GENOME EVOLUTION AND SYSTEMATICS OF THE PAENUNGULATA (AFROTHERIA, MAMMALIA)

AMANDA T. PARDINI

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Promoter: T. J. ROBINSON

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

Increases in taxonomic sampling and the numbers and types of markers used in phylogenetic studies have resulted in a marked improvement in the interpretation of systematic relationships within Eutheria. However, relationships within several clades, including Paenungulata (Hyracoidea, Sirenia, Proboscidea), remain unresolved. Here the combination of i) a rapid radiation and ii) a deep divergence have resulted in limited phylogenetic signal available for analysis. Specifically i) a short internode separating successive branching events reduces the time available for changes to occur, while ii) the longer the time since divergence, the greater the opportunity for signal to be negatively affected by homoplasy. This is evident in both molecular and morphological data where an overall consensus on paenungulate relationships is lacking. Morphological analysis of anatomical and fossil evidence favours the association of Sirenia (S) and Proboscidea (P) (Tethytheria) to the exclusion of Hyracoidea (H); further, support for uniting these three taxa as Paenungulata is contentious. In contrast, molecular data provide strong support for Paenungulata but intra-ordinal relationships are ambiguous. Although results from mitochondrial DNA sequence data favour Tethytheria, there is no consensus of support for this clade from nuclear DNA. Nuclear DNA is typified by node instability but favours H+P in the largest concatenation of sequences. Due to the expected increased effect from homoplasy and consequently the increased likelihood for misleading signal, it is unclear which result is most likely to represent the "true" tree.

An analysis of available and added intron sequences to characterise signal heterogeneity among nuclear DNA and mitochondrial DNA partitions indicated that the phylogenetic utility of partitions varies considerably. Subpartitioning of the data according to similar evolutionary processes/characteristics (e. g., mtDNA vs. nDNA and codon position) revealed new insights into the signal structure of the data set; specifically i) that nuclear DNA first codon positions, and to a lesser degree second codon sites, provide convincing support for H+P, and ii) that support for S+P by faster evolving sites within mtDNA suggests that this may be the result of misleading signal. If H+P represents the "true tree", then support for this clade indicates that phylogenetic signal has been reduced over

time as a result of multiple hits, which explains the presence of (hidden) support in slower evolving sites where homoplasy is less likely to occur, in contrast to faster evolving sites where no support for H+P was observed.

In an attempt to provide further resolution from an alternative perspective to that possible with DNA sequence data, chromosomal rearrangements were identified among the three paenungulate lineages. Using comparative chromosome painting, unique changes within each order and specific to Paenungulata were characterised, however, intra-ordinal synapomorphies were not recovered. Although this may suggest a hard polytomy, the slow to moderate rate of evolution estimated from the data is likely not sufficient relative to the rapid radiation associated with the paenungulate node. Further examination of chromosomal rearrangements at a higher level of resolution may yet reveal informative changes.

Opsomming

‘n Toename in die aantal taksonomiese monsters sowel as die aantal en soort merkers wat in filogenetiese studies gebruik word, het tot ‘n merkbare verbetering in die vertolking van sistematiese verwantskappe binne die Eutheria gelei. Desondanks bly ‘n aantal klades (stamlyne), met inbegrip van Paenungulata (Hyracoidea, Sirenia, Proboscidea), steeds onopgelos. By laasgenoemde het die kombinasie van i) ‘n vinnige radiasie en ii) ‘n diep divergensie die filogenetiese sein wat vir analise beskikbaar is, beperk. Meer spesifiek sal i) opeenvolgende vertakkings wat deur kort internodusse geskei word die beskikbare tyd waartydens veranderings kan intree, verminder, terwyl ii) ‘n toename in tydsverloop sedert divergensie die kans dat die sein deur homoplasie nadelig beïnvloed sal word, vergroot. Dit word in sowel molekulêre en morfologiese data, waar ‘n oorhoofse konsensus t.o.v. verwantskappe van Paenungulata ontbreek, waargeneem. Morfologiese analise van anatomiese en fossielbewyse ondersteun die samevoeging van Sirenia (S) en Proboscidea (P) (Tethytheria) ten koste van Hyracoidea (H). Ondersteuning vir die samevoeging van dié drie taksa as Paenungulata is egter aanvegbaar. In teenstelling hiermee word Paenungulata sterk deur molekulêre data ondersteun, al bly die verwantstkappe op intra-orde vlak, steeds onduidelik. Alhoewel die resultate van mitochondriale DNA op Tethytheria dui, word die klade nie deur data van kern-DNA ondersteun nie. Kern-DNA word gekarakteriseer deur node instabiliteit maar verkies H+P in die grootste samevoeging van geen volgordes. Na aanleiding van die verwagte toename in die effek van homoplasie en die gevolglik groter kans op ‘n misleidende sein, is dit nie duidelik watter van die resultate die meer korrekte filogenetiese stamboom verteenwoordig nie.

Analise van beskikbare en nuut toegevoegde intron-volgordes om sein-heterogeniteit tussen kern- en mitochondriale DNA verdelings te karakteriseer, toon dat die filogenetiese nut van verdelings beduidend verskil. Onderverdeling van die data op grond van soortgelyke evolusionêre prosesse/karaktereienskappe (bv. mtDNA vs. nDNA, en kodonposisie) het na nuwe insigte in die seinstruktuur van die datastel gelei. Meer spesifiek dat i) kern-DNA se eerste kodonposisies, en tot ‘n mindere mate die tweede

kodonposisies, H+P oortuigend ondersteun en ii) dat ondersteuning vir S+P deur posisies binne mtDNA wat vinnig verander, op ‘n misleidende sein mag dui. As H+P die korrekte stamboom verteenwoordig dui ondersteuning vir die klade op ‘n filogenetiese sein wat met verloop van tyd as gevolg van veelvuldige seinvoorkomste verklein het. Dit verklaar die aanwesigheid van versluierde ondersteuning in stadig-veranderende posisies waar die neiging tot homoplasie klein is, in teenstelling met posisies wat vinniger verander en waar ondersteuning vir H+P nie waargeneem is nie.

Op soek na verhoogde resolusie vanuit ‘n ander perspektief as DNA-volgordebepaling, is chromosomale herrangskikkings in die drie stamlyne van Paenungulata nagevors. Met behulp van vergelykende chromosoomkleuring is unieke veranderings binne elke orde en spesifiek binne Paenungulata gekarakteriseer, maar geen sinapomorfe kenmerke is op die intra-orde vlak gevind nie. Alhoewel dit op ‘n onopgeloste politomie mag dui, is die stadige tot matige evolusietempo wat van die data afgelei word, relatief tot die vinnige radiasie wat met die Paenungulata-nodus geassosieer word, waarskynlik onvoldoende vir ‘n oplossing. Verdere navorsing oor chromosomale herrangskikkings met ‘n hoër resolusievlak mag addisionele insiggewende veranderings aantoon.

Dedication

To my grandfather, Verdun Pardini

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CHAPTER 1 - GENERAL INTRODUCTION

1.1 Preamble

The mammalian clade Paenungulata ("almost ungulates"), comprising the orders Proboscidea, Sirenia and Hyracoidea, was first suggested by Cope (1884, in: Springer, Cleven et al., 1997) subsequently formally described by Simpson (1945) on the basis of morphological characteristics. An alternative interpretation of the relationships between these three orders exists favouring an association between Proboscidea and Sirenia forming Tethytheria (McKenna, 1975) and with Hyracoidea more closely affiliated to Perissodactyla (McKenna, 1975). However, in contrast to the Hyracoidea-Perissodactyla hypothesis, Paenungulata receives overwhelming support from molecular data suggesting the validity of this clade. When considering relationships among the three paenungulate lineages, however, there is no clear consensus from morphological and molecular characters. Individual DNA segments vary considerably with respect to which of the three possible associations within Paenungulata they support. Moreover, concatenations of individual partitions are sensitive to the addition of new data. Consequently, analyses remain equivocal over associations within Paenungulata. The extensive data currently available and their inability to provide resolution among the lineages indicates a need for additional, independent markers that may provide a new perspective on the paenungulate polytomy. To this end this study aims to use a combination of chromosomal and sequence data to address the systematic relationships and genome evolution within Paenungulata.

1.2 Background

1.2.1 Morphological data

The central issue in the morphological debate on systematic relationships between Hyracoidea, Sirenia and Proboscidea concerns the monophyly of Paenungulata. Specifically, one may ask whether Simpson's (1945) uniting of these three orders is more justifiable considering the data than McKenna's (1975) hypothesis of Tethytheria with Hyracoidea aligned more closely to Perissodactyla? Although the monophyly of Paenungulata is not in question here, consideration of this debate is important as it implicitly considers relationships among the three paenungulate lineages. Clearly, the

exclusion of Hyracoidea from Paenungulata suggests that the closer association of Proboscidea with Sirenia (Tethytheria) based on morphology is more acceptable.

The discussion of paenungulate monophyly and specifically that of Tethytheria is considered one of the most ongoing and contentious debates in eutherian phylogenetic relationships (Gheerbrandt, Domning et al,. 2005, p. 84; Amrine and Springer, 1999; Waddell, Okada et al. 1999). A comprehensive discussion of the validity of the different morphological arguments is beyond the scope of this thesis, but examination of the relevant literature highlights certain important points concerning the difficulties with resolving this issue. Few characters can be characterised as potential synapomorphies in support of a particular hypothesis and those that are, are very often reassessed as homoplasious. For example, a proposed synapomorphic character for Tethytheria concerns the absence of the splenius colli muscle (Amrine and Springer, 1999). However, this character state is also present in Tubulidentata, Cetacea and Pholidota (Shoshani, 1993) and since Tubulidentata, on the basis of molecular evidence, is a likely a sister taxon to paenungulates (Amrine-Madsen, Koepfli et al., 2003; Springer, Amrine et al., 1999; Stanhope, Smith et al., 1996), this character may be plesiomorphic. Further, a reassessment of an auditory character considered one of the most striking synapomorphies for Tethytheria (Fischer, 1990) was also subsequently found to be homoplastic (Court, 1994). Similar re-evaluations of potential synapomorphies for a Hyracoidea-Proboscidea or Hyracoidea-Sirenia association are also evident (Tassy and Shoshani, 1988, p.305). For example, initial suggestions of a Hyracoidea-Sirenia synapomorphy for placenta type has subsequently been invalidated (Enders and Carter, 2004, A. M. Carter pers. comm.)[1]. Earlier suggestions of a close relationship between Proboscidea and Sirenia by Gregory (1910, in: Tassy and Shoshani, 1988, p. 286) combined both derived, plesiomorphic, as well as convergent characters in support of this relationship. Consequently, following review of the validity of these characters, many have fallen away. In general, as new fossils are found and relationships are reassessed, characters may no longer be considered applicable (Lavergne, Douzery et al., 1996). To date though, Tethytheria receives more support from morphological evidence than either of the two other possible associations within Paenungulata (Gheerbrandt,

[1] A. M. Carter, Institut for Medicinsk Biologi, Syddansk Universitet, Denmark

Domning et al,. 2005, p. 102). However, there are large gaps in the paenungulate fossil record (Gheerbrandt, Domning et al,. 2005, p. 102) which may still yield important findings. An example is a very recent report of additional material from the oldest known proboscidean, *Phosphatherium escuilliei* which provides support for Paenungulata (Gheerbrandt, Sudre et al., 2005).

The problem of a lack of reliable synapomorphies is largely the result of the extensive morphological diversification within each paenungulate lineage. For example, sirenians have evolved a fully aquatic lifestyle and although hyraxes and elephants are both terrestrial, they have considerably different life-histories. It is noteworthy that a semi-aquatic ancestry has been suggested for ancestral proboscideans based on ontogenetic characters in *Loxodonta* (West, Fu et al., 2003; Gaeth, Short et al., 1999). The extent of anatomical differentiation is reflected (most strongly) in extant lineages. The progression of these morphological radiations can be traced through the fossil record with adaptations to the differing niches readily apparent. The earliest sirenian fossil found to date, *Pezosiren portelli* (early Middle Eocene) retains quadrupedal capability but shows changes to the rib cage, nasal openings and sinus morphology indicative of aquatic specialisations (Domning, 2001). Fossils from the Middle Eocene that follow *P. portelli* in temporal sequence, including *Prorastomus sirenoides* (Savage, Domning et al., 1994) and several protosirenids (Domning, 1994) exhibit reduction in hind-limbs, mandibular adaptations to bottom-feeding and associated dental changes. Most notable of the anatomical changes within Hyracoidea is the extensive size diversity and dietary adaptations evident in fossil specimens from the Paleogene, with body size variation ranging from that of extant hyracoids to those exceeding several hundred kilograms (De Blieux and Simons, 2002; Thewissen and Simons, 2001 and references therein). Likewise, proboscidean evolution is characterised by considerable body-size increases, the development of a trunk, and extensive variation in dental characteristics (see Shoshani, 1998 for review). Hence, the overwriting of synapomorphies established during the early stages of evolution by subsequent morphological changes (Madsen, Scally et al., 2001) is likely given the extensive morphological diversification in paenungulates.

1.2.2 Molecular data

The contribution of molecular data to the paenungulate debate was notable for its strong support of paenungulate monophyly to the extent that it is considered one of the most well supported supraordinal clades (Gheerbrandt, Domning et al,. 2005, p. 99). An early indication of an association of Hyracoidea with Tethytheria over Perissodactyla was provided through serological work which showed a close relationship between the hyrax and elephant (Weitz, 1953). Subsequent studies using immunological distances (Shoshani, 1986), amino acid sequences (Kleinschmidt, Czelusniak et al., 1986; Miyamoto and Goodman, 1986; Shoshani, 1986; Rainey, Lowenstein et al., 1984; De Jong, Zweers et al., 1981) and the first studies using nucleotide sequence data from a single DNA segment (Greenwood, Englbrecht et al., 2004; Madsen, Deen et al., 1997; Lavergne, Douzery et al., 1996; Porter, Goodman et al., 1996; Stanhope, Smith et al., 1996; Springer and Kirsch, 1993; Stanhope, Czelusniak et al., 1992; Irwin, Kocher et al., 1991) continued this trend, all providing support for Paenungulata. However, intra-paenungulate relationships were inconsistent with the different markers used, and despite the addition of new taxa and DNA regions, the outcomes continued to vary in their interpretation of relationships between Proboscidea, Hyracoidea and Sirenia (Eizirik, Murphy et al., 2001; Liu, Miyamoto et al., 2001; Waddell, Kishino et al., 2001; Amrine and Springer, 1999; Springer, Amrine et al., 1999; Stanhope, Madsen et al., 1998; Stanhope, Waddell et al., 1998; Springer, Burk et al., 1997; Springer, Cleven et al., 1997).

As eutherian molecular systematic studies began to include more taxa and additional markers (e.g., Amrine-Madsen, Koepfli et al., 2003; Murata, Nikaido et al., 2003; Murphy, Eizirik et al., 2001b; Murphy, Eizirik et al., 2001a; Eizirik, Murphy et al., 2001; Madsen, Scally et al., 2001; Nikaido, Kawai et al., 2001; Waddell, Kishino et al., 2001; Waddell, Okada et al., 1999), two important developments became apparent. First is that the increase in data, including whole mitochondrial genomes, did not lead to increased resolution within Paenungulata with the result that this node became one of the few remaining ambiguous nodes in eutherian evolution (Murphy, Pevzner et al., 2004; Waddell, Kishino et al., 2001; Waddell, Okada et al., 1999). For example, despite the very large size (16397bp) of the Murphy, Eizirik et al. (2001b) data set, the statistical support for the sirenian-hyracoid node

using both maximum likelihood (ML) and Bayesian inference (BI) was weak. Consequently, the sensitivity of this node to the addition of another 1.3kb to this data set (~8% sequence data increase) that resulted in a change to a hyracoid-proboscidean association (Amrine-Madsen, Koepfli et al., 2003) was not unexpected. Studies that have targeted different DNA regions and analysed the data using diverse analytical approaches (Douady, Scally et al., 2004; Asher, Novacek et al., 2003; Waddell and Shelley, 2003; Malia, Adkins et al., 2002; Scally, Madsen et al., 2001) have not resulted in improved resolution, confirming the instability of the intra-ordinal relationships within Paenungulata. Interestingly, when considering the number of synapomorphies supporting one of the three possible intrapaenungulate relationships among the 19 nuclear protein-coding genes at the amino acid level, Hyracoidea-Proboscidea is clearly favoured (Nishihara, Satta et al., 2005). Analyses that take account of secondary structures and protein signatures have also identified particular synapomorphies. Comparison of 16S rRNA secondary structure shows positional covariance for Hyracoidea-Sirenia, and also for Hyracoidea-Proboscidea, although the latter character also occurs in Pecora and Phocidae (Burk, Douzery et al., 2002). Considering the phylogenetic distance, however, it is not unreasonable to expect convergence in this case. Protein signatures in the apolipoprotein B gene yield one synapomorphy for Hyracoidea-Proboscidea (Amrine-Madsen, Koepfli et al., 2003) and a recent analysis of SINEs yielded a synapomorphy for Hyracoidea-Sirenia (Nishihara, Satta et al., 2005). Hence despite the relatively large amount of data available to assess intrapaenungulate systematic relationships, no congruence is apparent. A pattern has emerged showing a discrepancy between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) with the largest concatenations of mtDNA protein coding segments favouring Tethytheria (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003). Results from nDNA are varied although amino acid sequence comparisons favour Hyracoidea-Proboscidea (Nishihara, Satta et al., 2005; Waddell, Kishino et al., 2001).

An important point emerging from the molecular data is that, like the morphological analyses, there are relatively few potential synapomorphic characters while many more characters are autapomorphic within each lineage. This is likely an indication that the paenungulates underwent a rapid radiation reducing the time for changes to occur between divergence events (Nishihara, Satta et al., 2005; Amrine and Springer, 1999; Waddell,

Okada et al., 1999). This would certainly help explain the inability of the large molecular data sets to convincingly resolve intra-paenungulate relationships.

The second major development arising from the increase in data and taxonomic sampling for mammalian phylogenetic analyses involved considerable changes ("..dynamiting the tree..", Waddell and Shelley, 2003) to the conventional interpretation of eutherian mammal systematic relationships (Novacek, 1992). The larger molecular data sets revealed new relationships between taxa which were not previously suspected with morphological data. With the reorganisation, four new supraordinal groupings emerged, Xenarthra, Laurasiatheria, Euarchontoglires (= Supraprimates[2]) and Afrotheria. One of the surprising insights suggested by the molecular data is Afrotheria (Springer, Cleven et al., 1997), so named (Stanhope, Waddell et al., 1998) because of a presumed African origin of the member taxa (Gheerbrandt, Sudre et al., 1996; Carroll, 1988) most of which are still confined to this continent. The uniting of Macroscelidae, Afrosoricida (Chrysochloridae and Tenrecidae), Paenungulata and the monotypic *Orycteropus* (Tubulidentata) is contentious because it requires the merging of orders for which no morphological synapomorphy exists (Novacek, 2001; Asher, 1999). Furthermore, the established clades Anagalida, Ungulata and Insectivora are disrupted (Robinson and Seiffert, 2004; Stanhope, Waddell et al., 1998; Springer, Cleven et al., 1997). In some cases, such as a comparison of the “insectivorous” afrotherians (tenrecs and golden moles) to the “ungulate” members (paenungulates and aardvark), morphological differences are so considerable that without genetic evidence it is unlikely that these taxa would have been grouped together (Asher, 2001). However, prior suggestions of similar morphological characters do exist. All afrotherian taxa with the exception of the aardvark and certain members of Tenrecomorpha, have intra-abdominal (nondescended) testes (Whidden, 2002; Werdelin and Nilsonne, 1999). While this excludes the aardvark and is variably present within Tenrecomorpha, a morphological character uniting the majority of the smaller insectivorous members of Afrotheria with Paenungulata is compelling. It is important to note that intra-abdominal testes have previously been regarded as primitive (Freeman, 1990). Although it is unclear

[2] Priority is unclear between Euarchontoglires (Murphy et al. 2001b) and Supraprimates (Waddell et al. 2001); the former will be used here following Waddell and Shelley (2003).

based on comparisons to marsupials and monotremes what state represents the primitive form (Werdelin and Nilsonne, 1999), the view taken by Freeman (1990) would negate this character as a morphological synapomorphy for this clade. Additional morphological characters include dental features from the earliest fossil macroscelideans which show a closer affinity to paenungulates and “condylarths” (primitive extinct "ungulates", Seiffert, 2003; Tabuce, Coiffait et al., 2001; Simons, Holroyd et al., 1991) and, early morphological studies which describe features that show a strong similarity between Hyracoidea, Proboscidea and *Orycteropus* (Le Gros Clark and Sonntag, 1926). Particular astragalar features which unite hyraxes, elephants, elephant shrews, aardvarks, and the extinct *Plesiorycteropus* (but excludes extant sirenians due to the absence of hind limbs) have been suggested as possible synapomorphies requiring further consideration, although these characters are not exclusive to these taxa (Helgen, 2003; McPhee, 1994).

Within Afrotheria, Paenungulata is the most well supported clade. However, relationships among afrotherians, and particularly the sister-taxa to Paenungulata, are unclear. Despite the persuasive fossil evidence supporting a paenungulate-macroscelidean association (reviewed in Seiffert, 2003), these data are not conclusive. From a molecular perspective, a variety of hypotheses have been proposed but vary with the particular DNA markers used. Afroinsectiphillia (Murphy, Eizirik et al., 2001b; Waddell, Kishino et al., 2001) which includes all afrotherians except paenungulates, has found support from several molecular studies including cytogenetic data (Robinson, Fu et al., 2004). Within this clade, Afroinsectivora (which excludes the aardvark, Waddell, Kishino et al., 2001) has also been described though with varying support (Amrine-Madsen, Koepfli et al., 2003; Waddell and Shelley, 2003; Murphy, Eizirik et al., 2001b).

Traditionally, the radiation of eutherian mammals is believed to have begun on northern continents (Carroll, 1988 p. 449) approximately 65 million years ago (MYA) coinciding with the demise of the dinosaurs around the Cretaceous-Tertiary boundary (K/T) and the concomitant sudden availability of a broad range of adaptive niches (Carroll, 1988 p. 449). The K/T radiation hypothesis is borne out by the fossil record. Following the abrupt decline of the dinosaur fossil fauna in the Cenozoic (65 MYA), a considerable increase in the

diversity of mammalian fossils is apparent compared to the preceding Mesozoic (245-65 MYA, Rich, Vickers-Rich et al., 1997) where few fossils have unequivocally been described as ancestors to modern mammalian orders (Rage and Cappetta, 2002; Bromham, Phillips et al., 1999; Nessov, Zhegallo et al., 1998). The 'bush-like' phylogeny of mammals was interpreted as a sign of a rapid radiation (Explosive Model, Archibald and Deutschman, 2001; Bromham, Phillips et al., 1999) and this was generally accepted prior to the reshuffling of mammalian relationships suggested by molecular data that proposed a considerably different interpretation, prompted in large part by the basal placement of Afrotheria. It is necessary to mention that although Afrotheria is well supported and is favoured as the basal placental mammalian clade (as Exafroplacentalia) (Waddell, Kishino et al., 2001), other hypotheses do receive some support from molecular studies. These include Atlantogenata (Afrotheria + Xenarthra, Waddell, Cao et al., 1999b) and the morphological hypothesis of Xenarthra (Epitheria, McKenna, 1975). The molecular divergence of Afrotheria from the remaining three placental mammal supraordinal clades (Murphy, Eizirik et al., 2001b; Murphy, Eizirik et al., 2001a; Madsen, Scally et al., 2001) has more recently been estimated at 103 MYA (103-108 MYA, Springer, Murphy et al., 2003a; Murphy, Eizirik et al., 2001b, but see Waddell, Cao et al. 1999b and Waddel, Kishino et al. 2001). This divergence coincides with the separation of South America and Africa in the Cretaceous (~95-110 MYA Waddell, Cao et al., 1999a), following which Africa was isolated for approximately 90 MYA. Based on the molecular divergence patterns a subsequent dispersal from Gondwana to Laurasia yielded the split between Xenarthra and Boreoeutheria (88 to 100 MYA) eventually forming the northern hemisphere supraordinal groups Laurasiatheria and Euarchontoglires (Murphy, Eizirik et al., 2001b). The vicariant event caused by the opening up of the Atlantic ocean was also invoked by an earlier hypothesis in support of one of the alternative basal mammalian clades, Atlantogenata (Waddell, Cao et al. 1999b). The maintenance of small, insectivorous/generalist species retaining purported primitive placental characteristics in each of the four placental clades suggests that early lineages of these small mammals were isolated following continental breakup (Eizirik, Murphy et al., 2001; Waddell, Okada et al., 1999). Parallel adaptive radiations into modern orders, including specialisations for ant-eating and aquatic lifestyles, occurred in different geographic locations (Madsen, Scally et

al., 2001) and the increased availability of ecological niches following the dinosaur extinctions may have contibuted to the remarkable morphological diversification (Murphy, Eizirik et al., 2001a; Eizirik, Murphy et al., 2001). This pattern of mammalian radiation is more consistent (Springer, Murphy et al., 2003b) with what has been described as the Long-Fuse Model (Archibald and Deutschman, 2001) in which the majority of intraordinal divergences (like the preceding Explosive model) occur after the K/T boundary, but all interordinal divergences occur well within the Cretaceous.

The new hypothesis lacks convincing support from fossil evidence (but see Springer, Murphy et al., 2003b). However, in terms of paenungulate origins, this is not controversial since the majority of the fossils are found in (North) Africa supporting a southern hemisphere origin. In particular, the earliest fossil for Paenungulata, a primitive proboscidean *P. escuilliei*, was discovered in Morroco (57.9-55 MYA, Gheerbrandt, Sudre et al., 1996). It is worth noting that although paenungulates form part of the basal mammalian clade Afrotheria with a divergence of ~80 MYA (Springer, Murphy et al., 2003b), the newer molecular estimate of the paenungulate divergence of ~62.5 MYA (Springer, Murphy et al., 2003b) is similar to the morphological estimate. Although morphological data are unclear about the placement of Hyracoidea and places all three Paenungulate taxa in the most derived position in extant placental mammals, their divergence based on fossil and anatomical evidence is estimated at just preceding the K/T boundary (Novacek, 1992) an interpretation that dates from Gregory (1910: in Waddell, Cao et al., 1999a, p.120) at least, and has consistently held sway with most authors (e.g., De Jong, 1998).

1.3 Molecular analysis

1.3.1 DNA sequence data

DNA sequence comparison has been the principal molecular approach used in the investigation of mammalian systematic relationships, facilitated by the increasing availability of, and accessibility to, prospective marker fragments. Initially, phylogenetic analysis was dominated by mtDNA and in particular protein-coding fragments. The power of mtDNA has been ascribed to the rapid evolutionary rate (Creer, Malhotra et al., 2003;

Brown, Prager et al., 1982), maternal inheritance, and lack of recombination, although the latter two features have been shown to have exceptions (but also see Sato, Nakada et al., 2005; Kraytsberg, Schwartz et al., 2004; Piganeau, Gardner et al., 2004; Rokas, Ladoukakis et al., 2003; Gyllensten, Wharton et al., 1991). The phylogenetic utility of the non-coding control region, the 13 protein-coding, two rRNA, and 22 tRNA segments that comprise mammalian mtDNA has been demonstrated repeatedly at different levels. Due to representing a single linkage group, however, mtDNA segments reflect a single evolutionary history. The addition of nuclear DNA fragments to phylogenetic analysis has circumvented this and made available many more markers. Initially, like mtDNA, protein-coding regions were the marker of choice but non-coding regions, such as introns and untranslated regions (UTRs) have subsequently been included in mammalian studies (e.g., Castillo, Cortinas et al., 2005; Willows-Munro, Robinson et al., 2005; Gaubert and Veron, 2003; Murphy, Eizirik et al., 2001b; Murphy, Eizirik et al., 2001a; DeBry and Seshadri, 2001; Matthee, Burzlaff et al., 2001; Matthee and Davis, 2001).

The testing of systematic relationships with diverse markers may produce different “gene” trees (Hudson, 1983). This is due to variation in the performance of different markers in recovering phylogenies. Faster evolving sites will be more useful over shorter (Moore, 1995) and more recent time-scales, while slower, more conserved sites perform better at recovering deeper level phylogenies. Due to the overall increased rate of evolution in mtDNA (Vawter and Brown, 1986; Brown, Prager et al., 1982) it has been found to be more effective at tracking relatively recent changes (<15 MYA) consequently limiting the utility of mtDNA when applied to deeper level relationships (Halanych, Demboski et al., 1999; Lara, Patton et al., 1996; Kraus and Miyamoto, 1991). This has been documented in studies that have found that mtDNA markers perform better when compared among species and genera within the same family (Matthee, van Vuuren et al., 2004; Matthee, Burzlaff et al., 2001) than for deeper nodes. The limits of resolution of different markers/sites is a result of the different functional constraints imposed on them. Those genes and regulatory regions whose functions are most likely to be disrupted by mutations will have reduced rates of substitution (Moritz, Dowling et al., 1987), although for mtDNA an inefficient

DNA repair system also contributes to the elevated substitution rate (Moritz, Dowling et al., 1987).

In combined data sets, different markers will vary in their contribution to resolving a phylogeny and, although the effects of homoplasy from less useful markers may have a negative affect, it has generally been found that increasing data set size results in increased resolution. In contrast, although the data set size for Paenungulata is large, this has not resulted in improved resolution with marked disagreement between different data sets and partitions. A lack of congruence can be a result of both biological and analytical factors. Different inheritance pathways (eg mitochondrial versus nuclear) can lead to biological processes such as lineage sorting, gene duplication/deletion (e.g., Martin and Burg, 2002) , natural selection, genetic drift and lateral transfer of genetic material, producing different gene and species trees. Analytical factors which have been shown to affect phylogenetic reconstruction include among others taxon sampling (e.g., Graybeal, 1998), optimal model choice (Nylander, Ronquist et al., 2004), limited data (Cummings, Otto et al., 1995), model assumption violations (Nylander, Ronquist et al., 2004; Waddell and Shelley, 2003), base composition differences (Gibson, Gowri-Shankar et al., 2005; Foster and Hickey, 1999) and rate variation (site and lineage, Reyes, Pesole et al., 2000; Yang, 1996). Furthermore, there is much opportunity for subjectivity on the part of the researcher to influence the readers, for example, through choice of data set(s), model assumptions, method of tree inference and criterion of statistical support used for the results presented (Waddell and Shelley, 2003).

Results from previous phylogenetic studies including Paeunugulata give an indication as to why relationships within this clade have remained ambiguous. The problem of an unresolved paenungulate node can be characterized by conflicting associations between mtDNA (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003) and nDNA (Amrine-Madsen, Koepfli et al., 2003; Murphy, Eizirik et al., 2001b) and within the latter, an unstable node as evidenced by topological fluctuations with the addition of new sequence data and/or differences in analytical approaches (Amrine-Madsen, Koepfli et al., 2003; Waddell and Shelley, 2003; Springer, Amrine et al., 1999). Further, earlier studies have

generally found a reduced number of synapomorphies in relation to the number of autapomorphies in each of the three lineages. The lack of congruence between markers (in particular between mtDNA and nDNA) and low number of informative characters point to a rapid radiation of Paenungulata resulting in a short internal edge (Nishihara, Satta et al., 2005; Amrine and Springer, 1999; Waddell, Okada et al., 1999). The difficulties associated with a short internal edge are further exacerbated by the relatively deep divergence (~62.5 MYA) between Hyracoidea, Proboscidea and Sirenia (Springer, Murphy et al., 2003a) which will further reduce an already limited signal through homoplasy.

The two factors, a deep split followed by a rapid radiation, will affect the potential utility of nuclear and mitochondrial DNA to resolve this particular node. As a result of the maternal and haploid mode of inheritance of mtDNA, the effective population size is reduced to one quarter that of nDNA and hence it is more likely to track changes through a short internal edge (Moore, 1995). However, the advantages of using mtDNA over short intervals is likely to decrease as time since divergence increases because sites within mtDNA (such as third codon position and ribosomal RNA loop regions) experience multiple hits which can obscure the signal (which is already restricted in the case of a rapid radiation). Further, with the use of mtDNA (and faster-evolving markers overall) in conjunction with increasing evolutionary distance between taxa, comes the issue of larger variation in lineage-specific substitutional processes such as base compositional bias and among-site rate heterogeneity (Kelsey, Crandall et al., 1999).

The importance of these factors to phylogenetic analysis is best depicted in the development of mammalian systematic relationships using mtDNA. As mitochondrial data sets increased both in sequence length and sample size a considerable lack of congruence between different data sets (Gibson, Gowri-Shankar et al., 2005; Lin, Waddell et al., 2002; Waddell, Cao et al., 1999b) as well as tree inference methods was evident (Arnason, Gullberg et al., 1999; Waddell, Cao et al., 1999b). Particular lineages and their associations stood out as problematic. These include, the basal position of the hedgehog (Arnason, Adegoke et al., 2002; Arnason, Gullberg et al., 1999; Arnason, Gullberg et al., 1997; but see Waddell, Kishino et al., 2001, Sullivan and Swofford, 1997) the monophyly and

position of Murids (Arnason, Gullberg et al., 1997, but see Lin, Waddell et al., 2002; Waddell, Kishino et al., 2001), the status of Glires (Lin, Waddell et al., 2002; Arnason, Gullberg et al., 1997) and Lipotyphla (Lin, McLenachan et al., 2002; Mouchaty, Gullberg et al., 2000), and the affect of outgroups (Lin, McLenachan et al., 2002). The addition of new species helped improve resolution by breaking up long edges (Lin, McLenachan et al., 2002; Waddell, Cao et al., 1999b) where this was problematic and stabilising the position of certain taxa (e.g., the elephant as more afrotherian taxa were added). However, critical analysis of the lack of congruence in eutherian systematics through more senstitive tests highlighted the true extent of nonstationarity of evolutionary processes among species (Penny, Hasegawa et al., 1999a; Waddell, Cao et al., 1999b) and the inadequacy of then current analytical methods to deal with this (Lin, McLenachan et al., 2002; Mooers and Holmes, 2000; Penny, Hasegawa et al., 1999a; Waddell, Cao et al., 1999b; Waddell and Steel, 1997). The exclusion of problematic taxa (e.g., Lin, Waddell et al., 2002; Waddell, Kishino et al., 2001; Reyes, Pesole et al., 2000) and the use of more appropriate analyses gradually resulted in progress towards a more congruent mtDNA mammalian tree (Gibson, Gowri-Shankar et al., 2005; Kitazoe, Kishino et al., 2005; Reyes, Gissi et al., 2004; Hudelot, Gowri-Shankar et al., 2003; Waddell, Kishino et al., 2001). These approaches emphasised the importance of using a suitable model relative to the particular dataset and that high bootstrap support in the face of (considerable) model violation was not unlikely to be misleading (e.g., persistent basal hedgehog, Arnason, Adegoke et al., 2002; Arnason and Janke, 2002).

While nDNA is not exempt to problems arising from the saturation of sites (Springer, Amrine et al., 1999) and variation in substitution processes between taxa and markers, the slower rate of evolution is more appropriate for use over longer evolutionary time-scales. Further, some investigations indicate that when assessed on a per residue basis, the phylogenetic performance of nDNA surpasses mtDNA (Springer, DeBry et al., 2001; Springer, Amrine et al., 1999). Since the presence of a polytomy in combination with a deep divergence introduces particular difficultes to phylogenetic analysis, the anticipated merits or disadvantages of particular markers need to be evaluated in context of the specific study and set of taxa included.

The presence of limited signal in a data set poses particular issues for resolving the node in question. There are two scenarios to consider in this situation. First, if the internal edge is too short and/or if the original signal has been appreciably reduced over time, such that there is insufficient signal for phylogenetic analysis, then the node will be characterised as unresolvable. Alternatively, the signal may comprise sufficient informative characters for supporting a particular systematic association but is obscured by a low signal to noise ratio. Under this second scenario, the identification of emergent or hidden support (Gatesy and Baker, 2005; Gatesy, Amato et al., 2003; Gatesy, O'Grady et al., 1999; Barrett, Donoghue et al., 1991) may be possible for example, by analysing the data to maximise the phylogenetic signal by reducing the input from homplasious characters.

The addition of new sources of data to the analysis of a problematic node is a further means of improving resolution. The data available for assessing the radiation of paenungulates are extensive (Amrine-Madsen, Koepfli et al., 2003). However, the majority of markers comprise protein-coding regions with non-coding fragments represented by four UTRs. Introns are the transcribed non-coding spacer regions located between exonic regions in genes and, although they have been used successfully in phylogenetic studies of other mammalian taxa, their utility in Paenungulata has not yet been tested. These regions have been regarded as free from functional constraints and so all sites are considered potentially phylogenetically informative, with lower levels of homoplasy and reduced transition:transversion biases (Creer, Malhotra et al., 2003). This is in contrast to exons, where varying constraints on codon positions in particular will limit phylogenetic utility (Creer, Malhotra et al., 2003). Due to differing constraints on substitutional processes in comparison to exons, introns represent an alternative marker which can be used to test previous hypotheses (Waddell and Shelley, 2003). Further, introns have been shown to contain indels of varying size, that if shared between two or more taxa often contribute to phylogenetic resolution (e.g., Eick, Jacobs et al., 2005; Matthee, Burzlaff et al., 2001). Although an absence of functional constraints has been assumed for intron regions, it is becoming apparent that this may not be entirely correct. Higher than expected levels of sequence conservation in cross-species comparisons (Hare and Palumbi, 2003), non-random spatial differences that show higher conservation in sequences closer to flanking

exons than the interior of the intron (Hare and Palumbi, 2003), and nucleotide compositional bias at the fragment ends all indicate a level of functional constraint (Chamary and Hurst, 2005). It is not clear to what degree this may apply generally for intron sequences as differences in intron position within a gene have been shown to be a factor too (Levy, Hannenhalli et al., 2001; Venter, Adams et al., 2001). Although this is of concern in studies where it is assumed that neutral rates persist across a DNA segment for phylogenetic reconstruction, it is likely that similar constraints will be operating on orthologous regions across taxa and the negative effects of these "unknown" constraints will therefore be minimised.

1.3.2 Molecular cytogenetic data

The ability to accurately and confidently characterise cytogenetic changes between karyotypes at finer levels has facilitated their increasing use in comparative studies. Karyotypes were originally characterised at a basic level according to number, gross morphology (e.g. centromere position and size) and type (autosomal, sex). Through developments in staining methods which target specific areas on chromosomes, specific banding patterns (e.g., GTG-, Reverse-banding) are produced which enable the differentiation of individual chromosomes, and also delimit areas of constitutive heterochromatin (CBG-banding). However, comparison of banding patterns is an indirect means of establishing homology and particularly when chromosomal evolution is extensive, may be confounded. A more recent contribution to cytogenetic studies is the application of fluorescence *in situ* hybridisation (FISH) which allows for direct assessment of homology. Due to the relative ease of producing probe DNA to use in the hybridisation experiments, the method has considerably increased the range of potential markers for comparison. The effectiveness of this approach is evident in that it is now a commonly used application in many areas of research and clinical diagnosis.

The advantages of FISH in molecular cytogenetic analysis extend to phylogenetic studies where a wide range of markers have been identified and developed for use. These include whole chromosomes (and Frönicke, 2005 for recent reviews; see Weinberg, 2004), specific functional regions such as telomeres (Dobigny, Ozouf-Costaz et al., 2003; Meyne, Baker et

al., 1990; Meyne, Ratliff et al., 1989), targeted loci (Dobigny, Ozouf-Costaz et al., 2003; Waters, Kirby et al., 2001), and subregional probes through microdissection (Raudsepp and Chowdhary, 1999) as well as classes of DNA (e.g., LINEs, Waters, Dobigny et al., 2004). The majority of these markers delimit a part of the genome. In contrast, however, a coarse genome-wide characterisation of chromosomal changes is possible with the use of whole chromosome probes. This approach depends on generating a suite of probes consisting (preferably) of individual chromosomes. The isolation of individual metaphase chromosomes is made possible by the use of fluorescence activated cell sorting (FACS) which separates individual chromosomes on the basis of their size and nucleotide composition (Ferguson-Smith, Yang et al., 1998). This bivariate sorting procedure is performed by a dual laser beam system with associated dyes (Hoecsht 33258 and chromomycin A3). The chromosome pools generated from the sorting procedure require amplification and labeling with a fluorescent molecule, which is performed simultaneously using degenerate oligonucleotide-primed PCR (DOP-PCR, Telenius, Carter et al., 1992; Telenius, Pelmear et al., 1992). The development of multi-coloured probe systems such as SKY (spectral karyotyping e.g., Sawyer, Lukacs et al., 1998), have enabled higher levels of resolution to be obtained.

The first cross-species hybridisation experiments using fluorescently-labeled flow-sorted chromosomes, or comparative chromosome painting as it was soon termed, “painted” primate metaphase chromosomes with human probes (Jauch, Wienberg et al., 1992; Weinberg, Jauch et al., 1990). Refinements to this technique made it possible to increase the taxonomic distance of hybridisation experiments to the ordinal level (zoo-FISH, Scherthan, Cremer et al., 1994). Chromosome libraries have since been generated for representatives from almost all placental mammal orders (Frönicke, 2005) facilitating interordinal comparisons of chromosomal evolution.

1.3.2.1 Phylogenomics

Chromosome painting enables the characterisation of chromosomal changes and conserved regions through the identification of breakpoints and syntenic regions between taxa. This has enabled the identification of ancestral syntenic associations which have been detected

across different placental orders (HSA3/21, 7/16, 12/22, 14/15, 16/19)[3]. The extensive comparative studies have also enabled insight into the evolution and organisation of the mammalian genome through the reconstruction of an ancestral mammalian karyotype with the most recent estimates of diploid chromosome number ranging from 2n=44-50 (Frönicke, Wienberg et al., 2003; Murphy, Frönicke et al., 2003; Richard, Lombard et al., 2003; Yang, Alkalaeva et al., 2003). Normally, the establishment of ancestral versus derived characters is dependent on comparison to an outgroup. However, chromosome painting success between placental mammals and marsupials is limited to the X chromosome (Glas, Marshall Graves et al., 1999). The process of inferring the ancestral mammalian karyotype is consequently dependent on commonality where the most common character state of a specific character is assumed to be ancestral.

Although chromosomes, as independent genetic units that are subject to Mendelian patterns of inheritance, satisfy important requirements for phylogenetic analysis, they remain under-used in phylogenetic studies (Dobigny, Ducroz et al., 2004). Further, chromosomal rearrangements belong to a class of marker called rare genomic changes (RGCs, Rokas and Holland, 2000) which offer specific advantages over sequence based studies to particular problems. RGCs are large-scale and infrequent in comparison to nucleotide changes in sequence data (Rokas and Holland, 2000) and in addition to chromosomal rearrangements include for example, large indels, protein sequence signatures, and gene duplications (Rokas and Holland, 2000). As with morphological characters, chromosomal rearrangements can be scored on a simple presence-absence basis and hence avoid a complex analytical approach required for sequence data (Amrine-Madsen, Koepfli et al., 2003; Waddell, Kishino et al., 2001). With the inclusion of an appropriate outgroup, potential synapomorphic characters can be identified for phylogenetic reconstruction.

Previous chromosome painting studies on afrotherians have compared aardvark and elephant with human (Frönicke, Wienberg et al., 2003; Yang, Alkalaeva et al., 2003) and

[3] Ancestral syntenic associations are denoted according to their homology to human chromosomes. For example, adjacent chromosome segments of different mammalian species have been shown to be homologous to human (HSA) chromosomes 3 and 21. Here the notation HSA3/21 is used to indicate the presence of this homology.

the golden mole and elephant shrew with human (Robinson, Fu et al., 2004; Svartman, Stone et al., 2004). Syntenies detected from these comparisons have yielded the first non-DNA sequence data in support of this supraordinal clade and include HSA1/19p and an extension on the ancestral synteny HSA3/21, to HSA5/3/21. HSA1/19p has also been found in a primate, the galago (Stanyon, Koehler et al., 2002). However, in addition to the large genetic distance between afrotherians and primates, a comparison of G-banded chromosomes of the galago, aardvark and elephant indicates that the segments on HSA1 to which the afrotherians and the galago hybridise to are not orthologous (Frönicke, Wienberg et al., 2003). Hence although HSA1/19p is likely specific to afrotherians, fine-scale analysis of breakpoints would confirm this synteny. Syntenic segmental combinations were found in support of Afroinsectiphillia (HSA2/8p/4), while a further two segmental combinations, (HSA10q/17 and HSA3/20) indicate a closer association for the aardvark and elephant shrew (Robinson, Fu et al., 2004).

1.4 Aims

The aims of this study were:

1. To determine if phylogenetic signal informative for resolving the paenungulate polytomy can be distinguished from other (non-phylogenetic) signals present within the DNA sequence data. A hierarchical analysis was used to examine signal heterogeneity within existing sequence data supplemented with intron sequences to enable the identification of a limited or hidden, phylogenetic signal expected to result from a rapid radiation.

2. In an attempt to identify chromosomal synapomorphies that delimit paenungulate intra-ordinal relationships, I set out to delineate the pattern of chromosomal evolution among the three paenungulate lineages and outgroup taxa. Reciprocal cross-species chromosome painting (FISH) and standard cytogenetic staining techniques were used to characterise chromosomal rearrangements. It was hoped that these data would enable the reconstruction of an ancestral paenungulate karyotype from which insight into the genome evolution of this superordinal clade would be made possible.

CHAPTER 2 - SEQUENCING AND ANALYSIS OF NUCLEAR AND MITOCHONDRIAL MARKERS

2.1 INTRODUCTION

A polytomy represents a node in a phylogenetic tree where the expected bifurcating relationships among taxa are absent and the relationships among two or more taxa are unresolved. A node may be difficult to resolve for a variety of reasons, for example, it may represent the biologically realistic condition, i.e., a “hard” node where the divergence events among lineages are simultaneous (Cao, Adachi et al., 1994; Hoelzer and Melnick, 1994). In this case there is no shared phylogenetic signal and no further steps can be taken to improve the outcome. Alternatively a polytomy may be considered “soft” when it is difficult to resolve due to the presence of limited signal as a result of rapid successive branching events. Under certain circumstances, a soft polytomy may be perceived as a hard node. The data available may not contain sufficient signal needed to resolve the node and uncertainties will be encountered during phylogenetic reconstruction (McCracken and Sorenson, 2005).

Relationships among paenungulates have proven difficult to resolve (see Chapter 1) with the result that this clade is considered one of the remaining ambiguous nodes of placental mammal relationships (Murphy, Pevzner et al., 2004; Springer, Murphy et al., 2003b; Waddell, Kishino et al., 2001). Although a large amount of data has been applied to this question, the continued lack of resolution may be considered indicative of insufficient signal for phylogenetic reconstruction, suggesting a hard polytomy. Distinct associations among paenungulates are evident from previous studies such as the strong support for Sirenia-Proboscidea using mtDNA (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003) but, given the problematic behaviour of this marker at this level of divergence (e.g., Arnason, Adegoke et al., 2002; Arnason, Gullberg et al., 1999; D'Erchia, Gissi et al., 1996; Graur, Hide et al., 1991), it is unclear if these results are likely to represent the “true” tree or are misleading. Further, there is also the possibility that the true signal has been overlooked as a signal from a short internal edge will be limited and will not receive high

support. Although the inherent difficulties in resolving paenungulate relationships suggest that this is a hard polytomy, the possibility also exists that this node may be soft but due to the increasing effects of homoplasy appears hard. The question of whether Paenungulata is best characterised as a hard or soft polytomy can therefore be addressed by means of a comprehensive investigation aimed at identifying a limited phylogenetic signal.

In molecular systematics, the term 'signal' refers to the signature of phylogenetic history in the data. However, many other signals will be present in the data set including those resulting from multiple hits, positive selection, alterations to the mutational process and functional constraints (Gatesy and Baker, 2005 and references therein). Although it is often assumed that the signal from shared history is dominant, there is no substantiation for this. Consequently, the (phylogenetic) signal to noise ratio is an important factor in data analysis, particularly where the signal is small and likely to be obscured by homoplasy. How the signal, or characters of differing phylogenetic utility are dispersed throughout the data will also have an impact on the analysis, particularly with concatenated data sets. Incongruence within or among partitions[4] is unlikely to be evenly distributed across a finite set of sequences (Gatesy and Baker, 2005; Naylor and Brown, 1998), as a result removal or downweighting of data may result in the inadvertent loss of informative characters (Gatesy, Amato et al., 2003). The characterisation of these two factors, i.e., the relative proportion and distribution of phylogenetically useful sites, are summarised in this study as signal structure.

Variation in signal structure between partitions is determined by the gain and loss of informative characters at different sites due to diverse evolutionary processes acting at these positions. The effect of these processes may be homoplasious, for example where changes among sites are not independent such as in structural constraints in rRNA stem regions (Burk, Douzery et al., 2002), or where increased rates of evolution have resulted in saturation. However, under certain evolutionary scenarios, particular biological processes will be more likely to produce phylogenetically informative characters. For example,

[4] Definition of "partition" in this study – set of finite sequences analysed as a unit; may comprise a single segment, e.g., exon or intron, a set of combined segments or subset of segments.

constraints at slower evolving sites have facilitated the resolution of deep divergences. Consequently, similarity of evolutionary processes among sites may result in a similar phylogenetic signal and where the opportunity for signal to be established and subsequently retrieved is limited, a narrower set of sites may be expected to reflect synapomorphic changes. This is not to suggest that in each case a single set of characters will be appropriate. In fact, due to the many possible interactions between different character types (e.g., coding, non-coding) and evolutionary processes, several character sets may be useful. The identification of legitimate phylogenetic signal will provide support for a paenungulate soft polytomy; however, due to the restricted interval available for phylogenetically informative characters to be established as a result of the rapid radiation, these may be more likely to be present within a specific category of sites. As the signal degrades further (or the polytomy "hardens"), fewer phylogenetically informative characters with a gradually more diffuse and random distribution will remain. Hence the detection of a "non-random" signal (i.e., one associated with a particular group of characters) may provide evidence against a paenungulate hard polytomy. With a less favourable signal to noise ratio associated with polytomies, the ability of different data partitions to provide resolution at the appropriate level will vary due to the increased effects of stochastic error. Consequently, assessment of which regions of the data are less homoplasious, and hence more likely to contain a signal, is required.

The approach used to analyse large data sets composed of smaller partitions has attracted much discussion in the form of the "combined versus separate analysis" debate. Briefly, the combined approach has been advocated due to the expected improvement in resolving power with an increase in the number of characters. But this approach, particularly in its most extreme form (total evidence with unweighted parsimony, Kluge, 1997), has been criticised for ignoring potential heterogeneities among data sets which may generate misleading results (De Queiroz, Donoghue et al., 1995; Bull, Huelsenbeck et al., 1993). An advantage of separate analysis of data partitions is that this problem will be circumvented, but with a reduction in number of characters, resolving power may be diminished (De Queiroz, Donoghue et al., 1995). Congruence among partitions analysed separately is also often used as evidence of support for a particular hypothesis and where this is not apparent

can serve as an indication of incongruence (Miyamoto and Fitch, 1995; Hillis, 1987). Recent studies have shown that results obtained with separate and combined approaches can differ considerably (Gatesy, Amato et al., 2003; Barrett, Donoghue et al., 1991), even to the point for example, that a novel result, not evident through separate analysis, becomes apparent in a combined analysis as "hidden" support (Gatesy and Baker, 2005; Gatesy and Arctander, 2000). These results suggest that signal structure within a data set (separate and combined) is not clear-cut which has implications for phylogenetic analysis. Given that separate and combined approaches are able to provide different perspectives on conflict and support within a particular data set, the simultaneous use of these methods will be more informative than either alone (Gatesy and Baker, 2005). Where phylogenetic signal is small, this is particularly useful.

To determine which parts of the data are likely to contain phylogenetic signal appropriate for resolving a soft polytomy, signal heterogeneity is examined by determining whether phylogenetically informative or misleading signal is present. A heuristic approach is used to explore signal structure through hierarchical partitioning of the data set, facilitating identification of areas of conflict and support. This approach encompasses both combined and separate analysis of partitions, as well as subdivisions comprising similar evolutionary characteristics, i.e., mtDNA versus nDNA, coding versus non-coding, and within coding regions, codon position. This provides a more comprehensive means of surveying the data and consequently an increased chance of exposing potentially hidden signal to ascertain whether Paenungulata is likely to represent a hard or soft polytomy. To address this objective an expanded data set comprising 29904 characters from 15 mtDNA and 24 nDNA fragments is analysed. Signal structure is assessed by variation in topology and node support using two methods which differ fundamentally in their implementation, MP and BI analysis. Briefly, MP, is a cladistic approach and as the name indicates, searches for the most parsimonious outcome among competing phylogenetic hypotheses by optimising tree length to require the least number of steps. In contrast, BI is model-based and estimates different parameters in the process of generating a posterior probability distribution of trees sampled using a Metropolis-coupled Markov Chain Monte Carlo algorithm (MCMCMC, Ronquist and Huelsenbeck, 2003).

It must be made clear that in the present study, phylogenetic reconstruction is principally used as a tool to characterise signal structure (favourable or misleading) across different data partitions, rather than to search for the optimal, phylogenetically-favourable result in each partition. In phylogenetic analysis, the search for the true tree may involve the removal or downweighting of potentially homoplasious characters. Although characters are sometimes excluded in the present study, this is specifically performed to assess the phylogenetic contribution (positive or misleading) of the remaining characters and differs from the optimising approach in that all characters are ultimately considered. Following characterisation of phylogenetic signal across the data, the observed changes in topology and/or node support across different partitions are investigated in conjunction with expectations of data performance based on patterns of character evolution (e.g., coding versus non-coding, mtDNA versus nDNA). Furthermore, although the difference in approach to phylogenetic reconstruction by MP and BI can be considered as an advantage to assessing whether a result is misleading or not (i.e., convincing support of a node by both methods can be regarded with higher confidence), it is not improbable that both methods may be positively misled, for example, where sufficient violation of the model has occurred during BI (Nylander, Ronquist et al., 2004; Douady, Delsuc et al., 2003; Waddell, Kishino et al., 2001). Hence examination of the data set for areas where high support and expectations of the characters for providing resolution differ, is necessary.

The availability of an extensive data set comprising 39 partitions also enables the simultaneous assessment of the effects of i) increasing data, and ii) the use of consensus among individual partitions (McCracken and Sorenson, 2005) on improving the resolution of a polytomy. In particular, i) the increase in resolution associated with an increase in data (e.g., Gatesy and Baker, 2005; Creer, Malhotra et al., 2003; Murphy, Eizirik et al., 2001a; Madsen, Scally et al., 2001; Gatesy, O'Grady et al., 1999) is used as an indication that there has been sufficient time for a signal to be established (soft polytomy) in comparison to a hard polytomy where this has not been possible (McCracken and Sorenson, 2005). In the case of ii), the chance of encountering incongruence in the presence of a short internal edge is increased as incomplete lineage sorting is more likely to confound resolution under this condition (McCracken and Sorenson, 2005). Variation among topologies obtained with

separate analysis of different loci reflect polymorphism in the ancestral population and the strength of this effect is dependent on the length of the internal edge; as the edge length increases so does the likelihood of congruence between different data partitions, indicating that a polytomy is soft (McCracken and Sorenson, 2005). The inclusion of the combined and separate approaches enables the effect of increasing data and consensus among partitions, to be assessed respectively.

2.2 MATERIALS AND METHODS

2.2.1 Taxon sampling

Introns: Sequence data were generated from seven afrotherian species representing each of the six orders (Proboscidea, Hyracoidea, Sirenia, Tubulidentata, Macroscelidea and Afrosoricida) including specimens from each of the suborders within Afrosoricida, Tenrecomorpha and Chrysochloridea. Two outgroup species were chosen from each of the supraorders Xenarthra (Order Pilosa, *Tamandua tetradactyla* and *Bradypus tridactylus*) and Laurasiatheria (Order Carnivora, *Genetta genetta* and Cetartiodactyla, *Tragelaphus imberbis*). Nucleotide sequence data was generated for three of these species with the fourth (*Tragelaphus imberbis*) available from GenBank (AF165732, AF165733, AF165735, AF165737). Tissue was obtained either from cell pellets harvested from fibroblast cultures or fresh tissue stored in either 100% ethanol or 20% DMSO/saturated salt solution. Specimen details are listed in table 2.1.

Concatenation: Nucleotide sequences from previously published data were obtained from the publisher's website as supplementary information (Murphy, Eizirik et al., 2001b) or directly from GenBank (Apolipoprotein B and mtDNA; see table 2.2 for list of species and accession numbers). Taxon representation for the published sequence data did not always match the exact species used for the intron data. For example, in the multi-partition data set of Amrine-Madsen, Koepfli et al. (2003), *G. genetta* was not available and the next closest appropriate taxon (*Crocuta crocuta*) was selected from those available from electronic databases.

Table 2.1 New intron sequences generated for all afrotherian representatives and outgroup taxa in this study

Species name	Common name	Material	Region	Sample ID
Loxodonta africana	African savanna elephant	Fibroblast cells	MGF, PRKCI, STAT5A, THY	LAF-NAMIBIA
Procavia capensis	Cape rock hyrax	Tissue, DMSO	MGF, PRKCI, STAT5A, THY	PCA03-TUSRIV
Trichechus manatus latirostris[1, 2]	Florida manatee	Fibroblast cells	MGF, PRKCI, STAT5A, THY	TMA01-FLORIDA
Chrysochloris asiatica	Cape golden mole	DNA	MGF, PRKCI, STAT5A, THY	CAS03-STBSCH
Macroscelides proboscideus	Round-eared elephant shrew	Fibroblast cells	MGF, PRKCI, STAT5A, THY	MPR04-TANKWA
Microgale cowani[3]	Cowan's shrew tenrec	Tissue	MGF, STAT5A	MCO04-OLSEN
Echinops telfairi[4]	Lesser hedgehog tenrec	Tissue	PRKCI, THY	ETE04-SUN1256
Orycteropus afer	Aardvark	Tissue, DMSO	MGF, PRKCI, STAT5A, THY	OAF03-TUSRIV
Tamandua tetradactyla	Southern tamandua	DNA	MGF, PRKCI, STAT5A, THY	TTE-FRGUYANA
Bradypus tridactylus	Pale-throated three-toed sloth	DNA	MGF, PRKCI, STAT5A, THY	BTR-FRGUYANA
Genetta genetta	Small spotted genet	DNA	MGF	SUN1161

[1] Fibroblast cell culture provided by: Robert Bonde, Sirenia Project, U.S. Geological Survey, Florida Integrated Science Center, 2201 NW 40th Terrace, Gainesville, Florida 32605-3574.
[2] Federal Research Permit: MA-791721; CITES Export Permit: US808447.
[3] Sample tissue provided by: Dr Link Olson, Curator of Mammals, Assistant Professor of Systematic Biology, University of Alaska Museum, 907 Yukon Drive, Fairbanks, AK 99775-6960.
[4] Material provided by: Dr Heinz Künzle, Institute of Anatomy, University of Munich, Germany.

MGF – stem cell factor;
PRKCI – protein kinase C, iota;
STAT5A – signal transducer and activator of transcription 5A,
THY - thyrotropin

Table 2.2 Sequences obtained from previously published studies. The Murphy et al. (2001b) data set was downloaded directly from the publisher's website as supplementary information. (APOB - apolipoprotein B).

Species	Common name	Study	GenBank/EMBL* no.	Region
Genetta genetta	Small spotted genet	Eick et al. 2005	AJ866335*	PRKCI
Genetta genetta	Small spotted genet	Eick et al. 2005	AJ865444*	STAT5A
Genetta genetta	Small spotted genet	Eick et al. 2005	AJ865689*	THY
Tragelaphus imberbis	Lesser kudu	Matthee et al. 2001	AF165732	MGF
Tragelaphus imberbis	Lesser kudu	Matthee et al. 2001	AF165733	PRKCI
Tragelaphus imberbis	Lesser kudu	Matthee et al. 2001	AF165735	STAT5A
Tragelaphus imberbis	Lesser kudu	Matthee et al. 2001	AF165737	THY
Bradypus tridactylus	Pale-throated three-toed sloth	Amrine-Madsen et al. 2003	AF548427	APOB
Tamandua tetradactyla	Southern tamandua	Amrine-Madsen et al. 2003	AF548426	APOB
Llama glama	Llama	Amrine-Madsen et al. 2003	AY243381	APOB
Crocuta crocuta	Spotted hyena	Amrine-Madsen et al. 2003	AF548422	APOB
Echinops telfairi	Madagascar hedgehog	Amrine-Madsen et al. 2003	AF548412	APOB
Amblysomus hottentotus	Hottentot golden mole	Amrine-Madsen et al. 2003	AF548413	APOB
Elephantulus rufescens	Long-eared elephant shrew	Amrine-Madsen et al. 2003	AF548408	APOB
Orycteropus afer	Aardvark	Amrine-Madsen et al. 2003	AF548409	APOB
Loxodonta africana	African savanna elephant	Amrine-Madsen et al. 2003	AF548406	APOB
Procavia capensis	Cape rock hyrax	Amrine-Madsen et al. 2003	AF548411	APOB
Dugon dugon	Dugong	Amrine-Madsen et al. 2003	AF548410	APOB
Dasypus novemcinctus	Nine-banded armadillo	Arnason et al. 1997	Y11832	Complete mtDNA
Tamandua tetradactyla	Southern tamandua	Arnason et al. 2002	NC_004032	Complete mtDNA
Canis familiaris	Beagle dog	Zhu et al. (unpublished)	AY729880	Complete mtDNA
Llama pacos	Alpaca	Arnason et al. 2004	AJ566364	Complete mtDNA
Echinops telfairi	Madagascar hedgehog	Mouchaty et al. 2000	AJ400734	Complete mtDNA
Chrysochloris asiatica	Cape golden mole	Murata et al. 2003	AB096866	Complete mtDNA
Elephantulus sp.	Elephant shrew	Murata et al. 2003	AB096867	Complete mtDNA
Orycteropus afer	Aardvark	Arnason et al. 1999	Y18475	Complete mtDNA
Loxodonta africana	African savanna elephant	Hauf et al. 2000	AJ4821	Complete mtDNA
Procavia capensis	Cape rock hyrax	Murata et al. 2003	AB096865	Complete mtDNA
Dugon dugon	Dugong	Arnason et al. 2002	DDU421723	Complete mtDNA

2.2.2 Laboratory procedures

Total genomic DNA was extracted from tissue following standard phenol-chloroform protocols. DNA quality and approximate yield was estimated by visualizing the DNA using agarose gel electrophoresis and ethidium bromide staining.

Four nuclear intron regions, namely stem cell factor (MGF) intron 3/4, protein kinase C iota (PRKCI) intron 9/10, signal transducer and activator of transcription 5A (STAT5A) intron 15/16 and thyrotropin (THY) intron 2/3 were selected from a suite of genetic markers characterised by Matthee, Burzlaff et al. (2001) which have been shown to be useful for resolving phylogenetic relationships at both higher and lower taxonomic levels (e.g., Eick, Jacobs et al., 2005; Willows-Munro, Robinson et al., 2005; Matthee and Davis, 2001). Each intron region was amplified by polymerase chain reaction (PCR) under the following conditions: initial denaturation at 94˚C for 3 minutes; denaturation at 94˚C for 30 seconds, annealing at 48-56˚C for 30 seconds and extension at 72˚C for 45 seconds (30 cycles); and a final denaturation at 72˚C for 8 minutes. A combination of intron primers was used in the study. These were derived from primers developed for cetartiodactyls (Matthee, Burzlaff et al., 2001), leporids (Matthee, van Vuuren et al., 2004) and chiropterans (Eick, Jacobs et al., 2005). Two new afrotherian specific primers for MGF and STAT5A (table 2.3) were developed as part of the present investigation. A negative control (to test for contamination) and a positive control (from the species group the particular primer was designed from) were included each time. PCR products were visualized under UV light after agarose gel (1%) electrophoresis and ethidium bromide staining. The selected products were subsequently purified with the Wizard® SV Gel and PCR clean-up system (Promega) and cycle-sequenced using Big Dye terminator chemistry (version 3.1, Applied Biosystems, Inc). Sequences were obtained by analyzing the samples on an ABI 3100 automated DNA sequencer. The accuracy of the sequence data obtained was checked in most cases by sequencing both strands and confirming exact homology in the region of overlap. Sequence identity was further verified by GenBank BlastN searches. All sequences generated in this study were deposited in GenBank (table 2.4).

Table 2.3 Afrotherian-specific intron primers developed in this study

Region	Primer	5' – 3' sequence
MGF	MGF AFROTH F	AGTGATTGTGTGCTTTCTTC
MGF	MGF AFROTH R	TCAGTGTCACAAAACCATT
STAT5A	STAT AFROTH F	TCAGAAATCGGGGGCATCAC
STAT5A	STAT AFROTH R	AACGGCTTCAGGTTCCACAG

Table 2.4 GenBank accession numbers for intron sequences generated during this study

Species name	GenBank no. MGF	GenBank no. PRKCI	GenBank no. STAT5A	GenBank no. THY
Loxodonta africana	DQ211550	DQ211554	DQ211561	DQ211574
Procavia capensis	DQ211546	DQ211553	DQ211565	DQ211572
Trichechus manatus latirostris	DQ211547	DQ211557	DQ211566	DQ211573
Chrysochloris asiatica	DQ211545	DQ211558	DQ211564	DQ211569
Macroscelides proboscideus	DQ211544	†	DQ211563	DQ211571
Microgale cowani	DQ211549	*	DQ211567	*
Echinops telfairi	*	DQ211551	*	DQ211568
Orycteropus afer	DQ211542	DQ211552	DQ211559	DQ211570
Tamandua tetradactyla	DQ211543	DQ211555	DQ211560	DQ211575
Bradypus tridactylus	DQ211548	DQ211556	DQ211562	DQ211576
Genetta genetta	DQ279098			

† missing sequence data

* due to technical difficulties with tissue material two tenrec DNA sources were tested and the best result included

2.2.3 Alignment and processing of sequences

Sequences generated in this study and obtained from other sources were aligned separately according to the following partitions: intron data (this study), APOB (Amrine-Madsen, Koepfli et al., 2003) and mtDNA (GenBank, see table 2.2 for details). The Murphy, Eizirik et al. (2001b) sequences are available as an aligned data set and did not require further modification.

All sequences were initially aligned using T-COFFEE (Notredame, Higgins et al., 2000) with the default settings. The intron regions were manually optimized in MacClade 4.0 (Maddison and Maddison, 1989) to reduce gaps resulting from indels which ranged in size from 1-488bp across taxa. Exon sequence remaining from primer annealing sites was identified by comparison to published sequences and removed with the exception of several codons at each end which were available to anchor the alignment. Indels larger than 20bp were always found to be unique to a particular species and due to the autapomorphic nature of this character, omitted from the alignment. All remaining indels were treated as missing data for the computational analysis. Nucleotide sites that are heterozygous were identified as a double peak in the chromatogram and were recoded using an IUBMB ambiguity code.

The mtDNA sequence partition was formed by concatenating the 12 heavy-strand proteins. ND6, which is the only mtDNA protein encoded on the light strand and consequently has different evolutionary properties to the other proteins, was excluded (Murata, Nikaido et al., 2003; Nikaido, Kawai et al., 2001; Waddell, Cao et al., 1999b). The mtDNA 12S rRNA, 16S rRNA and the intervening tRNA valine form part of the aligned Murphy, Eizirik et al. (2001b) data set and therefore were not included with the mtDNA aligned data set. Remaining tRNA sequences are unavailable for any species from Hyracoidea and consequently, tRNA fragments, with the exception of tRNAvaline, were not included in the analysis for any species. Overlapping positions between ATP8/ATP6 and ND4L/ND4 were excluded. The mtDNA and APOB alignments consist entirely of coding DNA and were converted to amino acids using McClade to confirm that nucleotide alignments did not have codon frameshifts. Regions that could not be aligned unambiguously in each partition were excluded from the analysis.

2.2.4 Data analysis

2.2.4.1 Data partitioning

The data were partitioned as follows:

- each individual region or gene was analysed separately to determine (as far as possible) a relative level of signal and consequently its contribution in a combined analysis;
- to account for the different evolutionary processes and histories of mtDNA and nuclear DNA each of the individual partitions was grouped according to its genomic origin;
- to compare the potential affects of functional constraints between coding and non-coding sequences, individual genes and regions within each genomic partition were grouped according to functionality i.e., coding or non-coding;
- and at a further selective level within coding partitions, genes were divided according to codon position and these were merged across partitions;
- finally, all data were merged into a single large data set as a total molecular evidence approach in this study.

2.2.4.2 Base composition

Large variation in nucleotide composition has been shown to negatively affect phylogenetic reconstruction (Collins, Wimberger et al., 1994, Swofford, Olsen et al. 1996) and may be more of a concern for deeper divergences (Kelsey, Crandall et al., 1999). The base composition for each partition and combination thereof was first determined using PAUP* 4.0b10 (Swofford, 2002) and then tested for homogeneity of nucleotide composition using a chi-squared test of nucleotide frequencies across all taxa as implemented in PAUP* 4.0b10 (this test ignores correlation due to phylogenetic structure).

2.2.4.3 Congruence between data partitions

PAUP*4.0b10 was used to carry out pairwise comparisons of heterogeneity among individual DNA segments using the incongruence length difference test (Farris, Kallersjo et al., 1994). Uninformative characters were excluded and 1000 replicates were used for each comparison.

2.2.4.4 Phylogenetic analyses

All partitions were analysed using equal weights maximum parsimony (MP) in PAUP* 4.0 b10 (Swofford, 2002). Due to the small number of taxa in the study, the exhaustive search option could be used which evaluates every possible tree topology (Swofford, Olsen et al. 1996). To assess phylogenetic utility the number of parsimony informative sites (PI), consistency index (CI, Kluge and Farris, 1969) and retention index (RI, Farris, 1989) as well as the number of equally parsimonious trees for each data set was determined. A measure of support for each node was determined using 1000 replicates of nonparametric bootstrapping (Felsenstein, 1985), each with 100 random addition of taxa and TBR branch-swapping. Although the branch-and-bound option would have been more appropriate for smaller data sets, this option was too computationally intensive for the larger partitions. To maintain consistency across all data sets, a TBR branch-swapping approach was applied. To enable an assessment of the relative contribution of individual partitions to combined data sets, partitioned Bremmer support (Baker, Wilkinson et al., 2001) was calculated using TreeRot (version 2, Sorenson, 1999). Alternative topologies were assessed using the Kishino/Hasegawa test (1989), implemented in PAUP* 4.0 b10.

In contrast to treating sequence data as one, large, homogeneous partition as with the unweighted parsimony approach used above, the sequences were also analysed using a model-based approach. Here the evolutionary characteristics of nucleotides, such as substitution rate are taken into account and, using newer analytical methods (Huelsenbeck and Ronquist, 2001), can be estimated for separate data partitions simultaneously. For partitions with very different characteristics, as is expected with different markers and deeper divergences (Kelsey, Crandall et al., 1999), the efficiency of phylogenetic reconstruction may be hampered by various factors e.g., long branch attraction and/or among site rate variation. Model-based approaches to phylogenetic analyses, such as Bayesian inference, have been shown to be particularly useful where data partitions show a great degree of heterogeneity (Kelly, 2005; Nylander, Ronquist et al., 2004). The computer program MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) was used to analyse the data using a Bayesian inference and MCMC approach. Each run was initiated from a random tree. To better search tree-space and avoid becoming trapped on local optima, four Metropolis-

coupled MCMC (Huelsenbeck and Ronquist, 2001) chains (one cold and three heated) were implemented and the runs repeated (and compared) to determine convergence to the same topology. Four separate runs of 1 million generations, sampled at intervals of 50 generations, were conducted for individual genes or regions. Combined data sets were run for 2 million generations and sampled at intervals of 100 generations. Trees and parameter estimates sampled before the ln likelihood scores reached equilibrium were determined empirically by plotting ln likelihood scores against generation time in Excel (Microsoft) and confirmed using the *sump* command in MrBayes. This "burn-in" phase of the process was discarded prior to calculating the posterior node probabilities. The specification of an appropriate model for model-based analysis is imperative (Kolaczkowski and Thornton, 2004; Huelsenbeck and Crandall, 1997) and particularly so for more complex models (Nylander, Ronquist et al., 2004). For all data sets, an optimal model was determined by Modeltest 3.06 (Posada and Crandall, 1998) using the Akaike information criterion (Kishino and Hasegawa, 1989, Akaike, 1973). Priors were specified for the number of substitution types (nst=2 or 6) and among-site rate variation (gamma, G; invariant sites, I; or G+I) and where a gamma distribution was specified, four rate categories were used (Swofford, Olsen et al., 1996). Default settings were maintained for estimation of base frequencies (Dirichlet distribution) and topology (uniform).

In addition to the BI tree inference method, maximum-likelihood (ML) was included as an additional model-based approach to provide a more comprehensive analysis of the new (intron) sequences. The optimal nucleotide substitution model was selected (see above) using Modeltest 3.06 (Posada and Crandall, 1998) and searches using both the AIC and likelihood ratio test (LRT) were included where these differed. Maximum likelihood analyses were performed using PAUP* 4.0 b10 (Swofford, 2002). Starting trees were obtained using neighbour-joining and this was followed by TBR branch-swapping. To determine nodal support, 100 ML bootstrap replicates were performed using TBR branch-swapping on each topology.

2.3 RESULTS AND DISCUSSION

2.3.1 Data

2.3.1.1 Data description and partition variability

The combined total dataset of 29904bp consisted of 12 mtDNA protein-coding segments (10760bp), three mitochondrial rRNA regions (1640bp), 16 protein-coding genes (13857bp) and eight non-coding regions of nuclear origin. The eight non-coding nuclear regions were subdivided into four intron segments (1987bp) and four 3' untranslated regions (UTRs, 1660bp). Comparison of the different levels of functional divisions (e.g., coding versus non-coding, mtDNA versus nDNA) of the above partitions corresponds to the proportion of variable characters (% Var, table 2.5). Hence, the highest level present is found in the faster evolving mtDNA genome and specifically in ATP8 (87.56). This value is considerably higher than values for other mtDNA regions in the same class (protein-coding; 41.73-67.37, average 49.63) and may be a stochastic effect as a result of the short length of this partition (201bp). Despite this high outlier value, intron regions (50.66-69.84; average 60.16) have on average a higher level of variability than mtDNA protein segments even when including ATP8 (average 52.16). UTR partitions (16.98-56.36; average 36.05) fall within the range of variability of nuclear genes (16.45-59.68; average 36.27) and mitochondrial rRNAs (27.34-35.16; average 31.75) exhibit the lowest overall levels of variability. This pattern is not unexpected considering the higher substitution rate in intron and mtDNA regions in comparison to nuclear exons. The higher proportion of variable characters in introns, compared to mtDNA is, however, notable. Extending this analysis to codon positions within genes similarly conforms to expectations of substitution rate with third codon positions having a greater number of variable characters than first or second codon positions.

Comparison of the percentage of parsimony informative characters (%PI, table 2.5) between different partitions shows that overall mtDNA (39.27) has a far greater proportion of PI characters than nuclear DNA (17.59) and within the mitochondrial genome itself, protein coding segments (34.39-56.72; average 43.70) exceed ribosomal RNA regions (14.49-18.51; average16.68). Introns (18.06-32.65; average 24.48) contain the greatest number of PI characters within the nuclear DNA partition, followed by exons (8.30-27.02; average 17.21) and UTR regions (4.32-24.92; average 11.07).

Table 2.5 Characteristics of individual and combined regions (nDNA, mtDNA, coding, non-coding, codon position) used in this study. Values for each segment are given for the total number of characters, percentage parsimony informative characters (%PI), percentage variable characters (%Var), Retention Index (RI), MP tree length and the number of equally parsimonious trees (EPT). The percentage composition for Adenine (A), Cytosine (C), Guanine (G) and Thymine (C) are also presented.

Region	Total Sites	% PI	% Var	RI	MP TL	EPT	%A	%C	%G	%T
ND1	954	40	52	0.253	1569	1	31	28	12	29
ND2	1042	53	68	0.276	2275	3	35	28	9	28
ND3	345	45	58	0.266	618	4	31	27	12	30
ND4L	297	48	55	0.296	541	1	30	26	12	32
ND4	1377	45	59	0.242	2608	1	32	28	11	29
ND5	1746	51	60	0.236	3340	1	32	28	11	29
CO1	1541	34	42	0.240	2148	1	28	25	17	30
CO2	681	39	51	0.278	1013	1	33	26	14	27
CO3	783	36	46	0.256	1143	2	27	28	15	30
CYTB	1135	38	51	0.257	1732	1	30	29	13	28
ATP8	201	57	88	0.302	522	3	37	26	8	29
ATP6	679	40	60	0.263	1183	1	31	28	11	30
12SrRNA	672	17	33	0.341	458	3	33	23	21	22
16SrRNA	914	15	27	0.302	521	2	33	21	23	23
tRNAval	54	19	35	0.500	28	2862	31	23	20	25
ADORA3	321	21	42	0.373	253	5	21	24	19	36
A2AB	1083	17	38	0.498	698	17	15	35	29	21
ADRB2	827	11	17	0.497	363	4	21	29	25	25
ATP7a	675	16	38	0.535	398	2	34	20	19	27
BDNF	561	10	25	0.440	241	1	26	25	30	19
BRCA1	2791	23	60	0.425	2748	1	36	19	22	23
CNR1	990	12	24	0.386	439	3	22	28	25	25
EDG1	978	12	27	0.473	428	34	20	31	23	26
IRBP	1176	20	44	0.510	859	3315	19	31	31	19
PNOC	276	20	45	0.447	207	2	26	29	29	16
RAG1	774	14	31	0.516	401	255	26	24	28	22
RAG2	444	11	34	0.500	208	51	26	21	23	30
TYR	426	17	39	0.420	286	17	23	23	24	30
VWF	1236	27	52	0.400	1250	1	21	30	31	18
ZFX	204	8	22	0.615	58	510	36	22	21	21
APOB	1095	13	46	0.457	793	1	32	22	18	28
APP	630	10	34	0.474	306	1	28	20	17	35
BMI1	324	4	17	0.696	70	1275	28	17	15	40
CREM	391	10	37	0.465	207	6	26	18	26	30
PLCB4	316	25	59	0.453	303	3	34	23	18	25
MGF	569	23	61	0.494	561	1	33	17	17	33
PRKC1	452	18	51	0.575	340	17	31	14	15	40
STAT5A	441	33	70	0.453	552	3	18	29	26	27
THY	525	25	60	0.509	512	7	30	18	18	34

Table 2.5 continued.

Region	Total Sites	% PI	% Var	RI	MP TL	EPT	%A	%C	%G	%T
MtrRNA	1640	16	30	0.295	1024	1	33	22	22	23
MtDNACOD	10760	42	54	0.238	18925	1	31	28	12	29
MtDNAAll	12400	39	51	0.240	19773	1	31	27	14	28
MtCOD1	3589	33	46	0.265	4471	1	32	25	20	23
MtCOD2	3586	13	23	0.286	1812	1	19	27	12	42
MtCOD3	3585	83	95	0.227	12613	1	42	30	5	23
MtCOD12	7175	23	35	0.266	6301	2	26	26	16	32
NucAll	17504	18	45	0.430	12565	1	26	25	25	24
UTR	1660	11	34	0.426	901	2	29	20	19	32
Intron	1987	25	60	0.468	1888	2	29	18	19	34
NucCod	13857	17	41	0.419	9768	1	26	25	25	24
NucCOD1	4619	12	31	0.457	2305	1	31	22	29	18
NucCOD2	4619	10	26	0.452	1901	1	30	23	18	29
NucCOD3	4619	30	65	0.413	5518	1	18	32	27	23
NucCOD12	9238	11	28	0.452	4212	2	31	22	23	24
Total	29904	26	46	0.296	32353	1	27	24	23	26
TotalExMtCod3	26319	19	41	0.356	19996	1	27	25	21	27

Two indels larger than 2bp and shared between two or more taxa were scored from the intron sequences generated in this study (table 2.6). A further five large indels, between 79-488bp were found to be autapomorphic (table 2.6). All large indels were BLAST searched on GenBank and two of the five were identified as AfroSINEs (Nikaido, Nishihara et al., 2003) whereas the remaining three indels showed no significant homology to any other sequence present in GenBank. To avoid ambiguous assignment of indels, only regions that shared exact edges and were non-overlapping with other indels were scored. These are all reported in table 2.6.

2.3.1.2 Base composition

Base compositional differences corresponded to functional partitions (see figure 2.1 and table 2.5). Mitochondrial regions were characterised by a high percentage of adenine (A) and showed the typical mammalian bias against guanine (G, Reyes, Gissi et al., 1998) with varying levels of cytosine (C) and thymine (T) observed between species. Although the percentage of A in rRNA regions corresponds to that from the protein-coding genes, C, G and T deviated from the above pattern by displaying similar proportions to each other. Overall base composition was roughly equivalent when considering nuclear genes and UTRs; however, there was considerable variation among the individual partitions in this sample. Three of the introns, MGF, PRKCI and THY showed equivalent proportions of each base to each other with higher levels of A and T over C and G. In contrast, STAT5A deviated from this trend with approximately equal proportions of C, G and T and levels of A that were lower than the other three bases. The base composition of STAT5A apparent in this study was compared to sequences available in GenBank (AF165631, AF165647) which were found to show similar proportions.

Homogeneity of base composition was rejected in six (ND1, ND2, ND4, ND5, CO1, CYTB) of the twelve mtDNA protein coding genes, five nuclear genes (A2AB, BRCA1, IRBP, RAG1, VWF) and one intron (STAT5A) at $P < 0.05$ level (data not shown). Combined data sets all showed deviations from homogeneity except for mtDNA and nDNA second codon positions, nDNA variable first codon positions and the combined intron data set. It is likely that at deeper levels of divergence differences in base composition are more pronounced.

Table 2.6 Indels scored from intron sequences generated in this study. To avoid ambiguous assignment of indels, only regions that shared exact edges and were non-overlapping with other indels were scored. N/A – not applicable.

Taxon	Intron	Indel description	Reference
Paenungulata	MGF	14bp insertion	N/A
Xenarthra	MGF	5bp deletion	N/A
Golden mole	PRKCI	162bp, of which 67bp corresponds to AfroSINE Anc subfamily	Nikaido et al. 2003
Golden mole	PRKCI	488bp	N/A
Elephant shrew	MGF	172bp	N/A
Elephant shrew	MGF	204bp	N/A
Elephant	MGF	83bp, AfroSINE HSP subfamily	Nikaido et al. 2003

Base composition among taxa show little variation for nDNA and levels of similarity corresponded to established phylogenetic groupings (e.g., Xenarthra, Paenungulata). This is in stark contrast to that for mtDNA where considerable variation was present among sister taxa, in particular within Paenungulata (see fig. 2.2). The base composition of each mtDNA codon position was determined and then tested for homogeneity of nucleotide composition using a chi-squared test of nucleotide frequencies across Paenungulata as implemented in PAUP* 4.0b10. A highly significant difference was apparent for third codon positions which is expected due to the more exaggerated differences at these sites due to the reduced constraints in comparison to first and second codon positions. Consequently, third codon sites will evolve rapidly according to particular base composition preferences apparent in the genome (Inagaki, Simpson et al., 2004).

2.3.1.3 Nucleotide evolution within Paenungulata

2.3.1.3.1 Sequence divergence

Comparison of nDNA and mtDNA sequence divergences (corrected and uncorrected) between paenungulate species yields two distinct patterns that are consistent across partitions derived from each genome (table 2.7). In nDNA, sequence divergence estimates among paenungulates are lowest between manatee and elephant (M+E), followed by manatee and hyrax (M+H) and lastly hyrax and elephant (H+E). In contrast, mtDNA divergences between these three taxa are lowest in M+H, increasing with M+E and highest in H+E. By taking the relative substitution rates for each species within both mitochondrial and nuclear genomes into account, the difference in sequence divergence pattern of M+E between mtDNA and nDNA can be assessed. Specifically, substitution rate differences can be compared between different partitions by examining the edge lengths of the phylograms which give an indication of the number of changes leading to a particular terminal taxon. When topologies originating from both nuclear and mtDNA partitions are compared, there is a trend showing consistently shorter edge lengths for the manatee (92% of comparisons in mtDNA, 69% nDNA) relative to the hyrax and elephant, indicating a relatively slower rate of evolution in the manatee across both genomes. At nuclear sites the elephant has shorter edge lengths than the hyrax; however, in mtDNA topologies, the elephant has the longest edge length among paenungulates in 93% of cases which is consistent with an elevated mitochondrial

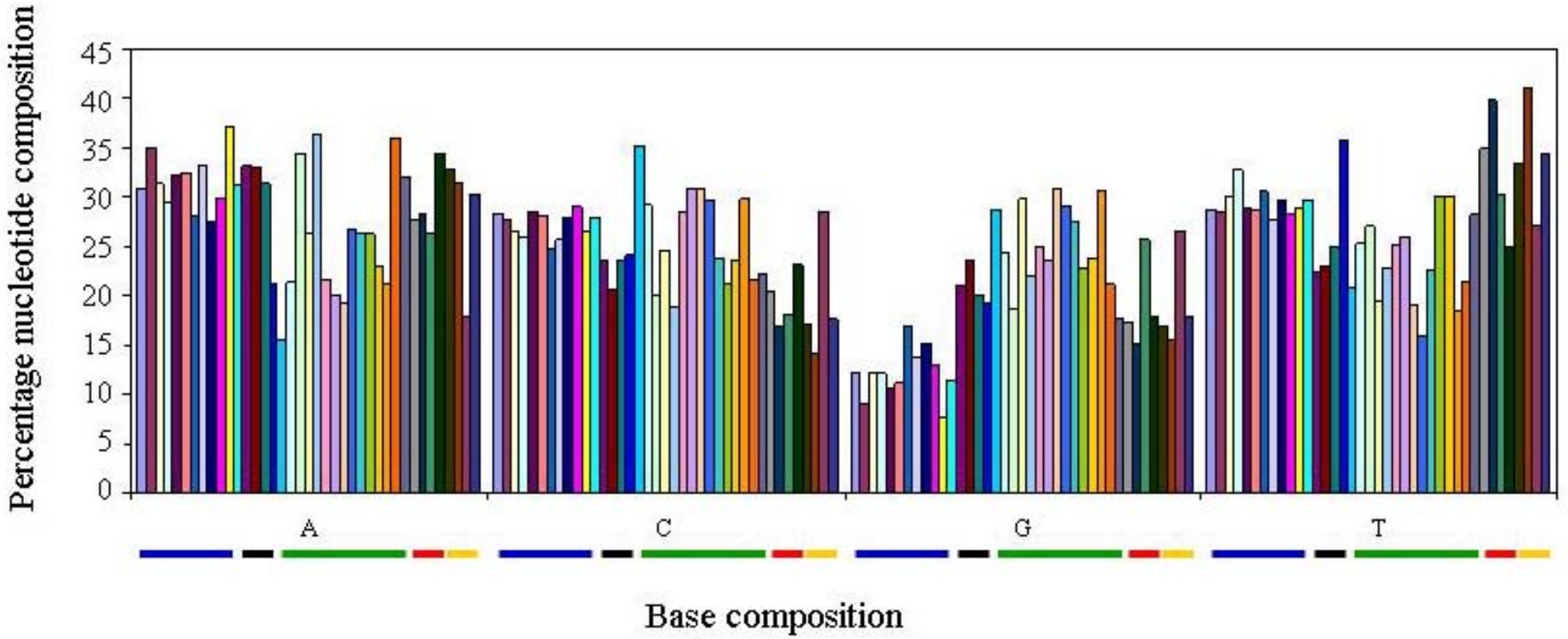


Figure 2.1 Nucleotide base composition differences between partitions. Individual bars represent separate partitions (e.g., ND2, BRCA1, MGF) and are grouped according to mtDNA protein-coding regions (blue), mtDNA rRNA regions (black), nDNA protein-coding regions (green), nDNA UTR fragments (red) and nDNA intron segments (yellow).

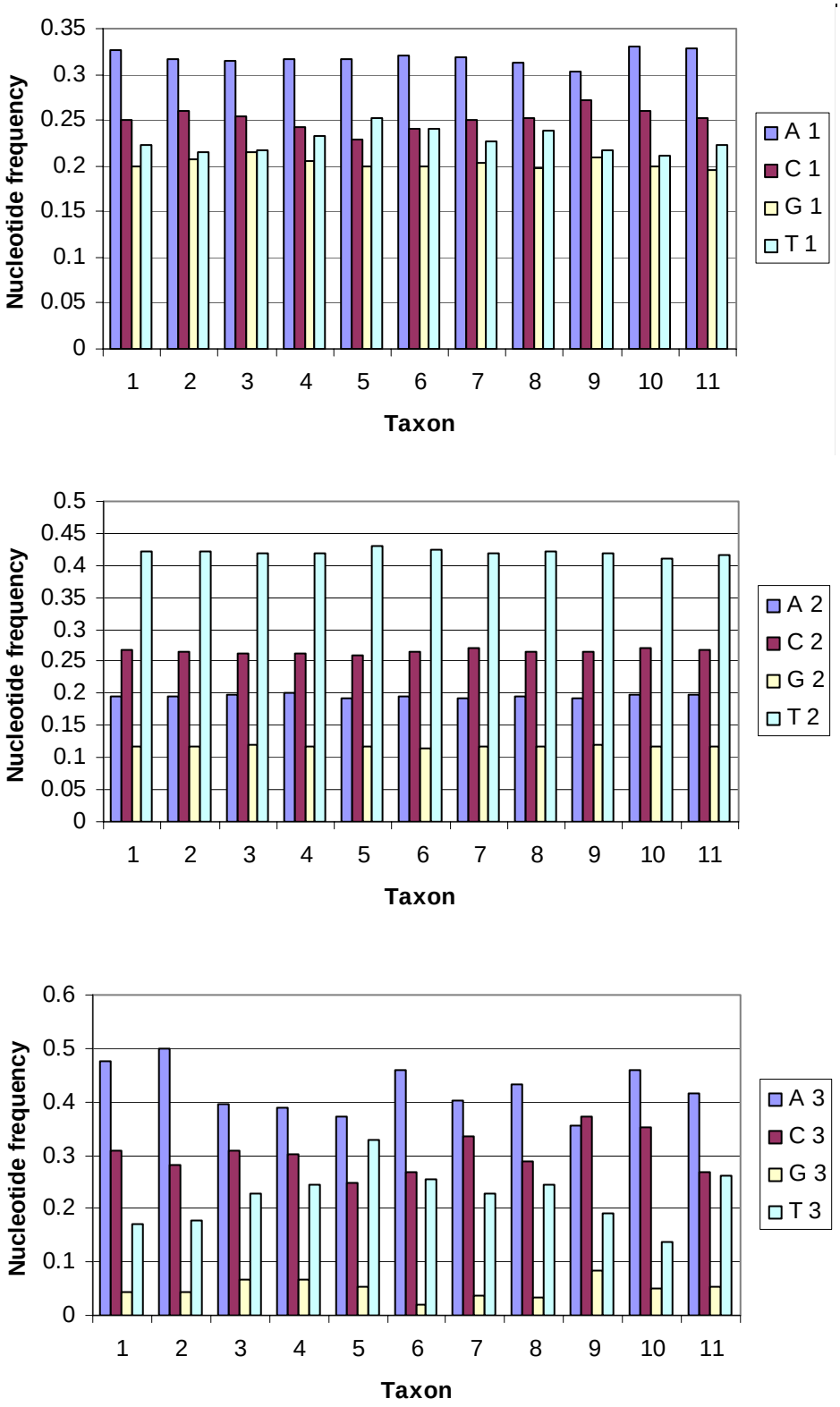


Figure 2.2 MtDNA base composition at each codon position of the taxa included in this study.1- sloth, 2- anteater, 3- llama, 4- caniform, 5- tenrec, 6- golden mole, 7- elephant shrew, 8- aardvark, 9- sirenian, 10- hyrax, 11- elephant

Table 2.7.1 Percentage sequence divergences for the total combined data set. Divergence values below the diagonal are uncorrected-p distances. GTR+G+I values are shown above the diagonal.

	Sloth	Anteater	Tenrecid	Golden mole	Sirenian	Hyrax	Elephant	Ele shrew	Aardvark	Llama	Caniform
Sloth		0.23	0.46	0.34	0.31	0.38	0.34	0.40	0.32	0.34	0.33
Anteater	0.15		0.46	0.35	0.33	0.39	0.35	0.41	0.33	0.34	0.33
Tenrecid	0.22	0.22		0.39	0.39	0.48	0.43	0.47	0.40	0.47	0.46
Golden mole	0.19	0.19	0.20		0.27	0.34	0.31	0.36	0.28	0.38	0.35
Sirenian	0.18	0.18	0.20	0.17		0.26	0.22	0.34	0.25	0.34	0.32
Hyrax	0.20	0.20	0.23	0.19	0.16		0.30	0.40	0.32	0.42	0.39
Elephant	0.19	0.19	0.21	0.18	0.14	0.18		0.37	0.28	0.37	0.35
Ele shrew	0.20	0.21	0.22	0.19	0.19	0.20	0.20		0.34	0.42	0.40
Aardvark	0.18	0.18	0.20	0.17	0.15	0.18	0.17	0.19		0.35	0.33
Llama	0.19	0.19	0.22	0.20	0.19	0.21	0.20	0.21	0.19		0.29
Caniform	0.18	0.18	0.22	0.19	0.18	0.20	0.19	0.21	0.18	0.17	

Table 2.7.2 Percentage sequence divergences for the combined nuclear data set. Divergence values below the diagonal are uncorrected-p distances. GTR+G+I values are shown above the diagonal.

	Sloth	Anteater	Tenrecid	Golden mole	Sirenian	Hyrax	Elephant	Ele shrew	Aardvark	Llama	Caniform
Sloth		0.11	0.27	0.20	0.17	0.24	0.18	0.25	0.19	0.20	0.19
Anteater	0.09		0.27	0.21	0.17	0.24	0.18	0.25	0.19	0.20	0.19
Tenrecid	0.18	0.18		0.23	0.21	0.29	0.22	0.29	0.23	0.28	0.27
Golden mole	0.15	0.15	0.16		0.13	0.19	0.14	0.21	0.15	0.23	0.21
Sirenian	0.13	0.13	0.15	0.11		0.13	0.08	0.18	0.12	0.19	0.18
Hyrax	0.17	0.17	0.19	0.14	0.10		0.14	0.24	0.18	0.27	0.24
Elephant	0.13	0.14	0.16	0.11	0.07	0.11		0.19	0.12	0.20	0.19
Ele shrew	0.17	0.17	0.19	0.15	0.14	0.17	0.14		0.20	0.27	0.25
Aardvark	0.14	0.14	0.16	0.12	0.10	0.13	0.10	0.14		0.22	0.20
Llama	0.15	0.15	0.19	0.16	0.14	0.18	0.15	0.18	0.16		0.17
Caniform	0.14	0.14	0.18	0.15	0.14	0.17	0.14	0.17	0.15	0.13	

Table 2.7.3 Percentage sequence divergences for the combined mtDNA data set. Values below the diagonal are uncorrected-p distances. GTR+G+I values are shown above the diagonal.

	Sloth	Anteater	Llama	Caniform	Tenrecid	Golden mole	Ele shrew	Aardvark	Elephant	Hyrax	Sirenian
Sloth		1.29	1.58	1.69	2.34	1.66	1.91	1.65	2.07	1.74	1.71
Anteater	0.21		1.62	1.65	2.34	1.71	2.11	1.72	2.18	1.80	1.91
Llama	0.23	0.24		1.23	2.18	1.73	1.91	1.63	2.01	1.81	1.58
Caniform	0.24	0.24	0.22		2.20	1.64	1.95	1.58	2.04	1.78	1.65
Tenrecid	0.27	0.26	0.27	0.26		1.91	2.21	2.02	2.62	2.27	2.00
Golden mole	0.23	0.23	0.24	0.23	0.25		1.70	1.41	2.00	1.74	1.54
Ele shrew	0.24	0.25	0.25	0.25	0.26	0.24		1.74	2.34	1.91	1.82
Aardvark	0.23	0.23	0.24	0.23	0.25	0.22	0.24		1.89	1.68	1.35
Elephant	0.26	0.26	0.26	0.26	0.28	0.26	0.27	0.25		1.76	1.37
Hyrax	0.24	0.24	0.25	0.24	0.27	0.24	0.24	0.24	0.25		1.36
Sirenian	0.24	0.25	0.24	0.24	0.26	0.23	0.25	0.23	0.24	0.23	

substitution rate in this species, particularly found to be higher than any other mammalian order in CYTB and 12S rRNA (Gissi, Reyes et al., 2000). It is noteworthy that the elevated rate in elephant mtDNA is contrary to the generation time hypothesis (Waddell, Cao et al., 1999a) and may indicate involvement from the many interrelated factors that could potentially affect substitution rates in mtDNA (Bromham, Rambaut et al., 1996). The increased sequence divergence of M+E present in mtDNA relative to nDNA, taking into account the slow rate in manatee across both genomes, is therefore likely a consequence of the increased rate of evolution of elephant mtDNA relative to the nDNA rate for this taxon.

2.3.1.3.2 Substitution processes

A comparison of transition/transversion (ti/tv) estimates against distance enables a further assessment of differences in substitution processes (Waddell and Steel, 1997) between nDNA and mtDNA. Ti/tv ratio estimates for pairs of taxa obtained with different corrections (gamma, G; invariant sites, I; or G+I; calculated in PAUP* 4.0b10, Swofford, 2002) were plotted against the observed distance for nDNA (figure 2.3) and mtDNA (figure 2.4). Transitions evolve faster than transversions (Jukes, 1987; Brown, Prager et al., 1982), consequently as the distance between taxa increases, the ratio will decrease as a result of multiple substitutions occurring at a site (Waddell and Steel, 1997). This is evident in nDNA (figure 2.3a). When ti/tv for each codon position is estimated separately (figure 2.3b-d), this effect is greater at faster-evolving third codon positions as expected. Ti/tv ratios estimated with a more parameter rich correction (e.g., GTR+G+I, Waddell and Steel, 1997) show a reduced leveling-off suggesting that these models are affecting a degree of correction for multiple hits. In contrast, the ti/tv estimates for mtDNA (figure 2.4a-c) show an increase with distance which appears to increase further as more parameters are included in the model suggesting an over-correction and/or strongly non-stationary process of evolution. Additionally, the range of distance values for mtDNA is narrower in comparison to nDNA which together with the trend of increasing ti/tv estimates, indicates that the models are not able to compensate for mtDNA substitution processes. Above all, the ti/tv estimates for M+E (for mtDNA) are clearly elevated in contrast to other taxon pairs particularly when third codon position are included

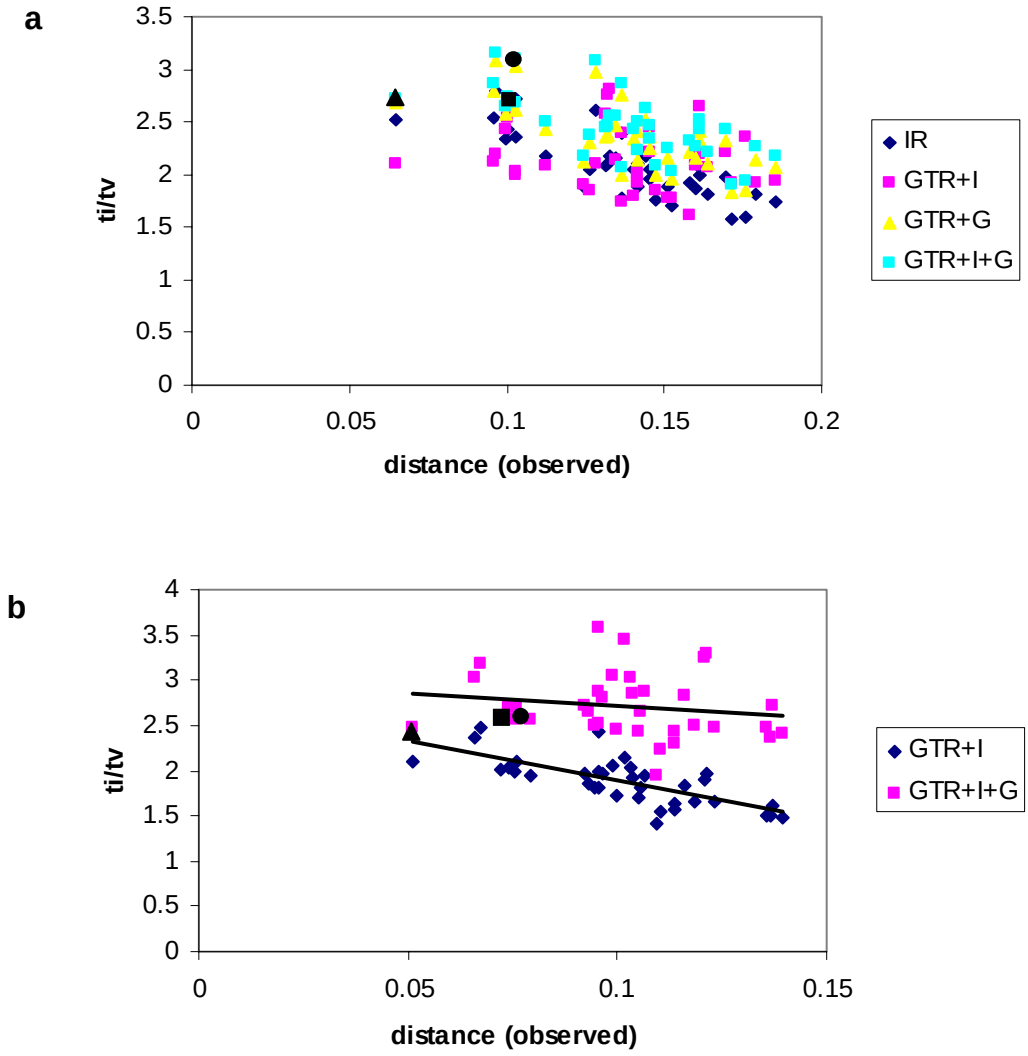
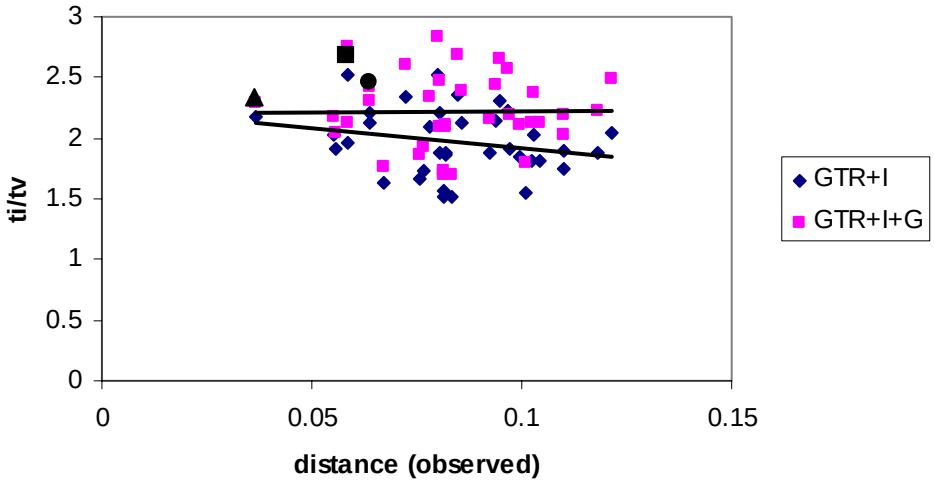


Figure 2.3 a b, (c,d overleaf) Transition versus transversion ratios in a) nuclearcod, b) nuccod1, c) nuccod2, d) nuccod3 plotted against the observed distance. IR - identical rates; ▲M+E, ● H+E, ■ M+H for GTR+G+I. Only one representative of each outgroup was included. Estimates for G and I were obtained from Modeltest 3.06 (Posada and Crandall, 1998), using the AIC and where a GTR+I+G model was not selected (nuccod3, nuccod1) the missing parameter estimates were obtained from MrBayes 3.1 (see appendix 3).

c



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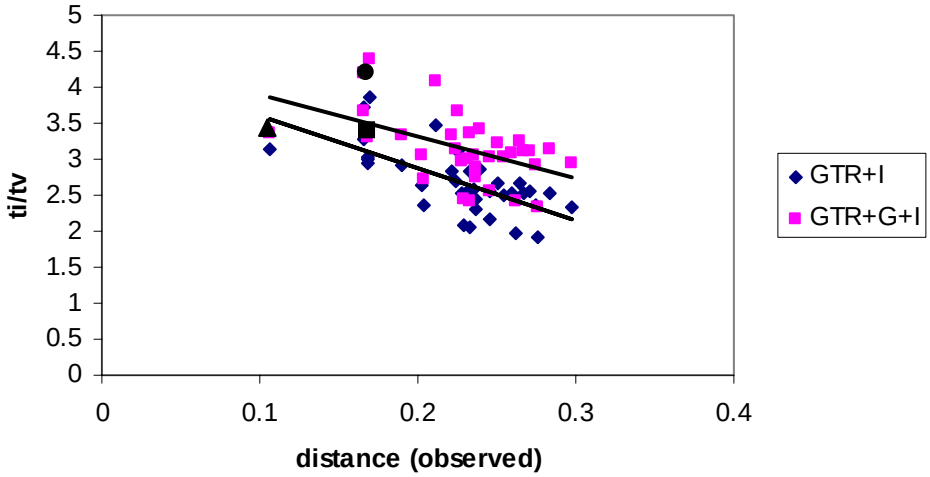

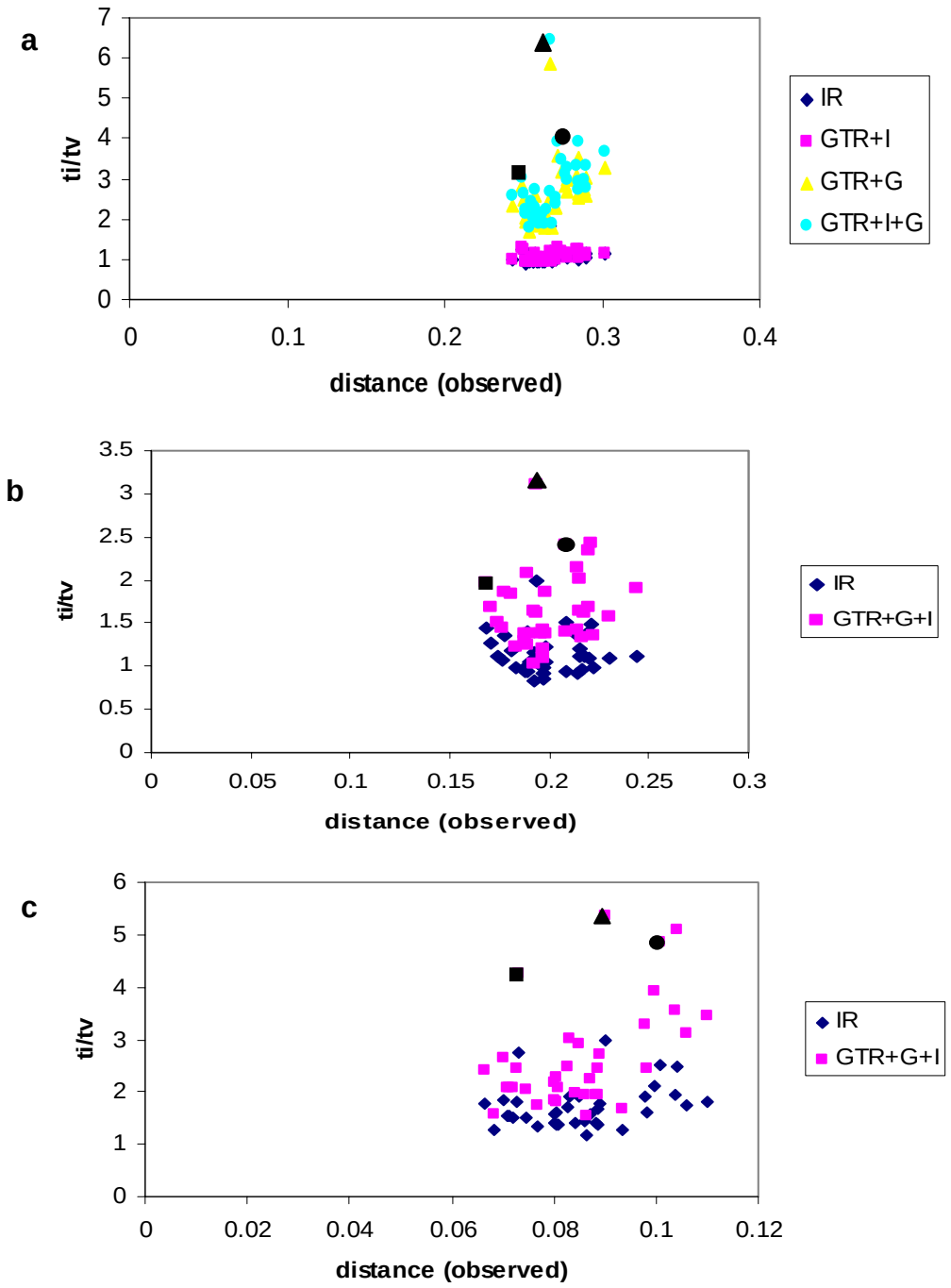

Figure 2.4 (a-c) Transition versus transversion ratios in a) mtDNAcod, b) mtDNAcod1, c) mtDNAcod2 plotted against the observed distance. MtDNAcod3 is not shown due to disproportionately high values (e.g. ti/tv = 9015 for elephant and caniform) and in many cases (including IR) were not defined by PAUP. Note IR (identical rates) points are obscured by GTR+I in mtDNAcod.▲M+E,●H+E,■M+H for GTR+G+I. Only one representative of each outgroup was included. Estimates for G and I were obtained from Modeltest 3.06 (Posada and Crandall, 1998), using the AIC (see appendix 3). Larger ti/tv ratios generally correspond to pairwise estimates with the elephant.

(fig. 2.4a) and estimates are obtained with more parameter-rich corrections. Although elephant pairwise ti/tv estimates overall are elevated in comparison to other taxon pairs (data not shown), the large difference of M+E to other elephant pairwise comparisons is noteworthy.

To determine if particular substitution biases are in effect with the elevated ti/tv estimate for M+E, a manual scan of the mtDNAcod dataset was performed using the trace character option in MacClade to directly characterise and quantify nucleotide substitutions shared between two paenungulates. Although Afrotheria receives considerable support in the basal position within the eutherian tree, alternative hypotheses are also considered feasible (see section 1.2.2). An ambiguous basal position can result in the incorrect assignment of the polarity of a character. Consequently, given the relatively small number of taxa used in this study a conservative approach was taken and the changes scored were limited to the following three groups; i) synapomorphic changes within Paenungulata, ii) those shared by two paenungulates and any single (non-paenungulate) taxon in the data set, and iii) those shared by two paenungulates and any two other (non-paenungulate) taxa in the data set. Changes were scored according to substitution type (e.g., A to T, G to A) and codon position and are summarised in table 2.8. A comparison of the number of changes counted in each of the three groups shows concordance in all cases except one (group i, T to C, M+H) indicating that the three groups are likely representative subsamples. Additionally, there is agreement in the proportion of changes in different substitution classes obtained from the manual scan (shared changes) and those present in the mtDNAcod data set (total changes per taxon, appendix 1 and 2) as calculated in PAUP* 4.0b10 (Swofford, 2002).

On first examination of the changes scored from the manual scan (table 2.8), the following results are of note: i) a large number of transversion differences for pairwise manatee changes from A to C and A to T; ii) an increase in first codon changes from C to T and A to C for M+E relative to the average number of first codon changes overall; and, iii) an increase in second codon changes from T to C present in H+E relative to the average number of second codon changes overall. Furthermore, a trend between the

Table 2.8 Pairwise nucleotide substitutions in mtDNAcod for Paenungulata. The tree used to trace the character changes corresponds to that in figure 2.6 with the root positioned between Afrotheria and Xenarthra. Group1-3 refers to group designations described in section 2.3.1.3. Cod1-3 refers to codon positions 1, 2 and 3.

		Group 1	Group 2	Group 3	Total	cod1	cod2	cod3
C to T	**MH**	3	8	13	24	4	1	19
	ME	8	16	20	44	***12***	3	29
	HE	4	14	18	36	5	5	26
T to C	**MH**	4	12	33	49	4	5	40
	ME	5	5	10	20	5	3	12
	HE	11	10	18	39	5	***12***	22
A to G	**MH**	15	7	4	26	8	1	17
	ME	13	9	5	27	9		18
	HE	7	4	3	14	4	1	9
G to A	**MH**		2	2	4	4		
	ME	1		3	4	4		
	HE	5	2	2	9	6	2	1
C to A	**MH**	2	3	3	8	2	1	5
	ME	3	3	5	11	2		9
	HE	5	8	12	25	7		18
T to A	**MH**		2	4	6		1	5
	ME		1	2	3			3
	HE	2	2	2	6	3	1	2
A to C	**MH**	9	13	10	***32***	7	1	24
	ME	12	16	24	***52***	***13***		39
	HE	6	10	2	18	3	2	13
A to T	**MH**	2	5	9	***16***	3	1	12
	ME	14	12	11	***37***	4	4	29
	HE		3		3			3
G to T	**MH**							
	ME	1		1	2	2		
	HE	2				2		
G to C	**MH**			1	1	1		
	ME							
	HE							
T to G	**MH**							
	ME			1	1		1	
	HE							
C to G	**MH**							
	ME							
	HE							

number of pairwise changes (i.e., M+H, H+E, M+E) of each substitution class and base composition is evident. An example of the latter concerns the number of A substitutions (A to N, N to A) scored for manatee pairwise associations (M+H, M+E) and the proportion of A in manatee mtDNA (fig.2.2). All changes scored for A to N are the largest for M+H and M+E, while the reverse is true for N to A with H+E showing a greater number of changes in this direction. The manatee has the lowest percentage of A relative to all other nine taxa in the mtDNAcod data set as well as to the majority of other documented eutherian mitochondrial genomes (OGRe database, sirenian representative *Dugon dugon*, Jameson, Gibson et al., 2003). This indicates a strong bias against A which agrees with the reduced number of manatee N to A pairwise changes and the increased number of A to N changes, resulting in a reduced percentage of A in mtDNA overall for this species. Furthermore, the relatively higher proportion of A present in the hyrax suggests a bias in favour of A which may also contribute to the opposite trend of hyrax pairwise comparisons to the manatee for this nucleotide. The correspondence of pairwise shared substitutions to base composition also applies to the higher proportion of G in the manatee, the lower percentage of T in the hyrax and a higher proportion of T in the elephant. When plotting the proportion of changes scored in each taxon against base composition (figure 2.5, table 2.9), there is a significant correlation for the proportion of A changes (both directions) and the proportion of T changes (T to N) but not for C. This is not unexpected given that the only two available comparisons involving C are with T and A and since these have opposite affects on H and M, they produce a counter effect in the regression canceling out the correlation.

The distinct pattern of pairwise shared sites shown here is likely due to the particularly strong base compositional biases present within Paenungulata and the way these differ with respect to each other. Base composition varies considerably between paenungulates with at least one taxon at the limit of the range of values in this data set. This pattern extends to a broader comparison with 128 placental mammals in the OGRe mtDNA database (Jameson, Gibson et al., 2003). Specifically, the hyrax lies within the lowest 4% and the elephant within the top 25% for T, the manatee has the lowest percentage of A overall while the hyrax is within the top 25% for this nucleotide, and the manatee is within the top 4% for G.

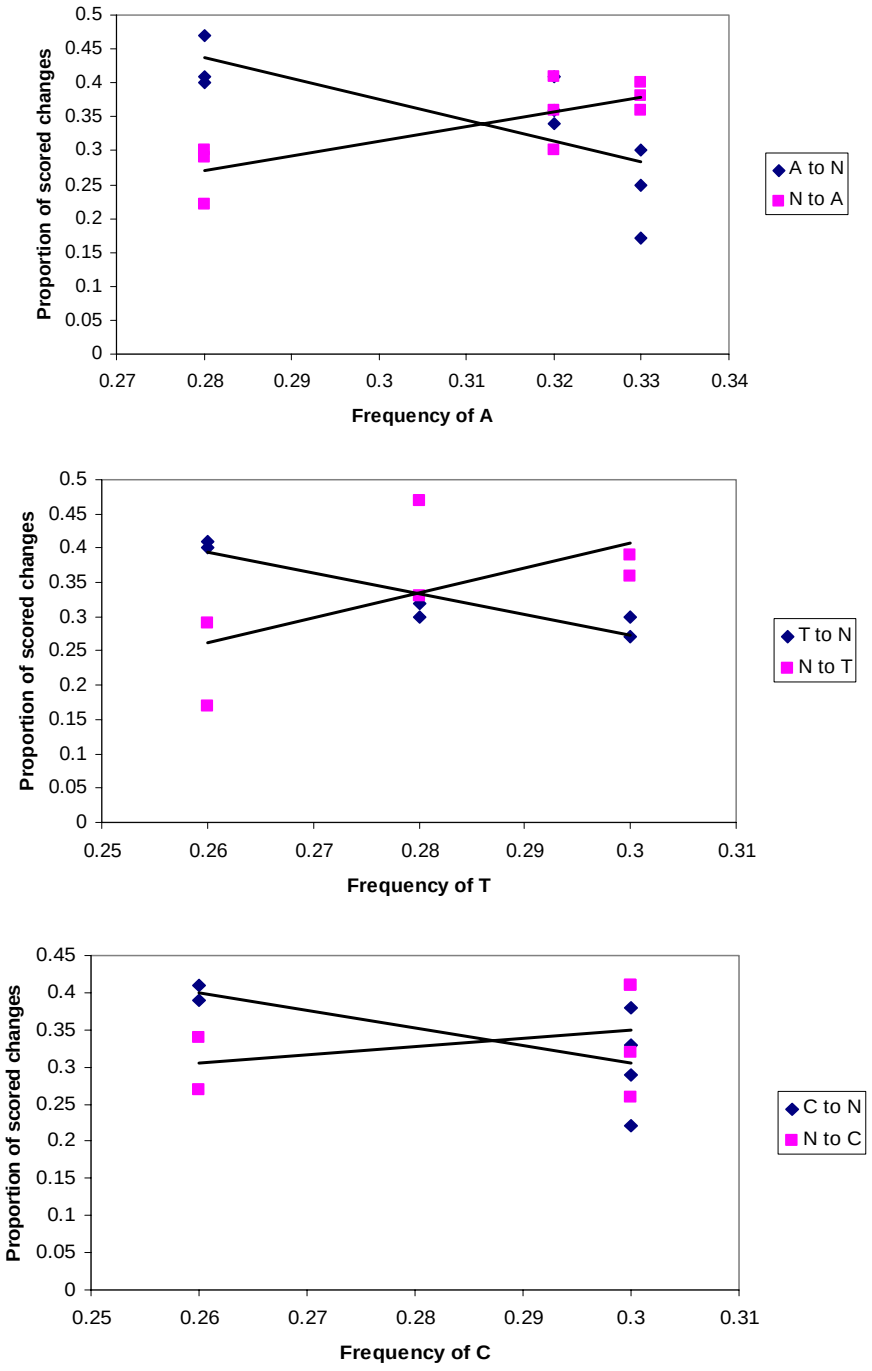

Figure 2.5 The proportion of changes scored in each taxa correlate with the base composition of A and T (T to N). Although a similar trend is seen for C substitutions and N to T substitutions, these were not found to be significant (table 2.9). Due to the lower numberof G substitutions, these could not be assessed (see table 2.8) except for A-G changes included with A above.

Table 2.9 Regression line formulas, correlation coefficients, and *P* values of lines plotted in figure 2.5.

Substitution class	Regression line	R^2	*P*
A to N	y = -3.0714x + 1.2977	0.5740	0.018*
N to A	y = 2.1905x - 0.3435	0.6538	0.008*
T to N	y = -3x + 1.1733	0.8606	0.017*
N to T	y = 3.625x - 0.68	0.4110	0.008*
C to N	y = -2.375x + 1.0175	0.4640	0.136
N to C	y = -2.375x + 1.0175	0.1265	0.490

* $P < 0.05$

Furthermore, the majority of the differences among paenungulates involve a divergent bias between two taxa (e.g., manatee versus hyrax for A, hyrax versus elephant for T) leading to different substitution patterns. As a result, since Paenungulata only comprises three taxa, the third taxon (with intermediate base composition) will show a similar pattern to either one of the other two "by default" since it shares a more similar base composition to both of them than they do with each other.

The pattern of shared substitutions shown here (e.g., A to N for M+E) likely accounts for the inflated ti/tv ratio seen in M+E in comparison to other estimates. The ti/tv estimate for M+E is due to both an elevated number of transitions and a reduced number of transversions in comparison to other taxa (appendix 1 and 2) which can be accounted for by the higher number of shared transversions (A to N) and lower proportion of shared transitions (T to C, G to A) demonstrated for M+E (table 2.8, appendix 1 and 2).

2.3.1.4 ILD tests

Significant partition heterogeneity as a potentially confounding factor in combining individual partitions was assessed by applying the incongruence length difference test (appendix 4). Tests between mitochondrial partitions were significant between 12S rRNA and ND4L ($P = 0.036$) and 12S rRNA and ATP6 ($P = 0.032$). Four groups can be identified: i) those which show two or less significant results when compared to other partitions (ND1, ND2, ND4, CO1, CYTB, CNR1, IRBP, RAG2, PNOC), ii) those that show relatively more conflict with mtDNA partitions than nuclear (ADRB2, A2AB, BRCA1, APOB, STAT5A, THY), iii) those which show most conflict with nuclear partitions (ND3, ND4L, ND5, CO2, CO3, ATP6, ATP8, 12S rRNA, 16S rRNA, tRNA APP, BDNF, CREM, PLCB4, TYR) and iv) those which show approximately even numbers of significant results between mtDNA and nDNA (ATP7, BMI1, EDG1, RAG1, MGF, PRKCI).

In considering significant heterogeneity between two particular partitions, the approach typically taken is to compare topologies and determine if conflict of well supported nodes between the topologies exists. Direct comparisons of topological variation between

partitions in this study was most often not possible due to the poor resolution of trees generated using a parsimony approach (table 2.10; see section II below for more detail). However, even when comparisons where made between topologies that were considered homogenous according to the ILD test, lack of resolution and/or conflicting nodes were evident. For example, BRCA1 pairwise comparisons to mtDNA segments returned significant P-values for all but three partitions, ND2, 12S rRNA and 16S rRNA. Comparing the BRCA1 topology to these three mtDNA partitions revealed no nodes in common between BRCA1 and 16SrRNA, only three out of nine shared nodes with 12S rRNA and five out of nine nodes in common with ND2. Consequently, the link between the extent of topological variation in pairwise comparisons and a significant result for the ILD test is tenuous in this study. Two factors may account for this variability. Firstly, the limiting effect of topological differences between partitions may impact on the capacity of the ILD test to detect incongruence (Darlu and Lecointre, 2002) and secondly, in cases where the added data generate few nodes with statistical support, it has been suggested that there is unlikely to be significant alteration of nodes previously supported in the original data set (Matthee, van Vuuren et al., 2004).

The use of the ILD test for the purpose of assessing combinability is not without criticism (Darlu and Lecointre, 2002; Yoder, Irwin et al., 2001; Cunningham, 1997; Huelsenbeck, Bull et al., 1996; Sullivan, 1996). In addition to the factors mentioned above, under conditions with increased homoplasy due to differences in substitution rate or where the number of informative characters is low, the ILD test loses power to detect incongruences (Darlu and Lecointre, 2002). Numerous studies have found that incongruence between data partitions should not prevent data set combination (e.g., Yoder, Irwin et al., 2001; Cunningham, 1997; Sullivan, 1996). Evidence is seen in the form of hidden support from combined data analysis (see Gatesy and Baker, 2005 and references therein) which likely explains why using a combined approach in cases of significant data conflict has lead to increased character support (Gatesy, Amato et al., 2003). Furthermore, the increasing evidence of hidden support indicates that non-inclusion of data sets in analysis because of conflict may be excessive, as many suitable characters might be missed (Gatesy, Amato et al., 2003). Differences in topologies with identical taxa, but generated using different

data partitions, can indicate that partition combination is questionable. However, if the conflict is due to limitations of the reconstruction method used (Cunningham, 1997), or other methodologically related factors, this does not preclude a combined approach (Huelsenbeck, Bull et al., 1996; Sullivan, 1996). Due to the overall poor resolution of the trees generated by parsimony and differences in topologies between the parsimony and model approaches for many of the partitions and some combined data sets in this study (see section 2.3.2), inadequacy of the phylogenetic method is likely a contributing factor and thus combining partitions is not problematic in these cases. However, when biological factors cannot be excluded as a potential cause of conflict, for example from lineage sorting, caution should be exercised. Due to the rapid radiation of Paenungulata (Waddell, Kishino et al., 2001; Amrine and Springer, 1999) the presence of a short internal edge is more likely to lead to lineage sorting among different partitions (McCracken and Sorenson, 2005) and consequently conflict among topologies.

2.3.2 Hierarchical (phylogenetic) analysis

To assess the level of resolution obtained from a particular topology each partition was considered according to the usefulness of the data in defining two (benchmark) clades, Afrotheria and Paenungulata. These particular nodes were selected as they are well supported by molecular data (Nishihara, Satta et al., 2005; e.g., Murphy, Eizirik et al., 2001b) and encompass evolutionary events at both temporal limits of this supraordinal clade (Springer, Murphy et al., 2003a). All subsequent phylogenetic results refer to table 2.10 and figure 2.6.

2.3.2.1 Comparison with published data

Due to the considerable differences in taxon sampling between this study and those previously published, analyses were initially performed using identical sequence data to confirm that the trees obtained in the present study were similar to those published. The Murphy, Eizirik et al. (2001b) data set consisting of 19 segments (16 nuclear, three mitochondrial) was analysed using ML (GTR+G+I, nonparametric bootstrapping) and BI (GTR+G+I) with the settings specified by the authors. Both analytical approaches agreed with that of the single published tree both with respect to topology and node support.

Although the Paenungulata node in the Murphy, Eizirik et al. (2001b) ML tree was constrained, associations among taxa within this group resulted in a polytomy when analysed here.

Amrine-Madsen, Koepfli et al. (2003) reanalysed the Murphy, Eizirik et al. (2001b) data set with the addition of a 1.3kb portion of the apolipoprotein B (APOB) gene. Both the ML (GTR+G+I) and BI (GTR+G+I) approaches were used for analysis on the gene segment alone and concatenated to the Murphy, Eizirik et al. (2001b) data set. Topologies obtained in this study were identical to those in Amrine-Madsen, Koepfli et al. (2003) with the following exceptions. In the APOB ML tree, the aardvark did not join with Paenungulata (unsupported in Amrine-Madsen, Koepfli et al., 2003) but was unresolved at the base of Afrotheria. For the ML concatenated tree, Amrine-Madsen, Koepfli et al. (2003) retrieved M+H (unsupported) whereas Paenungulata remained unresolved in this study. The BI concatenated tree and that obtained in this study were identical in topology and node support.

With regards to the mitochondrial DNA data set, two previous works (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003) have been published that include all members of Paenungulata for the 12 heavy-strand protein sequences. These were analysed using amino acid data and consequently cannot be compared to the mtDNA analysis in this study as the analytical approach differs considerably. However, with regards to associations within Paenungulata, M+E is favoured here and with the previously published result.

2.3.2.2 Taxon sampling

One of the most contentious debates in phylogenetic analysis is the question of the importance of taxon sampling versus increasing data size for efficient construction (Pollock, Zwickl et al., 2002; Hillis, 1998). By today's standards, the 11 taxa analysed here may be considered small (Sullivan, Swofford et al., 1999) and of concern. However,

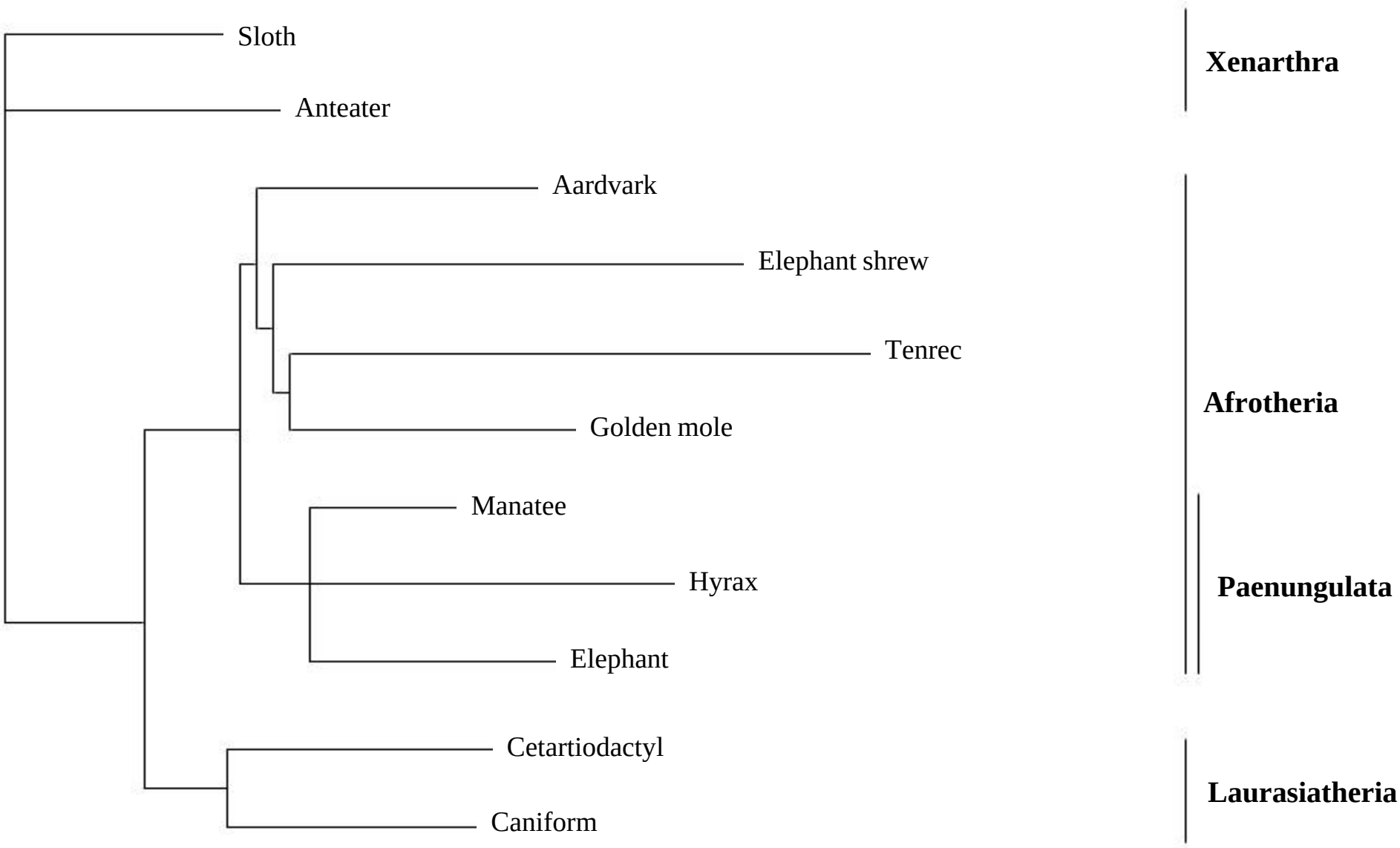

Figure 2.6 Bayesian inference tree obtained from the concatenated data set, all nodes have a posterior probability of 1. Paenungulate node represented as a polytomy due to mixed results with different BI analyses.

Table 2.10 Congruence of topologies resulting from analysis with MP and BI of individual partitions and combined data sets. Nodes in bold reflect the topology of the total evidence BI tree. A numerical value indicates (in most cases) that a particular node was statistically supported (>70% MP; >95% posterior probabilities) and two values for BI are shown where posterior probability values differed between runs. An asterisk indicates that a node was retrieved but not supported; '?' indicates that there was missing data for this node. **A** - Afrotheria, M - manatee, H - hyrax, E - elephant, **P**- Paenungulata, **I** - GM+T, **II** - Afroinsectivora, **III** - Afroinsectiphillia, IV - ES+T, V - ES+Aa, VI - ES+GM, VII - Aa+GM, VIII - Aa+T, IX - Aa+P (GM - golden mole, ES - elephant shrew, T - tenrec, Aa - aardvark). See appendix 5 for paenungulate edge lengths of combined data sets.

Region	Analyses	**A**	MH	ME	HE	**P**	**I**	**II**	**Node III**	IV	V	VI	VII	VIII	IX	**% congruence**
ND1	**MP**			*							*					0
	BI			*							*					0
ND2	**MP**	*	*			79		*								60
	BI	100		*		100				*						40
ND3	**MP**															0
	BI				*											60
ND4L	**MP**			*				*	*	*						40
	BI			96, 97				*		*						20
ND4	**MP**						*									20
	BI			*												0
ND5	**MP**															0
	BI			*			*									20
CO1	**MP**						*									20
	BI			*						*						0
CO2	**MP**	*	*			*		*	*	*						80
	BI	100	98,99					*	*	96,97						60
CO3	**MP**			*			*				*					20
	BI			*										*		0
CYTB	**MP**	*		*						*			*			20
	BI	*		96		*									*	40
ATP8	**MP**															0
	BI															0
ATP6	**MP**			*					72							20
	BI															0
12SrRNA	**MP**			*		*			*		*					40
	BI	100		*		100		*			99					60

Region	Analyses	A	MH	ME	HE	P	I	II	Node III	IV	V	VI	VII	VIII	IX	% congruence
16SrRNA	**MP**		*			*							*			20
	BI		*			95,94	*									40
tRNAval	**MP**															0
	BI															0
	% obtained	27	17	50	3	27	17	20	17	27	17	0	7	3	3	
	% supported	13	3	7	0	13	0	0	3	7	3	0	0	0	0	
ADORA3	**MP**	*		76			*									40
	BI	*		95		*	*									40
A2AB	**MP**		*			*		?	?	?	?	?				33
	BI	100				100	99	?	?	?	?	?				67
ADRB2	**MP**	81		*		74	70									60
	BI	*				*	*									60
ATP7A	**MP**	100			77	92	84				*					60
	BI	100			*	100	94				*					60
BDNF	**MP**															0
	BI						*									20
BRCA1	**MP**	100			*	98	84		83		*					80
	BI	100			*	100	*	*	100							100
CNR1	**MP**					*						*			*	20
	BI	*			*	*									*	40
EDG1	**MP**												81			0
	BI	100		*									100			20
IRBP	**MP**		*			*	?	?	?	?		?	?	?		50
	BI	100		*			?	?	?	?	*	?	?	?		50
PNOC	**MP**	*				*		*	*	*						60
	BI	100				98		98		97,96						60
RAG1	**MP**		?	*?	?	?	?	?	?			?	?			0
	BI		?	100?	?	?	?	?	?			?	?			0
RAG2	**MP**	*	?	83?	?	?	*				*					50
	BI	94	?	99?	?	?	*									50
TYR	**MP**		*			*										20
	BI	*	98			97				*						40
VWF	**MP**	87	*			*							*			40
	BI	100			*	100										40

Region	Analyses	A	MH	ME	HE	P	I	II	Node III	IV	V	VI	VII	VIII	IX	% congruence
ZFX	**MP**					*	?	?	?	?	*	?	?	?		50
	BI	95	*			*	?	?	?	?	*	?	?	?		100
APOB	**MP**	94			*	*	*	*								80
	BI	100			*	100			*						*	60
APP	**MP**	80					71									20
	BI	100			*	95,93	*		*							80
BMI1	**MP**		?		?	?	?	?	?			?	?			0
	BI	*	?		?	?	?	?	?	*		?	?			100
CREM	**MP**			*										*		0
	BI			*		*						*		*		20
PLCB4	**MP**	*				*		*		76						60
	BI	97,98				*										40
MGF	**MP**	94			76	79	*	*	*							100
	BI	100	*			100		95				*				60
PRKC1	**MP**	*			*	*		?	?	?	?	?				40
	BI	100			*	100		?	?	?	?	?		*		40
STAT5A	**MP**	*			*	93					*					40
	BI	*			*	100					94					40
THY	**MP**	93														20
	BI	100	*			99										40
	% obtained	73	19	16	33	79	40	19	17	15	21	8	8	7	6	
	% supported	44	2	5	5	41	13	3	6	5	2	0	5	0	0	
MtrRNA	**MP**	*	*			77		*			*					60
	BI	100		*		100	96		98		*					80
MtDNACod	**MP**	80		90		89				71						40
	BI - 1	100		99		100		*				*		*		60
	BI - sep	100		96		100		99				*				60
	BI - codon	100		100		100		100		*			*			60
MtDNAAll	**MP**	90		90		96		83	77	73						80
	BI - sep	100		100		100	*	100			*					80
MtDNACod12	**MP**	99		*		86		*		*			*			60
	BI	100		*		100				*						40
MtDNACod3	**MP**			*		*		*	*	*						60
	BI			100		*				*						20

Region	Analyses	A	MH	ME	HE	P	I	II	Node III	IV	V	VI	VII	VIII	IX	% congruence
MtDNACod2	**MP**	77			80	83		*		*			*			60
	BI	100				100										40
MtDNACod1	**MP**	92		78		*	*	*	*							100
	BI	100		*		100				*						40
UTR	**MP**	97			*	78		*	*							80
	BI - 1	100			*	100		*	*			*				80
	BI - indiv	100				100		*	*			*				80
NuclearAll	**MP**	100			54	100	*	*	*							100
	BI	100	57			100	100	100	100							100
NuclearCod	**MP**	100	*			100	*									60
	BI	100			75*	100	100	98	100							100
NucCod12	**MP**	100			89	100	92	70	*							100
	BI	100			98	100	100	100	*							100
NucCod3	**MP**	100		*		99						*				40
	BI	100	*			100						*			*	40
NucCod2	**MP**	100			*	98	*				*					60
	BI	100		*		100	*		100		*					80
NucCod1	**MP**	100			78	93	91	*	*							100
	BI	100			98	100	100	*							*	80
Introns	**MP**	100			*	100					*					40
	BI - 1	100	*			100					*					40
	BI - indiv	100	*			100					*					40
Total	**MP**	100		78		100		85	89	74						80
	BI - 1	100		100		100	100	100	100							100
	BI - 3	100				100	100	100	100							100
	BI - 5	100				100	100	100	100							100
TotalExMtDNA3	**MP**	100			50	100		*	73	*						80
	BI - 1	100		64		100	100	100	100							100
	BI - 3	100	92			100	100	100	100							100
	BI - 5	100	86			100	100	100	100							100

the results obtained from analysing previous data sets (see previous section, Amrine-Madsen, Koepfli et al., 2003; Murphy, Eizirik et al., 2001b) with the reduced number of taxa as used in this study, display good concordance. All topologies and levels of support were found to be identical for the combined partitions (16397-17492bp) as well the comparison with APOB alone (1095bp) when using BI, while minor differences were obtained when using ML. Despite this, the ingroup used in this study comprises all extant afrotherian taxa which precludes attempts at increasing the number of taxa at the ordinal level.

2.3.2.3 Individual partitions/separate analysis

This level represents the lowest or foundation level of the hierarchical analysis with the smallest partition sizes overall. Parsimony resolution across all individual partitions, based on nodes with bootstrap support, is limited to a single mtDNA partition (ND2), and ten nDNA regions (ADRB2, ATP7A, BRCA1, VWF, APOB, APP, MGF, PRKCI, STAT5A, THY) producing trees with either one or both of the benchmark clades supported. It is noteworthy that when adopting a less conservative standpoint in which unsupported topologies are considered, five mtDNA and 20 nDNA partitions retrieve Afrotheria and/or Paenungulata. Only three nDNA partitions showed support for associations within Paenungulata. However, when considering topologies alone, six nDNA partitions favour H+E, three M+E and four M+H and within mtDNA six partitions yield M+E, and three M+H.

When a model approach to phylogenetic reconstruction of the individual partitions was applied (BI), the extent of topological resolution and node support improved to varying degrees. However, two partitions (ATP6, ADRB2) showed reduced support while five regions (CO3, ATP8, tRNAval, ND1, ND4) remained unchanged. In agreement with the MP result, most mtDNA partitions favour M+E over M+H and H+E using BI, however, only two of these partitions show a well supported M+E node and neither of these topologies support either Afrotheria or Paenungulata. H+E continues to be favoured over M+E and M+H with nDNA, however, BI does not yield any significant posterior

probabilities for this association. Although RAG1 and RAG2 support M+E, they are not considered as the hyrax is missing from both these data sets.

Although the focus of this study concerns Paenungulata, a brief description of the results for the remaining afrotherians is included. The limited level of resolution seen for the previously discussed nodes extends to those within Afroinsectiphillia. Individual partitions retrieve a variety of associations however these also differ between mitochondrial and nuclear genomes. With both a MP and BI approach, nuclear partitions retrieve golden mole + tenrec (GM+T), Afroinsectivora and Afroinsectiphillia and consequently are more congruent with the total combined topology than are mtDNA partitions, which in contrast to nDNA regions, favour an elephant shrew + tenrec (ES+T) association.

In all but four of the topologies, the next most parsimonious tree was found to be one step away from the shortest tree. Numbers of mtDNA MP trees varied from 1-4 excluding tRNAval which produced 2862 trees, a result likely due to the very short sequence for this particular partition. Nuclear DNA partitions that did not have the full complement of taxa yielded greater numbers of MP trees than partitions with all eleven taxa. When excluding those partitions with missing taxa, the number of MP trees from nuclear partitions varied from 1-7, which is marginally larger than for mtDNA. The CI/RI estimates are lowest in mtDNA protein coding genes (CI: 0.44-0.57, RI: 0.24-0.50), intermediate in nuclear coding regions (CI: 0.54-0.67; RI: 0.37-0.62), and highest in introns (CI: 0.62-0.68, RI: 0.45-0.57) and UTRs (CI: 0.58-0.73, RI: 0.45- 0.70). In comparison to other mtDNA regions the ribosomal RNA segments tended toward the higher range of values (CI: 0.50-0.56, RI: 0.30-0.50).

Although few individual topologies had well supported nodes, it is clear that at the level of individual partitions there was no overall convincing support favouring a majority consensus of any one of the three possible associations within Paenungulata. This is also evident following assessment of the two possible alternative paenungulate topologies using the Kishino/Hasegawa (KH) test for individual partitions. The best tree was not

found to be significantly different to both of the alternative trees and only significantly different to one of the two alternatives in four partitions (ND1, CO2, 12S rRNA, TYR). In analyses of mtDNA and nDNA sequences, M+E and H+E were found to predominate respectively, suggesting consensus among mtDNA partitions and to a lesser degree, among nDNA regions. However, considering the rapid radiation of Paenungulata (Amrine and Springer, 1999) it is likely that lineage sorting is a factor. Where the internal edge is very short and under certain conditions i.e., the effective population size (*Ne*) is large, and sufficient time has passed for lineage sorting to have completed, it is expected that about 33% of gene trees from independent, polymorphic loci will support each of the three possible clades (McCracken and Sorenson, 2005; Waddell, Kishino et al., 2001; Hudson, 1983). Although support for M+E, H+E and M+H was not proportionate among nDNA partitions, and tended to favour H+E, the support for H+E was not substantial and consequently an affect from sampling bias cannot be disregarded. Based on consensus from individual partitions, support for H+E is not convincing. Since mtDNA is inherited as a single linkage group, it is not unexpected that the majority of the individual partitions share a similar result.

Resolution of Afrotheria and Paenungulata differed among individual partitions demonstrating variation in phylogenetic utility and consequently signal content among partitions. Both of these clades were supported to a greater degree by nuclear partitions and by extending the same comparison to codon position, it is apparent that the higher substitution rates in third codon positions of mtDNA coding regions have had a negative effect on the ability of individual coding partitions to resolve deeper nodes, with an unsupported topology for a single benchmark clade (Paenungulata) evident.

2.3.2.4 Intron data

The benchmark clades, Afrotheria and Paenungulata were retrieved with significant support across all four introns with five exceptions (tables 2.10. and 2.11). Three of these exceptions concern MP (PK and STAT), and one each ML (THY) and BI (STAT). Intrapaenungulate relationships in contrast were poorly resolved with only one node (MGF with MP) producing significant support for H+E. However, agreement among

model-based approaches for a particular association within an intron was noted for MGF (M+H), PK (H+E) and THY (M+H) while MP favoured H+E which is also evident in the combined intron analysis. Relationships within Afroinsectiphillia were not well resolved with a single significantly supported node (ES+A) obtained with STAT5A. The overall poor level of resolution for all nodes except Afrotheria and Paenungulata, also extended to the combined intron analyses. In the combined analyses, a manatee-hyrax association is favoured by the different model-based approaches while H+E is obtained with MP. It is noteworthy that M+E was not retrieved in any individual intron or combined analysis. The lack of resolution among paenungulates with this data set indicates that, despite the anticipated increased propensity for resolving short edge lengths, there is insufficient phylogenetic signal at the level of this analysis. Examination of indel events among the eleven taxa included (table 2.6), revealed a synapomorphic deletion specific to Xenarthra (synapomorphy implied) as well as an insertion that was informative for Paenungulata.

2.3.2.5 Combined partitions and subpartitions

Individual partitions were combined according to genome (mtDNAAll, nuclearAll), mtDNA or nuclear protein-coding loci (mtDNAcod, nuccod), non-coding regions (UTRs, introns, mtrRNA) and within protein-coding fragments, codon position (mtDNAcod1, mtDNAcod2, mtDNAcod3, mtDNAcod12, nuccod1, nuccod2, nuccod3, nuccod12). The combination of individual partitions yielded improved resolution of topologies and increased support for the benchmark clades with trends differentiating topologies derived from individual mitochondrial and nuclear loci carried through to the combined analysis. The mtDNA combined data sets show convincing support for M+E within paenungulates and favour ES+T within Afroinsectiphillia more often in comparison to GM+T. Deviation from an M+E association is found with both mtDNAcod2 and with the mtrRNA data set. It is worth noting that although mtDNAcod3 shows support for M+E, it is the only node, using either a MP or BI approach, that this data set can resolve. This is further apparent from Partitioned Bremmer Support analyses of the MP data (table 2.12) which indicate that most support (5<PBS>10) is from CYTB, CO3, ND1, ND4 and ND5 but in particular that this is largely limited to faster evolving sites, as support for this association is dominated by third (PBS = 27) and first (PBS=8) codon positions.

Table 2.11 Congruence of topologies resulting from analysis with MP, ML and BI of each of the four intron partitions separately and when combined. Nodes in bold reflect the topology of the total molecular evidence BI tree (table 2.10). A numerical value indicates (in most cases) that a particular node was statistically supported (>70% MP; >95% posterior probabilities). An asterisk indicates that a node was retrieved but not supported; '?' indicates that there was missing data for this node. **A** - Afrotheria, M - manatee, H - hyrax, E - elephant, **P**- Paenungulata, **I** - GM+T, **II** - Afroinsectivora, **III** - Afroinsectiphillia, IV - ES+T, V - ES+Aa, VI - ES+GM, (GM - golden mole, ES - elephant shrew, T - tenrec, Aa - aardvark).

									Node			
Region	**Analyses**	**A**	**MH**	**ME**	**HE**	**P**	**I**	**II**	**III**	**IV**	**V**	**VI**
MGF	MP	94			76	79	*	*	*			
	ML-AIC	100	*			98		*				*
	ML-hLRT	100	*			98						*
	BI	100	*			100		*		*		*
PRKC1	MP	*			*	*		?	?	?	?	?
	ML-AIC	99			*	96		?	?	?	?	?
	ML- hLRT	99				96		?	?	?	?	?
	BI	100			*	100		?	?	?	?	?
STAT5A	MP	*			*	93					*	
	ML-AIC	82	*			92					82	
	ML- hLRT	83				99					84	
	BI	*92			*	100					94	
THY	MP	100				93						
	ML-AIC	100	*			*						
	ML- hLRT	100	*			74						
	BI	100	*			99						
Combined	MP	100			*	100					*	
	ML	100	73			100						
	BI – 1	100	*			100					*	
	BI - indiv	100	*			100					*	

Table 2.12 Partitioned Bremmer support for mtDNA. Positive values indicate support for a particular node.

MtDNAAll	Tethytheria	Tenrec+EShrew	Afroinsectivora	Afroinsectiphillia	Paenungulata	Afrotheria	Laurasiatheria
COD1	8	2.5	2	13	11	11	24
COD2	1	2	-2	4	7	14	15
COD3	27	25	34	7	25	-10	50
ATP8	3	3	2	3	2	1	1
ATP6	5	0	3	0	14	-4	10
CO1	-6	4	3	-4	1	1	13
CO2	-6	9	12	6	3	0	-6
CO3	8	-11	-12	-2	13	-3	1
CYTB	10	5	6	-1	-7	4	8
ND1	10	2	0	4	9	7	6
ND2	-3	5	5	0	20	7	17
ND3	-3	0	2	-1	5	3	6
ND4L	4	10.5	14	10	5	1	10
ND4	7	6.5	11	-1	-2	1	6
ND5	7	-4.5	-12	10	-20	-3	17
12SrRNA	4	-1.5	-5	4	9	9	-3
tRNAval	-1	1	1	1	0	0	-1
16SrRNA	-5	-2	-3	3	3	-2	2

In contrast to mtDNA, combined nDNA data sets did not provide support in favour of a particular relationship within Paenungulata overall. However, assessment at the level of codon position provided convincing support for H+E. This association (H+E) was present (either supported or unsupported) in all nDNA combined partitions with BI and/or MP, except nuccod3. KH tests of the concatenated data sets found that H+E and M+E were significantly different in mtDNAcod ($P = 0.016$), mtDNAcod3 ($P = 0.009$), nuccod12 ($P = 0.023$) and nuccod1 ($P = 0.004$). Further, M+E and M+H differed significantly at the 95% level in mtDNAcod1 and mtDNAcod2. Well supported nodes for relationships within Afroinsectiphillia appear to be limited to the larger combined data sets (mtDNAAll, mtDNAcod, nucAll and nuccod), with limited resolution obtained with partitions less than 5Kb in size.

A further increase in data set length by combining all partitions (total) led to improved resolution in several parts of the tree; however, within Paenungulata stability remained problematic. An MP analysis favoured the mtDNA associations of M+E and ES+T but this did not apply to BI. In the latter, three different approaches to partition specification were applied. Initially all data was analyzed as a single partition. This was subsequently subdivided into three subsets: all nuclear DNA partitions combined (nuclearAll), mtDNAcod and mtrRNA and subsequently into five partitions: nuccod, UTRs, introns, mtDNAcod and mtrRNA. Dividing the total combined data set into 39 individual partitions is not possible with the (then) current version of MrBayes due to the specified limit of 30 partitions (see Material and Methods). All three methods produced identical topologies apart from relationships within Paenungulata which contrasted considerably between the single data set and the three and five subset analyses. The former maintained an M+E association as was obtained for the MP analysis, while the two latter approaches yielded polytomies.

An additional analysis was performed on the total data set excluding mtDNAcod3 with MP and the three BI approaches to partitioning the data set as implemented for the total combined data set (above). Topologies were similar in all respects except for associations within Paenungulata. The MP topology and BI one partition topology favoured H+E and

M+E respectively. Both the BI three and five partition data sets retrieved an M+H association within Paenungulata. In all cases documented above none of the nodes within Paenungulata were supported.

Using a combined approach a gradual improvement in the resolution of certain nodes was evidenced by the present study. In the case of nuclear DNA, a trend of increasing support for nodes was seen starting with Afrotheria and Paenungulata followed by relationships within Afroinsectiphillia as the different data sets were merged. Although relationships within Paenungulata were well supported in some combined data sets, the actual phylogenetic associations differed between partitions of similar size (e.g., nuccod and mtDNAcod comparisons) and continued to vary as data sets merged. This was also apparent with GM+T/ES+T but showed less variation than associations within Paenungulata when considering analysis of the total data set. A similar improvement with increasing data set size and support for the two benchmark clades was also seen when using mtDNA sequences. Comparisons between mtDNAcod and mtrRNA showed limited overlap of relationships within Paenungulata and Afroinsectiphillia but within mtDNAcod, M+E was retrieved most often with the larger data sets. Across different nuclear combined data sets, H+E was the predominant association that received support but this association was not seen with the total and totalExmtDNA3 data sets. The paenungulate node showed much variability at this level with the different analytical approaches. Although different combined data sets did provide good support for particular nodes, increasing the amount of sequence data to form the total data set did not result in convincingly resolved relationships within Paenungulata, providing evidence against a paenungulate soft polytomy. Alternatively, the presence of different signals in each genome indicates that the lack of resolution of Paenungulata may be due to incongruence/conflict among mtDNA and nDNA partitions.

2.3.3 Hyrax and Elephant

The hierarchical analysis of the first codon positions of nDNA (nuccod1) identified a well supported H+E node. Notably, this node received the most support in any nDNA combined partition for a paenungulate association using both BI and MP. The retrieval of

a highly supported H+E by nuccod1 and to a lesser degree nuccod2, as well as with several individual nDNA partitions, strongly suggests an H+E association within Paenungulata may be a biological reality. This result echoes a previous amino acid level analysis of a similar nDNA data set which also retrieved a supported H+E (Waddell, Kishino et al., 2001).

The topologies generated by both MP and BI analysis of the three nuclear codon partitions indicate that nuccod1 (which also shows good concordance with the total molecular evidence topology) shows a greater number of well supported nodes in comparison to nuccod2 or 3. By combining first and second codon positions (nuccod12), a further increase in support is obtained. However, BI analysis of a partition comprising all three coding sites results in an increase in support for all nodes except H+E. It is noteworthy that a previous study with considerably greater taxon sampling was also unable to provide support for H+E when analysing nuclear protein-coding fragments (supplementary information, Murphy, Eizirik et al., 2001b). Using an MP approach to analyse all three codon positions combined, a more dramatic change is seen producing a topology supporting only Afrotheria and Paenungulata. Substitution rates at nuccod1 are intermediate to the other two codon positions and thus substitutions at these positions may be more likely to resolve short internal edges and less likely to suffer from multiple hits in comparison to nuccod2 and 3, respectively. Whether rates in general at the first codon position are in fact appropriate for resolving the paenungulate node is unknown, but the combination of a well resolved topology across different analytical methods and which shows good concordance with the total evidence tree suggests that an H+E sister relationship merits consideration.

If this topology is considered as representative of the true tree then the improved resolution at this functional level indicates that homoplasy is proportionately lower at nuccod1 (and nuccod2) sites and misleading characters from other sites are obscuring the signal. This is indicated by the analysis of the nuclearAll data set using PBS (table 2.13) where the majority of support (5<PBS>10) is derived from three partitions: APP, ATP7A and BRCA1. However, APP is non-coding and was not included with the analysis of

nDNA protein coding sequences. The greatest support for H+E from nDNA is derived from nuccod1 (PBS = 10; nuccod2 PBS = 7) which suggests that this data set (comprising 16 partitions) contains hidden support for H+E. The negative value from nuccod3 further indicates that this partition negatively affects the retrieval of this node.

In phylogenetic analysis the justification for a particular hypothesis is largely based on statistical support and node stability across different partitions; however, it is difficult to judge the plausibility of H+E using these criteria since by definition, a soft node would not be expected to have strong support. Despite the large number of individual DNA segments included, there is the possibility that overall greater support for H+E is due to sampling error. However, support for H+E is also apparent from other sources including protein signatures which are amino acid replacements restricted to the clade in question (Van Dijk, Madsen et al., 2001) scored on a presence-absence basis similar to morphological characters. Amrine-Madsen, Koepfli et al. (2003) identified a single protein signature unique to H+E in APOB which involves the substitution of alanine for threonine at position 2396. Further, following a manual scan of the Murphy, Eizirik et al. (2001b) data set, a deletion of a single amino acid in the BRCA1 gene (at position 12710) specific to H+E was found. Recently, the amino acid sequences of 19 nuclear genes (all included in this study) were examined for Paenungulata and six other species used as outgroups (human, mouse and all remaining afrotherian taxa) to determine the number of phylogenetically informative characters supporting each of the three possible paenungulate sister relationships (Nishihara, Satta et al., 2005). Significantly with every outgroup comparison over half of the informative sites supported H+E. In the mitochondrial genome, additional support is seen using more conservative sites of mtDNAcod2 which yields a well supported H+E association with MP. Although not a completely reliable comparison, it is noteworthy that mtDNAcod2 exhibits roughly similar levels of variable sites and proportions of parsimony informative characters to nuccod1. Additionally, by analysing the data according to codon position, the effect of lineage sorting is effectively reduced as characters from different DNA segments are included. Finally, individual partitions within nDNA that favour H+E (ATP7A, BRCA1, APOB, PRKCI, STAT5A) also recovered well supported benchmark clades.

Table 2.13 Partitioned Bremmer support for nDNA. Positive values indicate support for a particular node.

NuclearAll	Hyrax+Elephant	Tenrec+GMole	Afroinsectivora	Afroinsectiphillia	Paenungulata	Afrotheria	Laurasiatheria
ADORA3	1	1	1	1	-6	7	7
ADRB2	1	2	0	0	-3	2	2
APP	5	3.5	2	2	-2	10	5
ATP7A	8	6.5	5	5	9	12	11
BDNF	1	1.5	4	4	3	2	8
BMI1	0	0	0	0	3	6	6
CNR1	-3	-3.5	-1	-1	8	-4	-4
CREM	1	0.5	2	2	0	-1	-3
EDG1	-3	-2	-16	-16	-11	-5	-7
PLCB4	-6	-6.5	-5	-5	-1	2	2
PNOC	-4	-3.5	0	0	5	4	7
RAG1	0	0	-3	-3	12	0	2
RAG2	0	0	-1	-1	-1	5	4
TYR	-7	-5	-3	-3	-2	1	6
ZFX	0	0	1	1	2	0	4
VWF	3	2.5	-4	-4	0	6	-5
BRCA1	6	6.5	19	19	16	45	44
IRBP	-1	-0.5	6	6	24	0	13
A2AB	-2	-1	-3	-3	0	5	8
NUCCOD1	10	9	21	21	12	58	49
NUCCOD2	7	5	9	9	17	21	32
NUCCOD3	-17	-11	-24	-24	36	4	17
APOB	0	-1.5	1	1	9	3	-2
MGF	-1	-1.5	0	0	14	10	17
PRKCI	2	1	2	2	1	6	10
STAT5A	2	2	2	2	12	0	12
THY	0	1	-1	-1	-2	9	13

2.3.4 Manatee and hyrax

Support for M+H was apparent both from mtDNA and nDNA markers although never in the majority in either case. Support for this clade by CO2 represented the only other well supported paenungulate node obtained from the separate analysis of mtDNA partitions and from a topological consideration alone was retrieved considerably more often than H+E. However, within nDNA M+H and M+E were apparent to similar extents but M+H was present in the combined analysis of intron as well as nuccod and nuclearAll. In the BI (partitioned) analysis of the total data set excluding mtDNAcod3, M+H was retrieved. Although M+H is present in several topologies, this clade is only supported by two data sets, CO2 and TYR. Unlike H+E, there is no convincing support at any other level and it is unclear to what extent there is hidden support for M+H.

2.3.5 Manatee and elephant

Support for M+E with mtDNA is evident from both the combined and separate analysis of partitions with particularly strong support from third codon positions and to a lesser degree, first codon positions. Furthermore, previous analysis of mtDNA protein-coding genes at the amino acid level, also provide support for M+E (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003). However, that signal for M+E is present in the fastest evolving sites, in particular mtDNAcod3 and apparently negligibly in mtDNAcod2 is a cause for concern. Since signal from third and first codon positions evolve at a faster rate it is expected that problems such as heterogeneous evolutionary processes and multiple hits which are associated with using mtDNA to resolve deep divergences, will be exacerbated (Kelsey, Crandall et al., 1999). Additionally, as a result of the short internal edge, any phylogenetic signal present will be eroded at a higher rate at faster-evolving sites and so are more likely to be obscured by a misleading signal. Consequently, there is an increasing probability that signal from third and first codons of mtDNA is likely to be compromised by homoplasy.

Although M+E is supported by mtDNA analyses together with high bootstrap values and significant Bayesian posterior probabilities, this does not discount the possibility of these results being misleading. A signal from a non-historical source may be sufficiently strong

to overwhelm the weaker but biologically real signal, as well as the statistical evaluation of the result (Bos and Posada, 2005; Naylor and Brown, 1998; Sullivan and Swofford, 1997). BI may be more sensitive to signal in the sequence data (Alfaro, Zoller et al., 2003); however, there is also a greater chance of incorrectly providing higher support for a short internal edge where non-Bayesian measures of support are low (Lewis, Holder et al., 2005) due to the stochastic nature of the model of evolution (Alfaro, Zoller et al., 2003; Erixon, Svennblad et al., 2003). Inflated levels of support for mtDNA data sets, particularly for protein-coding sequences, have been documented in previous mammalian systematic studies (Arnason, Adegoke et al., 2002; Penny, Hasegawa et al., 1999b) where high bootstrap support was associated with the incorrect placement of the root of the tree and also of certain clades (Lin, Waddell et al., 2002; Waddell, Cao et al., 1999b). Although Bayesian posterior probabilities are likely to be unbiased estimators of the probability of recovering a particular clade (Waddell and Shelley, 2003), the parameters of the model applied must be as similar as possible to those under which the sequences evolved (Nylander, Ronquist et al., 2004). If this is not the case, for example due to strong biases, then convincing support for the incorrect topology may result (Douady, Delsuc et al., 2003; Waddell, Kishino et al., 2002; Waddell, Kishino et al., 2001). This may account for the high support of the M+E node from BI when, with mtDNAcod3, a posterior probability of 1 was obtained. Furthermore, posterior probability values from the total data set analysis also show a well-supported M+E when all data partitions are merged. However, when the data are subdivided into three and then five partitions, a substantial change is noted with BI producing a polytomy at this level. In the totalExmtDNA3 analysis, a similar trend of reduced support for M+E is seen as the data is partitioned. Specification of individual partitions in a combined BI analysis enables heterogeneity among data partitions to be better accounted for (Brandley, Schmitz et al., 2005; Nylander, Ronquist et al., 2004). If this is applicable to the partition BI analysis here, the decreased support for M+E further supports that this signal is misleading. Furthermore, the over-correction evident with the ti/tv estimates obtained with different corrections demonstrates that the models are not able to account for the evolutionary processes in mtDNA.

In the presence of pronounced base composition heterogeneity, there is an increased chance for the accuracy of phylogenetic inference to be negatively affected (Gibson, Gowri-Shankar et al., 2005; Foster and Hickey, 1999; Lake, 1994; Lockhart, Steel et al., 1994). In particular, faster evolving sites which evolve rapidly to reflect particular biases in base composition (Inagaki, Simpson et al., 2004) potentially result in strongly misleading signals if the models used assume stationarity of the nucleotide substitution process (Foster and Hickey, 1999; Galtier and Gouy, 1998). The presence of variable nucleotide frequencies among paenungulates and a link between these and the proportion of pairwise shared substitutions (see section 2.3.1.3.2) is a clear indication that the signal favouring M+E is questionable. Moreover, it can be shown that the presence of an M+E association rather than M+H or H+E is a result of two specific factors: i) the presence of particular (divergent) biases in each of the three paenungulates and the interaction between these in different substitution classes; and ii) that the interaction between the biases takes place specifically under a three-taxon condition. In the case of ii), when considering a trichotomy with a divergent bias between two taxa (T1 and T2), the third taxon T3 will, as a result of being intermediate to T1 and T2, be more likely to share a similar substitution profile with either T1 or T2, than T1 and T2 would with each other. Consequently, the number of shared sites between T3 and either T1 (T3+T1) or T2 (T3+T2) will tend towards the bias particular for each divergent taxon, with the number of shared sites for T1+T2 intermediate to T3+T1 and T3+T2. As a result, the relative number of shared sites between two taxa, for example T1 and T3 (or T2 and T3), is due to divergent substitution biases in T1 and T2 rather than T1 and T3 sharing similar sites due to shared history (or conversely, sharing relatively fewer sites due to greater evolutionary distance). In the case of Paenungulata, a particular taxon pair (M+E) shares a greater number of changes across several different substitution types than other pairwise associations. M+E changes (table 2.8) dominate the data set with respect to both the total number of changes (M+E-200, M+H-166, H+E-150) and the number of different substitution classes (3 out of 6)[5] where M+E counts exceed M+H and H+E. This is accounted for by considering point i) above.

[5]The classes of substitution change where sufficient numbers of changes were detected in the manual scan include all transitions, C to A, T to A, A to T and A to C. Of these, a particular pairwise association showed a majority of shared sites in all classes except A to G and C to A (see table 2.8).

Using the previous example but taking into consideration the direction of divergent taxon bias, if the direction of change favours the bias present in T1 (i.e., a bias to G will favour changes in the direction of N to G), then logically T1 will share the most changes with T3 (intermediate taxon), while the taxon which does not favour the direction of change, T2, will share the least number of changes with T3. This is evident for divergent biases between both the hyrax versus the manatee and hyrax versus elephant. Significantly, the hyrax shares a clear divergent bias with either the manatee or elephant for seven of the eight substitution classes considered. Furthermore, since the majority of cases where the direction of change is favoured by the manatee or elephant (i.e., greater number of changes) coincides with substitution classes where a greater number of changes occur (e.g., T to C versus G to A, table 2.8), a larger number of M+E shared sites results overall. Consequently, the presence of a larger number of shared changes between M+E can be accounted for by the particular combination of biases in effect between these three taxa and differences in the relative proportion of changes present within different substitution classes, indicating that the signal associated with M+E is likely not due to shared history. Of note, although there are a majority of first and third codon sites which are shared between M+E, second codon sites do not show the same trend (with the exception of one substitutional class, A to T). This may be a result of sampling error with the low frequency of changes expected at second codon positions but may also indicate that these positions are less susceptible to the nucleotide bias and consequently may contain phylogenetic signal.

Although the previous discussion refers to analyses with all codon positions included, significant correlations obtained with these sites held for third and first codon positions separately. However, the latter found an additional significant correlation for N to T substitutions which is likely due to the elevated number of C to T sites for M+E. A relative increase at first codon positions was also present for A to C substitutions in M+E. In both cases the majority of sites code for leucine (A to C, 9 of 13 sites; C to T, 9 of 12 sites) which is the most abundant amino acid in mtDNA and one of only two amino acids that experience synonymous changes (C to T) at first codon positions. Strong biases in mtDNA nucleotide composition have previously been shown to be significantly

correlated with amino acid composition and negatively affect protein-level phylogenetic analysis (Gibson, Gowri-Shankar et al., 2005; Schmitz, Ohme et al., 2002; Singer and Hickey, 2000; Foster, Jermiin et al., 1997). This is contrary to the assumption that protein sequences are generally considered to be free from the effects of bias in nucleotide sequence due to the presence of greater functional constraints (e.g., Loomis and Smith, 1990). However, the presence of a significant correlation between base composition and N to T and A to N pairwise changes at first codon positions indicate that protein-level analyses for Paenungulata are also affected by nucleotide bias and may explain why previous studies using mtDNA protein-coding sequences analysed at the amino acid level also supported an M+E node (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003)[6].

It has been recently reported that random sequences can bias phylogenetic reconstruction (Susko, Spencer et al., 2005). Random sequences occur when a proportion of sites is independent of sequence data for other taxa, for example due to incorrect alignment or when sites evolve at a considerably increased rate in some portions of the tree; this phenomenon is of particular relevance when deep divergence times are involved (Susko, Spencer et al., 2005). With the considerably increased mtDNA substitution rate of elephant relative to manatee and most other mammalian orders (Gissi, Reyes et al., 2000; Hauf, Waddell et al., 2000; Waddell, Cao et al., 1999b), it is not unlikely that DNA segments from the elephant may contain random sites which negatively influence phylogenetic reconstruction.

2.4 CONCLUSION

In the present study, a hierarchical analysis was used to increase understanding of signal heterogeneity within the 29904kb data set. By taking advantage of the different approaches to phylogenetic reconstruction used by MP and BI, the identification of areas of conflict and support not otherwise evident with a strictly separate or total evidence

[6] Although the test for homogeneity of nucleotide composition (PAUP*4.0b10, section 2.3.1.2) was not significant for first codon sites ($P = 0.114$), the results discussed here indicate that this may be due to this test being conservative (Waddell, Cao et al., 1999)

combined approach was possible. This enabled the use of an exploratory approach to examine and interpret node support to gain insight into the paenungulate polytomy.

The M+E evolutionary relationship has received support from previous molecular studies (Murata, Nikaido et al., 2003; Nikaido, Cao et al., 2003) as well as from morphological characters as the Mirorder Tethytheria (McKenna, 1975). Additionally, evidence for an aquatic ancestry for Proboscidea (Gaeth, Short et al., 1999) is considered as further confirmation; however, convergent evolution of this character cannot be disregarded (Seiffert, 2003). As demonstrated here, there are several lines of molecular evidence to suggest that there are misleading factors contributing to M+E, most notably the correlation of base composition bias and the proportion of pairwise shared sites. Although this does not discount M+E altogether, it introduces a caveat for the use of these data to support M+E. The Waddell, Kishino et al., (2001) study was the first using a large, combined data set to suggest a H+E sister relationship for Paenungulata. In the present study, this association received convincing support from a specific set of characters sharing similar evolutionary properties. Although H+E is not robustly supported by larger data sets, this is not an expectation of a signal from a short internal edge (soft polytomy). Additional molecular evidence supports this result (e.g., Nishihara, Satta et al., 2005; Amrine-Madsen, Koepfli et al., 2003). The association of M+H was evident in several nDNA and mtDNA individual markers, though never in the majority, and together with H+E was retrieved by several combined partitions. This may indicate hidden support for M+H, however, due to the absence of a defined set of characters providing convincing support (as evident for H+E), precluded further investigation of the M+H association in this study.

A definitive solution to the paenungulate polytomy is not possible from the analysis presented here. However, given the likelihood that the M+E association is affected by misleading signal and the comparatively lower support for M+H, these associations are considered less probable than the H+E association. H+E fits the pattern expected for a small signal from a soft polytomy and if real, then Paenungulata is best characterised as a soft polytomy. If H+E is incorrect, the data based on this analysis are then in favour of a

paenungulate trichotomy, whether due to an essentially simultaneous radiation or subsequent hardening of a soft node. Finally, this study empirically demonstrates that increasing data does not necessarily result in more biologically accurate trees but that consideration of signal structure, particular in the case of a short internal edge, is an important element in combined analyses of increasingly larger and likely heterogeneous data sets.

CHAPTER 3 – COMPARATIVE CYTOGENETICS

3.1 INTRODUCTION

An option available in phylogenetic reconstruction to address the problem of an ambiguous node is the inclusion of alternative markers. For DNA sequence data this choice is in effect limitless, made possible by the increasing availability of new markers through comprehensive genome sequencing projects. A less frequently used approach to phylogenetic reconstruction is the comparison of chromosomal differences between taxa using cytogenetic methods. This approach provides a broad, genome-wide assessment of evolutionary changes in contrast to that of the relatively narrow but fine-scale analysis at the nucleotide level. Consequently, the phylogenetic analysis of problematic nodes may benefit from an alternative perspective on evolutionary relationships – such as the one provided by cytogenetic methods.

The relationship among the three extant orders (Proboscidea, Hyracoidea and Sirenia) comprising Paenungulata remains unresolved despite several comprehensive nucleotide-level analyses. Although various chromosomal data exist for several species from each paenungulate order (see Chapter 1), the potential of a cytogenetic approach to resolve this trichotomy has not previously been investigated. Accordingly, the delineation of chromosomal rearrangements among paenungulates and outgroup reference taxa permits the use of phylogenetic analyses to determine if synapomorphic changes are present (Dobigny, Ducroz et al., 2004; Qumsiyeh and Baker, 1988).

The choice of phylogenetic character (and character state) has varied across chromosomal phylogenetic studies and the validity of these choices have been much debated (e.g., Dobigny, Ducroz et al., 2004; Borowik, 1995; Qumsiyeh and Baker, 1988). Whole chromosomes (Viegas-Péquignot, Dutrillaux et al., 1983) or chromosome segments (Otells, 1995) as well as rearrangements (De Oliviera, Neusser et al., 2005; Frönicke, 2005) have previously been selected as the appropriate character for phylogenetic reconstruction. Here, I followed Dobigny, Ducroz et al. (2004) and defined individual chromosome rearrangements as characters and the presence or absence thereof as the

character state. Consequently, characters could be coded using a binary approach for phylogenetic analysis. The use of rearrangements as phylogenetic characters avoids multiple scoring which may be problematic if the whole chromosome, or part thereof, is used as a character. Further, this ensures that characters are independent (Dobigny, Ducroz et al., 2004). For example, a chromosomal segment that has undergone a fission is unlikely to be independently rejoined in various evolutionary lineages (Weinberg, Stanyon et al., 1997).

The characterisation of specific characters as derived rather than ancestral requires comparison with an outgroup reference (Dobigny, Ducroz et al., 2004; Qumsiyeh and Baker, 1988) following cladistic methodology (Farris, 1978). Here the inclusion of outgroup taxa enabled the polarisation of characters for phylogenetic analysis. In this study, the aardvark and the human were selected as outgroups. Although the sister taxon of Paenungulata is uncertain, the aardvark has received support in this position (Amrine-Madsen, Koepfli et al., 2003). Further, from a cytogenetic perspective, this species is regarded as conserved within eutherian evolution (Yang, Alkalaeva et al., 2003). Consequently, the inclusion of a closely related species as outgroup should filter “noise” that may result from using an outgroup that is too distantly related (Müller, Hollatz et al., 2003). It may have been additionally informative to include an outgroup to Afrotheria. This supraordinal clade is generally considered basal within eutherian phylogenetics (Springer, Murphy et al., 2003a, but see section 1.2.2 for discussion on alternative hypotheses) and would require comparison to a metatherian representative; however, data for a eutherian-metatherian assessment are currently limited to the X chromosome (Glas, Marshall Graves et al., 1999). Characterisation of homologous segments between paenungulates and human were indirectly mapped through existing data between the human and aardvark, and human and elephant (Frönicke, Wienberg et al., 2003; Yang, Alkalaeva et al., 2003). The human syntenic patterns were included to enable comparisons with existing data on paenungulates (Frönicke, Wienberg et al., 2003; Yang, Alkalaeva et al., 2003) and other eutherian taxa.

Two techniques were used to characterise karyotypic rearrangements: standard cytogenetic staining techniques (G- and C-banding), and cross-species comparative painting using fluorescence *in situ* hybridisation (FISH). Previous applications of cytogenetic data to phylogenetic questions were initially based on inferred homologies using chromosome morphology and staining results. However, the usefulness of this approach is restricted due to uncertainty regarding true homology, especially with highly rearranged chromosomes (Borowik, 1995) resulting from elevated levels of chromosomal evolution (Müller, Hollatz et al., 2003; Bininda-Edmonds, Gittleman et al., 1999) and/or from deep divergence times where it may be virtually impossible to infer homologies. The development of FISH for cytogenetic analysis has enabled the direct assessment of homology between DNA segments, circumventing this shortcoming associated with staining techniques. The application of FISH through cross-species chromosome painting using fluorescently-labeled whole chromosomes facilitates the identification of karyotypic rearrangements but not rearrangements within a particular conserved block (e.g., inversions). By including differences in G-banding patterns of homologous segments that have been verified through FISH, finer-scale changes such as inversions or translocations, may sometimes be identified.

In addition to the characterisation of phylogenetic characters for a systematic analysis, chromosomal rearrangement data in conjunction with banding patterns may provide insight into karyotypic evolution and genome organisation within Paenungulata. Specifically, estimates of rates of chromosomal change and the reconstruction of an ancestral paenungulate karyotype are used to provide a glimpse into the succession of evolutionary events involved in paenungulate evolution.

3.2 MATERIALS AND METHODS

3.2.1 Taxon sampling and cell culture

A representative specimen from each of the three paenungulate orders (Hyracoidea, Proboscidea and Sirenia) as well as from the monotypic Tubulidentata (included as an outgroup) were used in this study (table 3.1). Cell cultures for all four taxa were available at the start of the study. New specimens for the hyrax and aardvark were subsequently

Table 3.1 Species used as part of the comparative cytogenetics analysis in this study.

Species name	Common name	Material source	Diploid number (2n)
Loxodonta africana	African savannah elephant	Existing fibroblast culture	56[c]
Procavia capensis capensis	Cape rock hyrax	Fibroblast culture, intercostal muscle	54[d]
Trichechus manatus latirostris	Florida manatee	Fibroblast culture[a, b]	48[e]
Orycteropus afer	Aardvark	Existing fibroblast culture	20[f]

[a] Fibroblast cell culture provided by: Robert Bonde, Sirenia Project, U.S. Geological Survey, Florida Integrated Science Center, 2201 NW 40th Terrace, Gainesville, Florida 32605-3574.
[b] Federal Research Permit: MA-791721; CITES Export Permit: US808447.
[c] Houck, Kumamoto et al. 2001.
[d] Hungerford and Snyder 1969; Hsu and Benirschke 1971.
[e] Loughman, Frye et al. 1970.
[f] Yang, Alkalaeva et al. 2003.

included and fibroblast cell cultures were established from intercostal muscle and ear biopsies, respectively.

Tissue from external tissue sources used to generate primary cell cultures were thoroughly cleaned under sterile conditions with 70% ethanol (EtOH) and placed in tissue culture medium (Dulbecco's Modified Eagle Medium supplemented with 15% (v/v) foetal calf serum, FCS). Prior to processing the biopsy further it was incubated overnight at 37^{o}C with 5% CO_2 to ensure the tissue was contaminant-free. All samples were minced, placed into 25cm^2 tissue culture flasks, enough medium was added to almost submerge the tissue fragments, and the cultures incubated at 37^{o}C. Flasks were maintained until confluent using standard techniques (Schwarzacher, Wolf et al., 1974) for subsequent analysis. Cultures generated from new samples were also cryopreserved (FCS with 10% (v/v) DMSO) in liquid nitrogen and added to the existing SUN (Stellenbosch University) collection.

3.2.2 Chromosome and standard karyotype preparation

3.2.2.1 Chromosome metaphase suspension

Cells were harvested by arresting cell division at metaphase using 30 µl colcemid (10 µg/ml; Gibco). Prior to harvesting, manatee cell cultures in particular required synchronisation using a thymidine block (300 µg/ml) followed by BrdU (bromodeoxyuridine, 3mg/ml) treatment for seven hours before trypsinising. Subsequent to trypsin treatment, all cells were incubated in a hypotonic solution (0.075 M KCl) at 37^{o}C for 16 minutes and then fixed with 3:1 methanol-acetic acid. Slides for subsequent analysis were prepared by dropping a single drop of the fixed cell suspension which was then overlaid with a drop of 3:1 (methanol: glacial acetic acid) fresh fixative on a clean microscope slide.

3.2.2.2 Giemsa-banding (GTG-banding)

GTG-banding (Seabright, 1971) by trypsin digestion was used to enable the identification of homologous chromosomes. Slides for G-banding were aged overnight by baking in a 65 ^{o}C oven. Prior to trypsin treatment, slides were briefly rinsed in Hanks' balanced salt

solution (HBSS, excluding Ca^{2+} and Mg^{2+}). Chromosomes were trypsin-digested (0.05%) with the length of treatment varying between 1-6 minutes depending on the age of the slide. The enzymatic action was stopped by rinsing the slides in 0.025 M phosphate solution (pH 7.0) with 0.02% FCS followed by a brief rinse in H_20. A 2% (v/v) phosphate buffered Giemsa solution (pH 6.8) was used to stain the digested chromosomes for 3-4 minutes.

3.2.2.3 Constitutive heterochromatin banding (CBG-banding)

Regions of constitutive heterochromatin were identified using CBG-banding (Sumner, 1972). Unbaked, freshly prepared metaphase slides were used. Treatment consisted of 15-30 minutes in 0.2 N HCl, a brief rinse in H_20 followed by ~1 minute in saturated $Ba(OH)_2$ at 55 ^oC. The slides were rinsed thoroughly under running water to remove residual $Ba(OH)_2$ and then left for 1 hour in 2xSSC at 65 ^oC. Following a brief rinse with water, the slides were stained for 4-5 minutes in a 4% Giemsa solution (phosphate buffered, pH 6.8).

3.2.3 Standardisation of karyotypes

G- and C-banded karyotypes for the elephant, manatee and aardvark were arranged according to previously published karyotypes (aardvark, Yang, Alkalaeva et al., 2003; manatee, Gray, Zori et al., 2002; elephant, Houck, Kumamoto et al., 2001). Although a G-banded karyotype for the rock hyrax has previously been reported (Prinsloo, 1993), it has not been published and is presented here. Autosomes for *P. capensis* were grouped on the basis of the position of the centromere (meta/submetacentric and acrocentric) and then ordered by decreasing chromosome size.

3.2.4 Chromosome painting using fluorescence *in situ* hybridisation

3.2.4.1 Flow-sorting and generation of chromosome-specific probes

Chromosome-specific painting probes for all four taxa were generated from chromosome suspensions flow-sorted by fluorescence activated cell sorting (FAC) using a dual laser sorter (Ferguson-Smith, Yang et al., 1998; Yang, Carter et al., 1995) and subsequently amplified by degenerate oligonucleotide-primed PCR (DOP, Telenius, Carter et al.,

1992). DOP-PCR amplifications followed Yang, Carter et al. (1995) with minor modifications to the amplification protocol (F. Yang, pers. com.); these involved an initial denaturation at 94^{o}C for 3 minutes (1 cycle), followed by 25 cycles of 94 ^{o}C for 1 minute, 62 ^{o}C for 1 minute and 72 ^{o}C for 1.5 minutes. This was followed by a final extension step of 72 ^{o}C for 8 minutes. Probes were labeled with biotin-16-dUTP (Roche).

3.2.4.2 Fluorescence in situ hybridisation

This technique was applied in two different ways:

1. to characterise chromosomes separated by flow-cytometry for each of the paenungulate species (homologous or intra-FISH),
2. to delimit homologous chromosomal segments by reciprocal chromosome painting among paenungulates (heterologous or zoo-FISH) and unidirectional painting of paenungulate painting probes to aardvark chromosomes.

3.2.4.3 Reciprocal chromosome painting

Reciprocal hybridisations between taxa were performed using FISH with 100-150 ng of chromosome-specific paints made up to 12 µl with hybridisation buffer (50% deionized formamide, 10% dextran sulfate, 2X SSC, 0.5 mol/L phosphate buffer pH 7.3, and 1× Denhardt's solution). The probes were denatured at 70°C for 15 minutes and then preannealed by incubation at 37°C for a minimum of 15 minutes and up to 60 minutes.

Prior to hybridisation, the freshly prepared slides underwent a series of treatments to facilitate the annealing process between probe and target DNA:

- 1:1 fixative (methanol: acetic acid) for 5 minutes, slides allowed to air-dry,
- 100% ethanol for 5 minutes, slides air-dried,
- 0.02% pepsin (Sigma P-7000, dissolved in 10 mM HCl) for 5 minutes,
- 2x SSC repeated twice for 5 minutes each,
- 70, 80, 90, 100% ethanol series, 1.5 minutes in each, slides air-dried.
- slides were aged in 100% ethanol at 65°C for 2 minutes and air-dried.

Metaphase slides were denatured by incubation in 70% formamide/30% 2x SSC solution at 65°C for 1.5–2 minutes, quenched in ice-cold 70% ethanol, and dehydrated through a

70, 80, 90 and 100% ethanol series. The pre-annealed paints were applied to slides, covered with 22 x 22 mm^2 cover-slips, sealed with rubber cement and incubated for 48-72 hours at 37°C in a humid chamber. Post-hybridisation washes involved two 5-min incubations in 50% formamide/50% 2x SSC (v/v) at 40-42°C followed by two 5-min incubations in 2x SSC at 40-42°C. Biotin-labeled probes were visualised using Cy3-avidin (1:500 dilution, Amersham). After detection, slides were mounted in Vectashield mounting medium containing DAPI (4'6-diamidino-2-phenylindole, Vector Laboratories).

Chromosome preparations examined in this study were visualised using an Olympus BX60 fluorescence microscope with suitable excitation filters (Reichman, 1998). Images were captured using a cooled CCD camera and analysed with the Cytovision®/Genus™ system (version 2.7, Applied Imaging). Hybridisation signals were assigned to specific chromosome regions as defined by DAPI banding.

FISH with either two or three colours was applied where the identification of DAPI-banded chromosomes was problematic and required verification, and during intra-FISH hybridisations. Further, probes that were found to perform well during interspecific hybridisations were combined during subsequent FISH trials. Probes used in two-colour hybridisations were labeled with either biotin- or digoxigenin (DIG)-dUTP (described previously) and visualised with avidin-CY3 or anti-DIG-FITC (1:500 dillution, Amersham), respectively. Where three colour FISH was employed (intra-FISH), equal volumes of the biotin and digoxigenin were combined to produce the third colour.

3.2.4.4 Intra-FISH hybridisations

Characterisation of paenungulate flow-sorted chromosomes was identical to that for reciprocal chromosome painting. Exceptions to the procedure used above are outlined below.

The identity of the flow-sorted chromosomes was determined by hybridisation to G-banded metaphase spreads of the same species using FISH. After G-banded karyotype

images were captured, the slides were sequentially destained by immersion in 100% ethanol and 100% methanol each for 10 minutes and then baked at 65°C for 1-3 hours. The stringency of chromosome denaturing was reduced to 60°C for 10-30 seconds and the probes were allowed to hybridise overnight at 37°C.

3.2.4.5 Analysis

The patterns of chromosomal rearrangements detected between the paenungulates and the aardvark were scored according to the presence or absence of discrete chromosomal homology characters. Each chromosomal rearrangement (character) was defined as either a fusion or fission (character state) based on comparison to the aardvark and additionally to data from the human which is available indirectly for paenungulates through comparison to the aardvark and elephant (Yang, Alkalaeva et al., 2003). Although the human is not considered basal to afrotherians (Murphy, Eizirik et al., 2001b; Murphy, Eizirik et al., 2001a; Waddell, Kishino et al., 2001) the use of this species and the aardvark (which has a highly conserved karyotype see Robinson, Fu et al., 2004) enables polarisation of the character states for subsequent interpretation.

All rearrangements were characterised against aardvark chromosomes and this nomenclature was maintained across all comparisons to avoid scoring a particular character multiple times. The data matrix was analysed by maximum parsimony (MP), using the exhaustive search option as implemented in PAUP* 4.0b10 (Swofford, 2002). All characters were weighted equally based on the premise that the likelihood of each rearrangement occurring was the same. Characters supporting the monophyly of retrieved clades, as well as those autapomorphic for individual lineages, were subsequently mapped on to the topology obtained.

3.3 RESULTS AND DISCUSSION

3.3.1 Karyotype analysis

3.3.1.1 G- and C-banding

The diploid number obtained for *L. africana*, *P. capensis*, *T . m. latirostris* and *O. afer* corresponds to those previously published (table 3.1). G- and C-banded karyotypes generated in this study for species for which this data have already been published are presented in figures 3.1 (a and b), 3.4 (not including C-bands), 3.7 and 3.8. The karyotypes produced in this study do not show any discernable difference with those previously published.

In agreement with a previous (but unpublished) report on the G-banded karyotype of *P. capensis* (Prinsloo, 1993) the autosomal chromosomes (figure 3.2) are characterised by 21 acrocentric/telocentric pairs (1-21), three submetacentric pairs (22-24) and two metacentric pairs (25, 26). The X chromosome is a large submetacentric and the Y a small metacentric chromosome. The C-banded preparation of the hyrax (figure 3.3) indicates that heterochromatin is largely centromeric and not present on all chromosomes. Aardvark C-banding (figure 3.4) showed that heterochromatin was most often located in the vicinity of the telomeres (chromosomes 3, 4, 5, 6, 7 and 9). Exceptions include the presence of centromeric and pericentromeric heterochromatin in the two largest chromosomes (1 and 2) with an apparently almost completely heterochromatic p-arm of chromosome 1. Further, darker interstitial areas were evident on chromosomes 5 and 8, with the Y chromosome entirely heterochromatic. Interspecific comparisons among paenungulates indicate that patterns of C-banding overall are similar to that seen in the hyrax with heterochromatin restricted to centromeric and pericentromeric regions. The amounts of C-banding present in the chromosomes of the elephant and hyrax vary considerably with some chromosomes showing almost no staining (including the Y chromosome). In contrast, C-banding within the manatee is evident at all centromeres at relatively similar amounts and with a C-positive Y chromosome.

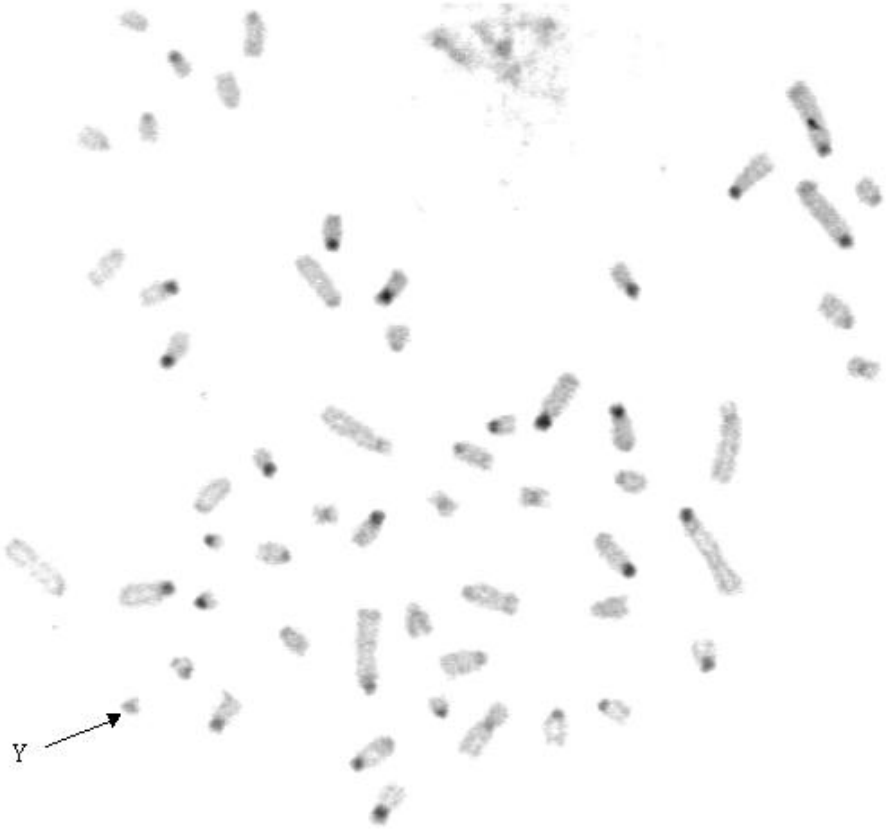

Figure 3.1a Constitutive heterochromatin staining in *L. africana*. Y chromosome identified.

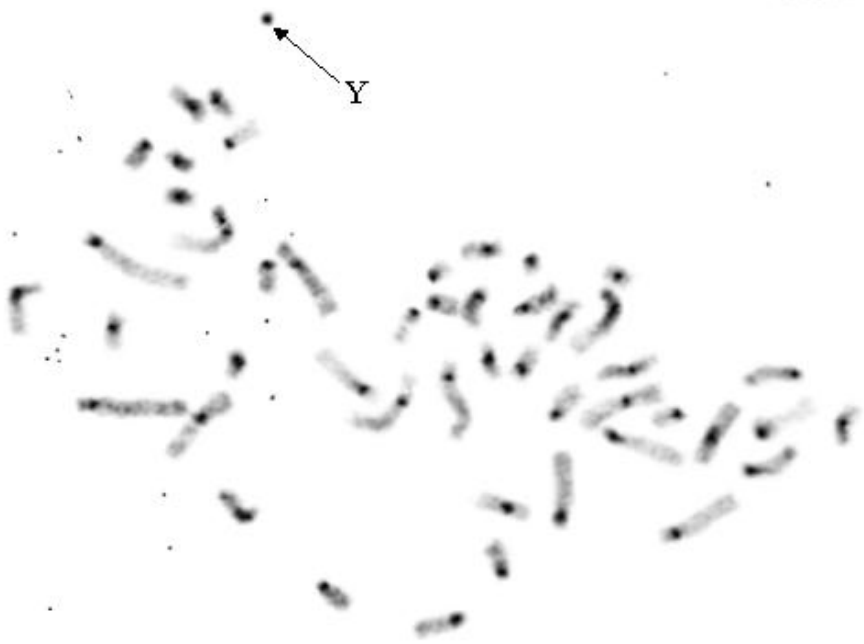

Figure 3.1b Constitutive heterochromatin staining in *T. m. latirostris*. Y chromosome identified.

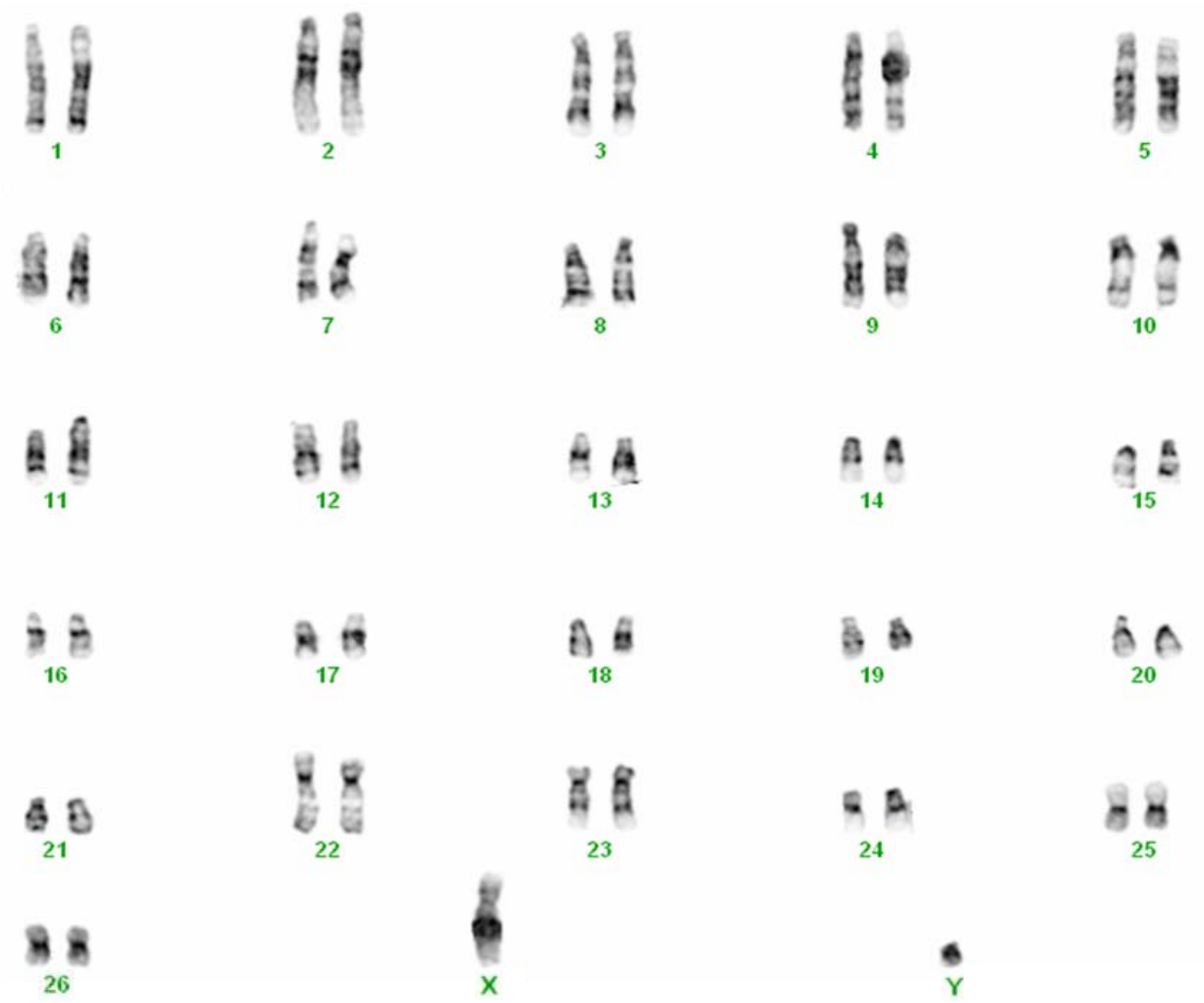

Figure 3.2 G-banded karyotype of a male cape rock hyrax, *P. capensis* (2n=54, XY). Chromosomes are arranged firstly according to the position of the centromere and then size of the chromosome.

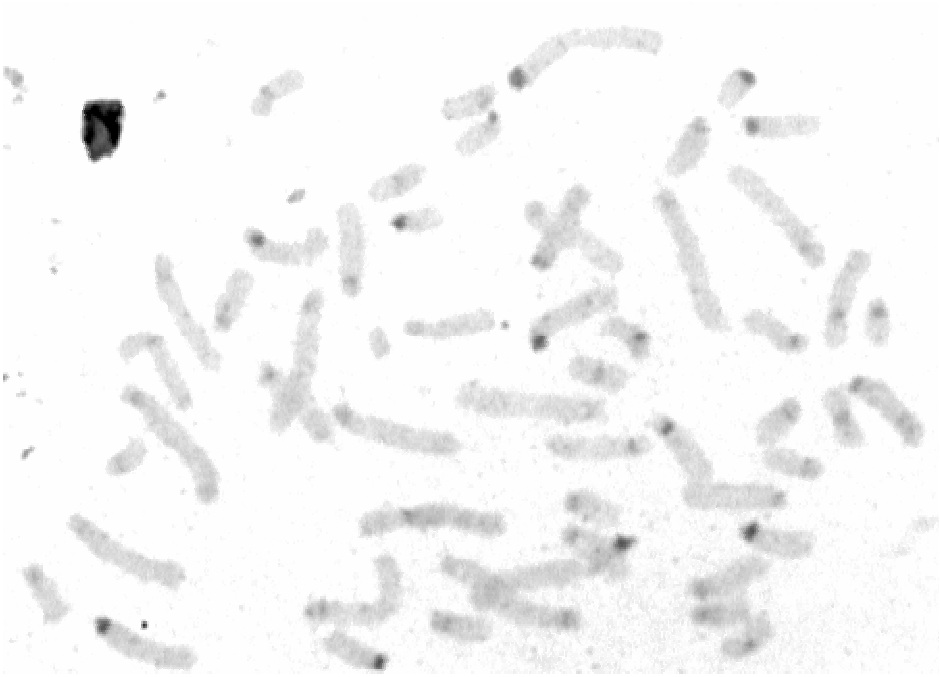

Figure 3.3 Constitutive heterochromatin staining in *P. capensis*.

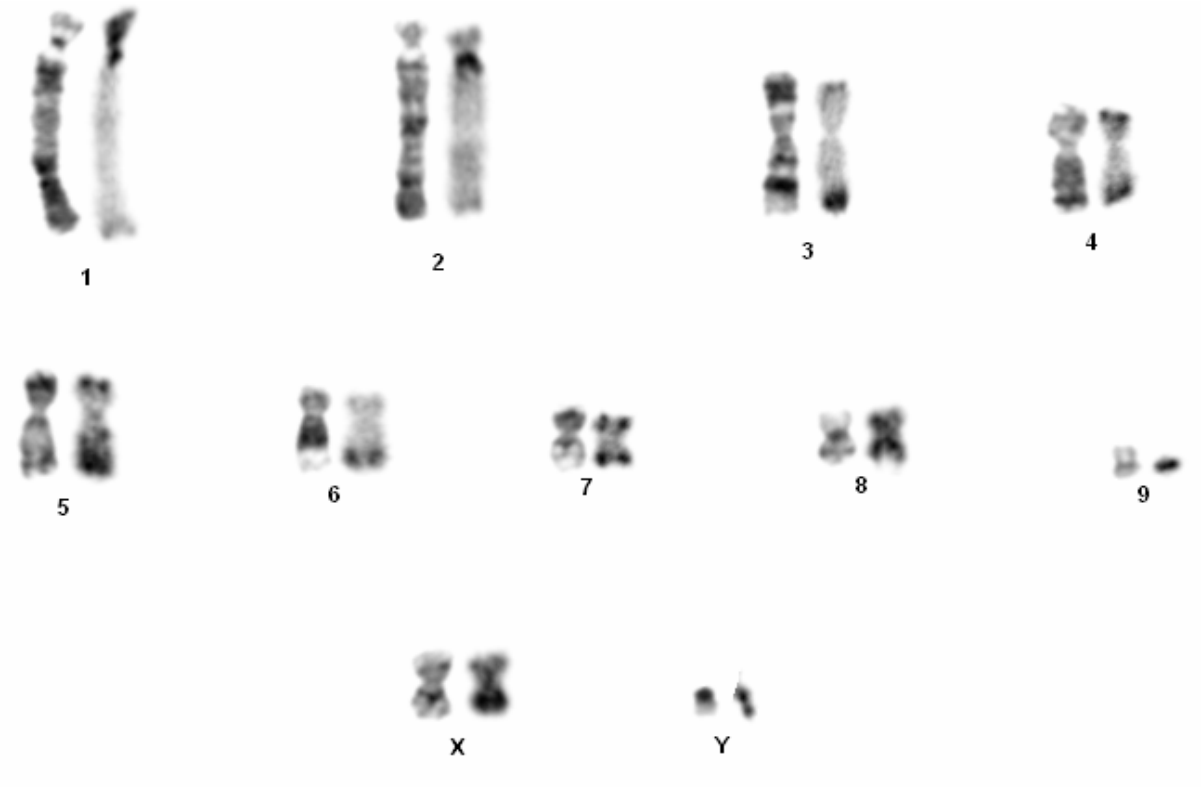

Figure 3.4 Side by side comparisons of G-banded and C-banded chromosomes of the aardvark *O. afer* (2n=20, XY).

3.3.2 Fluorescence *in situ* hybridisation

3.3.2.1 Flow-sorting and assignment of paenungulate chromosomes

3.3.2.1.1 Elephant[7]

Chromosomes from *L. africana* were flow-sorted into 29 different paint probes (table 3.2) of which 23 hybridised to a single elephant chromosome (2, 3, 5, 6, 7, 7, 8, 10, 11, 13, 15, 16, 17, 20, 21, 23, 24, 24, 25, 26, 27, X and Y) with the remaining six paints producing a signal on more than one chromosome. In three of the latter cases (1+3, 4+5, 9+16) one of the double signals was also available in pure form (i.e., sorted independently) facilitating further use of these probes in FISH experiments. However, chromosomes 19 + 22 (flow peak 21), and 12 + 14 (flow peak 27) could not be characterised as single probes and were limited to use as a double signal. The homologues of chromosomes 7 and 24 were each sorted separately reflecting a difference in size and/or nucleotide content which may have been due to heterochromatic differences.

3.3.2.1.2 Hyrax

Of the 24 painting probes generated from the set of flow-sorted chromosomes of the rock hyrax (figure 3.5), 19 hybridized to a single chromosome (no.s 1, 2, 4, 6, 7, 9, 11, 12, 13, 14, 15, 17, 18, 20, 21, 22, 23, 25, Y). Once again, homologues of certain chromosomes (15, 16, 25) segregated into different peaks. Pure sorts of chromosomes 15 and 25 were also isolated and pairs containing these chromosomes were consequently resolvable. Both homologs of chromosome 16 were isolated with different chromosomes (15 and 24) allowing for their characterisation as single markers using two-colour FISH. Two flow-

[7] 1) The results of the flow-sort characterisation of the elephant form part of a paper previously published: Yang F., Alkalaeva E. Z., Perelman P. L., Pardini A. T., Harrison W. R., O'Brien P. C. M., Fu B. Graphodatsky A. S., Ferguson-Smith M. A., Robinson T. J. (2003) Reciprocal chromosome painting among human, aardvark, and elephant (supraorder Afrotheria) reveals the likely eutherian ancestral karyotype. Proceedings of the National Academy of Sciences, USA. 100: 1062-1066.

2) Comparisons of chromosomes between species included in this study are done using the nomeclature adopted by the by the International System for Human Cytogenetic Nomenclature, ISCN (1978); hence elephant (*L. africana*), hyrax (*P. capensis*), manatee (*T. m. latirostris*) and aardvark (*O. afer*) are abbreviated to LAF, PCA, TMA and OAF, respectively.

Table 3.2 Flow-sort characterisation of chromosomes from the African elephant, *L. africana* (2n=56). See also Yang, Alkalaeva et al. (2003).

Peak	Chromosome	Peak	Chromosome
1	1+3	16	15
2	2	17	9+16
3	5	18	13
4	4+5	19	23
5	3	20	21
6	11	21	18+19+22
7	X	22	25
8	6	23	16
9	8	24	20
10	Y	25	10
11	7	26	18+20
12	27	27	12+14
13	24	28	17
14	7	29	24
15	26		

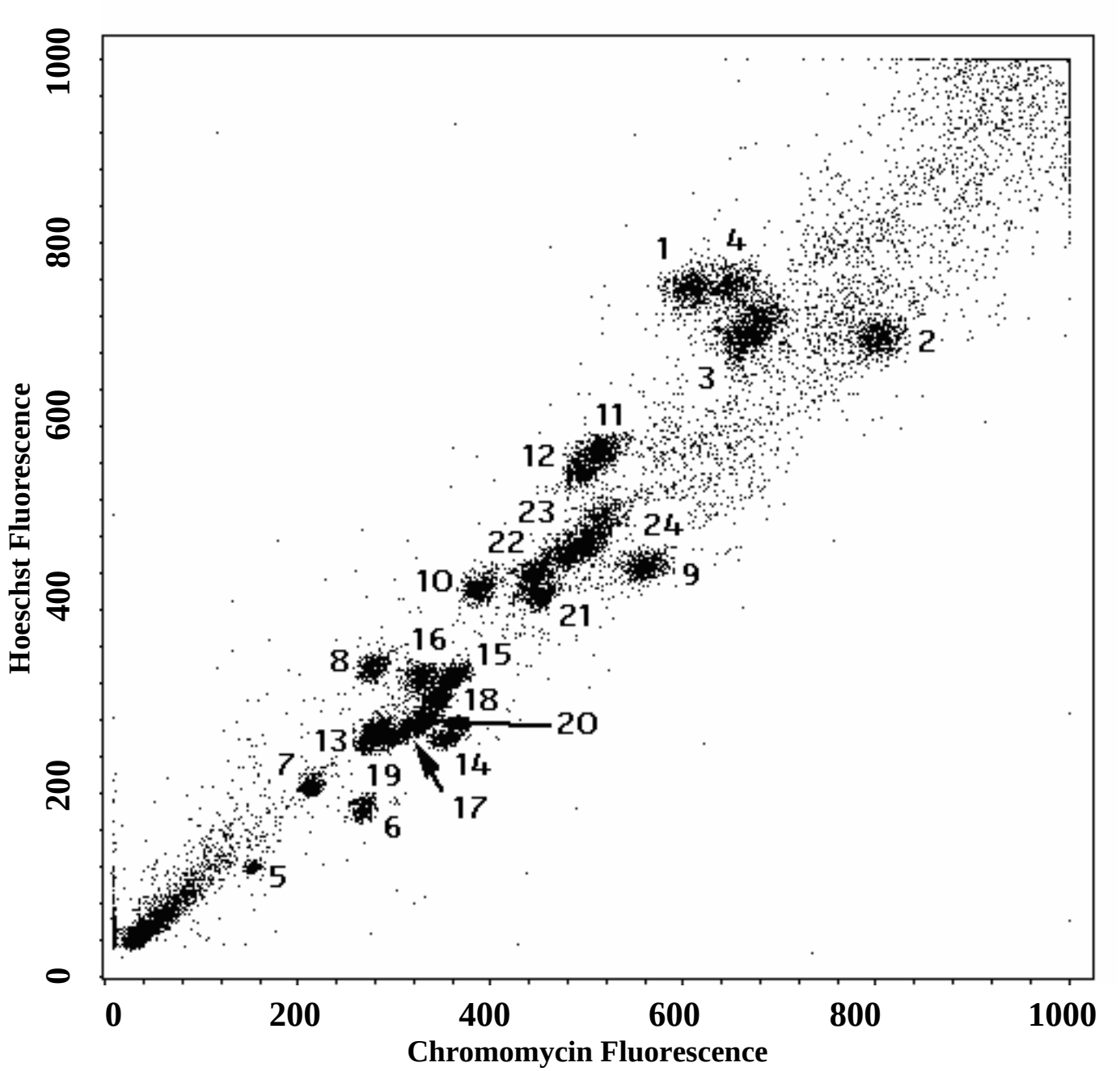

Figure 3.5 Assignment of bivariate-sorted chromosomes of the hyrax, *P. capensis* (2n=54, XY) using FISH to G-banded chromosomes.

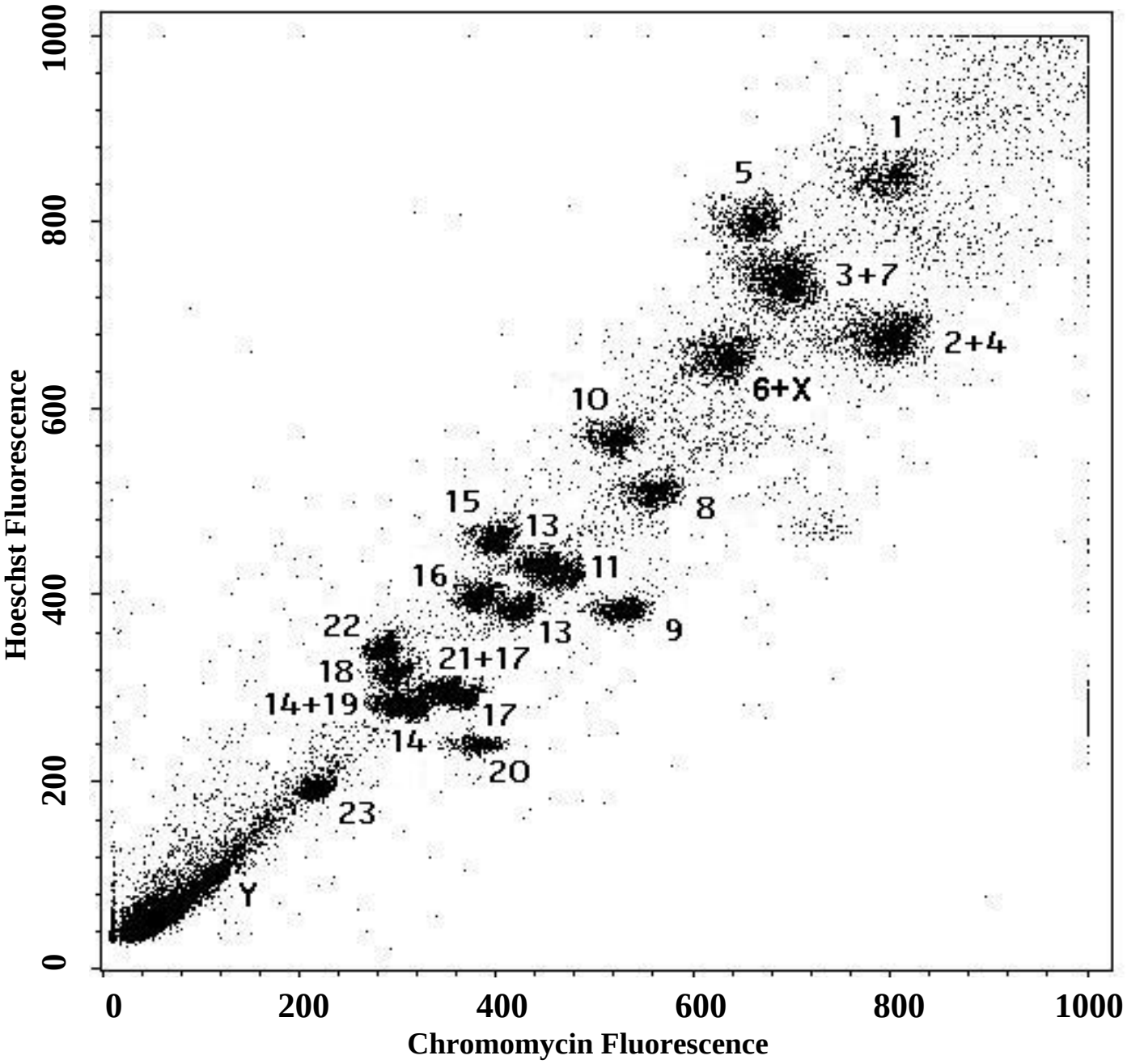

Figure 3.6 Assignment of bivariate-sorted chromosomes of the manatee, *T. m. latirostris* (2n=48, XY) using FISH to G-banded chromosomes.

peaks containing chromosomes 3+5 and 8+10 could not be resolved further. Chromosomes 26 and the X were not present in the flow-sorted chromosomes.

3.3.2.1.3 Manatee

The 48 chromosomes of the manatee separated into 23 peaks (figure 3.6) of which 17 comprised single chromosomes (1, 5, 8-12, 14-18, 20, 20, 22-23, Y). Homologues of chromosome 20 were present in two separate peaks. One homologue of chromosome 17 cosegregated with chromosome 21, while chromosome 19 was present in two peaks (13+19 and 14+19). Although chromosome 19 exists with another chromosome in each peak (13 and 14) a pure form of 14 exists which allows characterisation of chromosome 19 in interspecific hybridisations. Chromosomes 3+7, 2+4 and 6+X could not be resolved further as single chromosome probes.

Chromosomes that were only present in mixed peaks, or not present in the flow-sort (e.g., hyrax X chromosome), were characterised in the subsequent zoo-FISH trials involving multi-species comparisons. For example, hyrax chromosome 26 was not isolated during flow-sorting yet the elephant and manatee probes that hybridise to PCA26 also hybridised to each other, confirming that these homologies exist between all three paenungulates.

3.3.2.2 Reciprocal chromosome painting

3.3.2.2.1 Elephant

The G-banded chromosomes of the elephant with areas of homology from zoo-FISH experiments with the hyrax and the manatee are shown in figure 3.7. The hyrax and manatee painting probes delineated 33 and 32 conserved homologous autosomal segments in the elephant, respectively. Twenty (2, 3, 5-9, 11-15, 17, 19-21, 23-26) and sixteen (1-3, 7, 10-14, 16-18, 20-23) autosomal syntenic groups were apparent between the hyrax and elephant, and the manatee and elephant, respectively. The remaining five hyrax chromosomes (1, 4, 10, 16, 18, 22) delineated 2-3 segments each while LAF12q-prox, LAF21q-dist and LAF20q-dist did not show any hybridisation signals when painted with hyrax chromosomes. Seven (4, 5, 6, 8, 9, 15, 19) manatee chromosomes produced 2-3 signals each when painted to the elephant.

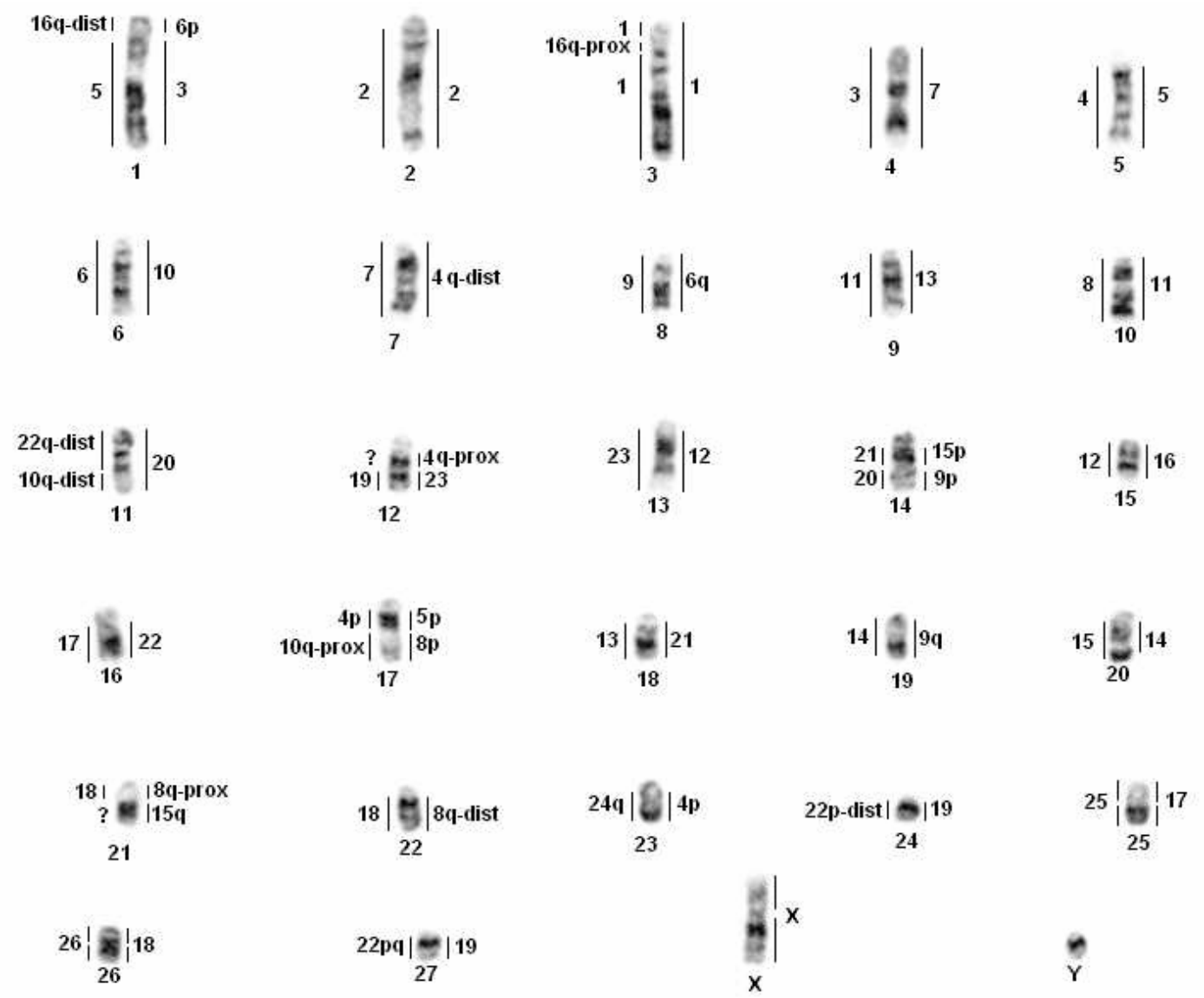

Figure 3.7 G-banded metaphase chromosomes of the African elephant, *L. africana* with regions homologous to the manatee (right) and hyrax (left) mapped using whole chromosome painting with FISH.

LAF20q-dist did not appear to hybridise with any manatee probe. Manatee flow-peak 6+X hybridised to autosomal segments in addition to the elephant X chromosome.

3.3.2.2.2 Manatee

Areas of homology between elephant and hyrax probes to manatee metaphase chromosomes are described in figure 3.8. Thirty-three segments (2-4, 6-13, 15, 16, 18-20, 22-27) of synteny are evident between the elephant and manatee, and 29 regions (1-9, 11-15, 17-21, 23-26) were found between the hyrax and manatee. Elephant chromosomes 1, 5, 12, 14, 17 and 21 and hyrax chromosomes 10, 16 and 22 each produced signals on two different chromosomes in the sirenian. Only the elephant X chromosome hybridised to the manatee equivalent as this chromosome was not isolated during hyrax flow-sorting of chromosomes. Hybridisation experiments with hyrax did not produce signals on regions TMA4q-prox and TMA15q.

3.3.2.2.3 Hyrax

Hybridisation experiments of elephant and manatee painting probes to hyrax chromosomes are depicted in figure 3.9 and yielded 32 and 29 autosomal conserved segments, respectively. Seventeen manatee (2, 3, 5, 7, 10-19, 21-23) chromosomes and 22 elephant autosomes (2, 4-10, 12, 13, 15, 16, 18-27) and the X chromosome hybridised in their entirety, while the remaining six manatee (1, 4, 6, 8, 9, 20) and five elephant (1, 3, 11, 14, 17) probes produced two signals each. The manatee X chromosome was flow-sorted with chromosome 6. When this painting probe was applied to hyrax chromosomes, a signal was produced covering the X chromosome in addition to the autosomal segments. There are three regions present on hyrax chromosomes (10q-prox, 23p and 24p) for which no signal was detected with respect to hybridisations with both elephant and manatee probes and may represent the missing signals on TMA15q, TMA4q-prox, LAF12q-prox, LAF21q-dist and LAF20q-dist.

3.3.2.2.4 Aardvark

Hybridisations of painting probes of each of the three representatives of Paenungulata to metaphase chromosome preparations of the aardvark produced similar numbers of

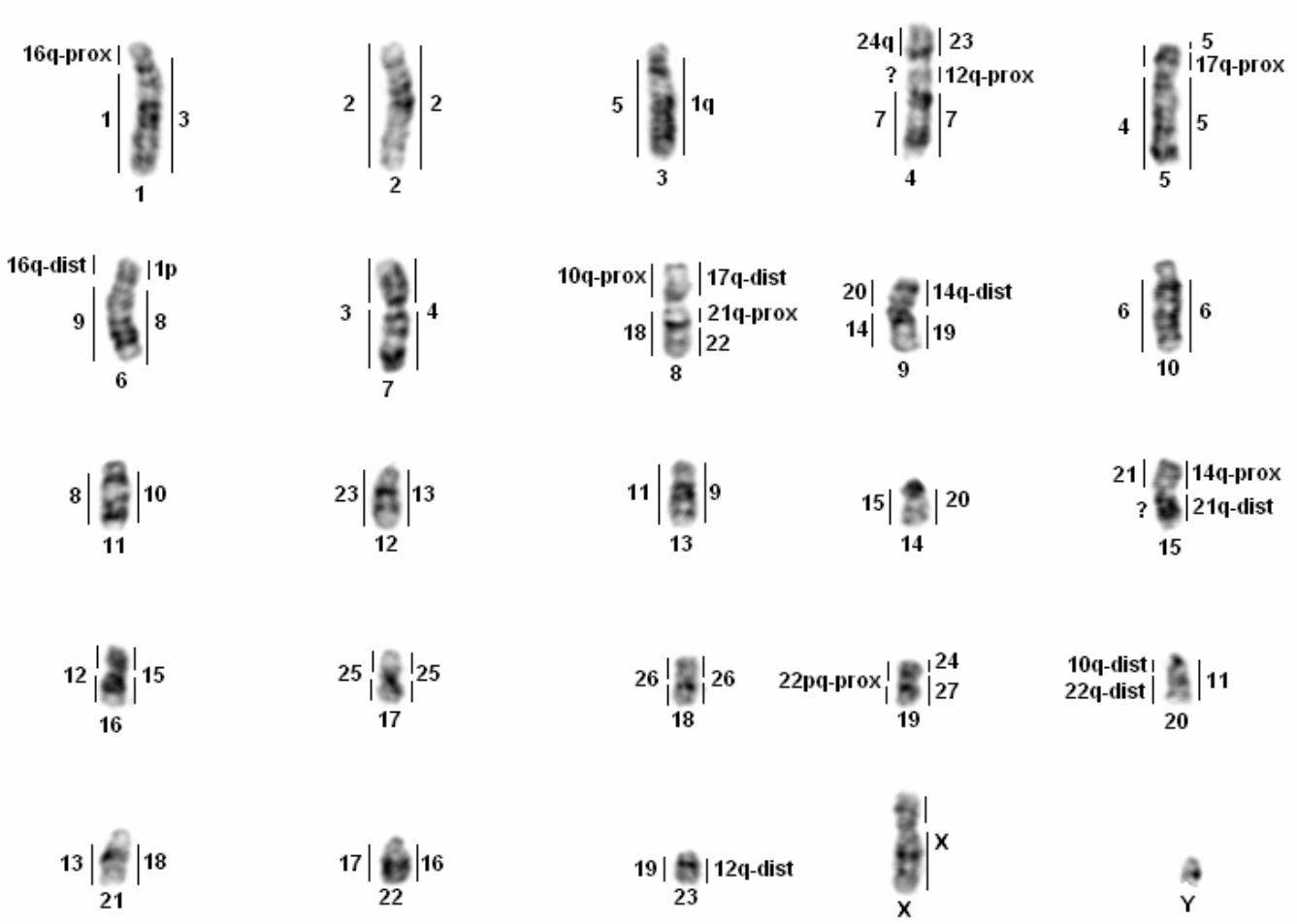

Figure 3.8 G-banded karyotype of the Florida manatee *T. m. latirostris* with regions homologous to the elephant (right) and hyrax (left) mapped using whole chromosome painting with FISH.

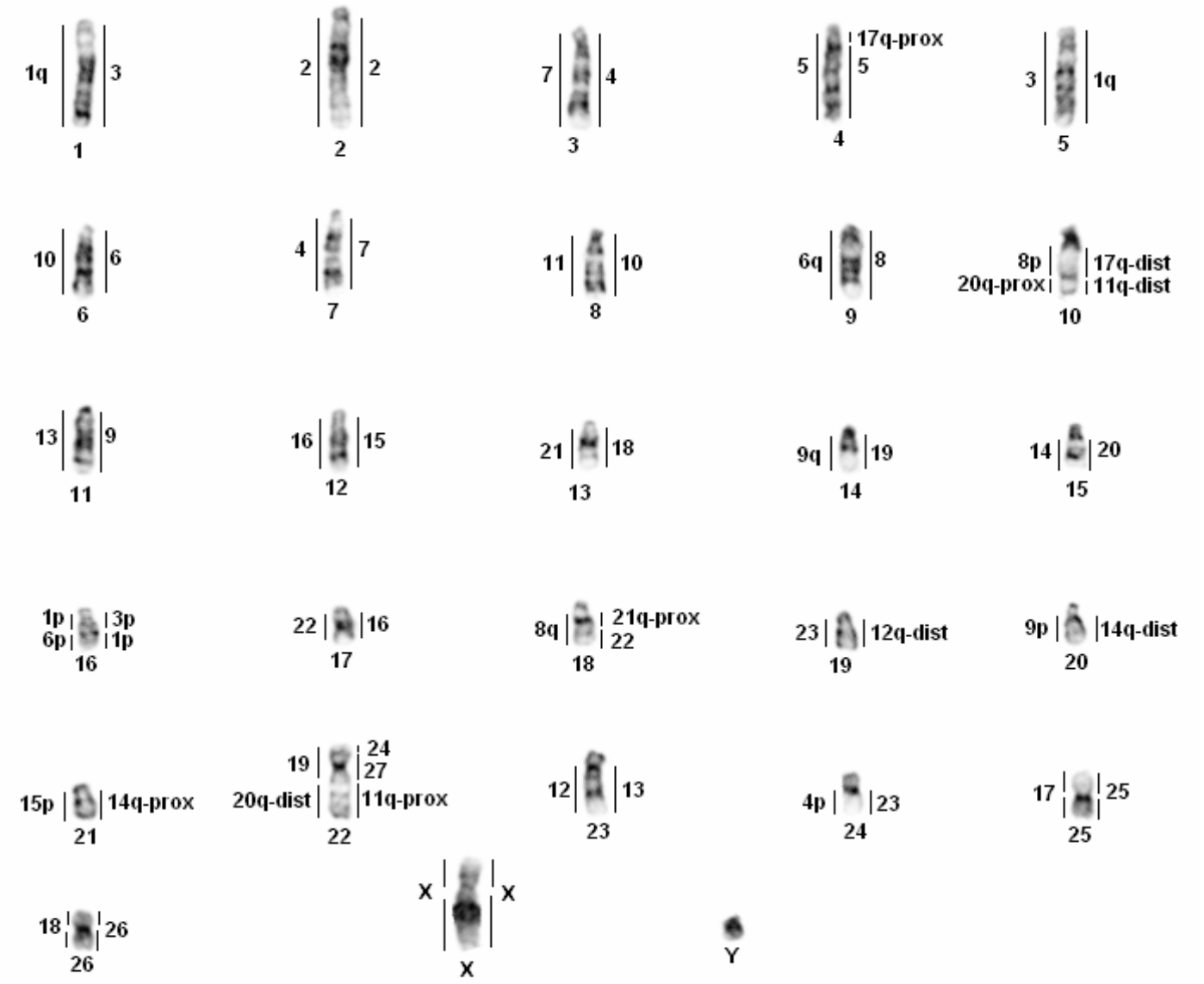

Figure 3.9 G-banded metaphase chromosomes of the hyrax *P. capensis*, with regions homologous to the manatee (left) and elephant (right) mapped using whole chromosome painting with FISH

conserved segments. These are mapped to G-banded chromosomes of the aardvark (figure 3.10) with homologies previously obtained from human probes also shown (Yang, Alkalaeva et al., 2003). Of the 36 different signals produced by elephant probes, 18 were present as single signals (2-11, 15, 16, 18-20, 22-24) while the remaining nine (1, 12-14, 17, 21, 25-27) occurred in two different segments. Three (16c, 26c, 1c) of the 36 signals delineated by the elephant were not clear from direct hybridisations but were confirmed through multispecies comparisons. A further two signals (21i, 17i), expected from painting results with other taxa (hyrax and manatee), were not found and also inferred from results with other species.

Of the 32 homologous segments obtained from the hybridisation of manatee probes to aardvark chromosomes, fourteen (1-3, 5, 7, 10, 11, 13, 14, 16, 20-23) show conserved synteny while the remaining nine (4, 6, 8, 9, 12, 15, 17-19) yielded two separate signals. Hybridisations of hyrax probes to aardvark delineated 33 conserved segments of which 20 (1-9, 11-15, 17-21, 24) produced a single signal, five (10, 16, 23, 25, 26) produced two signals and a single chromosome (22) was found to hybridise to three different aardvark chromosomes. Three different segments (26i, 26i, 17i) were not obtained directly during hybridisation trials; chromosome 26, like the X chromosome was not isolated during flow-sorting for the hyrax while 17 was inferred through multispecies comparisons. A further signal which was not clear through direct hybridisations was confirmed through multi-species comparisons (16c).

All three paenungulate species did not hybridise to a small section on the q-arm of aardvark chromosome 1; and in addition, paints showed limited hybridisation to the syntenic section of OAF3p. Confirmation of the latter result was through previous work (Yang, Alkalaeva et al., 2003). A segment on OAF2q corresponding to the homologous regions of LAF21q-dist and TMA15q which did not yield signals during hybridisation trials with the hyrax, was also not detected during hyrax to aardvark hybridisations. The p and q arms of TMA15 differed considerably in the intensity of their hybridisations to elephant chromosomes. Since these chromosome arms form part of the same painting probe and hence are subject to similar technical procedures, their differences in

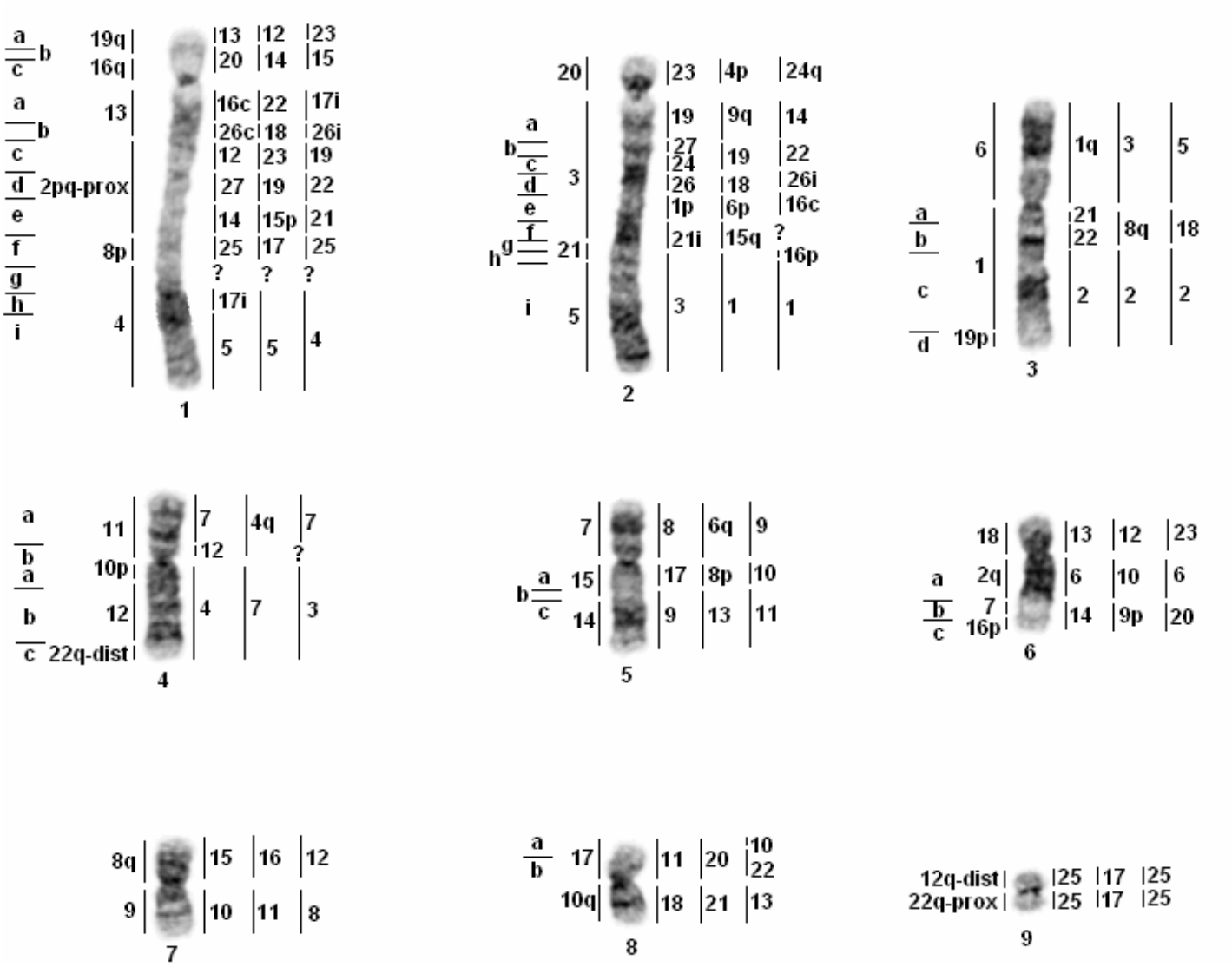

Figure 3.10 G-banded chromosomes of the aardvark, *O. afer* (2n=20) with regions of homology (right of aardvark chromosomes) delimited by FISH to the elephant, manatee and hyrax (from left to right). Correspondence with human chromosomes is shown to the left of the aardvark chromosomes and is taken from Yang, Alkalaeva et al. (2003). Positions of break-points are indicated to the left of human and each subdivided region is marked (a-i).

hybridisation efficiency are more likely to reflect differences in homology. Consequently, the absence of a signal between hyrax and TMA15q may be indicative of a further reduction in homology in comparison to the elephant and manatee. A similar situation is also apparent with regards to the missing signal to OAF1qg (from all paenungulates). Based on Yang, Alkalaeva et al. (2003), the missing signal is expected from LAF20 which also produces a signal on OAF1pbc. In contrast to the missing signal, the hybridisation to OAF1qbc produces an intense signal, suggesting problems of homology as indicated in the previous example between hyrax and TMA15q. Differences in hybridisation efficiency may be due to biological (homology) or technical factors such as differential amplification and labeling during DOP-PCR. However, that the missing hyrax hybridisations were evident among all paenungulate comparisons, suggests that biological reasons are the predominant factor leading to reduced hybridisation efficiency.

Examination of the chromosome painting data (character matrix, appendix 6) revealed no synapomorphies for intra-paenungulate relationships, as indicated by the polytomy obtained from the MP analysis (figure 3. 11). However, synapomorphic changes specific to Paenungulata and unique rearrangements for each of the three paenungulate orders (described below) were characterised and mapped to the MP tree.
Examples of cross-species chromosome painting using painting probes developed specifically for this investigation are presented in figure 3.12.

3.3.2.3 Comparison to previous studies

Comparison of the results obtained in this study to previous comparative painting trials (Frönicke, Wienberg et al., 2003; Yang, Alkalaeva et al., 2003) show differences in the identification of specific chromosomes as well as variation in homology assignments (table 3.3). Differences in the identification of G-banded chromosomes are likely to occur, particularly if there are strong similarities in the banding patterns. Alternative assessments (second opinion) of the ambiguous G-band assignments in this study were obtained to confirm identification. Regarding zoo-FISH experiments however, an important concern is that within a particular study there is consistency between

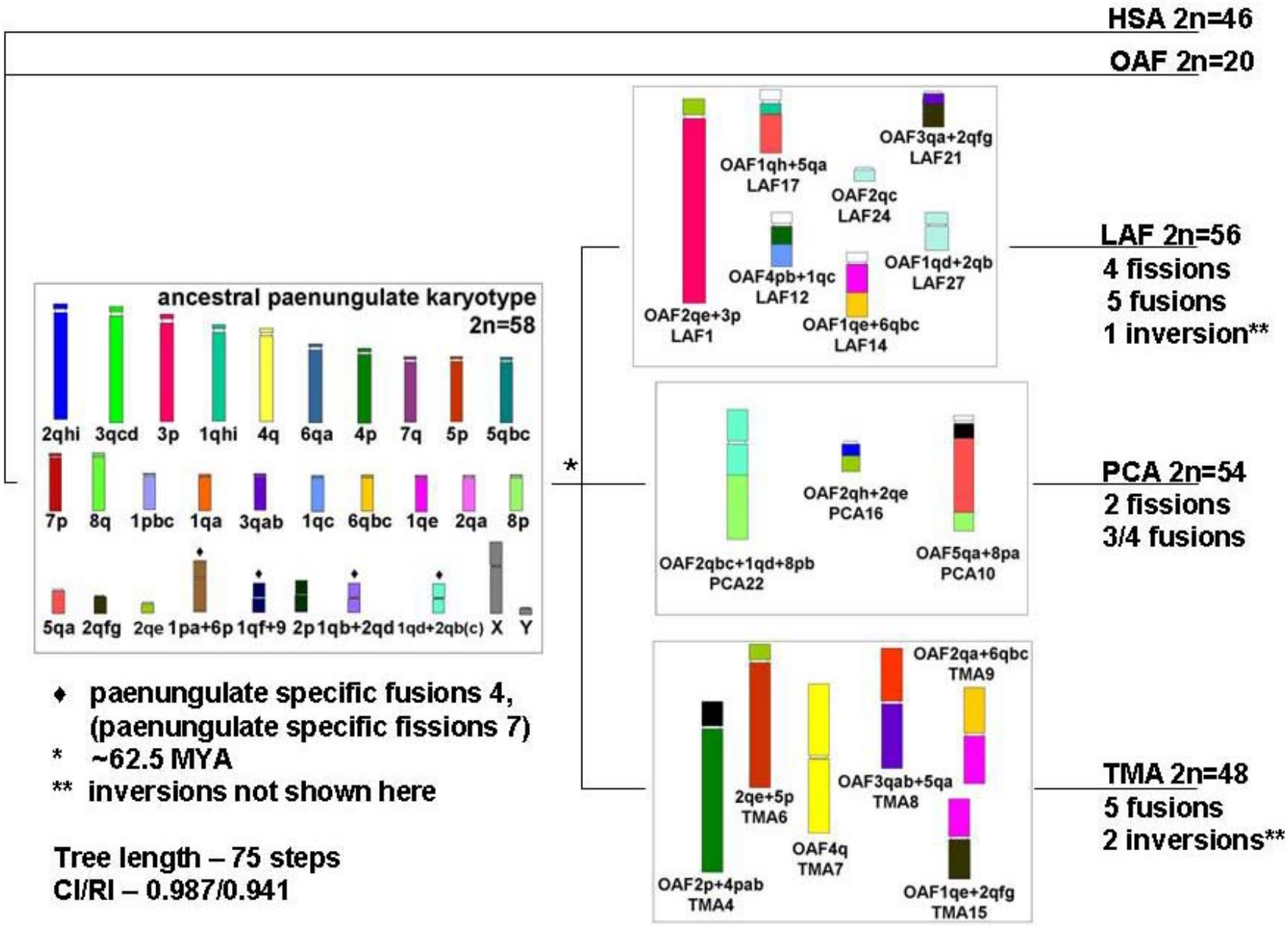

Figure 3.11 Reconstruction of chromosomal phylogeny using Maximum Parsimony (MP). Chromosome pairs deemed to have been present in the ancestral paenungulate and subsequent autapomorphic rearrangements within each lineage are described. The binary character matrix comprising 82 characters is included in appendix 6.

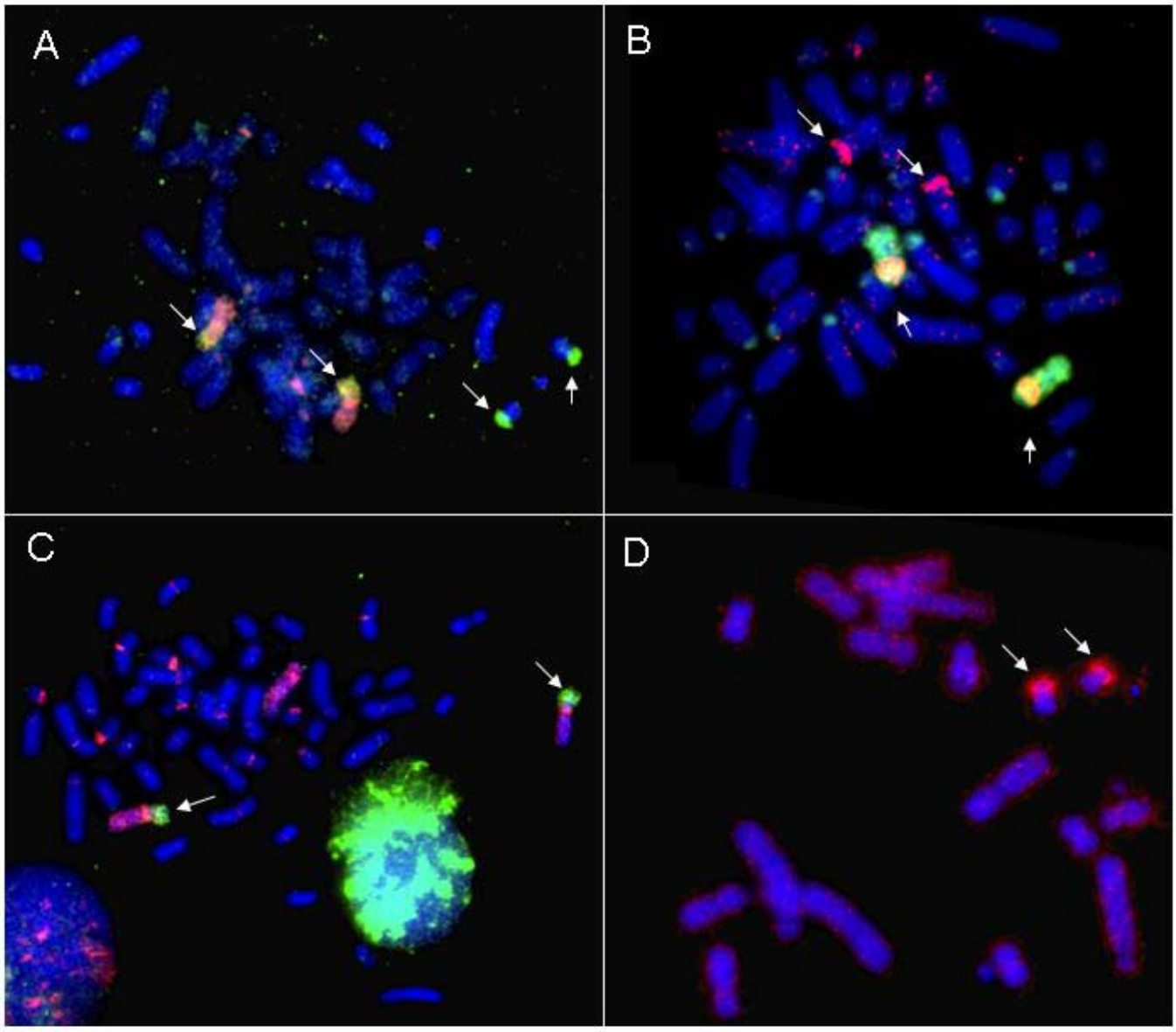

Figure 3.12 Examples of cross-species chromosome painting using painting probes developed specifically for this investigation: A – two-colour FISH on LAF metaphase chromosomes using biotin-labelled LAF17 and DIG-labelled TMA8; B – two-colour FISH on PCA chromosomes using DIG-labelled PCA22 and biotin-labelled TMA20; C- two-colour FISH on TMA metaphase chromosomes using biotin-labelled TMA3+4 (mixed peak) and DIG-labelled LAF23; D – OAF metaphase chromosomes painted with biotin-labelled TMA20.

assignment of chromosome flow-sorts and the identification of these based on G-banding. Both processes were performed here, reducing the likelihood of this problem.

The majority of discrepancies between the hybridisation results among the three studies involve small hybridisation signals (table 3.3, no.s 8-13). The level of resolution and quality of signal obtained between zoo-FISH experiments may vary considerably due to differences in experimental approach, chromosome preparations and quality of painting probes, for example. A possible solution to resolving differences might be to consider the result favoured by two of the studies to be the most likely outcome. However, zoo-FISH is not a perfect technique and with further cross-species painting and improvements in techniques, it would be expected that the discrepancies evident here will also be resolved.

The majority of differences in homology designations between the three different studies are not of great consequence in terms of identifying synapomorphies, with the exception of LAF27 + HSA2/3 (table 3.3 no.13, figure 3.13). LAF27/24 corresponds to TMA19 and PCA22pqprox, and when comparing among paenungulates following the Yang, Alkalaeva et al. (2003) result, one possible interpretation of the chromosomal rearrangements is as a synapomorphy for a manatee-hyrax association. Specifically, the presence of fused segments OAF1qd and OAF2qc in both the manatee and hyrax but not the elephant (remembering that OAF2qc does not contain LAF27 according to Yang, Alkalaeva et al., 2003). However, this does not take into account the polarity of the change, and when considering the outgroups (and the putative paenungulate ancestor, see below), this result can be explained differently. Here the most parsimonious explanation for the order of events that lead to the three respective paenungulate karyotypes is a fusion of segments OAF1qd and OAF2qc in the paenungulate ancestor, followed by a fission in the elephant, an additional fusion in the hyrax and no apparent further change in the lineage leading to the manatee.

Given that the part of TMA19 that is in conflict with Yang, Alkalaeva et al. (2003) borders a centromere, there is the possibility that non-specific hybridisation with repetitive DNA may have occurred. However, amplification of species-specific repetitive

	Yang et al. 2003 (Y)	Frönicke et al. 2003 (F)	Present study (Y/F)
Chromosome differences:			
1	LAF12	LAF12	LAF14
2	LAF14	LAF14	LAF12
3	LAF19	LAF22	F
4	LAF22	LAF19	F
5	LAF5	LAF6	Y
6	LAF6	LAF5	Y
Hybridisation differences:			
7	LAF3 + HSA5	LAF3 + HSA5/21	F
8	LAF9 + HSA15/14	LAF9 + HSA15/14/15/14	Y
9	LAF12 + HSA2/16/7	LAF12 + HSA2/11/16/7	Y (LAF12-LAF14)
10	LAF14 + HSA2/11	LAF14 + HSA1/11/2/1	Y (LAF14-12)
11	LAF21 + HSA1/21/3	LAF21 + HSA1/3/21/3	*
12	LAF26 + HSA6?/13/3	LAF + HSA13/3	F
13	LAF27 + HSA2	LAF27 + HSA2/3	F

Table 3.3 Comparison of hybridisation differences between this study and two previous studies that included *L. africana* (Frönicke, Weinberg et al. 2003; Yang, Alkalaeva et al. 2003). * resolution of this difference between human and elephant cannot be inferred indirectly as it concerns positional variation.

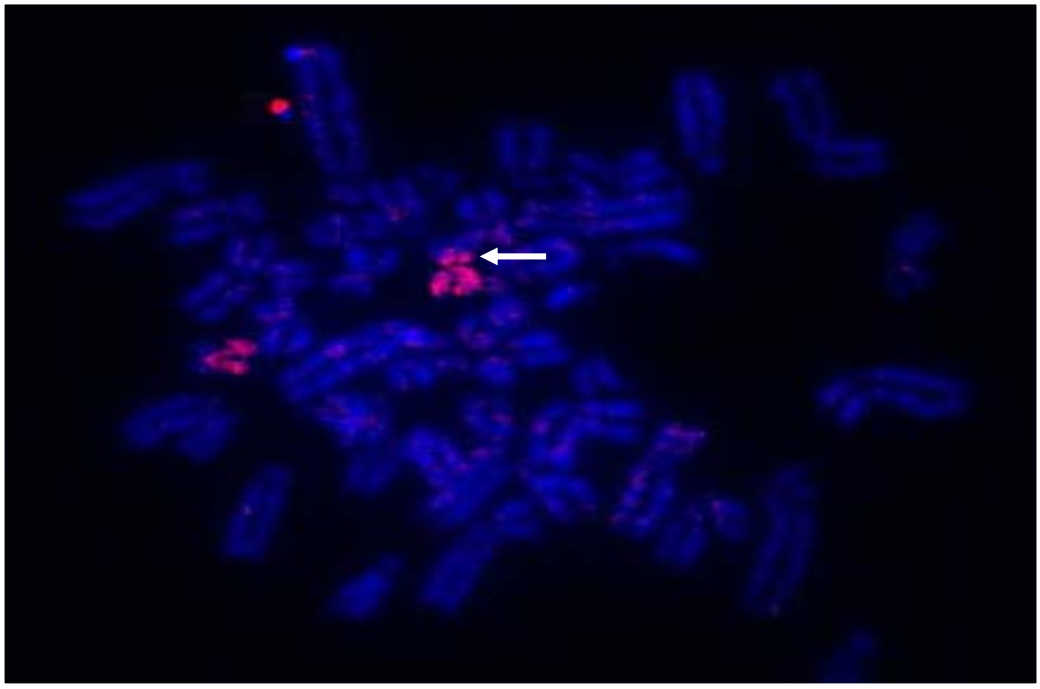

Figure 3.13 Hybridisation of LAF27 to TMA19. The presence of a signal on both the p and q arms (rather than one arm of TMA19 as would be interpreted from the Yang, Alkalaeva et al. 2003 result) agrees with Frönicke, Wienberg et al. (2003), supporting a HSA2/3 hybridisation in the elephant.

DNA is unlikely due to the deep separation time among paenungulates. Further, although repetitive DNA hybridisations were clear during intraspecific hybridisations during characterisation of flow-sorted chromosomes, similar patterns were not evident during zoo-FISH trials.

3.3.2.4 Chromosomal evolution within Paenungulata

3.3.2.4.1 The ancestral karyotype

Comparative analysis of the distribution of chromosomal rearrangements between the paenungulates and the two outgroup taxa (aardvark, human) enabled the construction of a putative ancestral paenungulate karyotype (APK; figure 3.11). This karyotype is representative of a paenungulate ancestor just prior to the divergence of Proboscidea, Hyracoidea and Sirenia, and allows for inferences on chromosomal evolution within this group relative to non-paenungulate afrotherian taxa. It is hypothesised that the APK has a higher diploid number of chromosomes (2n=58) than any of the extant representatives of this group studied to date. Comparison of rearrangements across all three lineages observed in this study reveals that there are a majority of fusions (13/14) in contrast to fissions (6). Although closest in diploid number to the APK, *L. africana* has undergone four fissions and five fusions to form the 2n=56 karyotype. Hyrax chromosomal changes display a majority of fusions (3/4) over fissions (2) relative to the APK; however, due to the uncertainties, the final number of changes using this approach cannot be completely determined. Changes between the karyotype of the APK and the manatee are characterised by five fusions and one change involving an inversion/centromere repositioning (Amor, Bentley et al., 2004; Ventura, Weigl et al., 2004; Montefalcone, Tempesta et al., 1999).

3.3.2.4.2 Paenungulate-specific syntenies

To obtain an estimate of the extent of chromosomal evolution occurring in the lineage leading to Paenungulata, a comparison of the APK to that of the last common ancestor of all other afrotherians would be required. However, construction of an afrotherian ancestral karyotype, based on data from comparative painting, is not yet possible as data for Tenrecomorpha is outstanding. Although the aardvark represents an extant

tubulidentate karyotype, it has been proposed that this species has retained a karyotype that is likely most similar to that of the last common eutherian ancestor (Yang, Alkalaeva et al., 2003), suggesting that it is conserved. Phylogenetic reconstructions of mammalian phylogenetic relationships using nucleotide sequence data (Kitazoe, Kishino et al., 2005; Amrine-Madsen, Koepfli et al., 2003) place the aardvark as the sister taxon to Paenungulata. However, previous studies also indicate a close relationship between the aardvark and elephant-shrew (Robinson, Fu et al., 2004), the elephant-shrew and Paenungulata (Waddell and Shelley, 2003), the aardvark with a paraphyletic Afrosoricida (Waddell and Shelley, 2003), the aardvark as sister-taxon to Afroinsectivora (Murphy, Eizirik et al., 2001b; Waddell, Kishino et al., 2001) and the aardvark with Afrosoricida (Nishihara, Satta et al., 2005). Consequently, the identification of a sister-taxon to the paenungulates is uncertain. The aardvark was selected as the paenungulate sister-taxon, based on the results from the largest sequence concatenation (including all three paenungulate taxa) available at the onset of this study (Murphy, Eizirik et al., 2001b), to estimate the extent of chromosomal evolution between Paenungulata and Afroinsectiphillia. Paenungulate-specific synapomorphies were identified and their specificity to this group was checked by previous comparisons with golden mole and elephant shrew (Robinson, Fu et al., 2004). Confirmation of these syntenies is pending examination of the final member of Afrotheria, Tenrecomorpha, with comparative chromosome painting, and missing data from elephant shrew-aardvark comparisons.

A minimum estimate of 10 chromosomal changes (six fissions and four fusions) specific to Paenungulata were identified. The syntenic associations (see figures 3.10 and 3.11, appendix 6) include OAF6p+1pa (HSA18/19q), OAF9q+1qf (HSA8p/22q), OAF1qd+2qb (HSA2pqprox/3) and OAF1qb+2qd (HSA3q/13) and the fissions OAF1pa/1pb, OAF1qa/1qb, OAF 2qd/2qe, OAF2qe/2qf, OAF3qb/3qc and OAF5qa/5qb. These were verified against data for other mammalian taxa (Frönicke, 2005). OAF6p+1pa represents a fusion between the short arms of aardvark chromosomes 1 and 6, OAF9q+1qf a fusion of a small segment on aardvark chromosome 1q and the whole of chromosome 9, while the third synapomorphy unique to this group involves a fusion of segments on the long arms of aardvark chromosomes 1 and 2. These overlap with three of

the eight previously reported (Yang, Alkalaeva et al., 2003) elephant-specific segmental associations (HSA 3/6, 18/19, 4/15, 2/16/7, 2/11, 4/16/19, 8/22 and 6/13/3). Further, segmental combinations (HSA3/21, 7q/16, 12/22a, 14/15, 16q/19q) that are found to be conserved across the four mammalian supraordinal groupings were present in all three paenungulate taxa. The segmental associations of HSA3/5/21 and 1/19p, were reported by Robinson, Fu et al., (2004) as afrotherian-specific syntenies with a loss of chromosomes five in the elephant. HSA1/19p was evident in all three paenungulates in this study however, a fission within the HSA21 portion of the HSA3/5/21 synteny was found as by Frönike, Weinberg et al., (2003), modifying this syntenic association to HSA3/21 + HSA21/5 (OAF2qhi + OAF2qfg). This rearrangement was also present in the manatee and hyrax[8] indicating that this fission likely represents a change that occurred in the APK and hence, is an additional synapomorphy for Paenungulata.

Within *L. africana*, *P. capensis* and *T. m. latirostris*, five, three and five specific associations were found respectively. Elephant-specific syntenies HSA3/6, 4/15, 2/16/7, 2/11 reported in Yang, Alkalaeva et al. (2003) were confirmed but not 6/13/3. The presence of HSA6 on LAF26 was ambiguous in the original study and was not found here. Further, 2/11 involves an unclear designation in the hyrax and may be excluded with the addition of new data. Syntenies found in the hyrax include OAF5qa+8pa (HSA15/17), OAF1qd+8pb (HSA2pqprox/17) and OAF2qh+2qe (HSA3/21) and those specific to the manatee comprise OAF2p+4pb (HSA11/20), OAF2qe+5p (HSA3/7), OAF5qa+3qab (HSA1/15), OAF6qc+2qa (HSA7/16p/3) and OAF1qe+2qg (HSA2pqprox/21). It is important to note that these are inferred through hybridisations between human and aardvark, hence the exact (positional) homologies require confirmation. These comparisons are of interest to gain insight into chromosomal evolution within Afrotheria but are also reported here, using human chromosome syntenies, in order to follow standard nomenclature and to allow for comparisons to previous studies including afrotherians.

[8] The hyrax displays a further (derived) fission within HSA21/5; data corresponding to HSA3/21 (LAF1p/21) is missing for this taxa and hence inferred.

3.3.2.4.3 Rates of chromosomal evolution

Characterisation of the number of unique changes within each paenungulate lineage facilitates the approximation of taxon specific rates of evolution within Paenungulata (Springer, Murphy et al., 2003b; Waddell, Cao et al., 1999a). Although estimates of evolutionary rate among taxa are dependent on several factors (e.g., generation time) calculation of the number of changes observed over a defined period of time enables a comparison of the tempo of chromosomal evolution among different lineages (Dobigny, Aniskin et al., 2005). Using this approach, a comparison of these rates indicates an elevated rate for the elephant (0.14 changes per MY) in comparison to the hyrax (0.09/MY) and manatee (0.08/MY) and are in agreement with the "default rate" of mammalian chromosomal evolution estimated at one change per 10 million years (Weinberg, 2004; O'Brien and Stanyon, 1999), although a more recent calculation sets this value at 1.9 changes per 10 million years (Frönicke, 2005). However, estimates of chromosomal rates deduced from zoo-FISH experiments vary considerably within Eutheria. Elevated rates (5.8-33.3 changes/MY) have been observed within, for example, Carnivora (Nash, Menninger et al., 2001), Perissodactyla (Yang, Fu et al., 2003), primates (Müller, Hollatz et al., 2003), Cervidae (Yang, Obrien et al., 1997) and Muridae (Volobouev, Aniskin et al., 2002). In contrast, much reduced rates of evolution have been described from Rhinocerotidae (one change over 17 MY, Trifonov, Yang et al., 2003) and Xenarthra (0.2-0.4 changes/MY, Dobigny, Yang et al., 2005), to which rates estimated within Paenungulata are similar. Consequently, the chromosomal rates of evolution within this clade are best described as slow to moderate in comparison to several other mammalian groups.

3.3.2.4.4 G-band chromosome comparison based on FISH homology

A comparison of homologous chromosomes and chromosomal segments among paenungulates (figure 3.14) indicates that the majority of whole chromosomes or chromosome arms have also maintained good G-band homology. Where differences occur, the changes, based on the level of resolution here, appear to be characterised by minor rearrangements. Chromosomes that do not show characterisable G-band similarity, often comprise autapomorphic rearrangements, for example, PCA16 and LAF12.

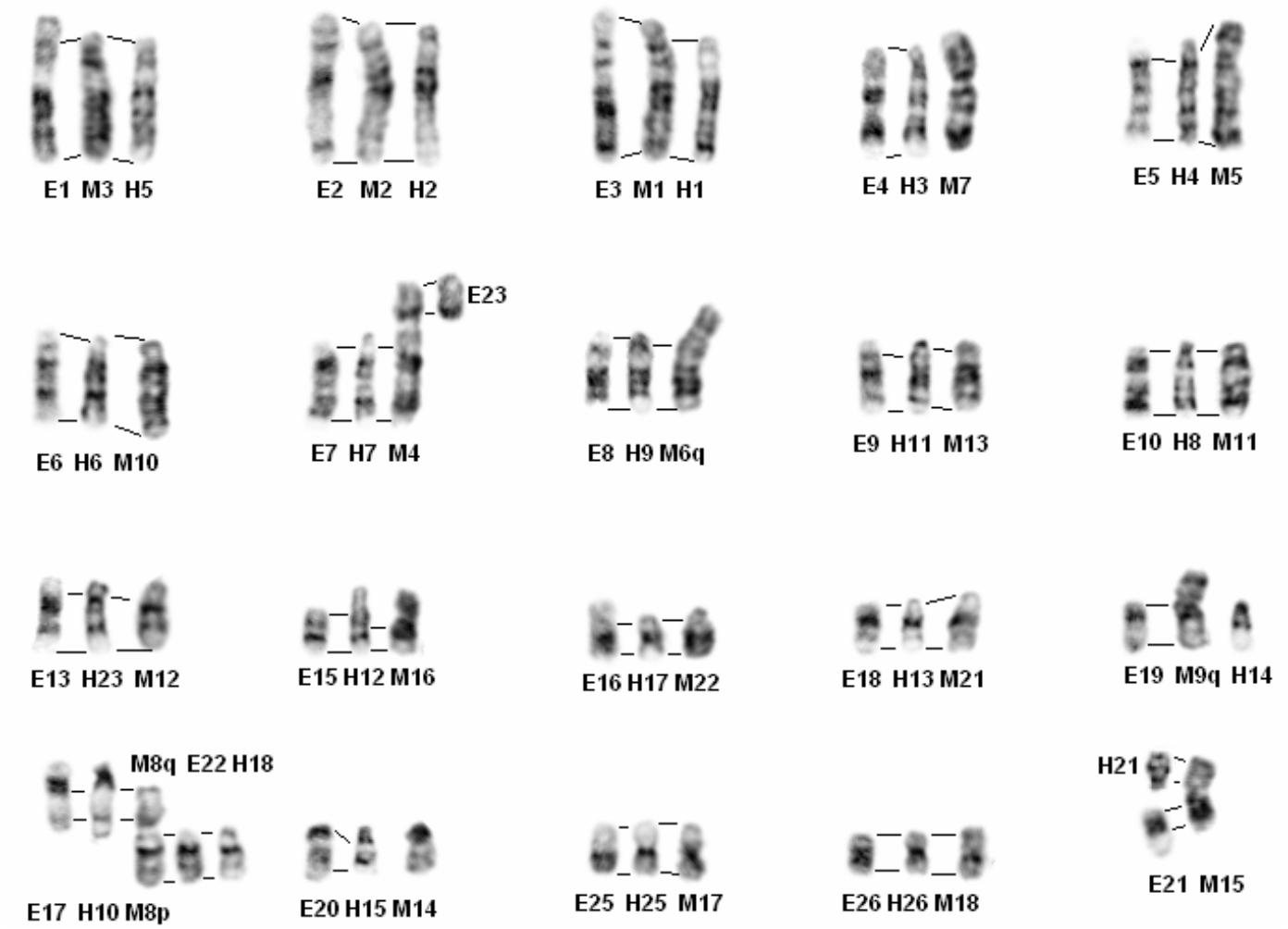

Figure 3.14 Comparison of G-banded chromosomes of the elephant (E), hyrax (H) and manatee (M). Numbers correspond to chromosomes in the standard karyotypes of the respective species. Horizontal bars indicate homologies as assessed by FISH. Where no bars are indicated, the G-band similarity is not obvious although chromosomal homology has been defined by FISH.

Further, in the comparison between LAF6, PCA6 and TMA10 there appears to be a difference between the number of bands between TMA10q-dist and the corresponding region of the other two paenungulates. The FISH results indicate complete homology of the q-arm across all three taxa and furthermore, no C-positive regions were detected. This suggests that this difference may be due to variation in spatial organisation, differences in the relative compression of the chromosomes, or minor, undetectable heterochromatic variation. Additionally, rearrangements detected with FISH that appear to be inversions could not be unambiguously defined as such. These may potentially involve centromere repositioning (Amor, Bentley et al., 2004; Ventura, Weigl et al., 2004; Montefalcone, Tempesta et al., 1999) without an inversion since the banding patterns do not convincingly support the latter. Although G-banding is an indirect assessment of homology and differences have been shown not necessarily to reflect underlying gene order evolution (Raudsepp and Chowdhary, 1999), the degree of homology that appears to be maintained at the intrachromosomal level across all three taxa may be considered further evidence of a low rate of evolution within Paenungulata.

3.3.2.4.5 Genome evolution within Paenungulata

Although FISH was performed on a single representative of each paenungulate order, the availability of cytogenetic data for other extant paenungulates enables an approximate intraordinal assessment of chromosomal evolution relative to the APK. In addition to *Loxodonta*, Proboscidea comprises the genus, *Elephas*, represented by the Asian elephant, *E. maximus*. The G-banded karyotype (2n=56) of *E. maximus* (Houck, Kumamoto et al., 2001) is very similar to that of *L. africana* with differences limited to heterochromatic and minor intrachromosomal variation indicating that almost no large chromosomal changes have occurred since their divergence approximately six million years ago (Vignaud, Duringer et al., 2002).

Within Hyracoidea, G-banded karyotypes exist for *Heterohyrax brucei* (2n=54) and *Dendrohyrax arboreus* (2n=54, Prinsloo and Robinson, 1991) and show good correspondence to the karyotype of *P. capensis*. In particular, the chromosomes of *P. capensis* and *H. brucei* show a high degree of similarity both with G- and C-banding

(Prinsloo, 1993). In contrast *D. arboreus* displays differences most apparent in the distribution of heterochromatin. Whereas *P. capensis* and *H. brucei* C-positive material is limited to the centromeres, several *D. arboreus* autosomes have prominent heterochromatic short arms as well as terminal blocks of heterochromatin (Prinsloo and Robinson, 1991). Although a number of mechanisms have been suggested to explain the evolution of heterochromatic DNA (e.g., replication slippage, rolling circle replication, conversion-like mechanisms, Redi, Garagna et al., 2001), it is unclear without further investigation how heterochromatin evolution might have proceeded in *D. arboreus*. Based on the earliest fossil finds, a middle-late Miocene origin (~10-14 MYA) has been suggested for the origin of the modern family Procaviidae which includes all three extant genera and the extinct genus *Gigantohyrax* (Rasmussen, Pickford et al., 1996). Within the radiation of modern hyracoids, a basal position is indicated by the fossil record for *Heterohyrax*, with *Dendrohyrax* as the derived genus (McKenna and Bell, 1997, p. 491). Further, estimates based on molecular data suggest a similar divergence time of 12.5-13.6 MYA between *Procavia* and *Dendrohyrax* (Springer, 1997). The level of karyotype conservation between *H. brucei* and *P. capensis* apparent since their divergence is consistent with the reduced rate of chromosomal evolution within Hyracoidea as indicated by the chromosome painting data. However, a middle-late Miocene origin provides ample opportunity for *Dendrohyrax* specific changes to occur. That these changes may be associated with the divergence event (e.g., Wichman, Payne et al., 1991) is also a consideration as although satellite sequences can remain dormant for extended periods, they are also capable of dynamic evolutionary changes (Ugarkovic and Plohl, 2002).

Of all extant paenungulates for which cytogenetic data are available, the sirenians exhibit the greatest variation in chromosome number (2n=48-56). In addition to the Florida manatee (2n=48) examined in this study (Gray, Zori et al., 2002), cytogenetic data are available for a further two of the four extant species comprising Sirenia: the amazonian manatee, *Trichechus inunguis* (2n=56, Assis, Best et al., 1988) and the dugong, *Dugon dugon* (2n=50, White, Harkness et al., 1976). Both G- and C-banding for the Amazonian manatee are available for comparison to the Florida manatee. Although C-banding

patterns are restricted to the centromere for both species (Gray, Zori et al., 2002; Assis, Best et al., 1988), differences between G-banding patterns indicate more extensive changes. In particular the difference in chromosome number indicates that at least four chromosomal changes separate these two taxa. Phylogenetic analysis of mitochondrial DNA control region sequence data shows that *T. inunguis* and *T. manatus* diverged approximately four million years ago which is also in agreement with fossil evidence (Catanhede, Da Silva et al., 2005)[9]. This indicates that a far greater degree of chromosomal change (1 change/MY) is evident over the last four million years in Sirenia in comparison to that seen within each of the paenungulate lineages since their divergence. Although the step-wise pattern of rearrangements occurring between *T. manatus* and *T. inunguis* is unknown, there is evidence from interstitial telomeric signals (ITSs) to suggest that this repatterning is more likely to be associated with *T. inunguis*. ITSs have been interpreted as relics of previous chromosome rearrangements in mammals (Dobigny, Ozouf-Costaz et al., 2003; e.g., Nash, Menninger et al., 2001; Meyne, Baker et al., 1990); consequently, the lack of interstitial telomeric signals in *T. manatus* (this study, data not shown) does not favour chromosomal change in this lineage.

A minimal estimate of the rate of chromosomal evolution in the ancestral paenungulate, i.e., prior to the divergence of Hyracoidea, Proboscidea and Sirenia, was calculated using the minimum number of changes uniquely present in Paenungulata (11) and the 17.5 million year time period separating the divergence of the ancestral paenungulate from Afroinsectiphillia (~80 mya) and the radiation of the paenungulates (Springer, Murphy et al., 2003b). A rate of 0.63 changes/MY is obtained which, although not as high as estimates apparent in other placental mammals, is approximately 4 to 5 times that of the fastest rate seen within paenungulates. Using these estimates of rates of change, a pattern of paenungulate chromosomal evolution spanning 80 million years can be described. This entails a reduction in the rate of chromosomal evolution within Paenungulata relative to the ancestral paenungulate which is maintained across all three lineages with the

[9] A more recent estimate based on cytochrome b places the *T. manatus* and *T. inunguis* divergence within the last million years (Vianna, Bonde et al., 2005). Although this is considerably different to ~4 MYA estimate it still indicates a recent increase in rate of chromosomal evolution within Sirenia.

exception of increased repatterning evident in the the last four million years of sirenian evolution.

The maintenance of a similarly slow to moderate rate of chromosomal evolution in all three paenungulate lineages is surprising in light of their extensive diversification. An association between rates of chromosomal evolution and molecular evolution has been observed (Marques-Bonet and Navarro, 2005; Eichler and Sankoff, 2003); specifically, genomic regions with increased repatterning present higher rates of synonomous and non-synonomous substitution (Marques-Bonet and Navarro, 2005). A comparison of molecular rates of evolution within nuclear DNA between paenungulates and eight other mammalian orders using relative differences in edge lengths indicates slowest rates overall in the elephant and manatee with intermediate rates in the hyrax (Chapter 2). Although this provides an approximate indication of overall molecular rates within Paenungulata, it may explain the slow to moderate rates of chromosomal evolution estimated by zoo-FISH. Furthermore, there is the proposed negative correlation between rates of molecular evolution and generation time and body size at four-fold degenerate nucleotide sites (but see Springer, Murphy et al., 2003b; Bromham, Rambaut et al., 1996). Increases in body size are evident in all three paenungulate lineages (Shoshani, 1998; Gheerbrandt, Sudre et al., 1996; Rasmussen, Pickford et al., 1996) relative to that estimated for the ancestral paenungulate (Gheerbrandt, Sudre et al., 1996) and may account for the reduction and subsequent maintenance of a slower rate across all three orders. Although changes in body size can be measured using fossil evidence, aspects of biology/life-history such as generation time, are not as easily quantifiable and consequently difficult to assess. Understanding the potential contribution (and interconnectedness) of diverse factors to molecular evolution is not straightforward (Bromham, Rambaut et al., 1996) and is further complicated where, as in the case of Paenungulata, extensive differentiation has occurred following speciation.

In contrast to the apparent conservation of similar rates among the three paenungulate lineages, is the elevated chromosomal rate evident between the Florida and Amazonian manatees over the last four million years. A possible explanation concerns the pattern of

evolutionary change over time within a lineage. The presence of non-uniform evolutionary rates at different levels across the genome (Marques-Bonet and Navarro, 2005 and references therein) is compelling evidence against a clock-like mode of chromosomal evolution. Consequently, the rate of evolution will be variable over time, resulting in periods of increased chromosomal repatterning alternating with periods of reduced change. Information on the occurrence of these periods of increased or reduced chromosomal evolution is lost as the method of characterising chromosome rearrangements "averages out" this process. Despite this drawback, examples of extensive genome repatterning over short time periods have been recorded and as expected, most examples are apparent in lineages with relatively increased rates of evolution. In particular, these periods of elevated chromosomal evolution have been reported in association with, or leading to speciation events (Dobigny, Aniskin et al., 2005; Navarro and Barton, 2003; Volobouev, Aniskin et al., 2002; Nash, Menninger et al., 2001; Britton-Davidian, Catalan et al., 2000). Within Sirenia, the period of increased chromosomal evolution corresponds to the divergence of *T. inunguis* and *T. manatus* approximately four MYA and consequently might represent a phase of increased chromosomal evolution in contrast to a stage where fewer changes have occurred, providing an "evolutionary snapshot" of sirenid evolution.

3.4 CONCLUSION

Chromosome rearrangement data obtained using zoo-FISH were found to be constructive for building a profile of genome evolution within Paenungulata spanning a period of ~80 MY. Chromosomal rearrangements unique to Paenungulata as well as autapomorphic changes within each lineage were identified. However, synapomorphic changes uniting any two paenungulates were not detected. Based on the divergence estimate of ~62.5 MYA (Springer, Murphy et al., 2003b) and the appearance of the first primitive paenungulate a proboscidean, *Phosphatherium escuilliei* 55-58 MYA (Thanetian age, Gheerbrandt, Sudre et al., 1996), a period of 4-7 million years is estimated for the radiation of Paenungulata. In contrast, the divergence between *T. manatus* and *T. inunguis* (~4 MYA), displays a greater level of chromosomal rearrangement than that

evident within the paenungulate divergence. The absence of synapomorphic changes uniting any two paenungulates indicates that the divergence of Paenungulata is not associated with significant chromosomal repatterning and consequently does not seem to be coincident with a period/pulse of increased rate of chromosomal change as suggested for the recent trichechid divergence and as described in other mammalian taxa. However, this does not imply that chromosomal repatterning was completely absent but that it has not been characterised at the level of resolution applied here. The reconstruction of the APK allowing a rate of chromosomal change within the ancestral paenungulate to be estimated, indicates that rates of chromosomal evolution were likely elevated prior to the paenungulate divergence. Despite the extensive diversification within each lineage, the reduction in evolutionary rate following divergence appears to have been maintained across all three lineages and is also evident through conserved G-banding patterns. Further, a comparison to other mammalian taxa indicates that chromosomal evolution within Paenungulata is best characterised as slow to moderate.

Consequently, the delineation of chromosomal rearrangements among Hyracoidea, Proboscidea and Sirenia through chromosome painting does not provide further insight into phylogenetic associations within this polytomy. However, it has been possible to establish that this limitation is due to a reduced rate of chromosomal evolution (in comparison to both the ancestral paenungulate and other mammalian taxa) in combination with the rapid radiation (Nishihara, Satta et al., 2005; Waddell, Kishino et al., 2001; Amrine and Springer, 1999) suggested for this clade.

CHAPTER 4 - SUMMARY AND CONCLUDING REMARKS

Although much success has been obtained using molecular approaches to resolve mammalian ordinal relationships, a few nodes remain problematic including Paenungulata (Murphy, Pevzner et al., 2004; Springer, Murphy et al., 2003b; Waddell, Kishino et al., 2001). The monophyly of Hyracoidea, Proboscidea and Sirenia is contentious among morphologists with the hypothesis of Tethytheria (manatee and elephant - M+E, McKenna, 1975), resulting in the exclusion of Hyracoidea, being generally favoured. Paenungulata is well established on a molecular basis (e.g., Amrine-Madsen, Koepfli et al., 2003; Nikaido, Nishihara et al., 2003; Waddell and Shelley, 2003), however intra-ordinal relationships are ambiguous with conflicting results from mtDNA and nDNA data sets (Nishihara, Satta et al., 2005; Amrine-Madsen, Koepfli et al., 2003; Murata, Nikaido et al., 2003). The morphological hypothesis of Tethytheria is well supported by amino acid level analysis of 12 mtDNA protein-coding genes (Murata, Nikaido et al., 2003). In contrast, large molecular data sets comprising mostly protein-coding nDNA markers provide no further support for a sirenian-proboscidean relationship and are characterised by unstable nodes (Scally, Madsen et al., 2001), nonsignificant support of M+H (Murphy, Eizirik et al., 2001b) and variable support for H+E (Amrine-Madsen, Koepfli et al., 2003; Murphy, Eizirik et al., 2001b supplementary information; Waddell, Kishino et al., 2001). The lack of consensus among studies, despite extensive analyses, has led several authors to suggest that the paenungulate divergence is best characterised as a rapid radiation (Nishihara, Satta et al., 2005; Waddell and Shelley, 2003; Amrine and Springer, 1999) with the successive, short branching events limiting the establishment of synapomorphic characters. In particular, it is unclear whether the paenungulate trichotomy represents a hard, unresolvable polytomy, or a soft node where phylogenetic signal is present but is obscured due to the confounding effects of homoplasy. In the latter case, a comprehensive analysis of the data may facilitate distinguishing phylogenetic signal from the masking effects of homoplasy. Furthermore, the phylogenetic signal may be differentially preserved in diverse markers and the incorporation of new markers, and at different levels of resolution, may therefore be

informative. In this study, an attempt is made to provide further insight into relationships within Paenungulata and is addressed through two aims.

Issues of homoplasy are of particular concern to the resolution of the paenungulate node as, in addition to the limited signal resulting from a rapid radiation, the relatively deep divergence estimated for paenungulates ~62.5 MYA (Springer, Murphy et al., 2003b) provides ample opportunity for signal degradation through homoplasy. The effects of homoplasy will differ across data sets resulting in the variation of phylogenetic signal among markers. The examination of signal heterogeneity among partitions enables the identification of areas of conflict and support within the data. This is encapsulated in the first aim of the study: to characterise signal structure among diverse mitochondrial and nuclear DNA segments using a hierarchical approach to survey the data with the aim of identifying a signal representative of a soft polytomy. Here, existing data (Amrine-Madsen, Koepfli et al., 2003; Murata, Nikaido et al., 2003; Murphy, Eizirik et al., 2001b) was supplemented with intron DNA sequences generated in this study.

Second, an alternative marker offering a vastly different level of resolution to that in the first aim was investigated. Reciprocal chromosome painting using FISH was used to delineate patterns of chromosomal rearrangements among paenungulates and an outgroup taxon, to enable the identification of synapomorphic characters informative at the level of intraordinal relationships. Additionally, traditional cytogenetic banding techniques were used to provide a further level of comparison. The incorporation of both chromosomal and DNA sequence data provide a fine-scale, though narrow view (sequence data) complemented by a broad, genome-wide assessment (chromosomal data).

The separate and combined analysis of the total sequence data set comprising 39 individual partitions did not provide clear support for any particular paenungulate clade. However, when assessed by genomic origin (i.e., mtDNA or nDNA), support for an M+E association by mtDNA was confirmed. Further, the results from the hierarchical analysis and PBS indicate that support for M+E is predominantly derived from faster-evolving sites (third and first codon positions). Within nDNA, H+E was retrieved by the majority

of individual markers and, together with M+H, was obtained by several combined data sets. Although the predominance of H+E in individual nDNA partitions may suggest an overall consensus (albeit reduced), the effects of sampling and lineage sorting cannot be discounted. However, the hierarchical analysis demonstrates robust support for H+E by nuccod1 and to a lesser extent nuccod2 using both MP and BI. The addition of nuccod3 results in a loss of resolution, particularly evident in the MP topology, indicating a negative effect from these faster evolving positions.

M+E obtained from the phylogenetic analysis (with stationary base composition) is the predominant signal at mtDNAcod3 and mtDNAcod1 and is also the most common pairwise association observed from the manual count across the different substitutional classes. This correspondence of M+E between phylogenetic signal and proportion of shared sites linked with nucleotide composition bias strongly suggests that support from MP and BI is a result of a misleading signal in the presence of a limited phylogentic signal. With the relatively deep divergence and faster rates of substitution at these sites, the increased likelihood of nonstationarity of evolutionary processes and thus homoplasy, is not unexpected. Moreover, the presence of a short internal edge combined with base composition heterogeneity increases the difficulty of phylogenetic inference from sequence data (Jermiin, Ho et al., 2004). Although the use of MP and a more generic model for BI permitted the identification of the different signals (positive or misleading) as well as specifc confounding factors in the data set, the next step required is a direct optimal reconstruction using a model that takes into account base composition bias in particular.

The support for H+E fits the profile expected for a signal from a soft polytomy: it is limited and masked by homoplasy in combined analyses, and is well supported by a specific set of characters with a similar evolutionary profile. If the signal was further degraded, indicative of a hard node (i.e. less resolvable), a more random or diffuse signal would be expected. Further, comparison of the profile of support for H+E across nDNA codon positions corresponds to expectations of a signal established over a short internal edge and which has had a lengthy interval for signal to be degraded. The signal is more

prevalent at faster evolving sites, i.e., nuccod1 over nuccod2, however, is absent from the fastest evolving sites (nuccod3) as these are most likely to be affected by multiple-hits. This is evident from the absence of H+E with nuccod3 and the reduction of support for H+E when nuccod3 is combined with nuccod12. If H+E does not represent a phylogenetic signal, the support from nuccod1 and 2 would suggest bias or sampling effect. A bias may result from dependence or linkage, however, since nuccod12 is derived from several different markers this is expected to be less likely. Comparison of the results obtained here with additional, independent markers will enable assessment of these factors.

The use of a hierarchical approach to characterise signal structure within the data set draws attention to the issues/difficulties associated with the phylogenetic analysis of the reduced signal of a polytomy. For example, due to the likely effects of lineage sorting the use of a consensus approach to establishing relationships among paenungulates was limited, as was evidenced by the separate analysis of markers. In particular, is the considerable variation in phylogenetic utility among partitions (assessed through the recovery of established clades) observed in both the separate and combined analyses. It is noteworthy that overall, nDNA segments performed better than mtDNA in the former analysis. Subpartitioning through the hierarchical analysis of the data allowed for differences in evolutionary processes among markers to be taken into account (for example, rate differences between codon positions). This provided an additional dimension to the characterisation of signal structure (e.g., among mtDNA sites the identification of increased support for M+E from mtDNAcod3 and 1 in particular) and by enabling the detection of hidden or obscured support (e.g. H+E by nuccod12).

Comparative chromosome painting among Proboscidea, Hyracoidea and Sirenia yielded four syntenic associations and seven fissions specific to Paenungulata, and ten (5 fusions, 4 fissions, 1 inversion/chromosome repositioning), five (3 fusions, 2 fission) and seven (5 fusions, 2 inversions/chromosome repositionings) autapomorphic characters specific to *L. africana*, *P. capensis*, and *T. m. latirostris*, respectively. However, no synapomorphic changes characterising an association within Paenungulata were evident. This may be due

to the loss of specific syntenies that occurred around this time, or alternatively that the length of the internal edge between successive divergence events was too short relative to the rate of chromosomal evolution. Comparison of the paenungulate rates of chromosomal evolution to other mammalian taxa, and that estimated for the ancestral paenungulate, indicates that paenungulate rates of change are reduced and consequently that the latter situation is more likely to account for this result. A reduced rate of chromosomal repatterning is also supported by G-banding comparisons which, in combination with the FISH data, suggest conserved intrachromosomal homology. Although no synapomorphic changes were evident, this may be due to the level of resolution obtained in the present study and may change with increased resolution (e.g., Pevzner and Tesler, 2003).

The examination of Paenungulata using a combination of both cytogenetic and sequence data has contributed to providing a comprehensive picture of evolution within this clade. In particular, this is the first characterisation of chromosomal repatterning among the three lineages and provides confirmation of the monophyly of Paenungulata with cytogenetic characters. Although no further resolution of associations among paenungulates was possible with a cytogenetic approach, the sequence data analysis suggests H+E as a potential alternative to the contentious M+E (based on molecular data) and finds in favour of a paenungulate soft polytomy. Clearly, continued investigation of the paenungulate polytomy is warranted. This should benefit greatly from the sequencing of the elephant genome (Roca and O'Brien, 2005) thus enabling the contextualisation of paenunuglate genome organisation within Mammalia.

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APPENDIX 1

Base differences for pairs of sequences for the mtDNAcod data set.
Values obtained from PAUP* 4.0b10 (Swofford, 2002)

Sloth vs. Anteater:

	A	C	G	T	totals
A	2844	329	172	231	3576
C	378	2119	50	425	2972
G	153	31	1076	28	1288
T	245	425	27	2227	2924
totals	3620	2904	1325	2911	10760

Sloth vs. Llama:

	A	C	G	T	totals
A	2600	413	254	309	3576
C	306	2117	62	487	2972
G	134	42	1076	36	1288
T	213	401	44	2266	2924
totals	3253	2973	1436	3098	10760

Sloth vs. Canif:

	A	C	G	T	totals
A	2573	405	233	365	3576
C	306	2060	56	550	2972
G	146	36	1070	36	1288
T	214	395	39	2276	2924
totals	3239	2896	1398	3227	10760

Sloth vs. Tenrec:

	A	C	G	T	totals
A	2478	383	192	523	3576
C	316	1856	59	741	2972
G	150	44	1039	55	1288
T	216	356	38	2314	2924
totals	3160	2639	1328	3633	10760

Sloth vs. Golden mole:

	A	C	G	T	totals
A	2731	357	96	392	3576
C	383	1987	26	576	2972
G	155	48	1048	37	1288
T	220	385	27	2292	2924
totals	3489	2777	1197	3297	10760

Sloth vs. EShrew:

	A	C	G	T	totals
A	2593	445	155	383	3576
C	326	2094	43	509	2972
G	139	58	1045	46	1288
T	204	475	41	2204	2924
totals	3262	3072	1284	3142	10760

Sloth vs. Aardvark:

	A	C	G	T	totals
A	2674	390	123	389	3576
C	311	2064	41	556	2972
G	161	31	1055	41	1288
T	225	407	32	2260	2924
totals	3371	2892	1251	3246	10760

Sloth vs. Sirenian:

	A	C	G	T	totals
A	2474	522	289	291	3576
C	266	2212	73	421	2972
G	137	49	1067	35	1288
T	177	485	49	2213	2924
totals	3054	3268	1478	2960	10760

Sloth vs. Hyrax:

	A	C	G	T	totals
A	2717	464	193	202	3576
C	400	2150	54	368	2972
G	171	48	1035	34	1288
T	245	524	32	2123	2924
totals	3533	3186	1314	2727	10760

Sloth vs. Eleph:

	A	C	G	T	totals
A	2607	405	207	357	3576
C	349	1947	54	622	2972
G	188	40	1015	45	1288
T	234	446	33	2211	2924
totals	3378	2838	1309	3235	10760

Anteater vs. Llama:

	A	C	G	T	totals
A	2599	459	254	308	3620
C	288	2065	47	504	2904
G	160	40	1087	38	1325
T	206	409	48	2248	2911
totals	3253	2973	1436	3098	10760

Anteater vs. Caniform:

	A	C	G	T	totals
A	2595	426	227	372	3620
C	281	2028	35	560	2904
G	160	36	1091	38	1325
T	203	406	45	2257	2911
totals	3239	2896	1398	3227	10760

Anteater vs. Tenrecid:

	A	C	G	T	totals
A	2465	388	207	560	3620
C	307	1859	36	702	2904
G	174	50	1045	56	1325
T	214	342	40	2315	2911
totals	3160	2639	1328	3633	10760

Anteater vs. Golden mole:

	A	C	G	T	totals
A	2735	381	104	400	3620
C	346	1967	17	574	2904
G	184	54	1040	47	1325
T	224	375	36	2276	2911
totals	3489	2777	1197	3297	10760

Anteater vs. Ele shrew:

	A	C	G	T	totals
A	2554	512	154	400	3620
C	326	2062	39	477	2904
G	167	55	1050	53	1325
T	215	443	41	2212	2911
totals	3262	3072	1284	3142	10760

Anteater vs. Aardvark:

	A	C	G	T	totals
A	2674	414	125	407	3620
C	290	2032	32	550	2904
G	179	49	1056	41	1325
T	228	397	38	2248	2911
totals	3371	2892	1251	3246	10760

Anteater vs. Sirenian:

	A	C	G	T	totals
A	2453	566	293	308	3620
C	250	2144	54	456	2904
G	153	60	1080	32	1325
T	198	498	51	2164	2911
totals	3054	3268	1478	2960	10760

Anteater vs. Hyrax:

	A	C	G	T	totals
A	2698	514	194	214	3620
C	367	2131	39	367	2904
G	213	41	1036	35	1325
T	255	500	45	2111	2911
totals	3533	3186	1314	2727	10760

Anteater vs. Elephant:

	A	C	G	T	totals
A	2600	430	215	375	3620
C	312	1908	41	643	2904
G	230	48	1004	43	1325
T	236	452	49	2174	2911
totals	3378	2838	1309	3235	10760

Llama vs. Caniform:

	A	C	G	T	totals
A	2520	276	197	260	3253
C	291	2115	40	527	2973
G	229	47	1114	46	1436
T	199	458	47	2394	3098
totals	3239	2896	1398	3227	10760

Llama vs. Tenrecid:

	A	C	G	T	totals
A	2327	277	186	463	3253
C	338	1881	49	705	2973
G	242	66	1054	74	1436
T	253	415	39	2391	3098
totals	3160	2639	1328	3633	10760

Llama vs. Golden mole:

	A	C	G	T	totals
A	2536	287	93	337	3253
C	400	1993	28	552	2973
G	259	57	1060	60	1436
T	294	440	16	2348	3098
totals	3489	2777	1197	3297	10760

Llama vs. Ele shrew:

	A	C	G	T	totals
A	2427	385	133	308	3253
C	353	2081	42	497	2973
G	225	75	1072	64	1436
T	257	531	37	2273	3098
totals	3262	3072	1284	3142	10760

Llama vs. Aardvark:

	A	C	G	T	totals
A	2514	306	122	311	3253
C	362	2055	35	521	2973
G	247	64	1062	63	1436
T	248	467	32	2351	3098
totals	3371	2892	1251	3246	10760

Llama vs. Sirenian:

	A	C	G	T	totals
A	2339	391	254	269	3253
C	297	2231	61	384	2973
G	225	68	1104	39	1436
T	193	578	59	2268	3098
totals	3054	3268	1478	2960	10760

Llama vs. Hyrax:

	A	C	G	T	totals
A	2522	391	165	175	3253
C	424	2151	51	347	2973
G	280	58	1060	38	1436
T	307	586	38	2167	3098
totals	3533	3186	1314	2727	10760

Llama vs. Elephant:

	A	C	G	T	totals
A	2424	313	193	323	3253
C	385	1972	48	568	2973
G	307	56	1027	46	1436
T	262	497	41	2298	3098
totals	3378	2838	1309	3235	10760

Caniform vs. Tenrecid:

	A	C	G	T	totals
A	2311	310	184	434	3239
C	326	1844	52	674	2896
G	227	55	1048	68	1398
T	296	430	44	2457	3227
totals	3160	2639	1328	3633	10760

Caniform vs. Golden mole:

	A	C	G	T	totals
A	2543	300	88	308	3239
C	392	1972	17	515	2896
G	227	59	1062	50	1398
T	327	446	30	2424	3227
totals	3489	2777	1197	3297	10760

Caniform vs. Ele shrew:

	A	C	G	T	totals
A	2403	412	126	298	3239
C	360	2054	45	437	2896
G	201	57	1073	67	1398
T	298	549	40	2340	3227
totals	3262	3072	1284	3142	10760

Caniform vs. Aardvark:

	A	C	G	T	totals
A	2513	321	106	299	3239
C	343	2024	36	493	2896
G	218	44	1078	58	1398
T	297	503	31	2396	3227
totals	3371	2892	1251	3246	10760

Caniform vs. Sirenian:

	A	C	G	T	totals
A	2329	419	253	238	3239
C	282	2162	67	385	2896
G	204	65	1091	38	1398
T	239	622	67	2299	3227
totals	3054	3268	1478	2960	10760

Caniform vs. Hyrax:

	A	C	G	T	totals
A	2549	382	152	156	3239
C	413	2098	48	337	2896
G	221	63	1073	41	1398
T	350	643	41	2193	3227
totals	3533	3186	1314	2727	10760

Caniform vs. Elephant:

	A	C	G	T	totals
A	2447	311	189	292	3239
C	358	1920	52	566	2896
G	268	54	1015	61	1398
T	305	553	53	2316	3227
totals	3378	2838	1309	3235	10760

Tenrecid vs. Golden mole:

	A	C	G	T	totals
A	2482	265	97	316	3160
C	327	1838	32	442	2639
G	185	59	1039	45	1328
T	495	615	29	2494	3633
totals	3489	2777	1197	3297	10760

Tenrecid vs. Ele shrew:

	A	C	G	T	totals
A	2337	375	153	295	3160
C	317	1902	43	377	2639
G	189	57	1036	46	1328
T	419	738	52	2424	3633
totals	3262	3072	1284	3142	10760

Tenrecid vs. Aardvark:

	A	C	G	T	totals
A	2421	295	126	318	3160
C	306	1867	40	426	2639
G	192	52	1034	50	1328
T	452	678	51	2452	3633
totals	3371	2892	1251	3246	10760

Tenrecid vs. Sirenian:

	A	C	G	T	totals
A	2257	376	272	255	3160
C	235	2026	74	304	2639
G	184	64	1050	30	1328
T	378	802	82	2371	3633
totals	3054	3268	1478	2960	10760

Tenrecid vs. Hyrax:

	A	C	G	T	totals
A	2446	342	181	191	3160
C	354	1952	48	285	2639
G	219	50	1028	31	1328
T	514	842	57	2220	3633
totals	3533	3186	1314	2727	10760

Tenrecid vs. Elephant:

	A	C	G	T	totals
A	2348	313	197	302	3160
C	344	1772	53	470	2639
G	225	57	991	55	1328
T	461	696	68	2408	3633
totals	3378	2838	1309	3235	10760

Golden mole vs. Ele shrew:

	A	C	G	T	totals
A	2578	431	151	329	3489
C	298	2016	34	429	2777
G	88	27	1051	31	1197
T	298	598	48	2353	3297
totals	3262	3072	1284	3142	10760

Golden mole vs. Aardvark:

	A	C	G	T	totals
A	2684	327	131	347	3489
C	271	2006	33	467	2777
G	90	22	1053	32	1197
T	326	537	34	2400	3297
totals	3371	2892	1251	3246	10760

Golden mole vs. Sirenian:

	A	C	G	T	totals
A	2483	462	283	261	3489
C	221	2127	62	367	2777
G	85	26	1064	22	1197
T	265	653	69	2310	3297
totals	3054	3268	1478	2960	10760

Golden mole vs. Hyrax:

	A	C	G	T	totals
A	2658	424	199	208	3489
C	349	2078	38	312	2777
G	121	20	1036	20	1197
T	405	664	41	2187	3297
totals	3533	3186	1314	2727	10760

Golden mole vs. Elephant:

	A	C	G	T	totals
A	2565	364	215	345	3489
C	322	1880	47	528	2777
G	146	22	998	31	1197
T	345	572	49	2331	3297
totals	3378	2838	1309	3235	10760

Ele shrew vs. Aardvark:

	A	C	G	T	totals
A	2508	319	122	313	3262
C	407	2078	41	546	3072
G	142	42	1053	47	1284
T	314	453	35	2340	3142
totals	3371	2892	1251	3246	10760

Ele shrew vs. Sirenian:

	A	C	G	T	totals
A	2324	414	271	253	3262
C	338	2229	71	434	3072
G	142	42	1062	38	1284
T	250	583	74	2235	3142
totals	3054	3268	1478	2960	10760

Ele shrew vs. Hyrax:

	A	C	G	T	totals
A	2553	364	169	176	3262
C	460	2187	54	371	3072
G	158	46	1044	36	1284
T	362	589	47	2144	3142
totals	3533	3186	1314	2727	10760

Ele shrew vs. Elephant:

	A	C	G	T	totals
A	2407	326	203	326	3262
C	441	1977	50	604	3072
G	194	35	1003	52	1284
T	336	500	53	2253	3142
totals	3378	2838	1309	3235	10760

Aardvark vs. Sirenian:

	A	C	G	T	totals
A	2457	377	294	243	3371
C	229	2236	50	377	2892
G	115	34	1072	30	1251
T	253	621	62	2310	3246
totals	3054	3268	1478	2960	10760

Aardvark vs. Hyrax:

	A	C	G	T	totals
A	2628	375	189	179	3371
C	400	2128	36	328	2892
G	151	35	1038	27	1251
T	354	648	51	2193	3246
totals	3533	3186	1314	2727	10760

Aardvark vs. Elephant:

	A	C	G	T	totals
A	2533	332	215	291	3371
C	334	1943	46	569	2892
G	167	33	1003	48	1251
T	344	530	45	2327	3246
totals	3378	2838	1309	3235	10760

Sirenian vs. Hyrax:

	A	C	G	T	totals
A	2506	273	163	112	3054
C	455	2343	44	426	3268
G	300	60	1074	44	1478
T	272	510	33	2145	2960
totals	3533	3186	1314	2727	10760

Sirenian vs. Elephant:

	A	C	G	T	totals
A	2453	216	201	184	3054
C	371	2123	40	734	3268
G	339	49	1041	49	1478
T	215	450	27	2268	2960
totals	3378	2838	1309	3235	10760

Hyrax vs. Elephant:

	A	C	G	T	totals
A	2624	356	223	330	3533
C	361	2061	47	717	3186
G	211	45	1009	49	1314
T	182	376	30	2139	2727
totals	3378	2838	1309	3235	10760

APPENDIX 2

Summary of base differences for pairs of sequences obtained from the mtDNAcod data set (values obtained from PAUP* 4.0b10):

Taxa	Transistions		Transversions				Identical				prop. diff.	ti/tv	total
	AG	CT	AC	AT	CG	GT	AA	CC	GG	TT			
Sloth vs. Anteater	325	850	707	476	81	55	2844	2119	1076	2227	0.23	0.89	325
Sloth vs. Llama	388	888	719	522	104	80	2600	2117	1076	2266	0.25	0.90	388
Sloth vs. Caniform	379	945	711	579	92	75	2573	2060	1070	2276	0.26	0.91	379
Sloth vs. Tenrecid	342	1097	699	739	103	93	2478	1856	1039	2314	0.29	0.88	342
Sloth vs. Golden mole	251	961	740	612	74	64	2731	1987	1048	2292	0.25	0.81	251
Sloth vs. Ele shrew	294	984	771	587	101	87	2593	2094	1045	2204	0.26	0.83	294
Sloth vs. Aardvark	284	963	701	614	72	73	2674	2064	1055	2260	0.25	0.85	284
Sloth vs. Sirenian	426	906	788	468	122	84	2474	2212	1067	2213	0.26	0.91	426
Sloth vs. Hyrax	364	892	864	447	102	66	2717	2150	1035	2123	0.25	0.85	364
Sloth vs. Elephant	395	1068	754	591	94	78	2607	1947	1015	2211	0.28	0.96	395
Anteater vs. Llama	414	913	747	514	87	86	2599	2065	1087	2248	0.26	0.93	414
Anteater vs. Caniform	387	966	707	575	71	83	2595	2028	1091	2257	0.26	0.94	387
Anteater vs. Tenrecid	381	1044	695	774	86	96	2465	1859	1045	2315	0.29	0.86	381
Anteater vs. Golden mole	288	949	727	624	71	83	2735	1967	1040	2276	0.25	0.82	288
Anteater vs. Ele shrew	321	920	838	615	94	94	2554	2062	1050	2212	0.27	0.76	321
Anteater vs. Aardvark	304	947	704	635	81	79	2674	2032	1056	2248	0.26	0.83	304
Anteater vs. Sirenian	446	954	816	506	114	83	2453	2144	1080	2164	0.27	0.92	446
Anteater vs. Hyrax	407	867	881	469	80	80	2698	2131	1036	2111	0.26	0.84	407
Anteater vs. Elephant	445	1095	742	611	89	92	2600	1908	1004	2174	0.29	1.00	445
Llama vs. Caniform	426	985	567	459	87	93	2520	2115	1114	2394	0.24	1.17	426
Llama vs. Tenrecid	428	1120	615	716	115	113	2327	1881	1054	2391	0.29	0.99	428
Llama vs. Golden mole	352	992	687	631	85	76	2536	1993	1060	2348	0.26	0.91	352
Llama vs. Ele shrew	358	1028	738	565	117	101	2427	2081	1072	2273	0.27	0.91	358
Llama vs. Aardvark	369	988	668	559	99	95	2514	2055	1062	2351	0.26	0.95	369
Llama vs. Sirenian	479	962	688	462	129	98	2339	2231	1104	2268	0.26	1.05	479
Llama vs. Hyrax	445	933	815	482	109	76	2522	2151	1060	2167	0.27	0.93	445
Llama vs. Elephant	500	1065	698	585	104	87	2424	1972	1027	2298	0.28	1.06	500
Caniform vs. Tenrecid	411	1104	636	730	107	112	2311	1844	1048	2457	0.29	0.96	411
Caniform vs. Golden mole	315	961	692	635	76	80	2543	1972	1062	2424	0.26	0.86	315
Caniform vs. Ele shrew	327	986	772	596	102	107	2403	2054	1073	2340	0.27	0.83	327
Caniform vs. Aardvark	324	996	664	596	80	89	2513	2024	1078	2396	0.26	0.92	324
Caniform vs. Sirenian	457	1007	701	477	132	105	2329	2162	1091	2299	0.27	1.03	457

Taxa	Transistions		Transversions				Identical				prop. diff.	ti/tv	total
	AG	CT	AC	AT	CG	GT	AA	CC	GG	TT			
Caniform vs. Hyrax	373	980	795	506	111	82	2549	2098	1073	2193	0.26	0.91	373
Caniform vs. Elephant	457	1119	669	597	106	114	2447	1920	1015	2316	0.28	1.06	457
Tenrecid vs. Golden mole	282	1057	592	811	91	74	2482	1838	1039	2494	0.27	0.85	282
Tenrecid vs. Ele shrew	342	1115	692	714	100	98	2337	1902	1036	2424	0.28	0.91	342
Tenrecid vs. Aardvark	318	1104	601	770	92	101	2421	1867	1034	2452	0.28	0.91	318
Tenrecid vs. Sirenian	456	1106	611	633	138	112	2257	2026	1050	2371	0.28	1.05	456
Tenrecid vs. Hyrax	400	1127	696	705	98	88	2446	1952	1028	2220	0.29	0.96	400
Tenrecid vs. Elephant	422	1166	657	763	110	123	2348	1772	991	2408	0.30	0.96	422
Golden mole v Ele shrew	239	1027	729	627	61	79	2578	2016	1051	2353	0.26	0.85	239
Golden mole v Aardvark	221	1004	598	673	55	66	2684	2006	1053	2400	0.24	0.88	221
Golden mole v Sirenian	368	1020	683	526	88	91	2483	2127	1064	2310	0.26	1.00	368
Golden mole v Hyrax	320	976	773	613	58	61	2658	2078	1036	2187	0.26	0.86	320
Golden mole v Elephant	361	1100	686	690	69	80	2565	1880	998	2331	0.28	0.96	361
Ele shrew vs. Aardvark	264	999	726	627	83	82	2508	2078	1053	2340	0.26	0.83	264
Ele shrew vs. Sirenian	413	1017	752	503	113	112	2324	2229	1062	2235	0.27	0.97	413
Ele shrew vs. Hyrax	327	960	824	538	100	83	2553	2187	1044	2144	0.26	0.83	327
Ele shrew vs. Elephant	397	1104	767	662	85	105	2407	1977	1003	2253	0.29	0.93	397
Aardvark vs. Sirenian	409	998	606	496	84	92	2457	2236	1072	2310	0.25	1.10	409
Aardvark vs. Hyrax	340	976	775	533	71	78	2628	2128	1038	2193	0.26	0.90	340
Aardvark vs. Elephant	382	1099	666	635	79	93	2533	1943	1003	2327	0.27	1.01	382
Sirenian vs. Hyrax	463	936	728	384	104	77	2506	2343	1074	2145	0.25	1.08	463
Sirenian vs. Elephant	540	1184	587	399	89	76	2453	2123	1041	2268	0.27	1.50	540
Hyrax vs. Elephant	434	1093	717	512	92	79	2624	2061	1009	2139	0.27	1.09	434

APPENDIX 3

Values for proportion of invariant sites (I) and gamma shape parameter (G) used to calculate ti/tv estimates in section 2.3.1.3. All values were obtained from Modeltest 3.06 where a GTR+I+G model was selected except for the nuccod1 and nuccod3 data sets which returned a GTR+G model. Here MrBayes 3.1 was used to obtain values for I. The G values obtained from both Modeltest and MrBayes 3.1 are included in the table for comparison.

Data set	Invariant sites (I)	Gamma (G)
Nuccod	0.121	0.764
Nuccod1	BI 0.533	BI 0.439 (Mt 0.428)
Nuccod2	0.348	0.864
Nuccod3	BI 0.174	BI 1.642 (Mt 1.629)
MtDNAcod	0.047	0.195
MtDNAcod1	0.435	1.190
MtDNAcod2	0.556	0.496
MtDNAcod3	0.010	0.341

APPENDIX 4

Incongruent length differences (ILD) for the pairwise comparisons among the 39 fragments included in this study. Numbers in bold indicate statistical significance at $P<0.05$.

	ND2	ND3	ND4L	ND4	ND5	CO1	CO2	CO3	CytB	ATP8	ATP6	12SrRNA	16SrRNA	tRNAval	ADORA3	A2AB	ADRB2	ATP7a
ND1	0.549	0.335	0.630	0.630	0.311	0.700	0.212	0.676	0.901	0.428	0.423	0.582	0.755	0.993	0.935	0.724	0.322	0.663
ND2		0.333	0.775	0.706	0.302	0.891	0.381	0.198	0.854	0.582	0.163	0.634	0.976	0.903	0.993	0.986	0.994	0.891
ND3			0.563	0.755	0.789	0.372	0.658	0.795	0.450	0.865	0.596	0.081	0.251	0.814	0.200	**0.009**	**0.016**	**0.001**
ND4L				0.704	0.613	0.823	0.389	0.501	0.803	0.144	0.606	**0.036**	0.084	0.501	0.506	**0.022**	**0.045**	**0.002**
ND4					0.931	0.982	0.505	0.326	0.945	0.542	0.269	0.244	0.654	0.793	0.998	0.462	0.810	0.250
ND5						0.945	0.223	0.854	0.842	0.746	0.328	0.081	0.795	0.936	0.997	0.107	0.523	0.056
CO1							0.782	0.958	0.943	0.832	0.825	0.679	0.967	0.987	0.973	0.471	0.905	0.358
CO2								0.276	0.423	0.145	0.139	0.521	0.234	0.998	0.183	**0.044**	**0.045**	**0.004**
CO3									0.051	0.689	0.844	0.510	0.761	0.976	0.788	**0.003**	**0.033**	**0.007**
CytB										0.650	0.116	0.513	0.864	0.844	0.998	0.783	0.938	0.295
ATP8											0.052	0.190	0.564	0.650	0.326	0.092	0.066	**0.006**
ATP6												**0.032**	0.140	0.495	0.430	**0.006**	**0.029**	**0.003**
12SrRNA													0.254	0.942	0.254	0.093	0.056	0.062
16SrRNA														0.697	0.523	0.548	0.230	**0.044**
tRNAval															0.081	0.269	0.079	**0.011**
ADORA3																0.351	0.882	0.053
A2AB																	0.530	0.250
ADRB2																		0.071
ATP7a																		
BDNF																		
BRCA1																		
CNR1																		
EDG1																		
IRBP																		
PNOC																		
RAG1																		
RAG2																		
TYR																		
VWF																		
ZFX																		
APOB																		
APP																		
BMI1																		
CREM																		
PLCB4																		
MGF																		
PKRC1																		
STAT																		

	BDNF	BRCA1	CNR1	EDG1	IRBP	PNOC	RAG1	RAG2	TYR	VWF	ZFX	APOB	APP	BMI1	CREM	PLCB4	MGF	PKRC1	STAT	THY
ND1	0.753	**0.024**	0.944	0.242	0.998	0.964	0.976	0.963	0.897	0.305	0.991	0.157	0.956	0.641	0.921	0.895	0.769	0.966	0.939	0.255
ND2	0.828	0.734	0.847	0.215	0.999	0.999	0.164	1.000	0.982	0.445	1.000	0.953	0.959	1.000	0.817	0.991	0.997	0.999	0.985	0.996
ND3	0.143	**0.003**	0.070	**0.001**	0.430	0.143	0.077	0.640	0.165	**0.007**	0.574	0.089	0.082	0.737	0.682	0.079	**0.009**	0.063	**0.009**	0.075
ND4L	0.256	**0.001**	0.131	**0.015**	0.888	0.608	**0.033**	0.697	0.456	**0.001**	0.955	0.123	0.054	0.997	0.318	0.445	**0.031**	0.110	**0.012**	0.089
ND4	0.767	**0.028**	0.493	0.148	0.952	0.688	0.119	0.967	0.702	0.204	1.000	0.371	0.971	0.994	0.771	0.907	0.384	0.893	0.314	0.848
ND5	0.783	**0.001**	0.261	0.840	0.296	0.680	0.331	0.994	0.525	**0.027**	0.990	**0.049**	0.984	0.894	0.792	0.735	**0.048**	0.408	0.208	0.269
CO1	0.803	**0.038**	0.749	0.413	1.000	0.974	0.123	0.927	0.963	0.110	0.995	0.457	0.888	0.980	0.869	0.978	0.760	0.900	0.543	0.843
CO2	0.277	**0.005**	0.360	**0.015**	1.000	0.727	**0.004**	0.503	0.822	**0.035**	0.997	0.056	0.141	0.998	0.128	0.634	0.061	0.118	0.073	**0.031**
CO3	0.118	**0.001**	0.071	**0.041**	0.981	0.570	0.302	0.897	0.105	**0.002**	0.828	**0.002**	0.279	0.745	0.881	0.081	**0.004**	0.074	0.140	**0.008**
CytB	0.602	**0.019**	0.813	0.984	0.995	0.989	0.645	1.000	0.842	0.567	1.000	0.468	0.969	1.000	0.903	0.949	0.474	0.959	0.465	0.984
ATP8	0.083	**0.003**	0.093	**0.019**	0.796	0.231	0.237	0.499	0.082	0.100	0.899	**0.020**	0.246	0.638	0.445	0.113	**0.006**	0.285	**0.020**	**0.021**
ATP6	0.177	**0.001**	**0.018**	**0.001**	0.304	0.183	**0.033**	0.892	0.138	**0.002**	0.697	**0.014**	0.072	0.927	0.413	0.171	**0.004**	**0.016**	**0.012**	**0.005**
12SrRNA	**0.019**	0.091	0.417	**0.006**	1.000	0.407	**0.013**	0.755	0.065	0.335	0.803	0.064	0.289	0.548	0.155	0.116	**0.020**	0.192	0.160	0.067
16SrRNA	0.080	0.181	0.096	0.112	0.956	0.845	**0.002**	0.779	0.474	0.293	0.974	**0.041**	0.216	0.850	0.370	0.336	0.308	0.603	0.295	0.304
tRNAval	0.567	**0.025**	0.959	0.776	0.729	0.366	0.660	**0.023**	0.205	0.249	0.399	**0.026**	**0.024**	0.120	0.200	0.057	0.121	**0.039**	0.126	0.093
ADORA3	0.167	0.192	0.102	0.411	0.919	0.588	0.117	0.992	0.118	0.312	0.942	0.169	0.666	0.839	0.530	0.213	0.105	0.806	0.246	0.724
A2AB	0.070	0.328	0.332	0.157	1.000	0.624	**0.002**	0.846	0.369	0.906	1.000	0.345	0.293	0.640	0.181	0.450	0.728	0.255	0.831	0.539
ADRB2	0.081	0.064	0.261	0.147	0.855	0.472	0.191	0.949	0.182	0.522	0.964	0.271	0.205	0.875	0.104	0.133	0.335	0.776	0.293	0.582
ATP7a	**0.003**	1.000	**0.005**	**0.001**	0.951	0.136	**0.001**	1.000	**0.009**	0.208	1.000	0.158	0.223	0.402	**0.001**	**0.013**	0.189	0.671	1.000	0.325
BDNF		**0.010**	0.681	0.090	0.376	0.114	0.246	0.177	**0.026**	0.116	0.517	0.077	**0.004**	0.546	0.126	**0.018**	**0.012**	0.430	0.059	**0.025**
BRCA1			**0.011**	**0.001**	0.937	0.467	**0.001**	1.000	**0.028**	**0.006**	1.000	0.095	0.749	0.670	**0.012**	**0.038**	0.337	0.885	0.837	0.403
CNR1				0.215	0.966	0.453	0.885	0.405	0.144	0.482	0.861	0.568	0.056	0.461	0.558	0.256	0.111	0.166	0.302	0.063
EDG1					0.234	0.087	0.688	0.428	**0.003**	0.424	0.919	**0.003**	0.076	0.516	0.248	**0.014**	**0.001**	**0.044**	**0.007**	0.115
IRBP						1.000	0.958	1.000	0.871	0.928	1.000	0.916	0.709	0.936	0.743	0.973	1.000	0.987	1.000	0.965
PNOC							0.058	0.754	0.643	0.334	1.000	0.366	0.099	0.956	0.145	0.770	0.503	0.790	0.593	0.666
RAG1								**0.039**	**0.004**	**0.001**	0.966	0.051	**0.004**	**0.001**	0.231	**0.004**	**0.003**	**0.005**	0.170	**0.003**
RAG2									0.442	0.798	1.000	0.508	0.876	0.486	0.238	0.417	0.670	0.889	1.000	0.834
TYR										0.229	0.934	0.255	0.053	0.916	**0.025**	0.682	0.707	0.205	0.107	0.828
VWF											0.995	0.188	0.406	0.866	0.109	0.115	0.210	0.772	0.467	0.878
ZFX												0.970	0.783	0.866	0.655	0.962	1.000	1.000	1.000	0.992
APOB													0.074	0.968	0.188	0.766	0.627	0.853	0.502	0.413
APP														0.749	0.027	0.067	0.056	0.641	0.431	0.491
BMI1															0.218	0.942	0.938	0.857	0.873	0.962
CREM																0.073	**0.038**	0.502	0.119	0.118
PLCB4																	0.584	0.669	0.297	0.871
MGF																		0.687	0.656	0.551
PKRC1																			0.994	0.705
STAT																				0.638

APPENDIX 5 Edge lengths among Paenungulata for the concatenated data sets. Lengths in italics correspond to the internal edge.

Data set	Paenungulate edge lengths
Total -BI1	(Hyrax:0.149871,(Sirenian:0.089243,Elephant:0.122055):*0.009607*)
Total -BI3	(Sirenian:0.201863,Hyrax:0.413505,Elephant:0.309776) polytomy
Total -BI5	(Sirenian:0.205064,Hyrax:0.414272,Elephant:0.311259) polytomy
TotalExMtDNA3 - BI1	(Hyrax:0.094594,(Sirenian:0.042995,Elephant:0.070495):*0.003355*)
TotalExMtDNA3 - BI3	(Elephant:0.071680,(Sirenian:0.043247,Hyrax:0.095983):*0.003115*)
TotalExMtDNA3 - BI5	(Elephant:0.071883,(Sirenian:0.043381,Hyrax:0.095932):*0.002975*)
MtDNAAll	(Hyrax:0.686030,(Elephant:0.877384,Sirenian:0.444095):*0.076555*)
MtDNAcod	(Hyrax:2.575592,(Elephant:3.282944,Sirenian:1.731028):*0.287357*)
MtDNAcod1	(Hyrax:0.190642,(Sirenian:0.118533,Elephant:0.252993):*0.019125*)
MtDNAcod2	(Sirenian:0.038031,Hyrax:0.070599,Elephant:0.111953) polytomy
MtDNAcod3	(Hyrax:2.247486,(Sirenian:1.612792,Elephant:1.957302):*0.474396*)
NucAll	(Elephant:0.042681,(Sirenian:0.034845,Hyrax:0.090254):*0.002515*)
Nuccod	(Sirenian:0.034823,(Hyrax:0.086387,Elephant:0.037116):*0.002636*)
Nuccod1	(Sirenian:0.026299,(Hyrax:0.058745,Elephant:0.026768):*0.003853*)
Nuccod2	(Hyrax:0.048904,(Sirenian:0.017048,Elephant:0.024099):*0.001555*)
Nuccod3	(Elephant:0.060739,(Sirenian:0.060091,Hyrax:0.150551):*0.005055*)
Introns	(Elephant:0.067686,(Hyrax:0.134435,Sirenian:0.042629):*0.006853*)
UTRs	(Sirenian:0.031703,(Hyrax:0.069673,Elephant:0.060187):*0.003327*)

APPENDIX 6

Binary character matrix used in the phylogenetic analysis comprising 82 characters (1 – present, 0 – absent). '* 'indicates a synteny spanning a centromere; '?' indicates rearrangements associated with missing data; "i" denotes an inferred rearrangement; characters in bold represent paenungulate specific fusions; underlined characters represent paenungulate specific fissions.

No.	Character	LAF	PCA	TMA	OAF	HSA
1	**1pa + 1pb**	0	0	0	1	1
2	1pb + 1pc	1	1	1	1	0
3	1pc*1qa	0	0	0	1	0
4	**1qa + 1qb**	0	0	0	1	1
5	1qb + 1qc	0	0	0	1	0
6	1qc + 1qd	0	0	0	1	1
7	1qd + 1qe	0	0	0	1	1
8	1qe + 1qf	0	0	0	1	0
9	1qf + 1qg	?	?	?	1	0
10	1qg + 1qh	?	?	?	1	1
11	1qh + 1qi	0i	1	1	1	1
12	2p * 2qa	0	0	0	1	0
13	2qa + 2qb	0	0	0	1	1
14	2qb + 2qc	0	1	1	1	1
15	2qc + 2qd	0	0	0	1	1
16	**2qd + 2qe**	0	0	0	1	1
17	**2qe + 2qf**	0	0	0i	1	1
18	2qf + 2qg	1	1	1i	1	0
19	2qg + 2qh	0	0	0i	1	1
20	2qh + 2qi	1	0	1	1	0
21	3p * 3qa	0	0	0	1	0
22	4pb * 4qa	0	0	0	1	0
23	5p * 5qa	0	0	0	1	0
24	6p * 6qa	0	0	0	1	0
25	7p * 7q	0	0	0	1	0
26	8pb * 8q	0	0	0	1	0
27	9p * 9q	1	1	1	1	0
28	3qa + 3qb	0	1	1	1	1
29	**3qb + 3qc**	0	0	0	1	1
30	3qc + 3qd	1	1	1	1	0
31	4qa + 4qb	1	1	1	1	0
32	4qb + 4qc	1	1	1	1	0
33	4pa + 4pb	0	?	1	1	1
34	**5qa + 5qb**	0	0	0	1	1
35	5qb + 5qc	1	1	1	1	0
36	6qa + 6qb	0	0	0	1	0
37	6qb + 6qc	1	1	1	1	0
38	8pa + 8pb	1	0	1	1	1

39	INV LAF3	1	0	0	0	0
40	2qe + 3p	1	0	0	0	0
41	4pb + 1qc	1	?	0	0	0
42	6p + 1pa	1	1	1	0	0
43	1qe+ 6qb	1	0	0	0	0
44	1qh + 5qa	1	0	0	0	0
45	3qa + 2qg	1	0	0	0	0
46	1pc + 1qg	1	?	?	0	0
47	9q * 1qf	1	1	1	0	0
48	1qb * 2qd	1	1	1	0	0
49	2qb(c) + 1qd	1	1	1	0	0
50	PCA10pprox + 5qa	?/0	?	?	?	?
51	5qa + 8pa	0	1	0	0	0
52	2qh + 2qe	0	1	0	0	0
53	2qb * 1qd	1	1	1	0	0
54	1qd + 8pb	0	1	0	0	0
55	PCA24p * 2p	?/0	1	?	?	?
56	2p * 4pb	0	0	1	0	0
57	2qe + 5p	0	0	1	0	0
58	INV TMA7 *	0	0	1	0	0
59	5qa * 3qa	0	0	1	0	0
60	6qc * 2qa	0	0	1	0	0
61	1qe * 2qg	0	0	1	0	0
62	INV TMA5	0	0	1	0	0
63	3qb + 3qc inv	0	0	0	0	1
64	3qa + 3qb inv	0	0	0	0	1
65	1qe + 6qa	0	0	0	0	1
66	2qa + 2qc	0	0	0	0	1
67	2qb + 2qc inv	0	0	0	0	1
68	2qc + 2qb inv	0	0	0	0	1
69	2qa + 2qf	0	0	0	0	1
70	2qd + 2qe inv	0	0	0	0	1
71	6qb + 5pb inv	0	0	0	0	1
72	5p * 6qb inv	0	0	0	0	1
73	6qb + 5p inv	0	0	0	0	1
74	5p + 6qb inv	0	0	0	0	1
75	6qb + 5p inv	0	0	0	0	1
76	1qf * 7p	0	0	0	0	1
77	4qa * 8q	0	0	0	0	1
78	4qb + 9p	0	0	0	0	1
79	1qb + 1qa inv	0	0	0	0	1
80	6qc * 1pc	0	0	0	0	1
81	3qd * 1pb	0	0	0	0	1
82	9q + 4qc	0	0	0	0	1