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ORIGINAL RESEARCH

Evaluating the Phylogenetic Position of Chinese Tree Shrew (*Tupaia belangeri chinensis*) Based on Complete Mitochondrial Genome: Implication for Using Tree Shrew as an Alternative Experimental Animal to Primates in Biomedical Research

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ABSTRACT

Tree shrew (*Tupaia belangeri*) is currently placed in Order Scandentia and has a wide distribution in Southeast Asia and Southwest China. Due to its unique characteristics, such as small body size, high brain-to-body mass ratio, short reproductive cycle and life span, and low-cost of maintenance, tree shrew has been proposed to be an alternative experimental animal to primates in biomedical research. However, there are some debates regarding the exact phylogenetic affinity of tree shrew to primates. In this study, we determined the mtDNA entire genomes of three Chinese tree shrews (*T. belangeri chinensis*) and one Malayan flying lemur (*Galeopterus variegatus*). Combined with the published data for species in Euarchonta, we intended to discern the phylogenetic relationship among representative species of Dermoptera, Scandentia and Primates. The mtDNA genomes of Chinese tree shrews and Malayan flying lemur shared similar gene organization and structure with those of other mammals. Phylogenetic analysis based on 12 concatenated mitochondrial protein-encoding genes revealed a closer relationship between species of Scandentia and Glires, whereas species of Dermoptera were clustered with Primates. This pattern was consistent with previously reported phylogeny based on mtDNA data, but differed from the one reconstructed on the basis of nuclear genes. Our result suggested that the matrilineal affinity of tree shrew to primates may not be as close as we had thought. The ongoing project for sequencing the entire genome of Chinese tree shrew will provide more information to clarify this important issue.

KEYWORDS: Chinese tree shrew; mtDNA; Phylogeny; Animal model; Flying lemur

1. INTRODUCTION

Our knowledge of the pathogenesis of human disease has been facilitated by using animal models, though a large number of significant results from animal experiments have not been successfully translated into clinical practice (van der Worp et al., 2010). Due to ethical issues and protection for human health and environment, the use of animal models in biomedical research continues and will be our Hobson's choice in a long period. Considering the restrictions in practical usage of nonhuman primate animals, it is urgent and necessary to find an alternative animal to primate in biomedical research. Such an alternative should contain a variety of specific features, such as enormous resource, cost-effectiveness, easy maintenance, and most importantly, close affinity to human.

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Despite the fact that the exact phylogenetic status of tree shrews (Family Tupaiidae) has been under debate for many years, there seems to be a consensus that this species had a close affinity to primates (Peng et al., 1991; Murphy et al., 2001; Seiffert et al., 2003; Janečka et al., 2007). Morphologically, tree shrews resemble squirrels and rats in body size, with small adult body weight (80−204 g) (Wang, 1987), relatively short life span (9−12 years), and short reproductive cycle. They also had the highest brain-to-body mass ratio of any known mammals (Peng et al., 1991). All these unique features facilitate easy-to-handle experimental processes and cost-effectiveness. The natural habitats of Tupaiidae species are restricted to tropical and subtropical forests that extend from India to the Philippines and from Southern China to Java, Borneo, Sumatra, and Bali (Fuchs and Corbach-Söhle, 2010). In a recent study, Roberts and coworkers (2011) had determined the molecular phylogeny of tree shrews and found a deep divergence between the two families (Ptilocercidae and Tupaiidae), as well as, between *Dendrogale* and all other genera within Tupaiidae.

Chinese tree shrews are mainly distributed across Southwest China (including Yunnan, Guizhou, Sichuan, Guangxi, and Hainan Province) (Wang, 1987) and have been classified as *Tupaia belangeri* (Helgen, 2005). Wang (1987) suggested that Chinese tree shrews could be classified into six subspecies according to their geographical, morphological, and morphometric data. In our previous study, we found a remarkable long genetic distance between tree shrews from urban Kunming and the reported Northern tree shrews from GenBank based on mtDNA control region sequence variation (Chen et al., 2011). We noticed that the eight *T. belangeri* samples that were mainly collected from Vietnam, Myanmar, and Cambodia were also clearly separated in the tree by Roberts et al. (2011), which suggested that we should consider genetic difference between tree shrews from different regions when using them to establish animal models.

In this study, we determined the complete mitochondrial genome of three Chinese tree shrews (*T. belangeri chinensis*) to obtain more information regarding the phylogenetic status of this species. One Malayan flying lemur (*Galeopterus variegatus*) was also sequenced for the entire mtDNA sequence. Combined with the published data for species in Euarchonta, we intended to discern the phylogenetic relationship among representative species of Rodentia, Lagomorpha, Dermoptera, Scandentia, and Primates.

2. MATERIALS AND METHODS

2.1. Sampling, DNA amplification and sequencing

We collected muscle tissues from three Chinese tree shrews (samples H1, H5, and H1-HD) that were initially captured from urban Kunming and were raised at the experimental animal core facility of Kunming Institute of Zoology, Chinese Academy of Sciences. The sampling procedures were approved by the Institutional Animal Care and Use Committee of Kunming Institute of Zoology, Chinese Academy of Sciences. Tree shrew H1-HD had been selected for the entire genome sequencing by using the second-generation sequencing methods and the data will be published separately. A cell line of Malayan flying lemur (*G. variegatus*) was retrieved from the Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences.

Genomic DNA was extracted from tissue samples and cell line using the standard phenol/chloroform method. We designed eleven and eight pairs of primers to amplify the entire mtDNA genome of Chinese tree shrews and Malayan flying lemur, respectively (Table S1). PCR amplification was performed in a volume of 50 μL reaction mixture containing 100 ng of DNA, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 200 μmol/L of each dNTP, 10 pmol/L of each primer, and one unit of *LA Taq™* DNA polymerase (TaKaRa Biotech Co., Dalian, China). Amplification started with a denaturation cycle at 95°C for 4 min, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, and ended with a final extension cycle for 2 min at 72°C. PCR products were purified on spin columns and directly sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA) on an ABI 3730 DNA sequencer according to the manufacturer's manual. We used several inner sequencing primers, together with the primers for PCR amplification (Table S1), to cordially generate sequences so that each fragment will be read in both strands or has multiple reads for the light strand. Because the mtDNA control region of Chinese tree shrews and flying lemur contained a chunk of tandem repeats, we determined the PCR fragment covering this region by using TA cloning and sequencing. In brief, PCR products were ligated into pMD19-T vector (TaKaRa Biotech Co.) and transformed to DH5α cells (Tiangen, China). For each fragment, three to five plasmids were selected for sequencing. Plasmid sequencing was performed with universal primers M13F (-47) (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and M13R (-48) (5′-AGCGGATAACAATTTCACACAGGA-3′).

The entire mtDNA sequences of Chinese tree shrews and Malayan flying lemur have been deposited in GenBank under accession Nos. JN800721−JN800724.

2.2. Prediction of mtDNA gene organization and structure

The locations of 13 protein-coding genes and two ribosomal RNA (12S and 16S rRNA) genes in Chinese tree shrew and Malayan flying lemur were determined by comparing with the published mitochondrial genome of Northern tree shrew (GenBank accession No. NC_002521) and Sunda flying lemur (AF460846.1 and NC_004031.1), respectively. We predicted the transfer RNA (tRNA) genes using program tRNAscan-SE 1.21 (Lowe and Eddy, 1997). Some tRNA genes, e.g., tRNA-Ser (AGY) which was not predicted by the tRNAscan-SE program (http://selab.janelia.org/tRNAscan-SE/), were identified by their secondary structure and the relative position in the mtDNA genome, with a reference to available data.

2.3. Phylogenetic reconstruction

To infer the phylogenetic position of Chinese tree shrew and Malayan flying lemur, we retrieved 20 mtDNA genomes of 19 species from GenBank (Table S2). Phylogenetic analyses were performed on the basis of the amino acid sequences of 12 concatenated protein-coding genes on the mitochondrial H-strand. The *ND6* gene was excluded because it is encoded by the L-strand and its properties deviate from the other 12 genes (Waddell et al., 1999). The Monotremata animal echidna (*Tachyglossus aculeatus*) was used as the outgroup to root the phylogenetic tree. *A priori* test of the data for the

Table 1
Characterization of the mitochondrial gene organization and structure of Chinese tree shrew and Malayan flying lemur sequenced in this study

Gene name	Chinese tree shrew[a]		Malayan flying lemur	
	Position	Size (bp)	Position	Size (bp)
tRNA-Phe	1−66	66	1−70	70
12S rRNA	67−1014	948	71−1035	965
tRNA-Val	1015−1080	66	1036−1102	67
16S rRNA	1075−2652	1573	1103−2676	1574
tRNA-Leu (UUR)	2653−2727	78	2677−2752	76
ND1	2730−3686	957	2754−3710	957
tRNA-Ile	3685−3753	69	3710−3778	69
tRNA-Gln	3751−3821	71	3776−3847	72
tRNA-Met	3821−3889	69	3849−3917	69
ND2	3890−4933	1044	3918−4961	1044
tRNA-Trp	4932−4998	67	4960−5025	66
tRNA-Ala	5004−5073	70	5029−5098	70
tRNA-Asn	5075−5147	73	5100−5173	74
tRNA-Cys	5180−5247	68	5208−5274	67
tRNA-Tyr	5248−5314	67	5275−5339	65
COI	5315−6856	1542	5348−6901	1554
tRNA-Ser (UCN)	6859−6927	69	6889−6957	69
tRNA-Asp	6932−7000	69	6965−7033	69
COII	7001−7684	684	7034−7717	684
tRNA-Lys	7685−7749	65	7721−7787	67
ATP8	7752−7955	204	7789−7992	204
ATP6	7913−8593	681	7950−8630	681
COIII	8593−9378	786	8630−9413	784
tRNA-Gly	9378−9443	66	9414−9482	69
ND3	9444−9790	347	9483−9829	347
tRNA-Arg	9791−9856	66	9830−9895	66
ND4L	9858−10,154	297	9896−10,192	297
ND4	10,148−11,525	1378	10,186−11,613	1428
tRNA-His	11,526−11,594	69	11,564−11,633	70
tRNA-Ser (AGY)	11,595−11,652	58	11,634−11,692	59
tRNA-Leu (CUN)	11,652−11,721	70	11,692−11,762	71
ND5	11,722−13,534	1813	11,763−13,559	1797
ND6	13,535−14,056	522	13,556−14,080	525
tRNA-Glu	14,057−14,124	68	14,081−14,149	69
Cyt b	14,128−15,267	1140	14,153−15,292	1140
tRNA-Thr	15,268−15,332	65	15,295−15,362	68
tRNA-Pro	15,335−15,401	67	15,364−15,430	67
D-loop	15,402−16,805	1404	15,431−16,745	1315

[a] There are length differences for the complete mtDNA sequences of the three Chinese tree shrews. Compared with the one showed in this Table, the other two tree shrew mtDNAs harbored a 19_20insT in the tRNA-Phe gene and had a control region fragment with a size of 1387 bp and 1354 bp, respectively (Fig. S1).

presence of phylogenetic signal was evaluated by constructing a maximum likelihood (ML) tree using MEGA 5.0 (Tamura et al., 2011). The best-fit model for amino acid sequence of mitochondrial gene in vertebrates at different taxonomic levels is MtMam; the rate variation among sites for the gamma shape parameter was estimated by using ProTest 2.4 (Abascal et al., 2005) and the finally yielded gamma value is 0.538.

We used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) to obtain a phylogenetic tree. We employed the GTR + I + G model as the substitution model to assign different rates for different residues, and the MtMam model was set as the default amino acid model. The Bayesian Markov chain Monte Carlo method was used to obtain phylogenetic inference and to achieve convergence (when the two simultaneous MrBayes runs produce a good sample from the posterior probability distribution). The default run started with one cold chain and three heated chains for 20 million generations and every 10 samples were retained to get the final result.

3. RESULTS

3.1. Mitochondrial genome organization

The mitochondrial genomes of three Chinese tree shrews sequenced in this study showed length variation, with 16,805 nt, 16,791 nt and 16,758 nt, respectively. The mitochondrial genome of Malayan flying lemur was 16,745 nt in length. The arrangement of polypeptide-coding genes, RNA-specifying, and non-coding regions in these two species are listed in Table 1; the two species shared the general gene organization and structure with available mammals including human. We observed two different repeat motifs (((CA)nTACACG)nTACACGTA; (CA)nTACACG repeat) in the mtDNA control region of the three tree shrews, which presented length polymorphisms (Fig. S1).

To reveal the genetic difference at the intraspecies level for tree shrew and flying lemur, we counted the number of synonymous and non-synonymous variants according to the pairwise comparison of the 13 mitochondrial protein-encoding genes (Table 2). Among the ~11,400 bp fragment of tree shrew, a total of 838 variable sites were found between Chinese tree shrews and the reported Northern tree shrews (NC_002521), in which 737 are synonymous and 101 are non-synonymous. In contrast, up to 1656 synonymous variants and 474 non-synonymous variants were observed between Malayan flying lemur and Sunda flying lemurs, suggesting that the three lemurs had deeper divergence. The frequency of mutation significantly varied among different genes, with the highest value in the *MT-ND5* gene for tree shrews and in the *MT-ND6* gene for flying lemurs (Table 2).

3.2. Phylogenetic tree

The phylogenetic tree that contained representative species of the Euarchonta and the Glires is shown in Fig. 1. We included nine representatives of the primates (human, gorilla, Sumatran orangutan, gibbon, hamadryas baboon, pygmy chimpanzee, common chimpanzee, slow loris, and tarsier), two Sunda flying lemurs and one Northern tree shrew for comparison with our sequences. Species belonging to the Glires included pika, rabbit, brown hare, mouse, rat, cane rat,

Table 2
Intraspecific differences of 13 mitochondrial protein-encoding genes between Chinese tree shrews and reported Northern tree shrew and between Malayan flying lemur and Sunda flying lemur

Gene	Tree shrew				Flying lemur			
	CDS length (bp)		No. of variants		CDS length (bp)		No. of variants	
	CTS	NTS	S	NS	MFL	SFL	S	NS
ND1	957	957	57	7	957	957	110	22
ND2	1044	1044	59	11	1044	1044	134	54
COI	1542	1542	99	1	1554	1541	202	15
COII	684	684	44	0	684	684	91	10
ATP8	204	204	8	6	204	204	21	19
ATP6	681	681	55	6	681	681	90	24
COIII	786	786	42	1	784	784	101	17
ND3	347	347	22	1	347	347	43	6
ND4L	297	297	16	2	297	297	40	7
ND4	1378	1378	90	11	1428	1378	178	43
ND5	1813	1813	146	17	1797	1797	249	94
ND6	522	522	14	28	525	525	243	132
Cyt b	1140	1140	85	10	1140	1140	154	31
Total	11,395	11,395	737	101	11,438	11,379	1656	474

The synonymous (S) and non-synonymous (NS) variants in 13 mitochondrial protein-encoding genes were counted in three Chinese tree shrews (CTS, GenBank accession Nos. JN800722−JN800724), one Northern tree shrew (NTS, NC_002521), one Malayan flying lemur (MFL, JN800721), and two Sunda flying lemurs (SFL, AF460846.1 and NC_004031.1).

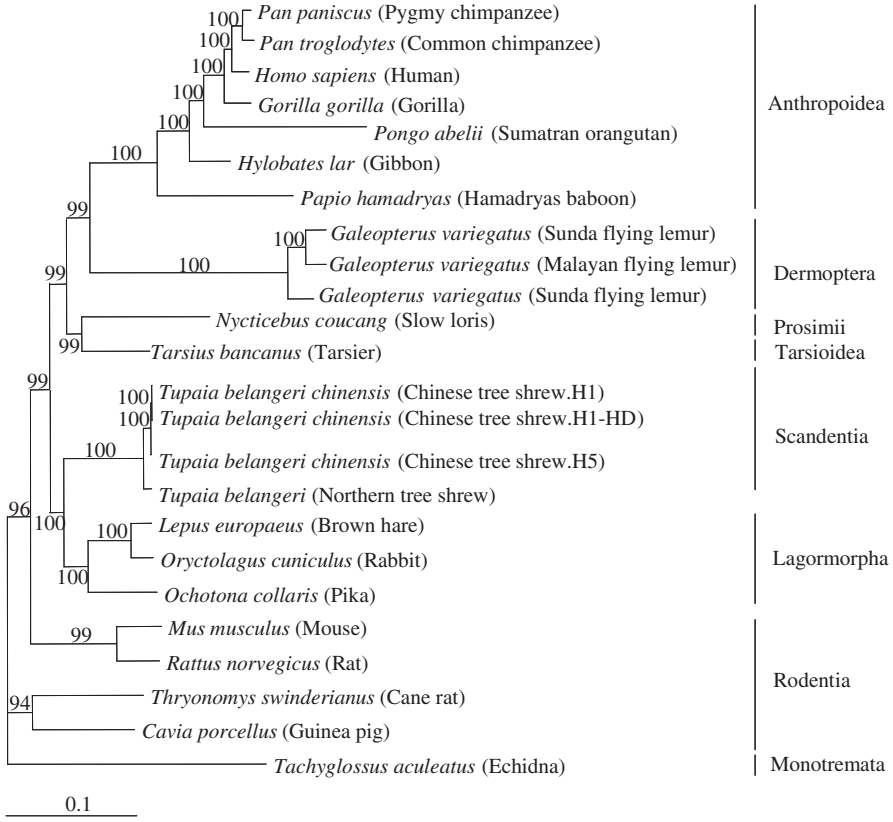

Fig. 1. Bayesian tree of 18 species of Euarchontoglires.
The tree was reconstructed on the basis of the amino acid sequences of 12 concatenated protein-coding genes on the mitochondrial H-strand. Detailed information for species belonging to Rodentia, Lagomorpha, Scandentia, Dermoptera, and Primates from published sources is listed in Table S2. The tree was rooted by the echidna (*Tachyglossus aculeatus*). The Bayesian posterior probabilities are indicated on the branches.

and guinea pig (Table S2). These species were considered to show the overall affinity of tree shrews (Scandentia) to species of Rodentia, Lagomorpha, Dermoptera, and Primates.

We observed a strong phylogenetic signal based on the maximum likelihood method for the amino acid sequences of 12 concatenated protein-coding genes, which indicated the suitability of the data set. In the maximum likelihood tree, species of Scandentia was first clustered with species of Lagomorpha (Fig. S2). We further inferred the phylogenetic relationship of the taxa in question using the Bayesian method. The Primates/Dermoptera and Lagomorphs/Scandentia clades were well supported, with 100% Bayesian posterior probabilities (Fig. 1). Note that the three flying lemurs formed a sister clade with species of Anthropoidea in both ML and Bayesian trees, whereas slow loris (Suborder Prosimii) and tarsier (Suborder Tarsioidea) diverged after the Dermoptera/Anthropoidea cluster. This pattern was also reported in a previous study for mammalian mitogenomic relationships (Arnason et al., 2002).

4. DISCUSSION

Scandentia belongs to the Euarchonta. The representative species of Scandentia, tree shrew, shares some characteristics of both the ancestral and modern primates (Peng et al., 1991; Murphy et al., 2001; Janečka et al., 2007). Tree shrews also harbor several unique features of experimental animals and have long been proposed to be an ideal substitute for the use of nonhuman primates in biomedical research and safety testing (Peng et al., 1991; Cao et al., 2003; Fuchs and Corbach-Söhle, 2010). However, there are some debates regarding the exact relationship among Scandentia, Dermoptera, and Primates. Previous studies based on mtDNA data showed that Scandentia had a closer relationship to Lagomorpha than to Dermoptera or Primates (Schmitz et al., 2000; Arnason et al., 2002). In contrast, analyses for nuclear genes showed that Scandentia had a close affinity to Primates (Murphy et al., 2001; Janečka et al., 2007), whereas molecular cytogenetic data supported for a Scandentia−Dermoptera sister clade (Nie et al., 2008). During the revision of our manuscript, Lindblad-Toh et al. (2011) performed a comparative analysis of 29 eutherian genomes and they showed that tree shrew had a close affinity to primates in their phylogenomic tree.

In this study, we revisited this hot topic by sequencing the entire mtDNA genomes of three Chinese tree shrews (Scandentia) and one Malayan flying lemur (Dermoptera) and compared to other species in Euarchontoglires, with an intention to tentatively answer the old question regarding the genetic affinity of tree shrews to primates. The entire mtDNA

data generated in this study will be supplemental to decipher the nuclear mitochondrial pseudogenes (NUMTs) in the entire genome sequence of Chinese tree shrew (The latter sequencing project was carried out by the Kunming Tree Shrew Genome Sequencing Consortium led by our group and BGI). Consistent with previous results based on mtDNA data (Schmitz et al., 2000; Arnason et al., 2002), our analysis of Chinese tree shrews also revealed a closer relationship of Scandentia to Glires. Intriguingly, the newly determined Malayan flying lemur was clustered with one reported Sunda flying lemur and diverged from another reported one. This pattern suggested that flying lemur has a relatively high intraspecific variation. Nonetheless, the Dermoptera clade was first clustered with species in Anthropoidea in the phylogenetic trees, suggesting their close matrilineal affinity. It should be mentioned that the phylogenetic alliances of tree shrew and flying lemur based on mtDNA genomes alone had a limitation, basically caused by the naive shortcomings of mtDNA molecule as a genetic marker (Galtier et al., 2009) and the current result should be received with caution. A careful examination of multiple unlinked genetic loci (best provided by the entire genome) in a phylogenetic context, as well as, morphological, behavioral, and ecological data is required to establish the reliable phylogeny (Galtier et al., 2009).

Due to morphological similarity of species in Scandentia, the exact phylogenetic relationship has not been unequivocally established at the species level. Most recently, Roberts et al. (2011) analyzed the phylogeny and divergence times among 20 currently recognized species in Tupaiidae based on the mitochondrial 12S RNA, $tRNA^{Val}$, and 16S RNA gene sequences. They found that eight *T. belangeri* samples from Vietnam, Myanmar, and Cambodia showed substantial genetic differentiation although they were considered as conspecific, and these differences were well corresponded to their geographical locations. Chinese tree shrews have been classified into six subspecies of *T. belangeri* according to the morphological and morphometric data (Wang, 1987). In our recent analysis for genetic diversity and matrilineal structure in Chinese tree shrews inhabiting Kunming, we noticed a remarkable difference between tree shrews from urban Kunming and the reported Northern tree shrew, and recognized several prevalent haplotypes (e.g., H1, H2, and H5) in our tree shrew samples (Chen et al., 2011). The newly determined mtDNA genome sequences of Chinese tree shrew will help us to further define the divergence between tree shrews from Kunming and the reported Northern tree shrews elsewhere, as well as, between different prevalent haplotypes in Chinese tree shrew population. In the phylogenetic tree, tree shrews from Kunming were clearly separated from the reported Northern tree shrew outside China. A simple counting for sequence variations in the 13 mitochondrial protein-encoding genes between our tree shrews and the reported Northern tree shrew showed a value smaller than that between Malayan flying lemur and Sunda flying lemur (Table 2), which would suggest a smaller intraspecific variation within *T. belangeri* than that of *G. variegatus*. However, as we were unable to sample all six subspecies proposed by Wang (1987), future study will be encouraged to testify their respective subspecies status.

In summary, we determined the entire mtDNA genome sequences for three Chinese tree shrews (in which one was undergoing the whole genome sequencing) and one Malayan flying lemur. Phylogenetic analysis of 12 concatenated mtDNA protein-coding genes showed that species of Scandentia and Lagomorpha were composed of a sister clade, whereas species of Dermoptera and Primates consisted of another sister clade. This pattern was in general agreement with previous studies based on mitochondrial gene analysis (Schmitz et al., 2000; Arnason et al., 2002), but was inconsistent with the analyses of multiple nuclear genes (Janečka et al., 2007; Lindblad-Toh et al., 2011). The phylogenetic divergence of tree shrews and Primates should be well considered when we attempted to develop them as alternative experimental animals to primates in biomedical research. We expect that the ongoing sequencing project for the whole genome of Chinese tree shrew will provide more information to clarify this important issue and to discern the biological uniqueness of tree shrew, as well as, to uncover whether it can be used as an alternative experimental animal to primates in biomedical research and drug discovery.

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SUPPLEMENTARY DATA

Fig. S1. Tandem repeats in the mtDNA control region sequence of three Chinese tree shrews (H5, H1 and H1-HD).

Fig. S2. Maximum likelihood tree of 18 species of Euarchontoglires. The tree was reconstructed on the basis of the amino acid sequence of 12 concatenated protein-coding genes on the mitochondrial H-strand and was rooted by the echidna (*Tachyglossus aculeatus*).

Table S1. Primer information for sequencing the complete mtDNA of Chinese tree shrew and Malayan flying lemur.

Table S2. Mammalian species (re-)analyzed in this study.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jgg.2012.02.003.

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Supplementary data

Table S1
Primer information for sequencing the complete mtDNA of Chinese tree shrew and Malayan flying lemur

Chinese tree shrew		**Malayan flying lemur**	
Primer name	**Primer sequences (5'→3')**	**Primer name**	**Primer sequences (5'→3')**
L14038	ACAAAAACACAATATAAGGCA [F1]	L10	GCTTAATTTGCCAAAGCAAGG [F1]
H15317	CTCCGTTTCTGGTTTACAAG [R1]	L435	AAAATAATCCACGAAAGTG [S]
L14038	ACAAAAACACAATATAAGGCA [F2]	L872	AGAATAGAGAGCTTAATTG [S]
L15041	TCCCATTCCTTCACACGTC [S]	L1316	GACAAAGAGAACTTTAGCC [S]
t15615	GTACTATGCACGTCTTC [R2]	L1780	TATATCAGACCGAATACCC [S]
L15493	CATTAATATATGGTACAG [F3]	L2044	GCAAGTCCCTAATTAAGGAAC [R1]
L15910	AATTGAAGATCGCCCACA [S]	L1963	AAGTATTAGAGGCACTGCCTG [F2]
L16446	CCCTACCCCCCCATCTTAAC [S]	L2455	TGCAACAGCTATTAAAGGTTC [S]
H70	TGTATGTTTATGGAGTCTATG [R3]	L 2854	GCAAAGGACCCAACATCGTAG [S]
L15910	AATTGAAGATCGCCCACA [F4]	L3349	ACCTAACAGAGGGTGAATC [S]
L256	ATGAACGAAAGTTTGAC [S]	L3778	AGAATTATGGGAATCGAAC [S]
L739	GTTAGGTCAAGGTGTAG [S]	H4015	GGGCTAATGTATTTATTTCCA [R2]
H1353	CACAGATGAATTTGCTC [R4]	L3885	CCGAAAATGTTGGTTCACATC [F3]
L1266	CATAATGAACTAGCCAG [F5]	L4318	AAAAACTAGCCCCCATCTC [S]
L1744	GCACAAGCCTATATCAG [S]	L4943	TCCCCCCATACTAGATTAG [S]
L2235	GGGTGACCTCGGAGAAC [S]	L5398	CTCTACCTCCTATTTGGTG [S]
L2712	AATCCTCTCCTTAACAC [S]	H5976	GTGTTTAAATTTCGGTCCGTT [R3]
H3144	GACTTCGTACGAGATTGT [R5]	L5849	TCATTAACATGAAGCCACCAG [F4]
L2968	GCCTGGCACTATCTATATG [F6]	L6307	TTCAGCTGACTTGCTACAC [S]
L3565	GCTTCCGATACGACCAACT [S]	L6814	ACAAACCTAGAATGACTGC [S]
L4071	TCACACAAGCCACAGCATC [S]	L7268	ATCGCTACGGATTCTTTAC [S]
L4526	ACAGCCGTATTCATGATC [S]	L7561	GACGTCTGAACCAAACAAC [S]
H5217	GAGAATCCATGTCGAATTG [R6]	H7876	GGGTCGGGATAATAAGTAAAT [R4]
L5010	ACTGCGAGACTCAACCTCAC [F7]	L7798	GGACACCTCAACATGATTCAC [F5]
L5480	GTCATCGTTACTGCTCAC [S]	L8182	ATTGGCTCAACAAATCTAC [S]
L5950	CGAAATCTAAACACGAC [S]	L8539	CTACTCACCATGCTTGAAC [S]
L6484	GGAGGCTTTGTCCACTG [S]	L8984	AGGCATCAATCCTCTCAAC [S]
H7017	CGTCCTGAAATCCTAGTTG [R7]	H9445	TTGACCAGATCCTATTGATTG [R5]
L6814	CCCCCTTACCATACATTTG [F8]	L8984	AGGCATCAATCCTCTCAAC [F6]

L7308	TCAATGATACTGAAGCTACG [S]	L9579	ATCAAGTCCATACGAATGTGG [S]
L7834	AATTATCCAGCTACTGCTAC [S]	L10262	TCGACAACTCACAGCCTAC [S]
L8390	CGGCTAACAGCGAACATCAC [S]	L10653	CCATACCACTATTAATTGC [S]
H8865	GCCTGCAAAGAAAAACACT [R8]	L11034	CTCTCATCGCATATTCATC [S]
L8753	CCATGTACCAATGATGACG [F9]	L11572	TTTAGACAAAACATCAAGC [S]
L9195	CACAGGATTCCACGGACTC [S]	L11930	TCACCTCCAACTGACACTG [S]
L9636	CTGTTCGACCTGGAAATTG [S]	H12396	GATTTACCTGCTGCTGCAAGT [R6]
L10079	AGGACTAGCCTTACTTG [S]	L12142	CTGTTCATCGGATGAGAAGG [F7]
L10469	ATCATGACATTCTCAGC [S]	L12770	GTTCCGGCTCCATTATCCA [S]
H11022	CACAAGGGCTATATGACTAA [R9]	L13117	ATAGTTGGCAGTATCTTCG [S]
L10469	ATCATGACATTCTCAGC [F10]	H14169	CAATGAATGAGTGGTTAAT [R7]
L11137	TAACTCAAACTACGAGC [S]	L15320	TCTTGTAAACCAGAAATGGAG [F8]
L11666	GAAGTAATCCGTTGGTC [S]	L15789	TAATCTGACAAGCTCCGAG [S]
H12254	GAATCCGATGTCACCAA [R10]	L16144	GGGGTTAATTCATTCATGC [S]
L12142	CTGTTCATCGGATGAGAAGG [F11]	L16509	AAGACGTACAACAAAACTC [S]
L12580	CTATTTACAGCCATCTGTGC [S]	H111	GCGGATACTTGCATGTGTAAT [R8]
L13032	CTTCCCACCTACCATTAC [S]	—	—
L13620	TAATCATAAATGGACGC [S]	—	—
H14169	CAATGAATGAGTGGTTAAT [R11]	—	—

The primers for amplifying the mtDNA genome were marked by “F” (forward) and “R” (reverse), together with the fragment number. The inner sequencing primers were denoted by “S”. The primer locations were scored relative to the mtDNA genome sequence of Northern tree shrew (GenBank accession No. NC_002521) and Sunda flying lemur (NC_004031), respectively. The “L” and “H” represented the light (L) strand and heavy (H) strand of mtDNA, respectively.

Table S2

Mammalian species (re-)analyzed in this study

Species	Common name	Reference	GenBank accession No.
Eutheria			
Euarchontoglires			
Euarchonta			
Primates			
Nycticebus coucang	Slow loris	Arnason et al., 2000	AJ309867
Tarsius bancanus	Tarsier	Schmitz et al., 2002a	AF348159
Papio hamadryas	Hamadryas baboon	Arnason et al., 1998	Y18001
Pan paniscus	Pygmy chimpanzee	Horai et al., 1995	D38116
Pan troglodytes	Common chimpanzee	Horai et al., 1995	D38113
Hylobates lar	Gibbon	Arnason et al., 1996a	X99256
Pongo abelii	Sumatran orangutan	Xu and Arnason, 1996a	X97707
Gorilla gorilla	Gorilla	Xu and Arnason, 1996b	X93347
Homo sapiens	Human	Arnason et al., 1996b	X93334
Dermoptera			
Galeopterus variegatus	Sunda flying lemur	Arnason et al., 2002	NC_004031
Galeopterus variegatus	Sunda flying lemur	Schmitz et al., 2002b	AF460846.1
Galeopterus variegatus	Malayan flying lemur	this study	JN800721
Scandentia			
Tupaia belangeri	Northern tree shrew	Schmitz et al., 2000	AF217811
Tupaia belangeri	Chinese tree shrew	this study	JN800722-JN800724
Glires			
Lagomorpha			
Lepus europaeus	Brown hare	Arnason et al., 2002	AJ421471
Oryctolagus cuniculus	Rabbit	Gissi et al., 1998	AJ001588
Ochotona collaris	Collared pika	Lin et al., 2002	AF348080
Rodentia			
Mus musculus	Mouse	Bibb et al., 1981	J01420
Rattus norvegicus	Brown rat	Arnason et al., 2002	AJ428514
Cavia porcellus	Guinea pig	D'Erchia et al., 1996	AJ222767
Thryonomys swinderianus	Cane rat	Mouchaty et al., 2001	AJ301644
Monotremata			
Tachyglossus aculeatus	Echidna	Janke et al., 2002	AJ303116

Supplementary Figure Legend

Figure S1. Tandem repeats in the mtDNA control region sequence of three Chinese tree shrews (H5, H1 and H1-HD).

Figure S2. Maximum likelihood tree of 18 species of Euarchontoglires. The tree was reconstructed on the basis of the amino acid sequence of 12 concatenated protein-coding genes on the mitochondrial H-strand and was rooted by the echidna (*Tachyglossus aculeatus*).

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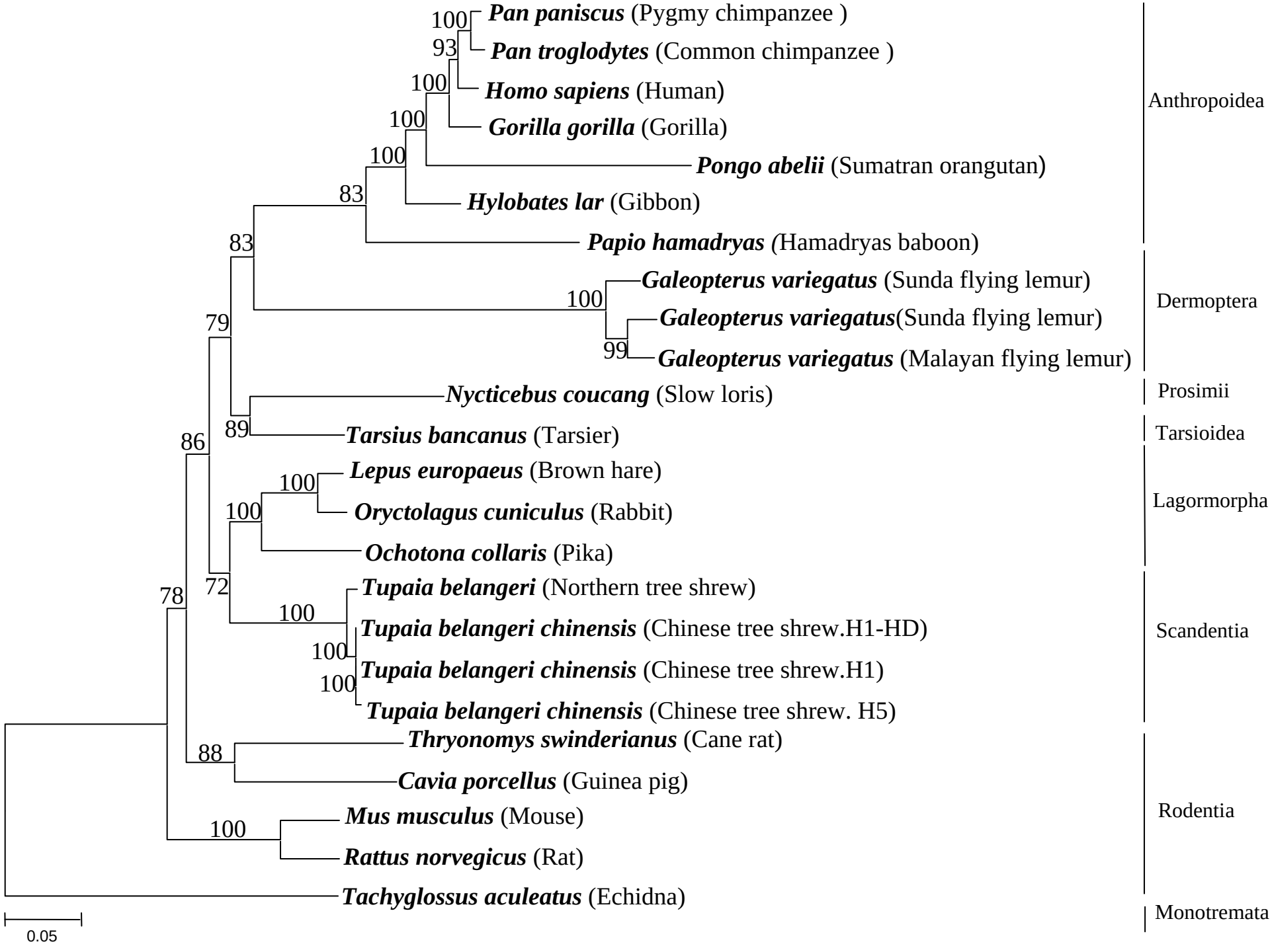
100
Pan paniscus (Pygmy chimpanzee)
93
Pan troglodytes (Common chimpanzee)
100
Homo sapiens (Human)
100
Gorilla gorilla (Gorilla)
100
Pongo abelii (Sumatran orangutan)
83
Hylobates lar (Gibbon)
83
Papio hamadryas (Hamadryas baboon)
Anthropoidea
Galeopterus variegatus (Sunda flying lemur)
100
Galeopterus variegatus(Sunda flying lemur)
79
99
Galeopterus variegatus (Malayan flying lemur)
Dermoptera
Nycticebus coucang (Slow loris)
Prosimii
89
Tarsius bancanus (Tarsier)
Tarsioidea
86
Lepus europaeus (Brown hare)
100
100
Oryctolagus cuniculus (Rabbit)
Ochotona collaris (Pika)
Lagormorpha
72
Tupaia belangeri (Northern tree shrew)
78
100
Tupaia belangeri chinensis (Chinese tree shrew.H1-HD)
100
Tupaia belangeri chinensis (Chinese tree shrew.H1)
100
Tupaia belangeri chinensis (Chinese tree shrew. H5)
Scandentia
Thryonomys swinderianus (Cane rat)
88
Cavia porcellus (Guinea pig)
Mus musculus (Mouse)
100
Rattus norvegicus (Rat)
Rodentia
Tachyglossus aculeatus (Echidna)
Monotremata
0.05