ความสัมพันธ์เชื่อมโยงระหว่างกันและกันทางวิวัฒนาการของสิ่งมีชีวิตในกลุ่มลอราเซียเทอเรียบนฐานข้อมูลอินทรอน 1 ของยีนทรานส์ไทรีติน

Transthyretin Intron 1 based Interordinal Relationships of Laurasiatheria

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บทคัดย่อ

ทรานส์ไทรีติน (transthyretin) เป็นโปรตีนหลักชนิดหนึ่งที่ทำหน้าที่ขนส่งฮอร์โมนไทรอยด์ในเลือด ยีนที่ควบคุมการสังเคราะห์ประกอบด้วยเอ็กซอน (exon) 4 ส่วน และอินทรอน (intron) 3 ส่วน ในปัจจุบัน มีการนำข้อมูลจากอินทรอนของยีนหลายชนิด รวมทั้งอินทรอนส่วนที่ 1 ของยีนทรานส์ไทรีติน (transthyretin intron 1) มาใช้เพื่อแยกแยะความสัมพันธ์เชื่อมโยงระหว่างกันและกันทางวิวัฒนาการของสิ่งมีชีวิต ในครั้งนี้ผู้วิจัยได้นำข้อมูลที่มีอยู่ในลำดับนิวคลีโอไทด์ของอินทรอนส่วนที่ 1 ของยีนทรานส์ไทรีติน มาใช้ในการแยกแยะความสัมพันธ์ทางวิวัฒนาการของสัตว์เลี้ยงลูกด้วยน้ำนม (mammals) ในกลุ่มลอราเซียเทอเรีย (Laurasiatheria) โดยลำดับนิวคลีโอไทด์ของอินทรอนส่วนที่ 1 ของยีนทรานส์ไทรีติน จากสัตว์และมนุษย์ จำนวน 36 ชนิด ได้จากการสกัดข้อมูลจากจีโนมที่มีอยู่ในยีนแบงค์ (GenBank) ส่วนลำดับนิวคลีโอไทด์ของอินทรอนส่วนที่ 1 ของยีนทรานส์ไทรีติน จากสัตว์อีก 2 ชนิด คือ ค้างคาวมงกุฎเทาแดง (*Rhinolophus affinis*) และค้างคาวเพดานเล็ก (*Scotophilus kuhlii*) ได้จากการเพิ่มปริมาณชิ้นยีนด้วยปฏิกิริยาลูกโซ่พอลิเมอเรส (polymerase chain reaction หรือ PCR) แล้วนำไปหาลำดับ จากการสร้างแผนภูมิความสัมพันธ์เชิงวิวัฒนาการของสิ่งมีชีวิต (phylogenetic relationship) โดยอาศัยโปรแกรม MEGA 6 โดยอาศัยข้อมูลจากอินทรอนส่วนที่ 1 ของยีนทรานส์ไทรีติน พบว่าแผนภูมิสามารถแยกและจัดอันดับทางวิวัฒนาการ (order) ของสัตว์ที่ศึกษาได้ ที่ความเชื่อมั่นบูตสแตรปมากกว่า 75 เปอร์เซ็นต์ (bootstrap >75%) ความสัมพันธ์กลุ่มวงศ์วานวิวัฒนาการเดี่ยว (monophyletic relationship) ของแต่ละอันดับมีค่าความเชื่อมั่นบูตสแตรปสูง ช่วงตั้งแต่ 67 เปอร์เซ็นต์ ในอันดับสัตว์กินแมลง Eulipotyphla ถึง 100 เปอร์เซ็นต์ ในอันดับซีตร้าทิโอแดกทีลา (Cetratiodactyla) และอันดับสัตว์กินเนื้อ (Carnivora) อย่างไรก็ตามการวางอันดับของสัตว์กีบคี่ หรือเพอริสโซแดกทีลา (Perissodactyla) เป็นพี่น้อง (sister group) กับอันดับสัตว์กินเนื้อ

มีค่าความเชื่อมั่นบูตสแตรปค่อนข้างต่ำ การจัดความสัมพันธ์แบบเกือบจะเป็นกลุ่มวงศ์วานวิวัฒนาการเดี่ยว (paraphyletic relationship) ของสัตว์ในอันดับไครอบเทอรา (Chiroptera) มีค่าความเชื่อมั่นบูตสแตรปสูงมาก คือ มากกว่า 85 เปอร์เซ็นต์ นอกจากนี้พบว่าค้างคาวมงกุฎเทาแดง มีความใกล้ชิดกับกลุ่มค้างคาวยักษ์ (megabats) มากกว่ากลุ่มค้างคาวขนาดเล็ก (microbats) โดยมีค่าความเชื่อมั่นบูตสแตรปสูงถึง 97 เปอร์เซ็นต์ ซึ่งผลการแยกแยะและจัดกลุ่มสัตว์ที่ได้เหล่านี้ชี้ว่าข้อมูลจากลำดับนิวคลีโอไทด์ของอินทรอนส่วนที่ 1 ของยีน ทรานส์ไทรีติน มีประโยชน์อย่างมากต่อการแยกแยะความสัมพันธ์ทางวิวัฒนาการของสัตว์ในกลุ่มลอราเซียเทอเรีย

ABSTRACT

Transthyretin (TTR), a major thyroid hormone distributor protein (THDP) in the blood, is coded by a gene which contains four exons and three introns. Nowadays, several nuclear gene introns have been successfully utilized to elucidate the relationships of organisms, including TTR intron 1 nucleotide sequence. In this study, the utilization of TTR intron 1 to resolve the interrelatedness of mammals in Laurasiatheria was explored. The complete TTR intron 1 sequences from 36 animal species including human (*Homo sapiens*) were extracted from the genome sequences retrieved from the GenBank. In addition, the sequences from two microbats e.g. intermediat horseshoe bat (*Rhinolophus affinis*) and lesser Asiatic yellow house bat (*Scotophilus kuhlii*) were amplified by PCR and sequenced. The phylogenetic relationship of the studied animals was analyzed using MEGA 6 program. Based on the TTR intron 1, the phylogenetic tree with most of major nodes was strongly supported by bootstrap >75% was successfully produced. The monophyletic relationships within each order were strongly supported, ranging from 67% (within Eulipotyphla) to 100% (within Cetratiodactyla and Carnivora) bootstrap. However, the placement of Perissodactyla as the sister group to Carnivora was supported at low bootstrap. The paraphyletic relationships of the members within Chiroptera was highly supported with >85% bootstrap. In addition, more closely relatedness of the microbat *R. affinis* to the megabats than to the other microbats was supported with 97% bootstrap. The results presented here indicated to the usefulness of TTR intron 1 in resolving the relationships of the animals in Laurasiatheria.

คำสำคัญ: วิวัฒนาการ อินทรอน ลอราเซียเทอเรีย วิวัฒนาการของสิ่งมีชีวิต ทรานส์ไทรีติน
Keywords: Evolution, Intron, Laurasiatheria, Phylogenetic, Transthyretin

INTRODUCTION

Thyroid hormones (THs) regulate growth, development and metabolism of mammals. Based on the high tendency to partition into lipid membranes (Hillier, 1970; Dickson et al., 1987; Mendel et al., 1987), THs require thyroid hormone distributor proteins (THDPs) to ensure their movement from the synthesis site to target cells. In most mammals, transthyretin (TTR) is one of the three major THDPs in the blood. TTR exists mainly as a tetramer of four identical subunits. Each TTR subunit is coded by a single-copy gene, which contains four exons and three introns (Sasaki et al., 1985; Sparkes et al., 1987). Among *vertebrates*, identity of TTR amino acid sequence is relatively high and highly conserved across almost all taxa.

Based on molecular phylogenetics, the animals in placentalia are classified into three major lineages, Afrotheria, Xenarthra, and Boreoeutheria in which the latter contains two major lineages, Euarchontoglires and Laurasiatheria. Within Laurasiatheria, the placement of subclades including Eulipotyphla, Perissodactyla, Chiroptera, Cetartiodactyla and Ferae (Pholidota and Carnivora) is still unclear; therefore, several models of Laurasiatheria interorder relationships were hypothesized (Murphy et al., 2001a; Murphy et al., 2001b; Nishihara et al., 2006; Prasad et al., 2008; Zhou et al., 2011a; Zhou et al., 2011b; Zhou et al., 2012). In addition, within the subclade Cetartiodactyla and Chiroptera in particular, their monophyletic relationships are still controversy (Agnarsson and May-Collado, 2008; Spaulding et al., 2009; Zhou et al., 2011a; Zhou et al., 2011b;). The elucidation of the interordinal relationship of Laurasiatheria and the position of its root are important for interpreting evolutionary processes involved in the early diversification of placental mammals.

Nowadays, several nuclear gene introns have been utilized to elucidate the relationships of organisms because their basic characters including a high conserve of positions in a gene are match to the requirements for an effective phylogenetic marker (Roy and Gilbert, 2005; Coulombe-Huntington and Majewski, 2007; Roy and Penny, 2007). Among these nuclear gene introns, TTR intron 1 has been successfully utilized to resolve the relationships of vertebrates including rodent (Walton et al., 2000), opossums (Steiner et al., 2005), and crocodile (Willis, 2009). In this study, we explore the utilization of TTR intron 1 sequence to resolve the interrelatedness of mammals in Laurasiatheria. The complete TTR intron 1 sequences from 36 animal species were extracted from the genome sequences retrieved from the GenBank, and those of two

microbats e.g. intermediat horseshoe bat (*Rhinolophus affinis*) and lesser Asiatic yellow house bat (*Scotophilus kuhlii*) were amplified by PCR and sequenced. The obtained phylogenetic relationship suggested the usefulness of TTR intron 1 sequence as a marker to elucidate the phylogeny of Laurasiatheria.

RESEARCH METHODOLOGY

Source of animals, blood and tissues

Livers of male *R. affinis* and *S. kuhlii* were removed and immediate frozen in liquid nitrogen and stored at -80°C until used. Bat capture and all experimental procedures were approved by the Department of National Park, Wildlife and Plant Conservation, and the Ethics Committee in Animal Experimentation of Prince of Songkla University, Thailand.

Genomic DNA isolation and amplification of TTR intron 1

Genomic DNAs were isolated from the livers with pancreatic RNase and proteinase K. The liver tissue (1 g) was ground into fine powder prior to the addition of a buffer containing 0.1 M Tris-HCl pH 8.0, 0.1 M EDTA, pH 8.0, 0.2 μg/ml pancreatic RNase and 0.5% SDS), and the homogenate was incubated at 37°C for 1 h. Proteinase K was added to a final concentration of 100 μg/ml and incubated at 50°C for 3 h. DNA was, then extracted with phenol/chloroform and precipitated with ethanol. The DNA pellet was dried, and dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0. The TTR intron 1 fragment was amplified using the purified genomic DNA as template with forward (5′-tggctgccctccgtctcctgctcctctg-3′) and reverse (5′-tcggcagtcttcttgaacaccttcacggc cacg-3′) primers. The reaction was carried out for 30 cycles at 94°C for 5 min, 66°C for 30 s, and 72°C for 1 min. The nucleotide sequence of the product was then determined by automated DNA sequencing.

Phylogenetic analysis

TTR intron 1 sequences of all the studied animals except *R. affinis* and *S. khulli* were manually identified from the retrieved nucleotide sequences of the animal genomes which are available in the GenBank.

The MEGA 6 program (Tamura et al., 2013) was employed to perform maximum likelihood (ML), maximum-parsimony (MP) and neighbor-joining (NJ) analyses of TTR intron 1 nucleotide sequences of the studied species. The sequences were aligned using MUSCLE (Edgar, 2004). Gaps were treated as additional characters. Support for specific nodes was evaluated by bootstrap analysis with 1,000 replicates. In the evolutionary analyses, the substitution models for nucleotide was performed based on the Tamura-Nei models (Tamura and Nei, 1993). Initial tree(s) for the heuristic search was obtained by apply the Neighbor-Joining method to a matrix of pairwise distances which was estimated using

the Maximum Composite Likelihood (MCL) (Tamura et al., 2004). In the evolutionary comparisons, the differences in the composition bias were also considered (Tamura and Kumar, 2002). A discrete Gamma distribution (shape parameter = 10), was used to model the rate differences among sites. All positions with less than 70% site coverage were eliminated.

RESULTS

Characteristics of TTR intron 1 from *R. affinis* and *S. kuhlii*

By PCR and in the presence of specific primers which designed based on the conserved regions flanking exon1/exon2 border of TTR precursor, the full length sequences of TTR intron 1 of *R. affinis* and *S. kuhlii* were obtained. On 1% agarose gel, the PCR products, covering TTR intron 1 and sections of exon 1 and exon 2, were approximate 900 bp. Alignment of the sequences with human TTR intron 1 revealed two homologous sequences to glucocorticoid receptor (GCR)-binding site in bat TTR intron 1 (Figure 1).

Phytogenetic analysis of TTR intron 1

To elucidate the relatedness of members in Laurasiatheria, nucleotide sequences of TTR intron 1 from *R. affinis* and *S. kuhlii* were put in analysis together with those of other 35 animal species from five orders including Eulipotyphla (families Erinaceidae, Soricidae, and Talpidae), Perissodactyla (families Rhinocerotidae and Equidae), Carnivora (families Canidae, Felidae, Ursidae, Odobenidae, and Phocidae), Cetartiodactyla (families Bovidae, Camelidae, Balaenopteridae, Physeteridae, Delphinidae, and Lipotidae), and Chiroptera (families Vespertilionidae and Pteropodidae) which were identified from the retrieved nucleotide sequences of the animal genomes that are available in the GenBank, using TTR intron 1 from human (*Homo sapiens*) as an outgroup. Sizes of the TTR intron 1 were ranged from 853 bp in *Vicugna pacos* to 1560 bp in *Bubalus bubalis* (Table 1). Of the 826 to 1560 bp examined which corresponded to 2778 characters including gaps, there were 1081 variable sites, 516 conserved sites, 778 parsimony informative sites, and 288 singleton sites. Analysis of the nucleotide sequence exhibited variation in base composition among the sequences (Table 2). High content of AT (>60%), was observed in the sequences from Eulipotyphla, Perissodactyla, and the megabats in Chiroptera.

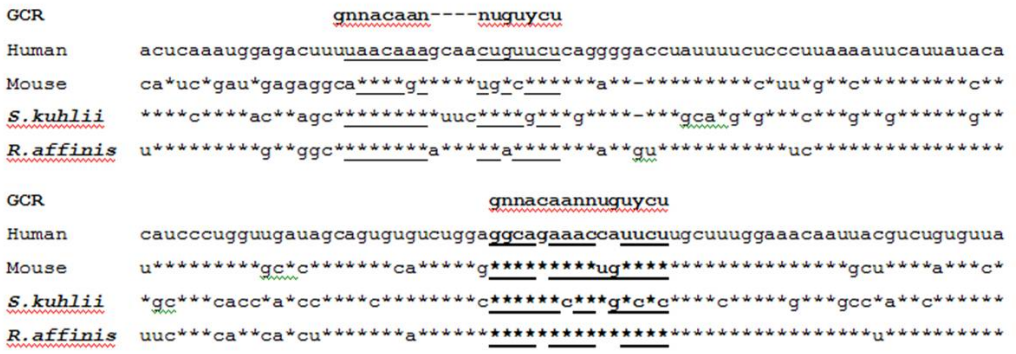

Figure 1 The alignment of nucleotide sequences at the 3′region of TTR intron 1 from *S. kuhlii* and *R. affinis* with those of human (Tsuzuki et al., 1985) and mouse (Wakasugi et al., 1986). The sequence of human glucocorticoid receptor-binding site (GCR) is given above the sequence of human TTR intron 1, and the sequence which is homologous to GCR is indicated by underlined. Asterisks show residue identical to that found in human TTR.

The nucleotide sequences of TTR intron 1 from all of the studied animals including *R. affinis* and *S. kuhlii* were aligned with MUSCLE (Edgar, 2004) which implemented in MEGA 6 program, followed by phylogenetic analyses using the MEGA program for maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) tree searches. All of the analyses, using *Homo sapiens* as outgroup, produced phylogenetic trees with the same interrelated patterns, and most of major nodes were strongly supported by bootstrap analyses (bootstrap values >75%). According to ML, Eulipotyphla was placed to the basement of Laurasiatheria which was then followed by Chiroptera. Carnivora was placed sister to Perissodactyla which was closely associated with Cetratiodactyla (Artiodactyla + Cetacea). Monophyletic relationships of the members in each suborder i.e. Cetratiodactyla, Chiroptera, Perissodactyla, Carnivora, but except Eulipotyphla, were supported with >85% bootstrap. Within Cetratiodactyla, the phylogenetic analysis showed a separation between the two families of Artiodactyla i.e. Bovidae and Camelidae. The animals of Bovidae showed more closely relatedness to Cetacea than to Camelidae, and the clade formed sister group with Cetacea (Figure 2).

Genetic distance analyses of the animal species by using uncorrected pair-wise (p-distance) values and maximum composite likelihood provided similar values. Based on the likelihood analysis, the mean distances between groups ranged from 0.204 (between

Artiodactyla and Cetacea) to 0.446 (between Eulipotyphla and Artiodactyla) (Figure 3). In addition, according to mean distance within group, the phylogenetic divergence was highest in Eulipotyphla and lowest in Cetacea (Figure 4). Within Chiroptera, *R. affinis* showed 0.173 and 0.174 divergent from *P. alecto* and *P. vampyrus*, respectively, whereas it was 0.301 to 0.319 deviated from the other studied microbats (Figure 5). This revealed that the microbat *R. affinis* is more closely related to the megabats than to the other microbats, and therefore, it indicated to the paraphyly of the members in Microchiroptera. Within Artiodactyla, the animals in family Bovidae formed a sister group with Cetaceans rather than with family Camelids, and the deviation of Bovidae from Camelidae was supported by pairwise distance. While the mean distance between Bovidae and Cetacea was 0.289 (ranged from 0.270, between *Bos mutus* and *Balaenoptera* acutorostrata, to 0.316, between Pantholops hodgsonii and *Lipotes vexillifer*), the mean distance between Bovidae and Camelidae was 0.309 (ranged from 0.308, between *Bubalus bubalis* and *Vicugna pacos*, to 0.331, between *Capra hircus* and *Camelus ferus*) (Table 6).

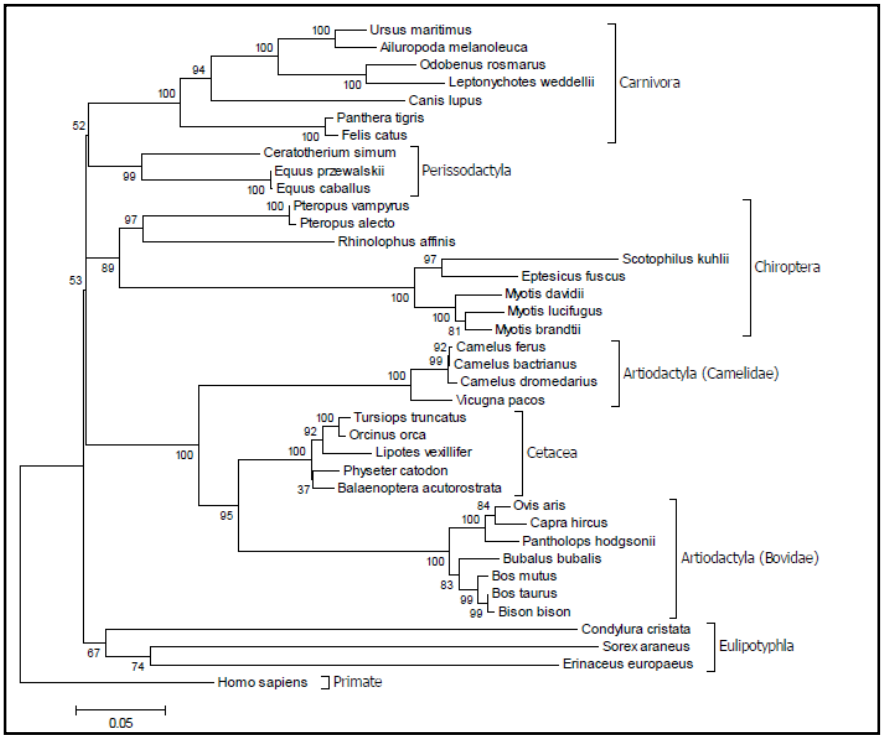

Figure 2 Phylogenetic tree of the studied animals in Laurasiatheria

DISCUSSION AND CONCLUSION

Nowadays, utilization of the nuclear gene introns as phylogenetic markers become more attractive for elucidate the relationships of organisms because of their unique characters that match to the basic requirements of the general phylogenetic markers including a high conserve of positions in a gene (Roy and Gilbert, 2005; Coulombe-Huntington and Majewski, 2007; Roy and Penny, 2007), a high variable but low rate change of the nucleotide at each positions in the nuclear intron, and independent and identically distributed of each nucleotides in the introns (Bulmer, 1987). Up to date, the intron sequences of several nuclear genes have been utilized to construct or reconstruct the phylogenetic relationships of mammalians (Hassanin et al., 2013; Nesi et al., 2013; Hassanin et al., 2015). Among of these nuclear gene introns, TTR intron 1 has been successfully utilized to resolve the relationships of several vertebrates including rodent (Walton et al., 2000), opossums (Steiner et al., 2005), and crocodile (Willis, 2009).

Table 1 Sizes of TTR intron 1 (base pair; bp) of all the studied animals in Laurasiatheria

Superorder	Order (suborder)	Family	Scientific name (Common name)	TTR intron 1 (bp)
Eulipotyphla	Erinaceomorpha	Erinaceidae	*E. europaeus* (European hedgehog)	1514
	Soricomorpha	Soricidae	*S. araneus* (Common shrew)	879
		Talpidae	*C. cristata* (Star-nosed mole)	1160
	Perissodactyla	Rhinocerotidae	*C. simum* (White rhinoceros)	888
		Equidae	*E. caballus* (Horse)	896
			E. przewalskii (Wild horse)	896
	Carnivora	Canidae	*C. lupus* (Dog)	1125
		Felidae	*F. catus* (Cat)	905
			P. tigris (Tiger)	904
		Ursidae	*A. melanoleuca* (Panda)	1100
			U. maritimus (Polar bear)	1094
		Odobenidae	*O. rosmarus* (Walrus)	1105
		Phocidae	*L. weddellii* (Weddell seal)	1110
	Cetartiodactyla (Artiodactyla)	Bovidae	*B. bison* (American bison)	1295
			B. mutus (Yak)	1294
			B. taurus (Cow)	1295
			B. bubalis (Buffalo)	1560
			C. hircus (Goat)	1283
			O. aris (Sheep)	1285
			P. hodgsonii (Tibetan antelope)	1283
		Camelidae	*C. bactrianus* (Bactrian camel)	864
			C. dromedaries (Arabian camel)	949

Table 1 Sizes of TTR intron 1 (base pair; bp) of all the studied animals in Laurasiatheria (continue)

Superorder	Order (suborder)	Family	Scientific name (Common name)	TTR intron 1 (bp)
		Camelidae	*C. ferus* (Wild Bactrian camel)	864
			V. pacos (Alpaca)	853
	Cetartiodactyla (Cetacea)	Balaenopteridae	*B. acutorostrata* (Common minke whale)	1011
		Physeteridae	*P. catodon* (Sperm whale)	1006
		Delphinidae	*O. orca* (Killer whale)	1010
			T. truncates (Bottlenose dolphin)	1011
		Lipotidae	*L. vexillifer* (Freshwater dolphin)	1009
	Chiroptera (Yangochiroptera; Megachiroptera)	Vespertilionidae	*E. fuscus* (Big brown bat)	874
			M. brandtii (Brandt's bat)	820
			M. davidii (David's myotis)	861
			M. lucifugus (Little brown bat)	861
			S. kuhlii (Lesser Asiatic yellow bat)	866
		Rhinolophidae	*R. affinis* (Intermediate horseshoe bat)	890
	Chiroptera (Yinpterochiroptera; Microchiroptera)	Pteropodidae	*P. alecto* (Black flying fox)	884
			P. vampyrus (Large flying fox)	899

Carnivora	0.347				
Cetacea	0.204	0.294			
Eulipotyphla	0.446	0.416	0.395		
Chiroptera	0.390	0.343	0.325	0.440	
Perissodactyla	0.290	0.243	0.226	0.345	0.275
	Artiodactyla	**Carnivora**	**Cetacea**	**Eulipotyphla**	**Chiroptera**

Figure 3 Evolutionary divergence over TTR intron 1 sequence pairs between groups

Mean of base substitutions per site from the overall TTR intron 1 sequence pairs between groups are shown. The analyses were conducted using the Maximum Composite Likelihood (Tamura et al., 2004; Tamura et al., 2013). The differences in the composition bias among sequences were considered in the comparisons (Tamura and Kumar, 2002). The analysis involved 37 sequences, and 869 positions in the final dataset.

Table 2 Base composition of TTR intron 1 nucleotide sequences

Specie	T(U)	C	A	G	A+T (%)	G+C (%)	Order/Suborder	
S. araneus	32.7	19.3	28.8	19.2	61	38.6	Eulipotyphla	
E. europaeus	32.6	19.6	29.2	18.6	62	38.2		
C. cristata	31.3	19.8	27.8	21.1	59	40.9		
P. vampyrus	31.3	21.7	30.1	16.9	61	38.6	Chiroptera	Megachiroptera
P. alecto	31.1	21.8	30.3	16.7	61	38.6		
R. affinis	31.3	20.8	29.2	18.7	61	39.4		Microchiroptera
S. kuhlii	23.6	27.6	22.7	26.1	46	53.7		
M. lucifugus	26.4	26.1	23.6	23.9	50	50.1		
M. davidii	26.9	25.5	23.5	24.2	50	49.7		
M. brandtii	26.8	24.9	24.8	23.5	52	48.4		
E. fuscus	24.4	28.8	22.9	23.9	47	52.7		
P. tigris	30.9	21.6	28.0	19.6	59	41.2	Carnivora	
F. catus	30.9	21.9	27.5	19.7	58	41.5		
C. lupus	30.9	23.9	25.5	19.6	56	43.6		
U. maritimus	28.8	23.9	27.3	20.0	56	43.9		
A. melanoleuca	28.8	23.8	26.6	20.7	55	44.5		
O. rosmarus	28.0	25.2	24.0	22.8	52	48.1		
L. weddellii	28.2	24.8	23.9	23.2	52	47.9		
E. przewalskii	32.4	21.0	28.3	18.3	61	39.3	Perissodactyla	
E. caballus	32.5	21.0	28.2	18.3	61	39.3		
C. simum	32.5	20.3	28.6	18.6	61	38.9		
C. ferus	28.2	23.8	26.2	21.8	54	45.6	Cetratiodactyla	Artiodactyla
C. dromedarius	28.0	24.1	25.6	22.2	54	46.4		
C. bactrianus	28.2	23.7	26.3	21.8	55	45.5		
V. pacos	28.5	23.4	26.3	21.8	55	45.3		
B.s bubalis	27.0	24.6	25.6	22.8	53	47.4		
P. hodgsonii	28.8	23.8	25.2	22.2	54	46.0		
O. aris	29.2	23.6	25.0	22.3	54	45.8		
C. hircus	29.1	23.9	24.9	22.1	54	46.0		
B. taurus	29.4	23.4	25.5	21.7	55	45.1		
B. mutus	29.4	23.3	25.5	21.7	55	45.1		
B. bison	29.5	23.3	25.4	21.8	55	45.1		
B. acutorostrata	28.5	23.2	28.1	20.2	57	43.4		Cetacea
P. catodon	27.4	23.9	28.6	20.1	56	43.9		
L. vexillifer	27.0	24.3	28.3	20.4	55	44.7		
T. truncatus	27.7	23.9	28.0	20.4	56	44.3		
O. orca	27.4	23.9	28.0	20.7	55	44.6		

	Average	S.D.
Artiodactyla	0.177	0.014
Carnivora	0.168	0.012
Cetacea	0.034	0.004
Eulipotyphla	0.463	0.032
Chiroptera	0.214	0.014
Perissodactyla	0.089	0.009
Primate	n/c	n/c

Figure 4 The average evolutionary divergence over TTR intron 1sequence pairs within groups Mean of base substitutions per site with standard deviation from the overall TTR intron 1 sequence pairs within each group are shown. The analysis covered 38 nucleotide sequences, and a total of 869 positions in the final dataset. n/c, not possible to estimate the distances.

R. affinis	0.374						
P. vampyrus	0.375	0.174					
P. alecto	0.369	0.173	0.004				
M. lucifugus	0.143	0.318	0.296	0.292			
M. davidii	0.150	0.315	0.286	0.287	0.051		
M. brandtii	0.150	0.301	0.282	0.282	0.035	0.046	
E. fuscus	0.135	0.319	0.300	0.299	0.103	0.105	0.100
	S. kuhlii	***R. affinis***	***P. vampyrus***	***P. alecto***	***M. lucifugus***	***M. davidii***	***M. brandtii***

Figure 5 The evolutionary divergence between TTR 1 nucleotide sequences within Chiroptera The numbers of base substitutions per site from between sequences are shown. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 8 nucleotide sequences. There were a total of 860 positions in the final dataset.

Hereinto, our analysis results supported the usefulness of TTR intron 1 to clarify the phylogenetic relationships of members in Laurasiatheria.

Laurasiatheria is a supraclade of mammals that the interordinal relationships within the superorder in particular are still controversial. Although many extensive molecular analyses have been performed, it could not be conclusively resolved. These include the placement of Perissodactyla (Murphy et al., 2001a; Murphy et al., 2001b; Matthee and Davis, 2001; Arnason, et al., 2002; Arnason, and Janke, 2002; Lin et al., 2002; Amrine-Madsen et al., 2003), the relatedness of the members in Cetratiodactyla (Hassanin and Douzery, 1999; Matthee and Davis, 2001; McGowen et al., 2009), and paraphyletic

relationship within Chiroptera (Teeling et al., 2002; Ruedi et al., 2013). Based on the TTR intron 1 nucleotide sequence, the relationships of the most animal groups in Laurasiatheria particularly that has been proposed either by traditional or recent molecular phylogenetic analyses were supported. These include monophyletic relationships within each suborder which were strongly supported ranging from 67% (within Eulipotyphala) to 100% (within Cetratiodactyla and Carnivora) bootstrap. In addition, paraphyletic relationships of the members within Chiroptera was highly supported with >85% bootstrap. The placement of Perissodactyla as the sister group to Carnivora was similar to the previous reports (Murphy et al., 2001a; Murphy et al., 2001b; Arnason, and Janke, 2002; Amrine-Madsen et al., 2003); however, the data of TTR intron 1 supported with low bootstrap.

The super clade Cetartiodactyla is one of the most diversified orders of mammals. According to the fossil and molecular evidences, only the animals of suborders Artiodactyla and Cetacea have been placed in the clade (Montgelard et al., 1997). Although many extensive efforts have been made to investigate the phylogeny of Cetartiodactyla (Gatesy et al., 1999; Arnason et al., 2004), the phylogenetic relationships including the monophyly and relationships between the suborders and among the families still are the issues of controversy (Gatesy et al., 1999; Arnason et al., 2000; Matthee et al, 2001; Spaulding et al., 2009); for reviews see (Price et al., 2005; May-Collado and Agnarsson, 2006). Traditionally, Artiodactyla was considered monophyletic and was divided into three major lineages: Suiformes (pigs, peccaries and hippopotamuses), Tylopoda (camels and llamas), and Ruminantia (bovids, deer, tragulids and giraffes). The recent molecular phylogenetic analyses revealed the paraphyly of Artiodactyla due to the sister-group relationship between Cetacea (whales and dolphins) and Hippopotamidae, and subsequently, Artiodactyla was placed into the same order as Cetacea, namely Cetartiodactyla (Montgelard et al., 1997). In our study, the data from TTR intron 1 nucleotide sequences of 16 animal species in Cetaceans (families Balaenopteridae, Physeteridae, Delphinidae and Lipotidaeand) and Artiodactyla (families Bovidae and Camelidae) supported the monophyletic relatedness of the members in Cetacea with 100% bootstrap, which is well agree with many recent reports (Gatesy et al., 1996; Gatesy, 1997; Ursing and Arnason, 1998; Gingerich et al., 2001). More closely relatedness of Bovidae to Cetacea than to Camelidae, and the sister group of Bovidae to

Cetacea were also strongly supported (95% bootstrap). Subsequently, the paraphyletic relationship of the members in Artiodactyla was confirmed. These should conceive to the usefulness of TTR intron 1 nucelotide sequence to clarify the phylogenetic relationships within Cetartiodactyla.

V. pacos	0.308														
T. truncatus	0.283	0.227													
P. catodon	0.278	0.217	0.044												
P. hodgsonii	0.059	0.310	0.295	0.291											
O. aris	0.055	0.311	0.289	0.285	0.029										
O. orca	0.282	0.222	0.010	0.041	0.293	0.286									
L. vexillifer	0.295	0.227	0.042	0.059	0.316	0.307	0.037								
C. hircus	0.064	0.327	0.301	0.298	0.035	0.024	0.296	0.319							
C. ferus	0.312	0.047	0.227	0.217	0.314	0.315	0.222	0.229	0.331						
C. dromedarius	0.311	0.049	0.227	0.217	0.313	0.314	0.222	0.229	0.329	0.007					
C. bactrianus	0.310	0.045	0.225	0.215	0.311	0.313	0.220	0.227	0.329	0.001	0.006				
B. taurus	0.039	0.297	0.287	0.285	0.052	0.049	0.286	0.299	0.058	0.300	0.300	0.298			
B. mutus	0.038	0.299	0.280	0.278	0.052	0.048	0.279	0.296	0.055	0.303	0.303	0.301	0.011		
B. bison	0.041	0.299	0.290	0.288	0.052	0.051	0.289	0.305	0.060	0.302	0.302	0.300	0.004	0.013	
B. acutorostrata	0.273	0.209	0.037	0.036	0.287	0.276	0.034	0.050	0.291	0.209	0.209	0.207	0.277	0.270	0.280
	B. bubalis	*V. pacos*	*T. truncatus*	*P. catodon*	*P. hodgsonii*	*O. aris*	*O. orca*	*L. vexillifer*	*C. hircus*	*C. ferus*	*C. drome-darius*	*C. bactri-anus*	*B. taurus*	*B. mutus*	*B. bison*

Figure 6 The evolutionary divergence of TTR intron 1 sequences from Bovidae, Camelidae and Cetacea

The numbers of base substitutions per site between sequences are shown. The analyses were conducted using the Maximum Composite Likelihood model. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 16 nucleotide sequences. All positions with less than 70% site coverage were eliminated. There were a total of 986 positions in the final dataset.

Chiroptera is the clade of bats. Among small mammals, bats have evolved special body structure and adaptive behaviors. Although they are the only mammals having the capable to fly for locomotion similar to birds, their evolution and adaptations are much distinguishable from birds. Therefore, the evolutionary relationships among populations and to the closest mammalian relatives of the members in Chiroptera are difficult to be elucidated solely on morphological features associated with flight or fossil records. According to molecular genetic data, the members in Chiroptera was divided into two suborders, Yangochiroptera (includes the *M. brandtii* and other echolocating bats) and Yinpterochiroptera (includes megabats, and four of echolocating bat species i.e. Rhinopomatidae, Rhinolophidae, Hipposideridae, and Megadermatidae) (Springer, 2013). In addition, Chiroptera was placed in Laurasiatheria among other mammals which currently includes Eulipotyphla (hedgehogs, shrews, and moles), Perissodactyla (rhinoceroses, horses, and tapirs), Carnivora (carnivores), Cetartiodactyla (artiodactyls and cetaceans), and Pholidota (pangolins) (Lin et al., 2002; Wildman et al., 2006; Hallström et al., 2011; Nery et al., 2012; Zhou et al., 2012). However, its position in the Laurasiatheria is still not conclusive. Based on the data of TTR intron 1 nucleotide sequences, the monophyletic relationship of the members in Chiroptera was strongly supported with > 90% bootstrap. The suborders Yinpterochiroptera (represented by *P. alecto* and *P. vampyrus*) and Yangochiroptera (represented by *S. kuhlii* and all of the studied Myotis species) were distinctly grouped. The monophyletic grouping of all the studied microbats except *R. affinis* was supported by all of the analyses i.e. ML, MP and NJ with 100% bootstrap. In addition, more closely relatedness of the microbat *R. affinis* to the megabats than to the other microbats was strongly supported with 97% bootstrap. Consequently, the paraphyletic relationship of the microbats was confirmed. The results presented here thus indicated to the usefulness of TTR intron 1 as a phylogenetic marker for resolving the relationships of members in the order Chiroptera.

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