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Preface

Luca Sineo, Roscoe Stanyon

Molecular studies at all levels from sequencing to cytogenetics on an increasing number of genomes provide ongoing insights into the fundamental aspects of the organization and evolution of Eutheria and primates. Comparative genomics has the potential to build *a new understanding of the evolutionary history and functional biology of mammals.* Studies of the genome are now entering a phase where the emphasis will be on understanding our genes, their regulation, expression and complex interactions. As the study of genomes becomes less descriptive, it will be essential to determine what drives chromosomal rearrangements. Current technologies are beginning to reveal the potential to interpret the dynamics of gene organization within chromosomes, to reveal the forces promoting chromosomal rearrangements and the conservation of chromosomal synteny such as the paper in this volume by Mora, Garcia and Ponsà as well as by Hirai and Hirai.

In particular, the increasing effort in comparing primate genomes and their chromosomal organization (syntenies) can put light on different and unexpected aspects of their organization, regulation, and function that appeared during the primate radiation (a great amount of serendipity is the complementary motif of this scientific endeavor). At this purpose, highly conserved aspects of genome architecture will not be accurately identified nor will the lineage-specific changes be identified as such, without the comparison to more evolutionary distant mammals and eventually other vertebrates. The contribution by Sineo and Romagno details the evolution of chromosome 7 in apes, monkeys and other mammals.

Chromosomes have long been used as phylogenetic markers. Chromosomes are inherited as Mendelian traits, are generally conserved within species, and mutations which become fixed in a species karyotype are considered rare events. Chromosome banding was an effective method for comparison between closely related species, but was prone to error in comparison between distantly related species or when the rate of chromosome evolution was particularly marked. In these cases molecular methods now allowed chromosomes to be compared at the DNA level and homology and convergence can be more easily identified. Homology with the human genome of even very distantly related species can be established by chromosome painting. Over the last two decade

molecular cytogenetics has added a new dimension of ease, economy and precision to comparative genomics. Fluorescence activated flow sorting followed by PCR amplification produces chromosome paints. Chromosome specific probes can be made from cultured cells of any species. Chromosome paint sets from two or more species makes reciprocal or multi-directional chromosome painting possible as demonstrated in the contributions in this volume by Dumas and Sineo as well as by Bigoni et al.

We now know that large syntenic tracts of the human genome are conserved across mammalian evolution and that chromosomes are important phylogenetic markers, because rearrangements fixed in various evolutionary lines are rare events. The principles of parsimony and outgroup comparisons are used to distinguish between conserved and derived syntenies and to identify the landmarks of mammalian genome evolution.

All primates are useful in order to understand genomic organization and human origin but, in consideration of the well known relationship (from Huxley, 1863, we must know that apes are our closest relatives), and the fact that we do not know very much about the functional aspects of our genome, primates remains the best model of study.

There was almost universal agreement among cytogeneticists since the 1960s that African apes and humans shared a number of traits that indicated a common period of evolution after the divergence of the orangutan (Chiarelli, 1962). In addition to the fusion of human chromosome 2, nine pericentric inversions are the most conspicuous karyotype differences between humans and the great apes. Pericentric inversions may have been important for the establishment of reproductive isolation and speciation of the hominoids as they diverged from a common ancestor. Chromosome banding suggested two phylogenetic possibilities: either humans and chimpanzees could be phylogenetically linked by inversions to chromosomes 4, 7 and 9 or alternatively the chimpanzee/gorilla were linked by inversions to chromosomes 12 and 16. Molecular cytogenetics now strongly supports the chimpanzee/human relationship. Inversions on 7, 9 and the Y are clearly link these two species while the rearrangements in homologs to 12 and 16 in the African apes were shown to be the result of independent mutations. The gorilla and chimpanzee homologs exhibit similar but not identical derived pericentric inversions for both while humans have conserved the ancestral form.

Analyses of chromosomal rearrangements that have occurred during the evolution of the hominoids can reveal much about the mutational mechanisms underlying primate chromosome evolution. An area of investigation that can yield rapid results is the comparison of the human genome sequence with that of the chimpanzee (http://www.nhgri.nih.gov/11509418). The initial assembly of the chimpanzee genome is available on the NIH-run, public database, *GenBank*. To facilitate biomedical studies comparing regions of the chimpanzee genome with similar regions of the human genome, the draft version of the chimpanzee sequence has been aligned with the human sequence. These alignments can be scanned using the National Center for Biotechnology Information's *Map Viewer*.

Humans differ from chimpanzees not only in the fusion origin of chromosome 2, but in at least 11 major inversions on chromosomes 1, 4, 5, 7, 9, 12, 13, 15, 16, 17,

and 18. This areas of the genome can be compared and compare the chimpanzee and the human sequences to analyze the breakpoints involved in the major structural rearrangements as well as others to discover further submicroscopic chromosomal rearrangements. These researches will help determine the events that differentiated human and chimpanzee genomes and have helped define what it is to be human.

Further, many species of primates face risks of extinction; yet the knowledge of their genomes could provide a deeper understanding of primate adaptations, human origins, and provide the framework for discoveries anticipated to improve human medicine. The "Critically endangered list" of animals in peril, recently recognized 55 primates that fit the IUCN criteria. The list first published in 2000, including 25 species. The top 25 there named "were merely the tip of the iceberg", and following rapid changes in field knowledge was revised (Beijing, 2002 IPS congress) and doubled in Turin (IPS 2004).

The great apes are among the most vulnerable and most important for human medical studies. However, apes are not the only species whose genomic information will enrich humankind. Comparative genomic studies of "less important species" can benefit conservation efforts on their behalf. Over the last 15 years molecular cytogenetics has revealed the genome composition of almost 50 species of primates, but many species remain to be studied even at the most rudimentary level.

It has often been speculated that there is a correlation between chromosome rearrangements in disease and evolution. Even more provocative is the hypothesis that the evolutionary history of chromosome rearrangements provides a causal link behind at least some cancers. This link seems probable since genome rearrangements do not represent random events, but instead, reflect higher order genomic features. Current research is beginning to show how the structure, function and fluidity of the genome and the relationship between evolution and disease. Indeed, recent work suggests that the genome consists of chromosome segments that are ancestrally conserved and have discrete boundaries defined by recombination hot spots (i.e. segmental duplications). Other lines of evidence suggest that ancestral viral integrations during primate evolution influence chromosome rearrangements. Recent research shows that the organization and replication of DNA render fragile sites (FSs) prone to breakage, recombination as well as becoming preferential targets for mutagens-carcinogens and integration of oncogenic viruses. The discoveries that chromosome translocations, amplification of proto-oncogenes, deletion of tumor suppressor genes and integration of oncogenic viruses all result from the specific breakage of genomic DNA at FSs provide compelling support for a causative role for FSs in cancer. A comparative study of the evolution of these regions in primates such as the paper in this volume by Ruiz-Herrera et al is an important development in cancer research. It is probable that the molecular characteristics of fragile sites that are conserved during chromosome evolution compared with fragile sites, which are not conserved or expressed, will provide clues to the mechanisms underlying fragile site formation and their relationship to cancer. Future comparative research on fragile sites in higher primates as should be an effective means to enhance our understanding of the role of fragile sites in both evolution and disease.

Data on genetic zoogeography can protect wildlife health management, and provide insights into demographic management of small populations in the wild or insure a right genetic variability. Genome sequencing studies cannot be expected to directly influence habit sufficiency for endangered species. But, the ability to evaluate genetic variation and demographic history in populations *via* genomic analyses may find application in Conservation, as like increase the effectiveness of residual habitat fragments for population persistence, and provide more quantitative evaluation of levels of threat.

Increased knowledge of genome makeup and variation (polymorphisms is a great issue never seriously approached in animal molecular biology) in endangered species finds conservation application in population evaluation, monitoring, and management, or identify risk factors for genetic disorders. We know an increasing number of genetic driven diseases that can have efficacious models in primates that are resistant, or immune, or differently responding, in presence of the homologous genetic trait. Species must have sufficient suitable habitat. This is the only real limitation they face. Human impact, a part from extraordinary case of pollution or intensive hunting, is determined just by "need of room". Primates must have ecosystem resources that allow for population existence over extended periods. Normally, these periods are in geological time scales. Currently, concern for many species is focused on the next few generations.

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Dedication

Roscoe Stanyon, Luca Sineo

One purpose of this symposium and book is to honor Brunetto Chiarelli's contribution to primate genetics and in particular primate cytogenetics. We want to make a brief review of his publication record in primatology and his university career to demonstrate that he can rightly be viewed as the "father" of primate cytogenetics.

Cytogenetics before the introduction of various technical advances such as tissue culture, discovery of mitogens and hypotonic treatments in the mid 1950s was an extremely difficult and very tenuous endeavor. The first demonstration of the correct dipoid number (2n=46) in human occurred in 1956 (Tijo and Levan, 1956) only after some 40 publications over almost three decades had repeatedly found 48 chromosomes. Cytogenetic workers were concerned with counting chromosomes and classifying them according to relative length and centromere position. For instance, patients with Down syndrome were discovered to have an additional copy of a small chromosome, chromosome number 21 (Lejeune *et al.*, 1959).

Chiarelli was awarded the laurea in Natural Sciences (Doctor of Natural Sciences, University of Florence) in 1957 with a thesis on Bantu osteology and three years later (1960) he was awarded a second laurea in genetics (Doctor of Biology, University of Florence) with a thesis on human chromosomes. Chiarelli's cytogenetic research began shortly after the human diploid number was finally known.

By 1961 Chiarelli had published the chromosomes of the orangutan in Nature (Figure 1). The following year he published a three-way comparison of the humans, chimpanzees and orangutan karyotypes. Within another two years (1963) Chiarelli traveling all over Europe had collected biological materials and already published on the karyotypes of 55 species of primates, a truly remarkable feat even by any standard. The majority of Chiarelli's work on primate cytogenetics deal with classically stained chromosomes. However, he published one of the first papers on trypsin banding (Chiarelli *et al.*, 1972) and one of the first (if not the first) chromosome banding comparisons of human and ape chromosomes (Chiarelli and Lin, 1972). Later various students of Chiarelli were involved in molecular cytogenetics such as FISH (fluorescence *in situ* hybridization).

In the period from 1958 to 1969 about half of Chiarelli's scientific publications dealt with primate genetics and cytogenetics. Over the following decades the percentage

of his publications dealing with primate genetics fell and Chiarelli turned his attention to other anthropological field. Altogether, Chiarelli is author of 240 citations currently found in the bibliographical database for primatology maintained by the University of Wisconsin: http://primatelit.library.wisc.edu/

If we examine the total articles currently reported in PubMed (44) more than half deal with primate genetics. Clearly, his work in primate cytogenetics accounts for the largest part of his impact on science (Figure 2).

Books on Primatology as author or co-author

In addition to publication in international journals Chiarelli also authored or edited a number of landmark books in primatology. Among the books he authored or co-authored:

1972 – Taxonomic Atlas of Living Primates. Academic Press, London

1973 – Evolution of the Primates: An Introduction to the Biology of Man. Academic Press, London

1971– Comparative Genetics in Monkeys, Apes and Man. Academic Press, London

1974 – Perspectives in Primate Biology. Plenum Press, New York

1979 – Comparative karyology of Primates. Mouton, The Hague

Edited books

1973 – Cytotaxonomy and Vertebrate Evolution. Academic Press, London

1968 – Taxonomy and phylogeny of the Old World Primates. Rosenberg e Sellier, Torino

1981 – Origin of the New World Monkeys and Continental Drift. Biogeographic and phylogenetic considerations. Plenum Press, New York

1982-83 – Proceedings of the VIII Congress of the International Primatological Society (3 volumi). Springer-Verlag, Berlin-Heidelberg-New-York

1986 – Sexual dimorphism in living Primates, Il Sedicesimo, Firenze

University Career

Chiarelli's University career was intimately connected to primatology. He became Professor of Anthropology in 1962 at the University of Turin and the Professor of

Primatology in Turin from 1969 to 1979 when he moved to assume the chair of Anthropology at the University of Florence. He was also visiting professor of Anthropology at the University of Toronto from 1970 to 1974.

Editorial and Congressional Activities in Primatology

He also organized symposium and congresses dedicated to primatology including:

- Taxonomy and Phylogeny of the Old World Primates (Torino, giugno 1967)
- NATO A.S.I. su: Comparative Genetics in Primates and Human Heredity (Erice, luglio 1970)
- Conference and Workshop on Comparative Karyology of Primates (con A. Koen; Detroit, agosto 1973), nell'ambito dei IX Congress dell'International Unions of Anthropological and Ecologicas Sciences (IUAES)
- VIII Congresso Internazionale di Primatologia (Firenze, luglio 1980)

Finally we should not forget that he founded and directed the Journal of Human Evolution, Academic Press, London 1972-1985 for 1972 until 1985. This journal under his direction published many papers on primate cytogenetic and genetics, even if this journal today under different editorship deals mainly with human paleontology.

Publications in Primate Cytogenetics and Genetics by B. Chiarelli

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1962

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1963

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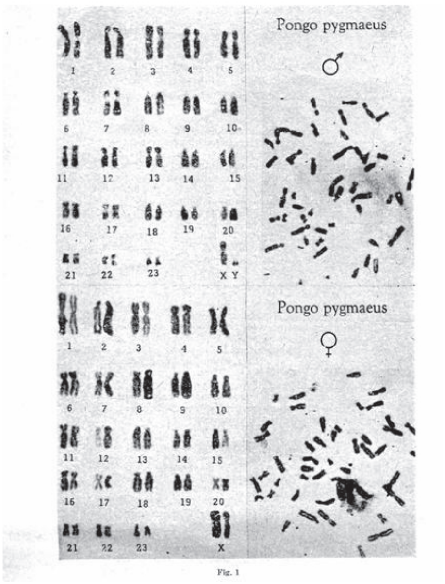

Figure 1 – Chromosomes and karyotype of the orangutan modified from a figure of Chiarelli published in Nature 1961.

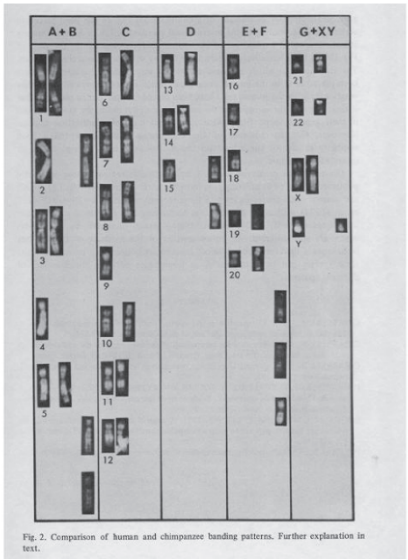

Figure 2 – Comparison of human and chimpanzee banding patterns. Further explanation in text (Lin & Chiarelli, *Genen en Phaenen*, 1972).

1962
Pan troglodytes, *Pongo pygmaeus*, *Pan paniscus*, *Macaca* (11 species), *Papio* (6 species), *Cynopithecus niger*, *Theropithecus gelada*, *Cercocebus* (4), *Cercopithecus* (9), *Erythrocebus patas*, *Presbytis obscurus*, *Colobus polykomos*, *Hylobates lar*, *H. agilis*, *H. moloch*, *Lemur catta*

1963
Lemur fulvus, *Callithrix jacchus*, *Cebus capucinus*, *Cebus apella*, *Ateles aracnoides*, *A. trivirgatus*, *Saimiri sciureus*, *Cercopithecus* (6 additional)

1966
Nasalis larvatus
Gorilla gorilla

1968
Cebuella pygmaea
Tupaia glis

1972
Hylobates syndactylus

Figure 3 – List of Primate's species karyotyped in ten years.

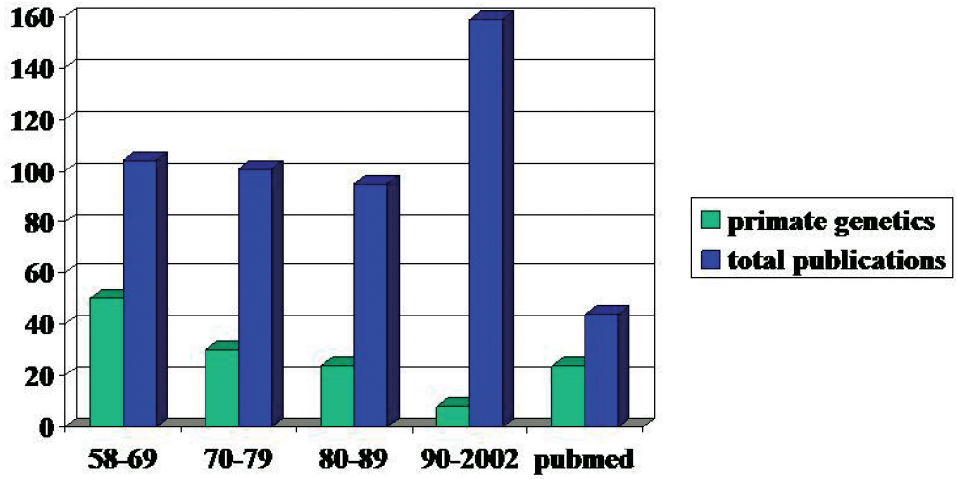

Figure 4 – Chiarelli's publications over the years (all types including abstracts, congresses etc.).

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Cytotaxonomy of Colobinae Primates with Reference to Reciprocal Chromosome Painting of *Colobus guereza* and Humans

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ABSTRACT: *Colobine phylogeny and evolution is little understood and poorly studied. We established* C. guereza *(2n=44) chromosome paint probes by flow sorting and reciprocally hybridized them to human chromosomes. The bivariate flow karyotype of* Colobus guereza *was resolved into 22 peaks. Paints of* C. guereza *were hybridized to human metaphases and 32 clear signals were detected. The reciprocal painting data allowed us to assign subchromosomal homologies between* C. guereza *and human chromosomes. A comparison of these data with previous chromosome painting and banding supported the monophyly of Colobinae and their division in an African and an Asian clade.* Pygathrix nemaeus *is karyologically the most conservative colobine species studied and it probably diverged early after the separation of Asian and African colobines. In contrast, chromosome painting shows that* Nasalis larvatus*, often considered the most primitive and isolated colobine, is karyologically derived and phylogenetically nested within Asian colobines. Both the painting and banding data support the taxonomic grouping of* Trachypithecus, *but would exclude the purple faced leaf monkey and align this species with the Hanuman langur in* Semnopithecus.

KEYWORDS: *molecular cytogenetics, chromosome rearrangements, genome evolution, phylogeny.*

INTRODUCTION

Colobine monkeys, also known as leaf-eaters, are a group of morphologically highly specialized catarrhines. Their latin name Colobinae, is derived from the Greek word for mutilated (kolobos), because these monkeys are characterized by a very short and in some case absent thumb. In contrast, the other fingers, especially the third and the fourth are longer then in cercopithecines (Strasser and Delson, 1987). Their dimensions vary from 4 kg in the African olive colobus (*Procolobus verus*), to 20 kg of the adult

males of the proboscis monkey in Borneo (*Nasalis larvatus*). Their diet consists primarily of mature leaves, but may also include other parts of plants, fruits, seeds and flowers, with variations among different species (Kay and Davies, 1994). Sometimes animal proteins in the form of insects, larvae and spiders are also included in the diet.

Their adaptation to the leaf eating niche explains their specialized morphology as well their digestive physiology. The colobine digestive system is unique among primates. Salivary glands are overdeveloped and produce a high quantity of saliva. The stomach is large and multi-chambered and symbiotic bacteria are present in the forestomach to digest cellulose (Oates *et al.*, 1994), therefore these monkeys are often indicated as 'foregut fermenters'. The stomach is divided in four chambers (*presaccus*, *saccus gastricus*, *tubus gastricus* and *pars pilorica*). In the first two chambers symbiotic bacteria are present and it appears that the saliva also acts as a buffer keeping the acidity in these first two chambers at an acceptable level for the survival of the bacteria. These bacteria are then digested by various enzymes in the next two stomach sections. The lysosomes involved are a striking case of adaptive convergence with ruminants (Stewart *et al.*, 1987). Recently, digestive RNases were studied in a colobine (*Pygathrix nemaeus*) and a clear case of duplicate gene evolution was found (Zhang, 2003).

The number of teeth and the dental formula are the same as in the other Catarrhinae (2-1-2-3), but important differences are present. The incisors are smaller then in cercopithecines and a high frequency of underbite was found. The masticatory system is overall powerful with teeth characterized by high cusps and cutting crests, also molars have high and sharp cusps linked by transversal crests (*bilophodontia*) (Richard, 1985). Colobines also differ from Cercopithecinae because they do not have cheek pouches.

Despite a recent accumulation of data on various features of these monkeys, Colobinae phylogeny and evolution are still not well understood, partly because they do not survive well in captivity, and there is no consensus on the taxonomy. Groves (1989) proposed a basic division between *Nasalis* (including *Simias*) and the other species, while Strasser and Delson (1987) preferred a split between African and Asian colobines, a scenario confirmed by molecular studies and accepted in recent taxonomy (Collura *et al.*, 1996; Collura and Stewart, 1995; Disotell, 1996; Messier and Stewart, 1994; Page *et al.*, 1999; Sarich, 1970). The African group was divided by Oates, Davies and Delson (1994) in two genera: *Colobus* (black and white colobus monkey) and *Procolobus* (red and olive colobus monkeys). *Colobus* included five species: *satanas*, *angolensis*, *polykomos*, *guereza* and *vellerosus*. *Procolobus* comprehended the subgenus *procolobus* (olive colobus, one species) and *piliocolobus* (red colobus, also monospecific). Groves (2001) described three genera of African colobines (*Colobus*, *Piliocolobus* and *Procolobus*) and a total of 15 species.

Asian colobines taxonomy has gone through even more extensive changes. Groves (1989) described five genera *Nasalis*, *Pygathrix*, *Presbytis*, *Trachypithecus* and *Semnopithecus*. Oates, Davies and Delson (1994) separated the genus *Simias* from *Nasalis*. In 2001 Groves agreed on the generic status of *Simias*. Groves also took in account morphological studies (Jablonski and Yan-Zhang, 1993) supporting the recognition of *Rhinopithecus* as full genus and not just as subgenus of *Pygathrix*, for a total of seven

Asian colobine genera: *Nasalis*, *Pygathrix*, *Presbytis*, *Trachypithecus*, *Semnopithecus*, *Simias* and *Rhinopithecus*. The number of species listed in the taxonomy of Asian colobines was also dramatically increased from 24 in Oates *et al.* (1994) to 43 according to Groves (2001).

Cytogenetics of Colobines

It is widely recognized that chromosomal events are linked to molecular divergence and to the speciation process (Navarro and Barton, 2003; Rieseberg and Livingstone, 2003). Therefore data on colobine karyotypes should provide hints for phylogenetic reconstruction and help to tease out evolutionary relationships. From studies using classical staining, the diploid number of both African (genus *Colobus*) and Asian (genus *Presbytis*) colobines was found to be 2n=44 (Chiarelli, 1963; Ushijima *et al.*, 1964). The karyotype of the genus *Colobus* was composed by all metacentric and submetacentric chromosomes, including one pair bearing the nucleolar organizer region (NOR). The karyotypes of the three species studied (*Colobus polykomos*, *C. badius* and *C. kirkii)* appeared similar if not identical (Chiarelli, 1963). Through classical staining, the karyotype of the genus *Presbytis* appeared to be almost the same as the karyotype of the African colobines with the only difference of a small acrocentric pair of chromosomes.

Banding techniques introduced in the 1970s improved the possibility of identifying differences between karyotypes, but only a few species of Colobinae have been studied with banding. A report on G- and Q-banding on *Trachypithecus cristatus* (*Presbytis cristata* in the original pububication) was reported with the description of two variant forms of chromosome 1 in the same female studied (Ponsa *et al.*, 1983). Three studies also present the R-banding of various species of of *Colobus* and *Trachypithecus cristatus* making clear that many differences were present among these species despite the same diploid number and apparent similar chromosome morphology (Dutrillaux *et al.*, 1981; Dutrillaux *et al.*, 1984; Muleris *et al.*, 1986). In particular a translocation involving an autosome and the Y chromosome was described in *T. cristatus*, the only case reported in catarrhine primates (Dutrillaux *et al.*, 1984).

Chromosomes are a useful tool for evolutionary studies and phylogeny when homologous structures are compared. Unfortunately, banding techniques, while providing good indicators of the morphology of chromosomes, are sometimes insufficient when used for comparisons between species. Banding provides a hypothesis of chromosomal homology between species, which should then be confirmed at the DNA level. Chromosome painting is a now well-established method for determining chromosomal homology. By firmly establishing homology, modern molecular cytogenetics offers powerful tools to investigate and clarify phylogenetic relationships by tracing the genome evolution of species. Recently we used flourescent *in situ* hybridization (FISH) of human chromosome paints on metaphases of various colobines to establish chromosomal homology at the DNA level between human chromosomes and chromosomes of four colobine species. These studies included *Colobus guereza* (Bigoni *et al.*, 1997b),

an African colobine (2n=44), and three species of Asian colobines: *Trachypithecus cristatus* (2n=44) (Bigoni *et al.*, 1997a), *Nasalis larvatus* (2n=48) (Bigoni *et al.*, 2003) and *Pygathrix nemaeus* (2n=44) (Bigoni *et al.*, 2004). Other authors have also reported on the hybridization of human chromosome paints to Asian colobines *T. francoisi* and *T. phayrei* (Nie *et al.*, 1998).

These studies supported the monophyly of Colobinae and their division in an African and an Asian clade. *P. nemaeus* is karyologically the most conservative of the Colobinae studied and possibly splitting soon after the divergence of Asian and African colobines (Bigoni *et al.*, 2004). On the other hand, chromosome painting shows that *N. larvatus*, often considered the most primitive and isolated colobine, is karyologically derived and phylogenetically nested within Asian colobines (Bigoni *et al.*, 2003). *T. cristatus* appears to be karyologically the most derived among the Asian colobines. This colobine has a reciprocal translocation of human 6 and 16 and is one of the very few primates and the only catarrhine showing a reciprocal translocation involving the Y chromosome and an autosome (Bigoni *et al.*, 1997a).

Detailed data of chromosomal homology are necessary to delineate karyological events and, to provide a worthwhile contribution to the evolutionary history and phylogeny of species. However, unidirectional painting (i.e. human probes hybridized to monkey metaphases) does not provide any information on subchromosomal homology, which can be particularly important when translocations have transformed the karyotype. Reciprocal chromosome painting in which chromosomal paints from two species are hybridized to metaphases of the other species can assign subchromosomal homology and helps locate breakpoints. Such information is helpful in determining if disrupted chromosomal synteny or syntenic associations found in two or more species derive from the same cytogenetic event. Therefore in this study we used reciprocal chromosome painting, a technique that allows a more precise localization of breakpoints and the detailed identification of segments involved in chromosome rearrangements.

There are only three reports available on reciprocal chromosome painting between humans and Old World monkeys: *Chlorocebus aethiops* (Finelli *et al.*, 1999), *Erythrocebus patas* and *Cercopithecus neglectus* (Stanyon *et al.*, 2005). Here we present the first report of reciprocal chromosome painting between a colobine monkey and humans. We established a set of whole chromosome painting probes of *Colobus guereza* by fluorescence activated chromosome sorting and DOP-PCR (degenerate oligonucleotide primed PCR). We then hybridized the *C. guereza* painting probes to human metaphases to define subchromosomal homology of the rearranged monkey chromosomes.

Materials and Methods

Chromosome preparations of a female *Colobus guereza* (CGU) were obtained by standard procedures from fibroblasts established by a skin biopsy kindly provided by Dr S. O'Brien (Laboratory of Genomic Diversity, National Cancer Institute-Frederick). Chromosomes of *C. guereza* were numbered according to Bigoni *et al.* (1997a).

C. guereza chromosome specific-probes were obtained by DOP-PCR from flow sorted chromosomes by PCR amplification and labeling conditions as previously described (Telenius *et al.*, 1992; Wienberg and Stanyon, 1998). Chromosome sorting was performed using a dual laser cell sorter (FACSDiVa) that allows a bivariate analysis of chromosomes by size and base-pair composition. From each peak in the flow karyotype about five hundred chromosomes were sorted directly in PCR tubes containing 30 l of distilled water. The 6MW primer (5'-ccgactcgagnnnnnnatgtgg-3') described by Telenius *et al.* (1992) was used in the primary reaction and to label the chromosomal DNA with biotin dUTP or digoxigenin-dUTP in a secondary PCR for indirect detection. These paints were first hybridized to *C. guereza* metaphases to identify the chromosome content of each peak of the flow karyotype and then to human chromosomes. Common FISH procedures were followed performing *in situ* hybridization and probe detection. About 300 ng of each PCR product per probe, together with 10 g of human Cot-1 (Invitrogen) were precipitated and then dissolved in 14 l hybridization buffer. After hybridization and washing of the slides, biotinylated DNA probes were detected with avidin coupled with fluorescein isothiocyanate (FITC, Vector). Digoxigenin-labeled probes were detected with antidigoxigenin antibodies conjugated with Rodamine (Roche).

Results

The bivariate flow karyotype of *C. guereza* was resolved into 22 peaks (Figure 1). Flow sorting and DOP-PCR provided chromosome paints from each peak. These paints

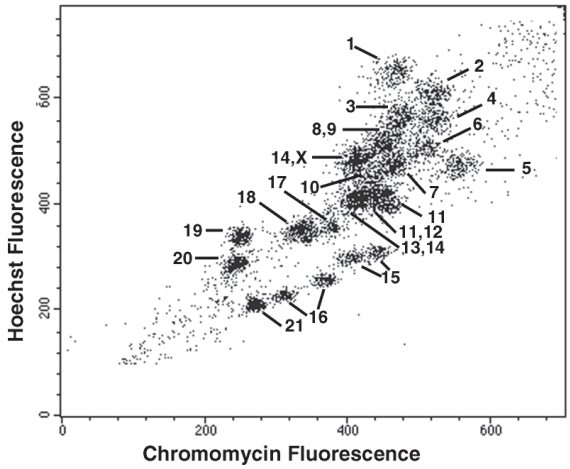

Figure 1 – This figure shows the flow karyotype of *Colobus guereza*. Chromosome were stained with a combination of Hoechst and Chromomycin-A which allowed a bivariate plot. The chromosomes were distributed in 22 peaks.

were then hybridized to *C. guereza* metaphases to identify the chromosome content of each peak of the flow karyotype. All but four peaks contained a single chromosome. Chromosomes 8 and 9 were contained in a single peak. Chromosomes 11 and 14 were present in two different peaks. Chromosome 11 once alone and the other in combination with chromosomes 12. Chromosome 14 was found once with 13 and the X-chromosome. Chromosomes 15 and 16 were each present in two different peaks. All peaks provided good chromosome paints. Colobine paints were then used to hybridize human metaphases and 32 clear signals were detected on the human karyotype (Figures 2 and 3). We had no Y-chromosome probe since a female cell line was used for sorting.

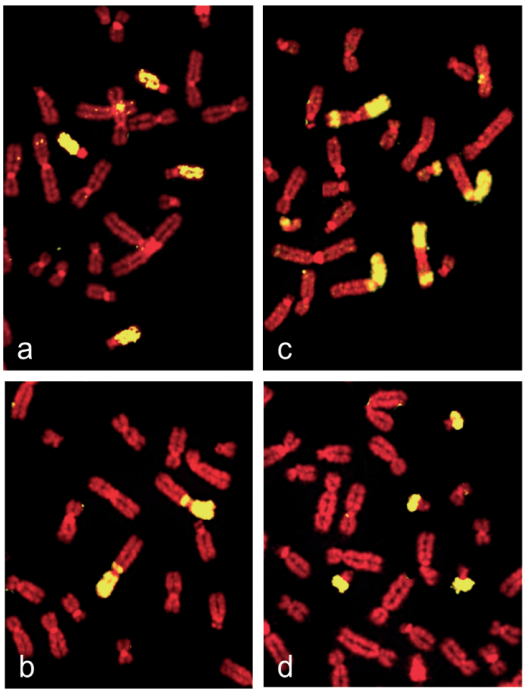

Figure 2 – Examples of hybridization of colobine paints to human metaphases a) CGU 6 hybridized to HSA 14 and 15; b) CGU 17 hybridized to HSA 2p and small part of 2q; c) CGU 18 hybridized to HSA 3 and 19; d) CGU16 hybridized to HSA 21 and 22.

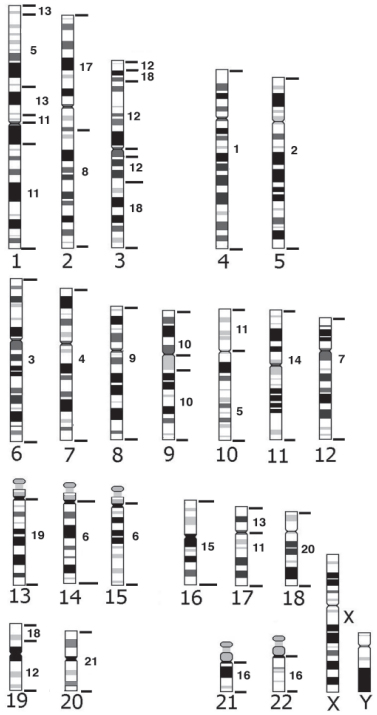

Figure 3 – Human idiogram, numbered below, with hybridization pattern of *Colobus guereza* chromosome paints to the left.

Twelve *C. guereza* chromosome paints hybridized completely a single human chromosome: CGU1 (HSA4), CGU2 (HSA5), CGU3 (HSA6), CGU4 (HSA7), CGU7 (HSA12), CGU9 (HSA8), CGU10 (HSA9), CGU14 (HSA11), CGU15 (HSA16), CGU19 (HSA13), CGU20 (HSA18), CGU21 (HSA20). Each of the following two *C. guereza* chromosome paints hybridized completely two human chromosomes: CGU6 (HSA14 and HSA15) and CGU16 (HSA21 and HSA22). Six human chromosomes

where painted by two or more *C. guereza* probes. HSA2, HSA10, HAS 17 and HSA19 where divided in two segments, HSA1 and HSA3 in four segments. On each human chromosome 1, 3 and 9 a subcentromeric heterocromatic band is present and was not painted. The following associations of *C. guereza* chromosomal segments were found on the human karyotype: 5/13 and 11/13 on HSA1, 8/17 on HSA2, 12/18 on HSA3, 5/11 on HSA10, 11/13 on HSA17 and 12/18 on HSA19.

Discussion

The reciprocal hybridization of *C. guereza* chromosome paints to human metaphases allowed us to assign the subchromosomal homology and breakpoints of fissioned chromosomal syntenies. The reciprocal painting also supported the conclusion previously reported on hybridizing human paints to *C. guereza* metaphases. In our previous report on hybridization of human probes on *C. guereza* metaphases (Bigoni *et al.*, 1997b) we showed that chromosomal synteny between humans and this species of colobine is generally well conserved, but with some exceptions. In *C. guereza* there are six human chromosomes (1, 2, 3, 10, 17 and 19) that are fragmented (the synteny is disrupted). These breaks and translocations have produced: CGU5 (HSA 1/10), CGU11 (HSA 17/1/10), CGU12 (HSA 3/19/3/19), CGU13 (HSA 1/17), and CGU18 (HSA 3/19). The alternating pattern of human segments 3 and 19 on CGU12 is best interpreted as a pericentric inversion that followed a translocation.

To draw phylogenetic information from the hybridization patterns found in colobine monkeys we must compare the chromosomes with the ancestral catarrhine karyotype (Stanyon *et al.*, 2004). There is good agreement that the ancestral catarrhine karyotype had a diploid number of 2n=46 with the following chromosomes: 1, 2a, 2b, 3-13, 14/15, 16-22, X and Y.

We can then compare the ancestral catarrhine karyotype with that of *C. guereza* and other colobines established through chromosome painting and secondarily by banding comparisons. In a previous study (Bigoni *et al.*, 2004) we established the chromosomal homology of *Pygathrix nemaeus* (douc) with human and other primates by *in situ* hybridization of human chromosome paints to douc metaphases. Our results indicated that *P. nemaeus* is karyologically the most conservative colobine species studied and that it probably diverged early after the separation of Asian and African colobines. These data reinforced the monophyly of the Colobinae and their division into an African and an Asian clade. When a human paint is found divided in two or more segments the genetic synteny is not maintained. The FISH data showed that three human syntenic groups are fragmented, as human paints of chromosomes 1, 2 and 19 are each present on two different douc chromosomes. Human chromosome paint 2 was divided on two douc chromosomes (12 and 13) as expected, since it is well known that an apomorphic tandem fusion gave origin to human chromosome 2. The other human syntenic groups fragmented are homologous to human chromosomes 1 and 19. The fragmentation and association of human chromosomes 1 and 19 can be explained with a reciprocal

translocation that produced the douc chromosomes. This association was found in all Asian colobines studied, but not in the African species *Colobus guereza*, where different translocations are present (Bigoni *et al.*, 1997a, 1997b). However, the karyotypes of *T. cristatus*, *T. francoisi*, *T. phayrei* and *N. larvatus* showed a more complicated pattern of four alternating segments of human chromosome paints 1 and 19 on the same colobine chromosome (Bigoni *et al.*, 2003, 2004; Nie *et al.*, 1998). The most parsimonious explanation is that a reciprocal translocation occurred in the lineage of the Asian colobines and distinguishes this group from the African colobines. Other rearrangements such as inversions may provide distinguishing traits between Asian and African colobines. *P. nemaeus* showed the primitive reciprocal translocation between 1 and 19 that was followed by a pericentric inversion linking the genus *Trachypithecus* with *Nasalis*.

The probe specific for the human chromosome 6 painted only one chromosome in the African colobine species *C. guereza* (Bigoni *et al.*, 1997b), and in *P. nemaeus* (Bigoni *et al.*, 2004). G-banding analyses and comparisons demonstrated that human chromosome 6 is also maintained in some other species of Asian colobines including *Semnopithecus entellus*, *Presbytis comata* and *Semnopithecus vetulus* (Bigoni, 1995). In *T. cristatus*, *T. francoisi* and *T. phayrei* the probe specific for human chromosome 6 painted two segments of two different chromosomes, but they are associated with a segment homologous to human chromosome 16, following a reciprocal translocation that involved human homologs 6 and 16.

It can be noted that the reciprocal translocation of 6 and 16 appears to be a distinguishing characteristic of the genus *Trachypithecus.* This rearrangement was found in all *Trachypithecus* species published so far. The only exception would be the purple-faced langur (*T. vetulus*): our unpublished FISH data show that this rearrangement is not present and a single syntenic homolog to human chromosome 6 was found. Therefore the cytogenetic data do not support the inclusion of the purple-faced langur in the genus *Trachypithecus* as suggested by Groves (1989).

Karyological data supporting a closer relationship between *S. entellus* (which also has a syntenic chromosome 6, unpublished data) and *vetulus* are not in contrast with geographical distribution of these two species (*entellus* in India and Sri Lanka, *vetulus* in Sri Lanka) and with observations on the color of infants, an important and variable character in colobines. In fact *vetulus* could be excluded from *Trachypithecus* on the basis that infants are not orange, but gray. On the same basis of a blackish color of newborn infants Groves (1989) argued that *entellus* is more primitive than *Trachypithecus*.

The exceptional diploid number of *Nasalis*, 2n=48 (Chiarelli, 1963, 1966; Soma *et al.*, 1974; Stanyon *et al.*, 1992) has played a pivotal role in phylogenies which view the proboscis monkey as the most primitive colobine, and a long isolated genus of the group (Giusto and Margulis, 1981; Groves, 1989; Peng *et al.*, 1993). Groves (1989) considered *Nasalis* primitive for a relevant number of morphological characters for the most part linked to the lack of masticatory specialization seen in other colobines and for the diploid number. He considered *Nasalis* as a sister species to all other African and Asian colobines and he divided the Colobidae into two subfamilies: Nasalinae and Colobinae. Harvati (2000) found support for Groves on the basis of colobine dental

eruption sequences. Peng *et al.* (1993) also claimed that *Nasalis* is the most primitive colobine genus on the basis of morphological measurements and again for the chromosome number. On the other hand, molecular studies support a monophyletic Asian clade including four lineages: *Nasalis*, *Rhinopithecus*/ *Pyghatrix*, *Semnopithecus entellus*/ *vetulus*, *Trachypithecus*/ *francoisi*/ *obscurus*/ *cristatus*. Zhang and Ryder (1998) supported the existence of a monophyletic Asian clade and suggested the possibility of a lineage including *Nasalis*, *Rhinopithecus* and *Pygathrix*.

In a previous report (Bigoni *et al.*, 2003) we used molecular cytogenetic methods to map the chromosomal homology of the proboscis monkey *(N. larvatus*) in order to test these hypotheses. The use of *in situ* hybridization allowed us to establish homologies between the chromosomes of humans and the *N. larvatus* karyotype. Comparisons with molecular cytogenetic data in other primates show that the proboscis monkey genome is derived and not primitive. The diploid number of 2n=48 can be best explained by derived fissions of a segment of human chromosome 14 and the fission of human chromosome 6. Consequently the higher diploid number found in *Nasalis* is not, as mistakenly assumed, a primitive character. Our results supported the view that the *N. larvatus* karyotype is not primitive, but derived in respect to other colobines and most other Old World monkeys. In fact this karyotype is derived not only in chromosome number, but also for the syntenies present. In spite of these derived apomorphic characters, *Nasalis* is closely related to and nested within other species of Asian colobines.

We cannot exclude the possibility that the fission of homologs chromosome 6 links *N. larvatus* with some *Trachypithecus* species after the divergence of *Presbytis* and *Semnopithecus*. If this is the case, then *N. larvatus* would show an intermediate stage between all the colobine species with intact human syntenic group 6 and the group *T. cristata, T. phayrei* and *T. francoisi* bearing the reciprocal translocation involving human chromosomes 6 and 16. According to this hypothesis chromosome 6 would have been fissioned in a common ancestor of *Nasalis* and *Trachypithecus*. After the divergence of *N. larvatus* two fusion events involving chromosome 6 and 16 homologs would have occurred in the phylogenetic line leading to *Trachypithecus*. This hypothesis is a less parsimonious explanation then the alternative hypothesis, which we favor here: the fissions of chromosome 6 homologs in these taxa are independent events. However, to distinguish between these hypotheses we need to know if the breakpoints in homologs to chromosome 6 in *Nasalis* and *Trachypithecus* are the same or not and that the resulting segments are truly homologous. To test these different hypotheses, more detailed studies are necessary such as reciprocal in situ hybridization, use of subregional probes, cloning and eventually sequencing of the breakpoints.

Integration of Chromosome Painting and Banding

The number of segments or hybridization signals is a good indicator of the evolution of the karyotypes with regard to interchromosomal rearrangements. The human paints were split into 26 segments in *P. nemaeus*, 30 segments in *N. larvatus* and *T. cristatus*

and 32 segments in *Colobus guereza* (always considering the female karyotype). The same diploid chromosomal number 2n=44 shared by *P. nemaeus*, *C. guereza* and *T. cristatus* is the result of different interchromosomal rearrangements. Additionally, *T. cristatus*, *T. francoisi* and *T. phayrei* karyotypes are derived for a reciprocal translocation between homologs to human 6 and 16 (Bigoni *et al.*, 1997a; Nie *et al.*, 1998). The karyotype of *N. larvatus* is also derived for two fissions of the homologs to human chromosomes 14 and 6 (Bigoni *et al.*, 2003). Our results suggest that *P. nemaeus* is the most conservative of the Asian colobines and is phylogenetically basal to all other Asian colobine studied up to now.

We can integrate the molecular cytogenetic data with banding comparisons. Particular chromosomes can provide additional clues to Colobinae phylogeny which however should eventually be confirmed by molecular methods (Figure 4). There is no equivalent to HSA1 in any colobine species. In African colobines there are at least two translocations present yielding two derived syntenic associations 1/10 and 1/17: in Asian colobines 1/19 is present. The synteny of the chromosome homologous to HSA 3 is maintained in Asian colobines and the banding pattern is similar to that found in macaques. A derived pericentric inversion distinguishes this chromosome in the genus *Trachypithecus*. *C. guereza* is distinguished by a 3/19 reciprocal translocation.

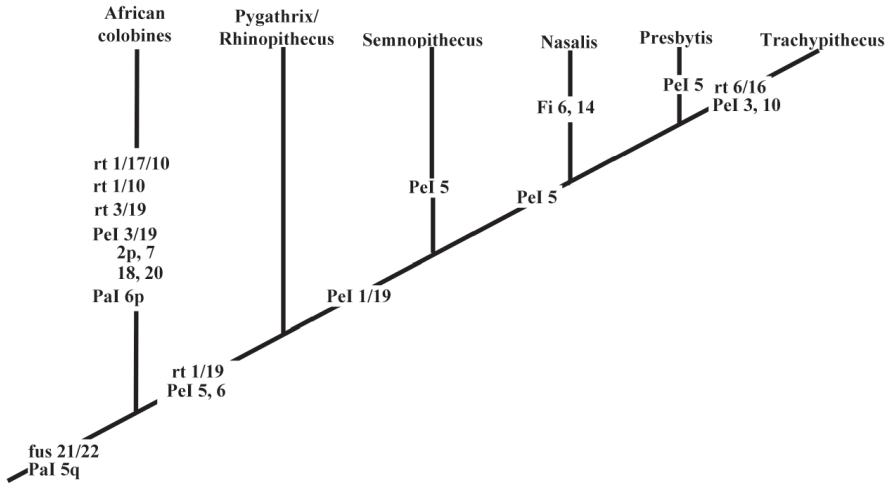

Figure 4 – A working hypothesis of the cytogenetic phylogeny of colobines monkeys based on a combination of chromosome painting and banding (rt=reciprocal translocation, PeI=pericentric inversion, PaI=paracentric inversion, fus=fusion, Fi=fission.

The banding pattern of chromosome 5 appears to provide phylogenetically relevant data. The banding pattern of HSA5 appears to be ancestral for catarrhines. A paracentric inversion on the terminal part of 5q appears to link both Asian and African

colobines. A subsequent pericentric inversion links all Asian colobines. Another pericentric inversion appears to link the genera *Nasalis* and *Trachypithecus*. Another pericentric inversion seems to link *S. entellus* and *S. vetelus*. Providing additional support that *vetulus* does not belong in *Trachypithecus*. Apparently apomorphic inversions derive the homologous chromosome in other Asian colobines.

The homolog to chromosome 10 is involved in a reciprocal translocation in the African colobines. A pericentric inversion derives the banding found in all *Trachypithecus* (but again not in *vetulus*). Different pericentric inversions of chromosomes 12, 18 and 20 appear to distinguish Asian and African colobines. In all Asian colobines, chromosome 19 is reciprocally translocated with chromosome 1, while a reciprocal translocation between 3 and 19 is found in African colobines. Finally, in both Asian and African colobines a derived fusion of 21 and 22 forms the marked (NOR bearing) chromosome.

Many of the hypotheses developed here should be tested with molecular methods. Further use of reciprocal painting in Asian colobines especially in *Trachypithecus* and *Nasalis* and additional African species could be highly informative concerning the phylogeny of leaf-eaters. Such data could help to clarify the exact position of *Nasalis* within the Asian colobines.

Use of subchromosomal probes of decreasing size such YACs, BACs and cosmids would effectively contribute to the study of colobines and help define breakpoints, which may have phylogenetic significance. It would be particularly interesting to have such data on chromosome 5 homologs as this chromosome appears to be rich in phylogenetic information. Finally there are no molecular cytogenetic data on *Rhinopithecus*. Such data could help clarify the phylogenetic position of the snub-nosed langurs and in particular their relationship to *Pygathrix*. Indeed the data presented here are only the beginning of the contribution modern cytogenetics can provide to probe colobine evolution.

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Cytogenetics Studies in the New World Monkeys

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ABSTRACT: *With the introduction of different molecular cytogenetic approaches for analysing genome evolution, the comparison between the karyotypes of primates species has became more precise. These molecular methods together with classical cytogenetics permit us to propose hypotheses on primate phylogeney, especially when species are characterized by a high rate of genomic change during evolution. The phylogenetic arrangements of taxa belonging to the New Word monkeys are still controversial and object of debate. To better define the real contribution of comparative cytogenetics in the clarification of taxonomic and phylogenetic issues in Platyrrhinae we have reviewed the available data on classical and molecular cytogenetics. Data have been discussed in the light of several issues first of all the taxonomic and phylogenetic relationship in between NWM families.*

KEYWORDS: *Platyrrhinae, Phylogeny, Evolution, Comparative Genomics, Chromosome painting.*

INTRODUCTION

Many publications have attempted to better understand the mechanisms of genome evolution, and the phylogenetic relationships between primate taxa. At present, in consideration of their complexity, the evolution of genomes can be efficaciously analysed by comparative molecular cytogenetics. In fact, comparative cytogenetics was previously limited by difficulties in establishing chromosomal homology between species. With the introduction of molecular methods, such as gene mapping and chromosomal painting it became possible to more precisely establish chromosomal homology, and to obtain data on inter-chromosomal rearrangements (translocations). Molecular cytogenetic results can be analysed utilizing cladistic taxonomic approaches such as parsimony, because chromosomal rearrangements are rare events, and convergence is limited. Therefore, common derived (synapomorphic) rearrangements are useful markers to phylogenetically link species (Rokas and Holland, 2000) given that the ancestral (symplesiomorphic) condition is distinguished from derived chromosomal rearrangements by reference to appropriate "outgroup" species.

The most common probes used for *in situ* fluorescence hybridization, in cytogenetic comparisons, are chromosomes paints. They are specific for a single chromosome and hybridize to the entire chromosome or to different chromosome segment in target metaphases of other species.

Chromosome sorting by Flow cytometry (FACS) is the main source of chromosome-specific DNA for the production of painting probes (Ferguson-Smith *et al.,* 2005), but they can also be produced by micro-dissection of chromosomes (Lengauer *et al.,* 1991). It has been also possible to obtain chromosome paints or chromosomes subregions probes from somatic cell hybrids (Muller *et al.*, 1996). Recently cloned DNA probes of different complexity such as yeast artificial chromosome (YAC_s) (Arnold *et al.*, 1966), bacterial artificial chromosomes (BAC_s) and derived artificial chromosomes (PAC_s) were applied to study genome evolution (Cheung *et al.*, 2001).

The use of sub chromosomal probes and reciprocal chromosome painting not only gives direct confirmation of chromosomal homology in two independent experiments, but also provides additional information about sub regional homologies and intra-chromosomes rearrangements. New molecular cytogenetics techniques such as spectral karyotype hybridization (SKY) (Ried *et al.*, 1992) and interspecies comparative genomic hybridization ($_I$CGH) permit in closely related species to easily define chromosomes rearrangements. In consideration that high resolution GTG banding comparisons in primates can be compared with molecular data, a tandem discussion between classical and molecular cytogenetcs data could be a very useful tool to understand the dynamics of New World monkey (NWM) genomes.

It has been already showed that New World and Old world monkeys have a common ancestry (Sineo and Stanyon, 1985; Stanyon *et al.*, 2001) and these data confirms the monophyletic origin of the primates order. However, NWM systematic relationships have been contentious for many decades. Their evolutionary history extends back over more than 30 million years. They constitute of an adaptive radiation, both in morphology and behaviour due to the fact that they evolved in the absence of other competing groups of Primates. (Fleagle, 1988).

Morphological studies among Platyrrhinae have shown that there is a large range of variation in morphological and ethological features between species that makes it sometimes difficult to discriminate homologies (Fleagle, 1988). On the other hand, karyological analysis reveals the presence of sister species with identical morphological feature, but with marked genetic divergence (Stanyon *et al.*, 1995). This situation is reflected in the difficulties to discriminate homologies and in the number of different phylogenetic trees produced when morphological and ethological characteristics are used (Ford, 1986; Kay, 1990; Rosenberger, 1981).

Palaeontology data are too limited and do not permit neither well founded proposals on Platyrrhinae evolution nor the deduction of secure phylogenetic relationship among Platyrrhinae monkeys (Horovitz, 1999; Kay, 1990). Due to the limitation of both palaeontological and morphological data, molecular cytogenetics assumes an important role in the study of the evolution and phylogenetics of these species. Moreover, karyological studies can help clarify the role of chromosomes rearrangements in primate speciation,

NWM TAXONOMY

The New World main families distinguishable by clear morphological characteristics are Cebidae and Callitrichidae, (Atelidae) (Fleagle,1988). Studies on morphological characteristics often placed *Callimico goeldii* in an intermediate position, Callimiconidae, between Cebidae and Callitrichidae families (Hershkovitz, 1977). In fact, *Callimico* resembles tamarins and marmosets in small body size, claws and dental morphology, but, at the same time, it shares single births and the third molar with Cebidae. Further, *Callimico goeldii* may be regarded as a primitive Callitrichidae, and it is proposed that after the divergence of *Callimico*, subsequent lines gave origin to the genera *Sanguinus*, *Leontopithecus*, *Cebuella* and *Callitrix* (Rosenberger, 1981; Ford, 1986).

On the contrary, data based on molecular analysis of chromosome banding and multidirectional chromosome painting provided evidence for a taxonomic and phylogenetic integration of *Callimico* within Callithrichidae (Dutrillaux *et al.*, 1988, Neusser *et al.*, 2001, Schneider *et al.*, 2001).

In the most recent review about platyrrhine taxonomy by Schneider *et al.* (2001), based on DNA sequences, three families were recognized: Cebidae, Pithecidae and Atelidae.

In this study the classical classification of Hershkovitz (1977) has been followed.

CYTOGENETIC DATA

Molecular cytogenetic methods confirmed the hypothesis that Platyrrhinae have a high rates of chromosomal evolution, with diploid number ranging from 2n=16 to 2n=62. Chromosome painting demonstrates that the associations of segments homologous to human chromosomes 5/7, 8/18, 10/16 were derived associations common for all Platyrrhini species analysed up to now. *Cebus* genus seems to have the most conserved karyotype while the owl monkey appears to be karyologically one of the most derived species among Primates. Data on fluorescence in situ hybridization (FISH) are especially useful to clarify taxonomy in taxa, such as New Word Monkeys, that show high rates of chromosome evolution. The three different families have been analysed in the light of literature data.

FAMILY CEBIDAE

Cytogenetic analysis between individuals of different cebidae species showed that many rearrangements involve full chromosomes or full arm homologies (Mudry *et al.*, 1990; Clemente *et al.*, 1987). Literature data show that among Cebidae family there is intraspecific and interspecific variability in number and structure of the chromosomes (Koiffmann *et al.*, 1974; Garcia *et al.*, 1983).

NAME	2N=	METHODS	REFERENCES	DATE
Alouatta caraya,	52	G-banding	Mudry *et al.*	1990
Aotus azarea,	50			
Callithrix jacchus,	46			
Cebus apella,	54			
Saimiri sciureus	44			
Alouatta fusca clamitans,	50	Giemsa	Koiffmann and Saldanha	1974
Aotes trivirgatus,	52			
Cebus albifons,	52			
***Cebus* sp,**	54			
Ateles paniscus paniscus,	34			
Lagothrix lagothrica	62			
Cebus apella,	54	G, C banding	Clemente *et al.*	1987
Cebus albifrons,	54			
Lagothrix lagothricha	62			
Cebus apella,	54	C- banding	Garcia *et al.*	1983
Cebus albifrons,	52			
Lagothrix lagothricha	62			

Table 1 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports on Cebida.

Subfamily Alouattinae

The howler monkeys (genus *Alouatta)* have the most widespread geographic distribution with a range extending from Mexico to Argentina. There is no universal agreement either in the taxonomy or in the phylogenetic arrangements of the taxa within this genus.

Classical cytogenetic studies showed a large variation in chromosome number (from 43 to 54). The howler monkeys (Alouattinae) present two unusual karyological characters: various Y-autosome translocations (Mudry *et al.*, 1998, 2001; Rahn *et al.*, 1996) and microchromosomes (Lima and Seuanez, 1991). The differences in chromosome number at subspecies level, are due to the presence of microchromosomes. It is interesting that some populations considered to belong to the same species are karyologically different. A multiple sex chromosome system is found in most species and it is the result of the translocation between the Y chromosome and one autosomes (Armada *et al.*, 1987; Rahn *et al.*, 1996).

The first work on chromosome painting in New World monkeys was published by Consigliere *et al.* (1996). Molecular Cytogenetic studies permitted these authors to test chromosome homologies proposed in literature on the basis of banding patterns (Stanyon *et al.*, 1995). They studied two taxa *Alouatta seniculus arctoidea* and *A. seniculus sara.* Human specific probe were hybridized on metaphases of the two *Alouatta* subspecies and showed a high variability, much more than expected between subspecies. Human probes did not hybridize the microchromosomes supposedly composed of repetitive DNA. Comparing the hybridization patterns with banding patterns data has been showed that

only some homologies proposed by banding were correct. The chromosomal rearrangements separating the subspecies are two Robertsonian translocations, five tandem translocations, four other translocations and five intra-chromosomal rearrangements. Those rearrangements are sufficient to determine reproductive isolation between the taxa.

Human chromosome paints were also hybridized to metaphases of *Alouatta belzebuth* (Consigliere *et al.*, 1998). It was shown that *Alouatta belzebuth* has a highly rearranged genome, in fact, human homologous are fragmented. Comparing the karyotype of the two red howler and black and red howler monkey it is possible to note that *A. belzebuth* does not share any derived association with the red howlers while the two red howler are linked by seven derived associations. The number of apomorfic associations clearly shows that *A. belzebuth* karyotype is more conserved while the two red howlers are more derived. The syntenic associations 5/11 and 4/15 link the species of this taxa.

Fluorescence *in situ* hybridization with a multidirectional approach was performed employing *human, Saguinus oedipus* and *Lagothrix lagothricha* probe on other howler monkey: *A. fusca, A. caraya, A. seniculus macconnelli* (De Oliveira *et al.*, 2002). Chromosomal homologies detected by this research have been compared with previous data on other Platyrrhini species and have been used to construct a matrix. The matrix has been used for computer-assisted maximum parsimony analysis. With this approach a single parsimonious tree has been constructed where *Alouatta* is a monophyletic clade with two distinct species groups*: A. caraya* and *A. belzebuth* in one group and *A.s. macconnelli, A. sara*, *A.s. arctoidea* and *fusca* in the other.

NAME	2N=	METHODS	REFERENCES	DATE
Alouatta fusca, A. caraya, A. seniculus macconelli	45-49/50, 52	FISH multidirectional painting	de Oliveira *et al.*	2002
Alouatta seniculus arctoidea, A.s. sara	50 45	G banding	Stanyon *et al.*	1995
Alouatta seniculus sara, Alouatta seniculus arctoides	45 male, 50 male/ 44female	FISH	Consigliere *et al.*	1996
Alouatta belzebuth	49/ 50	FISH	Consigliere *et al.*	1998
Alouatta seniculus stramineus	47 48, 49,	G,C, Ag-NOR banding	Lima and Seuanez	1991
Alouatta seniculus, Alouatta seniculus arctoides	45 male, 50 male/ 44female	meiotic Analysis	Mudry *et al.*	2001
Alouatta caraya	52	Synnaptonemal complex and C-B banding	Mudry *et al.*	1998
Alouatta caraya	52	Mitotic karyotype	Rahn *et al.*	1996

Table 2 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports on genus *Alouatta.*

Subfamily Atelinae

Genus Ateles

The spider monkey (*Ateles*) is distributed from central to South America. In this genus the taxonomy is entirely based upon highly variable morphological features. Cytogenetic studies provide additional data to help clarifying *Ateles* evolution and taxonomy. The diploid number in the genus *Ateles* ranges from 32 and 34 (Pieczarka, *et al.*, 1989, Morescalchi *et al.,* 1997). The chromosomes of the Black-handed spider monkey, *Ateles geoffroyi* were studied using human chromosome probes (Morescalchi *et al.,* 1997). *Atles belzebuth hybridus* was also studied with chromosomes paints (Garcia *et al.*, 2002). It is noteworthy that *A. belzebuth* and *A. geoffroy* differ for just one fusion followed by one inversion karyotype. These data were then compared with chromosome painting data from other primates and non-primate mammals. The syntenies 15/14 and 3/21 are considered ancestral to all mammals, while the associations 8/18 and 10/16 are ancestral for all Platyrrhini.

Genus Lagothrix *and* Brachyteles

The first reciprocal chromosome painting between New World Monkey and humans was made between the woolly monkey (*Lagothrix lagotricha*) and humans (Stanyon *et al.* 2001). A translocation between the homolog of the human chromosome 4 and 15 link all Atelines.

Brachyteles (woolly spider monkey) is of the most endangered species among Platyrrhinae; it shares with *Lagothrix* the highest diploid number found among Platyrrhini. From molecular studies and G band analysis *Brachyteles* genus is considered to be the sister group of *Lagothrix* (Schneider *et al.*, 1993; Canavez *et al.*, 1999; Viegas Peguignot *et al.*, 1985). A cladistic reconstruction (De Oliveira *et al.,* 2005), based on the identification of the ancestral chromosomes forms, by chromosomes painting and G banding reveals an evolutionary branching for the following genera: *Alouatta*, *Brachyteles*, *Lagothrix* and *Ateles.* Comparing the karyotype of *Ateles* and *Alouatta* with the ancestral karyotype these authors concluded that no shared derived associations were found. On the other hand, *Brachyteles arachnoides* and *L. lagothricha* have conserved karyotype in regard to the Atelinae ancestral karyotype.

Within *Ateles* genus the evolutionary branching proposed *is:* The evolutionary braching is: *Atles belzebuth marginatus, A. paniscus paniscus, A.b. hybridus* and *A. geoffry.* On the other hand it has been supposed that within *Ateles* taxa *A. paniscus paniscus* is the most derived and probably derives from *A. belzebuth hybridus* (Medeiros *et al.,* 1997).

Subfamily Aotinae

Night monkeys or Owl monkeys (*Aotus*) are nocturnal simians with a geographic distribution from Panama to Northern Argentina. The taxonomy and phylogeny of

the genus Aotus is controversial due to the difficulties in determining bio-morphological homologies. Chromosome polymorphisms are noteworthy and the range of karyotypes in the genus is impressive with chromosome numbers ranging between 46 and 59 (Torres *et al.* 1998, Galbreath, 1983). There were differences in diploid number between males and females due to a Y-autosomal translocation in males (Ma *et al.*,1976, Pieczarka and Nagamachi, 1988). It has been supposed that from the ancestral karyotype (2n=54), all the karyotypes were originated by fusions, fissions, translocations and inversions (de Boer, 1974; Torres *et al.*, 1998; Mudry *et al.*, 1984; Pieczarka *et al.*, 1992, 1993; Ma *et al.*, 1976, 1981, 1985).

NAME	2N=	METHODS	REFERENCES	DATE
Lagothrix lagotricha	62	Reciprocal Painting	Stanyon *et al.*	2001
Brachyteles arachnoides, ***Ateles paniscus paniscus,*** ***Ateles b. marginatus***	62 32 34	Multidirectional painting	De Oliveira *et al.*	2005
Brachyteles arachnoides, ***Cacajao melanocephalus,*** ***Lagotrix lagothrica***	62 45 62	R-banding, BrdU, Q,C banding e NOR staining	Viegas Pequignot *et al.*	1985
Ateles geoffroyi	34	FISH	Morescalchi *et al.*	1997
Ateles paniscus paniscus	32	G, C, NOR-banding	Pieczarka *et al.*	1989
Ateles paniscus chamek	34	Ateles somatic cell-hybrids	Seuanez *et al.*	2001
Ateles paniscus chamek	34	Syntenic association	Canavez *et al.*	1998
Ateles paniscus chamek	34	Ateles somatic cell-hybrids	Canavez *et al.*	1999
Genus *Ateles:* ***A. paniscus paniscus,*** ***A. paniscus chemec,*** ***A. belzebuth hybridus,*** ***A.b. marginatus***	32 34	G, C, NOR-banding	Medeiros *et al.*	1997
Ateles belzebuth hybridus ***Cebus nigrivitatus***	32 52	FISH, G-banding	Garcia *et al.*	2002

Table 3 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports on genera *Ateles*; *Lagothrix* and *Brachyteles.*

On the basis of the variation of pelage coloration, chromosomal features and geographic distribution nine species and four subspecies were proposed. These species belong to two main groups, "the gray-neck group" from the north of the Amazon River and "the red-neck group" from south (Hershkovitz, 1983).

Multidirectional painting was performed between *Aotus nancymae* (Owl monkey) *Chiropotes. s. utahicki, C. israelita* (Sakis monkeys) and humans. It was shown that the

karyotype of the saki monkeys is close to the hypothesized ancestral Platyrrhine karyotype while that of Owl monkeys is more derived. The syntenic association of 10/11 segments found in *Aotus* and *Callicebus* may link these two genera (Stanyon *et al.*, 2004) while an inversion between homologs to human segments 10 and 16 suggests a weak cytogenetic link between *Callicebus* and *Chiropotes* (Stanyon *et al.*, 2004).

NAME	2N=	METHODS	REFERENCES	DATE
Aotus (from Colombia)		R-Q-G-NOR-banding	Torres *et al.*	1998
Aotus trivirgatus, Callitrix jacchus, M. fascicularis	56 46 42	G banding	Chiarelli *et al.*	1985
Aotus trivirgatus (from Peru)	49, 50 female	G e C banding	Pieczarka and Nagamachi	1988
Aotus		Review.	Galbreath	1983
Aotus trivirgatus (from Peru)	46,47,48	G banding	Ma *et al.*	1981
Aotus		chromosome evolution	Ma *et al.*	1981
Aotus (from Northern Argentina)	50 female, 49 male	C, G banding	Mudry *et al.*	1984
Aotus		Chromosome Nomenclature	Reumer and De Boer	1980
Aotus (from Bolivia)	50 female, 49 male	C, Q, G-banding,	Ma *et al.*	1976
Aotus (from Rondonia, brazil)	48	G, C, NOR-banding	Pieczarka *et al.*	1993
Aotus nancymae, A. vociferans	54 46	G, C, NOR-banding	Pieczarka *et al.*,	1992
Aotus			de Boer	1974
Aotus nancymae, Chiropotes utahicki, C. israelita	54 54 54	FISH	Stanyon *et al.*	2004

Table 4 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports involving genus *Aotus*.

Subfamily Pitheciinae

Neotropical primates of the subfamily Pithecinae comprises three genera: *Pithecia* (saki), *Chiropotes* (bearded sakis) and *Cacajao* (uakaris). Little informations about karyotypic features are available from the genus *Chiropotes*. A karyotypic comparisons between the G banding patterns between *Cacajao melanocephalus* (2n=45), *C. rubicundus* and *C. calvus*

showed that they are similar (Koiffmann and Saldanha, 1981). G banding comparison between subspecies of *Chiropotes satanas*: *C.s. utahicki* and *C.s. chiropotes* showed that their karyotype is very similar differing only by a pericentric inversion (Seuanez *et al.*, 1992).

On the other hand, Bonvicino *et al.* (2003b) studied a morphotype comparing it with the two *Chiropotes satanas* subspecies. They considered that morphologic, karyotypic, and molecular differences to be sufficient to elevate *C.s. utahicki* and *C.s. chiropotes* to species status and propose the morphotype as a valid species: *C. israelita.*

NAME	2N=	METHODS	REFERENCES	DATE
Chiropotes satanas utahicki, ***Chiropotes satanas chiropotes***	54 54	G-banging	Seuanez *et al.*	1992
Cacajao melanocephalus	45	G-banging	Koiffmann and Saldanha	1981
Chiropotes utahicki, ***C. isrealita,*** ***Aotus nancymae,*** ***L. lagotrica***	54 54 54 62	FISH e reciprocal painting	Stanyon *et al.*	2004
***Chiropotes* sspp.**	54	G banding	Bonvicino *et al.*	2003b

Table 5 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports involving genera: *Pithecia*, *Chiropotes*, and *Cacajao.*

Subfamily Cebinae

Capuchin monkeys (genus *Cebus*) comprise four different species, *C. apella, C. albifrons, C. capucinus and C. nigrivittatus.* This genus has a very wide geographical distribution, from the south of Mato Grosso, the southwestern region of Goiaz (Brazil) and the southwestern region of Bolivia through Paraguay to the north of Argentina (Mudry, 1990). The Taxonomic arrangement of these species has been analysed by different authors and there are sufficient differences in karyotypes and geographic distribution to propose the presence of subspecies. (Mudry, 1990; Matayoshi *et al.*, 1987; Martinez *et al.*, 1999; Ruiz-Herrera, 2004; Freitas, 1982).

Among Platyrrhini the karyotype of *Cebus* monkeys was considered quite conserved and the species *Cebus capucinus* was the most conserved (Dutrillaux *et al.*, 1986; Dutrillaux and Rumpler, 1980; Dutrillaux, 1988, 1979; Campa and Stanyon, 1992; Garcia *et al.*, 2002). Pericentric inversions have been proposed as the rearrangements that were responsible for the chromosome difference between *C. apella, C. albifrons* and *C. capucinus*. On the other hand, karyological research of different population of the genus *Cebus* has evidenced a more then expected amount of inter and intraspecific variation (Ruiz-Herrera *et al.*,1999, 2004; Martinez *et al.*, 1999; Freitas and Seuanez 1982; Matayoshi *et al.*, 1987; Mudry, 1990; Garcia *et al.*, 1983).

Cebus capucinus, C. apella, and *C. nigrivittatus* have been studied by chromosome painting demonstrating a complete homology between *C. capucinus* and human

chromosomes. *C. capuchinus* and *C. apella* share an identical karyotype while *C. nigrivittatus* has one derived fused chromosome (Richard *et al.*, 1996; Garcia *et al.*, 2000, 2002).

NAME	2N=	METHODS	REFERENCES	DATE
Cebus capucinus	54	G banding	Campa and Stanyon	1992
Cebus capucinus		Q, R,C, NOR banding	Dutrillaux	1979
Cebus capucinus		FISH	Richard *et al.*	1996
Cebus apella	54	FISH	Garcia *et al.*	2000
Cebus apella		C,Q e R banding	Mudry	1990
Cebus apella		G, C, NOR-banding	Freitas and Seuanez	1982
Cebus apella		Q,C e G banding	Matayoshi *et al.*	1987
Cebus nigrivittatus	52	C banding	Ruiz-Herrera *et al.*	1999
Cebus nigrivittatus		C banding	Martinez *et al.*	1996
Cebus nigrivittatus		G,C banding	Ruiz-herrera *et al.*	2004
Ateles belzebuth hybridus, ***Cebus nigrivitatus***	32 52	FISH ,G-banding	Garcia *et al.*	2002
Cebus		Q, R,C, NOR banding	Dutrillaux *et al.*	1986
Cebus capucinus	54	Q, R,C, NOR banding	Dutrillaux and Rumpler	1980
Cebus apella, ***Cebus capucinus,*** ***Lagotthrix lagothricha cana***	54 54 62	G,R, NOR banding	Garcia *et al.*	1983
Cebus		Q, R,C, NOR banding	Dutrillaux	1988

Table 6 – Classical Cytogenetics and Molecular Cytogenetic reports involving genus *Cebus*.

Saimiri sciureus

This genus was divided into four species (Herschkovitz, 1984): *S. sciureus*, *S. boliviensis*, *S. oerstedii* and *S. ustus*. The diploid number was constant for these South America monkey 2n=44, but pericentric inversions produced three geographically distinct karyotypes. Many reports were available in the cytogenetic literature for these species (Moore *et al.*, 1990; Jonas *et al.*).Chromosome painting (Stanyon *et al.*, 2000) was performed using human probe on *Callicebus moloch* and *Saimiri sciureus* chromosomes. The hybridization results show that both species have highly derived karyotypes and confirm that Platyrrhini, along with Hylobatidae, are one of the most karyologically derived group of primates.

NAME	2N=	METHODS	REFERENCES	DATE
Saimiri sciureus	44	Giemsa	Jones *et al.*	
Saimiri sciureus, Callicebus moloch	44 50	FISH	Stanyon *et al.*	2000
Saimiri sciureus	44	NORs e C-banding	Moore *et al.*	1990
Saimiri sciureus	44	Chromosomal characters	Herschkovitz	1984
Callithrix jacchus, Saimiri sciureus, Aotus trivirgatus	46 44	C- T- Q banding	Dutrillaux and Couturier	1981

Table 7 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports involving genus *Saimiri.*

Subfamily Callicebinae

The titi monkeys, genus *Callicebus* are neotropical primates distributed from the tropical forests of the Amazon to the atlantic forest of brazil, and also in Bolivia and Paraguay.

Van Roosmalen *et al.* (2002) in the latest revision of *Callicebus* (Dusky Titi) genus, recognized 28 species (and no subspecies) included in five species groups or clades.

Many publications report on the karyotypes of various species, *Callicebus moloch* (Pieczarka and Nagamachi, 1988), *C. brunneus.* (Minezawa *et al.*, 1989) and *C. torquatus* (Barros *et al.*, 2000).

The species of this genus have different highly derived karyotypes with diploid numbers ranging from 2n=50 (Rodrigues *et al.*, 2001) to 2n=16 (Bonvicino *et al.*, 2003a). *Callicebus lugens* is the specie with the most derived karyotype and the lowest diploid number. Chromosome painting showed that fusions are the predominant rearrangements involved in the genome evolution of *C. lugens (*Bonvicino *et al.*, 2003a; Stanyon, 2003).

Multidirectional chromosome painting was used to analyse the karyotype of *Callicebus cupreus* (2n=46) and *C. pallescens* (2n=50), (Platyrrhinae). The karyotype of *C. pallescens* were already studied in a previous work by Stanyon *et al.* (2000). All the associations already proposed in literature for the ancestral New World Monkey karyotype (Stanyon *et al.*, 2003) were present in the two *Callicebus* species studied. The rearrangements differentiating *C. pallescens* from *C. cupreus* are the result of one inversion, a fission and three fusions (two tandem and one Robertsonian) that occurred on the *C. cupreus* lineage (Dumas *et al.*, 2005)

The results were then compared with other data present in literature on two different species of *Callicebus* genus: *C. donacophilus pallescens* (2n=50) and *C. lugens* (2n=16). This comparison between the two *Callicebus* species showed that *C. cupreus* is more derived while *C. pallescens* is relatively more conserved. A comparison with <<*C. donacophilus pallescens*>> karyotype (Barros *et al.*, 2003) demonstrates that *C. pallescens* described by Dumas *et al.*, (2005), is a different taxon, and two species are presumably

present. This analysis supports the hypothesis of the tendency to the reduction of the chromosomes number in the *Callicebus* group. The most important rearrangements responsible of variation in *Callicebus* have been identified in the fusions even if also fission, inversions, and no robertsonian translocations had a role in this process (Dumas *et al.,* 2005).

NAME	2N=	METHODS	REFERENCES	DATE
Callicebus torquatus	20	G banding, CBG, Ag-NOR	Barros *et al.*	2000
Callicebus lugens	16	G-banding	Bonvicino *et al.*	2003a
Callicebus donacophilus pallescens	50	FISH	Barros *et al.*	2003
Callicebus hoffmannsii	50	C,G,Ag NOR, FISH	Rodrigues *et al.*	2001
Callicebus lugens	16	FISH	Stanyon *et al.*	2003
Callicebus moloch moloch	48	G, C, NOR-banding	Pieczarka and Nagamachi	1988
Callicebus moloch brunneus	48	giemsa, C e G banding	Minezawa *et al.*	1989
Callicebus cupreus, Callicebus pallescens	46 50	FISH	Dumas *et al.*	2005
Callicebus pallescens	50	FISH	Stanyon *et al.*	2000

Table 8 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports *Callicebus* genus.

Family Callitrichidae

Callitrichids (marmosets and tamarins) are small squirrel sized primates, whose morphological characteristics were considered to be either primitive (Hershkovitz 1977) or alternatively very derived (Ford, 1980). Four genera belonging to the South America primates of the family Callitrichidae: *Cebuella*, *Callithrix* (with two group of species, *C. jacchus* and *C. argentata*), *Leontopithecus* and *Saguinus*. Those species are divided in two groups by tooth morphology: the marmosets, *Cebuella* and *Callithrix*, have a modification in the lower anterior dentition and the tamarin, *Leontopithecus* and *Saguinus* have the primitive dentition (Ford, 1986; Hershkovitz, 1977).

There are many reports on the karyotypes of the callitrichidae: Nagamachi *et al.*, 1999; Sineo and Stanyon, 1985; Ardito *et al.*, 1983; Nagamachi and Ferrari, 1984; Pieczarka *et al.*, 1996; Nagamachi *et al.*, 1994, 1996; de Souza Barros *et al.*, 1990; Nagamachi *et al.*, 1997a; Canavez *et al.*, 1996; Margulis *et al.*, 1995; Seuanez *et al.*, 1988; Nagamachi *et al.*, 1990; Nagamachi and Pieczarka, 1988; Bedard *et al.*, 1978;

Dutrillaux and Couturier, 1981. The diploid number of chromosomes ranged from 44 to 46 and the karyotypes are highly conserved and apparently their phyletic radiation has been characterized by a limited number of chromosome rearrangement (Seuanez *et al.*, 1988).

NAME	2N=	METHODS	REFERENCES	DATE
Cebuella pygmaea, ***Callithrix jaccus,*** ***Leontopithecus rosalia***	44 46 44	G banding	Seuanez *et al.*	1998
Saguinus oedipus, ***Saguinus fuscicollis,*** ***Callitrix jaccus***	46	G-C banding , NOR	Bedard *et al.*	1978
Cebuella pygmaea, ***Callithrix jacchus etc.***	44x46 = 45	iCGH, cross-species FISH	Neusser *et al.*	2005
Callithrix jacchus, ***Saimiri sciureus,*** ***Aotus trivirgatus***	46 44	C- T- Q banding	Dutrillaux and Couturier	1981

Table 9 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports involving Callitrichidae.

Marmosets are widely distributed throughout the forested tropical parts of South and Central America.

Nagamachi *et al.* (1999) proposed a chromosomal phylogeny of Callitrichidae analysing rapresentatives from the four genera and using *Cebus* as outgroup. They proposed that the four genera form a monophyletic group and among Callitrichidae, marmosets form a subcalde (*Cebuella* and *Callithrix*) with *Cebuella pygmaea* and C. *argentata* being more related in respect to *C. jacchus*.

Tamarins seems to share a recent common ancestor with marmosets. The genus *Saguinus*, one of the four genera of the family Callitrichidae is the largest and more complex. (Nagamachi *et al.*, 1997b; Nagamachi and Pieczarka, 1988). A comparison of *Saguinus* with *Leontopithecus* shows that they have similar karyotype (2n=46) and that they are distinguished by a paracentric invesion and pericentric inversion on at least four pairs of acrocentrics autosomes (Nagamachi *et al.*, 1997b).

There are four works on the molecular cytogenetics of this taxon on *Callithrix jacchus* (Sherlock *et al.*, 1996) on *Cebuella pygmaea*, *Callithrix argentata, Callithrix jacchus* (Neusser *et al.*, 2005; Serreau-Gerbault *et al.*, 2004), *Leontopithecus chrysomelas,* (Neusser *et al.*, 2005). The associations of human chromosomes that link callitrichidae species are 13/17/20, 13/19/22, 1a/10b and 2a/15b.

Callithrix argentata and *Callithrix pygmaea* share identical chromosomal syntenies while *S. oedipus* and *C. jacchus* differ by single independent translocation. It has been supposed that S. *oedipus* would constitute the most basal clade, *C. goeldii* the sister clade to genus *Callithrix* and *Cebuella* (Neusser *et al.*, 2001).

NAME	2N=	METHODS	REFERENCES	DATA
Leontopithecus chrysomelas	46	FISH	Serreau-Gerbault *et al.*	2004
Leontopithecus rosalia, L. chrysomelas, L. chrysopygus, L. caissara	46	G-C and NOR banding	Nagamachi	1997b
Saguinus midas midas (sub. specie)	46	G-C and NOR banding	Nagamachi *et al.*	1990
Saguinus midas niger (sub.specie)	46	G-C-NOR banding	Nagamachi and Pieczarka	1988

Table 10 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports on Saguinus and Leontopithecus genera.

NAME	2N=	METHODS	REFERENCES	DATA
Callithrichidae		Chromosomal Phylogeny	Nagamachi *et al.*	1999
Callithrix jacchus	46	G banding	Sineo & Stanyon	1985
Callithrix jacchus	46	NOR-banding	Ardito *et al.*	1983
Callithrix jacchus	46	G-banding	Namagachi and Ferrari	1984
C. aurita, C. kuhlii, C. geoffroy, C. penicillata	46	G-C and NOR banding	Namagachi *et al.*	1997a
Callithrix argentata	44	C-banding	Pieczarka *et al.*	1996
Callithrix argentata	44	G-C and NOR banding	Nagamachi *et al.*	1996
Callithrix emiliae	44	C-G-NOR banding	De Souza Barros *et al.*	1990
Callithrix mausei	44	G-C and NOR banding	Nagamachi *et al.*	1994
C. argentata, C. humeralifer, C. emiliae	44	G-C banding	Canavez *et al.*	1996
Callitrix jacchus	46	FISH	Sherlock *et al.*	1996

Table 11 – Classical Cytogenetics and Molecular Comparative Cytogenetic reports on *Callithrix* genus.

Family Callimiconidae

Callimico exist in small, widely dispersed populations that range from southern Colombia to northern Bolivia (Izawa, 1979).

The *Callimico goeldii* karyotype presents a Y-autosome translocation, consequently males may have a chromosomes number of 47 or 48 (Margulis *et al.*, 1995). Multidirectional chromosome painting provided evidence for a taxonomic and phylogenetic integration of *Callimico* within Callithrichidae (Neusser *et al.*, 2001) sharing with Callithrichidae species the human chromosomes association that characterises this family.

NAME	2N=	METHODS	REFERENCES	DATA
Callimico goeldii	48	G/C banding	Margulis *et al.*	1995
Callitrichidae, Callimico goeldii	44-46 47	multicolor FISH	Neusser *et al.*	2001

Table 12 – Classical Cytogenetics and Molecular Cytogenetic reports on Callimiconidae.

Conclusion

The data here analysed let us to arrive at four main conclusions:

1. The chromosome studies indicate a monophyletic origin of the primate radiation in the New World, in spite of the difficulties determine the migratory events. In fact, molecular and classical cytogenetic approaches reveal a high level of chromosomal homology between Old and New Word Primates while a series of derived chromosomal rearrangements link all NWM (Sineo and Stanyon 1985; Stanyon *et al.*, 2001). The NWM radiation presumably arose from an ancestral karyotype characterized by a diploid number of 2n=54. Cytogenetics comparisons among Platyrrhinae karyotypes have permitted researchers to discriminate shared ancestral traits from derived characters by comparison with the proposed ancestral Platyrrhini Karyotype (Stanyon *et al.*, 2000; Neusser *et al.*, 2001). The common derived syntenic association 8/18, 10/16 and 5/7 link all Platyrrhini karyotypes.

2. It has been showed that the ancestral Platyrrhini karyotype is conserved in the *Cebus* genus (Family Cebidae), (Richard *et al.* 1996; Garcia *et al.*, 2000) while some genera such as *Alouatta*, *Aotus* and *Callicebus* are highly derived. For instance the chromosome differences between some *Alouatta* subspecies show that multiple species are hidden in a single taxa; these results indicates that the number of species recognized by the morphological approach could be underestimated.

3. It also been shown that Callitrichidae species have a monophyletic origin. Tamarins and marmosets, in fact, are cytogenetically related; and the derived chromosomes associations linking these species are 13/17/20, 13/19/22, 1a/10b and 2a/15b.

4. From the analyses of the date reported, it is clear that classical cytogenetics together with molecular cytogenetics methods has the potential to formulate new phylogenetics and evolutionary hypotheses and to test other hypothesis based on different approaches. It is important to further investigate the correlation between karyotype and geographic distribution. This aspect has been often deduced but never really ap-

proached by cytogenetics analysis and it would show important implications for the understanding of the speciation process.

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Born and Rise of Human Chromosome 7 Syntenies

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ABSTRACT – *In recent years, the development of molecular techniques like "chromosome painting" that allow the accurate identification of sequence homologies across different taxa, have pointed to strong conservation of syntenies among eutherian mammals. Procedures like locus-specific* in situ *hybridisation can provide far more precise identification of breakpoints and homologous rearrangements than was ever available before, but despite this situation, the evolutionary history of different human autosomes remains obscure. In this contribution we offer a critical review of current understanding of the evolution of human chromosome 7 orthologues in several Eutherian groups. Although the study focuses on primates (including 9 strepsirrhine, 25 platyrrhine and 51 catarrhine species), our analysis includes data on species belonging to 11 Eutherian orders. Two forms of chromosome 7 synteny, referred to as the 7a and 7b forms, were present in the common ancestor of living mammals. These chromosomes underwent lineage-specific rearrangements in the different orders. A particularly complex suite of rearrangements is evident in primates. We propose a model the evolution of these syntenies that accords with the timing of divergences as estimated from the fossil record, based on specific landmarks indicated by classical and molecular cytogenetics. High resolution banded chromosome analysis are also shown to be a valuable tool for the preliminary detection of fine-scale rearrangements, events that can be finely investigated by small genetic probes.*

KEYWORDS: *Human chromosome 7; Primates; Evolution; Eutherian Mammals.*

INTRODUCTION

Human evolution is a central theme in contemporary biology, and palaeontological, achaeological and genetic evidence is all important to the debate. From a zoological point of view humans are encephalised primates with a complex behaviour called "culture". Many of the biological characteristics of modern humans evolved in a common

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ancestor to humans and other primate species. The comparative ethology of primates and of the entire class of Mammalia provides clues to our behavioural phylogeny.

There are more than 200 species of primates currently in existence, comprising two infraorders: the Strepsirrhini (lemurs, lorises and bushbabies), and the Haplorhini (tarsiers, monkeys and apes). The primate order is surprisingly ancient, with roots possibly going back as far as the late Cretaceous (Martin, 1993; Tavaré *et al.,* 2002). As the fossil record is very incomplete, and in some cases absent altogether (as in the Cenozoic of Madagascar), evolutionary reconstruction must rely on a variety of disciplinary approaches, and the interpretation of chromosomal evolution is considered a valuable tool (Novaceck, 1992). The increasing refinement of the results obtained from chromosome banding and FISH techniques, together with the information from the human genome project, have provided deeper insights into the structure of human chromosomes.

FISH technology and comparative gene mapping have been widely applied to comparative studies of the chromosomes of humans and other mammal species. Much effort has gone into charting genome homology between primates from strepsirrhines to humans, and extensive work has also been done in the Perissodatyla Carnivora, Edentata, Artiodactyla, Lagomorpha, Cetacea and Proboscidea. These studies suggest that wide stretches of syntenic homology persist across mammalian orders (Wienberg and Stanyon, 1998; O'Brien *et al.,* 1999; Murphy *et al.*, 2001). Despite this finding, only a small number of mammalian species have been studied in any detail, and large tracts of the mammalian genome remains uncharted.

From a chromosomal point of view, human syntenic associations are clearly conservative; at the same time, the fine structure of many chromosomes remains to be defined, as the comparative analysis of high resolution GTG banding demonstrates (Romagno *et al.*, 2004). This review gathers together all available cytogenetic information concerning the evolution of the syntenic homologues of human chromosome 7 in eutherian taxa, with special attention to primates. We propose a chronology for the cytogenetic landmarks that characterise the evolution of these homologues throughout mammalian history.

Materials and Methods

We include data for representatives of 11 eutherian orders: Chiroptera (Volleth *et al.,* 1999, 2002), Cetacea (Bielec *et al.*, 1998), Artiodactyla (O'Brien *et al.*, 1999; Antoniou *et al.*, 2002; Caetano *et al.*, 1999; Fronicke *et al.*, 1997a; Fronicke and Wienberg, 2001; Schibler *et al.*, 1998; Goureau *et al.*, 1996; Bruch *et al.*, 1996), Tubulidentata, Perissodactyla (Richard *et al.*, 2001; Raudsepp *et al.*, 1996; Caetano *et al.*, 1999), Insectivora (O'Brien *et al.*, 1999; Svartman *et al.*, 2004), Rodentia (Richard *et al.*, 2000; Watanabe *et al.*, 1999; Carver and Stubbs, 1997; Stanyon *et al.*, 2003), Lagomorpha (Korstanje *et al.*, 1999), Edentata (Richard *et al.*, 2000), Carnivora (Nash *et al.*, 1998; Fronicke *et al.*, 1997b; Wienberg *et al.*, 1997; Caetano *et al.*, 1999; Yang *et al.*, 2000; Murphy *et al.*, 2000; Richard *et al.*, 2000; Graphodatsky *et al.*, 2001; Graphodatsky *et al.*, 2002; Cavagna

et al., 2000), Proboscidea (Yang *et al.*, 2000; Yang *et al.*, 2003; Fronicke *et al.*, 2003), and Scandentia (Richard *et al.*, 2000; Muller *et al.*, 1999).

Our primate sample included 9 strepsirrhine species (Cardone *et al.*, 2002; Richard *et al.*, 2000; Muller *et al.*, 1997,1999; Stanyon *et al.* 1987, 2002; Masters *et al.* 1987), and 25 platyrrhines (Neusser *et al.* 2001; Muller *et al.* 2001; Stanyon *et al.*, 2000, 2001, 2003; Garcia *et al.*, 2002; Richard *et al.*, 1996, 2000; Carlà Campa and Stanyon, 1992; Consigliere *et al.*, 1996; Consigliere *et al.*, 1998; Stanyon *et al.*, 2001; Seuanez *et al.*, 2001; Morescalchi *et al.*, 1997; Stanyon *et al.*, 2003). The Catarrhini were represented by 20 species of Papionini (Stanyon *et al.* 1988; Wienberg *et al.* 1992; Ruiz-Herrera *et al.* 2002a, 2002b; Muller and Wienberg, 2001), 8 species of Colobinae (Nie *et al.*, 1998; Stanyon *et al.*, 1992; Ponsà *et al.*, 1983; Kingsley *et al.*, 1997), 13 species of Cercopithecini (Finelli *et al.*, 1999; Sineo *et al.*, 1986; Richard *et al.*, 2000; Sineo, 1986; Clemente *et al.*, 1990; Ponsà *et al.*, 1981; O'Brien, 1993; O'Brien *et al.*, 1993; Stanyon and Sineo, 1983; Romagno *et al.*, 2004),10 species of Hylobatidae (Nie *et al.*, 2001; Jauch *et al.,* 1992; Stanyon *et al.*, 1987; DeSilva *et al.*, 1999; Koehler *et al.*, 1995a, 1995b; Muller and Wienberg, 2001), and three species of great apes (Pongidae, Hominidae).

Results

Eutherian mammals

In most non-primate species, with the exception of rat and mouse (Watanabe *et al.*, 1999; Carver and Stubb, 1997), the synteny of human chromosome 7 and the homologous sequences are distributed on two chromosomes. In at least one species for every mammalian order, there is a large segment homologous to most of HAS 7 as well as a small segment associated with HSA 16 sequences. The small segment of HAS 7 has proved difficult to detect, and sometimes goes unnoticed in chromosome painting. This segment was overlooked in the initial painting studies in cat (Wienberg *et al.*, 1997), pig (Goureau *et al.*, 1996) and horse (Raudsepp *et al.*, 1996). It is likely that in many of the reports the chromosome painting signal for this small segment has escaped detection: e.g., *Rhinolopus mehely* (Chiroptera), *Sores araneus* (Insectivora), *Ailuropoda melanoleuca*, *Tremarctos ornatus*, *Phoca vitulina*, *Mustelia putorius furo* (Carnivora), and *Tupaia belangieri* (Scandentia).

Analysis of reciprocal painting as well as gene and genomic maps demonstrates that, in general, this small fragment contains sequences found in chromosome bands HSA7p22, 7q11.2, 7q21.11 and 7q22. The sequences of HSA16 with which this segment is frequently associated derive from 16p. By definition, in humans, sequences belonging to bands 7q11.2, 7q21 and 7q22 are associated with the sequences of the larger segment of HSA7 to form a single syntenic chromosome. However, new data on BAC hybridisation and in silicio study comparing HSA7 and orthologous sequences in other primates (Muller *et al.*, 2004) indicate that in some species this synteny is secondarily fragmented, and may be associated with different human chromosomal

syntenies. For example, a number of intra-chromosomal rearrangements has been demonstrated in cattle (Antoniou *et al.*, 2002) and in the cat (Murphy *et al.*, 2000).

Primates: Stepsirrhini

Strepsirrhine painting data are available for two species of Galagidae and three species of Lemuridae. In *Otolemur crassicaudatus* there is possible evidence of the ancestral eutherian association HSA7/HSA16. In this species, in fact, most of the part homologous to HSA7 forms an acrocentric chromosome (OCR 6), similar in banding to PPY10q, whereas a small segment of HSA7 and part of HSA 16 are found together on another chromosome (OCR7) where they are separated by a segment homologous to HSA 12 (Stanyon *et al.*, 2002). The associations along OCR7 (HSA16/HSA12/HSA7/HSA12) make it a plausible hypothesis that an inversion disrupted the ancestral HSA7/HSA16 association, after a translocation with a segment of chromosome HSA12. Apparently, the HSA16 sequences on OCR7 derive from the 16p. The same situation may pertain in *Galago moholi* even if HSA7 sequences have only been detected in a single chromosome. In fact, Stanyon *et al.* (2002) suggest that the signal of the small segment of HSA7 associated with HSA16 may have escaped detection. A similar banding pattern in *Otolemur garnetti* (Masters *et al.*, 1987) and in *Nycticebus coucang* (Stanyon *et al.*, 1987) makes it reasonable to hypothesise that the 7/16 association will eventually be found in these species.

In Lemuridae, homologues to HSA7 constitute either an acrocentric chromosome (*Eulemur fulvus mayottensis*, *Lemur catta*), or an arm of a metacentric chromosome (*Eulemur macaco*). These chromosomes all have a banding pattern similar to PPY10 (Muller *et al.*, 1997, 1999; Cardone *et al.*, 2002). The remaining HSA7 sequences comprise a microchromosome in *Eulemur fulvus mayottensis* and *Eulemur macaco* (Muller *et al.*, 1997, 1999), and possibly on the same chromosome in *E. macaco*, Richard *et al.* (2000) detected the presence of another unspecified human synteny. The small signal relative to HSA7 found in EFM and EMA may have gone undetected by Cardone *et al.* (2002) in *Lemur catta,* since they did not obtain signals for HSA2 and HSA4 paints, and found no signals for some chromosomal regions and some micro-chromosomes. A small acrocentric in this species could be homologous to the small fragment of the HSA7 synteny associated with HSA16 sequences in eutherian mammals.

Primates: Platyrrhini

In almost all New World primates analysed by chromosome painting, most of HSA7 is represented by a single acrocentric chromosome with a PPY10q like banding pattern or, in Callithrichinae and *Saimiri*, a submetacentric chromosome. Further, in some species it constitutes a large chromosomal segment or a whole arm, associated with different syntenies (Consigliere *et al.*, 1996; Stanyon *et al.*, 2000). The remaining, smaller part of HSA7 is associated with HSA5 sequences (Richard *et al.*, 2000). A

single hybridisation signal has been detected in only three of over twenty analysed species (two in Alouattinae, Consigliere *et al.*, 1996; one in Callicebinae, Stanyon *et al.*, 2000); however, we cannot exclude the possibility in these taxa that the signal of the small part of HSA7 is actually associated with HSA5 sequences, but has escaped detection.

Saguinus oedipus WCP probes tested on Alouattinae genomes (de Oliveira, 2002) and *Lagothrix lagothricha* whole chromosome probes tested on *Callimico goeldii* genomes (Neusser *et al.*, 2001; Stanyon *et al.*, 2001) demonstrated the presence of segments homologous to HSA7p22/7q11.2-7q21. The mapping of the GUSB gene on *Cebus capucinus* chromosomes (O'Brien *et al.*, 1993) demonstrated the presence of HSA7p22, 7q11.2 and 7q21 bands in association with HSA5 sequences. The mapping of the PGP gene (HSA16p) on chromosome 16 in *Cebus capucinus* (O'Brien *et al.*, 1993) could be a symptom of an ancestral mammalian 7-16 syntenic retention not detected by chromosome painting. More potential evidence of this ancestral association derives from *Callicebus lugens,* where chromosome painting has revealed the alignment on the same chromosome of HSA5, HSA7 and HSA16 sequences (Stanyon *et al.*, 2003). However, the HSA7/HSA16 association is more likely to be a derived trait because this chromosome has several human syntenies and the HSA16 synteny is highly disrupted.

Primates: Catarrhini

In all Papionini, a karyologically conservative group, the syntenic association HSA7/HSA21 forms chromosome number 2 (Stanyon *et al.*, 1988; Wienberg *et al.*, 1992; Morescalchi *et al.,* 1997; Ruiz-Herrera *et al.*, 2002a, 2002b; Muller and Wienberg, 2001). The HSA7 homologous segment has a banding pattern very similar to PPY10.

In Cercopithecini, marked by high karyotypic variability, painting data are limited, and many species have been studied only using banding techniques. In *Chlorocebus aethiops* HSA7 sequences comprise the large acrocentric chromosome 21, with banding similar to PPY10q, and the small acrocentric chromosome 28 with banding similar to PPY10p. Reciprocal painting on HSA chromosomes has demonstrated the presence of HSA7p21-cen, 7q21, 7q31-qter sequences on CAE 21, and 7p22, 7q11.2, 7q22 on CAE 28 (Finelli *et al.*, 1999). Williams-Beuren locus mapping confirmed the presence of 7q11.23 sequences on chromosome CAE 28 (Romagno *et al.*, 2004). Other gene mapping data, however, reveal that sequences of HSA7q21.11 and of HSA7q22 are also present, respectively, on CAE 28 and CAE 21 (O'Brien, 1993). Reciprocal painting data have shown a very similar situation for chromosomes 21 and 25 in *Erythrocebus patas* (Stanyon, personal communication); in this case, however, HSA7q22 sequences are present on both chromosomes EPA25 and EPA21. In *Cercopithecus l'hoesti* the banding analysis shows two chromosomes that are similar to CAE 21 (or EPA21) and CAE 28 or (EPA 25). These data indicate a period of shared ancestry for these three species, as was previously hypothesised on the basis of R-banding (Dutrillaux *et al.*, 1982) and molecular data (Tosi *et al.*, 2003).

Chromosome painting in *Cercopithecus diana* shows a single signal on a submetacentric chromosome (Richard *et al.*, 2000) with banding identical to PPY10. The same situation, albeit without molecular validation, can be hypothesised for *Allenopithecus nigroviridis*, the guenon species thought to be closest to the ancestral cercopithecine stock on the basis of karyological (Dutrillaux *et al.* 1980), morphological and molecular evidence (Strasser and Delson, 1987; Tosi *et al.*, 2003). In *Cercopithecus neglectus* HSA7 synteny is conserved within a single submetacentric chromosome (Stanyon, personal communication) with a banding pattern that is surprisingly similar to the homologue in *Gorilla*. A chromosome with a very similar banding pattern is also present in *C. mona*, *C. cephus, C. ascanius*, *C. petaurista, C. nictitans* and *C. mitis* (Sineo, 1986). As these species are grouped together in phylogenetic reconstructions based on R-banding (Dutrillaux *et al.*, 1982; Clemente *et al.*, 1990) and molecular data (Tosi *et al.*, 2003), it is possible that they share a common pericentric inversion in an ancestral element that is homologous to HSA7, and similar to that of *Allenopithecus nigroviridis* (ANI) and *C. diana* (CDI). The same inversion has probably occurred in the lineage leading to African great apes and humans, in which case it would be an example of convergent evolution, at least at the light microscope level of resolution.

In the other species considered, it was not possible identify a clear banding homology. Colobine monkeys (*Trachypithecus*, *Colobus*, *Nasalis* and *Semnopithecus*) analysed using the painting approach show HSA7 synteny in a single chromosome, which is similar in banding pattern among the species (Nie *et al.*, 1998), but different from any HSA7 homologue of the other cercopithecid species. William-Beuren locus and subtelomeric HSA7p probe mapping in *Trachypithecus cristatus* and other primates (Romagno *et al.*, 2004; Kingsley *et al.*, 1997) reveal the presence of complex intrachromosomal rearrangements (Romagno *et al.*, 2004); in *Trachypithecus*, *Colobus* and *Semnopithecus*, a p-terminal area without any hybridisation signal was reported (Nie *et al.*, 1998).

Among the lesser apes, only *Hylobates lar* presents a single signal for HSA7 synteny, on the q-arm and the proximal part of the p-arm of a large metacentric chromosome (Jauch *et al.*, 1992). The banding pattern of this region is identical to PPY10. The WS probe maps in the pericentromeric region of the p-arm indicate that there are sequences of HSA7q11.23 in this area (De Silva *et al.*, 1999). The other gibbon species with 2n = 44 show identical banding patterns and probably the same hybridisation pattern. In *H. hoolock* most of the HSA7 synteny is associated with other human syntenies in a submetacentric chromosome (Nie *et al.*, 2001) and the banding pattern is similar to PPY10q. The small remaining part has been translocated onto another chromosome. In *H. concolor* and *H. syndactylus* HSA7 synteny was variously fragmented into three segments.

Once the synteny of human chromosome 7 (HSA7) was established as a chromosome similar to the *Pongo pygmaeus* chromosome 10, it was then subject to a pericentric and subsequently a paracentric inversion in the lineage leading to *H. sapiens*. Specifically, high resolution chromosome analysis indicates that chromosome 7 in *Homo* and *Pan* differ from the orthologue in *Gorilla* by a paracentric inversion, which in turn

differs from the ancestral form by a pericentric inversion. The gorilla chromosome therefore represents an intermediate stage in the evolution of hominid karyotypes (Yunish and Prakash, 1982).

Muller and colleagues (2004) recently demonstrated that pericentric and paracentric inversions characteristic of the hominoid lineage occurred in 7p22.1 and 7q22.1 breakpoints (respectively at 6.8 Mb and at 97.1 Mb on the reference sequence map) and 7q11.23 and 7q22.1 (respectively at 76.1-76.3 Mb and 101.9 Mb on the reference sequence map). The analysis drew attention to the importance of fine BAC mapping and, more importantly, revealed the presence of "large segmetal duplications" of low copy repeats (LCRs) flanking these hot spots. Segmental duplications have been described in association with several important rearrangements in primates (Samonte and Eichler, 2002), and a causative role in such rearrangements has been proposed.

While chromosome painting showed chromosome 7 synteny to be highly conserved in higher primates, banding comparison BAC mapping and single locus mapping (e.g., Williams-Beuren syndrome *locus; WS*-HSA7q11.23*)* among Hominoidea and Cercopithecoidea have indicated the occurrence of significant rearrangements (i.e., pericentric and paracentric inversions) that remained undetected using whole chromosome paints (Romagno *et al.*, 2004).

Discussion

An analysis of the data presented above allows us to hypothesise the presence, in the ancestral eutherian karyotype, of an acrocentric chromosome containing most of HSA7 (Richard *et al.*, 2000; Murphy *et al.*, 2001). A small, probably submetacentric, chromosome would contain the rest of HSA7 associated with HSA16p sequences. The latter chromosome presumably contained sequences related to chromosome bands HSA7p22, 7q11.2, 7q21.11 and 7q22.

The presence in Strepsirrhines of a large (7a) and a small (7b) acrocentric chromosome containing only HSA7 sequences (Muller *et al.*, 1997, 1999) could indicate that the ancestral HSA7/HSA16 association was disrupted very early during primate evolution. Muller *et al.* (1999) proposed, from painting data for *Tupaia belangieri,* the presence of a single submetacentric ancestral chromosome, while Richard *et al.* (2000) proposed two chromosomes in the ancestral primate that were homologous to segments of HSA7: a large and a small acrocentric chromosome. The small acrocentric element would probably have contained the sequences that are associated with HSA16p, in the ancestral eutherian karyotype. This hypothesis is based, first, on the presence of a large and a small acrocentric element entirely composed of HSA7 sequences in Strepsirrhini, and, second, on the detection of two signals produced by HSA7 probes in *Tupaia chinensis*, with the smaller acrocentric associated with HSA16 sequences. Since Toder *et al.* (1992) have affirmed that the banding patterns of *Tupaia belangieri* and *T. chinensis* chromosomes are identical, it is likely that the small signal concerning HSA7 sequences in *Tupaia belangieri* escaped detection by Muller *et al.* (2001).

The situation becomes even more complex if we consider recent data for species of Galagidae (Stanyon *et al.*, 2002). These data introduce the possibility that the small chromosome with HSA7/HSA16 association was present in the ancestral primate karyotype. This hypothesis implies two independent fission events which disrupted the HSA7/HSA16 association, one in the Malagasy lemurs, and one in the lineage leading to simians, as the association has not been found to date in any simian species. Reciprocal painting, and subchromosomal probe and mapping data on *Otolemur* and *Galago*, together with painting analysis on a larger number of Strepsirrhine species, will be necessary to elucidate the situation definitively.

In the ancestral karyotype of New World monkeys we can hypothesise the presence of an acrocentric chromosome with most of the HSA7 synteny, similar to that of the ancestral Strepsirrhine karyotype, and of a submetacentric chromosome with the remaining part of HSA7 associated with the HSA5 synteny, as a result of a translocation (Richard *et al.*, 2000; Neusser *et al.*, 2001; Stanyon *et al.*, 2003). This new syntenic association, which in some species comprises only a part of the human 5, secondarily underwent different translocations in various species. An inversion involving the HSA5/HSA7 in a common lineage leading to Atelinae and Alouattinae, or independlently in the two subfamilies, could have led to the alignment HSA7/5/7 observed in most of the Alouattinae an in all Atelinae species Reciprocal painting (Neusser *et al.*, 2001) and gene mapping (O'Brien *et al.*, 1993) demonstrated the involvement of HSA7p22, 7q11.2 and 7q21 bands in the association with HSA5 sequences; this could confirm the homology with the HSA7 segment associated with HSA16 sequences in the ancestral eutherian karyotype.

In the ancestral karyotype of Old World monkeys, HSA7 sequences constitute a single chromosome which was probably similar to the Pongo PPY10. This element could have been derived from the fusion of two ancestral primate chromosomes similar to those proposed by Richard *et al.* (2000). During the evolution of Cercopithecini, after the divergence of *A. nigroviridis* and *C. diana*, the ancestral chromosome underwent a pericentric inversion in the lineage leading to the other arboreal species, resulting in the formation of a chromosome similar to the HSA7 homologue in *Gorilla*, and a fission in the lineage leading to the terrestrial species. Further, even if the probability of back mutations at recurrent breakpoints is very low, we have to consider that this last rearrangement could have restored the ancestral primate condition in these species.

A very similar submetacentric chromosome, containing the whole HSA7 synteny, is present in all Colobinae studied, indicating that this is the ancestral condition for this subfamily. The chromosome has a peculiar banding pattern, possibly derived from the ancestral Catarrhine form by a pericentric inversion and other complex intrachromosomal rearrangements. In Papionini, the ancestral HSA7 chromosome has undergone a translocation to form an association with the homologue to HSA21.

In Hylobatidae the ancestral HSA7 chromosome has probably survived unchanged with respect to the ancestral Old World monkey condition. It underwent a translocation with a chromosomal segment containing HSA2 sequences in the *Hylobates lar* group, while in other species it fragmented into two (*H. hoolock*) or three segments (*H.*

syndactylus and *H. concolor*) which subsequently became associated with different human syntenies in different species. A chromosome similar to PPY10, and therefore similar to the ancestral catarrhine HSA7 chromosome, was probably present in the ancestral karyotype of the Hominoidea. This chromosome was involved in a pericentric inversion in the common lineage leading to *Gorilla*, *Pan* and *Homo* and a paracentric inversion in the lineage leading to *Pan* and *Homo*.

Chromosome 7 evolution and synteny dynamics

Even though reports of reciprocal painting and gene mapping in primates are few, all the data currently available lead us to hypothesise that the p arm of the ancestral chromosome in Catarrhini, the associated HSA7/HSA5 sequences in Platyrrhini, and the small chromosome constituted by HSA7 sequences present in the common ancestor of the Strepsirrhini, are homologous. Further, this segment, associated with HSA16p homologous segments, characterised the common eutherian ancestral genome. The most consistent weakness of this hypothesis is the lack of data for HSA7q22 sequences in the small segment homologous to HSA7 that is associated with HSA5 in Platyrrhini. Because there are frequent rearrangements in New World monkeys which involve this region, the limitations and incongruities of the available data must be considered.

In chromosomal evolution the recurrence of rearrangements at breakpoints that appear to be localised in the same regions are not rare, and chromosome 7 is not exceptional. In primates, repeated breaks at the same site could be explained by the presence of fragile sites in specific areas. For example, both the HSA7p22 band and its homologues in PTR, GGO and MFA, and HSA7q11.2 and the homologous bands in GGO and PPY (Smeets *et al.*, 1990) contain fragile sites. Duplicated segments may also promote chromosome rearrangements, and in humans and great apes there is a duplicated region in HSA7q22 (DeSilva *et al.*, 1999; Muller *et al.*, 2004). Improvements in analytical technology will shed light on these issues. As Muller *et al.* (2004) demonstrated by fine BAC mapping, "homologous" breakpoints may have a different localisation at a molecular level of resolution.

In general, the data on the evolution of chromosome 7 indicate that the conservation of syntenies in mammals is important from a functional point of view (Murphy *et al.*, 2001; Novelli *et al.*, 1999). It could be hypothesised that gene clusters (or groups of genes) share regulatory elements acting at the local level. As gene order inside conservative syntenies is often not the same, it is possible that the same regulatory elements determine a different pattern of gene expression and consequent phenotypic diversity in various species in the face of syntenic conservation.

A chronology of the events in the evolution of the HSA7 synteny can be hypothesised on the basis of fossil evidence and the chromosome constitutions of extant species (Fig. 1).

- A 7 (a) and a 7(b)+16p association characterised the ancestral mammalian chromosome in the Cretaceous. The 7-16 fission originated among the early primates dur-

ing the Eocene, even if a 7-16 association was either maintained or re-evolved in the extant Strepsirrhines.

• The 7a-7b fusion that characterised further primate evolution presumably resulted from the rise of simian (anthropoid) primates, perhaps in the Oligocene. The divergence between Platyrrhini and Catarrhini has been estimated at around 40 Ma (Ciochon and Chiarelli, 1980). This divergence is chromosomally marked by a 7b-5 translocation in New World primates and by the 7a-7b fusion in African monkeys. This fusion signals the origin of the human chromosome 7 synteny that remained unchanged since the Dryopithecine radiation (Pilbeam and Young, 2001), and is present in *Pongo* as an ancestral chromosome 10 homologous to HSA7.

• Human chromosome 7 evolved recently via a pericentric inversion in the African ape lineage, and a paracentric inversion prior to the *Homo*/*Pan* divergence. These rearrangements probably occurred (Sineo *et al.*, 2000) within the last 10-7 Ma ago.

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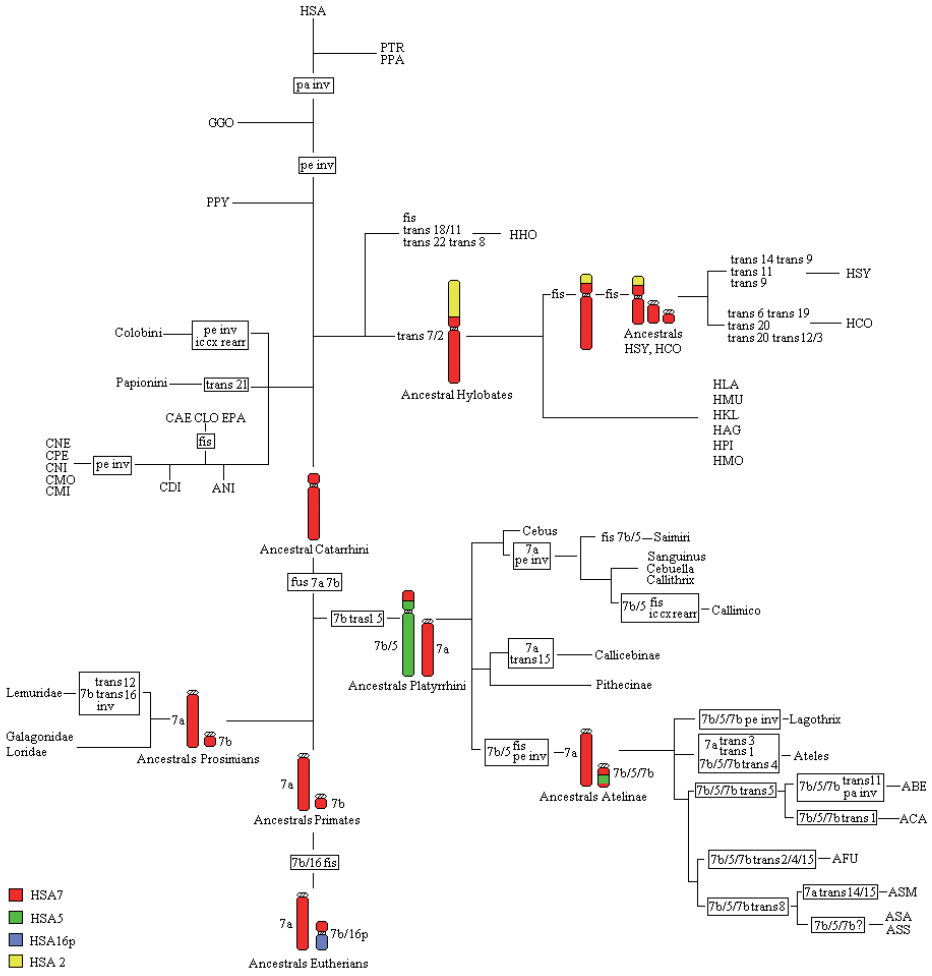

Figure 1 – The proposed sequence of the rearrangements occurred during Primate evolution. The tree starts from the presumed ancestral Eutherian chromosomes.

Legend: fis=fission, trans=translocation, pe inv=pericentric inversion, pa inv=paracentric inversion, ic cx rear=complex intra-chromosomal rearrangement.
ASS=*Alouatta seniculus sara*, ASA=*Alouatta seniculus arctoidea*, ASM=*Alouatta seniculus macconelli*, ABE=*Alouatta belzebul*, CNE=*Cercopithecus neglectus*, CMO=*Cercopithecus mona*, CPE=*Cercopithecus petaurista*, CNI=*Cercopithecus nictitans*, CMI=*Cercopithecus mitis*, CAE=*Chlorocebus aethiops*, CLO=*Cercopithecus l'hoesti*, EPA=*Erythrocebus patas*, HSY=*Hylobates symphalangus*, HCO=*Hylobates concolor*, HHO=*Hylobates hoolock*, HAG=*Hylobates agilis*, HKL=*Hylobates klossi*, HLA=*Hylobates lar*, HMO=*Hylobates molock*, HMU=*Hylobates muelleri*, HPI=*Hylobates pileatus*, HCO=*Hylobates concolor*, PPY=*Pongo pygmaeus*, GGO=*Gorilla gorilla*, PTR=*Pan troglodytes*, PPA=*Pan paniscus*, HAS= *Homo sapiens.*

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Chromosomal Territories in Evolutionary Rearranged Primate Chromosomes

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ABSTRACT: *Interphase chromosomes form distinct spatial domains called chromosome territories (CT). Different results show that the position of CTs in fibroblasts is related to chromosome size. In order to elucidate if the nuclear radial position of CTs is dependent on a "phylogenetic memory" of the ancestral position or it is conditioned to the relative chromosome size, we have analysed the CT positioning in* Lagothrix lagothricha *(LLA) fibroblasts of a large and a middle-sized chromosome (LLA6 and 11), resulting from different ancestral chromosome rearrangements, and their homologous chromosomes (HSA8, 18, 5 and 7) in human fibroblasts by three-dimensional fluorescence* in situ *hybridisation (3D-FISH). We have also compared the results obtained in each species to know if the cell lines studied have this size-dependent CT distribution. Our results indicate that the CT positioning in human fibroblasts is related to the chromosome size, while more LLA chromosomes must be analysed to confirm this hypothesis for this species, and that the radial distribution of a rearranged CT is also dependent on its chromosome size and not on its "phylogenetic memory".*

KEYWORDS: *Chromosome territories, evolutive cytogenetics, primates, 3D-FISH.*

1. INTRODUCTION

It is well known that genomes are arranged in the form of chromosomes during cell division and now it is accepted that the physical separation of genetic material also occurs throughout the entire cell cycle. In interphase, each chromosome occupies a distinct, spatially well-defined nuclear compartment called "chromosome territory" (CT). The arrangement of CTs in the interphase nucleus is non-random (Cremer and Cremer, 2001).

According to Croft *et al.* (1999), gene-dense CTs in human lymphocytes are located towards the centre of the nucleus, while gene-poor CTs are located in the nuclear periphery. This gene-density-dependent radial arrangement has also been reported in lymphoblastoid cell lines from different primate species. Tanabe *et al.* (2002) studied radial positioning of HSA18 and 19 in seven higher-primate species, finding a more peripheral position of gene-poor chromosome HSA18 when compared with gene-rich HSA19, indicating that this gene-density-dependent arrangement is evolutionarily

conserved in lymphoblastoid cell lines. However, chromosome gene-density appears not to be the only factor determinant of nuclear radial positioning. Chromosome size has also been correlated with radial positioning. In fibroblasts, several authors have given evidence that small chromosomes are positioned in the nuclear interior while larger chromosomes are in the periphery (in human, Sun *et al.*, 2000; Cremer *et al.*, 2001; in chicken, Habermann *et al.*, 2001; and in marsupial kidney cells, Rens *et al.*, 2003). Previous data from our laboratory (paper in preparation) show that phylogenetically conserved chromosomes in a single synthenic fragment conserve their radial distribution when fibroblast cell lines from different primate species are compared.

The aim of this work is to analyse whether the nuclear radial position of CTs is dependent on a "phylogenetic memory" of the ancestral position, or if it is conditioned to the relative chromosome size. For this purpose the CT positioning of a large and a middle-sized LLA chromosome resulting from different ancestral chromosome rearrangements and their homologous chromosomes in HSA has been analysed herein.

2. Materials and Methods

2.1. Cell lines and slide preparation

Human (HSA) dermal fibroblast and woolly monkey (*Lagothrix lagothricha*, Cebidae. LLA) skin fibroblast cell lines (Repository Number AG05356 from Coriell Institute for Medical Research), both cell types with a normal karyotype, were cultured on glass sterile slides previously treated with poly-L-lysine until confluency. Prior to fixation with 4% paraformaldehyde in PBS, cells were incubated in 1x PBS for 5 min in order to prevent shrinkage of the nucleus. Once fixed, cells were not allowed to dry out. Permeabilisation steps included treatments at room temperature with 1x Saponin-0.1% Triton X-100 (15 min for HSA and 10 min for LLA), HCl 0.1N-0.02% Triton X-100 (10 min for HSA and 15 min for LLA) and 10 min with Tris 0.1N (pH=7.8). Slides were incubated in 20% glycerol-PBS for 30-60 min at room temperature and then subjected to four repeated freeze-thaw cycles in N_2 liquid, soaking the slides in glycerol before each freezing. After two 5-min PBS washes, cells were treated with 0.002% pepsin-0.9N NaCl (pH=1.5) for 6-7 min at 37°C. After digestion with pepsin, cells were post-fixed with 1% PFA-PBS for 10 min and washed with PBS (3 x 5 min). Slides were kept in 50% formamide-2x SSC for 3-5 hours at room temperature, or at 4°C for approximately one month.

2.2. FISH on metaphase plates (2D-FISH)

In order to know which human chromosomes are homologous to *Lagothrix lagothricha* chromosomes 6 and 11 (chromosome numeration according to Clemente *et al.*, 1987), whole-chromosome probes homologous to LLA6 and LLA11 were hybridised on human metaphase plates. Both probes (kindly provided by Dr Stanyon) were generated by degenerated oligonucleotide primer PCR (DOP-PCR) from flow-sorted chromosomes (Stanyon *et al.*, 2001). Whole-chromosome probes were mixed

with salmon-sperm DNA and 3M sodium acetate, and the mixes were precipitated as described in Ruiz-Herrera *et al.* (2005).

2.3. Fluorescence in situ *hybridisation on 3D preserved nuclei (3D-FISH)*

Whole-chromosome probes homologous to HSA5, HSA7, HSA8, HSA18, LLA6 and LLA11 (all provided by R. Stanyon) were generated by DOP-PCR from flow-sorted chromosomes. Probe mix preparation and precipitation were performed as described above with the exception that we competitor DNA (Cot-1 human DNA) was added to human probe mixes. After precipitation, the probes were resuspended in half of the volume of hybridisation buffer in order to increase their concentration. Slides were denatured in 70% formamide-2x SSC at 73°C for 5 min. 3D-FISH was performed in a moist chamber at 37°C over 2-3 days and the slides were washed 10 min in 50% formamide-2x SSC at 43°C, and an additional three times in 2x SSC at 43°C. Detection of biotin-labelled probes (HSA5, LLA6 and LLA11) was performed using streptavidin-Alexa 488 (Molecular Probes) or avidin-FITC (Cambio), while detection of digoxigenin-labelled probes (HSA7, HSA8 and HSA18) was carried out by using anti-digoxigenin-FITC (Roche). To counterstain the nuclei, TO-PRO 3 or TOTO 3 (Molecular Probes) were used.

2.4. Confocal laser-scanning microscopy and position measurements

For each visual area at 63x, sections with an axial distance of 1?m were recorded using a confocal laser-scanning microscope (Leica TCS 4D). All measurements were performed using MetaMorph Imaging System 5.0 (Universal Imaging) at maximum projections of all sections. The perimeter of each nucleus as well as each chromosome signal were determined by local thresholding and the geometrical centre automatically determined by MetaMorph software. Distances from the centre of each nucleus to the centre of each signal were obtained and, in order to compare nuclei with different volumes and to correct the elliptic shape, radial distances were normalised to the local radius (the radius from the centre of the nuclei to the edge going through the centre of the analysed FISH signal). In nuclei with only one signal surrounded by nuclei with two signals, it was considered that both CTs are clustered and the data were counted twice. Roughly 100 nuclei were analysed for each hybridisation. Once radial distances were measured, the frequency of signals in intervals of 20% of the nuclear radius was calculated.

2.5. Chromosome size calculating

To calculate the size of each chromosome in each species in relation to the size of the other chromosomes within the same karyotype, the length of all chromosomes in 5 different metaphases of each species was measured. Because sizes are different depending on the state of compaction of the metaphase, chromosome sizes of each metaphase were normalized, converting the absolute values into a percentage of the total length of the karyotype, and the average relative size of each chromosome of all metaphases was

calculated. Chromosomes from the smallest to the largest and the order position of the chromosomes was divided by the total number of chromosome pairs in the karyotype in order to obtain a comparable value of each chromosome size in relation to the size of the other chromosomes in the karyotype of different species.

3. Results

3.1. Chromosomal homology between human and "Lagothrix lagothricha"

Fluorescent *in situ* hybridisation using a whole-chromosome painting probe from LLA6 on human metaphase plates indicates that it is homologous to the p-arm of HSA8 and to whole HSA18. Results using the LLA11 probe show that it is partially homologous to the q-distal segment of HSA5 and to several segments along HSA7 (Figure 1).

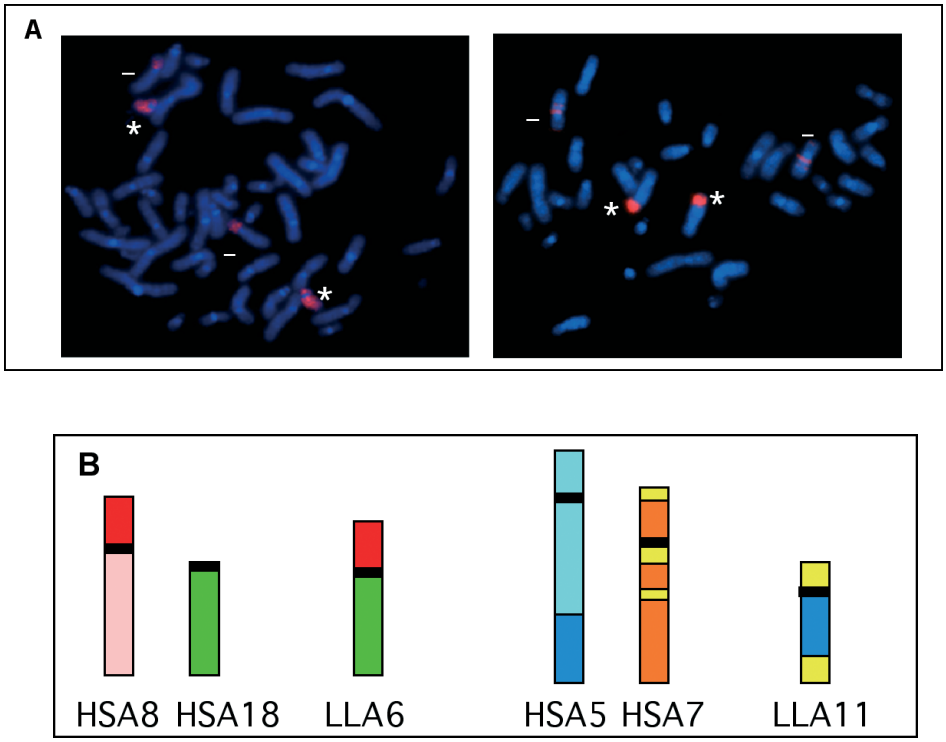

Figure 1 – (**A**) FISH on human metaphase plates using *Lagothrix lagothricha* whole-chromosome probes (LLA) (red). (*Left image*) Results when using LLA6 probe indicate that p-arm of human (HSA) chromosome 8 (arrow-head) and HSA18 (asterisk) are homologous to LLA6. (*Right image*) Results when using LLA11 probe indicate that its homologous human chromosomes are several regions on HSA7 (arrow-head) and the terminal region of the q-arm of HSA5 (asterisk). (**B**) Representation of the homologies between human (HSA) and *Lagothrix lagothricha* (LLA) chromosomes. Homologies data are results of FISH analysis on human metaphase plates using whole-chromosome probes from LLA6 and 11 and reverse-painting data not presented in this paper.

3.2. Chromosome size and radial distribution

Chromosome relative size for LLA6, LLA11, HSA5, HSA7, HSA8 and HSA18 is summarised in Table 1. The relative-size order for chromosomes included in this study is: HSA5>LLA6>HSA7>HSA8>LLA11>HSA18.

Results of 3D-FISH and radial distribution are shown in Figures 2 and 3, respectively. The mean radial position and standard deviation of HSA5, HSA7, HSA8, HSA18, LLA6 and LLA11 chromosome territories are represented in Table 1.

Chromosome target	Relative chromosome size	Mean radial position (% of the radius)	Standard deviation	Number of cells analysed
LLA6	23/31=0.74	62.56	18.19	96
LLA11	14/31=0.45	56.74	20.05	85
HSA5	19/23=0.83	68.32	15.55	105
HSA7	16/23=0.69	65.86	17.95	100
HSA8	15/23=0.65	66.02	17.50	106
HSA18	5/23=0.22	51.09	18.75	105

Table 1 – Relative size, mean radial position and standard deviation of *Lagothrix lagothricha* chromosomes (LLA) 6 and 11, and human chromosomes (HSA) 5, 7, 8 and 18. To calculate relative chromosome size, the ordering position of the chromosome (from the smallest to the largest) is divided by the total number of chromosome pairs in the karyotype. The numerator indicates the ordering position (the first being the smallest, and the largest the last one), and the denominator the total number of chromosome pairs in the karyotype of each species. Mean radial distribution and standard deviation have been calculated from data obtained from 3D-FISH.

Intraspecific comparison of radial distributions of CTs shows that LLA6 and LLA11 both have their maximum peak between 60%-80% of the nuclear radius, LLA11being slightly shifted to the nuclear centre. When comparing human CTs, HSA18 has a maximum peak at 40-60% of the nuclear radius (Figure 3), remarkably different of those of HSA5, HSA7 and HSA8, all with equivalent radial distribution, positioned more in the periphery with a maximum peak at 60%-80%.

To study interspecific radial distribution, LLA6 and LLA11 were compared to their homologous chromosomes in humans (HSA8 and 18 for LLA6 and HSA5 and 7 for LLA11). Results can be seen in Figure 4. We can see that HSA18 distribution is different to both HSA8 and LLA6, being placed in a more central position (peak at 40-60%) than LLA6 and HSA8 (peak at 60-80%). Results also indicate that LLA11 curve is clearly different to both HSA5 and 7, while HSA5 and 7 have equivalent distributions.

4. Discussion

4.1. Intraspecific comparison of radial distributions

The results of CT positioning in each species show that large chromosomes, like HSA5, 7, and 8 are situated near the periphery, with equivalent distributions, while

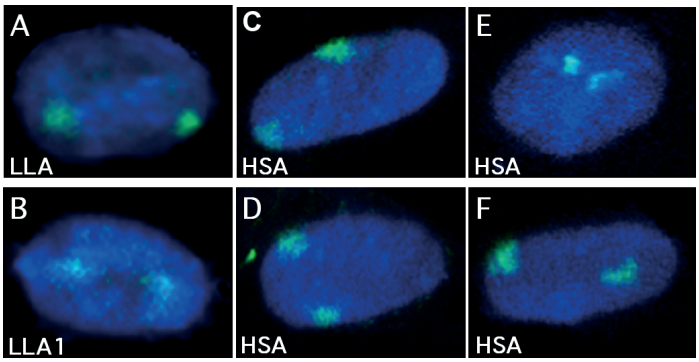

Figure 2 – Results of 3D-FISH using whole-chromosome probes (green) on *Lagothrix lagothricha* (A, B) and human (C, D, E, F).

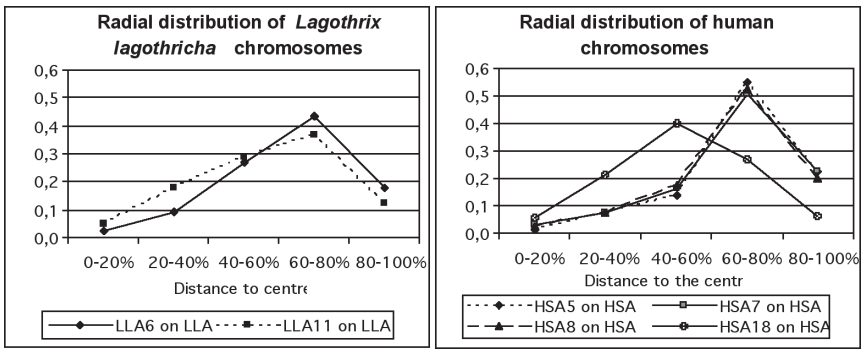

Figure 3 – Intraspecific comparison of radial distribution on 3D-preserved nuclei of *Lagothrix lagothricha* (LLA) fibroblasts using whole-chomosome probes of chromosome 6 and 11 (**A**), and (**B**) of human (HSA) fibroblasts using whole-chromosome probes of human chromosomes 5, 7, 8 and 18.

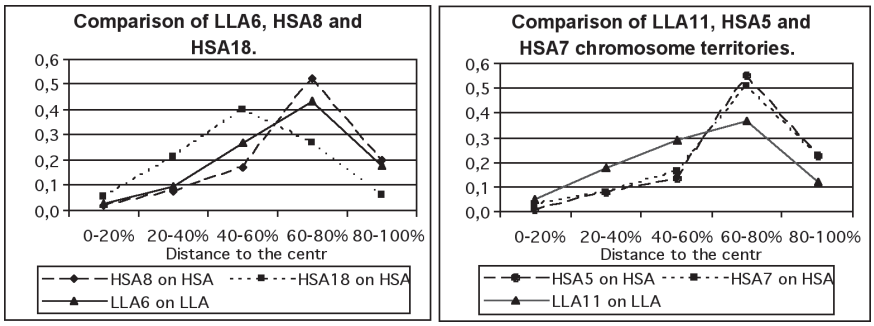

Figure 4 – Interspecific comparison of radial distribution on 3D-preserved nuclei of *Lagothrix lagothricha* (LLA) and human (HSA) fibroblasts. (**A**) Radial distribution of LLA6 chromosome territory is compared to its homologus HSA chromosome territories: HSA8 and 18. (**B**) Radial distribution of LLA11 compared to its homologus HSA chromosome territories: HSA5 and 7.

the small chromosome 18 is placed in a more central position, with a different distribution to all the other human CTs studied. These results are in agreement with previous published data in human fibroblasts that indicate that the radial position of chromosome territories is related to the chromosome size (Sun *et al.*, 2000; Cremer *et al.*, 2001). When the positioning of LLA6 and LLA11 (large-size and medium-size chromosomes, respectively) is compared, we can see that these CTs have similar radial distributions even they are not exactly the same (Figure 3). To confirm or discard the correlation between radial positioning and chromosome-size for the whole LLA karyotype further experiments analysing other chromosomes are needed.

4.2. Interspecific comparison of radial distributions

The aim of this work is to study whether the radial distribution of a CT is conditioned by a "phylogenetic memory" of the position it had in the ancestral nuclei or if it is dependent on the chromosome size. If we consider the "phylogenetic memory hypothesis", we would expect that the CT resulting from a translocation will have a similar or intermediate radial distribution of homologous ancestral chromosomes. On the other hand, if we focus on the "size-dependent hypothesis", we would expect that small-sized chromosomes will be in a central position.

When comparing LLA6 with its homologous forms: HSA8 and 18, we observe that LLA6 has a similar distribution to HSA8, both chromosomes with similar sizes, and HSA18, which has a smaller size, is situated in a more central position. Moreover, the radial distribution of LLA11 is significantly different from the large-sized chromosomes HSA5 and HSA7, LLA11 being placed in a more central position. All of these results are in agreement with the "size-dependent hypothesis"

Agreements

The authors would like e Microscòpia and Servei de Cultius of the Universitat Autònoma de Barcelona, and Mr Chuck Simmons for reviewing the English manuscript.

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Are Fragile Sites Breakpoints for Karyotype Evolution?

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ABSTRACT: *Fragile sites, as structural characteristics of mammalian chromosomes, have been related to human diseases and oncogenic processes. About their role in chromosomal evolution the evidence is much more scarce and restricted to primate species. In this paper we discuss the relationship between fragile sites and evolution.*

KEYWORDS: *Fragile sites, evolution, chromosomes, primates.*

1. HISTORICAL BACKGROUND

After the first cytogenetic observation of the expression of a fragile site in the mid-60's (Dekaban 1965), a huge number of papers has been published describing the presence of more than 100 fragile sites distributed along all human chromosomes, except in human chromosome 21 (see Sutherland and Richards, 1999 for revision). The initial demonstration of fragile sites as an inherent chromosomal characteristic (Lejeune *et al.*, 1968) as well as the first evidence of a relationship with some kind of mental deficiency (Lubs, 1969) promoted the scientific interest about these fragile regions because of their biomedical implications. Already in the late-70's, Heacht and Kaiser (1979) suggested some of the possible applications of the study of fragile sites, i.e.: (i) these regions could provide relevant information about the chromosome structure and architecture, as well as the possible function of some chromosomal bands, (ii) they would contribute to linkage cytogenetic studies and (iii) they would be useful for the elaboration of genetic maps. With the emergence of the results of the molecular characterisation of some fragile sites, there were reasons to suspect that their cytogenetic expression was actually the physical manifes-

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tation of a genomic instability underlying the molecular characteristics of these chromosomal regions. After more than 40 years of intensive research, the implication of fragile sites in human diseases is beginning to be elucidated and demonstrated, whereas their possible link with chromosome evolution remains an important subject for interpretation.

Given this picture, our interest is to investigate the implication of fragile sites throughout the evolutionary process within Primates. Fragile sites have been described in several mammalian species (Table 1) and, therefore, they have been considered structural characteristics of mammalian chromosomes (Sutherland and Richards, 1999). In the present study, our working hypothesis is twofold; on the one hand, are fragile sites "targets" for evolutionary reorganisation? And, on the other hand, since fragile sites are considered part of the chromosome structure, are the characteristics underlying their susceptibility to breakage conserved during evolution?

2. Fragile sites features

At the cytogenetic level, fragile sites are identified as non-stained gaps and breaks when cells are cultured under specific conditions (Sutherland, 1979). In general, fragile sites can be expressed by agents that delay or inhibit DNA replication or repair, as is the case of aphidicolin, BrdU and 5-azacytidine, among others (Sutherland and Baker, 2000). According to their frequency in the human population, as well as their mechanisms of expression, fragile sites are classified into two groups: common and rare (Sutherland and Richards, 1999). However, the expression of non-stained regions is characterised by an inter-individual variability and depend on the cell type analysed.

At the molecular level different situations have been described in both types of fragile sites. Whereas the expression of rare fragile sites is related to the amplification of repeat motifs (CCG repeats and AT-rich regions), the mechanisms underlying the breakage at common fragile sites are still poorly understood. So far, seven common human fragile sites have been cloned and/or characterised: FRA2G, FRA3B, FRA6F, FRA7G, FRA7H, FRA16D and FRAXB (Wilke *et al.*, 1996; Huang *et al.*, 1998; Mishmar *et al,.* 1998; Mangelsforf *et al.*, 2000; Arlt *et al.*, 2002; Morelli *et al.*, 2002; Limongi *et al.*, 2003). The characterisation of these regions has revealed that there are no simple repeat sequences responsible for instability. Moreover, the fragility has been observed over large regions (from 150 kilobases to 1 megabase) in which the DNA could adopt structures of high flexibility and low stability and they consist of AT-rich sequences (Mishmar *et al.*, 1998).

Recently, Casper and collaborators (2002) hypothesised a model for the expression of common fragile sites. They suggested that double-strand breaks are unlikely to be the primary cause of fragile site expression. On the contrary, the expression of these regions would be the result of stalled or collapsed replication forks that have escaped from the ATR intra S and G2/M checkpoints, resulting in single-stranded regions and subsequent cytogenetic expression of fragile sites as gaps or non-stained regions. Unfortunately, the reasons why fragile site regions are more prone to replication fork collapse remain unknown.

Species	Reference
Artiodactyla	
Sus scrofa	Riggs *et al.*, 1993; Yang and Long 1993; Ronne, 1995a
Bos taurus	Rodriguez *et al.*, 2002
Perissodactyla	
Equus caballus	Ronne 1992
Rodentia	
Mus musculus	Sanz *et al.*, 1986; Robinson and Elder, 1987 Djalali *et al.*, 1987; Elder and Robinson, 1989
Nesokia indica	Tewari *et al.*, 1987
Peromyscus maniculatus	McAllister and Greenbaum, 1997
Carnivora	
Canis familiaris	Stone and Stephens, 1993
Felis catus	Stone *et al.*, 1993, Ronne, 1995b Kubo *et al.*, 1998
Primates	
Gorilla gorilla	Schmid *et al.*, 1985; Smeets and Van de Klundert, 1990
Pan paniscus	Schmid *et al.*, 1985; Smeets and Van de Klundert, 1990
Pongo pygmaeus	Smeets and Van de Klundert, 1990
Macaca fascicularis	Ruiz-Herrera *et al.*, 2002
Mandrillus sphinx	Ruiz-Herrera *et al.*, 2005
Saimiri boliviensis	Fundia *et al.*, 2000
Alouatta caraya	Fundia *et al.*, 2000
Cebus apella	Ruiz-Herrera *et al.*, 2005
Cebus nigrivittatus	Ruiz-Herrera *et al.*, 2005

Table 1 – Fragile sites studies in mammalian species.

3. Implications for evolution

The biological significance of common fragile sites is still speculative since no direct implication in the development of human pathologies has been proved. The case of rare fragile sites is slightly different because some of them (FRAXA, FRAXE and FRA11B) are directly related to mental deficiency syndromes, as is the case of those fragile sites expressed in chromosome X.

Recent publications regarding common fragile sites have established a linkage between genomic instability, which characterises these chromosomal regions, and the predisposition to some types of cancer. In other words, the expression of fragile sites would increase the probability of initiation and progression of oncogenic processes (Ribas *et al.*, 1999). With the emergence of an increasing number of studies over the last decade, the link between fragile sites and cancer development has become more evident (see Popescu, 2003 for review). Indeed, fragile sites have been reported as regions with a high frequency to break and reorganise as well as sites for the integration of

oncogenic viruses (Wilke *et al.*, 1996; Huang *et al.*, 1998). But, what happens with the evolutionary implications of fragile sites?

In the middle 80's, the first cytogenetic studies on the conservation of fragile sites in Primates appeared (Yunish and Soreng 1984; Schimd *et al.*, 1985). These studies suggested that human fragile sites could be conserved in the homologous chromosome bands in the karyotypes of great apes. Subsequently, cytogenetic comparative studies performed in our laboratory among Primates revealed that a high proportion of chromosomal bands implicated in evolutionary reorganisations, centromeric shifts as well as limits of heterochromatin regions in the karyotypes of different Hominidae (Miró *et al.*, 1987), Cebidae (Clemente *et al.*, 1987) and Cercopithecidae species (Clemente *et al.*, 1990) were localised at chromosomal bands homologous to human fragile sites. Although these previous studies established the basis for the hypothesis of fragile-site conservation, it was not until the application of molecular cytogenetic methods when the conservation studies could be performed in more detail. At least, in Primates the hypothesis of fragile-site conservation has been supported, not only by G-banding comparisons, but also by molecular cytogenetic methods. In spite of the unquestionable advances that molecular cytogenetic tools have introduced in comparative genomic studies, the G-banding comparisons still have their importance for comparative studies of fragile-site location and characterisation.

The exhaustive comparative study performed by our group in different Old World monkey as well as in New World monkey species has shown that some fragile sites of different primate species have been conserved through evolution and that there is a correlation between fragile sites and evolutionary breakpoints (Ruiz-Herrera *et al.* 2002, 2005). The application of fragile site induction in primate cell cultures has allowed for the identification of chromosomal bands more prone to breakage. We were able to define the location of aphidicolin-induced fragile sites in the karyotype of different primate species: 95 fragile sites in the *Macaca fascicularis* karyotype, 50 fragile sites in *Mandrillus sphinx*, 53 fragile sites in *Cebus apella* and 16 fragile sites in *Cebus nigrivittatus* (Ruiz-Herrera *et al.*, 2002; 2005). *M. fascicularis* and *M. sphinx* belong to the Papionini tribe (Catarrhini), whereas *C. apella* and *C. nigrivittatus* are species of the same genera (*Cebus*, Platyrrhini).

Cytogenetic comparative studies have shown a high degree of conservation in the location of fragile sites among Catarrhini and Platyrrhini species. In Table 2 the percentage of coincidence among primate species studied by our group is summarised, taking into account the 76 human aphidicolin-induced fragile sites described in the literature (Human Gene Mapping, 1991). As expected, the high degree of coincidence observed was between evolutionarily related species; *M. fascicularis* vs. *Homo sapiens, M. fascicularis* vs. *M. sphinx*, and *C. apella* vs. *C. nigrivittatus*. If we extend the comparison to all species analysed (human, *M. fascicularis, M. sphinx, C. apella* and *C. nigrivittatus*), ten fragile sites are conserved in the corresponding homologous chromosomal bands in the five species: FRA1D, FRA4E, FRA4C, FRA7F, FRA7H, FRA8B, FRA14A, FRA16B, FRAXB, and FRAXC.

In order to demonstrate the hypothesis of fragile site conservation formulated on the basis of banding comparisons, we have tested the sub-chromosomal homology of regions harbouring fragile sites in the karyotype of two Papionini species (*M. fascicularis* and *M.*

	HSA	MFA	MSP	CAP	CNI
HSA		50%	25%	31.6%	7.9%
MFA	40%		42%	21%	4.2%
MSP	38%	80%		28%	10%
CAP	45.3%	37.8%	26.4%		18.9%
CNI	37.5%	25%	31.25 %	62.5%	

Table 2 – Percentage of coincidence in fragile site location among Primate species studied in our laboratory by cytogenetic comparative studies. Abbreviations: HSA, *H. sapiens;* MFA, *M. fascicularis*; MSP, *M. sphinx*; CAP, *C. apella*; CNI, *C. nigrivittatus.*

sphinx) and humans by using BAC/YAC probes. The fluorescence "in situ" hybridisation results demonstrated that human fragile sites FRA1B, FRA1D, FRA1H, FRA3B, FRA5A, FRA5C, FRA5E, FRA7D, FRA7F, FRA7H, FRA18A and FRAXB are conserved in the homologous chromosomal regions in both Catarrhini species (Ruiz-Herrera *et al.*, 2004). Of special relevance are the results obtained by using the genomic clones from human FRA3B (located at 3p14.2) and FRAXB (located at Xp22.3). The hybridisation of YAC clones containing the DNA sequences responsible for the fragility demonstrated that, not only the location, but also the molecular characteristics of the fragile site regions have been conserved during evolution. Thus, the application of the sub-regional probes has confirmed the chromosomal homologies previously described by G-banding comparisons.

On the other hand, we have also studied the implications of fragile sites in the karyotype evolution of Primates. Large-scale genomic rearrangements are the main force of evolutionary changes, so the verification of such events is fundamental for understanding the evolutionary history of chromosomes. The comparisons of primate karyotypes combining banding and molecular cytogenetic techniques allow for the assignment of evolutionary breakpoints when chromosomes are fragmented and reorganized. Combining G-banding comparisons with cross-species chromosome painting, we were able to define those chromosomal bands implicated in evolutionary reorganisations in the karyotypes of *M. fascicularis, M. sphinx, C. apella* and *C. nigrivittatus.* If we take into account the location of fragile sites, 66.67% of evolutionary chromosomal bands in the karyotype of Papionini species express fragile sites, whereas in *Cebus* species this percentage reaches 75% (Ruiz-Herrera *et al.*, 2005). In the same way, a high percentage of fragile sites described in the non-human primate species already mentioned are localised at evolutionary breakpoints.

Recently, mathematical modelling studies have supported the so-called "fragile breakage model" (Pevner and Tesler 2003; Zhao *et al.*, 2004), which considers that there are regions throughout the mammalian genomes more prone to break and reorganise, providing greater flexibility for chromosomal changes. This hypothesis, which claims

the existence of chromosomal regions with a great conservation in front of other ones with an extensive variation, support with previous cytogenetic studies herein discussed.

4. Concluding remarks

In the light of the results obtained in different Primate species, the evolutionary contribution of fragile sites is not a worthless issue. The existence of a relationship between fragile sites and evolutionary breakpoints suggests further questions: were those fragile sites responsible for evolutionary reorganisations or are they just the "scars" of those rearrangements that have been taking place during karyotype evolution? In this aspect we are just at the beginning of the road.

The demonstration that fragile sites are conserved as "fragile regions" in the non-human primate species opens new perspectives of fragile sites research. Evolutionary comparative studies will contribute to our understanding of the mechanisms responsible for the fragile site instability, as well as their role in the development of human diseases. It may also provide a key to initiate the investigations of chromosomal regions implicated in evolutionary breakpoints. If there are chromosomal regions with a high frequency to break and reorganise during the evolution of karyotypes, the study of fragile sites would provide the starting point.

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Chimpanzee Chromosomes: rDNA Silencing Due to Position Effects of Heterochromatin

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ABSTRACT: *In humans, transcriptional inactivation of nucleolus organizer regions (NORs) have long been described using the silver nitrate (Ag) staining technique, which detects proteins expressed in NOR activation. We investigated the repression mechanisms in humans and chimpanzees to elucidate its evolutionary aspects using FISH, Ag-NORstaining, C-banding, and* in situ *nick translation. Examination of 48 humans and 46 chimpanzees suggested that there are at least three different mechanisms that produce inactivation of NORs, elimination of rDNA, DNA methylation, and gene silencing due to position effects induced by heterochromatin. The elimination of rDNA and gene silencing are the most frequent event in humans and chimpanzees, respectively.*

KEYWORDS: *primates, gene silencing, NOR repression, transcription inactivation.*

1. INTRODUCTION

Silver nitrate (Ag) staining is an important technique to cytologically dissect chromosomes because this technique cleverly detects active nucleolus organizer regions (NORs) located at the secondary constriction of chromosomes (Sumner 1990). The silver binds to NOR proteins such as the RNA polymerase I subunit, the 1350kDa NOR protein, the 50-kDa UBF and SL1 present during mitosis (e.g., Roussel and Hernandez-Verdun, 1994; Whitehead *et al.*, 1997). In mammals, the proteins remain at the NORs throughout metaphase and anaphase even after the nucleolus has disappeared at prometaphase. Therefore, Ag-staining is the best procedure for detecting transcriptionally active NORs (Ag-NOR) at the cytological level.

On the other hand, it has long been admitted that not all the NORs were usually stained in species with multiple NORs. For example, in humans, no more than 7-8 out of the total 10 NORs were normally stained (reviewed in Sumner 2003). However, the mechanisms for NOR repression are as yet unknown (Guillén *et al.*, 2004). To elucidate these mechanims, we analyzed NOR repression mechanisms by comparing humans (HSA) and chimpanzees (PTR) chromosomes that have different chromatin structure around the NORs (Hirai *et al.*, 1999). Our report for these proceedings mainly deals with results that were obtained in chimpanzees.

2. Materials and methods

Blood samples of humans were obtained from 48 volunteers (23 females and 26 males) after informed consent. Blood samples of chimpanzees were obtained from a total of 46 captive individuals from the Primate Research Institute, Kyoto University, Japan (11 females and 4 males) and from the Kumamoto Primate Park, Sanwa Kagaku, Japan (17 females and 14 males). Whole-blood cell culture and chromosome preparation were carried out as previously described (Hirai *et al.*, 1999).

Fluorescence *in situ* hybridization (FISH) was performed with a previously described technique to detect the existence of rDNA in the NORs (Hirai *et al.*, 1999). The probes of 18S rDNA were kindly supplied by Dr Andrew K. Godwin, Fox Chase Cancer Center, USA.

To detect active NORs in rDNA locations and investigate the relationship between rDNA and C-band locations, Ag-NOR staining and C-banding were performed after washing off anti-fade solution used in FISH with running water, and rinsing in distilled water for 1h. Ag-NOR staining was carried out according to a previously described technique (Hirai *et al.*, 1996). Briefly, six droplets of solution I (1 g gelatin in 50 ml distilled water and 0.5 ml formic acid), and then six droplets of solution II (50% silver nitrate in distilled water) were applied to the washed chromosome preparation. The two solutions were covered with an adequately sized, fine nylon mesh. The solutions were heated from the back of the preparation with 70°C water for 90 s and, after cooling down the preparation to about 37°C at room temperature for approximately 30 s, the reaction was stopped by washing off the reacted solution with running water. C-band staining followed Sumner's method (1972).

To examine DNA methylation on negative Ag-NOR staining at sites containing rDNA, *in situ* nick translation analysis that detects the methylation at cytological level was conducted using minor modifications of a previously described method (O'Neill *et al.*, 1998). The chromosome preparation was frozen at -80°C and was placed in 99.5% ethanol for 5 min and air-dried before being rinsed with 10 mmol/L Tris-HCl (pH 7.4) for 5 min. Ten units of *Hpa* II (GibcoBRL) in 8μl of 10X enzyme buffer was applied to the slide and mounted with a coverslip (22 × 22 mm). The mounted slide was incubated in a moist chamber for 40 min at 37°C. This temperature was chosen after checking several possible temperatures. After removing the coverslip, the slide was rinsed in 10 mmol/L Tris-HCl (pH 7.4) for 5 min to stop the reaction, dehydrated with a series of 70% and 99.5% ethanol for 5 min each, and air-dried. A total of 10μl of a mixture of 10X dNTP containing biotin, 10X enzyme buffer and distilled water was placed on the slide, covered with a coverslip and incubated at room temperature for 15 min. The slide was washed in 10 mmol/L Tris-HCl and then in BI buffer (0.1 mmol/L sodium bicarbonate and 0.1% IGEPAL) for 5 min each (see also Guillén *et al.* 2004). Biotin incorporation was detected, after blocking with 5% non-fat milk at 37°C for 20 min, by incubation with FITC-avidin DCS (Vector) (1:100 dilution in 5% non-fat milk BI buffer) in a 37°C incubator for 1 h. Chromosomes were counterstained with DAPI (70 ng/ml) contained in anti-fade solution and mounted with a cover slip (see also Hirai and LoVerde, 1995).

To estimate more precisely the mechanisms of NOR repression, the spatial relationships between rDNA presence, Ag-NOR staining, DNA methylation, and C-band were observed using various combinations of sequential staining, e.g., *in situ* nick translation → FISH with rDNA probe → Ag-NOR staining or C-banding; DAPI staining → FISH signaling → Ag-NOR staining or C-banding.

For image analysis, fluorescence signals, Ag-particles, and Giemsa dye were observed and imaged using a Zeiss Axiophot microscope attached to a CCD camera system (Cool SNAP HQ, Photometrics) and an auto filter-wheel set. Images were analyzed on an Apple Power Mac G4 computer, using scientific imaging software (IPLab Spectrum, Scanalytics, Inc.).

3. Results

3.1. Aspect of rDNA localization and Ag-NOR activity

In accordance with many previous investigations, the humans and chimpanzees examined here essentially had rDNA at the short arm of the five acrocentric pairs. However, polymorphisms for the absence of rDNA were also observed in both species. Of the 48 humans analyzed, only 22 individuals (45.8%) showed positive hybridization on all ten acrocentrics, and the remaining 26 (54.2%) lacked rDNA on some acrocentrics, ranging from 1 to 3 negative chromosomes. In contrast, out of the 46 chimpanzees investigated, 41 individuals (89.1%) showed hybridization on all ten acrocentrics, and the remaining 5 (10.9%) had only one acrocentric chromosome that lacked the rDNA locus.

In addition, it was also frequently observed that some rDNA positive loci were Ag-NOR positive [rDNA(+)/Ag(+)], but others were Ag-NOR negative [rDNA(+)/Ag(–)]. Twenty-three individual humans (23/48, 47.9%), and 30 individual chimpanzees (30/46. 65.2%) were rDNA(+)/Ag(–) in at least one acrocentric. The rDNA(+)/Ag(–) class included two types of intra-individual variation of stable (–) and unstable (±) Ag-NOR negative. Namely, several individuals showed a fixed chromosome number that had rDNA(+)/Ag(–) in every cell, but other individuals were mosaic with the number of chromosomes showing rDNA(+)/Ag(+) or rDNA(+)/Ag(–) varying from cell to cell.

Moreover, comparison of each acrocentric pair between humans and chimpanzees revealed that there are species-specific differences in Ag-NOR staining as followings. First, humans had a higher (total average 7.1% of all acrocentrics) of rDNA(–)/Ag(–) than chimpanzees (average 1.1%), which is related to the frequency of rDNA loss. Second, the homologous chromosomes HAS-22 and PTR-23 were the most different, because the frequency of rDNA(+)/Ag(±) and rDNA(+)/Ag(–) on HAS-22 was 7.3%, but that on PTR-23 was 30.4%. The rDNA(–)/Ag(–) of both homologous chromosomes was 9.4% in humans and 0% in chimpanzees. The significant difference between the two species was the predominance of rDNA(–)/Ag(–) in humans and of rDNA(+)/Ag(–) in chimpanzees. That is, humans had a higher frequency of rDNA(–) and a

lower frequency of rDNA(+)/Ag(–), while chimpanzees had a lower frequency of rDNA(–) and a higher frequency of rDNA(+)/Ag(–).

3.2. DNA methylation

To investigate the cause of the rDNA(+)/Ag(–) we examined DNA methylation using *in situ* nick translation with *Hpa*II. As chimpanzees showed the rDNA(+)/Ag(-) condition more commonly, representatives of the species with the rDNA repression were examined for methylation. Treatment conditions used in the present study appeared to be adequate to detect methylation, because one homologue of the X chromosomes in females was not labeled with FITC, indicating hypermethylation. Similarly, short arms whose regions showed positive for rDNA but negative for Ag-NOR staining, were observed without FITC signals on the some acrocentrics. On the other hand, the greenish (FITC positive) short arms of other acrocentrics (hypomethylation) were positive with both of the procedures. The presence of Ag(–), however, did not always coincide with hypermethylation. For instance, both acrocentrics 14 and 17 of chimpanzees were positive for rDNA and incorporated FITC (hypomethylation), but only one was positive for Ag-NOR staining, and the other was negative. These data revealed that there are two distinct causes of rDNA(+)/Ag(–), involving two mechanisms of rDNA inactivation, only one of which may be attributable to DNA methylation (see figure 2 of Guillén *et al.*, 2004).

3.3. Relationship between rDNA and constitutive heterochromatin

What additional mechanisms, apart from DNA methylation, are there for rDNA inactivation? One candidate might be a position effect due to a variegation of heterochromatin, because characteristic chromatin structures and their variants were observed in chimpanzees. First of all, to examine the standard chromatin structure around the NOR in both species, HSA-13 and PTR-14 were compared with sequential staining. In humans, both rDNA and Ag-NOR staining were located only in the constriction, while C-bands were located only in the proximal region and did not overlap with rDNA. On the other hand, in chimpanzees the distal two-thirds of the short arm contained rDNA, which seemed to overlap with the satellite and proximal C-bands as well as the constriction, while Ag-NOR staining was located only in the constriction region (see figure 4 of Guillén *et al.*, 2004).

Several variant acrocentrics with different structural organization around the NOR were found in chimpanzees, though humans did not display such pronounced variation. The first case was a heterochromatic pair of PTR-14 in which the distal two-thirds of the short arm contained rDNA, and contained two large C-band blocks at the distal and proximal regions. In spite of the extensive distribution of rDNA, Ag(+) staining was observed only at the gap between the two C-band blocks (Figure 1a). The short arm of the homologue of this chromosome lost the distal C-band block and in additional, showed rDNA(+)/Ag(+) only at the euchromatic tip. The second case is a variant of

PTR-15 that showed a large block of rDNA in the middle of the short arm, but the center of the rDNA block was negative for Ag-staining, which corresponded to a distal intermediate C-band (Figure 1b). The third case involved a heterochromatic pair of PTR-22 in which one homologue showed a large rDNA signal and two C-bands, distally and proximally on the short arm. One homologue had an Ag(+) NOR, while the other homologue had a NOR which was Ag(–) and showed a smaller rDNA signal and C-bands (Figure 1c). Considered together, these cases suggest a relationship between rDNA repression and C-band blocks, because only the non-heterochromatic regions of the short arm were rDNA(+)/Ag(+).

4. Discussion

A negative Ag-NOR staining was observed in the present study associated with: (1) a loss of rDNA ; (2) a small amount of rDNA are likely to be totally negative for Ag-staining; (3) NORs with a large amount of rDNA are often partially negative for Ag-staining. These conditions differ between humans and chimpanzees. The differences may be exploited to help elucidate mechanisms of rDNA repression.

4.1. Elimination of rDNA

The loss and duplication of rDNA can be induced by unequal sister-chromatid recombination and by non-homologous recombination (hetero-site crossing over) in the germ cells. Unequal sister-chromatid recombination can explain loss and tandem duplication of rDNA regions between homologous chromosomes, while hetero-site crossing over can be responsible for rearrangements between non-homologous acrocentric chromosomes. In particular, non-homologous recombination is an important mechanism to relieve interlocking in associations constructed by the heterochromatic regions of chromosome ends (Imai *et al.*, 1986). This mechanism might explain the relocation of the NOR locus in whole or in part to other chromosome, resulting in the elimination of rDNA from a particular locus. A representative instance of such a relocation of rDNA is seen in the gorilla, where the NOR is related from arocentrics (22 and 23) to the terminal heterochromatic region of the metacentric gorilla chromosome 1 (Schempp *et al.*, 1998).

The differences in DNA organization in the distal region of the NOR locus between humans and chimpanzees is speculated to have induced species-specific variations, and humans have many more instances of rDNA loss than do chimpanzees. According to previous data (e.g., Meneveri *et al.* 1995; Assum *et al.* 1998; Hirai *et al.* 1999) and the present study, humans have non-heterochromatic DNA arrays consisting of b-satellite DNA and other repetitive sequences at the distal part of the short arm of NOR acrocentrics. In contrast, chimpanzees have heterochromatic DNA arrays consisting of different sequences at this region, which in chimpanzees is also occupied by rDNA sequences.

Chiasma analyses have shown that chromosomes have zones at both ends that cannot be exchanged between chromosomes (Wada and Imai, 1995; Hirai *et al.*, 1996; Imai *et al.*, 1999). The lack of exchange in these regions was already predicted by a theoretical analysis of karyotype evolution (Imai, 1975). The regions were regarded to be non-crossing over zones (NCOZ). From the chromosome end the chiasma-free zones span approximately average 0.6% of the total length of all haploid autosomes. Two chromosomes cannot interact structurally with each other in this area. The NCOZs in the acrocentric short arms of humans and chimpanzees contain distinct DNA structures. That of humans consists of non-heterochromatic reiterated DNA sequences such as b-satellite, while that of chimpanzees consists of both rDNA and heterochromatic repetitive DNA sequences. Thus as the human rDNA is outside the NCOZ, it may move to other chromosomes, but the chimpanzee rDNA cannot because it is within the NCOZ (Figure 2). This may account for the difference in frequency of rDNA elimination between the two species. Indeed, humans showed a much higher frequency of lost rDNA loci than did chimpanzees. The elimination of rDNA is the most frequent cause of Ag-NOR(–) acrocentric short arms in humans, and this trait is heritable in both humans and chimpanzees, as observed in comparisons of parents and offspring in our samples.

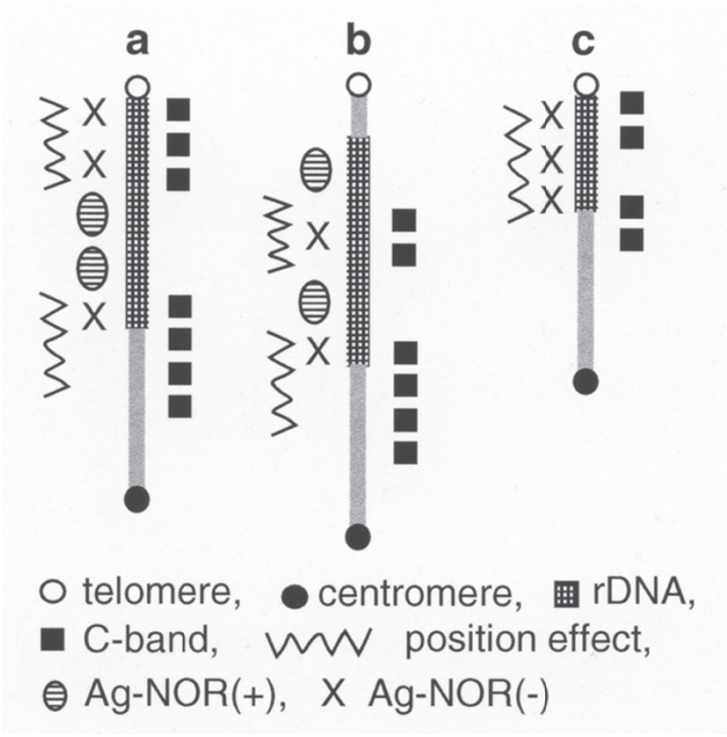

Figure 1 – Different mechanisms of NOR inactivation by position effects of heterochromatin observed in chimpanzees. See text for details.

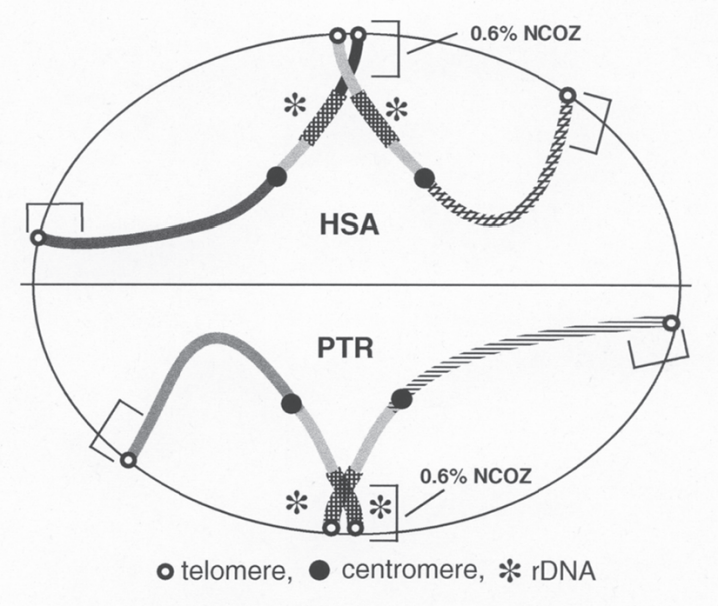

Figure 2 – Different mechanisms of interchromosome chromatin exchange in non-homologous recombination between humans (HAS) and chimpanzees (PTR). NCOZ, non-crossing over zone. See text for details.

4.2. Mechanisms of NOR repression

Previous papers have suggested that DNA methylation is the most likely candidate mechanism for the absence of Ag-NOR proteins on regions with rDNA (e.g., Tantravahi *et al.*, 1981; Ferraro & Prantera, 1988), even if, apparent exceptions to this relationship between gene activity and the level of DNA methylation have been reported (de Capoa *et al.*, 1991). Our *in situ* nick translation experiments with *Hpa*II restriction enzyme also showed that absence of Ag-NOR proteins was associated with DNA methylation. However, the experiments suggested that there might also be inactive NORs not undergoing methylation. We were not, in the present study, able to check all individuals who were rDNA(+)/Ag(–) using *in situ* nick translation. It is unknown how many cases of rDNA(+)/Ag(–) (in the 48% (23/48) of humans and 65% (30/46) of chimpanzees) were caused either by the DNA hypermethylation or by another as yet unknown mechanism. According to our preliminary results obtained from 14 chimpanzees (Primate Research Institute, Kyoto University, Japan) of 812 chromosomes with rDNA(+)/Ag(–) 184 chromosomes (22.7%) underwent methylation, but 628 chromosomes (77.3%) were negative for some other unknown mechanism (unpublished data). Though the causes remain uncertain, chromatin structure seems to be related to the unknown mechanism for rDNA (+)/Ag(–).

Three cases of variation that occurred around the NORs of chimpanzees suggest the existence of a relationship between rDNA inactivation and location of C-band blocks. The phenomena are summarized in Figure 1. In the first case, transcription of

rDNA located on the distal and proximal C-band blocks was suppressed (Figure 1a). The second case indicates that only the part of the rDNA area that forms a C-band is negative for NOR transcription (Figure 1b). The third case showed that in two homologous chromosomes with different sizes of C-band blocks in the short arm, the smaller short arm was negative for Ag-NOR staining (Figure 1c), but the larger was positive. These results indicate that the amount of rDNA sandwiched between the distal and proximal C-band blocks was critical. These observations could be examples of position effect variegation, which was originally described in the fruit fly (reviewed by Wakimoto, 1998; Wallrath, 1998; Sumner, 2003). That is, chromosome regions around constitutive heterochromatin are subjected to epigenetic gene suppression, the silencing of euchromatic genes. The higher frequency of rDNA(+)/Ag(–) in chimpanzees might be a result of these position effects. For example in PTR-23, where in 11 individuals and 369 chromosomes showed that the rDNA(+)/Ag(–) conditions 82% appeared due to the position effect and 18% were due to DNA methylation (unpublished data).

In conclusion, as mentioned above, plausible mechanisms of NOR inactivation observed in humans and chimpanzees can be summarized as follows (1) elimination of rDNA due to non-homologous crossing over; (2) DNA methylation; (3) or gene silencing due to position effect of heterochromatin.

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When, Where and How? Reconstructing a Timeline for Primate Evolution using Molecular and Fossil data

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ABSTRACT: *Non-human primates today are found throughout the forests and woodlands of the Old and New World tropics and subtropics, although in earlier phases of primate history, under different climatic regimes, their distribution was even wider. New fossil finds and a growing number of phylogenetic analyses based on nucleotide sequences require constant shifts in our thinking concerning the divergence times of the major primate lineages. Often the insights provided by these two data sources are incongruent with one another, and the source of the disagreement must be sought. In this contribution I summarise current information pertaining to major events in primate evolution stemming from both palaeontology and molecular biology, and review current ideas as to the time and place of the origin of the primate clade.*

KEYWORDS: *molecular phylogenies, fossils, divergence dates, primate origins.*

Non-human primates today are found mainly in the woodlands and forests of the tropical and subtropical regions in South America, Africa, Madagascar and South-east Asia, but in the past they occupied a much wider area of the Earth's surface. Euprimate fossils (i.e., fossils that display the clade-defining traits, e.g. petrosal bulla, postorbital bar) have been recovered from various localities in North America, and throughout the length of South America all the way down to the southernmost tip of Argentina. Primate fossil sites extend from western Europe through to eastern China, and include areas in Africa that are now barren desert. Understanding how they came to occupy their past and current distributions requires a well-supported timescale, and this is an area of primate history that is subject to constant revision as new fossils are discovered and more DNA sequences and ingenious methods of molecular analysis are brought to bear in the calculation of molecular clocks. In this contribution I summarise current knowledge concerning the timing of major events in primate evolution using both of these data sources, and review contemporary thinking as to where the primate clade originated.

One factor needs to be borne in mind with regard to estimating divergence times from molecular data. Because molecular substitution rates are not consistent from one lineage or one gene sequence to another, or even from one nucleotide position to another, they must be calibrated in some way. This is most often done in accordance with the estimated appearance times of the earliest fossils in a given lineage. Two of the most commonly employed calibration points in primate phylogeny are the appearance of the first cercopithecoid fossils, signalling the divergence of apes and monkeys, at 25 Myr, and the first appearance of platyrrhine fossils in South America at 26 Myr. It cannot be stated too strongly that these dates are too recent to record the actual lineage divergence. The probability that the fossil record actually documents the ancestor to any clade is vanishingly small, and these fossil calibration points will always be minimal divergence dates (Eizirik *et al.*, 2001). For example, Yoder and Yang (2000) have estimated the cercopithecoid-hominoid divergence date to be between 30 and 40 Myr, and Arnason *et al.* (1998) predicted it could even be ≥ 50 Myr. Nevertheless, the 25 Myr date continues to be employed (e.g., Page and Godman, 2001; Poux and Douzery, 2004), yielding unrealistically recent dates for the origins of other primate clades.

1. The origin of the primate clade

The time and place of the origin of the primate clade is controversial. Most palaeontologists (e.g. Gingerich & Uhen, 1994; Alroy, 1999; Benton 1999; Foote *et al.*, 1999) place the origin of the primates, along with those of other extant mammal orders, just after the Cretaceous-Tertiary (K-T) boundary, 65 Myr ago. The most commonly held view is that the mammal radiation was held in check up until the end of the Cretaceous because the potential ecospace was occupied, chiefly by dinosaurs. When the dinosaurs died out during the end-Cretaceous mass extinction event, a host of new ecological opportunities became available to the mammals, which rapidly radiated into the newly freed niches (Easteal, 1999). No undisputed primate fossils are known from sediments older than the K-T boundary. The plesiadapiforms, a group of highly diverse primate-like mammals that were common components of Northern Hemisphere faunas during the Palaeocene and early Eocene, were once referred to as "archaic primates". Their earliest representative, *Purgatorius*, was recovered during the unearthing of a 65 Myr old *Triceratops* in Montana (Van Valen & Sloan, 1965). Like strepsirrhine primates, plesiadapiforms came in a wide range of body sizes, from < 100 g to > 3 kg, and appear to have followed a diversity of diets, from insects to leaves and seeds, and even gum (Fleagle, 1999). Some were arboreal, while others were apparently terrestrial. However, the concept of plesiadapiforms as pleisomorphic primates has been challenged by fossil discoveries indicating that the living primate suborders, the Strepsirrhini and the Haplorhini, had already begun to diverge from one another in the Palaeocene, around the time that the plesiadapiforms were undergoing their own radiation (Godinot & Mahboubi, 1992). Furthermore, the osteological evidence linking the plesiadapiforms and the primates is no stronger than that linking the primates with the Dermoptera

(colugos) and Scandentia (tree shrews) (Fleagle, 1999). Thus, while most contemporary primate evolutionists agree to unite all three groups within a clade that includes the primates, the exact order of branching within the clade is contentious. The first undoubted primate fossil hypodigm, *Altiatlasius*, consists of ten isolated teeth recovered from Adrar Mgorn 1 locality in foreland basin deposits of the High Atlas Mountains, and dated at 60 Myr (Sigé *et al.*, 1990).

Studies in molecular biology have posed a major challenge to the palaeontological view of primate origins. Molecular clock estimates suggest that the primate clade diverged from its closest relatives well in advance of the K-T boundary, between 87 – 85 Myr (Eizirik *et al.* 2001; Springer *et al.* 2003; Murphy *et al.* 2004; Yoder & Yang 2004) or even earlier (Arnason *et al.* 1998; Kumar & Hedges 1998). There are three potential solutions to this apparent lack of congruence between palaeontology and molecular biology:

(a) The molecular divergence dates are essentially correct, but genetic divergence was not accompanied by morphological divergence discernible in the fossil record until after the K-T boundary, when new ecological opportunities opened up (Benton, 1999; Foote *et al.*, 1999; Eizirik *et al.*, 2001).

(b) The molecular divergence dates are essentially correct, but the Cretaceous phases of mammalian evolution took place in areas of the globe where there are no Cretaceous deposits, and these lineages suddenly dispersed in the Tertiary (Benton, 1999; Foote *et al.*, 1999); this is the Garden of Eden hypothesis of Foote *et al.* (1999).

(c) The molecular dates are overestimates of the divergence times, because the molecular clock sped up during the initial phases of the Tertiary radiation, as mammals radiated into free ecospace (Benton 1999; Foote *et al.*, 1999).

Statistical estimates of the extent of missing fossil history based on models of fossilization and recovery processes are contradictory in their findings. The model of Foote *et al.* (1999, p. 1310) indicates that "it is unlikely that many modern orders arose much earlier than their oldest fossil records". Other models are more in line with the molecular data, indicating that the primates diverged approximately 81.5 Myr ago (Martin, 1993; Tavaré *et al.*, 2002).

Finally, a strong signal is emerging from the molecular data showing that the extant mammalian orders did not all radiate around the same time period, as would be suggested by the palaeontological model, but have a well-supported hierarchical pattern (Easteal, 1999). The more basal branches of this hierarchical tree consistently diverge well before the K-T boundary.

2. The strepsirrhine-haplorhine divergence

The most fundamental split in primate biological organisation is reflected in two subordinal divisions: the Strepsirrhini (i.e., the living tooth-combed primates (Infraorder Lemuriformes) and their fossil allies) and the Haplorhini (i.e., the tarsiers, anthropoids, and their fossil allies). The fossil record indicates that this basal divergence oc-

curred during the Palaeocene (65-55 Myr; Sigé *et al.* 1990; Godinot & Mahboubi 1992). Recent phylogenetic analyses (Sieffert *et al.*, 2005; Jaeger and Marivaux 2005) indicate that the oldest known primate, *Altiatlasius* from the Palaeocene of Morocco (Sigé *et al.*, 1990), is a primitive stem anthropoid, as originally suggested by Godinot (1994). Thus, by the time we get our first glimpse of undoubted primates in the fossil record, the haplorhine-strepsirrhine divergence has already occurred. The divergence was certainly well established by the beginning of the Eocene, 55 Myr ago. This was a time of global warming, when moist tropical forests spread across Europe, Asia and North America (Fleagle, 1999), and supported a diverse fauna of euprimates. The vast majority of primate fossils recovered from this epoch have been classified into two distinct, but highly diverse, groups: the Adapiformes, which share several diagnostic characters with the lemuriforms and have hence been allocated to the Suborder Strepsirrhini (Kay *et al.*, 1997; Gebo, 2002), and the Omomyoidea, which have been allied with the extant *Tarsius* and are thus Haplorhini (Kay *et al.*, 1997; Fleagle, 1999).

Some molecular estimates of the timing of this divergence, based on calibration points external to the primate radiation, are somewhat older than the Palaeocene, i.e., ~ 80 Myr (Arnason *et al.*, 1998), or 77 Myr (Springer *et al.*, 2003; Murphy *et al.*, 2004). The estimations of Porter *et al.* (1997) and Goodman *et al.* (1998), using the 25 Myr calibration point for the cercopithecoid-hominoid divergence, place this event at 63 Myr. Poux and Douzery (2004), using 63 Myr as the date for the emergence of primates, calculate a haplorhine-strepsirrhine split at $\leq$ 60 Myr. Porter *et al.* (1997) and Goodman *et al.* (1998) further indicate that tarsiers and anthropoids shared a common ancestor 58 Myr ago. All of these dates calculated on the basis of calibration points within the primate clade are too recent from the point of view of the fossil record, and would be scaled back using a more realistic calibration point.

The important fact that emerges from both the palaeontological and molecular picture of early primate evolution, is that the two suborders diverged within a very short time of the origin of the primate clade.

3. The lemuriform radiation

The Infraorder Lemuriformes is made up of two superfamilies: the Lorisoidea (the galagos and lorises of Africa and Asia) and the Lemuroidea (the lemurs of Madagascar).

The lorisoid families appear to have originated in Africa (Yoder *et al.*, 1996; Sieffert *et al.*, 2003) and have a fossil record going back to ~ 40 Myr ago. Sieffert *et al.* (2003) described dental remains from the Fayum Depression that indicate the lorisid and galagid lineages had already diverged by this time, towards the end of the Eocene. More lorisoid material has been recovered from early Miocene deposits (20-15 Myr) of East Africa, testifying to the existence of a lorisoid radiation that did not yet bear the hallmarks of the living lorisoid families (Rasmussen and Nekaris, 1998; Masters *et al.*, 2005). Galagid dental remains discovered recently in Egypt suggest that the defining character of the living family, the molarised P4s, had evolved by 10,000 yr ago (Pickford, pers. comm.).

No fossil lemuroid older than 26,000 yr has yet been found on Madagascar (Simons *et al.*, 1995), making a palaeontological estimate of the timing of the lemuroid radiation very difficult. Molecular investigations have been more productive, and a slew of potential dates has been derived for the lorisoid-lemuroid divergence, as well as for the origins of the mainland and island radiations (Table 1). Most molecular studies have concluded

Authors	Lorisoid-lemuroid divergence	Lemuroid radiation	Lorisoid radiation
Yoder et al. (1996)	≥ 62	≥ 54	≥ 55
Porter et al. (1997)	50.2 – 50.9	41.3 – 47.7	23
Yoder (1997)	61.5 – 61.9	53.7 – 54.1	50.7 – 54.9
Arnason et al. (1998)	~ 68	~ 80	----
Goodman et al. (1998)	50	45	23
Poux and Douzery (2004)	45.4 – 46.7	39.6 – 40.7	13.8 – 14.2
Roos et al. (2004)	61 (50 – 80)	58 (47 – 76)	46 (37 – 60)
Yoder and Yang (2004)	68.5 (61.3 – 75.4)	62 (57.9 – 73.0)	39.1 (38.0 – 41.5)
Poux et al. (2005)	60 (69.6 – 51.6)	50 (58.5 – 41.1)	----

Table 1 – Divergence dates for the lemuriform, lemuroid and lorisoid radiations estimated from nucleotide sequences.

that the Malagasy lemuriforms are monophyletic, and that the invasion of Madagascar occurred once at some time between the divergence of the superfamilies and the radiation of the Lemuroidea. The lemuroid-lorisoid divergence is estimated to have occurred between 68 (Arnason *et al.*, 1998; Yoder and Yang, 2004) and 50 (Porter *et al.*, 1997; Goodman *et al.*, 1998) Myr ago, while derived ages for the lemuroid radiation range from 62 (Yoder and Yang, 2004) to 40 (Porter *et al.*, 1997; Poux and Douzery, 2004) Myr ago. Arnason and his colleagues have been alone in claiming that the lemuroid radiation began far in advance of the lemuroid invasion of Madagascar, perhaps as early as 80 Myr ago, so that the island was colonised twice by primates: once by the daubentoniids (aye-ayes) and once by the common ancestor of the remaining lemuroids. The lorisoid radiation appears to have been the most difficult to pin down of all the strepsirrhines, with estimates ranging from 55 (Yoder *et al.*, 1996) to 14 (Poux and Douzery, 2004) Myr ago. The fossil record seems to indicate that any date younger than 40 Myr is too recent, casting doubt on several of these estimates. Several molecular studies have also been unable to provide evidence in support of lorisid monophyly to the exclusion of galagids (e.g. see Goodman *et al.*, 1998, Masters *et al.*, 2005), which could indicate that the extant lorisid radiation has deep roots – certainly deeper than the extant galagid radiation.

4. The origin and radiation of the anthropoids

Following the first tantalising glimpse of stem anthropoids in Africa 60 Myr ago, the fossil record is mute until ~ 45 Myr ago, when undoubted early anthropoids existed contemporaneously in North Africa (*Algeripithecus*, Godinot and Mahboubi, 1992) and eastern China (*Eosimias*, Beard *et al.*, 1994). An Eocene tarsier, *Tarsius eocaenus*, was found alongside *Eosimias*, indicating that the tarsier-anthropoid divergence was well established, and making *Tarsius* the longest-lived genus of all primates, living and extinct.

A diverse anthropoid fauna is known from the late Eocene-early Oligocene (37-32 Myr) deposits of the Fayum Depression, Egypt, comprising at least three families: the Parapithecidae, the Propliopithecidae and the Oligopithecidae. Of these, the parapithecids and oligopithecids have been described as having "a platyrrhine grade of morphological organization that was substantially identical to that of living platyrrhines" (Kay *et al.*, 1997). They also had three premolars, while the Propliopithecidae had only two, and hence qualify as true catarrhines. Fleagle (1999, p.408) has indicated one late Eocene genus in particular, *Proteopithecus*, is very platyrrhine-like, and shows no specialisations that would preclude it from platyrrhine ancestry. What the Fayum anthropoid assemblage appears to be telling us is that the platyrrhine-catarrhine divergence had occurred by the end of the Eocene (33 Myr), and probably took place in Africa (Takai *et al.*, 2000).

Anthropoids appear for the first time in the South American fossil record in the late Oligocene, 27-26 Myr ago, despite the presence of highly productive fossiliferous deposits of Palaeocene and Eocene age on the continent. The oldest platyrrhine fossil is *Branisella*, which shows several intriguing similarities to *Proteopithecus* in its upper dentition (Takai *et al.*, 2000), and a phyletic relationship has been proposed between them. Where the taxa differ, *Proteopithecus* consistently shows the more ancestral morphology, as befits its greater geological age.

Molecular estimates of the catarrhine-platyrrhine split range from 40 Myr (Goodman *et al.*, 1998) to 48 Myr (Kumar and Hedges, 1998), and even 60 Myr (Arnason *et al.*, 1998), all of which could accord with an African origin. A date of 30 Myr, estimated by Sarich (1970) using immunological distances, is probably too young, since the propliopithecids are older than this. A more recent immunological study indicated a double invasion of South America by platyrrhines, once by the Cebidae and once by the Atelidae, placing a 52 Myr old date on the emergence of the cebid clade (Bauer and Schreiber, 1997). Platyrrhine paraphyly has not been supported by reconstructions based on sequence data, however (Porter *et al.*, 1997; Goodman *et al.*, 1998), and these studies have yielded a much younger date of *c.* 22 Myr for the cebid-atelid divergence. The true value is likely to lie between these estimates.

Cercopithecoid fossils have been recovered from early Miocene deposits in Africa, 25-20 Myr old, and they appear to predate the divergence of the living subfamilies, the leaf-eating Colobinae and the cheek-pouched Cercopithecinae. As a result, these fossils are classified in their own subfamily, the Victoriapithecinae (Benefit,

1993; Fleagle, 1999). The fact that these Miocene monkeys are absent from fossil faunas associated with dense forest, and probably preferred more open habitats, has led to the proposal that the cercopithcoid-hominoid divergence occurred as a result of differential adaptation of the two lineages to woodland/savanna and forest habitats, respectively.

As stated above, several molecular phylogeneticists have used this fossil date as a calibration point for the estimation of later divergence dates, although it is likely to be too young (Yoder and Yang, 2000). Hence, Arnason *et al.* (1998), using a calibration point outside the primates, have indicated an age of $\geq$ 50 Myr for the cercopithecoid-hominoid divergence, and 30 Myr for the Colobinae-Cercopithecinae split. In contrast, Page and Goodman (2001), using the 25 Myr cercopithecoid-hominoid calibration point, have estimated the colobine-cercopithecine split as occurring as recently as 14 Myr ago. Once again, the true value is likely to lie somewhere in between.

5. The hominoid radiation

The apes (Superfamily Hominoidea) diversified and dominated the primate faunas of African and Eurasia during the Miocene (23-5 Myr), at a time when monkeys were still relatively rare. Fossils representing 500-1,000 individuals have been recovered from an area spanning Eurasia from Spain to China, and almost the length of Africa from Egypt to Namibia (Fleagle, 1999). The earliest apes are found in Africa, and their radiation appears to have coincided with a period when global temperatures began to increase again after the episode of dramatic cooling that marked the Oligocene epoch. During the early Miocene it is likely that tropical lowland forest covered large tracts of Africa prior to the evolution of the savannas. When the African plate made contact with Eurasia, *c.* 15 Myr ago, apes moved northwards on to that landmass as well.

Given our present state of knowledge, it is not possible to link most fossil apes to particular living taxa. This makes the fossil estimation of hominoid divergences highly problematic. The fossil record of the gibbons (Family Hylobatidae) only extends as far back as the middle Pleistocene of China and Indonesia, but molecular data suggest that they diverged from the great apes at some point between 40 and 14 Myr ago (see Table 2). The 12 Myr old *Sivapithecus* (Kappelman *et al.*, 1991) shows cranial similarities to the living orangutan, but there are postcranial differences which argue against a close relationship between them. The fossil history of African great apes is almost entirely undocumented, with the exception of *Samburupithecus*, a 9.5 Myr old ape the size of a gorilla, from the Samburu Hills in Kenya (Ishida and Pickford, 1997).

A range of dates has been derived from molecular sequence comparisons, and a selection of these is presented in Table 2. The values vary widely, dependent on the calibration points employed. *Sahelanthropus*, the oldest fossil hypodigm claimed for the hominin lineage (Brunet *et al.*, 2002), if the claim is justified, places a minimum limit on the divergence of *Homo* from the great apes of 7-6 Myr.

Authors	Hylobatidae	Pongidae	Gorillini	*Homo-Pan*
Porter et al. (1997)	21.3	19.6	7.7	7.7
Arnason et al. (1998)	40	30	15 – 17	10 – 13
Goodman et al. (1998)	18	14	7	6
Kumar & Hedges (1998)	14.6 ± 2.8	8.2 ± 0.8	6.7 ± 1.3	5.5 ± 0.2
Yoder & Yang (2000)	----	----	7 – 9	4 – 6
Page & Goodman (2001)	18	14	7	6 – 5

Table 2 – Divergence dates for the hominoid clades estimated from nucleotide sequences.

6. The origins timeline and primate biogeography

Figure 1 summarises some recent molecular estimates of the ages of the bifurcations of the major primate lineages, along with the data concerning first fossil appearances and some of the other major geological events that have a bearing on the history of the landmasses currently occupied by primates. Virtually all of the land currently making up the tropics and subtropics of the Old and New Worlds was once part of the supercontinent of Gondwana. Approximately 160 Myr ago the supercontinent began to fragment, essentially rupturing into two parts: a western portion made up of South America and Africa, and an eastern portion made up chiefly of India, Madagascar, Antarctica and Australia (Reeves and de Wit, 2000). The separation of Africa and South America began in the south, with the final sundering of West Africa and Brazil occurring *c.* 120 Myr ago. South America and Antarctica remained connected until well into the Palaeogene, with the opening of the Drake Passage occurring at about 30 Myr. Indo-Madagascar maintained a connection with Antarctica by means of the Kerguelen plateau until 90 Myr at the latest (Reeves and de Wit, 2000). Separation between India and Madagascar was completed between 89 and 83 Myr ago, after which India drifted rapidly northwards to join with Asia (Reeves and de Wit, 2000; de Wit, 2003).

What is immediately clear from Figure 1 is that most of these events occurred well before either the molecular or the palaeontological estimates of the origin of the primate clade. The landmasses that make up the current geographic deployment of the primate order are separated from one another by vast stretches of ocean, and have been since the late Cretaceous and earlier. Where, then, did primates originate, and how did they come to occupy their current distribution?

This remains the single most puzzling aspect of primate evolution. Most reconstructions require one to several over-water dispersal events, whereby primates rafted from one landmass to another on mats of vegetation (e.g., Houlen 1999; Yoder *et al.*, 1996). Serious objections have been raised to the idea of rafting primates in terms of the animals' ability to survive an extended period of deprivation and exposure (Simons,

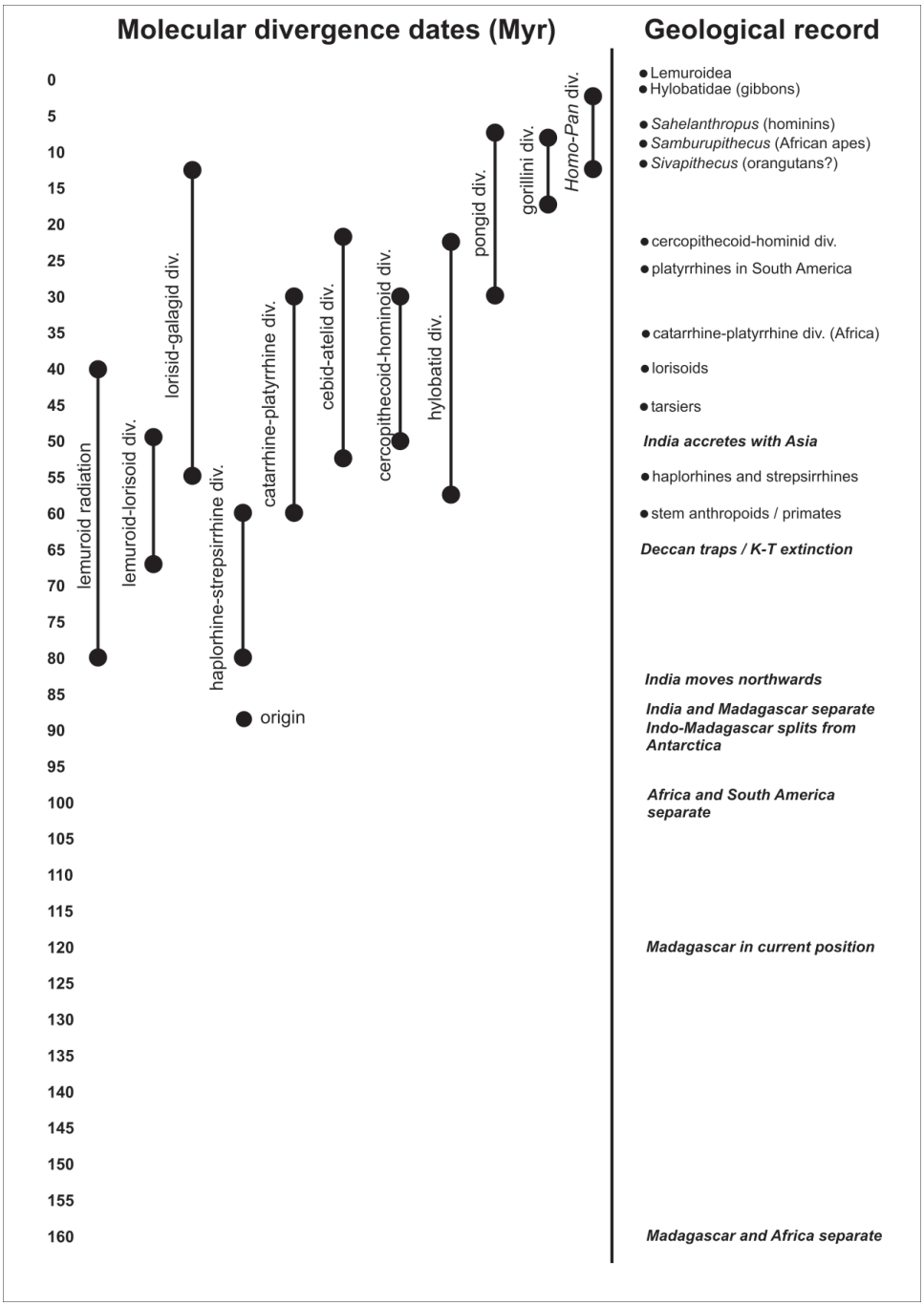

Figure 1 – Molecular estimates of the ages of the bifurcations of the major primate lineages, along with the data concerning first fossil appearances and other major geological events.

1976). The situation is particularly difficult to understand in the case of the invasion of Madagascar from Africa, for this appears to have occurred in defiance of current and wind directions (Masters *et al.*, 1995; Stankiewicz *et al.*, 2005). Nevertheless, in the absence of any more feasible alternative, these scenarios remain popular among primate evolutionists.

Africa was traditionally considered the birthplace of the primate clade, chiefly because the oldest primate fossils had been found there (Gingerich, 1990; Sigé *et al.*, 1990). It was also proposed as the place of origin for the anthropoid (Godinot and Mahboubi 1992; Godinot, 1994) and lemuriform (Yoder *et al.*, 1996; Sieffert *et al.*, 2003; Roos *et al.*, 2004) radiations. However, the observation that the primates do not form part of the clade of endemic African mammals (Eizirik *et al.*, 2001; Springer *et al.*, 2003; Murphy *et al.*, 2004), along with the fact that the sister taxa to the primates (Scandentia, Dermoptera, Plesiadapiformes) have apparently never been present on the African continent (Beard, 1998), have shifted attention from Africa to Asia as the source continent. All three of the sister taxa mentioned above either still occur in Asia, or did so in the distant past. However, Asia was isolated from other landmasses by considerable stretches of water until well after the primate radiation had begun. Until *c.* 55 Myr ago, Europe and Asia were separated by the Obik Sea (Smith *et al.*, 1994; Ni *et al.*, 2004), and for *Altiatlasius* to have inhabited Morocco 60 Myr ago, its ancestors must have crossed the not insubstantial Tethys Ocean that divided Africa from Eurasia (Smith *et al.*, 1994). At approximately this time, the lemuriform ancestors, too, would have had to make the journey not only to Africa, but to Madagascar as well. Either our early primate ancestors were extremely keen seafarers, or there is an important aspect of the puzzle missing.

Krause and Maas (1990) and Martin (2003) have suggested that primates originated either on India when it was adrift in the Indian Ocean, or on Indo-Madagascar when it was still a single landmass. The problem with both of these scenarios is that India docked with Asia only *c.* 50 Myr ago (Rowley, 1998), which makes it difficult to understand how primates might have arrived in North Africa by 60 Myr ago. A possible land bridge caused by the Deccan traps may account for this problem (de Wit, 2003; Masters *et al.*, 2005; Masters and de Wit under revision), but the fact that the primate sister groups have never been found on Madagascar needs to be explained for this model to be feasible.

Despite the phenomenal growth in molecular and fossil data that has occurred in recent years, major aspects of early primate evolution remain a mystery. The answer is surely out there, and as we continue to pick away at the traces and clues that have been left behind in karyotypes, molecular sequences and the fossil-bearing strata of the vast unexplored parts of the world, we must as surely find it.

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Personal remembrance: the origin and development of Primate Cytogenetics and evolutionary Karyology

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At my age I find myself rethinking the determining factors of my major scientific life choices and interests: studying Natural Sciences and being fascinated by Primate Evolution and Antropology.

Certainly the country place where I was born and spent my early youth (Quarto di Castello, near Florence) was the proper environment to stimulate my naturalistic interests. My mother's love for plants and gardening also played a great role. Another important stimulus for becoming an anthropologist and primatologist undoubtedly came from the frequent discussions I heard, when I was six/seven years old, between my father and the priest of the parish, don Raffaele Stiattesi. In spite of their different backgrounds and ideas, they both agreed in the uniqueness of Man due to his erect posture and the opposability of the thumb. They were responsible for my first experimental attempt: to impose the erect posture on my dear dog!

The wartime between 1942-1944 made some drastic change to my family life. My father was opposed to the fascist regime and I was strictly attached to him in his humanitarian and protective actions of Jewish and other escaped people against the nazi occupation. The basement of our house was filled of people of different origin.

For difficulties in getting to the regular school, I spent the last year of my elementary education (1944-1945) in a private institute close to home, where my mother was teaching. After three years of secondary school ('46-'49), spent at the "Scuole Pie Fiorentine", I spent the five years of high school ('48-'53) at the "Liceo Scientifico Leonardo da Vinci" when it was still located in via Masaccio. Then it seemed like unending hours of tedious history and philosophy interchanged with boring Latin lessons and memorizing silly poems. The only exciting discipline apart mathematics and physics was natural sciences taught by Ettore Tondini from whom I learned the basic principles of insect taxonomy and ethology.

My interest in Natural Sciences determined the choice of my enrolment at the University. My decision created some concern for my father who worried about my future. In the end I convinced him of my firm decision. Unfortunately he died when I was on my second year (1955).

The Faculty of Natural Sciences in Florence at that time (1950-1960) was stimulating for the high standard of its teachers: Valdo Mazzi, pupil of Edoardo Beccari, in Histology; Michele Dalla Corte in Physics; Ignazio Fazzari in Human Anatomy; Giuseppe Colosi in Zoology; Alberto Chiarugi in Botany and Genetics; Emanuele Padoa in Comparative Anatomy; Guido Merla in Geology, Guido Carobbi in Mineralogy; Cocco in Petrography; Augusto Azzaroli in Paleontology; Paolo Graziosi in Anthropology; Raffaele Petrini in Human Biology.

I was most fascinated by the lessons of Botany and Genetics by Chiarugi and I prepared notes of his lessons for our colleagues; this editorial work was approved and most appreciated by him. Chiarugi's interest in chromosomes and his efforts to utilise karyological information for plant taxonomy and phylogeny created a great appeal and I was on the point to enter in his laboratory for preparing my final degree dissertation.

Two independent events induced me to choose Anthropology. One was the meeting with Prof. Raffaello Parenti, at that time teacher of human biology at the University of Pisa and Florence, and the leading theologian in Florence (the subconscious memory of conversations between my father and don Stiattesi probably was still stimulating my mind). The other was the interest in applying genetic and chromosome data to a revision of Primate taxonomy and phylogeny.

At that time, by tradition, anthropological research was mainly dealing with human osteology. My dissertation was on a group of human Bantu skeletal remains collected by Lidio Cipriani in Mozambique. Part of my research was on statistical analysis of the osteological data. These procedures were important knowledge for my future studies on quantitative morphology and genetics. The dissertation was discussed on October 1957.

The premature death of my father caused financial difficulties in the family, therefore I started to teach sciences and mathematics in a school of Greve in Chianti. In the meantime I was accepted by Prof. Fazzari in his laboratory of human anatomy, with the position of voluntary assistant, spending also some time at the National Museum of Anthropology and Ethnology in Palazzo Nonfinito.

My interest in human and primate biology was constantly increasing. I updated the information collected for my "tesina" on primate and human chromosomes and I wrote a short note ["Tavole cromosomiche dei Primati"] which Chiarugi accepted for publication in "Caryologia" (1958) and this was my first publication. Encouraged by Chiarugi, I started to apply for different scholarships and to write to different Italian biologists and geneticists for suggestions and support. Giuseppe Montalenti invited me to visit his Institute in Naples, but just when I was planning to go to Naples in early February (1958) I received a letter from Prof. Adriano Buzzati Traverso offering me a job as secretary, with the possibility to work part-time on my research project. On the 20th February (1958) therefore I found myself working in the leading laboratory of Genetics of the University of Pavia.

Needless to say, my days in Pavia were exciting and have been terribly important for developing my scientific mentality. In April '58 I prepared the first Karyotype done in Italy and the chromosomes were mine from a tissue culture line of connective tissue I obtained from my own leg (f.1).

In the summer of 1958 I made a tour on several zoological gardens in Europe to have direct knowledge of the different species of Primates. During these visits, I developed the testing with PTC in order to survey the sensitivity to this substance in Primates, which have an hereditary basis in humans.

The PTC testing of Primate gave me a certain popularity both in the zoological gardens of Europe and at home. Buzzati wrote an article on my PTC testing on Primates on a weekly magazine ("L'Espresso"). I became popular among the colleagues also by an amusing cartoon, drawn by a technician of the Institute, on my adventure with the chimps. The results were presented at the Società Italiana di Antropologia ed Etnologia in Florence. The two years I spent in Pavia were very creative and formative for my scientific mentality. Buzzati, with his open mind, appreciated, encouraged and helped my research interest and I owe to him my results and the starting of my career.

The contacts with the Directors of Zoological Gardens in Europe gave further support to my research program. In the Zoological Garden of Basel in those days was born Goma, the first Gorilla born in captivity, and I had the great opportunity to follow her early biological and ethological development.

In 1959 Lejeune discovered the presence of the supplementary chromosome (21) in the Down's syndrome and I had the opportunity to be received for a few days in his laboratory. Human cytogenetics became a very popular subject for medical genetics in those years. In spite of several offers to be involved in medical research haring done some chromosome studies in pathological cases, I continued to concentrate my interest in Primate cytotaxonomy. I collected tissue sample from zoo of Turin and Rome making culture and the preparation in the laboratory of Pavia.

In March 1960 I obtained my second degree, this time in Biological Sciences, at the University of Firenze. In my thesis I described my own chromosomes with an attempt to use the electron microscope.

My results published in Italian on the PTC testing in Primates in the meantime was requested, translated and distributed in the US by the Genetic Department of the National Institute of Health.

In spring-summer 1961 I organised a campaign for collecting tissue samples in Chester and London Zoos, using as base the tissue cultures laboratory of David Hughes at the Cancer Laboratory of Fulham Road in London, and in Antwerp and Leiden Zoo, using the Human Genetic Laboratory facilities in Bruxelles of Lucien Koulisher. Other sample where collected in zoos of Switzerland and Germany, culturing the tissue and attaining the Karyotype in a portable laboratory I created on my car.

In April 1961, through the suggestion of Osman Hill, I was invited as main discussant at a conference on Primates organized by the Zoological Society of London, to present my PTC data. In that occasion I met Jane Goodall, who was just back from her first expedition in Africa, and a leading palaeanthropologist Louis Leakey who invited me for a visit at his excavation site in the Rift Valley in Kenya, which unfortunately I postponed for 20 years.

In late spring 1962 I received an invitation by my earlier professor of histology Valdo Mazzi to teach the course of Anthropology at the University of Turin which was

a great opportunity and a great honour at my age. I was the youngest professor in biology at the University of Turin and probably in Italy. In the rooms of the old Institute and Museum of Anthropology and Ethnology of via Accademia Albertina 17 from the fall of 1962, I created a karyological laboratory and continued studying the chromosomes of Primates collected previously. Thanks to the help of its owner Mr Arduino Terni and its director Prof. Alula Taibel I also create laboratory of Primatology in the Zoological Garden of Parco Michelotti, in homology with the Primate Center I started the previous years at the Zoological Garden of Rome. With Melchiorre Masali, we started researches on problems of primate posture (studying the Primate ear bone), following the ideas of cranial evolution of A. Delattre.

But it was at the Conference of the Italian Zoological Union in Bologna on the fall of 1963 that I obtained the first official mention to my work in Italy. Prof. Benazzi, at that time professor of Zoology at the University of Pisa, in his opening lecture, with my surprise, mentioned my chromosome research on Primates as a leading research project for reviving Anthropology. Anthropology in fact at that time was considered a deteriorated biological discipline dealing mainly with osteological remains for archaeological support. The ethnographic and anthropological collections existing in different Museum were left on a side with no care in spite of the internationally well known books of Renato Biasutti and Vinigi Grottanelli.

On September 1963 I attended the International Congress of Genetics in Den Haag, where I presented a poster with some results of my chromosome studies in Primates. In that occasion I had the opportunity to meet Jacob Warman who invited me to spent an year at his Genetic Department at the Hebrew University in Jerusalem.

On July 1964 I attended with other Italian colleagues the VII Congress of the International Union of Anthropological and Ethnological Sciences in Moscow. It was for me a great experience to meet important leaders in Primates and human evolution as Phillip Tobias, Alfonso de Garay, S. Kawamura. They were interested in my chromosome and genetic studies on Primates and we started to discuss the need for a publication focusing the information available on the subject.

From Moscow almost directly I went to Jerusalem. I stayed in Israel from August '64 to March '65. The Jerusalem experience was great for my enrichment of historical and archaeological information and for the anthropological knowledge. I had a real contact with the Jewish culture (I even met Moshè Bayan at his excavation at the Temple Mount) and interesting contacts with the people of the Arab triangle through Palestinian students of the Hebrew University. Moving with an Italian car I became familiar with practically the entire territory of Palestine and its problems. I spent also some days with a Bedouin group in the Negev desert. This anthropo-ethnological interest for the diaspora and the problems of the new state of Israel was not very productive for my chromosome studies except for a research project on the meiotic chromosome in a polymorphic species of *Gerbillus*.

From the 10th to the 25th October 1965, under the invitation of the Rector of the University of Warsaw, I was invited to visit and give lectures in different biological Institutes in Poland. From Poland I went directly to the US, invited by Dr H. Vagtborg

to visit the South West Foundation for Primate Research in San Antonio, Texas, and present chromosome data on Baboons. In that occasion, I took the opportunity to visit as many Primate Centres as possible. In only 25 days I was in New York, San Antonio, in Louisiana, in Denver, San Diego, Santa Barbara and Davis in California, and finally in Atlanta at the Yerkes Primate Center. I used to travel by internal flights and by night with the Greyhound bus. It was an exciting experience both at the tourist level and for the opportunity of meeting primatologists and anthropologists with whom I was in touch and I keep further contacts.

In the meantime the faculty of Science of the University of Turin in support of my activity opened a new course in Primatology, which was the first to be activated in Italy, and I think also in Europe.

In October 1966, under the invitation of his director, Bill Montagna, I went to the Oregon Primate Center in Beaverton to establish a tissue culture laboratory of comparative cytogenetics with a Spanish colleague, Dr José Egozcue. In that occasion I could visit again the Yerkes Primate Center in February '67 where I collected the chromosome of *Nasalis larvatus* and, with Arthur Falek, prepared a description of the human meiotic chromosomes.

In March of the same year I was invited by Alfonso De Garay to give lectures at the National Museum of Anthropology in Mexico City at the genetics laboratory of the Atomic Energy Commission where I studied the chromosome of a very special local race of hairless and dwarf dog (thexuili dog).

In June of the same year (1967), I organized in Turin a round table discussion on "Taxonomy and Phylogeny of the Old World Primate with reference to the origin of Man", with the contribution of Phillip Tobias, Ralph von Koenigswald, Hans Khun, Christian Vogel, Osman Hill, Gabriella Manfredi Romanini, Morris Goodman, Vincent Sarich and Neil Tappen, which resulted in the publication of a book by Rosenberg and Sellier (1968). This meeting was followed by three others, one in Erice in July 1970 on "Comparative Genetics in Primates and Human Heredity", another in June 1972 in Montaldo castle, near Turin, on "Perspective in Primate Biology" with cooperation of Chiara Bullo and in August 1973 in Detroit "Conference and Workshop on Comparative Karyology of Primates as session of the IX Congress of the IUAES.

It was in that occasion coming back from Canada that I visited the laboratory of Prof. Caspersson in Stockholm and I developed the idea of a circular model of chromosomes and of using trypsin for producing bands on chromosomes. But I spread the early results too much before publication and I lost the priority.

In the meantime, from 1971, I was nominated visiting professor to teach a course on "Problems of Human Evolution" at the University of Toronto. The teaching in Toronto promoted my cooperation with the Academic Press of London and I published my first English book "Evolution of Primates" which appeared on 1973. It was also in those years that I prepared "The Atlas of living Primates", just to try to unify the Primate nomenclature. It has been a great effort, which has been widely spread among the zoological gardens. The Atlas was planned as the first of a series in which to synthesize comparative biological data of different species of Primates. The others

dealing with skeletal morphology and biological data are however still waiting! To some of you to go on!

My teaching in Toronto, from 1971 to 1975, even if done for a short time every year (sometimes only for few weeks) create interesting contacts with Canadian students some of which actively cooperated with me also working in Turin and became later professional anthropologists as Anne Zeller, David Shafer, Shelly Saunders, Albert Molto both in U.S. and in Canada.

While in Toronto I took part to the Int. Biological Program "Man in the Biosphere" with an expedition organized by prof. David Hughes, to study the Eskimo community of Igloolik. In the winter 1972 I therefore organize the cytogenetic laboratory in the North East Territory of Canada, to study the eskimo chromosomes in order to detect environment contamination. Spending almost two winter months in an Eskimo settlement I made also some interesting observation on Eskimo and this was an important Ethnographic experience.

During my stay in Toronto I was invited to visit several Canadian and US Universities to give lectures or seminars. It was in one of these excursions at the Penn State University, invited by prof. P.T. Baker, that I met a young student, a certain Roscoe Stanyon and this was the start of a new story which seem to continue today!

These contacts on problems related to Primate and human evolution and the meetings and discussions which I could develop with different leading biologists at the time such as Theo Dhobzansky, B.G. Campbell, Ch. Washburn, Ernst Mayer, W.C. Osman Hill, Eduard Bonné S.J., A. Delattre, J. Itani, G.H.R. von Koenigswald, Sergio Sergi, P. Valentine Tobias helped me to formalize the idea of a journal devoted to "Human Evolution" and after contacts with different publishers, I found in the University Press of London a proper supporter. I started therefore the Journal of Human Evolution which published its first issue in 1972.

The International Primatological Society requested to have its VIII Congress in Italy, which was held in Florence in 1980.

In all these endevours I had a friendly support by my colleague in Turin Melchiorre Masali, who was always ready to assist the Turin students in my absence, apart the development of all the research work on the Egyptian skeletal material with the collaboration of Domenico Davide and Renato Grilletto. To them I owe a large part of this effort, as to Francesco Fedele with whom I developed prehistoric research in Piedmont and with Emma Rabino Massa with whom we started the research of mummified human tissue and human genetics in isolated populations of the Piedmont area.

In spite of having received an offer of a full position in Toronto and the chair of Anthropology in Paris an the retirement of George Olivier, my ambition was to come back to Florence at Palazzo Nonfinito to succeed Paolo Graziosi and to promote from there the early ideas of Paolo Mantegazza, the founder of Florence Institute, and the starting of Anthropology in Italy on 1865 and of my Master Raffaello Parenti, of Anthropology as the "Natural History of Man". This my ambition was frustrated by the interest of the colleagues who use to consider Anthropology only as a "service for archaeology" and this theoretical conflict was the origin of many difficulties which were connected

with my transfer to Florence and with the following situation. But this is a different history to write and clarify.

From 1981-1987, with the help of Roscoe Stanyon, Luca Sineo, Daniela Romagno, Andrea Camperio and other talented students, we developed a laboratory of comparative biology of Primates and on chromosomes studies the technical development on chromosomes of the last 50 years is the origin of these days conference. I really thank Roscoe and Luca for having request me to put together my early adventures with non Human Primates and my carrier as antropologist.