Phylogenetic relationships and the primitive X chromosome inferred from chromosomal and satellite DNA analysis in Bovidae

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The early phylogeny of the 137 species in the Bovidae family is difficult to resolve; knowledge of the evolution and relationships of the tribes would facilitate comparative mapping, understanding chromosomal evolution patterns and perhaps assist breeding and domestication strategies. We found that the study of the presence and organization of two repetitive DNA satellite sequences (the clone pOaKB9 from sheep, a member of the 1.714 satellite I family and the pBtKB5, a 1.715 satellite I clone from cattle) on the X and autosomal chromosomes by *in situ* hybridization to chromosomes from 15 species of seven tribes, was informative. The results support a consistent phylogeny, suggesting that the primitive form of the X chromosome is acrocentric, and has satellite I sequences at its centromere. Because of the distribution of the ancient satellite I sequence, the X chromosome from the extant Tragelaphini (e.g. oryx), rather than Caprini (sheep), line is most primitive. The Bovini (cow) and Tragelaphini tribes lack the 1.714 satellite present in the other tribes, and this satellite is evolutionarily younger than the 1.715 sequence, with absence of the 1.714 sequence being a marker for the Bovini and Tragelaphini tribes (the Bovinae subfamily). In the other tribes, three (Reduncini, Hippotragini and Aepycerotini) have both 1.714 and 1.715 satellite sequences present on both autosomes and the X chromosome. We suggest a parallel event in two lineages, leading to X chromosomes with the loss of 1.715 satellite from the Bovini, and the loss of both 1.714 and 1.715 satellites in a monophyletic Caprini and Alcelaphini lineage. The presence and X chromosome distribution of these satellite sequences allow the seven tribes to be distributed to four groups, which are consistent with current diversity estimates, and support one model to resolve points of separation of the tribes.

Keywords: Bovidae; phylogeny; primitive X chromosome; satellite DNA

1. INTRODUCTION

The Bovidae family is the most diverse family in the order Artiodactyla, with some 137 extant species (Wilson & Reeder 1983) in 13 tribes (Gentry 1992). It is difficult to systematize because of the rapid radiation and appearance of tribes and species in the fossil record, the morphological convergence and controversy surrounding its monophyletic origin (Allard *et al.* 1992; Gatesy *et al.* 1992; Franklin 1997). Domestic cattle with 58 acrocentric autosomes, X and Y ($2n=60$) are thought to retain the ancestral autosomal complement, although the diploid chromosome number ($2n$) of the Bovidae ranges from 30 to 60. The number of autosomal arms is almost constant at 58 for most karyotyped species (Gallagher & Womack 1992), with the occurrence of multiple centric fusion events (Wurster & Benirschke 1968; Buckland & Evans 1978a,b). The ancestral conditions of the Bovidae X and Y chromosomes remain to be determined (Gallagher *et al.* 1994, 1999) and there is only poor resolution in the phylogeny of Bovidae tribes because radiation was relatively rapid with little differentiation in the geological record. Outgroup comparisons to the Cervidae family indicate that the Bovinae subfamily (tribes Bovini,

Boselaphini and Tragelaphini) acrocentric X chromosome is nearer to the ancestral Bovidae condition than is the sheep (Caprinae subfamily) acrocentric X chromosome, even although the sheep condition is more common (Gallagher *et al.* 1999).

Satellite DNAs that reside in the pericentromeric regions of chromosomes are rapidly evolving and are valuable evolutionary markers (Saffery *et al.* 1999; Chaves *et al.* 2000a,b). The satellite I sequence has been studied in goat and sheep (the 1.714 family) and cattle (1.715 family) and caprine sequences have been compared (Buckland 1983, 1985; Chaves *et al.* 2000a). The presence of the 1.715 family satellite I DNA is considered a primitive condition for Bovidae species (Modi *et al.* 1993; Gallagher *et al.* 1999), found normally only at autosomal pericentromeric regions of chromosomes (excepting the X chromosome of river buffalo (*Bubalus bubalis*); Modi *et al.* 1993). Sika (*Cervus nippon*) and white-tailed deer (*Odocoileus virginianus*) in the Cervidae family also carry 1.715-like satellite I sequences on the X chromosome (cited as unpublished observation by Gallagher *et al.* (1999)) and these authors suggest that an acrocentric X chromosome with heterochromatin (and the 1.715 satellite I sequence) at the centromeres is the primitive condition. Modi *et al.* (1996) hybridized a sequence representative of

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Table 1. *In situ* hybridization patterns with probes sheep satellite I DNA (pOaKB9) and cattle satellite I DNA (pBtKB5) in all chromosome preparations of the 15 Bovidae species analysed.

((Hyb.) *in situ* hybridization detected in chromosome preparations, (No Hyb.) apparent absence of *in situ* hybridization in chromosome preparations, (+) positive *in situ* hybridization signal, (0) apparent absence of *in situ* hybridization signals, (♀) no Y chromosome analysed as female; (†) Y chromosome not identified; note $2n=60$ species have no biarmed autosomes.)

tribe	species name	2n	figure	X	Y	acrocentric autosomes	biarmed autosomes
1.714 (<i>Ovis aries</i> satellite I DNA)							
Bovini	<i>Bos taurus</i>	60,XY	no hyb.	0	0	0	
Tragelaphini	<i>Taurotragus oryx</i>	31,X1X2Y	no hyb.	0	0	0	0
	<i>Tragelaphus strepsiceros</i>	32,X1X1X2X2	no hyb.	0	♀	0	0
Caprini	<i>Ovis aries</i>	54,XY	figures 4 and 5	0	0	+	+
	<i>Ovis ammon</i>	54,XX	figure 1a	0	♀	+	+
	<i>Capra hircus</i>	60,XY	figure 4	0	0	+	
	<i>Ammotragus lervia</i>	58,XX	hyb.	0	♀	+	+
Reduncini	<i>Kobus leche</i>	48,XY	figures 1b and 4	+	0	+	+
Hippotragini	<i>Oryx dammah</i>	58,XY	figure 4	+	0	+	+
	<i>Oryx leucoryx</i>	58,XY	figure 4	+	0	+	+
	<i>Addax nasomaculatus</i>	58,XX	figures 1c and 4	+	♀	+	+
	<i>Hippotragus niger</i>	60,XY	figure 4	+	0	+	
Alcelaphini	<i>Connochaetes taurinus</i>	58,XY	figures 1d and 4	0	0	+	0
	<i>Damaliscus hunteri</i>	44,XY	figures 1e and 4	0	0	+	+
Aepycerotini	<i>Aepyceros melampus</i>	60,XX	figures 1f and 4	+	♀	+	
1.715 (<i>B. taurus</i> satellite I DNA)							
Bovini	<i>B. taurus</i>	60,XY	figures 2a and 4	0	0	+	
Tragelaphini	<i>T. oryx</i>	31,X1X2Y	figures 2b and 4	+	†	+	+
	<i>T. strepsiceros</i>	32,X1X1X2X2	figures 2c and 4	+	♀	+	+
Caprini	<i>O. aries</i>	54,XY	figure 5	0	0	+	+
	<i>O. ammon</i>	54,XX	hyb.	0	♀	+	+
	<i>C. hircus</i>	60,XY	hyb.	0	0	+	
	<i>A. lervia</i>	58,XX	hyb.	0	♀	+	+
Reduncini	<i>K. leche</i>	48,XY	hyb.	+	0	+	+
	<i>O. dammah</i>	58,XY	figure 2d	+	0	+	+
Hippotragini	<i>O. leucoryx</i>	58,XY	hyb.	+	0	+	+
	<i>A. nasomaculatus</i>	58,XX	figure 2e	+	♀	+	+
	<i>H. niger</i>	60,XY	hyb.	+	0	+	
Alcelaphini	<i>C. taurinus</i>	58,XY	figure 2f	0	0	+	0
	<i>D. hunteri</i>	44,XY	hyb.	0	0	+	+
Aepycerotini	<i>A. melampus</i>	60,XX	hyb.	+	♀	+	

satellite I from *Bos taurus* (1.715 family) to different artiodactyl metaphases, and they found that this sequence was present in all pecoran animals analysed.

Both Modi *et al.* (1993, 1996) and Chaves *et al.* (2000a) observed different satellite DNA *in situ* hybridization patterns within a small number of tribes in the Bovidae family, and we considered that the sequences had potential as phylogenetic markers to increase the resolution of the evolutionary tree at the base of the Artiodactyla (Chaves *et al.* 2000a). Here we aimed to analyse the presence and distribution of satellites to address evolutionary and phylogenetic questions with respect to the X chromosome (and its primitive state), karyotype, species and tribal evolution.

2. MATERIAL AND METHODS

Chromosomes were harvested from stimulated lymphocytes of 15 species (table 1) and male fibroblasts of *Damaliscus hunteri* using standard protocols. For G-banding, chromosomes were obtained only with late synchronization using bromodeoxyuridine (BrdU, Sigma) and Hoechst (H33258,

Sigma) before treatment with colcemid in the last 10 min. Sheep extended chromatin fibres were prepared from lymphocytes following routine procedures (Schwarzacher & Heslop-Harrison 2000). Air-dried slides were aged at 65 °C for 5 h or overnight and then were submitted to standard procedures of G-banding with trypsin. The chromosomes stained with Giemsa (GTG-banding) were photographed, fixed with 4% paraformaldehyde and sequentially C-banded. The chromosomes used for *in situ* hybridization were not stained at this stage but were refixed with paraformaldehyde (Chaves *et al.* 2002) before *in situ* hybridization, detection and staining with DAPI (the inverse image revealed the G-banding, GTD-banding). Karyotyping followed the standardization of the domestic bovids karyotypes (ISCNDB 2000). CBP-banding (by barium hydroxide treatment) followed the standard procedure of Sumner (1972) with minor modifications (50% time; propidium iodide as a counterstain).

For *in situ* hybridization on chromosomes and extended chromatin fibres, a satellite I sheep clone pOaKB9 (Chaves *et al.* 2000a, 2003b) and a satellite I cattle clone, (pBtKB5, GenBank AJ293510, Chaves *et al.* 2000b, 2003a) labelled

with biotin-16-dUTP (Sigma) or digoxigenin-11-dUTP (Roche) were used with standard protocols (Schwarzacher & Heslop-Harrison 2000). The most stringent post-hybridization washes were at 42°C in a 2×SSC and 50% (v/v) formamide. Labels were detected by FITC conjugated with avidin (Vector) and anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained with DAPI and mounted in Vectashield (Vector).

Metaphases were analysed with a Zeiss Axioplan 2 Imaging and Axiocam camera. Adobe Photoshop was used for image processing and analysis, using only contrast, overlaying and colour manipulation functions that affected the whole of the image equally; colours were chosen to maximize clarity. Variation within groups or tribes of the species analysed was low: for *B. taurus*, *Taurotragus oryx*, *Ovis aries*, *Capra hircus*, *Kobus leche*, *Oryx dammah*, *Oryx leucoryx*, *Hippotragus niger*, *Connochaetes taurinus* and *D. hunteri* both male and female individuals were investigated, with no differences in hybridization parameters analysed here (data not shown); indeed, no differences in hybridization patterns were notable between species belonging to the same genus (e.g. *O. aries* and *O. ammon*).

3. RESULTS

(a) *In situ* hybridization with 1.714 and 1.715 satellite DNA clones

The sheep satellite I clone (pOaKB9) (a member of the 1.714 sheep DNA satellite family) (figure 1) and the cattle satellite I clone (pBtKB5) (1.715 satellite DNA family, Chaves *et al.* 2000b) (figure 2), were hybridized *in situ* to metaphase chromosomes from the 15 Bovidae species; simultaneously, the constitutive heterochromatin was analysed with C-banding (figure 3) to correlate with the satellite DNA localization. Generally, it was possible to correlate the centromeric bands of the chromosomes in each species with the strength of hybridization of the 1.714 and 1.715 sequences, consistent with the suggestion that the heterochromatic bands consist largely of satellite I DNA. Where C-banding results have been reported previously, these were similar, although additional small bands were detected, many of which were significant for satellite DNA evolution (arrows only, figure 3). However, *C. taurinus* (tribe Alcelaphini; figures 1d, 2f and 3b) was unusual in showing no correlation of C-bands and satellite hybridization pattern: the large C-band and no satellite hybridization on chromosome one may reflect the presence of another satellite I variant without homology at the 80% hybridization stringency used here.

The 1.715 family showed hybridization to most autosomal chromosomes of the 15 species analysed (figure 2). The 1.714 DNA satellite family was not detected in the Bovini and Tragelaphini genomes, as expected (Chaves *et al.* 2000a). The 1.714 sequences showed centromeric or pericentromeric localization on acrocentric and, when present, on some biarmed chromosomes from the species belonging to Caprini (figure 1a) and Alcelaphini (figure 1d,e), but no hybridization was seen to the X or Y chromosome. In the Hippotragini (figure 1c), Reduncini (figure 1b) and Aepycerotini (figure 1f) tribes, the satellite hybridized to both autosomes and the X chromosome. The chromosomal localization of the two probes (1.714 and 1.715) was similar in all these species, although there were differences

in detail and hybridization strength. In particular, the hybridization signal on the autosomal acrocentrics was always terminal on the chromosomes as defined by DAPI-staining. On the autosomal biarmed chromosomes, the *in situ* hybridization signal was absent (e.g. *Connochaetes*), flanked the centromeric constriction (e.g. *Damaliscus*, *Kobus*) or overlaid the constriction (e.g. *Damaliscus*, *Tragelaphus*). Figure 2a–c shows hybridization of the 1.715 sequence in Bovinae metaphases of *B. taurus*, *T. oryx* and *Tragelaphus strepsiceros*. In *B. taurus* (figure 2a), only with acrocentric autosomes, the probe hybridized to the centromeric regions of all autosomes, but not to X and Y chromosomes. In contrast, the Tragelaphini X chromosomes (figure 2b,c) showed most hybridization at the terminal centromeric regions. The Tragelaphini autosomes showed weak hybridization signal at the centromeres of the mostly biarmed chromosomes.

Table 1 summarizes the *in situ* hybridization patterns with probes 1.714 *O. aries* satellite I DNA (pOaKB9) and 1.715 *B. taurus* satellite I DNA (pBtKB5) to the different chromosome groups in the 15 Bovidae species analysed. Individual chromosome types showed signals with characteristic strengths (figures 1 and 2) and there were significant differences between genera, reflecting variation in both copy number and sequence within each satellite family.

Figure 4 gives examples of satellite hybridization (where detected; 1.715 upper, 1.714 lower) and C-banding pattern of X chromosomes from species representing each tribe. As well as large C-bands correlating with parts of the hybridization sites of the satellite I sequences, C-bands along chromosomes arms were found, which did not correlate with hybridization sites (e.g. arrows in figure 4). X chromosomes from the tribes Tragelaphini, Reduncini, Hippotragini and Aepycerotini (figure 4) showed satellite DNA hybridization at (peri)centromeric sites, but this hybridization was not seen in the Bovini, Alcelaphini or Caprini tribes (figure 4). In contrast to the autosomal acrocentric chromosomes, the hybridization signal did not extend to the end of the chromosomes as defined by DAPI staining, except in the two Tragelaphini species.

In situ hybridization on sheep extended chromatin fibres with 1.714 and 1.715 satellite DNA clones let us examine organization of the sheep and bovine origin probes along chromosomes. Simultaneous *in situ* hybridization of the two satellite probes to fibres from sheep nuclei revealed an interspersed organization of the two satellites (figure 5), in agreement with the hybridization pattern seen on metaphase chromosomes where the two signals were colocalized.

4. DISCUSSION

The two related satellite I probes show less than 70% sequence similarity and are distinguished clearly by *in situ* hybridization with a stringent wash in 2×SSC, 50% formamide at 42°C. The 1.715 satellite from *B. taurus* and the 1.714 satellite from *O. aries* proved to be informative markers (Modi *et al.* 1996; Chaves *et al.* 2000a) for studying the nature and amplification of the satellite DNA families on the autosomes and the X chromosome of the Bovidae family (figures 1,2,4 and 5; table 1) and allowed phylogeny to be inferred. Where we investigated several genera within a tribe, hybridization patterns were similar

Figure 1. *In situ* hybridization of the sheep (*Ovis aries*) centromeric DNA satellite I-clone pOaKB9 (green-FITC) to metaphase chromosomes (chromosomal DNA stained with DAPI, presented in red pseudocolour) of the: (a) tribe Caprini, *Ovis ammon* (female, $2n=54,XX$), (b) tribe Reduncini, *Kobus leche* (male, $2n=48,XY$), (c) tribe Hippotragini, *Addax nasomaculatus* (female, $2n=58,XX$), (d) tribe Alcelaphini, *Connochaetes taurinus* (male, $2n=58,XY$), (e) tribe Alcelaphini, *Damaliscus hunteri* (male, $2n=44,XY$), (f) tribe Aepycerotini, *Aepyceros melampus* (female, $2n=60,XX$).

Figure 2. *In situ* hybridization of the cattle (*Bos taurus*) centromeric DNA satellite I-clone pBtKB5 (blue pseudocolour-rhodamine) to metaphase chromosomes (chromosomal DNA stained with DAPI, presented in red pseudocolour) of the: (a) tribe Bovini, *B. taurus* (male, $2n=60,XY$), (b) tribe Tragelaphini, *Taurotragus oryx* (male, $2n=31,X1X2Y$), (c) tribe Tragelaphini, *Tragelaphus strepsiceros* (female, $2n=32,X1X1X2X2$), (d) tribe Hippotragini, *Oryx dammah* (male, $2n=58,XY$), (e) tribe Hippotragini, *Addax nasomaculatus* (female, $2n=58,XX$), (f) tribe Alcelaphini, *Connochaetes taurinus* (male, $2n=58,XY$).

Figure 3. C-banded metaphases of: (a) tribe Hippotragini, *Addax nasomaculatus* (female, $2n=58,XX$), (b) tribe Alcelaphini, *Connochaetes taurinus* (male, $2n=58,XY$), (c) tribe Tragelaphini, *Tragelaphus strepsiceros* (female, $2n=32,X1X1X2X2$).

Figure 4. Figure that summarizes the C-banded X chromosome's pattern and the *in situ* hybridizations with the sheep (clone pOaKB9) and cattle (pBtKB5) DNA satellite I probes to the X chromosome of most representative species listed in table 1. The Bovinae subfamily shows only hybridization with the cattle satellite I and only in the X chromosome's centromeric regions of the tribe Tragelaphini was it possible to observe signal from the cattle satellite I. The metaphase preparations from the subfamilies Hippotraginae, Alcelaphinae and Caprinae show positive *in situ* hybridization signals with both sheep and cattle satellite I probes (cf. figures 1 and 2). However, only the X chromosome centromeric regions of the Tribes Reduncini, Hippotragini and Aepycerotini show positive *in situ* hybridization signals with both satellite probes. Finally, the constitutive heterochromatin characterized by C-bands is extremely heterogeneous; nevertheless all X chromosomes shows C-bands in the (peri)centromeric regions.

Figure 5. *In situ* hybridization of the two satellite I probes to extended chromatin fibres of sheep (blue, cattle probe pBtKB5, less abundant; green sheep probe pOaKB9, more abundant). Three different regions of the slide have been imaged and brought together in the figure. No arrays of exclusively blue or exclusively green hybridization sites were detected, indicating that the two variants of the satellite I sequence were interspersed. Assuming the DNA is extended to its full molecular length, the width of the picture represents about 8 kb of sequence (some seven copies of the satellite I repeat unit). Scale bar, 2 μ m.

with the exception of the biarmed chromosomes, where satellite DNA reshuffling occurs (Chaves *et al.* 2000b, 2003b).

In striking contrast to the morphological conservation of autosomal chromosome arms evident in the Bovidae family, the X chromosome shows considerable variation between tribes (Buckland & Evans 1978a,b; Gallagher *et al.* 1999; Robinson *et al.* 1996) in both sequence composition and arrangement, as has been shown using bacterial artificial chromosome (BAC) hybridization (Robinson *et al.* 1996; Piumi *et al.* 1998) to find regions of conserved synteny. The complex changes in satellite

composition are also illustrated by constitutive heterochromatin analysis by C-banding (figure 4), where X chromosomes from the species we analysed show (peri)-centromeric bands with different sizes, not entirely reflected by the *in situ* hybridization signals (e.g. *Aepyceros*) and polymorphisms in size and position between interstitial bands (arrows).

We found the 1.715 satellite hybridized to chromosomes of all tribes. The tribe Caprini is considered only distantly related to the Bovini and Tragelaphini tribes (i.e. the Bovinae subfamily; Buckland 1985) and, consistent with this, the latter tribes showed no hybridization of the 1.714

Figure 6. Evolutionary diagrams of the Bovidae tribes that assemble the data presented in the current paper: presence or absence (and suggested origin) of 1.714 satellite I DNA family in the genome of the different species analysed; presence and origin of 1.715 satellite I DNA family in the genome of all Bovidae species; X chromosomes with (X+) and without detectable (X-) satellite I DNA; and the diploid chromosome number of each species. Divergence times at the tribal level are from [Vrba \(1985\)](#), [Gentry \(1992\)](#) and, for the Bovini Tribe, [Groves \(1981\)](#). Inset left highlights a model for the origin of the 1.714 and 1.715 DNA satellite I families, while the tree shows how extant X chromosomes may be derived from inferred primitive forms.

satellite family, while the remaining tribes we sampled all showed presence of the satellite (figure 6, inset left). This suggests that the 1.715 satellite is evolutionarily older. [Modi et al. \(1996\)](#) dated the origin of 1.715 satellite family to 20–40 Mya, when the tragulinas and pecorans last shared a common ancestor. We infer that the 1.714 satellite DNA family dates from at least 15 Mya, when the radiation of the Bovidae tribes began with the separation of the Bovini/Tragelaphini from the other tribes.

While the cattle satellite sequence (1.715 family) is more ancestral than the equivalent sheep sequence (1.714 family), it is maintained in sheep genome during evolution. The sheep fibre-FISH (figure 5), with both satellite DNA families, shows the presence of both satellites with an interspersed organization. It has been suggested that the 1.714 family (sheep satellite) originated from 1.715 family (cattle satellite), but our results (figure 5) suggest that 1.715 satellite was maintained on this genome, with evolutionarily older variants of cattle satellite I being preserved during the origin of sheep satellite I; that is, they have been amplified, deleted or taken part of unequal exchange process with the new satellite I from sheep. These results provide support for one of the evolutionary mechanisms of satellite DNA proposed in cattle ([Nijman & Lenstra 2001](#)).

What was the structure of the X chromosome at the time of origin of the Bovidae family? Many have considered the acrocentric X chromosome with satellite I DNA as the primitive condition for the Bovidae ([Modi et al. 1993](#); [Gallagher et al. 1999](#)). Our results show both satellite I sequences on X chromosomes from the tribes Reduncini, Hippotragini and Aepycerotini (figure 4), in

contrast to the situation in the autosomal acrocentrics. Notably, the signal was (sub-)terminal only in Tragelaphini (with the 1.715 satellite only and in mostly biarmed autosomal chromosomes), suggesting a different structural organization of the satellite sequence in the X chromosomes in this branch and adding further evidence that this was the earliest branch of the Bovidae. The sheep acrocentric X differs from Bovinae acrocentric chromosomes in centromere placement and locus order ([Piumi et al. 1998](#); [Iannuzzi et al. 2000](#)). [Robinson et al. \(1998\)](#) demonstrated that the sheep X chromosome morphology is probably present in the 10 Bovidae tribes but not the Bovinae subfamily, although there is variation in the amount and position of X chromosome heterochromatin.

In both branches, some tribes have lost the satellite I sequences, a loss reflected in the much smaller C-bands which interestingly indicates there is no replacement by related repetitive sequences (figures 4 and 6). During evolution of karyotypes in the Bovidae, chromosome fusion events have occurred and these are frequently associated with loss and reshuffling of the centromeric satellites, suggesting that there are mechanisms for altering the repetitive sequence composition ([Chaves et al. 2003a](#)). Therefore, the multiple occurrence of satellite I loss or gain during evolution of the X chromosomes, which involves morphological rearrangement, is not surprising (figure 6). The analysis of satellite DNA sequence, organization and chromosomal distribution, which elucidates aspects of both genome and repetitive sequence evolution, is a valuable tool to reconstruct tribal phylogenies and the order of divergence where that is not found or is ambiguous in the fossil

record or other data. Finally, this work suggested the existence of two major subfamilial clades in Bovidae (Bovinae and Caprinae/Alcelaphinae/Hippotraginae clades), consistent with other molecular investigations based on ribosomal (Gatesy *et al.* 1997) or *cyt b* (Hassanin & Douzery 1999a,b; Matthee & Robinson 1999) mitochondrial sequences.

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