-Vidal E, Karasov WH, Chediack JG, Fasulo V, Cruz-Neto AP, et al. Paracellular absorption: a bat breaks the mammal paradigm. *PLoS ONE* (2008) 3: e1425.
24. Tracy CR, McWhorter TJ, Korine C, Wojciechowski MS, Pinshow B, et al. Absorption of sugars in the Egyptian fruit bat (Rousettus aegyptiacus): a paradox explained. *J Exp Biol* (2007) 210: 1726–1734.
25. Dean J.D., Goodwin P. H., Hsiang T. Comparison of relative RT-PCR and northern blot analyses to measure expression of b-1,3-glucanase in Nicotiana benthamiana infected with Colletotrichum destructivum. *Plant Mol Biol Rep*, (2002) 20:347-356.
26. Srinivasulu, C. and B. Srinivasulu. Bats of the Indian subcontinent. *Current Science* (2001) 80 (11): 1378-1380.
27. Zatkova A. An update on molecular genetics of Alkaptonuria (AKU). *J Inherit Metab Dis* (2011) 34, 1127-1136.
28. Magesh R & Priya Doss C G.. Computational Methods to Work as First-Pass Filter in Deleterious SNP Analysis of Alkaptonuria. *Scientific World J* (2012), 738423, 9.