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Molecular Phylogeny in 3-D

M. Milner[1], A.G. Bansode[2], A.L. Lawrence[3], S.A. Nevagi[1], V. Patwardhan[4], and S.P. Modak[1, 5] *

[1]Department of Zoology, Karnatak University, Dharwad, 580003, India
[2]Ahmednagar College, Ahmednagar, India
[3]Centre for Life Sciences Biotechnology & Bioinformatics, Poona Business School, C-5 Varsha Park, Baner Road, Pune 411019, India
[4]ARI, Pune 411004, India
[5]Open Vision, 759/75 Deccan Gymkhana, Pune 411004, India

Abstract

Molecular phylogenetic trees are constructed in three dimensions relative to the distribution of MW and pI classes and immunocrossreactivity against polyclonal antibodies to lens crystallins, as well as multiple sequence alignment between amino acid sequences, coding nucleotide sequences and the gene nucleotide sequences for β-globin. Euclidian distances are estimated to position species in x, y, z space by multidimensional scaling and merged with bootstrap-tested branching pattern of Fitch & Margoliash plots to obtain 3-D phylogenetic tree. Compared to single attributes, phylogenetic trees based on multiple parameters allow significant repositioning of rodents, chiroptera and primates.

Introduction

Since Darwin (1859), phylogenetic trees of living organisms have been constructed on the basis of palaeontological, morphological, embryological, physiological, ecological, genetic (Myer, 1970) and, more recently, molecular evidence, often dealing with single trait/character. The distinction between two nearest species depends on changes in multiple parameters before one can establish their near-neighborly or genealogical position. Although data are available for polypeptide amino acid sequences, their biochemical properties and immunocrossreactivity as well as nucleotide sequences of many genes and their RNA intermediates, two dimensional phylogenetic trees are constructed based on one attribute using unweighted pairs grouping (Fitch and Margoliash, 1967) or maximum likelihood (see, for review, Schadt *et al*., 1998). Thus, depending on the parameter, the same species/strain often places at different distance and position within the same test group (Patwardhan, 1992; Milner *et al*., 2003). For example, a tree based on manipulated or restricted nucleotide sequence offers number of solutions for even relatively simple genomes like HIV (Rambaut *et al*., 2001). The degenerate triplet code, coding sequences interrupted by introns, alternate splice sites and variable promoter sequences in genes make it difficult to fit sequence data in a uniparametric trees. Here we display and discuss and apply a multiparametric method (Milner *et al*., 2003) to construct phylogenetic trees based on (a) biochemical and immunocrossreactive properties of vertebrate lens crystallins and (b) sequence data for the gene, cDNA and polypeptide for β-globin. The 3-D trees can be viewed from any angle each furnishing better understanding of the relative position and evolutionary distance of each species.

Data and Methodology

Electrophoretic profiles of MW and pI of 18 vertebrate lens crystallins, their isoelectric focusing pattern and immunocrossreactivity to polyclonal antibodies against crystallins of the mouse, chiropterans *Rousettus leschnaulti* or *Megaderma lyra* (Bansode, 1985; Patwardhan, 1990; Patwardhan *et al*., 1990, 1992) were compared to estimate similarity coefficients (S value) (Brown *et al*., 1979) by the equation **[$S = Z / (X+Y-Z)$]**, where, the similarity coefficient S = fraction of shared bands, X = total number of bands in species A, Y = total number of bands in species B and Z= number of bands common to species A and B and the number of changes per molecule (P value) (Upholt, 1977; Brown *et al*., 1979) by the equation **$P = - [\ln S] / N$**, where N is 3 for α, β, and γ or δ crystallins, lnS is the natural logarithm of S value. The phylogenetic trees were constructed by unweighted pair groupings method (Fitch and Margoliash, 1967; King & Jukes, 1969; Goodman *et al*., 1971) using the software Mega (Molecular evolutionary genetics analysis software, MEGA version 2.1, S.Kumar, *et al*., 2001). In case of immunocrossreactivity the reactivity was estimated by the equation **$R = Z/X$** where R = reactivity, Z = the number of precipitin lines in the heterologous reaction and X = the number of precipitin lines in the homologues reaction. From the reactivity, P values were calculated by the equation **$P = - [\ln R] / N$**, where N is 3 for α, β, and γ or δ crystallins, lnR is the natural logarithm of R value. We compared vertebrate lens crystallins from Shark (*Scoliodon sorrakowah*, order Lamniformes, subclass Chondropterygii, Class Pisces), Pomfret (*Pampus argenteus*, order Perciformes, Subclass Teleostei, Class Pisces), Frog (*Rana tigerina*, order Anura, Class Amphibia), Garden lizard (*Calotes versicolour*, order Lacertilia, Class Reptilia), Chick (*Gallus domesticus*, order Galliformes, Class Aves), Mouse (*Mus musculus,* order Rodentia, Class Mammalia), Rat (*Rattus norvegicus,* order Rodentia, Class Mammalia), *Bandicoot* (*Bandicota indica,* order Rodentia, Class Mammalia), Squirrel (*Funambulus pennanti*, order Rodentia, Class Mammalia), 4 microchiroptera with 3 species under the Genus *Hipposideros* (*Hipposideros speoris, Hipposideros bicolor, Hipposideros cineraceus,* sub-order Microchiroptera, order Chiroptera, Class Mammalia), *Miniopterus* (*Miniopterus schreibersii,* sub-order microchiroptera, order Chiroptera, Class Mammalia),

*For correspondence. Email spmodak@rediffmail.com.

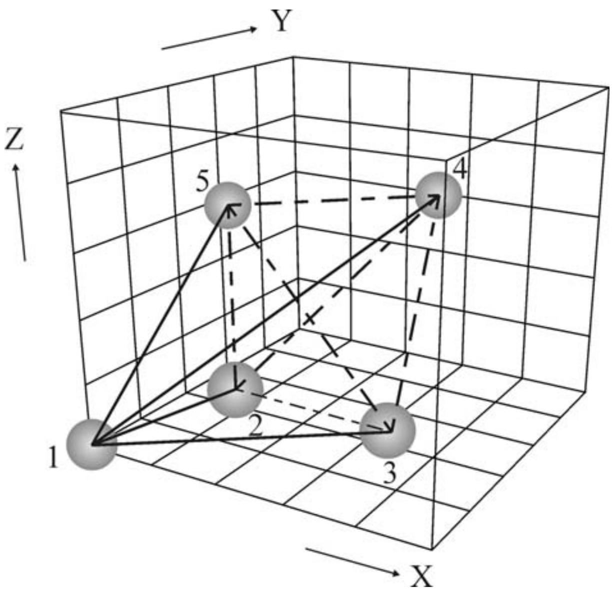

Figure 1. A schematic drawing showing "star" (____) Euclidian distances between the species (spheres) at the origin (0,0,0) and those in the 3-D space, and "network" (_ _ _) Euclidian distances among different species excluding that at the origin.

Megaderma (*Megaderma lyra,* sub-order Microchiroptera, order Chiroptera, Class Mammalia) and *Taphozous* (*Taphozous longimanus,* sub-order microchiroptera, order Chiroptera, class Mammalia), megachiroptera *Rousettus* (*Rousettus leschnaulti,* sub-order megachiroptera, order chiroptera, class Mammalia), *Pteropus* (*Pteropus giganteus,* sub-order megachiroptera, order chiroptera, class Mammalia), and *Cynopterus* (*Cynopterus sphinx,* sub-order megachiroptera, order chiroptera, class Mammalia) to obtain the similarity co-efficient [S] or R values and number of changes per protein [P]. The P values for MW and pI of 18 vertebrates were subjected to unweighted pair grouping as above to obtain the phylogenetic trees. The P values for precipitin lines after crossreacting against three polyclonal antisera in immunoelectrophoresis were plotted in X, Y and Z axes in a 3-D space (Figure 1b, c; *Megaderma,* not shown). Using the formula $\mathbf{D = \sqrt{(x_1-x_2)^2 + (y_1-y_2)^2 + (z_1-z_2)^2}}$, where D is the Euclidian distance between two species and (x_1,y_1,z_1) & (x_2,y_2,z_2) are the 3-D coordinates for any two species, "star" and "network" Euclidian distances were estimated from these 3-D plots as shown in Figure 1. Star distances are those between the species at origin (0,0,0) and others in the x, y, and z 3-D space while the "network" distances are those among all species within the x, y, and z space excluding the one at the origin. The data are expressed with SD whenever applicable (Table 1) Phylogenetic trees were constructed using Mega software as before and assessed by 1000 normal bootstrap trials (Efron, 1979; Felsenstein, 1985) to obtain estimates of the probability and confidence limit calculated by the formula $\mathbf{CL = P \pm 1.96\sigma}$, where CL is the Confidence limit, P is the probability of observing trees similar to mean distances tree and the $\sigma = \sqrt{P(1-P)/n}$ for n number of samples.

Table 1. Euclidean (star) distances in 3–D plots relative to distribution IC against polyclonal antibodies to lens crystallins from 3 mammals, pI and MW.

Species	Mouse	*Rousettus*	*Megaderma*
H. cineraceus	0.174	0.236	0.253
H. speoris	0.170	0.217	0.250
H. bicolor	0.187	0.248	0.262
Miniopterus	0.173	0.205	0.242
Megaderma	0.071	0.106	0.000
Taphozous	0.079	0.041	0.035
Rousettus	0.089	0.000	0.076
Cynopterus	0.076	0.076	0.090
Pteropus	0.076	0.076	0.072
Mouse	0.000	0.192	0.071
Rat	0.182	0.268	0.189
Bandicoot	0.142	0.292	0.139
Squirrel	0.162	0.193	0.246
Chick	0.902	1.090	0.948
Calotes	0.876	0.955	0.905
Frog	0.750	0.975	0.819
Shark	0.950	1.399	0.934
Pomfret	0.848	1.302	0.836

From the "star" distances a 3D plot was constructed using the "star" distances of between mouse antigen-antibody homologues reaction as reference (0,0,0), 17 heterologous anti-mouse reactions on x-axis, anti-*Rousettus* reaction on y-axis and anti-*Megaderma* reaction along z-axis. The Euclidian distances were measured between all pairs from these 3D plots and processed by unweighted pair grouping method to obtain phylogenetic relationship between 18 species. In the 3D plot the species were joined in stem-branch formation as in the phylogenetic tree (Milner *et al*., 2003). Phylogenetic trees were constructed from the network distances alone as well as "star + network" distances. Mean Euclidian network + star distances (Table 2) were subjected to MDS (version 1.13, http://www.let.rug.nl/0kleiweg/levenshtein, Multidimensional scaling) to fix individual species in an X, Y, Z space. The 3-D distances between all pairs were re-estimated from MDS plot and the stem-branch pattern obtained from the constructed phylogenetic tree was merged with the MDS plot. The Euclidian distances re-estimated from the MDS plots, were compared with the actual distances to determine the standard error. MDS plots give single measurement of Euclidean distance so that confidence limits were not estimated.

Amino acid sequence of β-globin polypeptide, the coding nucleotide sequence (cDNA) for β-globin and the gene nucleotide sequence were scanned from NCBI (http://www.ncbi.nlm.nih.gov/). Only 12 species yielded all 3 attributes for β-globin, which were then retrieved namely the primates the Human (*Homo sapiens,* order Primates, class Mammalia), Gorilla (*Gorilla gorilla,* order Primates, class Mammalia) and *Tarsius* (*Tarsius syrichta,* order Primates, class Mammalia), Rabbit (*Oryctolagus cuniculus,* order Lagomorpha, class Mammalia), the Ungulates namely Bovine (*Bos taurus,* order Artiodactyla class Mammalia) and Goat (*Capra hircus*, order Artiodactyla class Mammalia), the rodents including Mouse (*Mus musculus,* order

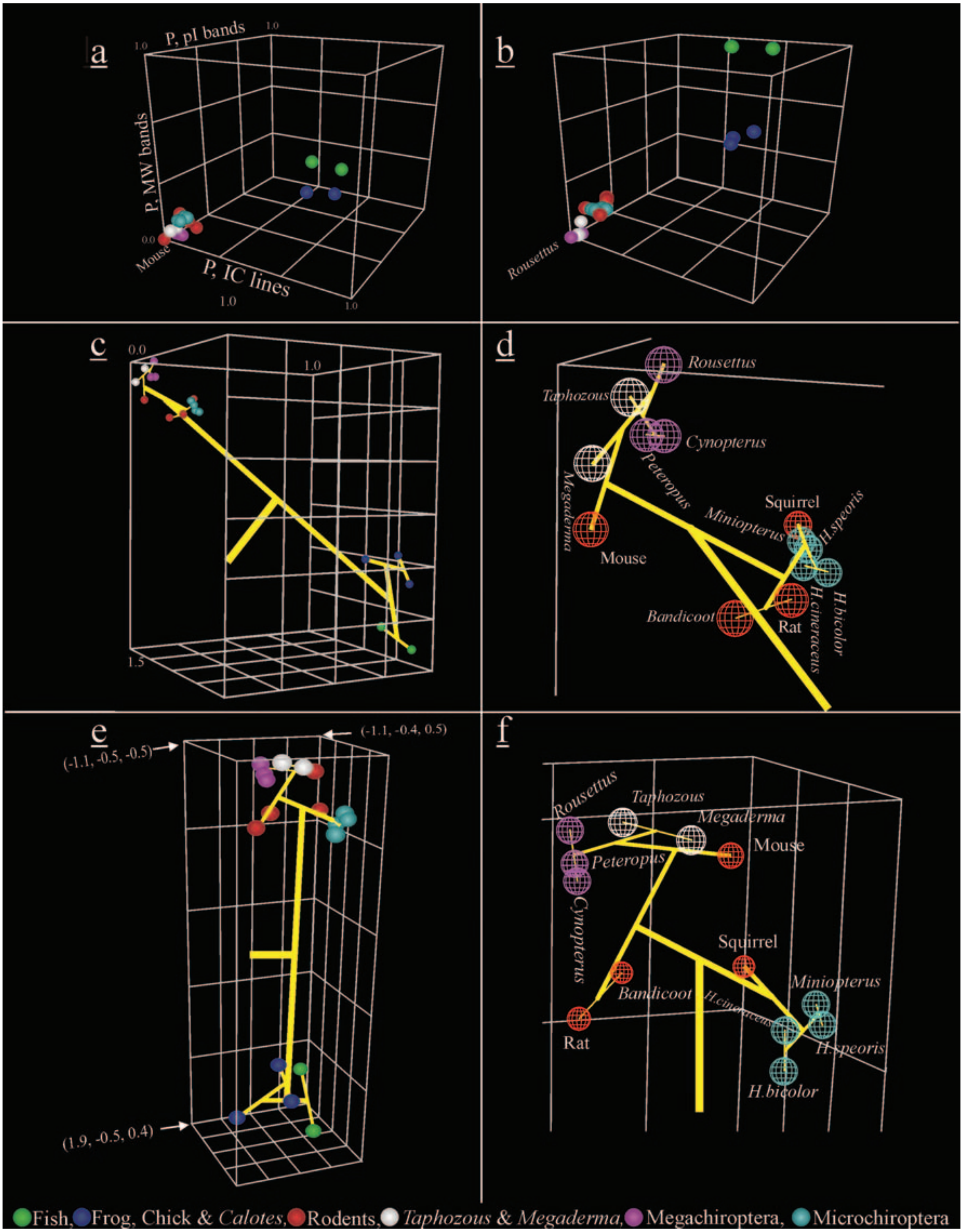

Figure 2. Three-dimensional molecular phylogenetic trees for lens crystallins (a-f). [a] positioning species in 3-D against P values for distribution of pI (y axis), crystallin immunocrossreactivity (IC) against antibody to mouse crystallin (x axis) and MW (Z axis) of crystallins; [b] positioning species in against P value of crystallin pI, MW and IC against antibody to *Rousettus* crystallin (x axis); [c] 3-dimensional repositioning of "star" Euclidian distances in a, b and anti-*Megaderma* plots along 'X', 'Y' and 'Z' axes, respectively. Species are joined by transposing the nearest-neighbor data in Figure 3c to form the tree with the stem positioned midway between mammals and lower vertebrates. The thickness of the Stem decreases as one moves towards mammalian species. [d] A magnified view of the distal portion of the 3-D tree in c and slightly rotated showing the right cluster with 3 megachiroptera, mouse and presumed microchiroptera *Taphozous* sp. and *Megaderma* sp., while 4 microchiroptera and squirrel form a separate cluster to the left. Notice the distinct blossoms with *Cynopterus* and *Pteropus* which is farthest away from those bearing *Bandicoot* and rat, or microchiroptera under genus *Hipposideros*,. Also notice that the diameter of a sphere (species position) is largest for the species immediately in the foreground and smallest when farther away. [e] a 3-D tree based on MDS of "network + star" distances in x, y and z space- again species positions were joined as in the 2-dimensional phylogenetic tree in Figure 3e; [f] magnified and slightly rotated view of the mammalian branches in 2e again showing a discrete clusters for mega- and microchiroptera as in d and distinct blossoms for *Taphozous* and *Megaderma* along with megachiroptera and away from that for microchiroptera. Notice the rat and *Bandicoot* pair near mouse and megachiroptera unlike in d.

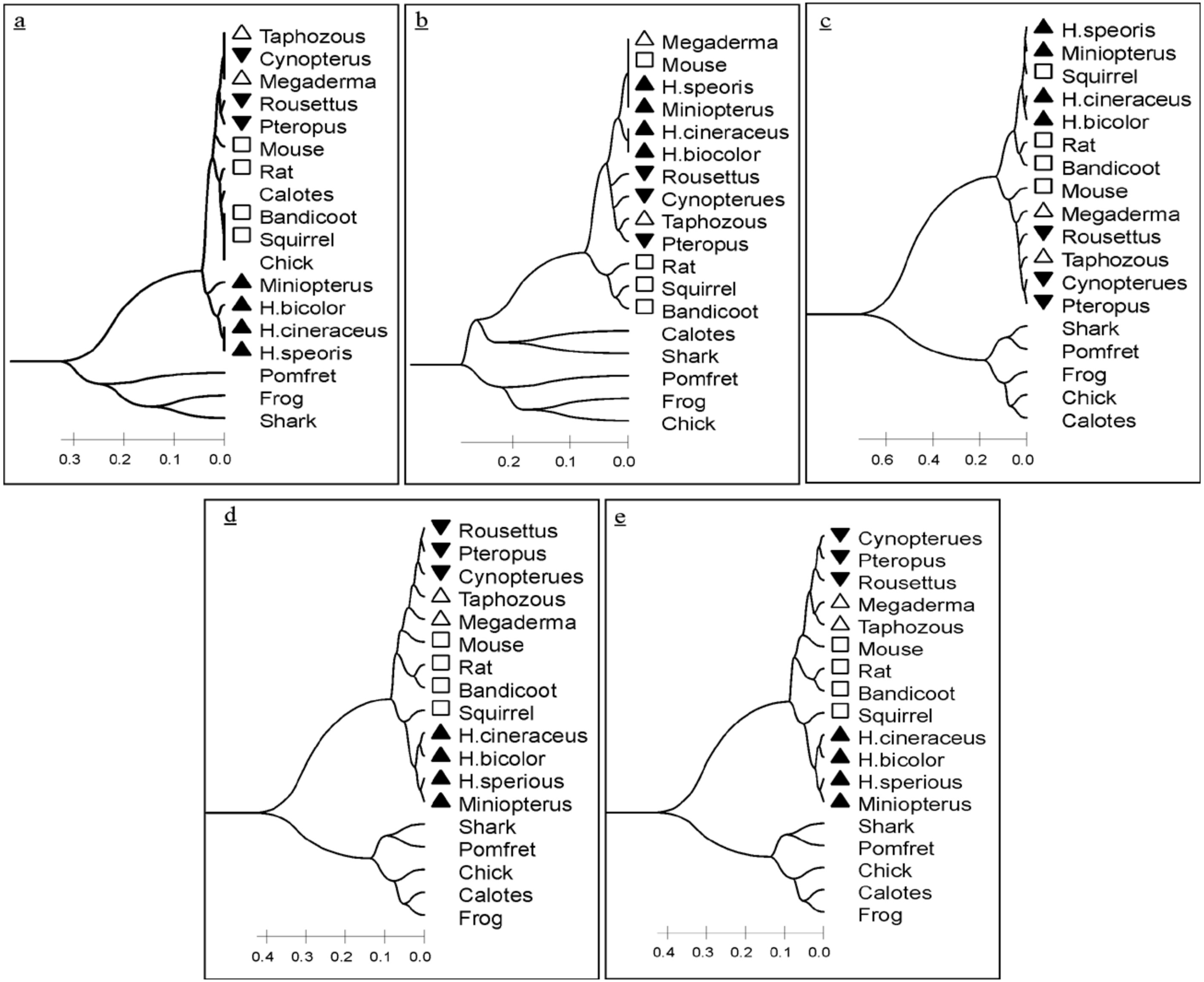

Figure 3. Phylogenetic Relationship by Unweighted Pairs Grouping method among species based on (a) P values of crystallin Isoelectric focusing, (b) P values of crystallin Molecular Weight, (c) Re-estimated Euclidian distances from 3-D for P value of pI, MW and IC, (d) Crystallin “network” distances as well as Euclidian distances re-estimated from “network-MDS” plot. (e) Crystallin “network + star” distances as well as those re-estimated from “network+star” -MDS plot, ❑ Rodents, ▼ Megachiroptera, Δ *Megaderma* and *Taphozous*, ▲ Microchiroptera,

Table 2. Euclidean “network + star” distances for lens crystallins.

Species	*H.cineraceus*	*H. speoris*	*H. bicolor*	*Miniopterus*	*Megaderma*	*Taphozous*	*Rousettus*	*Cynopterus*	*Pteropus*	Mouse	Rat	*Bandicoot*	Squirrel	Chick	*Calotes*	Frog	Shark	Pomfret
H.cineraceus	0.00																	
H. speoris	0.04 ± 0.01	0.00																
H. bicolor	0.02 ± 0.00	0.04 ± 0.00	0.00															
Miniopterus	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.00														
Megaderma	0.17 ± 0.08	0.16 ± 0.08	0.18 ± 0.07	0.16 ± 0.08	0.00													
Taphozous	0.19 ± 0.08	0.19 ± 0.07	0.20 ± 0.08	0.18 ± 0.07	0.05 ± 0.03	0.00												
Rousettus	0.21 ± 0.05	0.21 ± 0.05	0.22 ± 0.05	0.21 ± 0.04	0.09 ± 0.02	0.05 ± 0.02	0.00											
Cynopterus	0.18 ± 0.03	0.19 ± 0.02	0.20 ± 0.03	0.18 ± 0.01	0.09 ± 0.01	0.06 ± 0.02	0.05 ± 0.03	0.00										
Pteropus	0.20 ± 0.04	0.20 ± 0.04	0.21 ± 0.04	0.20 ± 0.04	0.08 ± 0.01	0.05 ± 0.02	0.04 ± 0.04	0.02 ± 0.04	0.00									
Mouse	0.14 ± 0.06	0.14 ± 0.07	0.16 ± 0.05	0.13 ± 0.08	0.08 ± 0.02	0.11 ± 0.06	0.13 ± 0.06	0.11 ± 0.06	0.11 ± 0.05	0.00								
Rat	0.17 ± 0.07	0.20 ± 0.06	0.18 ± 0.07	0.20 ± 0.06	0.18 ± 0.01	0.18 ± 0.05	0.16 ± 0.09	0.15 ± 0.06	0.15 ± 0.06	0.16 ± 0.03	0.00							
Bandicoot	0.15 ± 0.04	0.18 ± 0.03	0.16 ± 0.04	0.17 ± 0.03	0.15 ± 0.04	0.15 ± 0.09	0.15 ± 0.12	0.13 ± 0.09	0.13 ± 0.09	0.14 ±0.02	0.06 ± 0.02	0.00						
Squirrel	0.09 ± 0.03	0.11 ± 0.03	0.10 ± 0.03	0.11 ± 0.03	0.15 ± 0.08	0.16 ± 0.08	0.19 ± 0.05	0.15 ± 0.03	0.17 ± 0.06	0.16 ±0.03	0.15 ± 0.05	0.13 ± 0.05	0.00					
Chick	0.82 ± 0.04	0.84 ± 0.05	0.80 ± 0.04	0.85 ± 0.05	0.94 ± 0.07	0.94 ± 0.11	0.93 ± 0.14	0.92 ± 0.11	0.92 ± 0.11	0.91 ±0.02	0.80 ± 0.05	0.82 ± 0.03	0.84 ± 0.06	0.00				
Calotes	0.74 ± 0.01	0.76 ± 0.01	0.72 ± 0.01	0.77 ± 0.01	0.87 ± 0.04	0.88 ± 0.06	0.87 ± 0.08	0.86 ± 0.05	0.86 ± 0.05	0.84 ± 0.04	0.74 ± 0.01	0.76 ± 0.03	0.78 ± 0.02	0.14 ± 0.09	0.00			
Frog	0.67 ± 0.07	0.70 ± 0.07	0.66 ± 0.07	0.71 ± 0.07	0.80 ± 0.09	0.81 ± 0.13	0.80 ± 0.16	0.79 ± 0.13	0.79 ± 0.12	0.78 ± 0.03	0.67 ± 0.07	0.69 ± 0.06	0.71 ±0.09	0.17 ± 0.03	0.10 ±0.03	0.00		
Shark	0.92 ± 0.22	0.94 ± 0.21	0.90 ± 0.22	0.95 ± 0.22	1.05 ± 0.22	1.06 ± 0.28	1.05 ± 0.30	1.04 ± 0.28	1.04 ±0.28	1.02 ±0.18	0.92 ± 0.21	0.93 ± 0.19	0.95 ±0.25	0.25 ±0.21	0.23 ±0.25	0.28 ± 0.19	0.00	
Pomfret	0.82 ± 0.22	0.84 ± 0.21	0.81 ± 0.22	0.85 ± 0.21	0.95 ± 0.22	0.96 ± 0.28	0.95 ± 0.30	0.94 ± 0.28	0.94 ± 0.28	0.92 ±0.17	0.81 ± 0.21	0.82 ± 0.19	0.85 ± 0.26	0.31 ±0.16	0.30 ± 0.19	0.26 ± 0.19	0.19 ± 0.01	0.00

± Standard deviation in the mean

Table 3. Euclidean network distances of lens crystallins.

Species	*H.cineraceus*	*H.speoris*	*H.bicolor*	*Miniopterus*	*Megaderma*	*Taphozous*	*Rousettus*	*Cynopterus*	*Pteropus*	Mouse	Rat	*Bandicoot*	Squirrel	Chick	*Calotes*	Frog	Shark	Pomfret
H. cineraceus	0.00																	
H. speoris	0.02	0.00																
H. bicolor	0.02	0.04	0.00															
Miniopterus	0.03	0.01	0.05	0.00														
Megaderma	0.30	0.29	0.32	0.28	0.00													
Taphozous	0.31	0.29	0.33	0.28	0.07	0.00												
Rousettus	0.31	0.29	0.33	0.28	0.13	0.06	0.00											
Cynopterus	0.25	0.23	0.27	0.22	0.10	0.07	0.08	0.00										
Pteropus	0.26	0.25	0.28	0.23	0.08	0.05	0.08	0.02	0.00									
Mouse	0.26	0.25	0.27	0.24	0.13	0.17	0.21	0.14	0.14	0.00								
Rat	0.07	0.08	0.08	0.08	0.27	0.29	0.31	0.24	0.25	0.23	0.00							
Bandicoot	0.13	0.14	0.14	0.14	0.24	0.28	0.30	0.23	0.24	0.19	0.07	0.00						
Squirrel	0.04	0.03	0.06	0.02	0.28	0.27	0.27	0.21	0.23	0.24	0.10	0.15	0.00					
Chick	1.32	1.34	1.30	1.35	1.60	1.62	1.62	1.56	1.58	1.55	1.33	1.37	1.36	0.00				
Calotes	1.20	1.21	1.18	1.22	1.48	1.49	1.49	1.44	1.45	1.43	1.21	1.25	1.23	0.14	0.00			
Frog	1.10	1.11	1.08	1.12	1.37	1.39	1.39	1.34	1.35	1.32	1.10	1.14	1.13	0.23	0.15	0.00		
Shark	1.56	1.57	1.54	1.58	1.82	1.85	1.85	1.80	1.81	1.76	1.56	1.58	1.60	0.31	0.45	0.48	0.00	
Pomfret	1.39	1.41	1.37	1.42	1.65	1.68	1.69	1.63	1.64	1.59	1.39	1.41	1.43	0.25	0.35	0.34	0.17	0.00

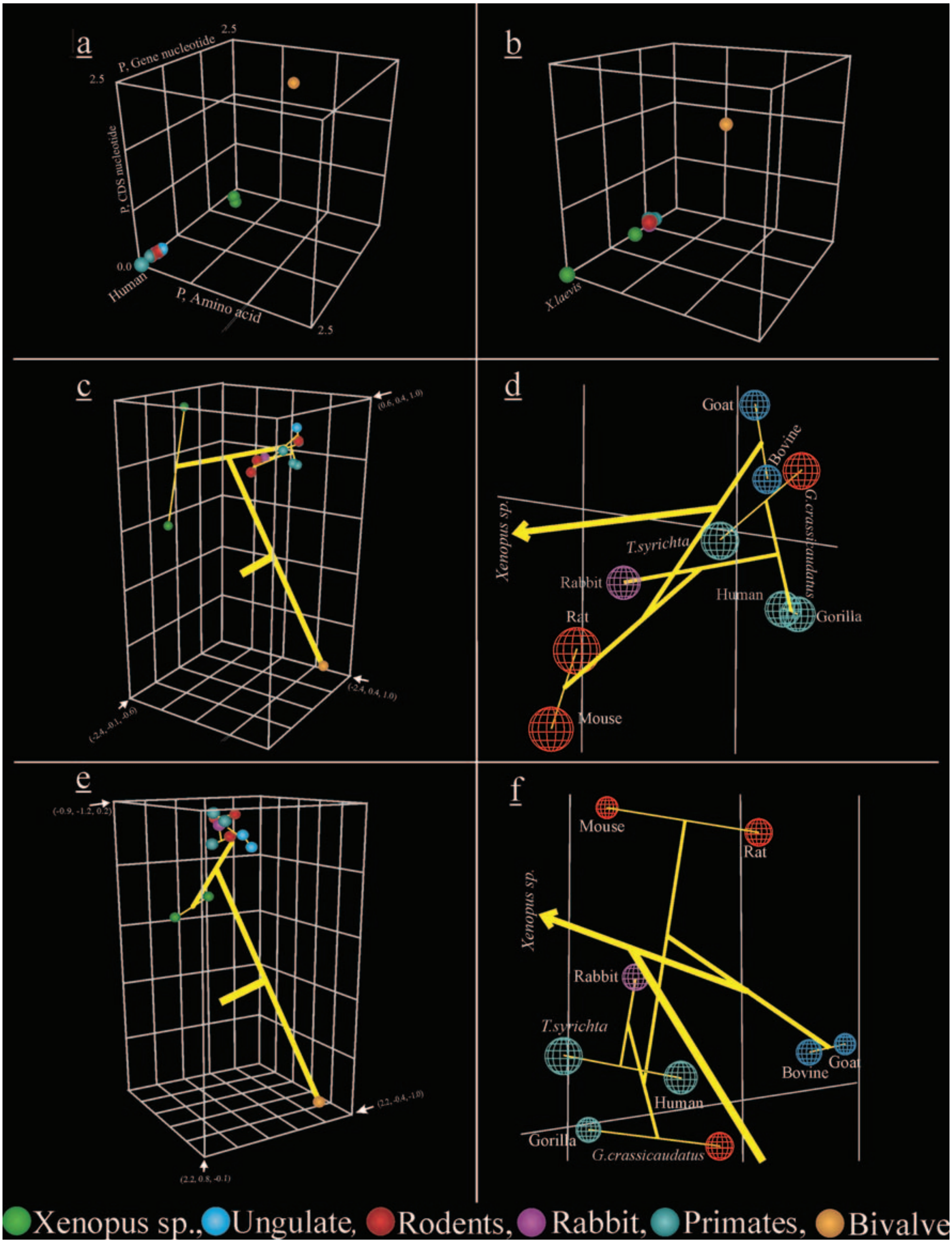

Figure 4. Three-dimensional molecular phylogenetic trees for β-globin. [a] P values derived from the MSA of human (positioned at 0,0,0) β-globin gene nucleotide sequence (y axis), β-globin coding nucleotide sequence (z axis) and β-globin amino acid sequence (x axis) against 11 other species; [b] MSA comparison in 3-D as in h but against *Xenopus laevis* β-globin (at 0,0,0); [c] 3-D plot of "star" Euclidian distances by MDS combined with the phylogenetic tree in Figure 5d. [d], a magnified and slightly rotated view of the mammalian branch and sub branches positioning primate-blossom with the rodent *G. crassicaudatus* and distant from rat-mouse, and cow-goat blossoms. [e] 3-D MDS of "network + star" Euclidian distances combined with phylogenetic tree in Figure 5h. [f] a magnified and slightly rotated view of mammalian branch with primate and *G. crassicaudatus* (rodent) blossoms distant from rat-mouse and ungulates.

Rodentia, Class Mammalia), Rat (*Rattus norvegicus,* order Rodentia, Class Mammalia) and Dormouse (*Graphiurus crassicaudatus,* order Rodentia, Class Mammalia), two species under the Genus *Xenopus* (*Xenopus laevis,* and *Xenopus tropicalis,* Order Anura Class Amphibia), and the bivalve *Anadara* (*Anadara trepezia,* order Arcoida, Class Pteriomorphia, Phylum Mollusca). The gene sequences were scored beginning with 5' proximal TATA box till the 3' polyadenylation site and inclusive of exons and introns. Data for all pairs were subjected to multiple sequence alignment (MSA) by CLUSTALW (Thompson *et al*., 1994) to estimate the percent similarity coefficient and P values as above. Similarly, coding nucleotide sequence and polypeptide amino acid sequences were subjected to MSA to obtain P values. Individual phylogenetic trees were constructed from P values for the amino acid sequence, gene nucleotide sequence and the coding nucleotide sequence. With a different reference species at the position (0,0,0), species were positioned in 12 different 3-D plots with P for amino acid sequence on x-axis, P for coding nucleotide sequence on y-axis and gene nucleotide sequence on z-axis and examples of human, mouse, *X. laevis* and *A. trepezia* are shown in Figure 1. From each plot Euclidian "star" and "network" distances were estimated as before (Figure 1a) along with standard deviation distances wherever applicable. The phylogenetic trees were constructed with "network" and "star +network" distances were tested by bootstrap as before. The "star" or "network + star" distances were then used to fix each species in a 3-dimensional space by MDS and, from re-estimated Euclidian distances, the branching pattern in the phylogenetic tree was merged with the MDS plot. The standard error was calculated as before.

Results and Discussion

Vertebrate lens crystallin homology

First, we have plotted P values among pI, MW immunocrossreactivity (IC) of vertebrate crystallins against polyclonal antibodies to mouse (Figure 2a) *Rousettus* (Figure 2b) or *Megaderma* (not shown). Euclidian distances were estimated and the "star" distance data are shown in Table 1 while the combined Euclidian "network" and "star" distances are shown in Table 2. From the network distances alone (not shown) and "network + star" distances (Table 1) phylogenetic trees were constructed (Figure 3d-e), tested by bootstrap which gave the probability of fit with confidence limit of 0.73± 0.039 for trees using "network" as compared to 0.61 ± 0.043 for "network + star" distances. The phylogenetic trees were also constructed for individual attributes pI (Figure 3a) and MW (Figure 3b) that exhibit the substantially different genealogical arrangement as compared to the trees based on multiple parameters. We then plot in 3-D Euclidean "star" distances (Table 1) for mouse (x-axis), *Rousettus* (y-axis) and *Megaderma* (z-axis) (Figure 2c). Euclidian distances were re-estimated among all pairs of species (Table 3) from which another phylogenetic tree is constructed (Figure 3c) and resulting connectivity is transposed over the 3-D plot in Figure 2c to obtain a 3-D phylogenetic tree. Thus, from the hypothetical vertebrate stem, mammals cluster on one branch, which is farther away from that leading to other vertebrates. A magnified view (Figure 2d) shows that the mammalian cluster further bifurcates with the closer one bearing the mouse, *Taphozous, Megaderma* and all megachiroptera while the Rat, *Bandicoot*, Squirrel and microchiroptera *Miniopterus* and 3 species under the Genus *Hipposideros*, are positioned further away over the second branch. The closer branch then bifurcates between mouse and the remaining chiroptera blossom separately in the sequence *Megaderma, Rousettus* and *Taphozous* on one hand, and the *Cynopterus-Pteropus* pair on the other. Thus, *Taphozous* and *Megaderma,* previously classified as microchiroptera on the basis of classical taxonomic and geographic considerations (Romer 1945, Suthers et.al. 1970, Honacki et.al. 1982), may actually be closer to the megachroptera & mouse and distant from the sub-branches with *Bandicoot* and rat, 3 species under Genus *Hipposideros,* and squirrel. The data in Table 2 are subjected to MDS in order to position the 18 species in x, y, z space (Figure 2e-f). The Euclidian distances (Table 4) from the resultant x, y and z coordinates were used to reconstruct the phylogenetic tree (Figure 3e) and the branching pattern was again transposed to visualize a 3-D tree (Figure 2e,f). This tree also exhibits segregation of mammals from other vertebrates and the mammalian stalk bifurcates into the closer one bearing squirrel, *Miniopterus* and 3 species under Genus *Hipposideros* while further away on the second branch are sequentially positioned the rodents followed by *Taphozous* and *Megaderma* while the *Pteropus*, *Cynopterus* and *Rousettus* (megachiroptera) blossom out further away. We again conclude that *Megaderma lyra* and *Taphozous longimanus* are probably closer to Megachiroptera than the Microchiroptera and probably constitute the link between the latter and Rodentia. Most microchiroptera are cave dwelling and insectivorous with a well developed echolocation apparatus. *Taphozous* and *Megaderma* do not share the last two attributes with microchiroptera in addition to being distant in terms of the distribution pI, MW and of crystallins and IC to 3 distinct antigens. We, therefore, reclassify *Taphozous* and *Megaderma* under the sub-order megachiroptera or as a separate suborder minichiroptera.

The phylogenetic tree based on "network" distances for 5 parameters or attributes, namely, pI, MW, and IC to mouse antiserum, *Rousettus* antiserum and *Megaderma* antiserum (Figure 3d) is in all essential features similar to that for MDS "network + star" distances, while those based on unique attributes such as MW (Figure 3a) or pI (Figure 3b) are considerably different. For example, from pI (Figure 3a), the lizard *Calotes* and the chick position on the rodent-megachiroptera branch while from MW (Figure 3b) considerable positional intermixing is found among rodents, megachiroptera and microchiroptera. Thus, unlike single attributes (Figure 3a,b) multiple attributes (Figure 3c-e) provide a more rational representation of parsimony and allow these to be viewed in three-dimensional plots.

β-globin homology

To build multiparametric 3-D phylogenetic trees based on sequence data for 12 species, we retrieved (see, materials and methods) complete amino acid sequence, coding nucleotide sequence and gene nucleotide sequence for β-globin from 11 vertebrates and a bivalve. In order to

Table 4. Euclidean distances re-estimated from “network + star”-MDS plot for lens crystallins*.

Species	*H.cineraceus*	*H.speoris*	*H.bicolor*	*Miniopterus*	*Megaderma*	*Taphozous*	*Rousettus*	*Cynopterus*	*Pteropus*	Mouse	Rat	*Bandicoot*	Squirrel	Chick	*Calotes*	Frog	Shark	Pomfret
H.cineraceus	0.00																	
H.speoris	0.04 ± 0.00	0.00																
H.bicolor	0.02 ± 0.00	0.04 ± 0.00	0.00															
Miniopterus	0.04 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.00														
Megaderma	0.16 ± 0.00	0.15 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.00													
Taphozous	0.19 ± 0.00	0.19 ± 0.00	0.20 ± 0.00	0.18 ± 0.00	0.04 ± 0.01	0.00												
Rousettus	0.22 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.09 ± 0.00	0.05 ± 0.00	0.00											
Cynopterus	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.00	0.08 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.00										
Pteropus	0.20 ± 0.00	0.20 ± 0.00	0.21 ± 0.00	0.20 ± 0.00	0.08 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.00									
Mouse	0.15 ± 0.00	0.15 ± 0.01	0.16 ± 0.01	0.13 ± 0.00	0.07 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	0.00								
Rat	0.17 ± 0.00	0.20 ± 0.00	0.17 ± 0.00	0.20 ± 0.00	0.19 ± 0.01	0.18 ± 0.00	0.16 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.00							
Bandicoot	0.15 ± 0.00	0.18 ± 0.00	0.16 ± 0.00	0.18 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.13 ± 0.00	0.14 ± 0.00	0.05 ± 0.00	0.00						
Squirrel	0.08 ± 0.00	0.11 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.16 ± 0.00	0.18 ± 0.02	0.19 ± 0.00	0.17 ± 0.02	0.18 ± 0.01	0.11 ± 0.06	0.14 ± 0.01	0.11 ± 0.02	0.00					
Chick	0.82 ± 0.00	0.84 ± 0.00	0.80 ± 0.00	0.86 ± 0.00	0.95 ± 0.01	0.95 ± 0.00	0.94 ± 0.01	0.90 ± 0.02	0.92 ± 0.00	0.93 ± 0.02	0.79 ± 0.01	0.82 ± 0.01	0.84 ± 0.00	0.00				
Calotes	0.74 ± 0.00	0.76 ± 0.00	0.72 ± 0.00	0.77 ± 0.00	0.88 ± 0.01	0.89 ± 0.01	0.89 ± 0.02	0.84 ± 0.01	0.86 ± 0.00	0.86 ± 0.02	0.73 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.14 ± 0.00	0.00			
Frog	0.67 ± 0.00	0.70 ± 0.00	0.66 ± 0.00	0.71 ± 0.00	0.81 ± 0.00	0.81 ± 0.00	0.81 ± 0.01	0.77 ± 0.02	0.79 ± 0.00	0.79 ± 0.01	0.65 ± 0.02	0.68 ± 0.00	0.69 ± 0.01	0.17 ± 0.00	0.10 ± 0.00	0.00		
Shark	0.91 ± 0.01	0.94 ± 0.00	0.90 ± 0.00	0.95 ± 0.00	1.06 ± 0.01	1.07 ± 0.01	1.07 ± 0.01	1.03 ± 0.01	1.04 ± 0.00	1.03 ± 0.01	0.91 ± 0.01	0.93 ± 0.00	0.93 ± 0.03	0.25 ± 0.00	0.23 ± 0.01	0.27 ± 0.01	0.00	
Pomfret	0.82 ± 0.00	0.85 ± 0.00	0.81 ± 0.00	0.85 ± 0.00	0.96 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.93 ± 0.01	0.94 ± 0.00	0.92 ± 0.01	0.80 ± 0.00	0.82 ± 0.00	0.82 ± 0.03	0.31 ± 0.00	0.30 ± 0.00	0.26 ± 0.00	0.19 ± 0.00	0.00

*distances estimated from Figure 2e.

± Standard error in the mean.

Table 5. Star distances from 3-D plots of multiple sequence alignment of β-globin.

Species	Human	Mouse	Rabbit	Bovine	*R.norvegicus*	Gorilla	*A.trepezia*	*X.laevis*	*X.tropicalis*	*T.syrichta*	*G.crassicaudatus*	Goat
Human	0.00											
Mouse	0.24	0.00										
Rabbit	0.20	0.31	0.00									
Bovine	0.22	0.54	0.33	0.00								
R.norvegicus	0.25	0.10	0.31	0.51	0.00							
Gorilla	0.01	0.61	0.41	0.41	0.53	0.00						
A.trepezia	2.67	2.54	2.53	2.61	2.79	2.67	0.00					
X.laevis	1.37	1.28	1.30	1.35	1.32	1.32	2.91	0.00				
X.tropicalis	1.36	1.23	1.31	1.30	1.37	1.38	2.34	1.22	0.00			
T.syrichta	0.15	0.26	0.21	0.26	0.28	0.15	3.01	1.42	1.36	0.00		
G.crassicaudatus	0.17	0.65	0.28	0.24	0.30	0.23	2.67	1.30	1.38	0.16	0.00	
Goat	0.28	0.67	0.37	0.12	0.52	0.45	2.81	1.34	1.35	0.28	0.29	0.00

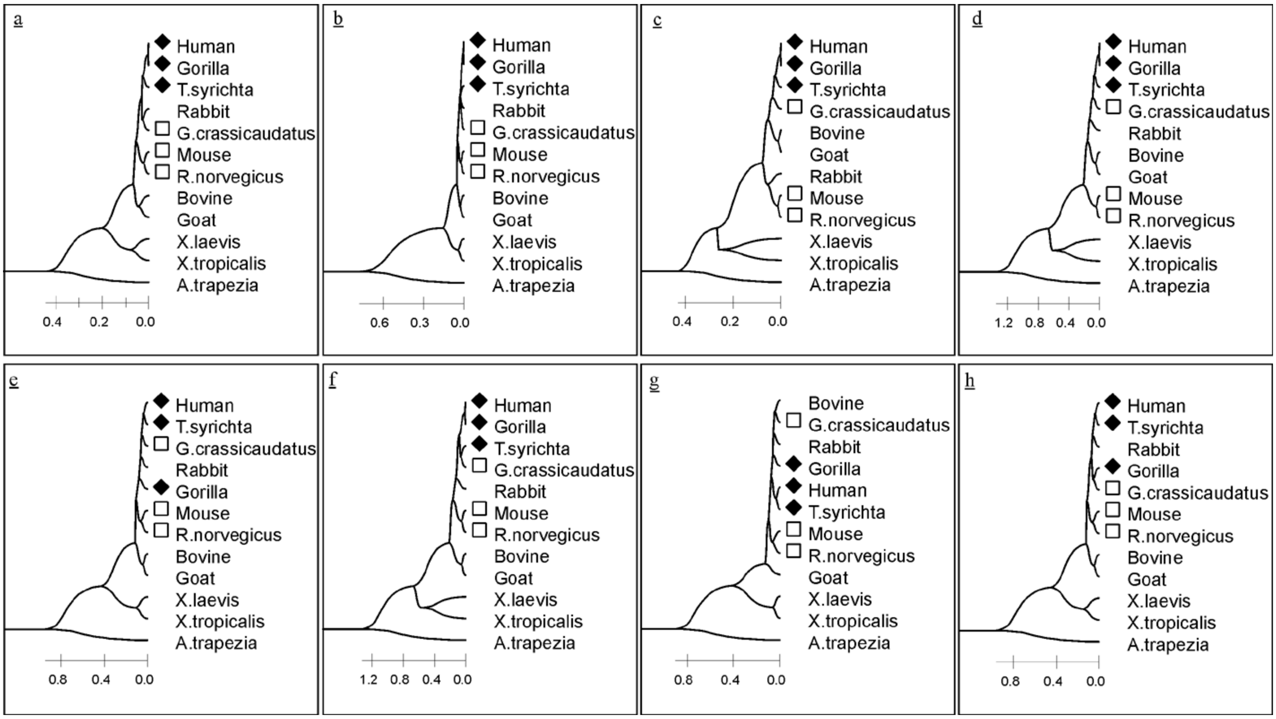

Figure 5. (a) P values of multiple sequence alignment score for β-globin Amino acid, (b) P values of multiple sequence alignment score for β-globin coding segments nucleotide sequence, (c) P values of multiple sequence alignment score for β-globin gene nucleotide sequence, (d) β-globin "star" distances (e) β-globin "network+star" distances (f) Re-estimated Euclidian distances from β-globin "star" MDS plot (g) Re-estimated Euclidian distances from β-globin "network" MDS plot (h) Re-estimated Euclidian distances from β-globin "network + star" MDS plot. ❑ Rodents, ◆ Primates.

Table 6. "Network+star" distances from multiple sequence alignment of β-globin.

Species	Human	Mouse	Rabbit	Bovine	*R.norvegicus*	Gorilla	*A.trepezia*	*X.laevis*	*X.tropicalis*	*T.syrichta*	*G.crassi-caudatus*	Goat
Human	0.00											
Mouse	0.25 ± 0.17	0.00										
Rabbit	0.13 ± 0.10	0.18 ± 0.12	0.00									
Bovine	0.17 ± 0.12	0.26 ± 0.22	0.15 ± 0.12	0.00								
R.norvegicus	0.21 ± 0.15	0.13 ± 0.18	0.17 ± 0.16	0.21 ± 0.21	0.00							
Gorilla	0.14 ± 0.13	0.26 ± 0.20	0.17 ± 0.14	0.18 ± 0.15	0.24 ± 0.24	0.00						
A.trepezia	2.19 ± 0.78	2.07 ± 0.73	2.13 ± 0.74	2.12 ± 0.75	2.12 ± 0.76	2.11 ± 0.77	0.00					
X.laevis	0.97 ± 0.44	0.84 ± 0.39	0.91 ± 0.39	0.88 ± 0.43	0.85 ± 0.42	0.89 ± 0.40	1.65 ± 0.71	0.00				
X.tropicalis	0.98 ± 0.43	0.82 ± 0.40	0.89 ± 0.41	0.89 ± 0.41	0.91 ± 0.38	0.88 ± 0.44	1.59 ± 0.57	0.30 ± 0.46	0.00			
T.syrichta	0.08 ± 0.10	0.27 ± 0.17	0.15 ± 0.15	0.18 ±0.12	0.20 ± 0.13	0.20 ± 0.15	2.20 ± 0.80	0.95 ± 0.45	0.99 ± 0.39	0.00		
G.crassi-caudatus	0.11 ± 0.12	0.25 ± 0.21	0.14 ± 0.12	0.13 ± 0.08	0.22 ± 0.17	0.14 ± 0.10	1.66 ±0.69	0.91 ±0.42	0.92 ± 0.43	0.13 ± 0.14	0.00	
Goat	0.22 ± 0.15	0.30 ± 0.25	0.21 ± 0.18	0.09 ± 0.10	0.20 ±0.22	0.22 ± 0.20	1.64 ± 0.71	0.84 ±0.45	0.89 ± 0.40	0.83 ± 0.41	0.17 ± 0.12	0.00

± Standard deviation in the mean.

Table 7. Euclidean distances re-estimated from MDS plot distances of β-Globin*.

Species	Human	Mouse	Rabbit	Bovine	*R. norvegicus*	Gorilla	*A. trepezia*	*X. laevis*	*X. tropicalis*	*T. syrichta*	*G.crassicaudatus*	Goat
Human	0.00											
Mouse	0.24 ± 0.12	0.00										
Rabbit	0.20 ± 0.05	0.31 ± 0.02	0.00									
Bovine	0.22 ± 0.05	0.54 ± 0.02	0.33 ± 0.03	0.00								
R. norvegicus	0.25 ± 0.08	0.10 ± 0.01	0.31 ± 0.02	0.51 ± 0.01	0.00							
Gorilla	0.01 ± 0.01	0.61 ± 0.23	0.41 ± 0.14	0.41 ± 0.13	0.53 ± 0.19	0.00						
A. trepezia	2.67 ± 0.05	2.54 ± 0.16	2.53 ± 0.11	2.61 ± 0.05	2.79 ± 0.01	2.67 ± 0.07	0.00					
X. laevis	1.37 ± 0.04	1.28 ± 0 .09	1.30 ± 0.12	1.35 ± 0.10	1.32 ± 0.07	1.32 ± 0.11	2.91 ± 0.11	0.00				
X. tropicalis	1.36 ±0.01	1.23 ± 0.03	1.31 ± 0.13	1.30 ± 0.10	1.37 ± 0.08	1.38 ± 0.01	2.34 ± 0.10	1.22 ± 0.07	0.00			
T. syrichta	0.15 ±0.01	0.26 ± 0.05	0.21 ± 0.02	0.26 ± 0.02	0.28 ± 0.04	0.15 ± 0.01	3.01 ± 0.28	1.42 ± 0.09	1.36 ± 0.00	0.00		
G. crassicaudatus	0.17 ±0.02	0.65 ± 0.21	0.28 ± 0.03	0.24 ± 0.00	0.30 ± 0.06	0.23 ± 0.03	2.67 ± 0.05	1.30 ± 0.09	1.38 ± 0.10	0.16 ± 0.02	0.00	
Goat	0.28 ±0.07	0.67 ± 0.07	0.37 ± 0.02	0.12 ± 0.00	0.52 ± 0.02	0.45 ± 0.09	2.81 ± 0.13	1.34 ± 0.11	1.35 ± 0.11	0.28 ± 0.79	0.29 ± 0.04	0.00

* Data estimated from Figure 4c.

± Standard error in the mean.

Table 8. Euclidean distances re-estimated from MDS plot of β-globin*.

Species	Human	Mouse	Rabbit	Bovine	*R. norvegicus*	Gorilla	*A. trepezia*	*X. laevis*	*X. tropicalis*	*T. syrichta*	*G. crassicaudatus*	Goat
Human	0.00											
Mouse	0.25 ± 0.01	0.00										
Rabbit	0.13 ± 0.02	0.18 ± 0.03	0.00									
Bovine	0.17 ± 0.01	0.26 ± 0.00	0.15 ± 0.00	0.00								
R. norvegicus	0.21 ± 0.01	0.13 ± 0.00	0.17 ± 0.03	0.21 ± 0.04	0.00							
Gorilla	0.14 ± 0.02	0.26 ± 0.01	0.17 ± 0.04	0.18 ± 0.01	0.24 ± 0.02	0.00						
A. trepezia	2.19 ± 0.13	2.07 ± 0.01	2.13 ± 0.10	2.12 ± 0.18	2.12 ± 0.05	2.11 ± 0.16	0.00					
X. laevis	0.97 ± 0.01	0.84 ± 0.04	0.91 ± 0.03	0.88 ± 0.00	0.85 ± 0.02	0.89 ± 0.01	1.65 ± 0.00	0.00				
X. tropicalis	0.98 ± 0.00	0.82 ± 0.02	0.89 ± 0.01	0.89 ± 0.02	0.91 ± 0.02	0.88 ± 0.03	1.59 ± 0.02	0.30 ± 0.00	0.00			
T. syrichta	0.08 ± 0.00	0.27 ± 0.01	0.15 ± 0.01	0.18 ± 0.05	0.20 ± 0.03	0.20 ± 0.00	2.20 ± 0.07	0.95 ± 0.07	0.99 ± 0.01	0.00		
G. crassicaudatus	0.11 ± 0.01	0.25 ± 0.03	0.14 ± 0.00	0.13 ±0.03	0.22 ±0.01	0.14 ± 0.03	1.66 ± 0.23	0.91 ± 0.02	0.92 ± 0.01	0.13 ± 0.04	0.00	
Goat	0.22 ± 0.04	0.30 ± 0.00	0.21 ± 0.02	0.09 ±0.01	0.20 ±0.03	0.22 ± 0.02	1.64 ± 0.18	0.84 ± 0.02	0.89 ± 0.04	0.83 ± 0.11	0.17 ± 0.02	0.00

* Data estimated from Figure 4e.

± Standard error in the mean.

restrict the analysis to the phene-relevant portion of β-globin, the beginning of the gene was taken from the 5' proximal TATA box and terminating with polyadenylation signal 3' of the coding sequence. We carried out the multiple sequence alignment (MSA) in a 12x12 matrix separately for the polypeptide, coding sequence and the gene to estimate the percent similarity and P values (see methods, computed data not shown). With reference to the species positioned at (0,0,0), the P values of remaining 11 species were plotted in a 3-D along the x (amino acid sequence)- y- (gene nucleotide sequence) and z- (coding nucleotide sequence) axes, thereby yielding 12 plots and, as representative plots we show those for human (Figure 4a), mouse (not shown) *Xenopus laevis* (Figure 4b) and the bivalve *Anadara trepezia* (not shown). From these, Euclidean "star" (Table 5) and "network" distances were estimated (see, methods). Table 6 summarizes "network + star" distances and 2-D phylogenetic trees were constructed (Figure 5d e); the relative species positions in the tree for "network" distances alone is similar to that in Figure 5e, however the estimated evolutionary distances differ between the two trees. Therefore, we have subjected both data sets to bootstrap analysis and find that the trees fit the data with probability of 0.84 ± 0.032 for "network + star" distances as compared to 0.80 ± 0.035 for "network" distances alone.

Euclidean distance data in Tables 5 and 6 were subjected to MDS (Figure 4f). and the re-estimated Euclidean distances (Table 7) were used to construct a phylogenetic tree (Figure 4d). However, the bi-directional "star" distances being identical, bootstrap was not necessary. After transposing the branching pattern (Figure 5f) to the MDS plot, we obtain a 3-D tree (Figure 4f) in which two branches originating from the stem reach the bivalve and vertebrates, respectively. The vertebrate branch bifurcates between *Xenopus* sp. and mammals with the latter branching off to ungulates, rodents, the rabbit, the dormouse and primates, in that order. Except for the switched positions between ungulates and rodents, the 2-D trees are similar for Euclidean "star" and "star-MDS" distances (Figure 5d, and e). When the mammalian branch is magnified (Figure 4d) it sub-branches sequentially into ungulates, rodents, rabbit and primates with Human and Gorilla as a separate blossom from the *Tarsius* sp. and dormouse. MDS based on Euclidean "network" distances alone (not shown) gave a tree (Figure 5h) which is considerably different from that based on original "network" distances (Figure 5e) data were Euclidean "star + network" distances (Table 6) were placed in x, y and z space by MDS and the 2-D tree constructed from the re-estimated Euclidean distances (Table 8) by unweighted pair grouping (Figure 5g) was transposed over MDS plot to obtain 3-D phylogenetic trees (Figure 4e,f). The mammalian branching pattern is somewhat different with 'star-MDS". Thus, ungulates lead to rodents and gorilla-dormouse pair, followed by the rabbit and primates *T. syrichta* and the human. Furthermore, the mammalian β-globin blossom exhibits ungulates, rat and mouse as distant from primates among whom the *Homo sapiens* share closeness with either gorilla or *Tarsius*.

Finally, the β-globin phylogenetic tree based on the amino acid sequence (Figure 5a) is similar to that using coding nucleotide sequence (Figure 5b). In contrast, the tree for gene nucleotide sequence (Figure 5c) is substantially different with the rat, mouse and rabbit positioned farther to primates than ungulates. Thus, as expected, mutations appear to change the gene nucleotide sequence, inclusive of 5' and 3' noncoding regions and introns, and determine the phylogenetic distance at molecular level.

One of the issue concerns the choice of Euclidean distances as compared to the Mahalanobis distance, which is a squared distance between two points in a multidimensional space. Earlier (Milner *et al*., 2003), we have examined the 3-D positioning of 8 vertebrates on the basis of the Mahalanobis distance that did not give satisfactory fits. However, for a heterogeneous population of organisms, this method should be kept in view. For positioning 8 vertebrates in 3-D space, we also used (Milner *et al*., 2003) a Sequential Positioning method, which gave good fits but requires extensive computations and the software program, is now being developed.

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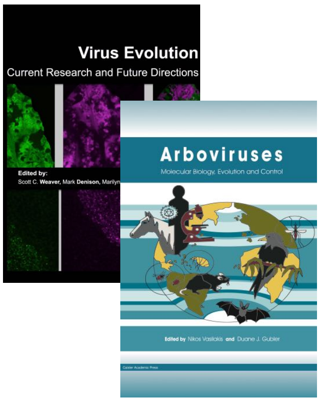

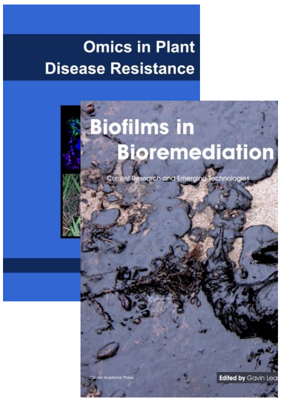

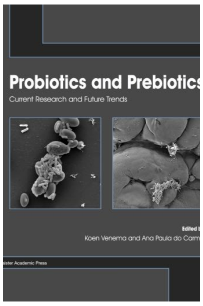

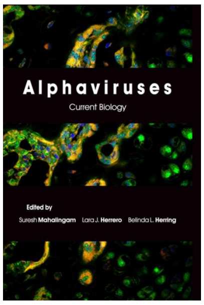

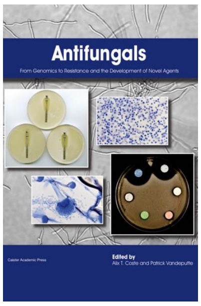

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