Chapter 4

# Applying Cytogenetics in Phylogenetic Studies

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Additional information is available at the end of the chapter

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**Abstract**

Cytogenetics, with its fundamental role in the field of genetic investigation, continues to be an indispensable tool for studying phylogenetics, given that currently molecular evolutionary analyses are more commonly utilized. Chromosomal evolution indicated that genomic evolution occurs at the level of chromosomal segments, namely, the genomic blocks in the size of Mb-level. The recombination of homologous blocks, through the mechanisms of insertion, translocation, inversion, and breakage, has been proven to be a major mechanism of speciation and subspecies differentiation. Meanwhile, molecular cytogenetics (fluorescence in situ hybridization-based methodologies) had been already widely applied in studying plant genetics since polyploidy is common in plant evolution and speciation. It is now recognized that comparative cytogenetic studies can be used to explore the plausible phylogenetic relationships of the extant mammalian species by reconstructing the ancestral karyotypes of certain lineages. Therefore, cytogenetics remains a feasible tool in the study of comparative genomics, even in this next generation sequencing (NGS) prevalent era.

**Keywords:** cytogenetics, comparative cytogenetics, fluorescence *in situ* hybridization, genomic *in situ* hybridization, zoo-CGH

## 1. Introduction: chromosomal evolution of mammals

According to fossil records, the radiation evolution of mammals diverged after the K-T boundary (approximately 65 Mya, between the Cretaceous and Tertiary periods, at which most of the dinosaurs were extinct). There are three hypotheses that try to explain such findings: (1) Explosive hypothesis: It is supported by most paleobiologists and states that the genesis and diversification of many phyletic groups ("Orders") diverged after the Cretaceous-Tertiary (K-T)

boundary; (2) Long Fuse hypothesis: It supports the view that Order diversification occurred after the K-T boundary but that genesis occurred in the Cretaceous period, i.e., before the K-T boundary; and (3) Short Fuse hypothesis: It considers the genesis and diversification of Orders to have diverged before the K-T boundary (**Figure 1**) [1].

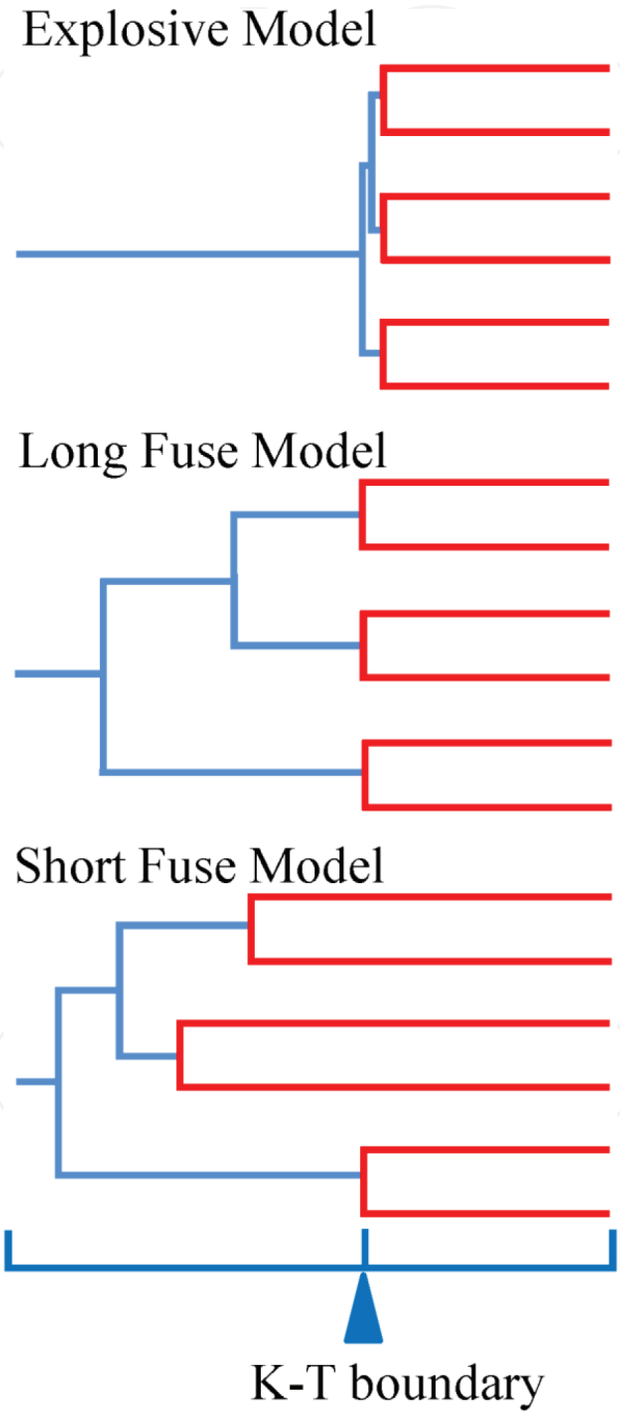


**Figure 1.** Three hypotheses of mammalian interordinal divergences, modified from Ref. [1].

Molecular data indicate that mammalian diversification began in the Cretaceous period, which supports the (2) Long Fuse and (3) Short Fuse hypotheses. However, these data have limitations, including the availability of a single temporal calibration point and the variable evolution rate of different phyletic groups. Due to the lack of representativeness of the samples, this inadequate taxon sampling restricts the use on some, but not all, placental mammals, and it makes the negative correlation between evolution rate and body size difficult to explain. William Murphy and Stephen O'Brien's team made a successful attempt at answering these questions with zoo-fluorescence *in situ* hybridization (zoo-FISH). Currently, the Long Fuse hypothesis seems to be a better match with the evolution of most phyletic groups, but not the orders *Rodentia* and *Eulipotyphla*, which better suit the Short Fuse hypothesis [1].

**Figure 2** presents the phylogenetic tree of placental mammals derived from 16,379 nucleotide sequences (including 19 nuclear genes and 3 mitochondrial genes published by the study team), where opossum is considered an outgroup using the maximal likelihood method, and placental mammals are considered to appear at 105 Mya. When the K-T boundary is labeled with red dashes, we find that "Order" genesis and diversification are events that occur before the boundary.

By comparing the chromosomal break point of multiple species, including the chromosomal rearrangement of loci discovered via comparative genomics and some genetic sequences from

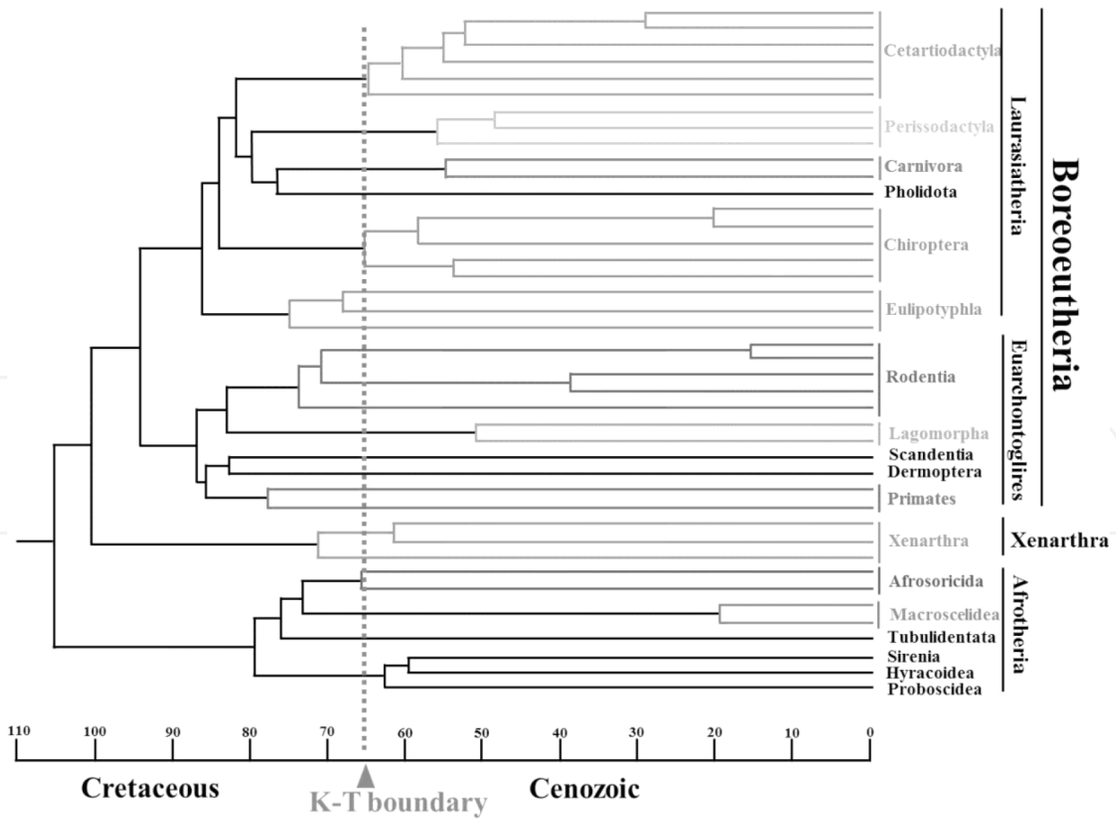


**Figure 2.** Phylogenetic tree of placental mammals derived from 16,379 nucleotide sequences, modified from Ref. [1].

fully sequenced species, we can clearly find that (1) Approximately 20% of chromosomal break points are repeatedly involved in the evolutionary process of mammals. (2) These repeatedly involved break points are primarily located at the centromere and telomere. (3) The number of genes within and near the break point blocks that are involved in chromosomal evolution is higher than the mean of the overall genome. (4) The unique break points unique in Primates are located at repeated segment regions and the ends are surrounded by reversed sequences. **Figure 3** refers to the rate of chromosomal breaking using the chromosomal break points involved in the evolution of mammals.

The result shows that the chromosomal rearrangement rate before the K-T boundary is 0.11–0.43/My, and this rate is doubled to quadrupled for Primates and increased fivefold for Rodentia [2, 3].

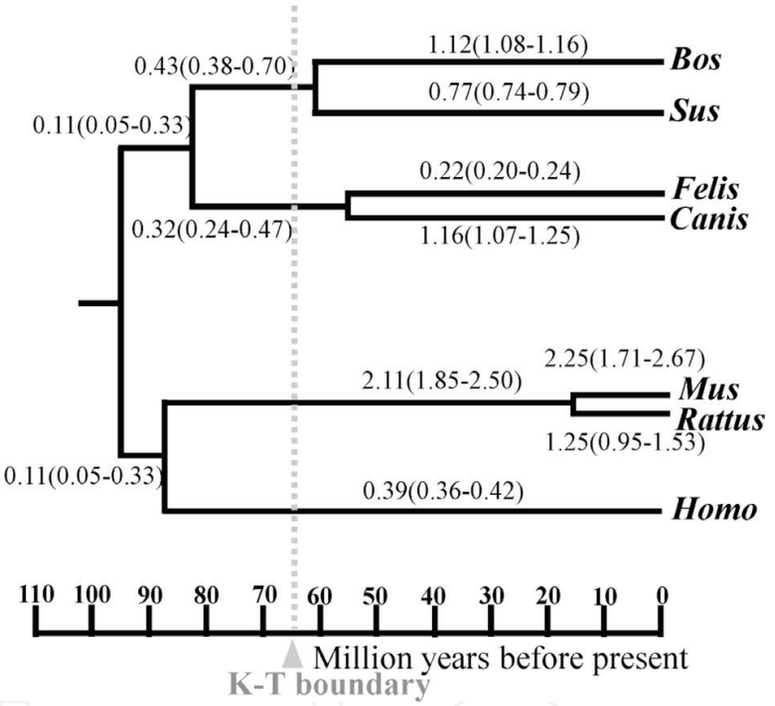


**Figure 3.** Rate of chromosomal breaking using chromosomal break points involved in the evolution of mammals, modified from Ref. [3].

## 2. How to apply molecular genomics in the study of evolution and parental relationships

### 2.1. Zoo-FISH

Comparative mapping: It is a method for comparing the location of homologous genes of different species to explore the evolution of genomes; zoo-FISH is an extension of such technology. This method assesses the overall chromosomal similarity among all mammalian

orders and becomes a powerful tool to study genomic evolution. The possible mechanism and factors related to mammalian genomic evolution can be understood with Metatheria and Eutheria studies.

When conducting zoo-FISH, partial or whole chromosomes are obtained through the sorting of fluorescence-labeled cells or microscopic extraction. DNA extracted from this specific chromosomal block is subject to degenerated oligonucleotide primed-PCR (DOP-PCR), then labeled with fluorescence to produce probes, and hybridized with the chromosome of the species of interest. Due to the resolution of zoo-FISH, which is approximately 10 Mbp (megabase pairs), this method may underestimate the real rearrangement events on the chromosome. However, zoo-FISH has revealed some interesting facts: many chromosome blocks of different species are rather conservative, and the similar chromosome blocks from a common ancestor are called synteny blocks. For example, one somatic chromosome of the gray-headed flying fox (*Pteropus poliocephalus*) possesses synteny blocks that are also found in *Homo sapiens* (HSA) chromosome 3 and HSA 21. These HSA3+21 synteny blocks form the primary synteny blocks of placental mammals, i.e., it is a characteristic that was present in a common ancestor and all researched Eutheria members [4].

One of the most important applications of zoo-FISH is to study the speed of chromosomal rearrangement when studying genomic evolution [5]. Using the phylogenetic tree that is based on fossil evidence, we can understand the rate of movement and rearrangement of synteny blocks in the chromosomes of two species. When there are difficulties in bi-directional zoo-FISH, monodirectional zoo-FISH can provide with key information or a new understanding. By comparing the chromosomal synteny blocks of indicator mammals and Aves, the occurrence rate of chromosomal rearrangement was found to be fixed at approximately 1–10/Mya [6]. The chromosomal rearrangement rate is shown in **Figure 4**, and the rate may differ with lineage genesis and at different evolutionary stages.

Three important stages of chromosomal rearrangement are found (**Figure 4**): The first stage (1–3 Mya) < 0.2/My, the second stage increased to 1.1/My, and in the third stage, the rearrangement rate greatly varied in nonrodents. For example, humans, *Carnivora* and *Soricidae* are of low rearrangement (< 0.1/My), swine, cattle, equine and dolphin are moderate (0.1–0.3/My), and large apes are relatively fast (1.5–2.3/My). The chromosomal evolution in Rodentia is the fastest, and the possible explanations include (1) population size (a larger population provides more genetic modification); (2) different genetic composition (more than 50% of the mammalian genome is repeated sequence, whereas it is only 15% repeated sequence in birds), and (3) different generation times (a short generation time indicates more mitotic events). From chromosomal evolutionary evidence, scientists believe that the evolution of mammalian genomes was inconsistent. The evolution was faster for Rodentia, bears, canines, cattle and few big apes, whereas it was relatively slow for cats, ferrets, badgers, dolphins and humans. In addition, it is worth noting that zoo-FISH, like other FISH-based methods, cannot identify intrachromosomal rearrangements (such as inversion). It was believed that the incidence of interchromosomal rearrangement events is higher than intrachromosomal events, but a sequential comparison revealed that it is the opposite for feline and cattle. In a zoo-FISH using human DNA as probe, some recombination events were lineage-specific. For

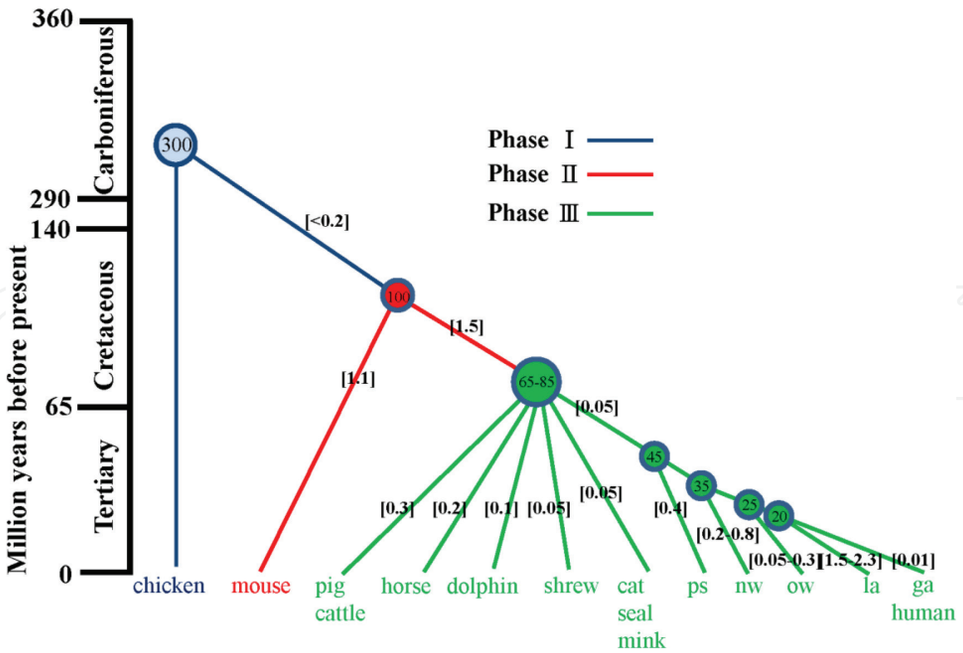


**Figure 4.** Three phases of chromosomal rearrangement rate. The numbers in the circles are the time (Mya) of divergence of common ancestors, and the numbers in the brackets indicate the rates of chromosomal rearrangement per Myr. (ps= prosimians; nw= new world monkeys; ow = old world monkeys; la = lesser apes; ga = great apes.) Modified from Ref. [6].

example, "15 + 19" (suggesting synteny blocks similar to HSA15 and 19) is Cetartiodactyla- and Perissodactyla-specific, "3 + 19" is Carnivora-specific, and "14 + 15" is widely seen in Aves and placental mammals other than Rodentia (**Figure 5**).

The other application of zoo-FISH is to reconstruct primitive karyotyping. **Figure 6** shows the estimates of ancestral placental mammal ($2n = 50$), primate ($2n = 50$), and Carnivora ($2n = 42$) karyotypes as well as each chromosome and its relationship with human syntenic-associated chromosomes.

It is worth noting that the study shows that the chromosomal karyotype of primitive placental mammals is $2n = 50$, while Svartman et al. [7] also found that the karyotype of Hoffmann's two-toed sloth (*Choloepus hoffmanni*), a Xenarthra member, possesses a karyotype close to the primitive one. This result suggests that the most primitive placental mammals may be Xenarthra, not Afrotheria. Both groups originated in the southern hemisphere, and this result does not violate Murphy's hypothesis on the origin of mammals. That is, when the part of supercontinent Gondwana in southern hemisphere had not yet separated and formed Africa and South America, placental mammals diverged and Xenarthra and Afrotheria appeared; later, the ancestors of Laurasiatheria and Euarchontoglires diverged and migrated to the northern hemisphere.

The karyotype of Hoffmann's two-toed sloth: The blocks that are syntenic to HSA are labeled on the left of each chromosome. For example, Chromosome 1 is syntenic to HSA1, but it is not syntenic to other HSA chromosomes, while Chromosome 6 contains synteny blocks that are similar to those found in HSA3 and HSA21 [7]. These karyotypes are presented in **Figure 7**.

| Superorder/order | Species | Syntenic block associations | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 12/22 | 16/19 | 14/15 | 3/21 | 7/16 | 4/8p | 5/19p | 1q/10q | 3/19p | 10p/12 | 5/21 | 18/22 | 11/19 | 2/8/4 | 3/20 | 18/19 | 2/8 | 7/10 | 4/20 | 2/20 | 1/10p | 20/15 | 12/8 | 10/16 |
| Cetartiodactyla | Dolphin | ● | ● | ● | ● | ● | | ● | | | | | ● | | | | | | | | | | | |
| | Indian muntjac | ● | ● | ● | ● | ● | | ● | | | | ● | ● | | | | | | | | | | | |
| | Pig | ● | ● | ● | ● | ● | ● | ● | | | | | | ● | | | | | | | | | | |
| | Camel | ● | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | | | |
| | Cow | ● | ● | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | | |
| Perissodactyla | Rhinoceros | ● | | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | | |
| | Zebra | ● | ● | ● | ● | ● | ● | ● | ● | | | | | ● | | | | | | | | | | |
| | Horse | ● | | ● | ● | ● | ● | ● | ● | | | | | ● | | | | | | | | | | |
| Carnivora | Cat | ● | ● | ● | ● | ● | ● | | | ● | ● | | ● | | | | | | | | ● | | | |
| | Dog | ● | | ● | ● | ● | ● | | ● | ● | | | | | | | | | | | | | | |
| | Giant panda | ● | ● | | ● | | ● | | | ● | | | | | | | | | | | | | | |
| Pholldota | Pangolin | ● | ● | ● | ● | ● | ● | | ● | | | | | | | ● | | | ● | | | | | |
| Chiroptera | Bat | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | | | | |
| Eullpotyphla | Shrew hedgehog | ● | | ● | ● | ● | ● | | | | ● | ● | | | | | | ● | | ● | | | | |
| | Common shrew | ● | ● | ● | ● | ● | ● | | | ● | | | | | | | | | | ● | | | | |
| Rodentia | Gray squirrel | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | ● | ● | ● | ● |
| Lagomorpha | Rabbit | ● | ● | ● | ● | ● | ● | | | | | | | | | | | | | | ● | ● | | |
| Scandentia | Tree shrew | ● | ● | ● | ● | | | | | | | | | | | | | | | | | | | ● |
| Primates | Howler monkey | | | ● | ● | | | | ● | | | | | ● | | | | | | | | | | |
| | Slow loris | ● | | ● | ● | ● | | | | | | | | | | | | | | | | | | |
| Xenarthra | Two-toed sloth | ● | | ● | ● | ● | ● | | | | | | | | | | | ● | ● | | | | | |
| | Anteater | ● | ● | ● | ● | ● | ● | | | | | ● | | | | | | ● | ● | | | | | |
| Afrotheria | Aardvark | ● | ● | ● | ● | ● | ● | | | | ● | ● | | | ● | ● | | | | | | | | |
| | African elephant | ● | ● | ● | ● | ● | | | | | ● | ● | | | | | ● | | | | | | | |
| | Golden mole | ● | ● | ● | ● | ● | ● | | | | ● | ● | | | ● | | | | | | | | | |

**Figure 5.** Human synteny block associations observed in other placental mammals by zoo-FISH, and positive results were indicated by the solid circles, modified from Ref. [4].

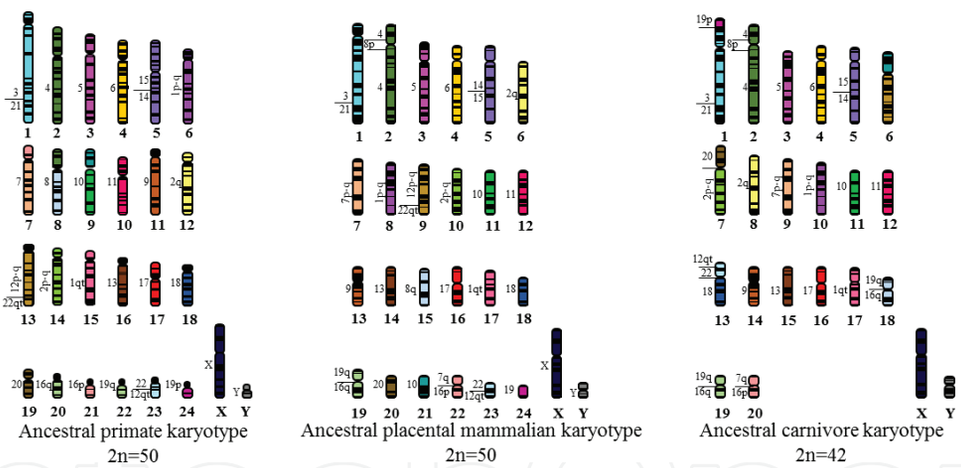


**Figure 6.** Assumed ancestral karyotypes. Numbers at the left side of the ideogram indicate the regions homologous to human karyotype segments, modified from Ref. [4].

## 2.2. How is chromosomal recombination fixed in evolution?

Theoretically, chromosomal rearrangement may lead to meiotic errors and reduced fertility. It is fundamentally a harmful genetic variation, and most rearrangements are difficult to pass on in a population. However, (1) genetic drift, (2) Muller's ratchet mechanism or (3) hitch-hiker make it possible to keep some chromosomal recombination (beneficial mutations may be eliminated due to the selection of other loci, whereas harmful mutations may be preserved due to the selection of other beneficial loci).

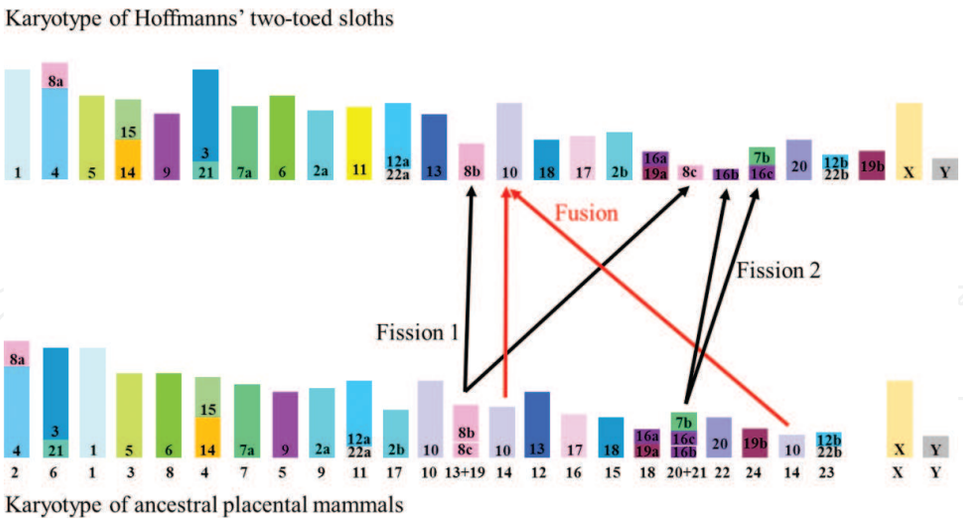


**Figure 7.** The karyotype of Hoffmann's two-toed sloth is arranged from left to right in the order of chromosomal number, the number in the column refers to the number of HSA it is syntenic to, and the diagram below karyotype of ancestral placental mammals describes the synteny blocks in sloth chromosome. We can find that both are quite similar but one is subject to two fissions and one fusion, modified from Ref. [7].

### 2.3. The importance of studying "the weird mammals"

The genome of most mammals contains approximately 3 billion nucleotides ($3 \times 10^9$ bp), but the number of chromosome varies greatly. For placental mammals, Indian muntjac possesses as few as $2n = 6$, while South America rodents possess $2n = 92$; and for opossum, swamp wallaby possesses as few as $2n = 10$, while rufous rat kangaroo possesses $2n = 32$. Long, conservative synteny blocks are found in placental mammals. For example, mice and humans share 116 synteny blocks, and it is estimated that approximately 94 rearrangement events have occurred.

Infraclasses Eutheria (placental mammals) and Metatheria (opossum) diverged at approximately 130 Mya, and their subclasses, Theria and Prototheria (i.e., monotreme), diverged at approximately 170 Mya. Fossil studies show that the radiation evolution of placental mammals (20 orders, including more than 4600 species) occurred in the Cretaceous period (approximately 60–80 Mya). By comparing the differences in the genomes of various animal populations, especially those that play specific roles in evolutionary history (Jennifer Graves, an Australian scholar, called them "the weird mammals"), such as monotreme, opossum and fast-evolving rodents, we can learn more about the evolution progress of mammals.

## 3. The innovative application of zoological comparative genomic hybridization (CGH) in phylogenetics

Placental mammals include four major lineages: (1) Afrotheria, which includes the orders Sirenia, Hyracoidea, Proboscidea, Tubulidentata, Macroscelidea and Afrosoricida; (2) Laurasiatheria,

which includes orders Eulipotyphla, Carnivora, Pholidota, Perissodactyla, Cetartiodactyla and Chiroptera; (3) Euarchontoglires, which includes Rodentia, Lagomorpha, Primates, Scandentia and Dermoptera; and (4) Xenarthra [8]. Currently, there are disputes and uncertainties in the phylogenetic relationships and the true origins of each order in these four lineages. We attempt to define the phylogenetic relationship of the orders Pholidota, Carnivora and Xenarthra using genomic *in situ* hybridization, which was used to determine such relationships for plants. In fact, there is a similar technology called "DNA-DNA hybridization," developed by Sibley and Ahlquist [9]. The basic premise of DNA-DNA hybridization is that a single strand is obtained from the DNA double helix of each species, and when the single strands are hybridized, the binding of the strands from two different species will be much stronger and their associated melting temperatures will be higher when they have a closer relationship. Radioisotope labeling is used to verify the binding as reformation of the double helix or the combination of single strands from two compared species. This technology was applied in the determination of the phylogenetic relationship between Primates and Aves. This technology revealed that in hominoids, humans are closer to chimpanzees than to gorillas or orangutans (**Figure 8**).

In this "DNA-DNA hybridization," the DNA of two species was cut into small chunks of 600–800 bp before mixing. Unfortunately, this technology was unable to prevent errors that were caused by the existence of paralogous sequences instead of orthologous sequences. The result was used for trending, similar to zoo-GISH, but it was not designed for accuracy. On the other hand, analyses that are focused on one or more genes that are present in the evolutionary history of only a few loci, lack a bridge to connect them. We are looking for a tool that is capable of not only whole genome and individual gene trending, but also larger block trending for

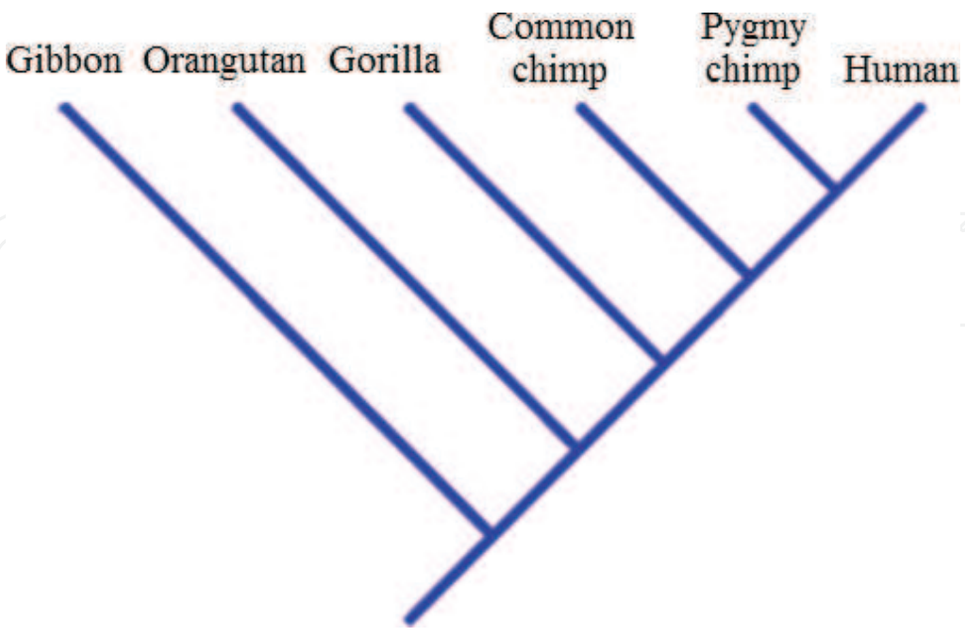


**Figure 8.** Phylogenetic relationship between Primates determined by DNA-DNA hybridization, modified from Ref. [9].

genomes, and even positioning. Therefore, the author chose to apply a mature technology from the study of human neoplasms called "metaphase comparative genomic hybridization (CGH)" to the study of phylogenetic history.

## 4. The history and prior applications of CGH

In 1992, Dan Pinkel's lab at UC San Francisco published an innovative technology named CGH [10]. In this method, tumor and normal cellular DNA probes were labeled with red and green fluorescence, respectively. They were then hybridized with normal cells in metaphase and competed with each other in incorporating with normal chromosomes. Yellow is observed when red and green fluorescence are mixed in equal amounts. A block with more tumor cell genome than the normal reference, i.e., with duplication, turns green, whereas deletion causes it to turn red. This innovative genome-wide technology not only allows positioning, but shows increase or decrease, making it a powerful tool in searching for tumor suppressing genes (which make the amount of tumor cells lower than those of normal reference) or oncogenes (which make the amount of tumor cells higher than those of normal reference), with a resolution of 5–10 Mbp. However, this technology is difficult to operate and requires specific photographic tools and image processing software to calculate the ratio of red and green fluorescence. Recently, gene chips have replaced this technology. Gene chips, formally known as array CGH (the original CGH was renamed as metaphase CGH), have designated probes that are fixed onto a chip [11]. The array CGH probes are derived from the known sequences of target organisms. Array CGH does not involve chromosomal preparation or microscope interpretation. Conversely, metaphase CGH is genome-wide and has chromosome-level resolution, and it is a useful tool when the full genome sequence is unknown. This technology can be applied in more than tumor research; it is also valuable for studying human genetic diseases that are related to repeated or deleted blocks, especially those that are caused by copy number variation [12]. The captured images and the last interpretation are presented in **Figure 9**, where (A) fluorescein (FITC) is used to provide green light; (B) rhodamine for red light; and (C) merged CGH results from one normal sample.

The fluorescence of the green-red ratio was analyzed with software.

We also applied this technology to report a rare case of missing human 13q31 without clinical symptoms [13]. In **Figure 10**, we can see that the human 13q31 block presents more red fluorescence in the block indicated by a straight red line (considered an increase when the green-red ratio is more than 1.2 and a decrease when the ratio is less than 0.8). The label $n$ =18 indicates that the number of Chromosome 13 samples is 18. Therefore, 13q31 is possibly a large polymorphic block in the human genome and this discovery is important in clinical genetic consultations.

Based on the experience of metaphase CGH in human medicine, the author considered the feasibility of applying this technology in interspecies exploration to characterize the

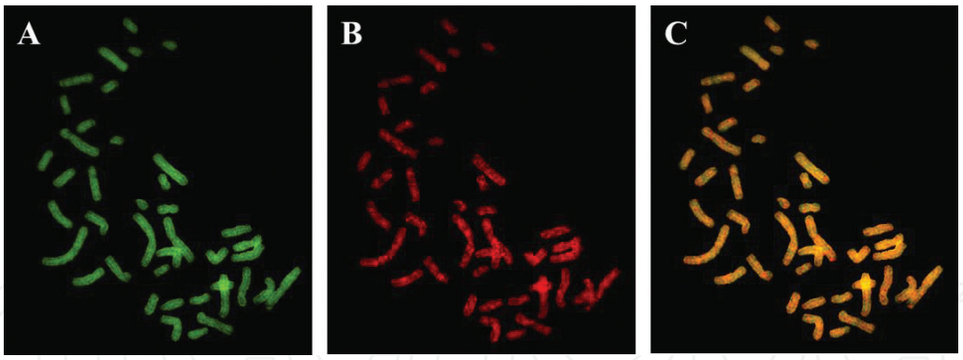


**Figure 9.** The result of metaphase CGH. (A) The signal of FITC-labeled probes. (B) The signal of rhodamine-labeled probes. (C) The merged CGH image of FITC and rhodamine.

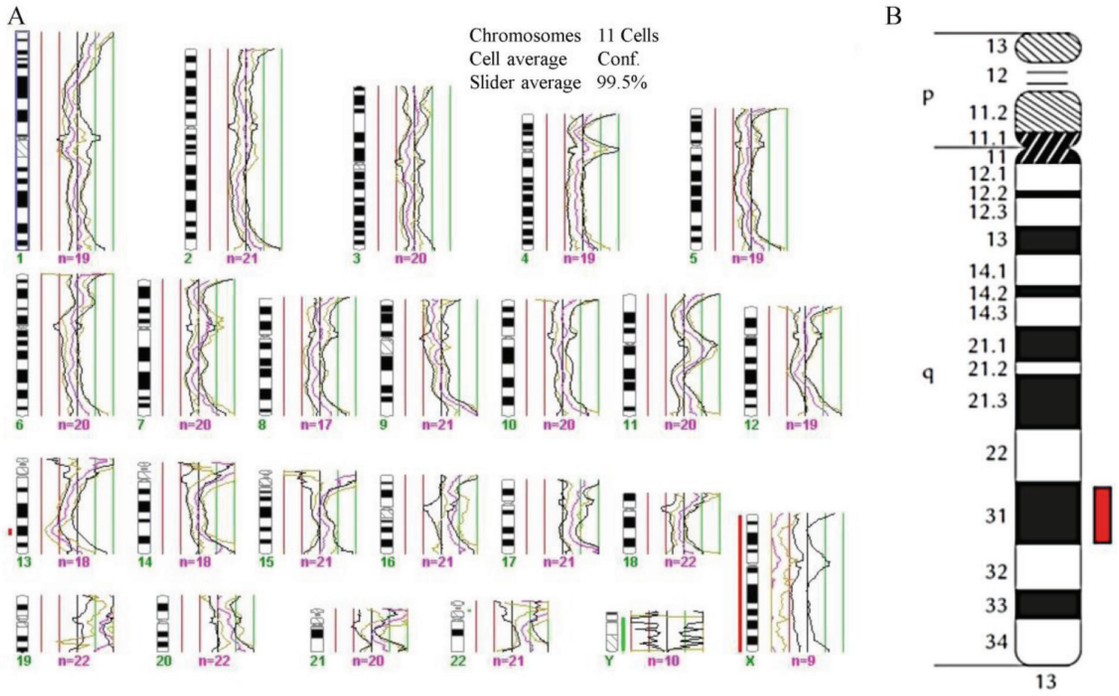


**Figure 10.** Metaphase CGH profiles of the 13q31 deletion case. (A) An interstitial deletion at band 13q31 was found (denoted as a red vertical bar beside chromosome 13). (B) An amplified ideogram of chromosome 13 with the deleted region marked by a red vertical bar on the right.

evolutionary relationships among extant eutherian mammalian taxonomic groups (orders/supraordinal clades). That is, to determine the sequence/genomic similarity of unknown-sequence species A and B with respect to species C, the DNA of species A and B would be labeled with molecules emitting different fluorescence dyes. The ratio of the labeled fluorescence intensities in each chromosome of species C should then reflect regions of sequence similarity to species A versus B. This is a brand-new application and the author named it "zoo-CGH" (**Figure 11**).

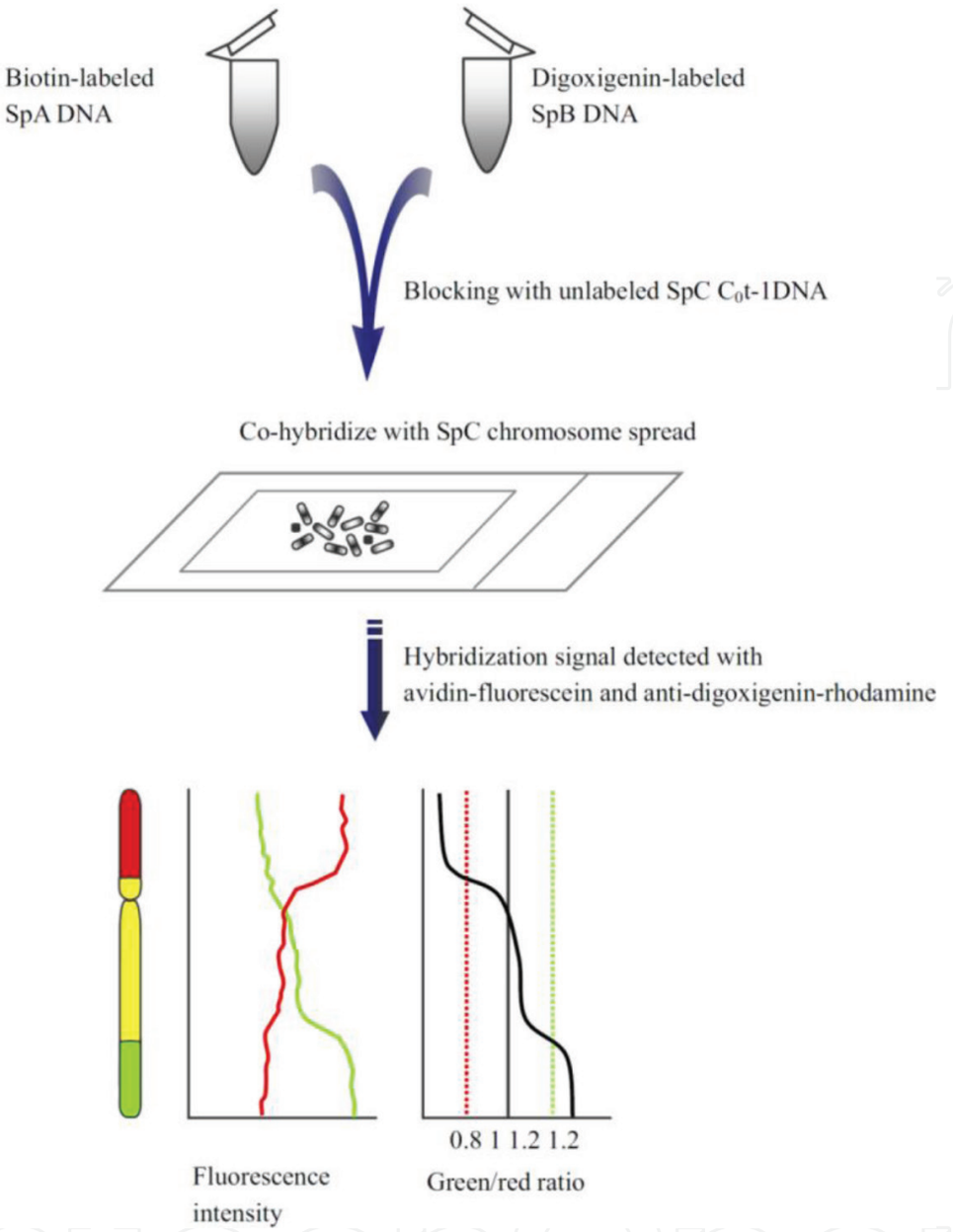


**Figure 11.** Schematic diagram of zoo-CGH. After calibration for genome size, equal amounts of genomic DNA from Species A (SpA) and Species B (SpB), labeled with a green and red fluorophore, respectively, were competitively hybridized to metaphase spreads of Species C (SpC).

## 5. Applying CGH in exploring the relationship between *Pholidota*, *Carnivora*, and *Xenarthra*

Myrmecophagy is a feeding behavior characterized by mainly or exclusively eating ants, termites, or both. This feeding specialization occurs in few eutherian mammals. Myrmecophagous species of Eutheria are in the orders Pholidota (e.g., pangolins, *Manis* spp, Manidae), Tubulidentata (e.g., aardvark, *Orycteropus afer*, Orycteropodidae) and Carnivora (e.g., aardwolf, *Proteles cristata*,

Hyaenidae), and superorder Xenarthra (e.g., anteaters, Myrmecophaga spp, Myrmecophagidae; armadillos, Dasypus spp, Dasypodidae) [14, 15]. These species share similar adaptations for this feeding specialization, including short teeth and jaws, a long sticky tongue, powerful forelimbs with strong claws, a rounded skull, and a low metabolic rate. In these species, the taxonomic status of Pholidota is a controversial issue. Morphological cladistics propose a close relationship between Pholidota and Xenarthra, whereas molecular evidence from mitochondrial and nuclear genes indicate that Pholidota is the sister taxa of Carnivora. However, it was recently noted that Pholidota lacks one of the lineage-specific karyotypic signatures of Carnivora. Zoo-CGH provided a genome-wide perspective on the relationship among Pholidota, Xenarthra, and Carnivora, even though the sequences of these animals are not fully determined. In the following example, DNA of the domestic dog (*Canis lupus familiaris*; Carnivora) and the two-toed sloth (*Choloepus didactylus*; Xenarthra) are labeled with different fluorophores and then hybridized with the metaphase chromosome spreads of Taiwanese pangolin (*Manis pentadactyla pentadactyla*; Pholidota).

### 5.1. Method and procedures

#### *5.1.1. Determine nuclear genome size*

The genome size of the two-toed sloth and domestic dog were determined to ensure that approximately equal numbers of nuclei (i.e., copy number of whole genomes in each species) are used in zoo-CGH analyses. The genome sizes were obtained after flow cytometry analysis of propidium iodide (IP)-stained nuclei from the target organisms.

#### *5.1.2. Extract DNA from the two-toed sloth and domestic dog*

Genomic DNA was isolated from leukocytes with a commercial kit (Gentra Puregene DNA Purification Kit, Qiagen, Hilden, German), used in accordance with the manufacturer's instructions.

#### *5.1.3. Prepare the mitotic metaphase slides of Taiwanese pangolin*

Fibroblast cell lines were established from lung tissues derived from Taiwanese pangolin, and metaphase cells were harvested following a 2-hour incubation with colcemid (at a concentration of 0.1 μg/ml).

#### *5.1.4. Produce two-toed sloth and domestic dog DNA probes*

The two-toed sloth and domestic dog DNA were labeled with biotin and digoxigenin (DIG) by nick translation, respectively.

#### *5.1.5. Prepare pangolin $C_0t$-1 DNA*

$C_0t$-1 DNA obtained its name from its isolation using a method called $C_0t$ analysis ($C_0$ denotes "DNA concentration," whereas t denotes "time"). Repetitive nucleotide sequences, which

constitute most of the $C_0t$-1 DNA, are abundantly distributed in most mammalian genomes. Blocking the repetitive sequences by $C_0t$-1 DNA can suppress nonspecific hybridization in FISH and CGH assays, and hence is a common step in such analyses. The genomic DNA of Taiwanese pangolin was sonicated to break the DNA into approximately 500-bp fragments, and the fragmented DNA was purified by ethanol precipitation. The purified DNA was dissolved to 500 ng/ml in TB buffer, denatured at 95°C for 10 minutes, and then chilled in ice for 10 minutes. A 1/10 volume of 12× SSC was then added to the fragmented DNA, which was reannealed at 60°C for 10 minutes. Then, S1-nuclease was used to digest the nonannealed DNA at 42°C for 1 hour. Thereafter, DNA was precipitated with ethanol and resuspended in TE buffer. Lastly, the acquired $C_0t$-1 DNA was quantified by spectrometry.

*5.1.6. Perform zoo-CGH*

Male Taiwanese pangolin chromosome spreads were prepared on a slide and denatured at 73°C for 5 minutes in 70% formamide and 2 × SSC, pH 7.0, followed by dehydration in a graded ethanol series. Next, equal genome copy numbers of biotin-labeled two-toed sloth DNA and DIG-labeled domestic dog DNA were coprecipitated with a 50-fold excess of Taiwanese pangolin $C_0t$-1 DNA, then redissolved in 10 μl of hybridization buffer (50% formamide, 10% dextran sulfate, and 2 × SSC), acting as the hybridization probe. Before hybridization, the probe was denatured at 80°C for 7 minutes, and then incubated at 37°C for 1 hour for preannealing of the repetitive DNA. The denatured probe was applied to the slide with the denatured and dehydrated metaphase spreads, covered with a cover slip, sealed, and incubated at 37°C for 72 hours. After hybridization, the slide was washed three times with 50% formamide and 2% SSC at 40°C for 5 minutes, and then washed twice with 2% SSC at 40°C for 5 minutes. The slide was kept undisturbed with 0.1% Tween 20 in 4 × SSC for 5 minutes, and the hybridization signal was detected with fluorescein-conjugated avidin (green fluorescence; for biotin-labeled probe) and rhodamine-conjugated ant-DIG antibody (red fluorescence; for DIG-labeled probe). Pangolin chromosomes were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) and fluorescence signals were visualized under a Leica DMLB microscope equipped with a cooled CCD camera. The profile of the fluorescein versus rhodamine fluorescence intensity ratio (F/R ratio) was estimated with CGHView image analysis software.

*5.1.7. Analyze image*

By comparing the fluorescence ratio on the longitudinal axis of pangolin metaphase chromosome, we estimated differences in the inter-species gene copy number and DNA sequence similarity. The means of the F/R ratios obtained from the heterologous hybridization, which represents DNA from different species labeled with different fluorophores that are competitively bound to probes obtained from a third species, were calculated for each pangolin autosome. Pangolin chromosomal segments with F/R ratios of < 0.8 (red fluorescence is more intense) and > 1.2 (green fluorescence is more intense) were considered to have significantly different hybridization strengths. When the F/R ratios were between 0.8 and 1.2 (showing yellow fluorescence), the DNA sequence difference or copy number of each pair was roughly equivalent. Means of the ratios were also calculated using a dye-swap design.

### 5.2. Result

In **Figure 12**, we can see red, green or yellow blocks on different parts of the chromosome. The overall homology between the pangolin and dog genomes was higher than that between the pangolin and sloth genomes. Analysis of pangolin chromosomes 14 and 15, which were the largest and most easily identifiable, showed that red fluorescence is dominant in euchromatin, i.e., more similar to the domestic dog (**Figure 12E**). When dye swapping was conducted, i.e., green fluorescence for the domestic dog and red fluorescence for the two-toed sloth, consistent results were obtained (**Figure 12F**).

**Figure 12** shows zoo-CGH for the domestic dog, two-toed sloth, and Taiwanese pangolin. In panel (A) genomic DNA from dog (labeled with DIG conjugated to the red fluorophore, rhodamine) and sloth (labeled with biotin conjugated to the green fluorophore, fluorescein) were mixed in equal quantities and competitively hybridized to metaphase spreads from the pangolin lymphocytes. In panel (B) individual chromosome analysis of the fluorescent ratio in (A) was presented where blue lines denote the ratio of F/R signal at each position of the pangolin chromosomes. Numbers in brackets represent the number of chromosomes analyzed. When the vertical bar between each chromosome and its ideogram appears red or green, the F/R ratio was <0.8 or >1.2, respectively. Overall, all chromosomes (except Y) appeared red. Panels (C) and (D) represent dye swap of (A) and (B), respectively. All chromosomes (except Y) appeared green.

From the results above, we found that all somatic chromosomes of *Manis pentadactyla* are more similar to the domestic dog (Carnivora) than the two-toed sloth (Xenarthra), providing evidence that *Pholidota* is more related to Carnivora than Xenarthra. For the Y chromosomes, which show the opposite results, we must eliminate the possibility of deletion of domestic dog's Y chromosome. We further analyzed the karyotype of this individual, but did not find such deletion. Therefore, it is possible that Y chromosome of *Manis pentadactyla* has a different evolutionary history than the somatic chromosomes [16]. The differences in the Y chromosome results can also be attributed to the size difference between the Y chromosomes of domestic dog and two-toed sloth. The large genomic blocks of somatic chromosomes lack structural rearrangements during evolution, making "richness" prevail in signal expression instead of "similarity," which is more desired. We performed molecular evolution analysis with the *Sry* gene, which is located on Y chromosome, and the results were combined with those from zoo-CGH; that is, two markers of different evolutionary history were used to answer the question. There is no doubt in the answer: in terms of extant mammal taxonomy, Pholidota has a closer relationship with Carnivora than Xenarthra. The new methods we developed can be used as a powerful tool for clarifying the phylogenetic relationships of orders under the Mammalia class, and they help answer some long-disputed taxonomical questions. For example, to which greater taxonomical category should Chiroptera belong: Laurasiatheria or Euarchontoglires? Zoo-CGH not only reveals the similarity trend of the whole genome but also individual gene blocks, making it the CGH technology with the highest resolution before the complete sequencing of each species; when it is combined with cross-species whole chromosome painting FISH (zoo-FISH), a new era of comparative genomics begins [17].

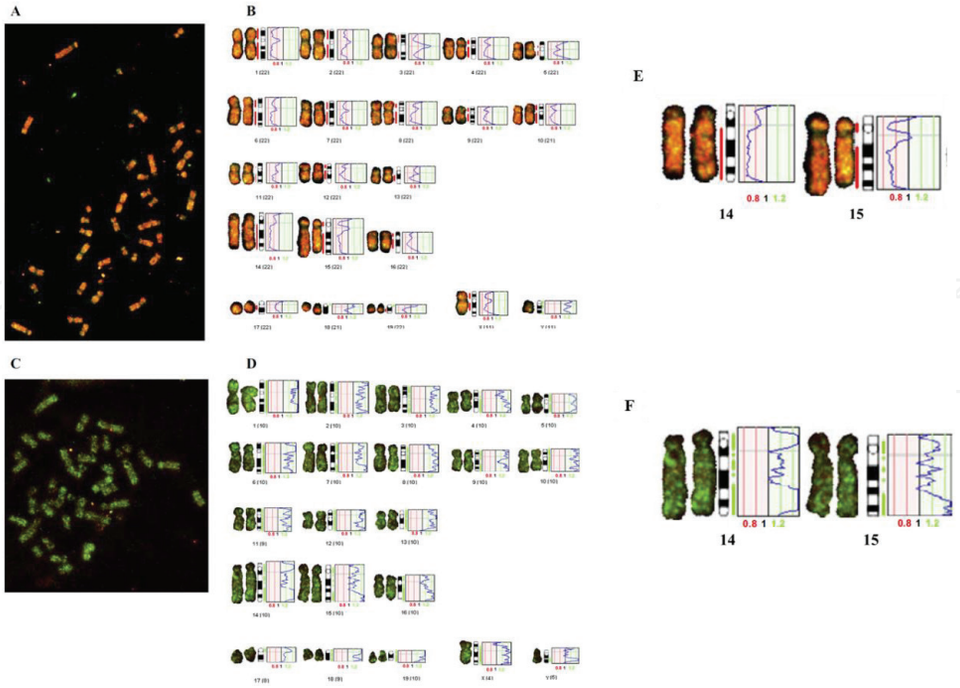


**Figure 12.** Cross-species CGH for the domestic dog (*Canis lupus*), two-toed sloth (*Chloepus didactylus*), and Taiwanese pangolin (*Manis pentadactyla pentadactyla*). (A) The competitively hybridization results of dog (rhodamine) and sloth (FITC) to metaphase spreads from pangolin lymphocytes. (B) Individual chromosome analysis of the fluorescent ratio in (A). (C) and (D) dye swap of (A) and (B), respectively. (E) and (F) Enlarge pangolin chromosomes 14 and 15 of (B) and (D), respectively.

## 6. Discussion

In early times, comparative genomics study between closely related species can only be done by comparing the karyotypes of the species and the techniques used are primitive, including Giemsa stain only, the G-banding techniques, and thus only the diploid number (2N), the functional number (FN, indicating the numbers of the chromosomal arms), as well as the classification of the chromosomes into metacentric, submetacentric, acrocentric, and telocentric according to the arm ratios can be provided. In addition, the special stains, such as the C-banding and Ag-nucleolus organizer region (NOR) staining, can be used to elucidate the constitutive heterochromatin (by C-banding), and the sites of secondary constriction and the active-transcribing ribosomal DNA genes (by Ag-NOR staining), can help to find the more trivial differences between species which may carry evolutionary significance [18, 19]. However, the advent of fluorescence *in situ* hybridization (FISH) technology greatly expanded the role of cytogenetics in studying the karyotypic evolution, not only in mammals but also in plants [5, 7, 20]. The authors therefore propose here a complete cytogenetic study in the light of karyotypic evolution that should include the conventional karyotyping, the special stains, as well as the fluorescence *in situ* hybridization (FISH)-based technologies such as genomic *in situ* hybridization (which is specific to plants), the chromosomal painting to study the movement and shuffling of the large genomic blocks in the Mb-level (in mammals), the telomere (TTAGGG)*n* FISH to demarcate the

chromosomal ends or to demonstrate the insertional translocation between species (in all vertebrates), mapping the locations of the gene of special interest with the FISH probe made by the gene segment cloned (in both animals and plants), and the innovative zoo-CGH we described in the previous section (in mammals), as our previous studies recently demonstrated [17–19].

## 7. Conclusion

Despite molecular evolution being made nowadays, by studying the homologous DNA sequences and using different evolutionary analytical models to reconstruct the phylogeny, which is the mainstream of comparative genomics [1–4], especially when sequencing the whole genome of each species has become more feasible through the powerful next generation sequencing (NGS) technology [21], cytogenetics remains an indispensible tool in studying the karyotypic evolution, which is one of the major mechanisms and thus is equally important as the molecular evolution to the processes involved in the speciation and subspecies differentiation. Conventional karyotyping, special stains to delineate the locations of heterochromatin, sites of active-transcribing ribosomal DNA genes, as well as molecular cytogenetics (namely, the fluorescence *in situ* hybridization (FISH)-based methodologies) can still provide insightful clues to solve the deficiencies that molecular evolution-based analyses cannot easily answer because in addition to point mutations and small insertion/deletions (indels), the movement of large genomic segments in the size of Mb-level, which is very difficult to analyze if by molecular methods, is also important in the evolution of the genetic complements of species deriving from a common ancestor in a specific lineage. The authors therefore propose a more balanced approach to study phylogenetics that is mandatory when considering using cytogenetics or molecular analyses as the major research tool. Evolutionary genetics will not be complete if the valuable insights obtained through cytogenetics are ignored or omitted in this NGS-predominant molecular era.

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