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Chapter 1

Cytogenetic Mapping and Contribution to the Knowledge of Animal Genomes

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Abstract

Decades before the recent advances in molecular biology and the knowledge of the complete nucleotide sequence of several genomes, cytogenetic analysis provided the first information concerning the genome organization. Since the beginning of cytogenetics, great effort has been applied for understanding the chromosome evolution in a wide range of taxonomic groups. The exploration of molecular biology techniques in the cytogenetic area represents a powerful tool for advancement in the construction of physical chromosome maps of the genomes. The most important contribution of cytogenetics is related to the physical anchorage of genetic linkage maps in the chromosomes through the hybridization of DNA markers onto chromosomes. Several technologies, such as polymerase chain reaction (PCR), enzymatic restriction, flow sorting, chromosome microdissection and BAC library construction, associated with distinct labeling methods and fluorescent detection systems have allowed for the generation of a range of useful DNA probes applied in chromosome physical mapping. Concerning the probes used for molecular cytogenetics, the repetitive DNA is amongst the most explored nucleotide sequences. The recent development of bacterial artificial chromosomes (BACs) as vectors for carrying large genome fragments has allowed for the utilization of BACs as probes for the purpose of chromosome mapping. BACs have narrowed the gap between cytogenetic and molecular genetics and have become important tools for visualizing the organization of genomes and chromosome mapping. Furthermore, the use of chromosome probes has permitted the development of chromosome painting technologies, allowing an understanding of particular chromosomal areas, whole chromosomes or even whole karyotypes. Moreover, chromosomal analysis using these specific probes has contributed to the knowledge of supernumerary chromosomes, sex chromosomes, species evolution, and the identification of

chromosomal rearrangements. Finally, the synergy between chromosomal and molecular biology analysis makes cytogenetics a powerful area in the integration of knowledge in genetics, genomics, taxonomy and evolution.

Chromosomes as a Tool for Understanding Genome Organization

The Chromosome History

The universal occurrence of chromosomes as genetic units of heredity suggests that such cell structures have appeared early in the history of life. The array of genetic material in chromosomes gives several advantages to cells and organisms, packing the genetic information into segregational units and reducing the probability of gain, loss or changes of genetic information. On the other hand, the chromosomes allow for the occurrence of specific and coordinated events of recombination and rearrangement in the genetic material that are of great value to the diversification and evolution of organisms. In this way, the “cytogenetics,” which deals with the science of chromosomes, represents a powerful area that aggregates knowledge of cell and molecular biology and can answer several questions on the biology of the species.

The second half of the 19th century is characterized as a remarkable time for genetics and science in general. The mechanisms of heredity began to appear in the works of Gregor Mendel and Charles Darwin, and then a series of significant discoveries involving chromosomes in the end of the 19th century and in the early 20th century allowed for a link between the inheritance of genetic traits and chromosomes. In this way, the cytogenetic science was born with the first analysis of the behavior of chromosomes during cell division, which was carried out at the end of the 19th century by Walter Flemming (Flemming 1882). Flemming provided the first information related to the segregation of chromatin during mitosis in animal cells. At the same time Eduard Strasburger (Strasburger 1875) observed the mitotic process in plant cells, and further demonstrated that nuclei arise only from pre-existing nuclei. Although Flemming and Strasburger had discovered the chromosomes, the term was coined few years later by Heinrich Waldeyer (Waldeyer 1890). Based on Flemming’s discoveries, Theodor Boveri (Boveri 1887) provided the first evidence that the number of chromosomes was reduced in germ cells and restored after the fusion of sperm and egg nuclei. Walther Sutton in 1902 postulated that all chromosomes have a stable structure, or "individuality," that is maintained through generations. With this statement, Sutton articulated the first step of the chromosomal theory of inheritance that was subsequently supported by Thomas Hunt Morgan's experiments with fruit flies, *Drosophila* (Morgan et al. 1915). Morgan made a significant contribution to the science of genetics, stating that genes are located on chromosomes. In the subsequent years the advances on biochemistry would allow for the development of new techniques, contributing to a better characterization of the chromosomal structure. The discovery of the double helix structure of DNA in 1953 (Watson and Crick 1953) gives new directions to the genetics, including cytogenetics. The chromosomes entered a new era with their structure being “molecularly dissected” by advanced molecular biology techniques. The cytogenetics moved from “past cytogenetics,”

which used a tissue section to identify the human chromosomes in the beginning of 20th century, to the "modern molecular cytogenetics," which utilizes chromosome painting and bioinformatics to recover the evolutionary history of karyotypes.

Cytogenetics Meets Genomics

During the first half of the 20th century the human chromosome number was the focus of several scientific papers, but the results obtained through tissue sections were imprecise and gave incorrect information (reviewed in Capanna 2000). The correct diploid number of human cells was only established as 2n=46 in 1956 (Tjio and Levan 1956). In later years new techniques have allowed for the obtention of high quality chromosomes, using cell suspension preparation and cell culture. In the second half of 20th century several methods of chromosome staining and banding allowed for expressive progress in the cytogenetic area. After 1980, with the advances in molecular biology and genomics, cytogenetics experienced a synergy with molecular biology, allowing significant advances in understanding genomes throughout the chromosomes. Nowadays, the advances in microscopy, the application of bioinformatics, and the integration of chromosome analysis and genomic data represent promising tools for the future of cytogenetics.

The availability in the last decade of hundreds of completely sequenced eukaryotic genomes opens new avenues for the cytogenetics, with more perspectives for physical chromosomal mapping of genes and comparative cytogenetics. Although the integration of cytogenetics and genomics seems to be a recent event, the chromosome took its place of importance in the emerging of genomics. The "genome" terminology appears for the first time in the book of Hans Winkler (Winkler 1920), with reference to the "haploid chromosome set." In this way, the chromosome set represented the base for the foundation of the new area that emerges as "genomics." The connection between genome and chromosome has been often conducted in an indirect way, using linkage mapping that is based on the determination of gene positions in chromosomes according to recombination frequencies among them. More recently, the complete nucleotide sequence of several genomes also allows the obtention of chromosome maps for some species. In all these cases, the "chromosome map" comes from indirect methods that do not involve "physical chromosome" analysis. Despite the intensive effort of studies using new genomic analyzer tools and bioinformatics, many of the genomes reported completely sequenced still present portions that remain as gaps due to the difficulty in correctly identifying the position and array of particular types of nucleotide sequences, such as the repetitive DNAs. In this way, the molecular cytogenetics comes to integrate nucleotide sequences with physical chromosome mapping of DNA sequences or genes. The cytogenetic can provide true "physical chromosome maps" that are of great value to anchor and support other genetic maps, such as linkage maps, restriction maps and nucleotide sequence maps (Figure 1).

The advances in bioinformatics permit the karyotype reconstructions of species based on *in silico* analysis of complete nucleotide sequences of genomes. The ancestral syntenies of nucleotide sequences across different groups can be established based on sequence orthologies among species. Such an approach allows the application of electronic chromosome painting (E-painting) and the foundation of "*in silico* cytogenetics" as a new perspective for analyzing chromosomes and karyotypes. Kohn and co-workers (Kohn et al.

2006) have applied *in silico* cytogenetics to a large data set of genes of humans, chickens, zebrafish and pufferfish, advancing in the reconstruction of the ancestral vertebrate protokaryotype comprising 11 protochromosomes. *In silico* cytogenetics permitted the identification of conserved linkage groups between very distant related animal groups. Ancient eumetazoan chromosomes have been found by comparing the human and the sea anemone (Cnidaria) genomes (Putnam et al. 2007).

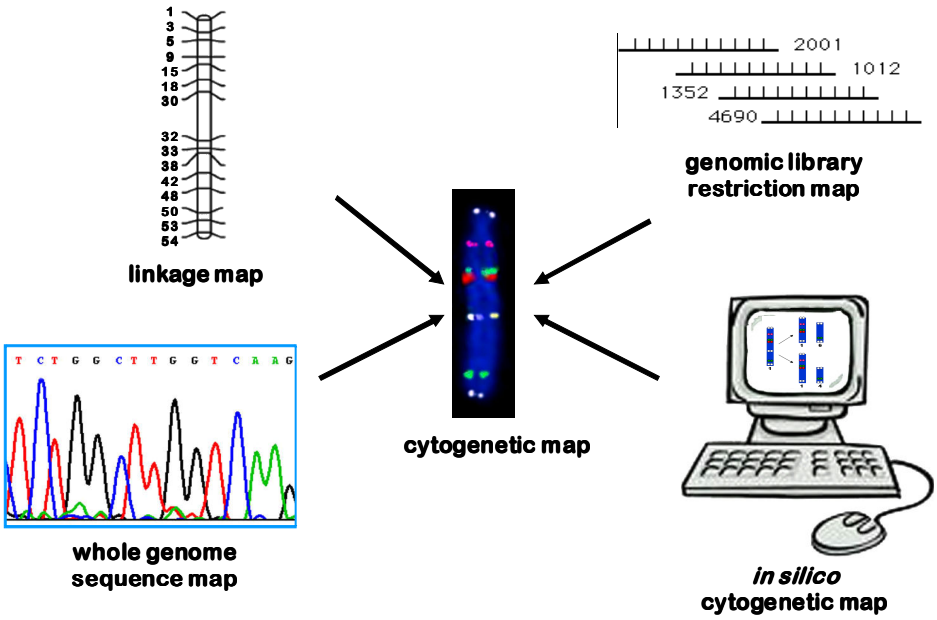

Figure 1. Integrative view of cytogenetics.

Conserved synteny between human and sea anemone chromosomes were identified in 40 large homologous segments, despite 700 million years of divergence among them (Putnam et al. 2007). On the other hand, some conflicts between *in silico* (bioinformatics) and cytogenetics analyses are apparent. This can reflect the genomic sampling, limited to a few species associated with specific algorithms applied by bioinformatic tools. The increasing taxa sampling and the development of more sophisticated bioinformatic tools will allow the match between the cytogenetic and bioinformatic models (Froenicke et al. 2006). The more precise integration of cytogenetics and bioinformatics, plus the inclusion of different genetic and genomic data, will allow for a reliable reconstruction of karyotypes and the evolutionary history of groups.

Applied Technologies in the Physical Mapping of Animal Chromosomes

For several decades the cytogenetic analysis were performed using classical karyotyping methods, which allowed a gross description of chromosome structure and organization, revealing chromosomal number and morphology, and sex chromosome systems in animals

and plants. Even with the advance on chromosomal banding techniques, such as C-banding, silver nitrate staining, G-banding and others, the chromosomal studies remained depending on the description of banding patterns along the length of each chromosome with limited resolution. The major advance in cytogenetics has come in the last two decades with the application of *in situ* detection of DNA sequences in the chromosomes, using specific DNA segments as probes. This technique defined the transition from the classical cytogenetic era to the molecular cytogenetic era, allowing more detailed studies in the cytogenetic field and enabling the integration of molecular information of DNA sequence to their physical location along chromosomes and genomes (Schwarzacher 2003, Jiang and Gill 2006). The molecular cytogenetics has gained a great importance in chromosomal studies by allowing the direct and physical location of a specific DNA segment in the chromosomes. The molecular cytogenetics can be applied in the detection of unique or repetitive sequences, specific chromosomal regions, entire chromosomes and even whole genomes. Such an approach allows the integration of cytogenetic maps to linkage maps, elucidation of chromosome structure, and genome organization and evolution.

The *in situ* hybridization technique is based on the capacity of denaturation and specific annealing of complementary DNA chains (adenine anneals with thymine and cytosine with guanine) through hydrogen bonds between the bases attached in the DNA sugar-phosphate backbone (Swinger and Tucker 1996, Wilkinson 1999). This technique depends on a labeled probe (sequence of interest) and chromosome spreads obtaining, denaturation of the probe and chromosomal DNA (target), hybridization (probe-target DNA annealing) and probe detection (Figure 2).

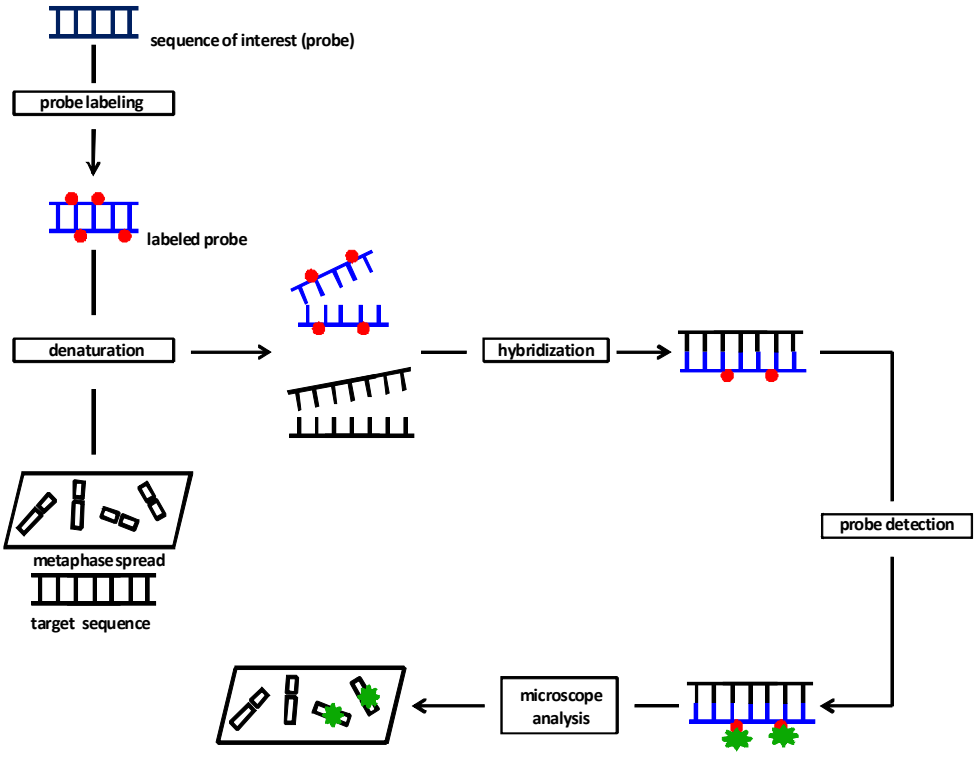

Figure 2. Basic steps of fluorescence *in situ* hybridization (FISH).

Since the first use of *in situ* detection of a DNA sequence using a complementary DNA as a probe in cytological preparations by Pardue and Gall (1969) in the toad *Xenopus laevis,* this technique has undergone modifications related to the hybridization process, probe labeling and detection, and analysis methods increasing sensitivity, specificity and the resolution of results. Initially, the probes for *in situ* hybridization were labeled directly by radioactive isotopes ^{32}P, ^{125}I, ^{3}H and ^{35}S, but since the beginning of the 1980 decade the probes started being labeled by non-radioactive molecules. Although several methods based on enzymatic reaction using alkaline phosphatase, beta-galactosidade or horseradish peroxidade were available, the most applied method in the subsequent years was based in the utilization of fluorescent elements, therefore the technique was named fluorescent *in situ* hybridization (FISH) (Pardue and Gall 1969, Forster et al. 1985, Pinkel et al. 1986, McNeil and Ried 2000, Schwarzacher and Heslop-Harrison 2000). The use of FISH permit a color era for cytogenetics and a substantial increase in the quality of the final results observed. Some information about the processes of probe obtaining, labeling and detection, and microscopic analysis for FISH experiments will be discussed along this section.

Routine Methods for Probe Obtaining

The advent of molecular biology, with advances in cytogenetics and cellular techniques and equipment, has permitted the generation of a range of probes to be used in chromosomal mapping. Among these techniques, the most used for this purpose are Polymerase Chain Reaction (PCR), enzymatic restriction, chromosome microdissection, flow sorting and BAC library construction (Figure 3).

These techniques have permitted the generation of probes of the whole genome of one species, whole chromosomes or sub chromosomal regions, such as centromere, telomere, specific arms, specific chromosomal locus or dispersed elements, constituted by highly and moderately repetitive DNAs or single copy sequences (Figure 4). These probes have been used to analyze condensed chromosomes in metaphases, interphasic nucleus, cells in initial division stages and in distended DNA fibers (Fiber-FISH), that are used to study the karyotypic ongoing in distinct animal groups.

The most common probes used in FISH experiments are composed of repeated DNAs organized *in tandem* or dispersed in the genome. The *in tandem* repetitive DNAs most applied in FISH experiments include the satellite DNAs (Figure 3d), multigenic families (Figure 3a-c), and telomeric (Figure 3l) and centromeric sequences. In general these probes provide well visible signals, due to their abundant repetition and distribution organized in large blocks along the chromosomes.

Some satellite DNAs, and the multigenic families of ribosomal DNAs (5S and 18S rRNA genes), have been isolated from distinct animal species, including invertebrates and vertebrates, providing good markers for chromosome identification. The dispersed repetitive DNAs are represented by the transposons and retrotransposons (Figure 3j) that are moderately or highly repeated sequences dispersed throughout the genomes, which can form viewable blocks, next to the genes, associated with a specific genomic area, such as ribosomal DNAs and heterochromatin, or dispersed in euchromatic regions of the chromosomes.

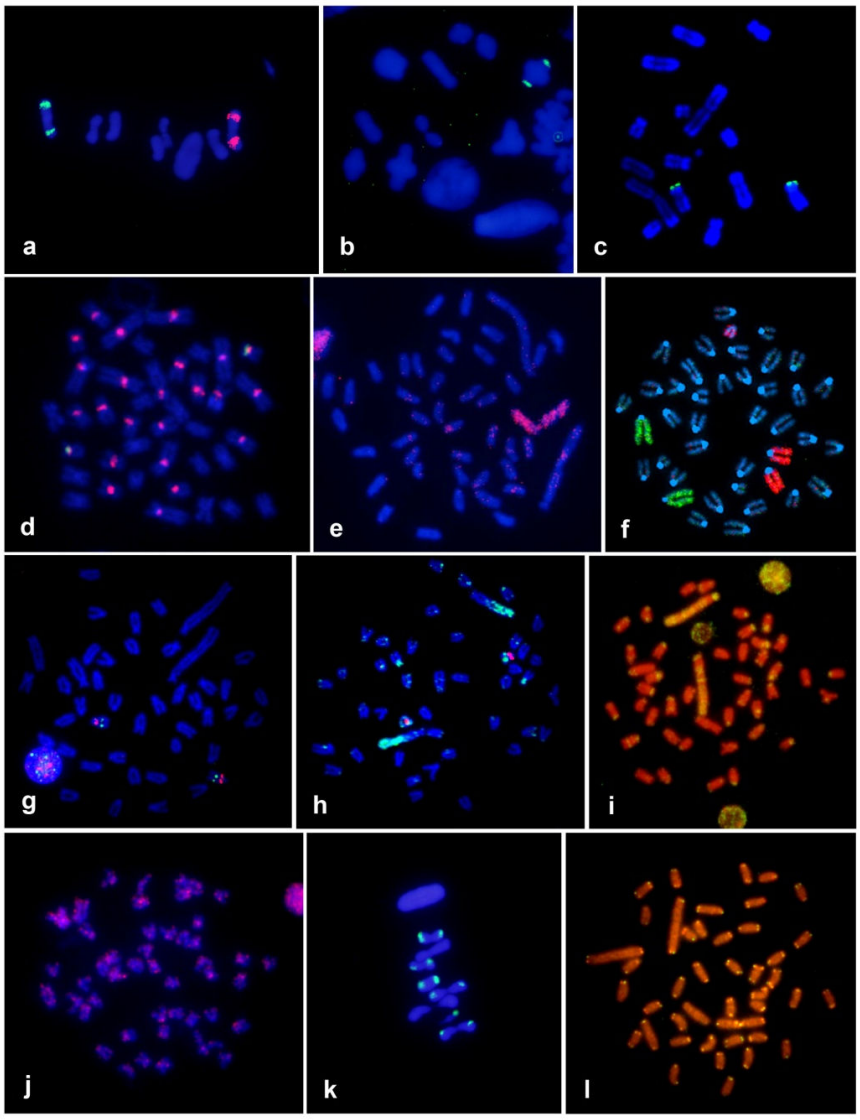

Figure 3. Fluorescence *in situ* hybridization results using different probes obtained by available routine methods. (a-d, j, l) probes obtained though PCR: (a) 18S rDNA (green) and 5S rDNA (red) in the beetle *Dichotomius bos*, (b) H3 histone gene in the grasshopper *Stiphra robusta*, (c) 18S rDNA in the bat *Tonatia saurophila*; (d) 5S (green) and 5S*Hind*III satellite (red) in the fish *Hoplias malabaricus*; (j) *Rex*3 transposable elements in the fish *Haplochromis obliquidens*; (l) telomeric probe in *Oreochromis niloticus*; (d) satellite DNA 5S*Hind*III (red) obtained by enzymatic restriction in the fish *Hoplias malabaricus*; (e) chromosome paint probe obtained by chromosome microdissection of chromosome pair 2 of the fish *Oreochromis niloticus*; (f) chromosome paint probes obtained by flow sorting of pairs 2 (green) and 3 (red) in mouse; (g, h) BAC-FISH in *O. niloticus* using BAC clones of green opsin (green) and blue/red opsin (red). Note the differences in the green signal intensity between "g" and "h"; (g) BAC with low quantity of repetitive DNA and (h) BAC for the same chromosomal region containing high amount of repetitive DNA; (i) BAC enriched of repetitive DNAs hybridized to *O. niloticus* chromosomes; (k) FISH of C_0t-1 DNA fraction in *Dichotomius bos*; The FISH figures "c" and "f" are courtesy of Sotero-Caio CG and Baker RJ (Texas Tech University, USA) and Yang F (Sanger Institute, England), respectively.

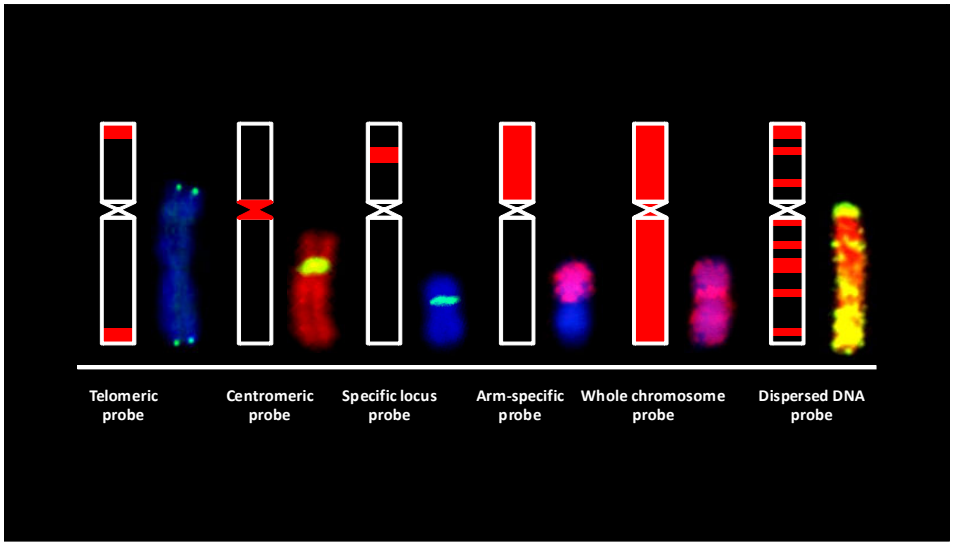

Figure 4. Most applied probes in fluorescence *in situ* hybridization experiments.

Among the methodologies for probe obtaining the most used technique is the PCR. Compared to the other methods PCR represents the easiest and cheapest procedure, and even laboratories with a minimum condition in molecular biology can use this technology to obtain probes (see latter in this topic and in Table 1). As a consequence of these advantages, the most part of manuscripts that have been published in the specialized literature use probes obtained by PCR (see published manuscripts on specialized journals, such as Chromosoma, Chromosome Research, Cytogenetic and Genome Research, Genetica, among others). In general the PCR is used to synthesize probes containing known repetitive DNAs, such as ribosomal DNAs (rDNA), histone genes, transposable elements, and telomeric sequences. This technique consists in the amplification of DNA strands from the genomic DNA using a specific small oligonucleotide primer that anchors the start of DNA polymerization by a DNA polymerase enzyme. There are some examples of conserved primers designated to obtain sequences for animal genomes through PCR reaction, i.e. for 5S and 18S rDNAs, histone genes, and transposable elements (Martins and Galetti 1999, Pineau et al. 2005, Zhang et al. 2007 Teixeira et al. 2009, Cabral-de-Mello et al. 2010a). For highly conserved sequences, such as RNA transcribing genes, the primers are universal, and primers designated to insects, fishes, mammals or other groups can be used to amplify the same sequences in distantly related taxa.

In relation to less conserved sequences, such as histone genes, it is possible to design degenerated primers to be used in DNA amplification of sequences in non related taxa. On the other hand, other sequences are more variable and specific primers need to be designated for restrict groups. That situation is common for transposable elements, which presents extensive variations among distinct taxa, caused by differences in the evolutionary dynamics of repeats with a consequent rapid sequence modification.

Table 1. Advantages and disadvantages of the probe obtaining routine methods

Probe Obtention Methodology	Advantages	Disadvantages
PCR	Low cost and rapid probe obtaining in relation to the other techniques; Facility in laboratory manipulation; Isolation of known sequences that facilitates the analysis; Use of the same primer/probe in non related taxa; Direct labeling of the sequence of interest;	Isolation of only known DNAs that limits the studies;
Enzymatic Restriction	Possibility the isolation of not described repetitive sequence; Useful for studies related to centromere, telomere, heterochromatin organization, and sex and B chromosome structure;	Laborious technique: it is necessary the test of different restriction enzymes, cloning and nucleotide sequencing; More expensive in comparison to PCR;
Chromosome Microdissection	Possibility the isolation of entire chromosomes or even specific chromosome regions; More useful for non mammalians animals;	Probes with low complexity, in general it is not useful for cross-species painting; Difficulty for distinction of specific target chromosomes;
Chromosome Flow-Sorting	Possibility of isolation of all chromosomes from interest species; Probes with high complexity, allowing the cross-species, cross-genus and cross-order painting;	Necessity of cell culture with high amounts of metaphases; Difficulty in chromosome separation in some groups, mainly non mammals;
BAC library	Possibility of mapping of functional gene sequences; Possibility of mapping single-copy genes; Facility in the manipulation of constructed libraries; Integration of linkage and cytogenetic maps;	Dependence in the maintenance of a genomic library or a laborious work in the construction of a library from the species of interest; Small signals, that sometimes difficult microscope analysis;

When the probe or primers of interest are not available, an easy and rapid way for obtaining a specific primer for less explored taxa is the use of DNA sequences deposited in the National Center for Biotechnology Information (NCBI). After searching NCBI, the retrieved sequences can be explored with programs available in the internet, such as ClustalW (www.ebi.ac.uk/clustalw) (Thompson et al. 1994) for alignment, and primer3 (www.frodo.wi.mit.edu) (Rozen and Skaletsky 2000) for primer design. These primers can be designed in conserved DNA regions and for more divergent DNA sequences it is more suitable the design of degenerated primers from reverse translation of protein sequences, increasing the success of these primers in non related groups.

The products obtained by PCR can be directly labeled (as explained latter in this topic) or can be cloned, using ordinary bacterial plasmids. The cloning gives the advantage of storage the sequence of interest for a long time and the obtention of large amount of probe that can be easily obtained by bacterial plasmid DNA extraction.

The enzymatic restriction consists in the cleavage of double DNA strands using restriction endonucleases that cuts the DNA in one specific nucleotide sequence. This molecular approach can be used primary to obtain probes of highly repetitive DNAs, such as satellite DNAs (satDNA) and transposable elements. The main difference related to the PCR is that in this technique, in general, it is isolated an unknown repetitive DNA sequence, and this sequence can be used mainly in related taxa, due the extensive variation in the profile of the repeated sequence even among related groups. This technique is more laborious and expensive than the PCR, due to the necessity of testing distinct enzymes to isolate a highly repetitive and useful sequence (Table 1). Moreover, the sequences obtained by enzymatic restriction need to be purified from agarose gel, cloned and sequenced to obtain specific information about the isolated element to be used in chromosomal studies. Southern blot experiments can also give good contributions in relation to the genomic organization of the isolated sequence. After the sequencing of the isolated repetitive DNA, primers can be designated to test the presence of this sequence in other taxa or to generate probes of this element for other species, facilitating the process of probe obtaining. This strategy has been most applied to obtain specific probes of satDNAs frequently used in studies of chromosome evolution, chromosome identification, B chromosome origin, centromere structure and sex chromosome evolution in insects (Cabrero et al. 2003a, Abdelaziz et al. 2007, Palomeque and Lorite 2008, Kuhn et al. 2008, 2009), mollusks (Biscotti et al. 2007, Petrović et al. 2009), fish (Phillips 2001, Caputo et al. 2009, Mazzuchelli and Martins 2009), and mammals (Adega et al. 2008, Matsubara et al. 2008, Acosta et al. 2009), among other groups.

Besides the isolation of repetitive DNAs using PCR and enzymatic restriction, another assay for this purpose is the use of *C_0t-1* DNA, a fraction of genomic DNA elements enriched for highly and moderately repeated DNAs (Figure 3k). This methodology is based in the reassociation kinetic of DNA strands, that is faster for repetitive sequences than to low copy number elements (Britten and Kohne 1968). It consists basically in the denaturation (at 95^{o}C) of fragmented genomic DNA of interest, reannealing in specific conditions (65^{o}C, for distinct times), and the treatment with the enzyme S1 nuclease (at 37^{o}C), that is active against single-stranded DNAs represented in this assay by low copy DNAs (Zwick et al. 1997). In fact the *C_0t-1* DNA fraction is used to "block" nontarget-specific DNAs and has been extensively applied in studies of chromosome painting and BAC-FISH to eliminate background. On the other hand, this methodology has shown to be a promise tool for isolation of non-specific repetitive sequences to be used as probes in animal cytogenetic, focusing in problems of B chromosome origin, heterochromatin differentiation, sex chromosomes evolution and genome organization (Ferreira and Martins 2008, Cabral-de-Mello et al. 2010a). In the same way, DOP-PCR (Degenerate Oligonucleotide Primed-PCR) that is based in the use of degenerated oligonucleotides can also be applied in the obtention of repeated sequences of the genome (Mazzuchelli and Martins 2009).

Another method used in the isolation of animal probes is the chromosome microdissection. This methodology was first applied in polythene chromosomes of *Drosophila* (Scalenghe et al. 1981) and some modifications were introduced in the application of the technique in mammals, birds, insects, fish and other organisms. This technique permits the isolation of whole chromosomes, or specific chromosomal regions directly from the metaphases plate using micromanipulation. The technology can be useful for studies of chromosomal rearrangements, chromosome evolution, sex chromosomes and origin of B chromosomes. When this method was developed chromosome probe

hybridizations were conducted from the construction of libraries though microcloning of isolated sequences, a laborious and expensive methodology (Guan et al. 1994, Saitoh and Ikeda 1997). Nowadays the microdissected elements are directly amplified in general by PCR using frequently a degenerate universal primer (DOP-PCR) and their products can be labeled to be used for painting (Wesley et al. 1990, Meltzer et al. 1992, Telenius et al. 1992). The most important improvement of the technique in the last years was the introduction of the inverted microscope (Senger et al. 1990), laser-based microdissector and the DOP-PCR reaction (Guan et al. 1992, Meltzer et al. 1992, Saioth and Ikeda 1997, Yang et al. 2009). These modifications allowed the decrease amount of DNA template of microdissected elements from 100-200 to 20-40 or even lesser, and higher precision in the chromosomal region to be microdissected (Yang et al. 2009). Moreover, some modifications have been done in the chromosome preparations improving the recognition of the target chromosome, including the use of G-banded and C-banded metaphases and meiotic plates.

Besides the use of microdissected chromosomes to generate chromosomal paint probes another methodology which can be used for this purpose is the flow sorting. The flow-sorting uses the flow cytometry and sorting for separation and purification of mitotic metaphasic chromosomes (Carrano et al. 1983). This technique is based in the separation of chromosome populations by the difference in their size, morphology and DNA content that produce specific fluorescence intensity for each chromosome, generating the flow-sorted karyotype. Individual chromosomes may be isolated using a high speed liquid stream (containing the chromosomes) that is converted into droplets. The specific droplets containing the chromosomes of interest are electrically charged and are deflected electrically by a passage through an electrostatic field (Givan 2001). The use of flow sorted chromosomes is most concentrated in studies of chromosome evolution of mammals, due the difficulty of the establishment of cell cultures and the precise separation of chromosomes in the other non mammalian groups. The difficult of the use of flow sorting in non mammalian species is related to the symmetry in the karyotype and the chromosome compartmentalization of some species, which causes the non precise separation of the chromosomes (Figure 5). On the other hand, some modifications applied in non mammal vertebrate groups have permitted the isolation of, at least, specific chromosome groups or part of the karyotype from fish, birds and reptiles, although the results are until scarce. These probes have been constantly synthesized for studies of mammalian chromosomal evolution and detection of chromosomal aberrations in cancers. Marketable probes are available for human (Telenius et al. 1992) and mouse (Rabbitts et al. 1995) karyotypes. The flow-sorting is more advantageous in relation to the microdissection generating probes with more quality and complexity, due the more efficiency in the quantity of chromosomes isolated, that permits studies using distantly related species, such as the cross-species painting, cross-genera paints even cross-order paints (Table 1).

A bacterial artificial chromosome (BAC) library is a collection of cloned inserts greater than 100 kilobases (kb) usually using *Escherichia coli*. The construction of these libraries with ability to accommodate such large inserts is advantageous and it provides an easy access to stable DNA for manipulation (Miyake and Amemiya 2004). This technology has been encountered application in genomic studies, such as genome sequencing, positional cloning, microsatellite and gene isolation, cDNA selection, transgenic construction and in the physical chromosome mapping through FISH using these elements as probes (Li et al. 1999, Beck 2001, Lander et al. 2001, Gong et al. 2003, Chen et al. 2004, Miyake and Amemiya 2004, Powers and Amemiya 2004, Romanov et al. 2005, Yasukochi et al. 2009).

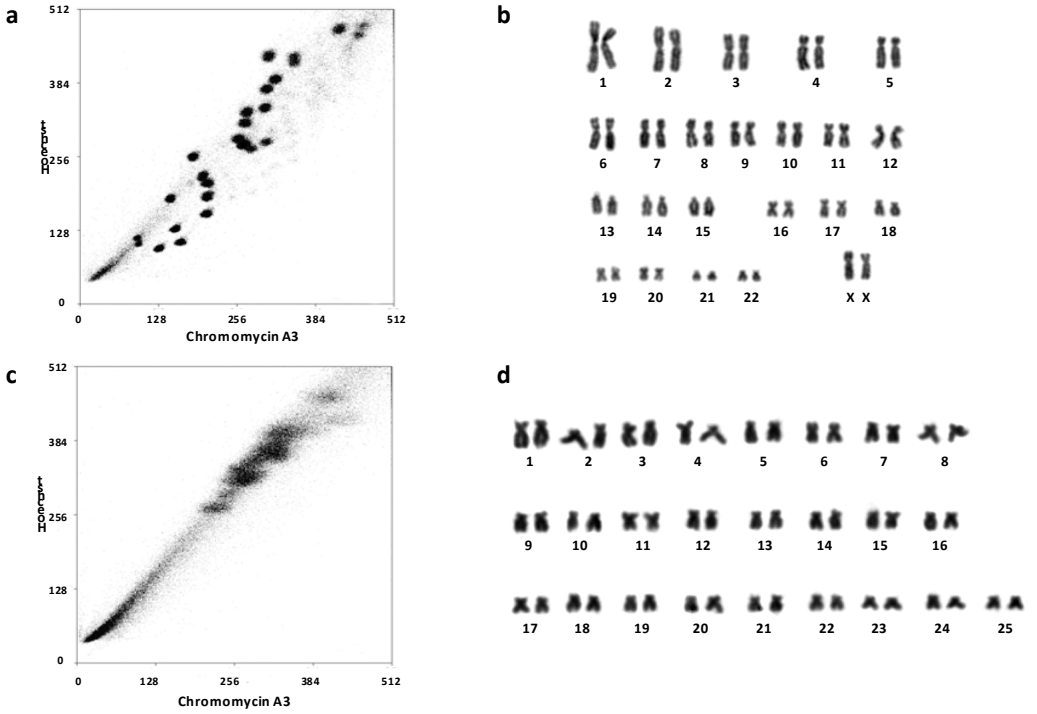

Figure 5. Chromosome flow sorting graphic of human female (a) and zebra fish (c) karyotypes showing the differences in chromosome separation patterns. Note the difference in the karyotype structure between humans (b) and zebrafish (d). The flow sorting graphics are courtesy of Yang F (Sanger Institute, England).

The use of BAC inserts has permitted the integration of linkage and cytogenetic maps and has been useful in comparative cytogenetic and genomics. In general the use of BAC-FISH is applied in studies of specific locus region related to a gene or linkage group of interest. Moreover, some studies have been conducted using BACs with high quantity of repetitive DNA in the understanding genome organization and evolution in vertebrates (see Figure 3i) (Ferreira and Martins 2008, Cheng et al. 2009, Poletto et al. 2010). Although most studies using BAC-FISH have been conducted in mammals, there are in the literature information concerning fishes, birds, mollusks, insects, and other groups (Aerts et al. 2003, Romanov et al. 2005, Cnaani et al. 2007, Huan et al. 2009, Ocalewicz et al. 2009, Yasukochi et al. 2009). The major difficult in the application of BAC-FISH is the availability of BAC clones, which in general depends of genome sequencing projects. Nowadays with the rapid advances in the genome sequencing technologies, the availability of BAC libraries for a range of species is increasing.

The probe obtaining methods described above are the most common used in molecular cytogenetic, although, there are other approaches also in use, such as the synthetic oligonucleotides, cDNA fragments and whole genomic DNA (Schwarzacher et al. 1989, Svartman and Viana-Morgante 1999, Schwarzacher and Heslop-Harrison 2000, Matsuda et al. 2005, Srikulnath et al. 2009, Valente et al. 2009). The synthetic nucleotides can be labeled and located by ordinary FISH procedures using a specific motif with abundant repetition, i.e.

the telomeric and microsatellite sequences. The oligonucleotides are relatively cheap and fast to order, and the labeling can be incorporated during the synthesis or by chemical modification currently by end labeling (Schwarzacher and Heslop-Harrison 2000). According to Schwarzacher and Heslop-Harrison (2000), in the future when *in situ* methods become more sensitive and allow the detection of single copy nucleotides sequences the use of synthetic probes will become more common and universal. The cDNA probe obtaining method consists in the use of cloned DNA sequences obtained by RT-PCR (Reverse Transcription-PCR) technique, and permits the chromosomal mapping of functional genes or libraries of EST (Expressed Sequence Tags). This method has been recently applied with success in reptiles and birds (Matsuda et al. 2005, Srikulnath et al. 2009). The use of whole genomic DNA from a species as probe can be useful and informative for analysis of hybrids, genome relationship, chromosomal evolution and introgression. Moreover, the genomic DNA is applicable to prevent unspecific probe hybridization in FISH experiments. Actually, the use of genomic DNA is a modification in the FISH technique called genomic *in situ* hybridization (GISH), explained in more details below.

Probe Labeling and Detection

In the FISH technique the probe detection and results obtaining are based in the observation of fluorescent molecules by means of an epifluorescence microscope. The fluorescent systems permit a better definition of hybridization signals in relation to the radioactive or enzymatic methods, facilitating the distinction between dirt, background and real signal.

The fluorescent molecules can be incorporated directly in the DNA sequence (direct labeling) or can be coupled to other small molecule that recognizes a marker molecule (hapten) bounded in the probe (indirect labeling). In the direct way of labeling method the probe is labeled with a nucleotide bounded to a fluorochrome, like Texas red or Cy5, which is incorporated into the probe sequence instead of an ordinary nucleotide. In this method none special immunocytochemical visualization procedure is necessary and the probe could be observed after the hybridization step, thus making this method faster and resulting in low background in the results, but slightly less sensitive, that is attributed to the presence of low number of fluorescent molecules in each probe fragment (Schwarzacher and Heslop-Harrison 2000).

In the indirect labeling method modified nucleotides are incorporated into the probes and a detection step is required, being this method more sensitive, but in the same time with more background. Although there are a variety of haptens for label and anti-hapten antibodies, the marker molecules more used in the indirect labeling are the biotin and digoxigenin (DIG) coupled to dUTP nucleotides, which are incorporated into the probes covalently in substitution of the thymine, as described in the method of direct labeling. The biotin is an H vitamin and the digoxigenin is a steroid isolated from the foxglove plant (*Digitalis purpurea* and *D. lanata*). Both molecules are coupled in the carbon five of the pyrimidine ring by a long carbon ring that is important to avoid the reduction in the efficiency recognition of the marker molecule and the stereochemistry interference between the target DNA and the probe. After the hybridization process the probes labeled with biotin or digoxigenin need to be detected by means of antibodies or a molecule with high affinity to the marker molecule

linked with an appropriate fluorochrome. In general it can be used to detect the biotinilated products the avidin, a molecule with high affinity to biotin, and to digoxigenin the anti-digoxigenin antibody coupled to a fluorochrome. The initial FISH experiments were carried out using the fluorescein isothiocyanate (FITC) as a fluorochrome, but nowadays the use of some distinct fluorochromes permits the use of distinct probes in the same metaphases offering precise and clear hybridization signals.

The most common fluorochromes used in FISH experiments bounded to the probes emit signals in red (Cy3, rhodamine, texas red) or green (FITC, Cy5) colors, and the chromosomes can be counterstained in red (propidium iodate) or blue (4',6-diamidino-2-phenylindole - DAPI). The chromosome counterstaining can be conducted either separated or directly mixed with the antifade solution, which prevents the brightness fluorochrome decay. Moreover, some other flurorochromes are available with other colors, and the combination in distinct equimolar proportions of the basic colors can generate probes with some distinct colors, frequently used in multi color FISH experiments (see below).

Independent of the type of probe labeling with direct or indirect methods, the modified nucleotides can be inserted in the probes by enzymatic methods through random priming, nick translation, end labeling, *in vitro* translation (used for probes of RNA) and PCR. The nick translation method uses simultaneously the activity of two distinct enzymes, the DNAse I and *Eschericia coli* DNA polymerase I. The DNAse I in the presence of the cofactor Mg^{2+} acts as a single stranded endonuclease, creating nicks randomly in both DNA strands of the probe; and the DNA polymerase I has exonuclease and polymerase activity polymerizing the cut strand in the 5'-3' orientation and removing the nucleotides in the 5'-3'and 3'-5' orientation, with proof-reading activity. In addiction to the enzymes, in the nick-translation are used the non-labeled four nucleotides (dATP, dCTP, dGTP and dTTP) and a labeled nucleotide, frequently dUTP or dATP that will substitute part of the dTTP in the probe. This process results in DNA strands with the same genetic information but with labeled nucleotides inserted (Figure 6a).

The PCR and the random priming labeling methods use the same principle of polymerase action and labeled probes are produced through the synthesis of a new DNA chain. Primers are used in the reaction with non-labeled nucleotides (dATP, dCTP, dGTP and dTTP) and a labeled nucleotide, which is randomly inserted in the new DNA sequences by polymerase action, obeying the complementarities with the DNA template, resulting in labeled probes. In the PCR labeling amplification multiple cycles of DNA denaturation, primer annealing and DNA replication using specific primers by polymerase I action, generates new labeled DNA chains (Figure 6b). In the case of random priming method it is used a mix of degenerated primers (each one composed by six nucleotides), that contain almost all combination of A, T, C and G, and initiates the new DNA chains in random positions in the template DNA chain, so that almost every bit of templates DNA is covered by these primers (Figure 6b). Another difference from the PCR labeling method is that in the random priming labeling the single-stranded DNA is synthesized using the Klenow fragment of *E. coli* DNA polymerase I, which has only the site responsible for the 5'-3' polymerase action (Figure 6c).

The end labeling and *in vitro* transcription are less used as labeling probe methods in FISH experiments. The former uses the enzyme terminal deoxynucletidyl transferase (TdT) and a DNA polymerase, which is responsible for addition of nucleotides on the terminal 3'-OH in a single or double DNA strand. In the *in vitro* transcription method it is generated a labeled RNA molecule (riboprobes) using the action of a RNA polymerase that transcribes a

DNA cloned sequence. The riboprobes are frequently used to RNA targets, and in few cases are used to hybridize against chromosomal DNA (Schwarzacher and Heslop-Harrison 2000).

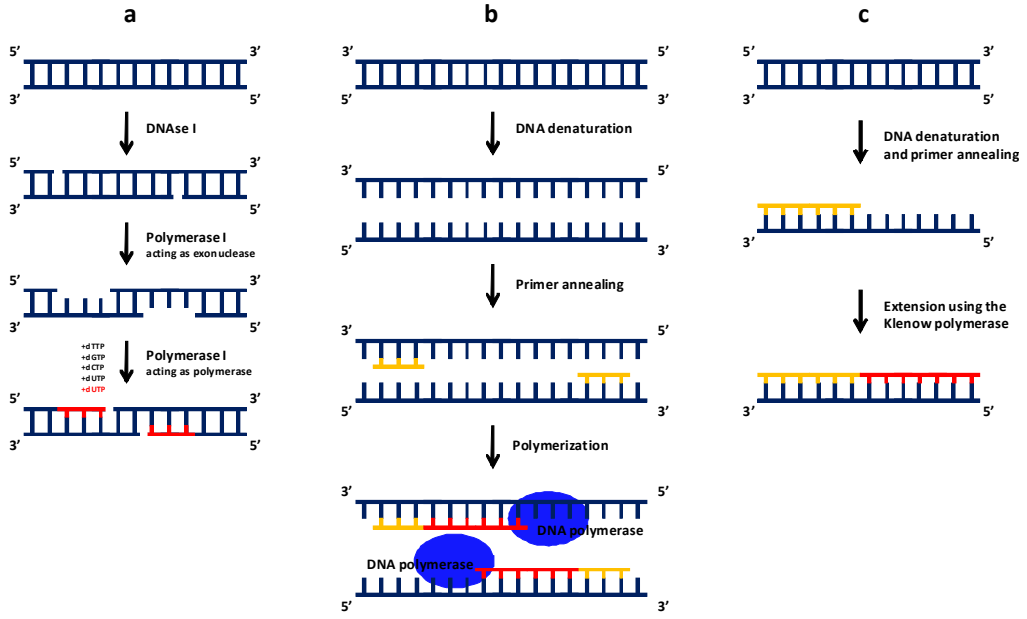

Figure 6. Most applied labeling methods for FISH. (a) Nick-translation; (b) PCR labeling; (c) Random priming. The primer is indicated in orange and the extended labeled DNA strand in red. For more details see the text.

The use of distinct methods of probe labeling described above basically depends on the size of initial DNA sequence and the amount of DNA in the reaction. For long DNA sequences it is useful the nick translation labeling method, that in general give probes with lengths about 200-300 bp. It is important the use of appropriate DNA quantity in this reaction, because the DNA molecule is not amplified along the nick translation reaction. The random priming and PCR methods are suitable for probes using low initial DNA amounts and for shorter fragments than the used in the nick translation. The size of the probe is an important parameter, due to influences in the hybridization results, where small probes have more facility of detachment from the target DNA, while the long probes can present difficult in the cell/nucleus penetration.

Recent Advances and Application of FISH Technique

During the recent years some modifications in the traditional FISH technique were introduced increasing the use of this methodology to solve distinct biological questions in basic research and clinical genetics. Among the advances, it can be referenced the increase of resolution in the identification of chromosomal rearrangements and the number of distinctly labeled probes that can be hybridized at the same time. Several technical modifications created to analyze human and plant chromosomes have been applied for animal chromosome

analysis, such as comparative genomic hybridization array (array CGH), multicolor-FISH, fiber-FISH, and genomic *in situ* hybridization (GISH).

The GISH technique is based in the extraction of total DNA of one species, labeling and application of this pool of DNA sequences in *in situ* hybridization experiments with cells from another related organism using cytological preparations (revised by Stace and Bailey 1999, Kato et al. 2005). This technique was first developed in animals for studies of hybrid cell-lines (Pinkel et al. 1986), and in plants it was first used in 1987 by researches from the Plant Breeding Institute, Cambridge (Schwarzacher et al. 1989). It has been mainly used for discrimination of parental genomes in interespecific and intergenetic hybrids and allopolyploid plant species. Since the first use of this methodology some research groups have applied this method for other purposes, such as in studies of chromosome positioning, analysis of B chromosomes, and comparative cytogenetic and genomics. Although most studies of GISH are concentrated in plants there are few but exciting examples for use of total genomic DNA as probes in animal chromosomes, i.e. in marsupials (Svartman and Viana-Morgante 1999), rodents (Houseal et al. 1995), fishes (Valente et al. 2009), salamanders (Bi et al. 2009) and insects (Bressa et al. 2009) showing that this methodology is a promising tool in the investigation of genome structure, chromosome evolution and intergenomic exchange in animals, related to autossomal complement and sex chromosomes. This methodology does not permit the generation of precise data about organization and evolution of specific chromosomes, but it allows a gross comparison of chromosomes and genomes of related species without the isolation of specific chromosomes or chromosomal segments (Svartman and Viana-Morgante, 1999). Moreover, this methodology is inexpensive and does not require DNA cloning and sequencing.

Also related to the overall genomic analysis the CGH array can be applied on chromosomes or in the genomic DNA. The CGH array on chromosomes was first described by Kalioniemi et al. (1992) and latter it was applied essentially in studies of gains and losses of genomic parts in human clinical cytogenetic, enabling high-resolution and genome-wide screening of segmental genomic copy number variations (CNVs). The principle of this technique for humans is based on the differential labeling of a normal and a pathological DNA (i.e. extracted from cancer cells) and competitive hybridization against a normal metaphase chromosome spread, detecting gains and losses of chromosomal/genomic parts by differences in signal intensity (Forozan et al. 1997). The standard pattern in this methodology is the label of the tumor DNA and the normal DNA on equal grounds with distinct haptens or fluorochromes, mix of the labeled probes and hybridization against normal metaphases. After the hybridization the chromosome spread is counterstained with DAPI and the profile of each chromosome is analyzed separately to investigate the gains and losses.

In fact in animal studies the CGH array technique can be applied in a similar path to that applied to humans, both in chromosomes or using only the genomic DNA. Studies of description of tumor conditions, genomic imbalance, chromosome aneuploidy or elimination were most frequently conducted in domestic animals and in animals with economic importance (Dunn et al. 2000, Thomas et al. 2007, 2009, Sakai et al. 2007, Hornak et al. 2009). In comparative and evolutionary studies this technique has been recently applied in the detection and measuring of structural variations in the genome, showing inter-specific genomic differences among related species, and could also find application in phylogenetic studies, but there are until now few results in the literature related to chromosomal analysis (Toder et al. 1998, Traut et al. 1999a, Griffin et al. 2008, Dávilla-Rodríguez et al. 2009, Mitra

et al. 2009). The use of CGH in animal chromosomes has been useful to visualize sequence homology of clusters in related species (Traut et al. 1999a), determination of species-specific chromosomal regions (Toder et al. 1998), and with most frequency in elucidation of sex chromosome establishment in vertebrate and invertebrate groups without strong differentiated sex chromosomes, such as fishes (Traut et al. 2001), crustaceans (Barzotti et al. 2000), insect groups (Traut et al. 1999b, 2001, Vítková et al. 2007), amphibians (Abramyan et al., 2009) and reptiles (Ezaz et al. 2005, 2006), being a powerful methodology for this purpose.

In studies about karyotype repatterning and chromosomal ongoing, the probes obtained by flow-sorting technique are frequently used in reciprocal cross-species chromosome painting assays to establish the chromosomal homeologies among related species. These studies have been conducted mainly in mammal representatives and improvements have been conducted along the recent years. One important improvement in the whole chromosome paints (WCP) was the use of some probes obtained from distinct chromosomes and hybridized together in the same metaphase spreads, the multicolor-FISH (mFISH) (Figure 7b).

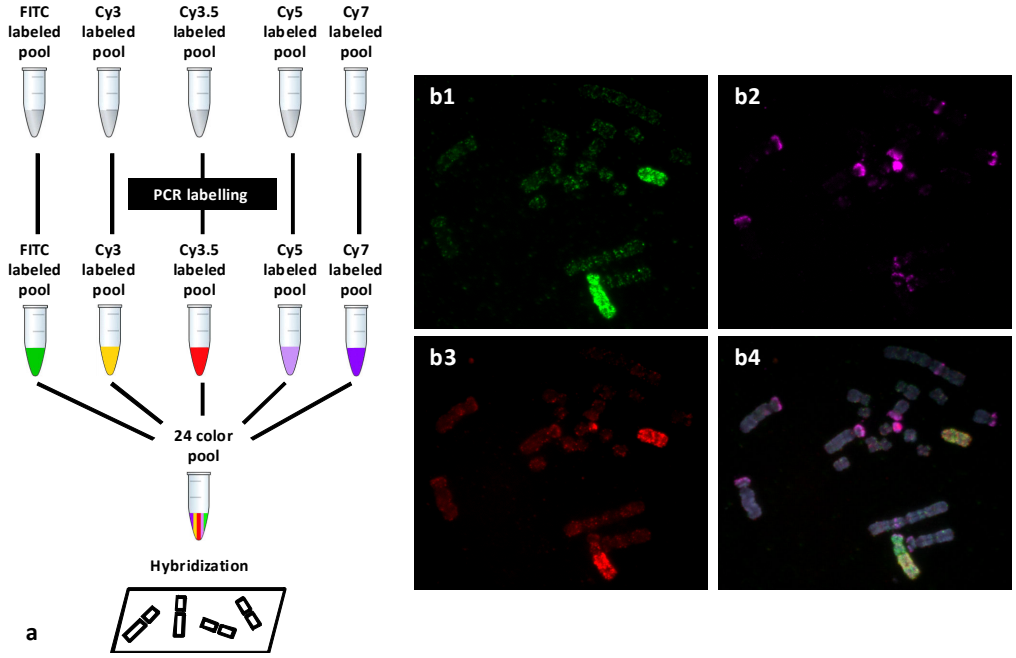

Figure 7. Multicolour FISH. (a) probe sets obtaining by combination of distinct fluorochromes for each chromosome; (b1-b3) sequential probe capture using the distinct probes labeled by three distinct fluorochromes for mFISH experiment in the bat *Carollia brevicauda*, (b4) overlapping of all chromosome probes in the same metaphases. In red Cy3 (Y2 chromosome probe), green FITC (X chromosome probe) and purple Cy5 (Y1 chromosome probe). Images b1-b4 are courtesy of Pieczarka JC (Universidade Federal do Pará, Brazil).

The mFISH assays are efficient in the description of chromosomal rearrangements and its principle was described in 1989 (Nederlof et al. 1989). Since 1996 this method has been applicable routinely in the simultaneous analysis of the 24 human chromosomes in mFISH paints and spectral karyotyping (SKY). The use of some probes in the same metaphase is advantageous due to the possibility of analysis of distinct probes in the same preparation,

generating economy of samples, reactives and time. Moreover the mFISH allows an easier and more precise description of the junction points of the different syntenic blocks in comparison with results obtained by single FISH.

The generation of probes with distinct colors is based in the combination of distinct fluorochromes for the same sequence of DNA (probe). For example, using two fluorochromes it is possible the labeling of three probes with distinct colors, two using the isolated fluorophores, i.e. green or red, and one with the equimolar combination of the two fluorochromes, green plus red which is yellow. The formula that determines the number of probes using combinatorial labeling assay is $C = 2^N-1$, where C is the number of different colors that can be distinguished when N different fluorochromes are used (Figure 7a). Using three fluorochromes it is possible the display of seven different colors and with five fluorochromes it is possible the hybridization of the 22 human chromosomes in the same metaphase. As described before multiple probes can be labeled directly with fluorophores or using available haptens.

For DNA sequences that appear overlapped in FISH experiments using metaphasic chromosomes it is useful the use of distended DNA fibers. This variation in the FISH technique is named DNA combing or fiber-FISH and consists in the extension of DNA fibers before the hybridization step. It is a very useful method in assessing the length of DNA probes, and in the analysis of the organization of sequences relative to one another and interspersed sequences. This method has been extensively used in the mapping of satellite DNAs and BAC probes providing maps with higher resolution than the generated using metaphasic chromosomes, or initial meiotic cells, such as pachytene (Schwarzacher and Heslop-Harrison 2000, Speicher and Carter 2005, Jiang and Gill 2006). Sequences separated by few kilobases can be resolved using the fiber-FISH, while the powerful resolution of FISH in metaphasic chromosomes is around 5-10 Mb for barley chromosomes (Pedersen and Linde-Laursen 1995). In less condensed chromosomes observed in prophasic-prometaphasic chromosomes the powerful resolution is increased to about 2 Mb (Cheng et al. 2002), and in pachytene chromosomes the signal resolution can be increased approximately to 500 Kb. For smaller genomic distances the signals appears overlapped, i.e. two probes, one green and another red will appears as a yellow signal (Schwarzacher and Heslop-Harrison 2000).

Microscope Analysis for FISH Assays

The analysis of FISH results requires the use of an epifluorescence microscope with a coupled photographic apparatus (Figure 8). Nowadays, the most part of images (almost all) are captured using digital cameras connected directly to a computer that facilitates the image acquisition, analysis, interpretation and increases the quality of the results for publication. The epifluorescence microscope apparatus consists basically of illumination, excitation and emission filters (that determines the wavelength to be observed), a dichroic beam-splitter (that shows the capacity of reflecting and transmission of light), and additional common parts present in light microscopes.

Briefly, the lamp emits a light with a range of wavelengths that pass through the excitation filter (Figure 8). This filter is responsible for blocking all light wavelengths except the wavelengths that excite the fluorochrome that will be observed.

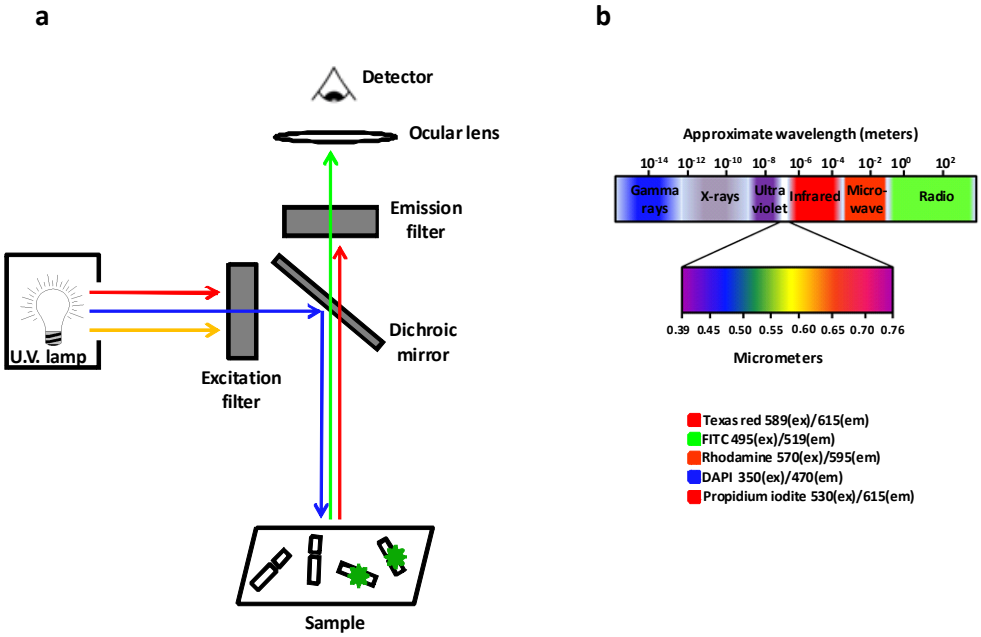

Figure 8. Microscope apparatus for FISH analysis. (a) internal epifluorescence system. (b) distinct wavelengths emitted by the fluorescence microscope lamp; excitation (ex) and emission (em) wavelengths for commonly used fluorochromes.

Then the wavelengths of interest pass to the dichroic beam-splitter reflecting some shorter light wavelengths that excite the fluorochrome linked to the probe or chromosome that will be observed. Moreover, this dichroic beam-splitter permits the transmission of longer wavelengths, which are emitted by the fluorophores in the chromosome preparations. The wavelengths that pass in the dichroic beam-splitter are filtered by the emission filter, which blocks the transmission of most wavelengths, allowing the passage of the wavelengths emitted by the fluorochrome and removing wavelengths from autofluorescence and reflected light. Finally, the light pass through the ocular lens or goes to the capture system (Figura 8a).

The process of signal emission (fluorescence emission) by the fluorochrome bounded to the probe or to the chromosomes consists basically in three steps. When an appropriate wavelength is directed to the chromosome slide a specific fluorochrome absorbs one photon (step one) and changes its state, becoming excited (step two). After this process causes the emission of one photon (step three), resulting in the fluororescent light emission that will be visualized. For a precision process it is necessary the presence of specific filters in the microscope for the fluorochromes of interest that are responsible to filter the wavelengths emitted by the microscope lamp and fluorochromes. The fluorochromes possesses distinct wavelength for excitation and emission, which may be similar between the distinct fluorochromes (Figure 8b). The use of more specific filters (that permits only the passage of specific wavelengths) results in FISH analysis with more quality and less background in the results. For more information about fluorochromes and technical approaches concerning the epifluorescence microscope and capture systems for FISH analysis it is useful to check the available catalogues of some distinct manufactures.

All approaches presented along this section have been extensively used in cytogenetic and have provided a good resolution for studies related to chromosomal evolution and

genome organization. Some examples of the application of these methodologies using repetitive DNAs, single copy sequences and chromosome paints will be presented in the next sections of this chapter, showing the importance and powerful of FISH technique for studies of karyotypic tracking in animal chromosomes.

Repetitive DNAs

Basic Features of Repetitive DNAs

The presence of a great amount of repetitive sequences is a common characteristic of eukaryotic genomes. These sequences are characterized by high variability and constitute families of repeated DNAs that represent a substantial component of eukaryote genomes, in some cases more than 80% of the DNA content of the cell (Charlesworth et al. 1994, Ridley 1996, Gregory 2005, Plohl et al. 2008) (Figure 9).

Repetitive DNA consists of sequences, identical or similar, which are in tandem (side by side) or dispersed throughout the genome. The satellite DNAs represent a typical example of tandem array repeated sequences whereas the transposable elements (TE) represent scattered sequences, although sometimes TEs can be organized in blocks in the chromosomes (Long and Dawid 1980, Charlesworth et al. 1994, Sumner 2003). Although today it is known that transposable elements and satellite DNAs can be transcribed, for a long time the general consensus established these sequence as non-encoding "junk DNA" (Figure 9). The "non-encoding" sequences are represented by satDNAs, minisatellite, microsatellite and transposable elements and the encoding are composed by the multigene families composed of hundreds to thousands of copies such the histone and ribosomal RNA (rRNA) genes (Kedes 1979, Flavel 1986, Prokopowich et al. 2003, Nagoda et al. 2005).

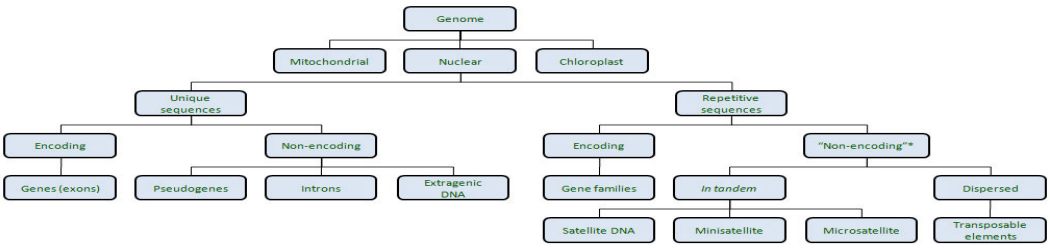

Figure 9. Scheme of genome organization in eukaryotes. The "non-encoding" nature of repetitive sequences (*) is explained in the text.

The large variation in genome size reported for eukaryotes is attributed, mostly, to the accumulation of repeated sequences (Petrov 2001, Kidwell 2002). The repeated DNA may vary between species with no obvious relation to the complexity of the organism, number of genes or ploidy level (Gregory 2005). The accumulation process involves repeated DNA amplification via gene conversion and unequal crossing-over (Charlesworth et al. 1994, Hancock 1999, Kidwell 2005).

Multigene Families

Most multigene families studied by fluorescence *in situ* hybridization are the rRNA and histone genes. Ribosomal DNA (rDNA) transcribes the rRNA component of ribosomes, which are essential for the protein synthesis process (Haeusler and Engelke 2006). Eukaryote genomes contain multiple copies of rRNA genes, presumably because exceptionally high quantities of RNA transcripts are necessary (Prokopowich et al. 2003). Ribosomal RNA genes are organized in tandem arrays containing transcriptional units coding for the 18S, 5,8S and 28S ribosomal RNAs (Figure 10a), being the three rRNA molecules transcribed from a single promoter by RNA polymerase I. The repeat units containing the codifying sequences for the 18S, 5,8S and 28S ribosomal RNAs are separated from each other by variable intergenic spacers (IGS) and an external transcribed spacer (ETS) (Eickbush and Eickbush 2007). Ribossomal DNA repeat units are evolutionarily dynamic and seem to be able to spread through the genome creating new rDNA loci (Iborra and Cook 2002). In eukaryotes 5S rRNA is also transcribed from tandemly repeated sequences, but are often of higher copy number than the others rRNA genes. The 5S rDNA repeats consist of a highly conserved transcribed sequence of 120 bp, which is separated from each other by a variable non-transcribed spacer (NTS) (Figure 10b).

These two families of genes are generally not at the same chromosomal location, although there are exceptions (Leitch and Heslop-Harrison 1993). 5S genes are transcribed by RNA polymerase III, the polymerase also responsible for synthesis of tRNAs and other small, non-translated RNAs (Bell et al. 1977, Haeusler and Engelke 2006).

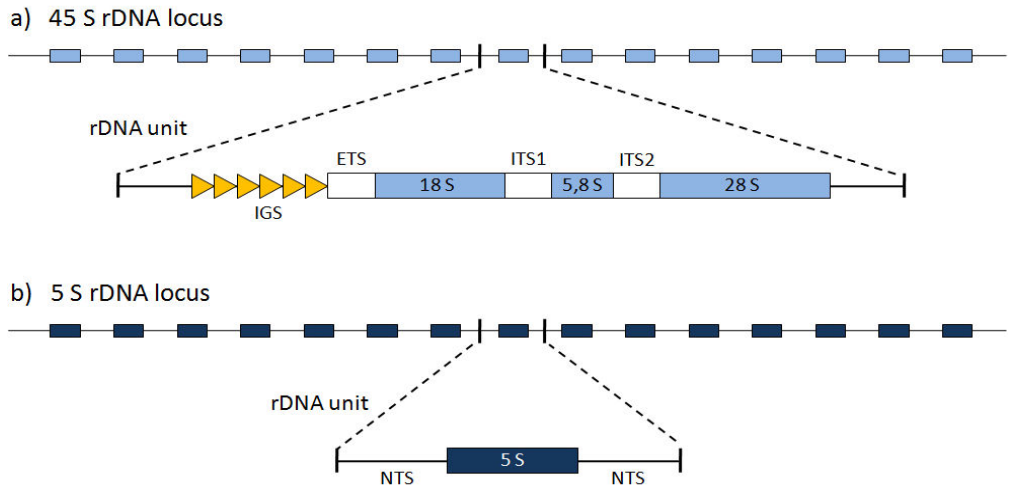

Figure 10. Organization of ribosomal RNA (rRNA) genes in eukaryotes. The genes are organized into tandemly repeated units as diagrammed at the top. A typical unit is shown in expanded detail. IGS, intergenic spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer; NTS, nontranscribed spacer.

Figure 11. Histone gene arrangements in the quintet repeating unit.

The second multigene family most explored by fluorescence *in situ* hybridization codifies the histones and are known to be a family of moderately repeated genes (Kedes 1979). The histone genes have an extraordinary organization in tandem arrays, are interspersed from each other with noncoding spacer sequences, and codifies for five histone proteins (Kedes and Gross 1969, Kedes 1979, Nagoda et al. 2005) (Figure 11). It has been discovered in some genomes a few atypical unit lacking every gene (Schienman et al. 1998, Nagoda et al. 2005). The organization and arrangement of histone genes has undergone expressive evolutionary change, but it is not yet clear how these different units originated and spread in the genome (Nagoda et al. 2005). Histones are a class of basic proteins that associate with each other and with nuclear DNA to form the nucleosome, the fundamental unit of chromatin structure. The structure of histones, particularly the H3 and H4 histones, generally is highly conserved between diverse animal phyla and even between the animal and plant kingdoms, although remarkable variations have been reported in the DNA sequence for these proteins (Ruberti et al. 1982, Maxson et al. 1983, Miller et al. 1993, Del Gaudio et al. 1998, Tsunemoto and Matsuo 2001, Albig et al. 2003).

Even among the most divergent clusters histone genes have several features in common (Kedes 1979, Maxson et al. 1983, Nagoda et al. 2005). The structural differences in the histone repeat units among diverse organisms suggest they originated by evolutionary amplification of a unique ancestral histone gene cluster. Alternatively, a single ancestral cluster may have undergone various rearrangements and sequence changes to produce the diversity of fine structures in the histone gene clusters found in different organisms (Maxson et al. 1983, Schienman et al. 1998).

DNAs Repeated in Tandem

The classification of repeated sequences organized in tandem is based on the size of the repeated unit and in the size of the cluster of repetitions. The first group is represented by the satellite DNA, composed of highly repeated sequences, grouped into one to several locations along one or more chromosomes and interspersed with single copy sequences. The repeated units range from 100 to 1.000 nucleotides, varying in structure, location and quantity within the genomes (Ridley 1996, Ugarković and Plohl 2002). Longer repeat size (even longer than 4 kb) can also be present in the genome and are named megasatellite DNAs (Gondo et al. 1998). Satellite DNAs represent the main component of the heterochromatin and are located preferentially in pericentromeric and telomeric regions (Yunis and Yasmineh 1971, John and Miklos 1979, Juan et al. 1993, Shapiro and Sternberg 2005). Satellite DNAs are generally AT-rich and show high variability in monomer size, nucleotide sequence, copy number and chromosomal organization (Charlesworth et al. 1994).

The second group consists of moderately repeated DNA, with about 10 to 100 bp, called minisatellites or sequences with variable number of repeats (VNTR - Variable Number of Tandem Repeats) (Jeffreys et al. 1985). Each cluster of repeats represents a minisatellite locus, whose alleles are distinguished by variations in their overall size and can be used as genetic marker in a technique called DNA fingerprinting (Mueller and Wolfenbarger 1999). One of the first minisatellites was described in an intron of the human myoglobin gene and is comprised of 33 bp tandem repeat units with some sequence similarities with other minisatellites discovered previously. It was flanked by a 9 bp direct repeat, a characteristic signature of transposable elements, suggesting that this minisatellite was able to transpose in some way (Jeffreys et al. 1985). The minisatellites were further identified in a wide variety of

organisms, such *Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster*, *Homo sapiens* and *Saccharomyces cerevisiae* demonstrating that such DNA class is common in the eukaryote genomes (Richard et al. 2008).

Another group of tandem repeated DNAs is composed of short repeats of 1 to 6 bp. They are widely distributed in the genome, highly polymorphic and therefore used as markers in studies of population genetics, conservation, epidemiology, testing and kinship mapping (Bowcock et al. 1994, Balloux et al. 1998, Röder et al. 1998, Schlötterer 2000). Among the functions assigned to microsatellites are its participation in chromatin organization, DNA replication, recombination and regulation of gene activity (Li et al. 2002a). The first microsatellite was characterized by Weller and colleagues as a polymorphic $(GGAT)_{165}$ repeat in the human myoglobin gene (Weller et al. 1984, Richard et al. 2008). Other example is the telomeric repeats, which are composed of the hexameric unit TTAGGG in all mammals and many animals (TTTAGGG in plants), and are essential for chromosome stability and regulation of replicative lifespan of somatic cells (Blackburn 2005, Fouché et al. 2006). Microsatellites were observed in a range of organisms, such *Arabidopsis thaliana, Drosophila melanogaster, Homo sapiens, Mus musculus*, *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Tetraodon nigroviridis* (Richard et al. 2008), being common and widespread in the prokaryote and eukaryote genomes.

Transposable Elements

The transposable elements (TEs) are differentiated from other genome sequences by having the ability to mobilize in the genome (Hartl et al. 1992, Kazazian 2004). An important feature of these elements is the polymorphisms generated as a consequence of the insertion and the variability in the number of copies that can arise within and between species (Shapiro and Sternberg 2005, Feschotte and Pritham 2007, Lankenau and Volff 2009). The classification of transposable elements is based on enzymology, structural similarities and sequence relationships (Kapitonov and Jurka 2001, Wicker et al. 2007). It includes classes, subclasses, orders, superfamilies, families and subfamilies (Figure 12). All eukaryotic TEs belong to two types (retrotransposons and DNA transposons) and are composed of five major classes: long terminal repeat (LTR) retrotransposons, non-LTR retrotransposons, cut-and-paste DNA transposons, rolling-circle DNA transposons and self-synthesizing DNA transposons (Figure 13). Each class of TE is composed of a small number of superfamilies or clades, and each superfamily consists of numerous families of TEs (Charlesworth et al. 1994, Hua-Van et al. 2005, Wicker et al. 2007, Kapitonov and Jurka 2008, Pritham 2009).

The mechanisms for implementation are related to the means used by transposable elements to insert into a new site within the genome. The means of implementation through DNA can be conservative or replicative. First, the TE is removed from one site and inserted into another, while in the second TE is duplicated before being transported to a new location, increasing the number of insertions in the genome (Kappitonov and Jurka 2001, Wicker et al. 2007). In the process of transposition via RNA, the RNA intermediate is reverse transcribed into a new copy of DNA, and thus basically replicating, and inserted in a new genomic location (Xiong and Eickbush 1990, Wicker et al. 2007).

UNIVERSAL CLASSIFICATION SCHEME OF TRANSPOSABLE ELEMENTS

Type 1: DNA transposons	Type 2: retrotransposons	
	Non-LTR retrotransposons	LTR retrotransposons
Superfamily	Superfamily	Superfamily
Chapaev	*CRE*	*Copia*
En/Spm (CACTA)	*NeSL*	*Gypsy*
hAT	*R4*	*BEL*
Harbinger (Pif)	*R2*	*ERV1*
ISL2EU (IS4EU)	*L1*	*ERV2*
Kolobok	*RTE*	*ERV3*
Mariner	*Jockey*	*DIRS*
Merlin	*CR1*	
Mirage	*Rex1*	
MuDR (MULE)	*I*	
Novosib	*Randl (Dualen)*	
P	*Tx1*	
PiggyBac	*SINE1*	
Rehavkus	*SINE2*	
Transib	*SINE3*	
Helitron	*Penelope*	
Politon (Maverick)		

Figure 12. The universal classification and nomenclature of eukaryotic transposable elements. Different classes of transposable elements (TEs) are differently colored.

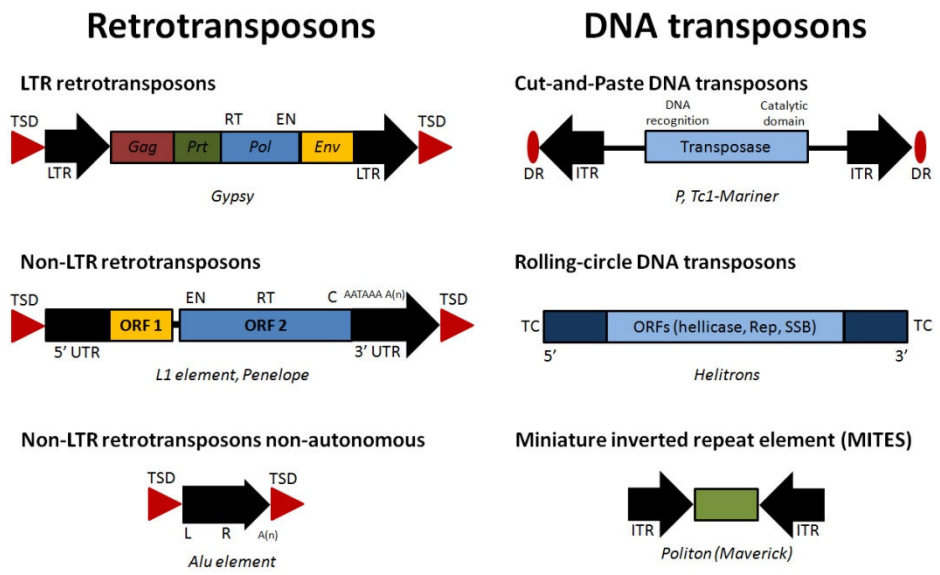

Figure 13. Generalized structures of the main types of transposable elements.

Mapping of Repetitive DNAs

Eukaryotic genomes contain vast amounts of repetitive DNAs, and the large-scale sequencing of these genomes has produced an unprecedented wealth of information about the origin, diversity, and genomic impact of the repetitive sequences. Repetitive DNAs represent great chromosomal markers that are very useful in studies of chromosome structure and function, evolution, identification of chromosomal rearrangements, supernumerary chromosomes and sex chromosomes (Goldman et al. 1984, Cabral-de-Mello et al. 2010a, Gross et al. 2009, Meštrović et al. 2009, Nakamura 2009). Fluorescence *in situ* hybridization, using repetitive DNAs as probes, is a powerful technique that can correlate molecular information of a DNA sequence with its physical location along chromosomes and genomes, and has been applied to chromosomes in various eukaryotes, including several animal groups (Fridolfsson et al. 1998, Nomoto et al. 2001, Vitturi et al. 2002, Volpi and Bridger 2008, Mazzuchelli and Martins 2009, Veltsos et al. 2009, Cabral-de-Mello et al. 2010a) (Figure 3a-d, j-l).

The application of fluorescence *in situ* hiybridization in animals frequently use rDNA, transposable elements or satellite DNA as probes, but there are also studies using histone genes and other sequences (Clabby et al. 1996, Barragán et al. 2002, Cabrero et al. 2003a, b, Odierna et al. 2004, Cabrero et al. 2009, Giovannoti et al. 2009, Mazzuchelli and Martins 2009, Teixeira et al. 2009, Cabral-de-Mello et al. 2010a, b). The rDNAs and some satDNA have been mapped in all vertebrate groups and in some invertebrates, such as annelids, mollusks, arthropods and echinoderms, while the histone genes were mapped in insects, mollusks and fishes (Bizarro et al. 2000, Vitturi et al. 2000a, b, c, Kubota et al. 2001, Colomba et al. 2002, Zhang et al. 2007, Cabral-de-Mello et al. 2010a, Nguyen et al. 2010).

Most studies using rDNA as probes in invertebrates have been conducted in insects and some variation for the chromosomal distribution of this marker has been described (Moura et al. 2003, Cabrero and Camacho 2008, Nguyen et al. 2010). Although some studies concerning the rDNA sites have been extensively conducted by means of silver nitrate staining, detecting only active nucleolar organizer regions (NOR), that can not correspond to the real genome organization or 45S rDNA clusters (for example, see Cabral-de-Mello et al. 2010a). There are few reports in the literature for chromosomal location of 5S rRNA genes in grasshoppers, beetles and other insects (Bizzaro et al. 2000, Cabrero et al. 2003b, Loreto et al. 2008, Cabral-de-Mello et al. 2010a, c). In grasshoppers this sequence was mapped in *Eyprepocnemis plorans* (Cabrero et al. 2003b), *Ramathocerus brasiliensis* (Loreto et al. 2008), *Locusta migratoria* (Teruel et al. 2010) and in four Proscopiidae species (Cabral-de-Mello et al. 2010c). The first three species presented some clusters of 5S rDNA, while in the Proscopiidae species a remarkable conservation for this sequence was observed in both number and location of sites. Moreover this sequence was observed in the B chromosome of *E. plorans* and in *R. brasiliensis* being an important marker to analyze B chromosome origin and evolution (Cabrero et al. 2003a, Loreto et al. 2008). The 5S rDNA was located only in one species of Coleoptera and one of Hemiptera. In *D. geminatus* (Coleoptera) this sequence was located in one chromosome distinct to the location of 18S rDNA, while in *Acyrthosiphon pisum* (Hemiptera) the two rDNA clusters were located in the same chromosome but with distinct positions (Bizzaro et al. 2000, Cabral-de-Mello et al. 2010a).

Among other invertebrates, the 18S-28S and 5S rDNAs were mapped in mollusks, Anelidae and Crustacea and these sequences were frequently detected in conspicuous blocks

in the same chromosome (Drouin and Moniz de Sá 1995, Colomba et al. 2002, Vitturi et al. 2002, 2004, Wang and Guo 2004). In the mollusk *Melarhaphe neritoides* (Caenogastropoda), for example, the repeated units of both rDNA classes are closely associated on the same chromosome pair, most probably interspersed due to overlapping of the two hybridization signals (Colomba et al. 2002).

The scenario of organization of rDNA clusters in vertebrates indicates a non association of the two rDNA classes, with most species presenting these sequences in distinct chromosomes, although in some species it was reported the presence of clusters for these sequences near each other or far in the same chromosome (Lucchini et al. 1993, Liu and Fredga 1999, Mandrioli et al. 2000, Sola et al. 2000, Martins and Galetti 2001). In general 5S rDNA occurs in interstitial regions of the chromosomes (Lucchini et al. 1993, Ferreira et al. 2007, Gornung et al. 2008), and apparently this pattern may represent an ancestral condition or could confer some advantage for the genome organization of these sequences (Martins and Galetti 1999, 2000). Another interesting characteristic has been described in fishes for 5S rDNA with the presence of two distinct sequence classes organized in different chromosomal regions or even in different chromosomes (Sajdak et al. 1998, Martins et al. 2000, Martins and Galetti 2001).

The variations observed for rDNA sites indicate a complex microevolutionary pattern that rules their organization in the genome. Ribossomal DNAs seem to be able to spread through the genome thus creating new rDNA loci (Castro et al. 2001), variant rDNA copies (Martins et al. 2006) and even association to other multigene families (Eirín-López et al. 2004, Cabral-de-Mello 2010a, 2010c). In the South American Erythrinidae fish *Hoplias malabaricus*, variant 5S rDNA repeat copies are spread in the centromeric area of several chromosomes (Martins et al. 2006, Ferreira et al. 2007, Cioffi et al. 2009). Similarly in other Erythrinidae species, *Erythrinus erythrinus*, 5S rDNA dispersion is associated to the distribution of the retroelement *Rex*3 (Cioffi et al. *in preparation*). Several spreading mechanisms have been suggested, including transposition, insertion of extrachromosomal rDNA amplified during oogenesis, the presence of repetitive elements facilitating nonhomologous chromosome exchange, and the amplification of minor rDNA loci (Phillips et al. 1988, Maggini et al. 1991, Dubcovsky and Dvörak 1995).

The use of histone genes in chromosomal mapping have been done with more frequency in invertebrates, but it is restrict to analysis of 19 chironomid midges, 11 fruit flies, five mollusks, 39 grasshopper and one beetle (Hankeln et al. 1993, Schienman et al. 1998, Ranz et al. 2003, Eirín-López et al. 2004, Zhang et al. 2007, Cabrero et al. 2009, Cabral-de-Mello et al. 2010a). In general these sequence have presented more conservation related to chromosomal location and number of clusters compared to the rDNAs (Cabrero et al. 2009, Cabral-de-Mello et al. 2010a), although small variability in cluster number have been reported (Ranz et al. 2003, Zhang et al. 2007). The recent study in grasshopper performed by Cabrero et al. (2009) revealed a strong association of H3 and H4 histones in grasshopper and intense conservation of chromosomal location of these sequences. This chromosomal conservation was also described for other insects (Cabral-de-Mello et al. 2010a, 2010c). Recently, Teruel et al. (2010) used this sequence for precise establishment of B chromosome origin in the migratory locust *Locusta migratoria*.

Some molecular analyses for histone gene sequences have revealed that these sequences form a block of tandem arrays in some groups. This pattern of histone organization has been detected for some invertebrates as cited above and in vertebrates it was observed in three fish

species, *Salmo salar* (salmon), *S. trutta* (brown trout) and *Oncorhynchus mykis* (rainbow trout) (Pendás et al. 1994). Moreover, histone sequences and 5S rRNA genes can be linked in the same cluster in animal genomes. This finding was reported in one mussel and two crustaceans (Drouin and Moniz de Sá 1995, Barzotti et al. 2000, Eirín-López et al. 2004). Moreover the FISH using probes for 5S and H3 histone genes in beetles (Cabral-de-Mello et al. 2010a) and grasshoppers (Cabral-de-Mello et al. 2010c) revealed a unique block for these two sequences, indicating a possible interspersed association of both genes.

There are several studies in some animal groups related to chromosomal location, molecular organization and possible functions of heterochromatin (Sumner 2003, Grewal and Jia 2007). However, despite the heterochromatin has been extensively studied, the mapping of satellite DNAs are restricted to a few groups (Phillips and Reed 1996, Odierna et al. 2004, Wang et al. 2001, Palomeque and Lorite 2008, Giovannotti et al. 2009). Usually satellite DNA families are species-specific (Arnheim 1983). However, there are a few exceptions in which a group of species share the same satellite DNA family, as observed for the centromeric alpha satellite DNA, which is preserved in the primate order most probably because of its centromeric function (Schueler et al. 2001). Alpha satellite-like sequences were also detected in other organisms, as chicken, primates and zebrafish (Li and Kirby 2003, Alkan et al. 2007).

Despite their sequence heterogeneity, the study of insect satDNAs indicates the evolutionary conservation of certain features, and within each taxonomic group of insects the satDNAs has been studied in only a few species (reviewed in Palomeque and Lorite 2008). Satellite DNA has been characterized in eight species of the *Formica* genus (Hymenoptera, Formicidae). This satellite DNA is organized as tandem repeats of 129 bp monomers (with the presence of internal inverted repeats) and is located in the pericentromeric regions of all chromosomes (Lorite et al. 2004). Although the evolution of the satellite DNA in ants could be similar to that in other organisms, there may be some particularities as a result of a haplodiploid system. Ant male haploids do not undergo meiotic recombination and redistribution of chromosomes to the next generation. In Hymenoptera the processes of molecular evolution of the satellite DNA would be altered by the special constraints imposed by the haplodiploid system (Bigot et al. 1990). It was suggested that satellite dynamic appears to be the outcome of both general molecular processes and specific organism traits (Luchetti et al. 2003).

Other examples of the use of satDNA in chromosome and genome structure in insects were performed in beetles from the family Tenebrionidae and in grasshoppers for analysis of B chromosome structure (Mravinac et al. 2004). In tenebrionids that present species with high amount of AT rich centromeric heterochromatin, the mapping of satDNA was applied in chromosomes of representatives from the genus *Tenebrio* and *Tribolium*, and the results were similar to observed in other groups with distinct satDNAs located in heterochromatic regions (Mravinac et al. 2004, Mravinac et al. 2005). For the genus *Tribolium* the satDNA families have conserved and variable segments and common characteristics, as short inverted repeats, nonrandom distribution of A or T≥3 tracts and a CEMP-B box-like motif (Mravinac et al. 2005).

For grasshoppers one satDNA with 180 bp was mapped in distinct population of the B harboring species *Eyprepocnemis plorans*. This sequence was present in the centromeric region of some autosomes and X chromosome with variations between the populations and subspecies representatives. Moreover this sequence was mapped in B chromosomes of some

populations and showed variability related to the presence/absence of this sequence that interestingly was correlated with the distinct populations (Cabrero et al. 2003a, Abdelaziz et al. 2007, López-León et al. 2008).

The physical mapping of satellite DNAs in mollusks provided an effective tool for oyster chromosome identification which is so difficult due their small size and similar arm ratio (Guo et al. 1996). Clabby et al. (1996) identified a highly repetitive satellite DNA, named *Cg*170, in the Pacific oyster. *Cg*170 is the first centromeric satellite sequence identified in bivalves and in mollusks as general. It is interesting to note that centromeric *Cg*170 in the Pacific oyster is about the same size as the centromeric satellite monomer in humans. Other satellite sequences have been reported in a few mollusks, which are apparently unrelated to *Cg*170 satellite, and provide important insights on the organization and evolution of mollusk genomes (Plohl and Cornudella 1997, Ruiz-Lara et al. 1992, Mary-Elizabeth et al. 1998, Wang et al. 2001, Petrović et al. 2009). In *Donax trunculus* a highly abundant satDNA named DTF2 was mapped on chromosomes and overlaped only partially with the GC-rich heterochromatin located in interstitial and subtelomeric regions (Petrović et al. 2009).

For vertebrates satDNAs were mapped mainly in fishes and mammal chromosomes, although there are results concerning amphibians and reptiles. The chromosomal distribution of satellite DNAs among fish species showed that these sequences are mainly located in the centromeric region of chromosomes. A *Hind*III satellite DNA family isolated from the sturgeon *Acipenser naccarii* genome was preserved in the pericentromeric regions of the chromosomes of six species of the genus *Acipenser* and one of the genus *Huso* (Lanfredi et al. 2001). Centromeric satellite DNA families were also isolated from the genome of the gobiid *Gobius cobitis* (Canapa et al. 2002) and the Nile tilapia *Oreochromis niloticus* (Ferreira and Martins 2008). Particularly in the Nile tilapia, the satellite family was present in the centromeric region of all chromosomes of the complement (Ferreira and Martins 2008). Particular attention has been directed to the identification of satellite DNAs related to sex and supernumerary chromosomes in fishes. Satellite DNAs have been isolated and mapped in sex chromosomes of several species, such as *Leporinus elongatus* (Nakayama et al. 1994), *Chiondraco hamatus* (Capriglione et al. 1994a), *Poecilia reticulata* (Nanda et al. 1990), and *Oncorhynchus tschawytscha* (Devlin et al. 1991, Stein et al. 2001), among others. A satellite DNA, named As51, correlated to a supernumerary chromosome was firstly isolated in *Astyanax scabripinnis*, had repeats of 51 bp and was located in the non-centromeric heterochromatins, and in the NORs and in the supernumerary chromosome. The symmetric distribution of As51 in both arms and its meiotic behavior suggest that this chromosome is an isochromosome (Mestriner et al. 2000). Satellite DNAs were also isolated from *Prochilodus lineatus* which presents 0 to 5 small supernumerary chromosomes. Two satellite DNA families, with monomeric units of 441 and 900 bp, were isolated from the genome of this species and were located in the pericentromeric region of several chromosomes of the A complement. The 900 bp satellite was also located in several supernumeraries demonstrating that the supernumerary chromosomes of this species have originated from A chromosomes that harbor the 900 bp satellite DNA family (Jesus et al. 2003).

For amphibians for example, studies of the *Pst*I satellite DNA family contributed to understanding the origin of tetraploidy in *Bufo viridis* (Amphibia, Anura), suggesting that this was an ancient event (Odierna et al. 2004). In green toads, a group of frogs, the diversification appears to occur without any chromatin change, including the localization and composition of heterochromatin and of the *Pst*I satellite DNA family (Birstein and Mazin 1982, Matsui et al.

1995, Odierna et al. 2004). In *Discoglossus pictus* (Amphibia, Anura), it was observed that there is an unusual chromosomal distribution of the Dp-sat1 satellite DNA. This satDNA is characteristic of the genus *Discoglossus* and represents a major repetitive DNA that accounts for about 6% of the *D. pictus* genome. Two interesting aspects are the non-random distribution of the Dp-sat1 DNA and its association in the interphasic nucleus. Most Dp-sat1 associated heterochromatin appears to be located only in one pole of the nucleus, showing evidence of ordered organization and location of the chromosomes in the nucleus during interphase (Amor et al. 2009).

There are few studies in reptiles with satellite DNAs, and they are concentrated in molecular analysis without chromosomal mapping (Caputo et al. 1994, Grechko et al. 2005, Giovannotti et al. 2009). A large number of satDNAs sequences are of recent origin and are only observed in related species (Capriglione et al. 1994b, Rudykh et al. 1999, Ciobanu et al. 2003, 2004, Grechko et al. 2005), whereas the most common ones are also highly conserved in unrelated species, maybe because any important function (Olmo et al. 2002). The first satellite DNA isolated and characterized in Scincidae was the *Ava*II satellite DNA isolated from the genome of *Eumeces schneideri* (Reptilia, Scincidae), one of the most diverse and species-rich families of squamate reptiles (Giovannotti et al. 2009). The mapping of this sequence revealed that this satellite is widespread in the genome of this lizard, occurring on most of the 16 chromosomes of this species with variation in signal intensity. Moreover the fiber-FISH using *Ava*II satDNA and telomeric sequence showed and overlapping of these sequences. Comparative nucleotide sequence analyses of *Ava*II satDNA with sequences of satDNAs deposited in GenBank revealed no similarity, indicating that *Ava*II satDNA constitutes an undescribed satellite (King and Cummings 1997). In *S. scincus*, considered the most closely species to *E. scheneideri*, it was also observed the presence of the *Ava*II satDNA. The occurrence of this satellite in phyletic lineages which split about 14 million years ago (Carranza et al. 2008) and the presence of a unique satellite family in *E. schneideri* indicates that these taxa are considered as chromosomal conservative lineages (Slamovits and Rossi 2002). Through the karyotype stability observed in *E. schneidei* and the closely related species *E. algeriensis*, *S. scincus*, and *S. hemprichii,* this hypothesis seems to be confirmed (Caputo et al. 1994).

The amount of pericentromeric satellite DNA is highly variable in mammals. In several species of the genus *Acomys* (Rodentia), for example, a family of centromeric satellite DNA represents between 0.88% of the haploid genome. In mouse the minor satellite represents about 0.5–1%, and the major satellite DNA approximately 6% of the genome (Kunze et al. 1999). Mammalian centromeres are composed of multiple families of satDNA, and the centromeric satellites are well characterized in humans and other primates and are usually characterized by the presence of tandem repeats of monomers from 169 to 170 bp, which are organized into chromosome specific high-order repeat units (Willard and Waye 1987, Choo et al. 1991, Wang et al. 2001).

The majority of satDNA families have an important role in mammal genome evolution by promoting chromosomal rearrangements (Wichman et al. 1991, Qumsiyeh 1994, Slamovits et al. 2001, Louzada et al. 2008, Ropiquet et al. 2008). The involvement of constitutive heterochromatin in events of chromosomal evolution is discussed in several studies, suggesting that these regions act as hotspots enabling structural chromosome rearrangements (Chaves et al. 2004, Louzada et al. 2008, Ventura et al. 2009). The location of evolutionary breakpoint regions is coincident with the location of regions rich in repetitive

sequences (Li et al. 2000, Locke et al. 2003, Ruiz-Herrera et al. 2006). The Indian muntjac (*Muntiacus muntjak vaginalis*) has a karyotype of 2n = 6 in the female and 2n = 7 in the male (Wurster and Benirschke 1970), and is a classic example of chromosome rearragement. The karyotype evolution of Indian muntjac via extensive tandem fusions and several centric fusions was well documented by molecular cytogenetic studies (Yang et al. 1995, Chi et al. 2005, and others). It has been characterized in the Indian muntjac three cervidae-specific centromeric satDNA sequences (Bogenberger et al. 1987, Lin et al. 1991, Qureshi and Blake 1995, Vafa et al. 1999, Li et al. 2002b, Liu et al. 2008). A segment of centromeric DNA identified revealed a complex organization of sequences, and only two regions have sequences homologous to previously identified cervid centromeric satellite DNA families, showing also homology to interspersed repetitive sequences and sequences scattered over this segment of centromeric DNA. It might be that Indian muntjac centromeric DNA segment represents characteristics of the centromere of Cervidae or is the result of chromosome restructuring during the process of karyotypic differentiation (Cheng et al. 2009).

Another important repetitive DNA class widely mapped in many karyotypes is the transposable elements (TEs) and in general, their pattern of distribution in heterochromatin and euchromatin is variable among different genomes. However it seems that they tend to accumulate in the centromeric and/or heterochromatic regions of animal chromosomes. Such patterns can be correlated with a role of the repeated sequences in the structure and organization of pericentromeric regions and heterochromatic areas. However, the distribution of TEs in human and mouse genomes is relatively uniform in both euchromatin and heterochromatin (Kidwell 2002, Volff et al. 2003, Fisher et al. 2004). An astonishing diversity of transposable elements not found in the human and the mouse has been observed in the genome of others animals.

In *Drosophila*, TE accumulates preferentially near centromeres and telomeres and account for 8% of heterochromatin and 4–5% of euchromatin (Bartolomé et al. 2002). Pimpinelli et al. (1995) show the distribution of 9 different transposable elements on *Drosophila melanogaster* mitotic chromosomes (copia, gypsy, mdg-1, blood, Doc, I, F, G, and Bari) and they are preferentially clustered into one or more discrete heterochromatic regions in chromosomes. Moreover, FISH analysis of geographically distant strains revealed that the locations of these heterochromatic transposable element clusters are highly conserved.

Analysis of the chromosomal location of various types of TEs in the compact genome of the pufferfish *Tetraodon nigroviridis* showed that these sequences are generally excluded from gene-rich regions (Dasilva et al. 2002). In the cichlid *Cichla kelberi* some retrotransposable elements (*Rex1*, *Rex3*, *Rex6*) were compartmentalized predominantly in the centromeric region in coincidence with heterochromatic areas and also as small dispersed signals along most chromosomes (Teixeira et al. 2009). The compartmentalization of *Rex1* and *Rex3* elements in the centromeric areas and heterochromatin was also observed in the fish *Notothenia coriiceps* (Ozouf-Costaz et al. 2004). Chromosomal mapping data on *Tc1*-like sequences in vertebrates is scarce, but in the cichlid *C. kelberi* this transposable element is observed in centromeric regions and dispersed along the chromosomal arms of most chromosomes (Teixeira et al. 2009). This pattern is similar to *Rana sculenta* (frog), a sequence that originated from a *Tc1*-like element was organized in tandem arrays in the centromeric area of few chromosomes. Another example of such remarkable compartmentalization was observed in *T. nigroviridis*, where the *Tc1*-like elements were

clustered in the heterochromatic short arms of six subtelocentric chromosome pairs (DaSilva et al. 2002).

The centromeres of a number of species have also shown TEs interspersed with satellite sequences (Hua-Van et. al. 2005). Fisher et al. (2004) show a heterochromatic co-localization of transposable elements with minisatellites in the compact genome of the pufferfish *T. nigroviridis*. The satellite DNA amplification together with retrotransposon accumulation in the centromeres indicates an important role for the expansion and stabilization of this chromosome region (Plohl et. al. 2008).

In mammals the transposable elements characterized to date appear to be nonrandomly distributed. While most TE were found to be exclusively restricted to specific chromosomal location, LINE elements and some retrovirus-like elements are preferentially accumulated in G-banding regions of the chromosomes, and in some cases in the sex chromosomes, while SINE elements occur preferentially in R-banding regions. Four mechanisms are presented which may explain the nonrandom genomic distribution of mammalian transposons: i) sequence-specific insertion, ii) S-phase insertion, iii) ectopic excision, and iv) recombinational editing. Some of the available data are consistent with each of these four models, but no single model is sufficient to explain all of the existing data (Wichman et al. 1992).

Besides the preferential distribution of TEs in noncoding regions, they have a different distribution among and within chromosomes, being most frequently associated with sex chromosomes than autosomes, probably due a higher concentration of heterochromatin in these elements (Kidwell 2002). These sequences have important role in sex chromosome differentiation and evolution. Together these data indicate that transposable elements are major structural components of heterochromatin and have played an important role in the chromosomal evolutionary history of host genomes.

Although the most applied FISH-probe class is related to the use of repeated DNAs, many questions about chromosome and genome evolution still remains to be answered. In this way, the application of other kinds of probes (single-copy mapping and chromosome painting, for example) represents additional strategies to advance in understanding the genomes (see the following topics in this chapter).

Mapping of Single-Copy Genes

Nowadays many studies have been conducted using dispersed or *in tandem* repetitive DNAs as probes for FISH cytogenetic mapping. In general these probes provide easy and well visible signals, due their abundant repetition and distribution pattern forming long blocks along the chromosome (see the topic "Mapping of repetitive DNAs" in this chapter). However, to determine small chromosomal rearrangements such as translocations and inversions or even to determine an exact position of small DNA sequence like single-copy genes, a more refined methodology based on single copy fluorescent *in situ* hybridization is required. It is now straightforward to combine methods that range from identifying gross structural changes in the genome down to single-nucleotide differences. Various technical modifications and in-house tricks have been developed by different laboratories to improve FISH sensitivity. Such modifications involve improvement in specificity and resolution of the

FISH technique, brought about by a better understanding of the chemical and physical properties of nucleic acids and chromatin, together with the advances in the fields of fluorescence microscopy and digital imaging, associated to the growing availability of genomic and bioinformatics resources.

But how can a small probe be visualized with FISH? Unfortunately, the usefulness of FISH in specific applications is still frequently limited by low detection sensitivity. For example, using current FISH techniques, a probe containing less than 10 Kb of single-copy DNA did not reliably reproduce detectable signals in metaphase chromosomes. To overcome difficulties in the chromosome localization of single-locus genes, two basic and different approaches have been developed. One is the use of genomic DNA clones, like large cosmid, yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) clones, containing single-copy genes to be mapped, as probes for FISH. Another one is a sequence-specific detection of nucleic acid *in situ* that did not require large and labeled hybridizing probes. This method is called primed *in situ* labeling (PRINS) and is based on rapid annealing of unlabeled DNA primers to complementary target sequences, followed by *in situ* primer elongation with *Taq* polymerase.

Besides, there is the possibility of combining these technologies on the extended chromosomes present in interphasic or meiotic chromosomes. It has become a usual practice as an alternative to mitotic chromosomes to map single or low copy sequences to resolve distances between sequences smaller than 1 Mb. Moreover there is the fiber-FISH technology that is one of the most powerful tools for mapping small DNA sequences onto specific regions of the genome, because it allows accurate sizing of gaps and overlaps between probes. These techniques are called high-resolution FISH and represent an alternative to achieve information on genetic collinearity that allows a better understand of the processes that are involved in normal genomic function, diseases, and chromosomal evolution and rearrangements.

Strategies for Mapping Single-Copy Genes

Various types of genomic DNA clones have been used in FISH mapping, including λ clones (Peterson et al. 1999), cosmid clones (Sadder and Weber 2002), YAC clones (Fransz et al. 2000) and BAC clones (Tör et al. 2002) that are powerful tools for detailed analysis of complex genomes. Among them the most recently used for cytogenetic mapping are the BACs, that although presents insert sizes (typically 100~300 kb) much smaller than those of YACs have several additional advantages over YACs such free from chimerism, higher stability of the insert and are very easily manipulated (Hasterok et al. 2006). Due this statement most part of studies have been conducted using BACs as probes as will be discussed below.

BACs have demonstrated useful for many aspects of molecular and genomic studies, such as the positional cloning of genes (Stiglec et al. 2007a), comparative studies of synteny and gene organization among different species (Goldammer et al. 2009), as well as for local or whole genome physical and genetic mapping and sequencing (Katagiri et al. 2005). In cytogenetic research and chromosome mapping, BAC clones also have been used successfully as probes in FISH (Jiang et al. 1995, Hoskins et al. 2000, Stiglec et al. 2007a). *In situ* hybridization of these sequences allows recognizing each chromosome pair individually,

integration of physical and genetic maps, and determining the chromosomal relationships among related species, as well as species belonging to different orders, through cross-species hybridization (Cheng et al. 2001, Pedrosa et al. 2003, Hasterok et al. 2006, Ferguson-Smith and Trifonov 2007, Griffin et al. 2007). The potential of FISH for studying a lot of animal genomes has increased considerably after the construction of a number of commercial and non-comercial BAC libraries: giardia*, trypanossoma*, fruitflyes*, lepidopterans (Wu et al. 2009), honey bee*, channel catfish (Quiniou et al. 2003), Atlantic salmon (Thorsen et al. 2005), stickleback*, zebrafish*, Nile tilapia (Katagiri et al., 2005), frog*, reptile green anole lizard*, turkey*, chicken*, mouse*, monkey*, bat*, rat*, dog (Werner et al. 1999), bovine*, caprine* and of course human*. (* indicates commercial BAC libraries available at http://bacpac.chori.org/home.htm).

From those libraries a number of molecular markers have been identified and sequenced and their chromosomal positions have been established. BACs have narrowed the gap between cytogenetics and molecular genetics and have become important tools for visualizing the organization of genomes because they produce bright, well defined signals on metaphase and interphase chromosome preparations (Figure 3g,h). BACs constitute a resource that provides the tools to integrate questions raised at the chromosomal level into answers at the molecular level (Korenberg et al. 1999).

There are some examples in the literature showing the powerful of BAC-FISH analysis. Korenberg et al. (1999) show how BACs provide molecular links for understanding human genomic duplications, meiosis, and evolution, as well as application for conducting genome-wide prenatal diagnosis at the molecular level and for detecting candidate genes associated with novel cancer breakpoints. Like in humans, a lot of experiments have been done using BACs as probes in animal genetics: Corradini et al. (2003) have performed FISH mapping of nine bacterial artificial chromosomes (BACs) carrying several predicted genes to correspond to protein-coding genes involved in important cellular functions in *Drosophila*. Experiments have been done in chicken, where 17 genes were mapped thought BAC probes onto female chicken metaphase spreads (Stiglec et al. 2007).

In the pufferfish *Fugu* and in the Antarctic fish (*Notothenia coriiceps*) it was demonstrated through BAC-FISH that two of the globin gene locus were located on separate chromosomes (Gillemans et al. 2003, Pisano et al. 2003). Likewise, Harvey et al. (2003) and Chiang et al. (2001) have demonstrated that the two aromatase genes (CYP19), which catalyses the conversion of androgens are located in distinct chromosomes in the Nilo tilapia *Oreochromis niloticus*, and zebrafish (*Danio rerio*).

The use of BAC-FISH on comparative cross-species hybridization has increased in the last years in animal cytogenetic research. A detailed comparative analysis of sheep, human, and cattle mapping data allowed the construction of a comparative map that confirms and expands the knowledge about evolutionary conservation and break points between the X chromosomes of the three mammalian species (Goldammer et al. 2009). In lepidopterans, recent advances in *Bombyx mori* genomics make comparative genomic studies feasible. The draft sequence information of this species has been deposited in public database and all genetic linkage groups have been successfully assigned to individual chromosomes (Yoshido et al. 2005). BAC-FISH mapping using selected BACs carrying orthologs genes are powerful tools for identification of conserved synteny between *B. mori* chromosomes and the chromosomes of other lepidopteran species (Sahara et al. 2007).

Comparative cytogenetics based on BAC-FISH between Atlantic salmon and rainbow trout was able to anchor genetic maps (linkage map) to chromosomes (physical map) (Phillips et al. 2009). This analysis provided strong evidence for conservation of large syntenic blocks in these species, corresponding to entire chromosome arms in rainbow trout. BACs containing sex linked markers of the Nile tilapia were mapped in the chromosomes and compared to the linkage map of the species (Cnaani et al. 2008). Another importance of these assays is related to the possibility of mapping the real distances between genes and marks in the chromosomes. This information cannot be predicted using linkage maps, because recombination rates vary along the length of chromosomes according to genomic content and, as a result, genetic distances are not directly proportional to physical distances.

In spite of the power of BAC mapping, one should be aware that these large-insert genomic clones as BACs, especially those from species with very large genomes, may contain an extensive amount of repetitive DNA sequences that could decrease the efficiency of localization of the single-copy sequences and also causes unspecific hybridization (see figure 3h). However, if repetitive sequences account for 90% of an animal genome, a 100 kb BAC clone should, theoretically, contain ~10 kb of unique sequence, which is enough to generate FISH signals. The cross hybridization from the repetitive DNA sequences can be minimized by pre-annealing the probe with C_0t-1 DNA or shared genomic DNA (Jiang and Gill 2006).

When an unpredicted location of a BAC clone is identified, additional BAC clones from the same genomic region should be mapped to understand whether observed results were caused by chimeric BAC structure, sequence similarity between different genes, or chromosomal insertions that were not detected previously (Kukekova et al. 2009). A critical step to obtain reliable results is the slide preparation with chromosome spreads and the pre-treatment of the slides for the FISH procedure. Difference in these steps may be the reason why FISH protocols are frequently different for different species.

In attempt to minimize some problems occurred through BACs hybridization due to the small signal generated, PRINS (primed *in situ* labeling) represents a different strategy for single-copy mapping. According to the PRINS method, which combines features of fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR), unlabeled short oligonucleotide primers are annealed and extended on chromosome preparations on microscope slides in the presence of labeled nucleotides. During primer elongation, labeled nucleotides are incorporated into newly synthesized DNA. After extension the relevant sequences are visualized by fluorescence microscopy (Koch et al. 1991).

The use of oligonucleotide probes allows the detection of much smaller targets than those accessible to conventional *in situ* hybridization and results in the higher sensitivity of the PRINS method and in its amazing discriminatory power for small sequence variations and detection of low and single copy DNA sequences (Pellestor et al. 1994, 1995). The signal intensity can be further increased by performing several cycles of the PRINS reaction (so-called cycling PRINS), which accumulates labelled copies of the target sequence at the site of synthesis (Terkelsen et al. 1993).

The ability to localize single copy genes via PRINS methodology has opened various applications in diagnosis and research. For example, PRINS was used to localize the FACTOR IX gene (a single copy gene on the X chromosome) (Cinti et al. 1993) and in the elucidation of specific genetic defects such as abnormal sex differentiation through the localization of SRY gene in XX men, in a woman with XY gonadal dysgenesis and in an

azoospermic man with Xp-Yp interchange (Kadandale et al. 2000). In addition, PRINS demonstrates efficiency, sensitivity and specificity in the diagnosis of microdeletions in groups of patients with Prader Willi/Angelman (PWS/AS) syndrome and DiGeorge/velocardiofacial syndrome (DGS/VCFS) (Tharapel et al. 2002).

Although the possibility of the use of PRINS for mapping of single copy sequences, this methodology has been most applied in mapping repetitive sequences as centromeric and telomeric sequences, *Alu* repeats and 5S rRNA genes (Koch et al. 1991, Gosden et al. 1991, Gosden and Lawson 1994, Martins and Galetti 1999). The PRINS was also applied for the detection of repeated DNA sequences in several domestic animals, as chicken (Coullin et al. 2005), pig (Pinton et al. 1998), horse (Wnuk et al. 2008) and frog (Freeman and Rayburn 2005).

PRINS proved to be useful because (i) it generates highly specific labeling and the sensitivity and specificity of PRINS allow localization of DNA segments that are too small to be detected by conventional FISH. Moreover the target-specific PRINS approach can overcome some FISH limitations such as the nonspecificity of the usual probes (FISH probes can contain overlapping DNA segments outside the targeted loci and can cross-hybridize to unintended targets); (ii) it can be performed rapidly and easily; PRINS results can be available within 2±4 hours, whereas a typical FISH procedure takes at least 12 hours. (iii) single-copy genes and small DNA segments in general can be detected simultaneously by combining PRINS and FISH; (iv) it is useful for analysis of chromosome rearrangements such as translocations or small intragenic deletions; (v) this method leads to considerable reduction of costs approximately 10 times less expensive than FISH (Cinti et al. 2002, Coullin et al. 2002, Tarapel et al. 2002, Wilkens et al. 1997). Although this methodology presents several advantages it has not been explored in basic research.

In order to achieve the goal of routine single-copy gene detection, in some cases is necessary to combine these methods with a completely new technique that would provide better target access into the chromosomal DNA like the use of the extended chromosomes present in meiotic interphase or pachytene.

Improving Single-Copy Mapping

In order to improve the resolution of metaphase chromosomes, FISH has been applied to interphase cell nuclei, meiotic chromosomes, mechanically stretched chromosomes (MSCs) and by fiber-FISH (Salinen et al. 2001). For examples, the use of meiotic chromosomes that are considerably longer than their mitotic counterparts can increase to 2 Mb the FISH resolving power (Cheng et al. 2002) and the resolution in the fiber-FISH method ranges from a few to 300 Kb (Volpi and Bridger 2008). This is named high-resolution FISH and includes hybridization of probes to “free chromatin”. These targets have widened the resolution of FISH to detect distances from the traditional cytogenetic resolution level down to a resolution of a few kilobases (Salinen et al. 2001). This method can help to map single-copy sequences directly on chromosomes and also to order the BAC contigs better solving problems associated with physical mapping from genome projects, e.g., ambiguous gene orders and intergenic distances, concerning the comparison of the human and mouse genomes (Salinen et al. 2001).

For example, birds, like other vertebrates with yolk-rich eggs, present lampbrush chromosomes during the diplotene stage of the first meiotic prophase (Hutchison 1987) and theses chromosomes can certainly provide excellent resolution to locate repetitive and single-copy sequences on avian chromosomes such as chicken (Hori et al. 1996, Solovei et al. 1998, Derjusheva et al. 2003, Galkina et al. 2005, 2006).

In certain organisms the use of FISH on pachytene chromosomes has become usual practice to map single or low copy sequences because they allow a higher degree of spatial resolution, and also display a clear differentiation of heterochromatin blocks, which helps the identification of individual chromosomes, rearrangements and translocations, and establishes repeat-rich domains (Pigozzi 2007).

However, the resolving power of pachytene FISH depends on three factors. First, the degree of chromatin condensation at the pachytene stage may vary significantly among different species. Second, the location of probes in euchromatic or heterochromatic regions will affect the FISH resolution. Third, the resolving power also depends on the different pachytene substages (early or late). Late pachytene chromosomes can be used to orient the telomere-centromere positions of the adjacent clones, whereas early pachytene chromosomes can be used to resolve even partially overlapped BAC clones (Cheng et al. 2002). Because of these three factors, a special attention should be payed to convert the microscopic distance, which separates the two DNA probes on pachytene chromosomes, into kilobases (Cheng et al. 2001).

Meiotic bivalents prepared for observation of the synaptonemal complexes (SCs) have been employed in plants and also among vertebrates (Peterson et al. 1999, Froenicke et al. 2002, Pigozzi 2007). The physical location of nine genes was established using FISH along the ZZ bivalent, using synaptonemal complex spreads from the avian male Zebra finches and it was possible to compare their positions in the mitotic and meiotic chromosomes (Pigozzi 2008). Single-copy sequences have also been mapped on mouse pachytene bivalents using SC and their positions compared with those obtained by genetic analyses (Froenicke et al. 2002). This kind of comparison seems to be very important due it shows that gene positions with respect to the centromere are different in mitotic compared to meiotic chromosomes in mice (Froenicke et al. 2002).

Similarly, several differences were observed in relative length and arm ratios between mitotic chromosomes and their SCs in human spermatocytes (Sun et al. 2004). These discrepancies lie in the differential packing of the DNA along the axial elements of the highly specialized meiotic chromosomes. The main components involved in this differential distribution of the DNA are repetitive sequences enriched in mammalian G or R bands and those sequences forming heterochromatin (Stack 1984, Zickler and Kleckner 1999, Hernandez-Hernandez et al. 2008).

Among fishes, FISH and DAPI staining of the synaptonemal complex of the Nile tilapia (*Oreochromis niloticus*) allow orientation of the unpaired region of bivalent 1 observed during early pachytene. This appears to be related to recombination suppression around a sex determination locus (Ocalewicz et al. 2009).

Mechanically stretched chromosomes (MSCs), prepared by cytocentrifugation of hypotonically treated cells, provide an alternative target for high-resolution FISH mapping. However, the morphology of MSCs is significantly distorted so that little if any banding pattern is discernible. Furthermore, the degree of stretching is variable from one slide to

another, from one metaphase to another, and from one chromosome to another. Thus, no quantitative information on precise probe distances can be obtained (Laan et al. 1995).

The application of extended chromosomes for the ordering YAC clones separated by less than 1 Mb and for the structural analysis of α-satellite DNA and proteins, has been reported in human (Haaf and Ward 1994a, b). Sallinen et al. (2001) have applied both MSCs and extended DNA fibers to the physical mapping of the mouse genome. At first, five mouse collagen genes were localized and the mutual order of the genes, centromere–*Col10a1–Col13a1–Col6a2–Col6a1–Col18a1*–telomere, was determined.

The fiber-FISH is another technique also applied to high-resolution FISH mapping approach of small DNA sequences onto specific regions of the genome and ordering of probes relative to one another can be also performed on released chromatin fibers. Chromatin can be released from interphase cell nuclei by various chemical or mechanical methods, and investigators tried to coin names that reflect their individual approach. Isolation of DNA from cell nuclei, extension, and preparation of chromatin or DNA fibers with diameters ranging in size from a few to several hundred nm improves the accessibility of the DNA targets for both probes and detection reagents (Weier 2001). This method is essential for defining gene order and organization, the physical architecture of specific genomic regions, allows accurate sizing of gaps and overlaps in contigs, and analysis of segmental duplications and copy number variants. The degree of resolution in the fiber-FISH method ranges from a few to 300 Kb (Volpi and Bridger 2008).

The method consists of removing the histones of chromatin and stretching and fixing of DNA on a slide prior to hybridization (Volpi and Bridger 2008). The sensitivity of the technique is increased, probably as a consequence of the high accessibility of naked DNA to probes and immunological detection reagents. Fiber-FISH has been used in various types of animal genome mapping projects, including analysis of structure and organization of repetitive DNA sequences, mapping of single-copy, large genomic locus, mapping of BACs and comparative analyses.

Theuns et al. (1999) determined the genomic organization of the human presenilin 1 gene, which was localized on chromosome 14q24.3 by fiber–FISH. Genes of the major histocompatibility complex (MHC) of rhesus macaque were localized to the long arm of the rhesus macaque chromosome 6 in 6q24, the orthologous region to human 6p21.3. Furthermore, centromere to telomere orientation of the rhesus macaque MHC as well as the internal order of the MHC genes tested is the same as in human. Fiber-FISH allows a rough estimate of distances between these MHC genes in the rhesus macaque, and, as in the human, the rhesus macaque MHC comprises about 3 to 4 Mb (Huber et al. 2003).

The Rhesus (Rh) blood group system in humans is encoded by two genes with high sequence homology. However, the genomic organization of Rh genes in chimpanzees and other non-human primates has not been precisely studied. Suto et al. (2003) analyzed the arrangement of the Rh genes of chimpanzees *(Pan troglodytes)* by two-colour fluorescence *in situ* hybridization on chromatin DNA fibers (fiber-FISH) and discovered intra- and interspecific genomic variations in the Rh gene locus in hominoids, so it would shed further light on reconstructing the genomic pathways of Rh gene duplication during evolution.

A comparative fiber-FISH on extended chromatin of cattle (*Bos taurus*, BTA, 2n = 60), river buffalo (*Bubalus bubalis*, BBU, 2n = 50), sheep (*Ovis aries*, OAR, 2n = 54) and goat (*Capra hircus*, CHI, 2n = 60), using the SMN gene, was performed on R-banded chromosome preparations. SMN was located on BTA20q13.1, OAR16q13.1, CHI20q13.1

and BBU19q13. These chromosomes and chromosome bands are believed to be homeologous, confirming the high degree of chromosome homeologies among bovids (Iannuzzi et al. 2003).

The fiber-FISH have been used to examine the structural organization of the mouse a2u-globulin locus, and found the genes to be arranged as an array of both direct and inverted repeats. The organization of the rat a2u-globulin genes differs from the mouse genes and suggests different evolutionary events that have acted reorganizing these homologous sets of genes (McFadyen and Locke 2000).

The high resolutions of the pachytene FISH method, together with the recently developed fiber-FISH techniques and MSCs, add new tools to the arsenal for fine physical mapping of single-copy genes, and, thereby, in positional cloning and genomic sequencing of animal species. Consequently, this would open new avenues in the field of animal comparative genomics.

Chromosome Painting in Comparative Cytogenetics and Genomics

Advances in molecular cytogenetics have provided great insights into chromosome evolution, being the chromosome painting a powerful technique to achieve this aim. This technology allows the application of whole chromosome (WCP-whole chromosome painting) or a partial chromosome (PCP-partial chromosome painting), that may be obtained by microdissection or flow sorting methods followed by an amplification and labeling step, as probes. Usually, this technique has been applied to the study of the evolution of whole karyotypes, or particular chromosomes, such as B chromosomes, specific chromosomes from A complement, and sex chromosomes. It is common to use these probes when is necessary to identify the homologies or homeologies among some chromosome -or all chromosomes- from distinct species. This kind of hybridization is named cross-species chromosome painting, or comparative chromosome painting or Zoo-FISH (revised by Yang and Graphodatsky 2009). The reciprocal cross-species painting allows the more exact identification of homologous chromosome segments. However, the Zoo-FISH is not successful among groups that diverged genetically more than 105 million year (Ferguson-Smith and Trifonov 2007).

The first use of chromosome painting in vertebrates was performed in a human in 1988 by Pinkel et al. (1988) and Lichter et al. (1988), and today it is very common to see the application of this technique in other mammals as well as in birds, fishes and some invertebrates. One of the first applications for comparative cytogenetic was conducted by Wienberg and colleagues (1990), who applied this tool for comparative analysis between human and Japanese macaque genomes. Thereafter, a great number of studies have allowed the comparison among genomes, mostly of eutherian mammals and birds (Wienberg et al. 1990, Jauch et al. 1992, Shetty et al. 1999, revised by Griffin et al. 2007, revised by Ferguson-Smith and Trifonov 2007, Stanyon et al. 2008, revised by Yang and Graphodatsky 2009, among others).

In the beginning, the chromosome paintings were obtained from libraries of human chromosome-specific DNAs cloned in BACs (revised by Yang and Graphodatsky 2009). Each BAC clone may be used to physical mapping and sometimes one or more BAC clones

may be able to paint completely or partially one chromosome. BACs obtained from one species can be used to screen genomic library of another species and thus, the homologous BAC clones of the latter species may be used to its own physical mapping, showing the regions of homology with the first species. This comparative BAC mapping is a kind of "cross-species chromosome painting" and this methodology is still used today when the species that are under study have high chromosomal DNA divergence such as the eutherian and monotremes or the marsupials and the other vertebrate classes (revised by Ferguson-Smith and Trifonov 2007).

The most common studies about chromosome painting were conducted in mammals because the difficulty in the probe achievement for other animal groups (see the topic "Applied technologies in the physical mapping of animal chromosomes" in this chapter). The achievement of sex chromosomes, B chromosomes, and other chromosome marker as probes are facilitated if the length, shape or heterochromatin patterns are different in relation to the other chromosomes from their own genomes. Nevertheless there are many papers in the literature that conducted chromosome painting in other groups such as invertebrates, birds, fishes, among others. It will be revised in this section data concerning chromosome painting for genomic comparison, sex chromosome and B chromosome studies in a range of taxonomic groups.

Before the advent of chromosome painting in mammal groups, the comparison among karyotypes was conducted based on classical cytogenetic methods (for example GTG-banding technique) as well as mapping of individual genes or other kind of DNA sequences. After the advent of chromosome painting the knowledge acquired in the field of classical cytogenetics were confirmed and refined. In birds, as discussed in the review of Griffin et al. (2007), the classical method of GTG-banding in general is limited, due the poor distinction of chromosome bands compared to mammals and it is not possible to visualize bands in the microchromosomes. Therefore, the great insights about chromosome evolution in birds were made after the introduction of chromosome painting. Due to the extent of data already published about genomic comparison using chromosome painting in mammals and birds, the next section is separated in two topics in attempt to describe the mammals and birds discoveries.

Mammals Karyotype Evolution

In recent years, the greatest revolution in comparative cytogenetic of mammals was obtained due to the increase of the number of species analyzed and the availability of probes for chromosome painting. Nowadays most cases of Zoo-FISH among orders of mammals have been conducted referring to the human genome. For example, chromosome painting technique in mammal groups was applied onto genomes of about 150 species, being the human probes applied onto the chromosomes of ~70 mammal species (revised by Ferguson-Smith and Trifonov 2007, revised by Graphodatsky 2007, revised by Yang and Graphodatsky 2009). In the review performed by Ferguson-Smith and Trifonov (2007), it is related that most of these studies revealed mapping homologies among chromosome segments thus allowing the identification of karyotype rearrangements among the main mammal lineages and their evolutionary interrelationship. In other words, Zoo-FISH using human probes in species of each mammal order helped to complement and extend the phylogenetic

relationships based on gene sequencing, as well as the establishment of an ancestral karyotype for primates, carnivores, cetartiodactyls, perissodactyls and rodents, and therefore the ancestral eutherian karyotype (AEK).

As already discussed it is unfeasible to apply the human chromosome probes onto monotreme (Prototheria, Monotremata) chromosomes. However, there are some studies using comparative BAC mapping and chromosome painting using monotreme chromosome probes onto their own chromosomes, generally in attempt to sex chromosome studies (Grützner et al. 2004, Rens et al. 2004, 2007, revised by Graves 2008, revised by Ferguson-Smith and Trifonov 2007). Other chromosome probes which were isolated by flow sorting of platypus genome (Rens et al. 2004, McMillan et al. 2007) may increase the extent of data about monotremes karyotype evolution. For example, Zoo-FISH experiments using platypus (monotreme) chromosomes as probes onto chromosomes of short-beaked echidna showed a conservation *in toto* of 10 autosomes between both species and besides, others platypus autosomes represent chromosomal segments in echidna (monotreme) (for more details see Rens et al. 2007). Although these results are interesting for monotreme karyotype evolution, the major discussion that includes this group are about monotremes and therian (marsupials and eutherians) sex chromosomes evolution, as it will be shown later in this section, in the topic "Chromosome painting in the sex chromosome studies".

The Marsupialia includes opossum and kangaroo species and the chromosome painting applied in this clade revealed homology between the X chromosomes of marsupials and eutherian mammals. In addition the autosome homologies data are still lacking for this group; however the chromosome painting using chromosomes of five marsupial species as probes showed that the Australian marsupials as well as American Didelphidae have similar genomes (Rens et al. 2001).

The Zoo-FISH using chromosome probes of eutherians (in general human chromosomes) onto monotreme or marsupial chromosomes is not as easy as aforementioned, thus the great karyotypic comparisons using this technique is restrict into Eutheria clade (include the Xenarthra, Afrotheria, Laurasiatheria and Euarchontoglires superorders). These Zoo-FISH findings have allowed the comparison among syntenic blocks (which involve a whole chromosome or chromosomal segments) in almost all eutherian orders often using the *Homo sapiens sapiens* chromosomes (HSA) as reference (revised by Ferguson-Smith and Trifonov 2007). Therefore, it was concluded that some syntenic associations (syntenic blocks) are specific of one group (they are the cytogenetic signatures of a clade) and other are conserved among two or more, or even so throughout eutherian clade. These analyses helped to construct the ancestral eutherian karyotype (AEK) (Chowdhary et al. 1998, Frönicke et al. 2003, Richard et al. 2003, Yang et al. 2003, Robinson et al. 2004, Svartman et al. 2004, Wienberg 2004, Froenicke 2005, Froenicke et al. 2006, for more details see review by Ferguson-Smith and Trifonov 2007).

Due to the great amount of data related to eutherian mammals (~90 species in ~18 orders), which are far outside the scope of this chapter, not all papers neither all discovery for all orders will be discussed here (the extent of information about this theme into this clade is worthy of its own chapter). In this way, the reader is advised to check for papers cited in this section as well as other papers available in the scientific literature (for example the list of articles in the Cambridge Resource Centre for Comparative Genomics, http://www.vet.cam.ac.uk/genomics/ or in others web search tools). Other information, such as the location of each ancestral eutherian synteny into some eutherian karyotypes, references

of the important papers in this area, karyotypes and GTG-banding for many eutherian species, and an extensive chromosome homology map, can be retrieved in the CHROMHOME (CHROMosome HOmology Mapping and E-painting) http://www.chromhome.org/. Thus, the great aim of this section is to show some of the conclusions obtained when the chromosome painting is applied in karyotype evolution, more specifically in the eutherian karyotype evolution.

The power of comparative chromosome painting, such as establishment of phylogenetic relationships of ancestral karyotypes and cytogenetic signatures of one group, as already mentioned, can be noticed in studies using the eutherian species. For example, the establishment of the syntenic block 1/19p as a shared synteny between Afrotheria (aardvark, elephants, golden mole, manatee and elephant-shrew) and Xenarthra (tree anteater) (revised by Ferguson-Smith and Trifonov 2007) could be present in the common ancestor for Afrotheria and Xenarthra (compose the South American armadillos, anteaters, three-toed sloths and two-toed sloths) (Yang et al. 2006). In the same way, the phylogenetic relationships were also showed between Eulipotyphla and Pholidota orders, in which four syntenic associations are shared between Javan Pangolin (Pholidota order) and long-eared hedgehog (Eulipotyphla) (Yang et al. 2006, Ye et al. 2006). Likewise, in Chiroptera order, Zoo-FISH and GTG-banding analysis corroborated the division of the families of this order in three lineages and showed a close relationship between two of these lineages, the Megachiroptera (Pteropodidae) and Rhinolophoidea (Microchiroptera) (for more details see Volleth et al. 2002, Ao et al. 2007).

Since the first Zoo-FISH performed by Wienberg et al. (1990) in one primate species, this technique have been applied in more than 50 species of primates under studies of comparative chromosome painting (revised by Stanyon et al. 2008). It is evident that the chromosome painting was very important to presume the putative ancestral karyotype in many primates (see Table 2) and in the establishment of phylogenetic relationships in this group (Muller et al. 1999, O'Brien and Stanyon 1999, Murphy et al. 2001, revised by Ferguson-Smith and Trifonov 2007, revised by Stanyon et al. 2008). As an example of karyotype evolution of primates, chromosome painting showed immense homologies between all human chromosomes and apes chromosomes, except for a centric fusion between two chromosomes, which formed the HSA 2 (revised by Ferguson-Smith and Trifonov 2007).

Interestingly, besides all results produced by Zoo-FISH and BAC mapping experiments, the comparative chromosome painting in hominoids karyotype evolution showed that the orangutan lineage diverged earlier than gorilla and chimpanzee lineage. After this, the gorilla lineage was the second to diverge and finally the human line acquired three inversions and one chromosome fusion (the chromosome 2), which reduced the number of chromosome from 2n=48 to 2n=46 (see Table 2) (revised by Ferguson-Smith and Trifonov 2007).

The insights about the evolution of one group (as noticed above in apes and human cases) using chromosome painting can be also observed in Cetartiodactyla order, in which it was corroborated the hypothesis that the Camelidae family was the first to diverge during the Cetartiodactyla evolution followed by divergence of Suina and finally of Cetacea/Hippopotamidae and Pecora groups (for more details see Kulemzina et al. 2009).

In addition, chromosome painting showed that the Afrotheria superorder (Fronicke et al. 2003, Yang et al. 2003, Robinson et al. 2004), Canidae order (Graphodatsky et al. 2002, Nie et al. 2002, Perelman et al. 2005, 2008, revised by Ferguson-Smith and Trifonov 2007), Perissodactyla order (include tapirs, rhinoceros, horses, onager, kulan, donkey, and zebras)

(revised by Trifonov et al. 2008), Eulipothyphla order (include hedgehogs, shrews, solenodons and moles) (revised by Ye al. 2006), Cetartiodactyla order (include cattle, sheep, deer, giraffes, pigs, camels, hippopotamus, dolphins and whales) (revised by Ferguson-Smith and Trifonov 2007) and Rodentia order (Froenicke et al. 2006) possess different rates of karyotype rearrangements into their evolutionary history (revised by Ferguson-Smith and Trifonov 2007).

Table 2. Predicted ancestral karyotypes for several primate groups based on the 23 human chromosomes. HSA, *Homo sapiens sapiens*; AHK, ancestral hominoid karyotype; ACK, ancestral catarrhine karyotype; APLK, ancestral platyrrhine karyotype; AAK, ancestral anthropoids karyotype; APK, ancestral karyotype of all primates; AEK, ancestral eutherian karyotype. On the bottom of the table are presented the diploid number of each karyotype. Data obtained from Ferguson-Smith and Trifonov (2007), and Stanyon et al. (2008)

HSA	AHK	ACK	APLK	AAK	APK	AEK
1	1	1	1a, 1b, 1c	1	1	1
2	2a, 2b or 2p-q, 2q	2a, 2b	2a, 2b/16q	2p-q, 2q	2p-q, 2q	2p-q12, 2q
3	3	3	3a, 3b, 3c/21	3/21	3/21	3/21
4	4	4	4	4	4	8p/4
5	5	5	5/7a	5	5	5
6	6	6	6	6	6	6
7	7	7	7b, 5/7a	7a, 7b	7b, 7a/16p	7a/16p, 7b
8	8	8	8a, 8p/18	8	8	8p/4, 8q
9	9	9	9	9	9	9
10	10	10	10p, 10q/16	10p, 10q	10p, 10q	10p/12pq/22qt, 10q
11	11	11	11	11	11	11
12	12	12	12	12	12a/22a, 12b/22b	10p/12pq/22qt, 12qt/22q
13	13	13	13	13	13	13
14	14	14/15	14/15a	14/15	14/15	14/15
15	15	14/15	14/15a, 15b	14/15	14/15	14/15
16	16	16	10q/16p, 2p/16q	16p/16q	7a/16p, 16q	19q/16q, 7a/16p
17	17	17	17	17	17	17
18	18	18	8p/18	18	18	18
19	19	19	19	19	19p, 19q	19p, 19q/16q
20	20	20	20	20	20	20
21	21	21	3c/21	3/21	3/21	3/21
22	22	22	22	22	12a/22a, 12b/22b	12qt/22q, 10p/12pq/22qt
X	X	X	X	X	X	X
Y	Y	Y	Y	Y	Y	-
23	24	23	27	25	25	23

In this way, the Canidae, Ursidae and Mephitidae families (all belonging to Carnivora order), and muroid superfamily (for example mice, hamster and rats) have higher rates of chromosome evolution (Nash et al. 1998, Yang al. 1999, Froenicke et al. 2006, revised by Ferguson-Smith and Trifonov 2007, Perelman et al. 2008), being the latter group the highest

evolutionary rate among mammal species (revised by Graphodatsky 2007). In fact, comparative reciprocal painting between mice and rats revealed that the rate of divergence between both species is tenfold higher than the one observed between a human and a cat, and represents a good example of the high number of rearrangements in muroids (Stanyon et al. 1999).

Furthermore, the chromosome painting in eutherian species allowed obtaining the cytogenetic signatures for the superorder Afrotheria and Xenarthra (Yang et al. 2003, 2006, Robinson et al. 2004, Gilbert et al. 2006, Kellogg et al. 2007, Pardini et al. 2007) and on the other hand, for Euarchontoglires (composed of Primates, Scandentia, Dermoptera, Lagomorpha and Rodentia orders) and Laurasiatheria (composed of Eulipotyphla, Chiroptera, Pholidota, Carnivora, Perissodactyla and Cetartiodactyla orders) cytogenetic signatures were not identified (revised by Ferguson-Smith and Trifonov 2007) (for more details, see the discussion of syntenic association shared into Laurasiatheria superorder by Yang et al. 2006). In addition, the establishment of their respective ancestral karyotypes was obtained for some orders (revised by Ferguson-Smith and Trifonov 2007).

Comparative chromosome painting was applied in ~90 species in ~18 orders of eutherian mammals in attempt to build an ancestral eutherian karyotype (Chowdrhary et al. 1998, Fronicke et al. 2003, Richard et al. 2003, Yang et al. 2003, Robinson et al. 2004, Svartman et al. 2004, Wienberg 2004, Froenicke 2005, Froenicke et al. 2006, for more details see review by Ferguson-Smith and Trifonov 2007). Throughout the years, this ancestral karyotype has been refined on the basis of cytogenetic studies as well as analysis of bioinformatics (Froenicke et al. 2006, Robinson and Ruiz-Herrera 2008).

Based on syntenic associations that are shared by 18 mammal orders (it is thought that conserved syntenic association blocks could be present in ancestral karyotypes), it was possible to hypothesize the most likely ancestral eutherian karyotype, which had 2n=46 chromosomes (Froenicke 2005, Froenicke et al. 2006, revised by Ferguson-Smith and Trifonov 2007). After the completion of this ancestral eutherian karyotype, it was observed that the human karyotype has many features of this ancestral genome. For example, it was suggested that many human chromosome segments were not disrupted in AEK, which formed entire chromosomes in the ancestral (notably the entire human chromosomes 1, 5, 6, 9, 11, 13, 17, 18, 20 and X are conserved intact in this putative ancestral karyotype). Likewise, entire human chromosomes were connected or linked with part(s) of other(s) human chromosome(s) that also formed entire chromosomes in AEK (Table 3) (revised by Ferguson-Smith and Trifonov 2007). Interestingly, the ancestral eutherian genomes models suggested by the bioinformatics and molecular cytogenetic have similar chromosome numbers, but surprisingly the numbers of conserved segments and the number of syntenic associations is greatly different. Thus the authors argued that the ancestral eutherian genome model suggested by cytogenetics may be a far more likely representation of this ancestral genome (Froenicke et al. 2006).

Interestingly, the analysis of whole-genome sequence assemblies between human and opossum (*Monodelphis domestica*), and between human and *Gallus gallus* (opossum and chicken are used as outgroups in this case), determined the regions of synteny among them. The analysis of these homologous syntenic blocks revealed the human chromosome 1, 5, 6, 9, 11, 17, 20, and the X (all intact chromosomes in AEK) are disrupted in opossum and chicken (Robinson and Ruiz-Herrera 2008). The meaning of this data is that these characteristics are monophyletic for eutherian clade.

Table 3. Ancestral eutherian karyotype and its relationship to human chromosome syntenies. AEK, Ancestral eutherian karyotype; (*) indicates the human syntenies conserved for most eutherians. Bioinformatics analysis revealed that some of these syntenies are also present in opossum and/or chicken, being thus probable synplesiomorphies for Eutheria (see Robinson and Ruiz-Herrera 2008). The syntenies into parenthesis are refinements of the syntenic associations. Data obtained from Froenicke et al. (2006), Yang et al. (2006), and Ferguson-Smith and Trifonov (2007). For more details see Robinson and Ruiz-Herrera (2008), among others

AEK	Human
1	1
2	* 8p/4 (4q/8p/4pq)
3	* 3/21 (3p/21)
4	5
5	* 14/15
6	6
7	* 10p/12pq/22qt
8	2q
9	7 (7b)
10	2p-q12
11	9
12	11
13	10q
14	13
15	8q
16	17
17	18
18	* 19q/16q
19	20
20	* 16p/7 (16p/7a)
21	* 12/22 (12qt/22q)
22	19p
X	X

Moreover, these sequence comparisons also concluded that the human chromosomes 13 and 18 (both also intact chromosomes in AEK) are also intact in chicken and opossum karyotypes, respectively, and it means that these karyotype features are symplesiomorphic (shared ancestral). Moreover, it was also concluded that the human segments 8q, 10q and 19p, which are also present in AEK (Table 3), are present as a single syntenic block in opossum. In the same way, the 8q and 10q are also present as a single block in chicken, and thus, these segments (8q, 10q and 19p) represent symplesiomorphic characteristics for Eutheria, but they are not monophylic characters for eutherian in relation to the outgroups (because of the sharing among these species) (for more details see Robinson and Ruiz-Herrera 2008).

It is remarkable that chromosome painting was a very important cytogenetic tool for the establishment of karyotype relationships or corroborating previous phylogenetics hypothesis in these eutherian orders as well as others not cited here. Moreover, chromosome painting refuted or corroborated many putative genomic blocks based in GTG-banding shared among mammalian groups. In other words, it means that chromosome painting has more resolution than classical cytogenetic methods. On the other hand, despite the great contribution of the

comparative chromosome painting about evolution of mammal karyotypes, it is needed caution in the analysis and comparisons of the syntenic associations because, as revised by Ferguson-Smith and Trifonov (2007), a syntenic association may be shared by two or more groups due to convergence, that may occur by centromeric fusion and/or fission, for example.

Avian Karyotype Evolution

Although most studies about comparative chromosome painting are related to mammals, there are also many studies applied to avian groups. The birds are separated in two large groups known as Paleognathae (ratites) and Neognathae (carinates), and in general the number of chromosomes range 2n=74-86 and 2n=66-74, around 63% and 24% of species, respectively (in general 2n~80 chromosomes). The karyotype characteristic in birds includes macrochromosomes, sex chromosomes and several microchromosomes. The general karyotype pattern of birds was maintained relatively constant along the diversification of the groups (revised by Griffin et al. 2007).

Shortly after the development of chromosome-specific probe for each *Gallus gallus domesticus* macrochromosome, including nine autosomes and the Z chromosome (Griffin et al. 1999), Shetty et al. (1999) conducted the first Zoo-FISH in birds. In this remarkable study, the 10 chromosomes of chicken (Neognathae) were used as probes onto the emu *Dromaius novaehollandis* (Paleognathae) chromosomes, both distantly related. In general, the results showed that each chromosome probe of chicken hybridized in the same chromosome in emu and these results revealed a great homology between two species, corroborating the hypotheses of karyotype conservation suggested by classical cytogenetic methods, which generally includes the macrochromosomes and the sex chromosome.

Some years after the first Zoo-FISH in birds, Guttenbach et al. (2003) conducted Zoo-FISH using chicken chromosome probes over the chromosomes of Paleognathae *Rhea americana* and the results also revealed an overall karyotypic conservation in avian groups. Thus, it was reported that the divergence of Paleognathae and Neognathae (the first great divergence in avian group) was not defined by remarkable and specific karyotype change in the macrochromosomes (except by arising of heteromorphic sex chromosomes in Neognathae). In fact, the thought that the bird karyotypes are stable, apparently higher than mammals (Burt et al. 1999), has been supported in recent studies (Shibusawa et al. 2004 a, b, revised by Griffin et al. 2007, Nanda et al. 2008). During birds' evolution, the karyotype conservation and the absence of intense chromosomal changes were also maintained after the divergence of Galliformes and Anseriformes (both Neognathae birds). Likewise, another important evolutionary event in birds was the divergence between 'higher land' and 'higher water' birds and this process was not followed by major karyotype changes, except a series of microchromosomal fusions which tended to reduce the diploid number in some groups (revised by Griffin et al. 2007). On the other hand some studies with Zoo-FISH using chicken probes has showed an excessive chromosome rearrangement in Falconiformes and Psittaciformes birds (for more details see Nanda et al. 2006, 2007, 2008).

Although the first Zoo-FISH was carried out in 1999 (Shetty et al. 1999), the first draft of the ancestral avian karyotype was described many years ago by Stock and Bunch (1982) examining banding patterns. Based in Zoo-FISH analysis, Shibusawa et al. (2004a) describes the first ancestral karyotype for some avian groups (Galliformes) and predicted a conserved

karyotype for the avian lineages. After this, the search for an ancestral karyotype of avian group continued to be refined and involved many studies of Zoo-FISH and even sequence comparison between chicken and soft-shelled turtles, and between chicken and human. Nowadays, it is thought that the ancestral karyotype of avian group is composed of 10 macrochromosomes and a Z chromosome (excluding the microchromosomes), being the *G. gallus domesticus* karyotype, excluding the microchromosomes, very similar to this ancestral karyotype. In fact, the only difference between both karyotypes is that the chicken chromosome 4 is a product of the fusion between 4 (4q of *G. gallus domesticus*) and 10 (4p *G. gallus domesticus*) ancestral avian chromosomes. In general, the studies reveal that the chicken chromosomes 1, 2, 3, 4q, 5, 6, 7, 8, 9, 4p and Z represents the ancestral chromosomes 1-10 + Z for all birds. Interestingly, based on sequence analysis, the ancestral avian chromosome 4 is also present in mammals and turtles, representing an ancient chromosome. Latter in the evolutionary history of vertebrates, the chromosomes 1, 2, 3, 5 and Z arise and the sharing of these chromosomes between turtles and birds suggests that they emergence occurred before the divergence of both animal groups (for more details see the review by Griffin et al. 2007).

Finally, in all cases aforementioned for birds karyotype evolution (for more details see the review by Griffin et al. 2007), the Zoo-FISH using chromosomal probes of *G. gallus domesticus* refined and corroborated the previous hypothesis to avian karyotype evolution proposed by classical cytogenetic methods.

All data available about chromosome painting in mammals and birds reveals that, in general, the mammals have more karyotypic changes than birds (Wienberg 2004), or in other words the birds have higher karyotype stability than mammals (Burt et al. 1999, Shibusawa et al. 2004 a, b, reviewed by Griffin et al. 2007, Nanda et al. 2008). Good examples of these affirmation may be noticed in the Chinese Muntjac (2n=46), Indian Muntjac (2n=6 and 7 in males and females respectively) (Yang et al. 1997), gibbons, Muridae rodents, caniforms (Wienberg 2004), equides (Trifonov et al. 2008), among others, which generally had many karyotype rearrangements during their evolutionary history. Some of these conclusions were achieved long time before chromosome painting advents, but any way, there are no doubts that painting methodology refined and confirmed these conclusions.

Chromosome Painting in the Sex Chromosome Studies

The sex determination is very important for reproduction, not only in being substantial for evolution and genome diversity (Manolakou et al. 2006). Thus, the sex chromosome has attracted the interest of many researchers, and many techniques have been applied for understanding their origin and evolution in mammals, birds, fishes, amphibians and invertebrates groups. It is important to note that chromosome painting is a powerful technique that has been applied to achieve knowledge on sex chromosomes in several animal groups.

There are many papers that discuss the sex chromosome evolution in mammals and in general. The Zoo-FISH using HSA X chromosome as probe, as well as others cytogenetic methods applied throughout Eutheria clade, revealed that this chromosome is conserved among all placental mammals and probably was present in the AEK (Table 3) (revised Ferguson-Smith and Trifonov 2007 see other papers cited in the begining of this topic). In fact, Ohno (1967) revealed that the gene content in the X chromosome is almost identical

among mammal species. An interesting fact was observed when the HSA X was used as probe onto two-toed sloth (Pilosa, Xenarthra), showing signals in the entire X and in part of Y chromosomes of this species, indicating the existence of some homology between Y and X chromosomes. It is surprising because these patterns of hybridizations using human probes had never been reported in mammals, with exception of the cross-hybridization to the pseudoautosomal region between closely related species (Yang et al. 2006).

Another interesting characteristic in relation to the mammal sex chromosomes was observed in the Monotremes species named duck-billed platypus (*Ornithorhynchus anatinus*). The chromosome painting of individual chromosomes of platypus revealed that they have ten sex chromosomes (X_1-X_5/Y_1-Y_5) and, interestingly, these chromosomes are organized in a chain configuration at male meiosis (Rens et al. 2004, revised by Grützner et al. 2004). These sex chromosome traits, as well as the enigmatic platypus genome qualities (Brown 2008), made the sex chromosome systems of Monotremes interesting for the comparative chromosome painting analysis.

As already discussed, mammal sex chromosome evolution is a theme that has attracted the interest of many researchers, and platypus sex chromosome took its place in this scenario. The comparative gene mapping analysis showed that the largest X chromosome of platypus (X_1) has homology with part of eutherian and marsupial X chromosomes, and the X_5 has homology to the birds Z chromosome. The whole chromosome painting of platypus sex chromosomes showed that the sex chromosome chain evolved from an original sex chromosome pair with homology to the avian ZW system and during the early evolution of mammals, four autosome pairs were translocated for this system (Grützner et al. 2004, revised by Graves 2008).

Many years before this conclusion, Ohno (1967) proposed that both mammals' and birds' sex chromosome systems have evolved from the same autosomal pair. Ohno's proposition was refuted after the demonstration that the chicken Z chromosome is homologous to the human chromosomes 9 and 5, and that the therian the X chromosome is homologous to chicken chromosome 4p and part of chromosome 1 (Graves and Shetty 2000, Nanda et al. 2000). Nevertheless, the recent insights about sex chromosome evolution in platypus evoked Ohno's theory (1967), because the observation of the homologies between sex chromosomes of platyplus with the therian XY and birds ZW system (Grützner et al. 2004). This 'link' was questioned when two new papers using comparative gene mapping about this theme were published (Rens et al. 2007, Veyrunes et al. 2008). These papers showed that platypus X_1 and X_3 have homology to the some chicken autosomes and Z chromosomes and, besides that, some chicken Z genes are present on platypus X_3 and X_5 and echidna X_3 and X_4. Either way,, X_1 of platypus and eutherian X chromosomes do not share homologies, and consequently it was proposed that the X_1 of platypus is more related to the avian chromosomes than to therian sex chromosomes (Rens et al. 2007, Veyrune et al. 2008). Taken together, the authors argued that these results may indicate that the monotreme sex chromosome system could be linked to the avian sex chromosome system by an ancestral sauropsid sex chromosome system (or an ancestral bird-like ZW sex chromosome) and that in fact, they are not a link between bird and mammal sex chromosome systems, as proposed before by Grützner et al. (2004) (Rens et al. 2007, Veyrunes et al. 2008). Consequently, it was proposed that the therian sex chromosome system evolved after the prototherian and therian divergence in which the amniote ancestor may has had a bird-like ZZ/ZW system and thereafter, on therian lineage the XY system has evolved (Rens et al. 2007, Veyrunes et al. 2008). In summary, it was concluded that the

monotremes and eutherian sex chromosomes had independent origins, such as currently observed for sex chromosome evolution in teleost fishes (revised by Mank et al. 2006).

Recently, the sex chromosomes of the short-beaked echidna (*Tachyglossus aculeatus*) and platypus were compared by Zoo-FISH experiments. At first, it was concluded that the echidna has 10 sex chromosomes in females (ten Xs) and 9 in males (five Xs and four Ys), and, similarly to the sex chromosomes of platypus, the sex chromosomes of echidna are organized in chain configuration at meiosis. Moreover the results revealed that some chromosomes of platypus (X_1-X_3/Y_1-Y_3) are conserved *in toto* on echidna karyotype and that some other sex chromosomes are homologous for some sex chromosomes and autosomes to the echidna and *vice-versa* (for more details see Rens et al. 2007). In addition, it was showed that the 'Y_5 chromosome' present in platypus but absent in echidna (it was proposed that the Y had been lost in this species) was incorporated into Y_3 in the latter species. Finally, other experiments (including some reciprocal chromosomal painting) showed that, although both platypus and echidna sex chromosomes are organized in meiotic chains, all data indicate differences in the constitution and order of the chromosomes between both chains, and they have probably continued to evolve after the divergence between echidna and platypus (for more details, see Rens et al. 2007).

In relation to the sex chromosome in birds, the first paper about comparative chromosome painting in the avian clade sheds light on their origins. It observed a karyotypic conservation between Paloegnathes and Neognathes species models (emu and chicken, respectively). Interestingly, the *G. gallus domesticus* Z chromosome (GGAZ) probe hybridized both Z and W chromosomes of emu (the sex chromosome is a homomorphic pair in this species), showing a conservation of the Z chromosomes between two species and maintenance of an ancient conservation between Z and W sequences in birds. Consequently, the results of these studies corroborated the theory of autosomal origin for the sex chromosomes in birds group (Shetty et al. 1999). Since this first paper (Shetty et al. 1999) many other studies have been published related to molecular cytogenetics in avian sex chromosomes. These papers used comparative mapping with GGAZ-orthologous genes, comparative chromosome painting with GGAZ-specific DNA libraries, Zoo-FISH with GGAZ as probes and other probes which were hybridized in some Paleognathes and Neognathes species. Except for some special particularities of each study, the results generally supported and corroborated the evidence of the conservation of Z chromosome and an extensive homology between Z and W chromosomes, although the W chromosome is not more conserved than Z (Shetty et al. 1999, Shibusawa et al. 2004a, Nishida-Umehara et al. 2007, revised by Griffin et al. 2007, revised by Stiglec et al. 2007b, for more details see Nanda et al. 2008, among others).

Thus the conservation of sex chromosomes into avian group may reflect the fact that birds have more of an overall karyotype stability than mammals (Nanda et al. 2008). It is also possible that the existence of some homology between Z and W chromosomes in birds and the absence of a general homology between X and Y chromosome (except for the sloth case) may reflect a higher karyotype stability of birds compared to mammals.

Some conclusions about the sex chromosome evolution of the Z and X chromosomes, in birds and eutherians, respectively, are similar. The sex chromosomes of homogametic sex (Z and X) shows little variability among some birds and mammals. On the other hand, several studies, as cited in the review by Griffin et al. (2007), did not find orthologues of the chicken W chromosome in other birds, and this statement have been also reported for the Y

chromosome, indicating that the heterogametic sex chromosomes could show higher evolutionary rates.

Although there are few articles in the literature about chromosome painting involving sex chromosomes of non tetrapoda vertebrates, some papers have been published in fish groups. For example, in the South American fish of the genus *Eignmannia*, popularly known as wekly-electric, the species *E. virescens* has XX/XY sex chromosome system (Almeida-Toledo and Foresti 2001) and another species, *E.* sp.2 has a $X_1X_2Y/X_1X_1X_2X_2$ sex chromosome system (Almeida-Toledo et al. 1988). In attempt to understand the evolution of both systems in this genus, assuming the hypothesis of independent origin, the X chromosome of *E. virescens* and Y chromosome of *E.* sp.2 were used as probes in chromosome painting experiments. In general the X chromosome of *E. virescens* is homologous to the Y chromosome and part of chromosome 8. In the same way, the Y chromosome of *E.* sp.2 had homology with both X chromosome of its own genome. The Zoo-FISH, using the Y chromosome of *E.* sp.2 (E2Y) as probe onto *E. virescens* chromosomes, revealed that the E2Y chromosome has high similarities with autosomal pairs of the *E. virescens*. The last results indicate a lack of homologies between both sex chromosome systems and corroborate the hypothesis of independent evolution of these systems in these populations (Henning et al. 2008). It is probable that the X and Y chromosomes of the *E. virescens* originated from an autosomal pair.

Triportheus is another South American fish that harbors a ZZ/ZW sex chromosome system. It is supposed that the Z chromosome is conserved in the genus *Triportheus* (Artoni et al. 2001, Artoni and Bertollo 2002), and cross-species chromosome painting was performed using the Z chromosome of *T. nematurus* as probe into the chromosomes of several species of this genus, as well as into the chromosomes of some species of some genera putatively related to *Triportheus*, in order to analyze the evolution of this system. The Zoo-FISH experiments corroborated the hypothesis of Z conservation into the *Triportheus* genus; however, the Zoo-FISH in species of other genus did not show signals as it was expected. The authors argue for an independent origin of sex chromosomes in *Triphortheus* in relation to other genera, corroborating the synapomorphism of the sex chromosome systems for this genus (for more details see Diniz et al. 2008).

The Nile tilapia fish, *Oreochormis niloticus,* is believed to have a XY sex chromosome system (largest chromosome pair) proposed by synaptonemal complex analysis (Carrasco et al. 1999). In this way, Harvey et al. (2002) created chromosome probes of X and Y chromosomes of the Nile tilapia and showed that these probes are specific to the largest chromosome pair, and that there are differences in sequence content between both chromosomes. Additional studies (Carrasco et al. 1999, Griffin et al. 2002, Harvey et al. 2003, Ferreira and Martins 2008) pointed out that this chromosome pair has characteristics of a real XY sex chromosome, but the linkage mapping analysis, using BAC probes with sex determining markers, revealed that the sex determination locus related to the XY system is located in another chromosome pair (a small chromosome pair). In addition, the largest pair has a ZW locus for sex determination (Lee et al. 2003, Lee and Kocher 2007, Cnaani et al. 2008). Therefore Cnaani et al. (2008) proposed that the *Oreochromis* sp. ancestral had a ZZ/ZW system and in the *O. niloticus* lineage the XY locus system took the control of the sex determination pathways.

Although chromosome painting has poorly explored the sex chromosomes among fish species, this animal group represents a potential model for understanding sex chromosome

origin and differentiation among vertebrates. Most fish species do not possess differentiated sex chromosomes, but a diverse number of sex chromosome systems (XX/XY, ZZ/ZW, $X_1X_1X_2X_2/X_1X_2Y$, XX/XY_1Y_2, ZZ/ZW_1W_2) occur and are observed in all major taxa of the group.

The studies about the origin and evolution of sex chromosomes are not only restricted to the vertebrate taxa. In fact, there are many papers in the literature about this theme in invertebrate groups, although most of these studies use traditional cytogenetic methods, such as conventional analysis and C-banding (e.g. Dutrillaux and Dutrillaux 2009, Cabral-de-Mello et al. 2010d). The few works that use chromosome paints in invertebrates are concentrated on insects, although the use of this methodology, until now, is frequently associated with other chromosomal marks (such as 18S rDNA, satDNA, heterochromatin, transposable elements), in attempt to increase the knowledge about chromosome evolution. For example, in the genus *Dysdercus* the origin of the neo-XY sex system was investigated using 18S rDNA probe, GISH and Zoo-FISH of X chromosome obtained from *D. albofasciatus* (neo-XY) against the karyotypes of *D. chaquensis* and *D. ruficolis* with XO sex system (Bressa et al. 2009). The chromosome paint of an X chromosome together with rDNA mapping corroborated the previous idea proposed by Bressa et al. (1999) concerning the fusion of the ancestral X chromosome in *D. albofasciatus* with ancient XO sex-system generating the neo-XY bivalent. Moreover, this work (Bressa et al. 2009) added some refined information about the insertion of an X chromosome in the autosomal pair harboring rDNA cluster, followed by inversion and transposition of rDNA sites from the neo-Y to neo-X.

A good example of another kind of chromosome painting was performed by Willhoeft et al. (1998) using partial chromosome painting (PCP) of the Y chromosome (five probes, medY1-medY-5) and X chromosome (medX1) of the fruit fly *Ceratitis capitata*. The results using the probes against the *C. capitata* chromosomes showed that the sex chromosomes in this species are composed of three regions in which: the region II (part of Yq, part of Yp and Xq) is the oldest region being thereby the most degenerated region; the region I (centromeric region of X and Y, Xp, part of Yp and part of Yq) show high homologies between the X and Y chromosomes; the region III (part of Yq and part of Xq) was the latter region to be inserted into the sex chromosomes. Moreover, Zoo-FISH using medY1, medY2 and medX1 probes onto chromosomes of *C. rosa* and *Trirhithrum coffeae* demonstrated that *C. capitata* is more related to *C. rosa* than to *T. coffeae*, and that at least some sequences of the Y chromosome of *C. capitata* were present in its own genome before the divergence between *C. capitata* and *C. rosa* (for more details see Willhoeft et al. 1998).

Another example is related to the ZW sex chromosomes in the clodling moth *Cydia pomonella*. The W chromosome of this species was used as a probe onto its own chromosomes, showing thus that this probe was a specific W chromosome probe. The absence of signals in the Z chromosome supports the evidence of high level of molecular differentiation between Z and W chromosomes (for more details see Fuková et al. 2007).

In *Ephestia kuehniella*, *Cadra cautella*, *Plodia interpunctella* and *Galleria mellonella* species (Lepdoptera, Pyralidae), the W chromosome was used as a probe to detect the similarities among W chromosomes in the family. Zoo-FISH with W chromosome probe of *E. kuehniella* revealed a partial homology between W chromosomes for other three species. The data are consistent with phylogenetic relationships among species of Pyralidae family, and it was concluded that they have an ancient sex chromosome system with a higher

molecular differentiation, which was revealed by low similarities among W chromosomes (Vítková et al. 2007).

Although there are few results using chromosome painting in non-mammalian groups, this approach proved to be a very satisfactory tool for understanding the origin and evolution of XY and ZW sex chromosomes in a range of animal taxa. Most of these studies are in agreement with many hypotheses that involve the sex chromosome evolution such as: autosomal origin of sex chromosomes; lack of homologies between Z and W or X and Y; conservation of Z as well as X chromosomes throughout some specific taxa. Moreover it was possible to establish phylogenetic relationships using chromosome painting approach.

Chromosome Painting in the B Chromosome Investigation

Before the advent of chromosome painting, the molecular composition of B chromosomes was investigated using techniques like gradient density ultracentrifugation, Southern blotting, enzymatic digestion, genomic libraries and *in situ* hybridization (revised by Camacho 2005). The first application of chromosome painting in B chromosome studies was conducted in the plant *Secale cereal* (Sandery et al. 1991). Among animals, B chromosomes were first studied under painting technology in the marsupial *Petauroides volans* (McQuade et al. 1994). After this, many studies were conducted in this field concluding, in general, that B chromosomes are rich in repetitive elements and, in some cases, harbor few gene sequences (revised by Camacho 2005).

In an attempt to elucidate the origins and composition of B chromosomes there are two recent examples in the literature for the grasshoppers *Locusta migratoria* and *Eyprepocnemis plorans*. In both species the B (for *E. plorans* the B_{24}) and X chromosomes were used as probes for chromosome painting. In *L migratoria* the B chromosome probe revealed that the B chromosome share sequences with pericentromeric regions of X and most of A chromosomes. Moreover, it was showed that the X chromosome shares sequences with pericentromeric regions of two A chromosomes, some non-centromeric regions of most A chromosomes and on most parts of the B chromosome. These results suggested an intraspecific origin of B chromosomes in *L. migratoria* and, even sharing some sequences with X chromosome, it was not possible to determine the specific A chromosomes from which the B originated (Teruel et al. 2009a). Although the use of B chromosome paint did not reveal the origin of the B chromosome in *L. migratoria*, the precise origin of this element was revealed using histone genes hybridization. The B chromosome of this species has H3 and H4 histone genes that are shared with the autosomal pair 8, indicating the origin from this chromosome (Teruel et al. 2010)

In the grasshopper *E. plorans,* previous studies on the B_{24} chromosome using rDNA and satelitte DNA as probes suggested that the B chromosome is derived of the X chromosome, despite the probe sequences being present in most of the autosomes. Thus, chromosome painting using B_{24} and X chromosomes was not able to reveal if the B chromosome is derived from the X chromosome or from one or more autosomes, because the probes hybridized the whole B_{24} chromosome and also the proximal regions of all A chromosomes (Teruel et al. 2009b). However, these experiments corroborated the observation that the X chromosome and B_{24} chromosome share repetitive elements (López-León et al. 1994, Cabrero et al. 1999, Teruel et al. 2009b). Although these results show the possibility of the use of chromosome

paints in the elucidation of origin of B chromosomes, it is clear that this methodology gives only general information concerning this analysis, and that the use of other chromosomal markers is necessary for attempting better clarification of this issue. The repetitive nature of B chromosomes causes hybridization in several chromosomes of the A complement, making difficult the precise establishment of B origin.

The Future of Chromosome Painting

Chromosome painting is an excellent approach for elucidation of origin, evolution and relationship between different chromosomes and genomes. The most notable contributions of chromosome painting seem to be the establishment of an ancestral karyotype, phylogenetic relationships and the probable origin of some specific chromosomes. Cross-species chromosome painting is very resolute to identify homologies among syntenic blocks, thus allowing the construction of karyotype phylogenies between species, families and orders, in addition to the establishment of their ancestral karyotypes, as observed in mammals and birds. Moreover, the data obtained by chromosome painting can be integrated to other data, such as the analysis of heterochromatin patterns, BAC-FISH mapping, FISH with other kinds of probes as well as whole- genome sequences comparisons.

The application of chromosome painting for comparative chromosome studies in diverse animal groups is expected, despite the difficulties for probe obtaining in some specific groups. Furthermore, the chromosome comparisons can be predicted comparing available nucleotide sequences of whole genomes. In this way, bioinformatics seems to be an important tool to be integrated to classical painting and applied in cytogenetics generating data on the chromosomal level based exclusively on nucleotide sequence data.

Conclusion

Important information about the genomes can be acquired from fundamental cytogenetic studies based on the identification of chromosome number and morphology, up to advanced molecular and bioinformatic approaches applied in cytogenetics. In this chapter we reviewed the contribution of this area to the knowledge of animal genomes. One of the most significant contributions of cytogenetics to genomics can be illustrated by the pufferfish *Tetraodon nigroviridis*, one vertebrate organism model for genomic studies. This species contain the most compact genome of a vertebrate species with 340 Mb. Molecular cytogenetics was applied to anchor nucleotide sequence data to specific chromosomes of the species, allowing comparative analysis of other vertebrates and inferences on the ancestral bony vertebrate, which was composed of 12 chromosomes (Jaillon et al. 2004). Furthermore, analysis of the *Tetraodon* and human genomes shows that whole-genome duplication occurred in the teleost fish lineage, subsequent to its divergence from mammals.

The most recent novelty in cytogenetics is the application of bioinformatics to generate information on the chromosome level (Kohn et al. 2006). The availability of complete nucleotide sequences of a large number of animal genomes brings the possibility of a new area for chromosome analysis, the *in silico* cytogenetics. On the other hand, the physical

analysis of the chromosomes and their visualization under a microscope constitute the most fascinating and promising way to understand genomes throughout cytogenetics.

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