ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 42

Comparative Genome Analysis in the Horse

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Akademisk avhandling som för vinnande av filosofie doktorsexamen kommer att offentligen försvaras i sal 1, Klinikcentrum, Ultuna, fredagen den 29 januari, 1999, kl. 9.00.

Abstract

Comparative genome analysis is one of the ways to investigate the equine genome. In the present work this was carried out by mapping specific genes to horse chromosomes and by detecting homologous chromosomal segments between horse and other species. Zoo-FISH on equine metaphase chromosomes using human whole chromosome painting probes (WCPs) for the first time delineated homologous segments between the karyotypes of the two species. The homology was further refined using microdissected chromosome arm specific paints (ASPs) for HSA2, 5, 6, 16 and 19. The Zoo-FISH studies serve as a basis to extrapolate and efficiently transfer gene mapping data from the advanced gene maps of human/mouse to that of the horse.

An important part of any genome mapping project is mapping of specific genes. In this thesis four genes were added to the horse physical map: *IGF2* using an equine specific probe, and *MC1R, KIT, PDGFRA* using heterologous (porcine) probes. Successful use of heterologous large insert genomic BAC clones for the assignment of individual genes across distantly related species was the first such report in farm animals, and opens new avenues for developing comparative maps in the absence of species specific gene probes. The comparative gene mapping work was further extended to donkey - another equid species. These results represent the first mapping data in the donkey and provide interesting comparative information with the closely related horse genome.

Comparison between the karyotypes of the two equid species was expanded by generating fifteen microdissected equine chromosome specific probes (all meta- and sub-metacentric autosomes and the sex chromosomes) and hybridizing them to donkey metaphase chromosomes. Eight of the equine WCPs showed one-to-one correspondence with the donkey chromosomes, one to a single arm, while six to 2-3 arms on separate chromosomes. The results provided an insight into how the two genomes are organized in relation to each other, with respect to the equine chromosomes used in the present study. An important outcome of the results was indirect deduction of homology between human and donkey karyotypes. The latter gives a basis for comparison of the donkey genome with other mammalian species.

Accumulating Zoo-FISH data between human and several non-primate species help to understand how different mammalian genomes are organized in relation to each other. Analysis of comparative chromosome painting data between distantly related species enabled identification of evolutionarily conserved whole chromosomes, large chromosomal segments and contiguous synteny combinations, that very likely comprise the karyotype of an eutherian ancestor. The equine genome comprises the focus for all investigations within the thesis, and therefore the results are discussed with special emphasis on the horse.

Keywords: horse, human, Zoo-FISH, gene mapping, heterologous FISH, chromosome microdissection, donkey, karyotype evolution.

Distribution:
Swedish University of Agricultural Sciences
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Box 7023, S-750 07 UPPSALA

UPPSALA 1999
ISSN 1401-6257
ISBN 91-576-5405-0

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1999

Acta Universitatis Agriculturae Sueciae
Veterinaria 42

ISSN 1401-6257
ISBN 91-576-5405-0

Tryck: SLU Service/Repro, Uppsala 1999

To the Donkey-
the symbol of stubbornness

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Raudsepp T. 1999. *Comparative genome analysis in the horse.*
Doctoral dissertation.
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Contents

Appendix I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals

I. Raudsepp T, Frönicke L, Scherthan H, Gustavsson I, Chowdhary BP (1996). Zoo-FISH delineates conserved chromosomal segments in horse and man. *Chromosome Res.* 4, 218-225.

II. Raudsepp T, Otte K, Rozell B, Chowdhary BP (1997). FISH mapping of the *IGF2* gene in horse and donkey - detection of homoeology with HSA11. *Mamm. Genome* 8, 569-572.

III. Chaudhary R, Raudsepp T, Guan X-Y, Zhang H, Chowdhary BP (1998). Zoo-FISH with microdissected arm specific paints for HSA2, 5, 6, 16, and 19 refines known homology with pig and horse chromosomes. *Mamm. Genome* 9, 44-49.

IV. Chowdhary BP, Raudsepp T, Frönicke L, Scherthan H (1998). Emerging patterns of comparative genome organization in some mammalian species as revealed by Zoo-FISH. *Genome Res.* 8, 577-589.

V. Raudsepp T, Kijas J, Godard S, Guérin G, Andersson L, Chowdhary BP (1998). Comparison of horse chromosome 3 with donkey and human chromosomes using cross species painting and heterologous FISH mapping. *Mamm. Genome* (in press).

VI. Raudsepp T and Chowdhary BP (1998). Construction of chromosome specific paints for meta-and submetacentric autosomes and the sex chromosomes in horse, and their use to detect homologous chromosomal segments in donkey. *Chromosome Res.* (in press).

Abbreviations used in the thesis

ASP - chromosome arm specific paint
BAC - bacterial artificial chromosome
bp - base pair
BTA - *Bos taurus* (cattle)
CATS - comparative anchored tagged sequences
cM - centimorgan
CSL - chromosome specific library
DNA - deoxyribonucleic acid
EAS - *Equus asinus* (donkey)
ECA - *Equus caballus* (horse)
EST - expressed sequence tag
FISH - fluorescent *in situ* hybridization
HSA - *Homo sapiens* (human)
ISH - *in situ* hybridization
kb - kilobase pair
LG - linkage group
Mb - megabase pair
mtDNA - mitochondrial DNA
Myr - millions of years
PAC - P1-derived artificial chromosome
PCR - polymerase chain reaction
RH - radiation hybrid
SCH - somatic cell hybrid
SSC - *Sus scrofa* (pig)
STS - sequence tagged site
TOAST - traced orthologous amplified sequence tag
UMP - universal mapping probe
WCP - whole chromosome paint
YAC - yeast artificial chromosome

Introduction

1. Mammalian genomes

1.1 Background

The evolutionary history of placental mammals (subclass Eutheria) dates back to Palaeocene, when the major adaptive radiation explosively started and gave rise to 18-19 extant eutherian orders with approximately 4,000 species. Thus, the genomes of some of the present day mammalian species, especially human, mouse and the livestock species, diverged 65-100 million years (Myr) ago, and have since then evolved separately (Benton 1990; Graur 1993; Arnason et al. 1996a). This evolution proceeded with disparate rates and mechanisms, in the different groups. For example, genome and karyotype evolution has been rapid with extensive inter- and intra-chromosomal rearrangements in some groups, e.g., rodents and equids (Ryder et al. 1978; Sumner 1990; Qumsiyeh 1994; Comparative Genome Organization...1996), while quite conservative in the others, e.g., bovids and cetaceans (Evans et al. 1973; Buckland and Evans 1978; Arnason 1977; Sumner 1990; Gallagher and Womack 1992; Gallagher et al. 1994).

Man started to domesticate animals around 10,000 years ago. Since then, livestock genomes have been manipulated without a clear idea of what lies behind phenotypic variation. The first steps to develop such an understanding were, however, taken almost a century ago, when simple experiments were conducted to test how "characters" are inherited. This was the beginning of a new era where several workers tried to comprehend the structure, organization and function of the inherited component. The work of G. Mendel led to the proposition that each heritable property of an organism is controlled by a factor, which today is called a "gene". Further, the idea that genes reside in chromosomes was proposed by W. Sutton. The suggestion received experimental support from T. H. Morgan and his colleagues who introduced one of the first concepts of gene mapping, i.e. "linkage" (Morgan 1910).

On the molecular level, the discovery of the biochemical nature and structure of deoxyribonucleic acid (DNA), and the pathway of flow of genetic information from nucleus to cytoplasm gave a new dimension to genome studies during the 1950s. However, on the cytogenetic level, understanding genome organization was influenced primarily by achievements in chromosome studies during the 1960s through the '70s. From then onwards, series of advancements in areas such as recombinant DNA technology, DNA sequencing methods, polymerase chain reaction (PCR), positional cloning, etc., have revolutionized mammalian genome analysis. The list of discoveries is constantly expanding. Consequently, there are now better possibilities to uncover hidden details of different genomes, and to utilize the information in a constructive way for the welfare of animals and mankind alike.

1.2 Genome organization

Despite millions of years of divergent evolution in thousands of eutherian species, the mammalian genomes appear to be highly conserved in size, gene number and general organization. It is now evident that about 99.99% of the genetic information of any mammalian cell lies in the nuclear DNA. The haploid genome comprises around 3 x 10^9 base pairs (bp), which are packed into complex but microscopically distinct structures - the chromosomes. The majority of the nuclear DNA consists of non-coding sequences (various kinds of repetitive DNA, introns, pseudogenes) which are variable between species. However, about 3% of the DNA comprises specific protein coding sequences - the genes. The number of genes in a mammalian genome is estimated to be between 70,000-100,000 (Cavalier-Smith 1985; Nowak 1994). Comparisons of various genes hitherto studied indicate a moderate to high degree of sequence homology even between distantly related species (Comparative Genome Organization...1996). In addition to the nuclear DNA, a small amount of genetic information lies also in the mitochondrial DNA (mtDNA), which encodes fewer than 40 genes, and is inherited maternally through egg cytoplasm (see Stoneking and Soodyall 1996). The mutation rate in mitochondrial genes is reported to be about 10-fold higher than in nuclear genes (Brown et al. 1979). Hence, in several studies mtDNA has been used for phylogenetic comparisons.

Chromosomes, which have long been considered as carriers of hereditary material, morphologically comprise a *centromere* somewhere along the length of the chromosome, and *telomeres* at either ends. The centromeric position helps to classify the chromosomes into three main categories: metacentric, submetacentric and acrocentric. Morphology (as decided by their size and centromeric position) and number of chromosomes are specific for a species. Therefore, each species has a unique karyotype, which represents specific organization of it's diploid set of chromosomes. During early 1970s, the arrangement of the chromosomes into a karyotype was further elaborated with the help of unique banding patterns for each pair of homologous chromosomes (see Sumner 1990).

In spite of similar genome size and gene number in mammals, chromosome number varies considerably among species. Most extreme examples range from Indian muntjac with 2n=6/7 to South American rodent (*Tympanoctomys barrerae*) with 2n=102 (see Qumsiyeh 1994). The highest diploid numbers among domestic animals are reported in dog (2n=78), followed by horse (2n=64) and cattle/goat (2n=60). Buffalo (2n=48-50) and sheep (2n=54) fall in the medium range while cat (2n=38) and pig (2n=38) are on the lower side of the count.

1.3 Genome conservation

It was as early as 1927 when J.B.S. Haldane (1927) observed that phenotypically similar traits were linked in more than one species forecasting an "ancestral togetherness" (later coined as "conserved syntenies/linkages"). However, first molecular/biochemical evidence to this effect was shown only during the '70s by comparative mapping. For example, two carbonic

anhydrase loci shown to be linked in guinea pig (Carter 1972), pig-tailed macaque (De Simone et al. 1973) and mouse (Eicher et al. 1976). Similarly, association between two amylase loci in humans and mouse (Merritt et al. 1972; Kaplan et al. 1973); and of different esterases in mouse and rat (Womack and Sharp 1976) was also reported. Since then, more and more illustrations of conserved syntenies and linkages became evident, though the data mainly comprised information from humans and rodents.

Until the 1970s, most evidence concerning evolutionary conservation of genome structure in mammals was adducted from karyotype studies. Almost identical banding patterns and gene content of the X-chromosome among a large cross-section of mammals suggested that at least some long-term evolutionary conservation of chromosome structure had occurred (Ohno et al. 1964, Pathak and Stock 1974). Chromosome banding studies in the autosomes too revealed conservation of chromosome organization not only among members within individual mammalian groups (e.g., carnivores, bovids, cetaceans etc.), but also between species belonging to distantly related groups (e.g., rodents and primates, lagomorphs and primates, rodents and carnivores, human and cattle, human and mouse, etc.; see Kiel et al. 1985; Sawyer and Hozier 1986; Sumner 1990, Rønne 1992). In some cases, the data were strongly supported with the presence of the same group of genes (e.g., humans vs. mouse; Lalley et al. 1978; Sawyer and Hozier 1986; see O'Brien et al. 1988). Similarities between banding patterns in euchromatic regions (regions where most of the genes are located) of certain chromosomal segments across species, in general, reflected homology in their genetic content (see Baker et al. 1987; Sumner 1990).

If genome organization is conserved among a wide range of mammalian species, why is there variation in the diploid number of their chromosomes? A plausible explanation to this is that during karyotype evolution, multiple rearrangements scrambled the ancestral synteny assemblages into a variety of combinations in different lineages. In cases where the ancestral configuration underwent a less degree of rearrangements, chromosome banding homologies (and sometimes even morphology) were conserved between species. However, when the rearrangements were extensive, both morphology and banding patterns were not comparable. These views, which emerged through cytogenetic analysis and early gene mapping data, appeared the most reasonable answer to conjoin karyotype diversity and conservation between studied mammalian species.

Detailed cytogenetic analysis and construction of gene maps in a variety of species have added to our curiosity about the comparative aspects of different mammalian genomes. The past decade has witnessed a significant progress in farm animal genome analysis. The number of loci hitherto mapped in some of the livestock species is now second only to humans and rodents. These achievements, together with new set of tools recently available, have significantly increased the power to obtain a broader comparative view of distantly related genomes. All this progress has transformed comparative genome analysis into a full fledged area of research.

2. Comparative mapping

Comparative genomics has become an integral part of present day mammalian genome analysis. The core concept of comparing genomes has not changed much over the years. However, the list of components defining a comparative map has considerably expanded. It would, therefore, be timely to put forward a clear definition of a "comparative map", where the components are distinctly identified.

2.1 What is a comparative map?

Comparisons between genomes of two species can only be carried out if each of them has a "map". For any meaningful comparison between two maps, it is important that the set of parameters measured should be the same. As pointed out earlier, the two major components of a genome are the *coding* and *non-coding* sequences. The latter cannot be used for comparison because of their low degree of conservation between species. The former, which represents specific genes, is thus the obvious choice for comparison because their sequences are moderately to highly conserved even between distantly related species (O'Brien 1991; O'Brien et al. 1993). The next issue is how to compare?

Chromosomes represent a basic image of a genome. Comparative location of the same set of genes in different genomes can be viewed as one of the definitions of a comparative map. It, however, needs to be stressed that "location" is a rather later addition to the concept of comparative map. During the early days of genome analysis, when accurate chromosome numbers were not known for most of the species, location was indirectly defined through tendency of a pair of genes to segregate together. Although detailed chromosome knowledge added a new dimension to comparative maps, the basic concept of co-segregation of genes continues to be one of the primary ways of comparing genomes.

To distinguish specific genes as the main landmarks of a comparative map from a number of other sets of markers, the term "Type I" markers was introduced (O'Brien 1991; O'Brien et al. 1993). Another set of markers, the Type II markers (e.g., microsatellites, minisatellites, short and long interspersed nuclear elements, random amplified polymorphic DNA, etc.), were initially considered unsuitable for cross species comparison. However, during recent years, it has also been possible to use Type II markers across species within a family (e.g., bovidae; Womack and Kata 1995; Prakash et al. 1997; Piumi et al. 1998) or order (e.g., Artiodactyls; Prakash et al. 1996). Thus, with regards to closely related species, these markers can be referred to as "comparable".

During the past 2-3 years, a new set of comparable markers has emerged. They are not necessarily defined genes but represent cognate sites across genomes, sometimes only 25-400 bp long. Generally, they all are conserved sequence tagged sites (STSs) derived from evolutionarily conserved regions of the genome. However, when they originate from coding-regions, they are referred to as expressed sequence tagged sites (ESTs). STSs/ESTs are

mapped either by direct filter/ *in situ* hybridization or using the PCR approach. A new term "Zoo-PCR" has been proposed to note PCR amplification of STSs across diverged species (Mazzarella et al. 1992).

Another class of comparative markers are the universal mapping probes (UMPs) which are DNA segments less than 15 kb in size, containing conserved sequences immediately adjoined by a highly polymorphic CA repeat site. The conserved region determines physical gene location, whereas the CA repeat facilitates genetic mapping (Hino et al 1993). Further, comparative anchored tagged sequences (CATS; Lyons et al. 1997) and traced orthologous amplified sequence tags (TOASTs; Jiang et al. 1998) represent PCR primer based comparative markers which have been used across species. Basically both types contain conserved and variable elements, which help in PCR based linkage, somatic cell and radiation hybrid mapping. All these new generation markers are gradually becoming an integral part of comparative maps, thus providing a new vision to correspondence between genomes.

2.2 Historical background

Ever since mankind started addressing questions regarding inheritance of traits from parents to offspring, either in humans or in different animals surrounding them, a basic sense of "genome comparison" kindled their inquisitiveness. Later, with the development of science, and beginning of an era of organized genetic studies to trace how phenotypic traits segregated from one generation to another, comparative genomics started getting a framework. The advent of linkage analysis during early 1900, enabled geneticists to initiate comparisons between the nascent gene maps of, for example, humans, rabbit and mouse (Castle 1924; see Sinnott et al. 1950). However, with limited mapping information available on different genomes, at this stage meaningful comparisons were not possible.

Correspondence between linkages on the X-chromosome among some mammals can always be looked upon as a landmark in the development of comparative maps (Sinnott et al. 1950; see Ohno 1970). Mapping of the same gene(s) on the X-chromosome in two or more species (e.g., hemophilia A and B both in humans and dogs; Hutt et al. 1948) provided first comparative status for loci on the X-chromosome. Linkage of M-N blood types and sickle cell anemia (Snyder 1949) marked the beginning of autosomal linkage among humans, which in turn provided basis for comparing autosomal linkage with other mammalian species. However, it was only early during this century that accurate chromosome numbers in different species became known. This consequently provided the stimulus to know "what maps where", thus initiating organized mapping in human and mouse. Later, gene mapping studies also started in rat, rabbit and cat, whereby a broader platform for comparisons between genomes became available.

Although initially, the rabbit and cat gene maps contained <50 loci (Fox and van Zutphen 1979; Echard et al. 1981; O'Brien and Nash 1982; see O'Brien 1990), the outcome was exciting because the synteny/linkage between genes very closely mimicked that observed in humans. In some

cases, the findings strongly supported earlier suggested banding homologies with human chromosomes (Nash and O'Brien 1982; see Lemieux and Dutrillaux 1992). The remarkable degree of synteny conservation between cat and human suddenly graded cat as a "model animal" for investigating various human conditions. Presently, there are over 30 feline analogues of human inherited diseases (O'Brien et al. 1997b). Since the mid '80s, however, only limited progress has been made in expanding the cat or rabbit gene maps. On the other hand, the human, mouse and rat maps have steadily developed over the years, and are therefore providing interesting comparative information with respect to each other, as well as other mammalian species (Levan et al. 1991; Levan et al. 1993; Szpirer et al. 1998). Presently, the human gene map is the most advanced, followed by mouse and rat. With over 7,000 and 1,000 specific genes mapped (GDB), respectively, in the latter two species, their comparative maps are fairly informative.

Among the livestock species, sporadic gene mapping data in pigs was already available during the early 60s. The work centered primarily around blood groups and their linkage relationships. For over two decades, only 20-25 new loci were added in pigs (see Chowdhary 1998a). The halothane linkage group was the highlight of the pig gene map because it was extensively studied by several groups around the world (Andresen 1971, 1979; Jörgensen et al. 1976; Rasmusen 1981). However, the overall genome information in pigs was too little for comparisons with the human gene map. In contrast, gene mapping in cattle got a more systematic start, thanks to the efforts invested in constructing a cattle x hamster somatic cell hybrid panel during the early '80s (Womack and Moll 1986). Of the 35 loci allocated to 24 syntenic groups in cattle, 32 represented homologous genes mapped in human and mouse. Within another 3-4 years, the number of loci as well as syntenic groups expanded. Consequently, numerous conserved syntenies were detected between human and cattle, indicating that the two genomes share larger regions of conserved synteny than that shared between human and mouse (Womack and Moll 1986; Fries et al. 1989). Thus, in terms of a true comparative map in farm animals, the cattle gene map provided a lead (see Womack 1993).

Organized gene mapping in pigs was initiated during the latter half of the '80s. Consequently, a basic comparative map in pigs was available only 5-6 years ago (see Chowdhary 1991). The present day pig gene map is undoubtedly the second most developed map among the livestock species. Gene mapping has also progressed in sheep and goat. Although the number of mapped loci in the two species remained low for a long period, concerted efforts by some laboratories during the past few years have considerably expanded their maps (Iannuzzi et al. 1997; Burkin et al. 1998; Schibler et al. 1998). The progress is attributed mainly to the gains made in cattle gene mapping. Similarly, success in developing gene map in buffalos also relies primarily on readily transferable information from cattle. Today, over 500 genes have been mapped in cattle, >220 in pigs, >200 in sheep and goats and around 50 in river buffalo (GDB; Iannuzzi et al. 1997; Schibler et al. 1998). The progress is significant in terms of furnishing comparative information with regards to the advanced gene maps in humans and mouse.

Among livestock, horse is one of the species which received attention of the geneticists very late. Autosomal linkage studies in early 1980s were among the first to provide comparative mapping data between horse and other mammalian species (Andersson et al. 1983a, b). Organized international efforts to initiate gene mapping in horse (The First Equine Gene Mapping Workshop 1995) started only during the past couple of years and have expanded the equine gene map by almost 10-folds. Presently, over 50 genes are mapped in horse. This serves as a ground-work for comparative studies with humans and other species with advanced gene maps. Lastly, it needs to be mentioned that gross molecular chromosomal comparisons (referred to as Zoo-FISH) is also one of the approaches which has greatly added to our knowledge about the comparative status of several livestock/mammalian genomes. These will, however, be discussed in details in the section below.

2.3 Approaches for constructing comparative maps

Several mapping approaches have contributed significantly towards the development of gene maps, which in turn has helped geneticists in relating one genome to another. Some techniques tell us about the relative order of the genes, while others simply assign genes to chromosomes or even to specific locations on them. Because each of these techniques facilitate genome comparison in a special way, they are very briefly discussed individually, to provide an overview about how they add to comparative maps.

2.3.1 Genetic linkage analysis

A linkage map shows relative order of loci within a genome. Distances between loci do not correspond to physical distances but to recombination frequency between the pair or set of loci investigated. Closer are the markers, greater are the chances of their co-segregation during meiosis. Linked loci can be tagged to a specific chromosome if one or more of them are physically mapped to a chromosome (see below). In a fairly well developed linkage map, all loci present on the same chromosome are "linked". Thus, as a map progresses, the number of linkage groups in a species corresponds to the number of haploid chromosomes. The basic prerequisites for constructing genetic linkage maps are i) good family material and ii) availability of polymorphic markers.

In the majority of the livestock species, linkage maps have been established using a range of polymorphic markers on family material generated by crossing as diverse breeds as possible. For example, in pigs, the family material comprises of F2 and F3 generation offspring from crosses between Large White (Swedish Yorkshire) x Wild pig (Andersson et al. 1994), Large White x Chinese Mehishan/Minzhu (Schook et al. 1994); in cattle, from crosses between *Bos taurus* x *B. indicus* and *B. taurus* x *B. gaur* (Womack 1993; see Lyons et al. 1994); in cat from crosses between *Felis catus* x *F. bengalensis* (Lyons et al. 1994), and so on (horse discussed in details later). During recent years, attempts have been made in pigs and

cattle, to merge maps generated from different sources. Also, an international mapping panel is being developed in each species, to co-ordinate mapping results from different groups around the world.

Of the various markers hitherto linkage mapped in different farm animals, a large proportion (ranging from 70-85%) are Type II, and therefore not the best suited for direct genome comparisons between species. Nevertheless, the remaining 15-30% of the linked markers, which encode for specific genes, are useful in comparing their relative order and map distances across species, thus detecting conservation and/or rearrangements. A classical illustration of the practical utility of comparative linkage maps is the study of malignant hyperthermia in humans and pigs. Comparison of linked markers in the two species demonstrated extensive conservation, which helped to discover a mutation in the ryanodine receptor gene as the cause of the disease (McCarthy et al. 1990; Fujii et al. 1991).

2.3.2 Somatic cell hybrid (SCH) analysis

Synteny means on the same chromosome, and a synteny map simply represents a list of loci known to reside on the same chromosome in a particular species. The basic method for building synteny maps is through the construction of a somatic cell hybrid panel by fusing cell lines of two species (Gross and Harris 1975), one of which is the species in which the map is to be made. Analysis of pairs of genes in a SCH panel reveals concordance or discordance of their retention, thus showing their synteny or asynteny, respectively. The main methods for analyzing SCH panel are enzyme electrophoresis, Southern blotting and PCR amplification with species specific primers (see Kao 1983; Dionne and Jaye 1993). The latter is nowadays the most extensively used approach. The precision of synteny mapping depends on how well the hybrid clones are characterized cytogenetically as well as through mapping of a sufficient number of markers. Once the chromosome segments are accurately characterized, it is possible to assign markers even to segments/bands of individual chromosomes.

At present several SCH panels are available for all main livestock species, and the PCR based mapping approach has revolutionized physical assignment of genes, ESTs, microsatellites and anonymous DNA segments in cattle (Ma et al. 1998), sheep (Burkin et al. 1998), pigs (Yerle et al. 1996; Zijlstra et al. 1996), horses (Shiue et al. 1998) and buffalos (El Nahas et al. 1996). Gene maps of some species, like cat, are almost exclusively based on SCH analysis (O'Brien et al. 1997 a, b). The main drawback of this method, however, is that it shows synteny but not gene order or genetic distances, as is revealed through linkage maps. Nevertheless, the possibility to map any kind of DNA sequences, including Type I or non-polymorphic markers, is an advantage of the method. Like linkage maps, syntenic maps have also contributed significantly in deducing comparison between genomes of different species.

2.3.3 Radiation hybrid (RH) analysis

RH mapping is basically a SCH technique with the difference that before fusion of cell lines, the whole or partial genome of the species of interest is exposed to high doses of X-ray irradiation that causes fragmentation of chromosomes (e.g., see Cox et al. 1990). RH mapping shows not only synteny between loci, but also helps to analyse physical distance between them. The farther apart two markers are on a chromosome, the greater are the chances that they will be separated by X-ray treatment and vice versa. The range of resolution of RH maps is dependent on the irradiation dosage applied.

RH mapping enables the integration of linkage maps based on polymorphic markers with non-polymorphic Type I markers. The technique has proved to be a powerful tool for high resolution mapping in human and mouse (McCarthy 1996; McCarthy et al. 1997; Stewart et al. 1997; Flaherty and Herron 1998). Among farm animals, whole genome RH panels have been recently constructed for cattle (Womack et al. 1997) and pigs (Alexander et al. 1998; Yerle et al. 1998). In pigs over 1,000 microsatellite and Type I markers have been mapped with the RH panel (Alexander et al. 1998). Because the resolution of RH maps exceeds that of linkage and cytogenetic maps, it provides a new perspective for constructing high resolution ordered comparative maps between species. The very recent comparison between HSA17 and BTA19 using RH maps is one of the best examples of the power of parallel RH mapping for comparative purposes (Yang and Womack 1998; Yang et al. 1998).

2.3.4 In situ *hybridization (ISH)*

In situ hybridization is a technique which is widely used in several branches of biology. However, with reference to gene mapping, the technique allows direct visualization of the location of specific genes or anonymous DNA segments on the chromosomes. The location is thus a reflection of the molecular constitution of the chromosome at that site. There are two major components of *in situ* hybridization, viz., chromomes, which are the targets, and probes, which are DNA segments of various lengths. Usually, the *target* is either metaphase or prometaphase chromosomes, but in cases where high resolution physical mapping is conducted, the chromatin fibre could either be from interphase cells or from mechanically stretched cellular DNA. The *probes*, however, vary considerably in size as well as origin. The size can range from a few base pairs (bp), e.g., the telomeric or centromeric repeat oligonucleotide sequences, to several hundred kilobases (kb) cloned in a yeast artificial chromosome (YAC) vector.

Depending on the type of ISH, the DNA probe can be labelled radioactively or non-radioactively. Radioactive ISH (RISH), which has now almost become a technique of the past, used radioactively tagged nucleotides as labels, of which tritium (^{3}H) was most extensively used (see Chowdhary 1998a). However, from the 1980s onwards, the non-radioactive approach has progressed significantly (Pinkel et al. 1986; Lawrence et al. 1988; Lichter et al. 1991; Trask 1991 a, b), and during recent years has become the

method of choice. In this approach, the DNA is labelled with nucleotides tagged with biotin, digoxigenin (DIG), di-/tri-nitrophenol, or with other labelling molecules. The hybridization is then detected with a variety of reporter molecules which have affinity to the labels. The detection can either be carried out *enzymatically* or with the help of *fluorochrome conjugates*. The latter approach, which is also referred to as fluorescent *in situ* hybridization (FISH), is the most widely used.

Although sensitivity is still a problem when small sized probes are used for FISH, several groups have successfully carried out band specific localization of 1-2 kb cDNA probes (see Trask et al. 1993; Chaudhary et al. 1997; Thomsen et al. 1998). Nevertheless, general experience shows that it is usually difficult to FISH map sequences < 2 kb in size. Conversely, targets larger than 15 kb, such as those cloned in λ phages, cosmids, BACs and YACs, can be detected with > 90% efficiency (Trask et al. 1993). The hybridization process itself, as well as the post-hybridization washing, can vary considerably depending upon the type and size of the DNA probes used. Details on individual aspects/components of ISH are described elsewhere (Lichter et al. 1991; Trask 1991 a, b; Lichter and Cremer 1992).

In the context of gene mapping, ISH normally means localization of a single probe. However, with the availability of a number of labels and reporting molecules, it has now been possible to hybridize two or more probes in one experiment (double- and multicolour FISH). The results enable ordering of the loci (if three or more are used), and even estimation of physical distances between them. Depending on the distance between the probes, ordering of the loci can be carried out by metaphase-FISH, interphase-FISH or fiber-FISH. There are reports where researchers have even been able to mechanically stretch a single chromosome and successfully order closely located clones (Claussen et al. 1994; Laan et al. 1995). The first double-colour FISH mapping experiment in farm animals was carried out to order *GPI-CRC-LIPE* loci on pig chromosome 6 (Chowdhary et al. 1995). Similarly, the first fiber-FISH experiments in farm animals were also carried out by the same group (Sjöberg et al. 1997a, Liu et al. 1998), and once again in pig. During the past 3-4 years, the two techniques, together with another development referred to as DNA combing, have added a new dimension to physical gene mapping (see Heiskanen et al. 1994; Palotie et al. 1996; Kraus et al. 1997).

The different FISH approaches discussed above make it necessary to give a brief overview of their resolution. The closest distance resolvable between two loci on metaphase chromosomes is 1-3 Mb (megabase pairs) (Lawrence et al. 1990; Lawrence et al. 1992). This range varies with the degree of contraction of the chromosomes. In interphase FISH, probes 25-50 kb apart can be readily resolved from each other (Lawrence et al. 1992). However, if a set of probes are separated more than 750 kb, the reliability of order is low due to the constant twisting of the chromatin fiber in the interphase stage. Fiber-FISH on the other hand enables to distinguish probes separated by 1-2 kb (optimistically). Probes more than 350-400 kb apart are less suitable to be studied using this approach. This is attributed to the tendency of the DNA fibers to break beyond the 400-500 kb level, when they are mechanically stretched on glass slides (Heiskanen et al. 1994; Palotie et al. 1996).

Among livestock, pig was the first animal where ISH was applied. Using the radioactive approach, the porcine major histocompatibility complex locus was assigned to specific chromosomal bands (Geffrotin et al. 1984; Rabin et al. 1985; Echard et al. 1986). However, it was not before 1988 that ISH was routinely applied for chromosomal localization of markers in pig, cattle and horse. The technique was gradually extended to other species like sheep, goat, buffalo, dog, fox etc., and has now been applied to almost all livestock species. As mentioned earlier, the initial phase (at least for five years in farm animals) of *in situ* work extensively used the radioactive approach. However, thereafter, the trend gradually shifted to the use of the FISH approach. Like in other species, today the latter has almost completely replaced RISH.

One of the major contributions of the ISH technique to gene mapping in farm animals is the assignment of syntenic and linkage groups to specific chromosomes. During the primary stages of gene mapping this was crucial in aligning the physical and genetic linkage maps. Thereafter, with the map building up, ISH data kept on showing how the two maps related to each other. Initially, the basic cytogenetic map in the farm species was constructed on a random mapping basis, i.e., the loci ISH mapped were randomly chosen. However, as the map grew, it started becoming evident which areas of the genome require mapping of more markers. Thus, ISH has been constantly needed to see whether i) coverage with markers is spanning the entire length of individual chromosomes, and ii) there is a homogenous distribution of markers on all chromosomes. Consequently, sufficient number of ISH localizations in pig and cattle helped to develop a consensus map (Ellegren et al. 1994; Rohrer et al. 1996; Ferretti et al. 1997).

As pointed out earlier, specific genes form a small proportion of the total loci mapped in each farm animal species. Like other maps (linkage or syntenic maps), this also holds good for the ISH based cytogenetic maps. At present, there are over 200 *in situ* mapped genes in cattle, sheep and goats (Schibler et al. 1998), more than 150 in pigs (see Chowdhary 1998a) and around 30 in horses (see section below). Nevertheless, the cytogenetic maps have provided a visual basis for detecting homologous segments/ chromosomes across species by demonstrating physical order and distances of syntenic/linked genes. The technique has thus made a vital contribution in enhancing our knowledge about comparative organization of different genomes.

2.3.5 Comparative chromosome painting and allied techniques

Before discussing comparative chromosome painting in details, it will be useful to briefly describe the sources - flow sorted and microdissected chromosomes - which contribute to probe preparation. The discussion of the sources is further essential because the approach which creates them also contributes to gene mapping in other ways.

Chromosome flow sorting: The technique separates individual chromosomes of a given species using a fluorescence activated cell sorter

system (FACS). Because the exhibited fluorescence of a chromosome is proportional to the DNA content and AT/GC rich regions, it is possible to separate individual chromosomes (Lebo 1982; Lebo and Bastian 1982). The procedure results in generating a flow karyotype, distinguishing each chromosome (Lebo 1982). Once separated and collected, DNA from individual chromosomes can be either directly amplified using, e.g., degenerated oligonucleotide primers (DOP; Telenius et al. 1992), or used for library construction (Collins et al. 1991). In both cases, whole chromosome specific DNA is available as a composite probe for FISH applications in clinical cytogenetics, comparative painting, cloning and gene mapping.

Flow sorting was first used to generate chromosome specific libraries (CSLs) in human (Collins et al. 1991; Vooijs et al. 1993). Over the years these libraries have been extensively used for genome analysis in humans. However, during recent years, human CSLs have also been used as composite probes to detect cross species chromosome homology. Besides humans, flow karyotypes have been generated for mouse (Rabbits et al. 1995), pig (Langford et al. 1993; Yerle et al. 1993); dog (Langford et al. 1996); sheep (Burkin et al. 1997a), cat (Wienberg et al. 1997), and some non-domestic species. Although, compared to humans, very little has been done with the CSLs generated in farm animals (see Chowdhary 1998a), the success in humans shows how animal geneticists can harness this resource.

Chromosome microdissection: An alternative to flow sorting for generating chromosome specific probes is chromosome microdissection. A schematic drawing showing the main steps and procedures of chromosome microdissection is presented in Fig. 1. The technique was already initiated 18 years ago by isolating DNA from Drosophila polytene chromosomes (Scalenghe et al. 1981). In times to come, the method was modified and improved by introduction of PCR (Lüdecke et al. 1989; Senger et al. 1990), use of universal primers (Telenius et al. 1992) and Topoisomerase treatment (Guan et al. 1993). Presently it has developed into one of the most direct means for isolating DNA from any chromosomal region of an organism (Cannizzaro 1996). Further, whole chromosome or even arms, regions or single band ranging from 5-100 Mb can readily be microdissected.

In humans, chromosomal microdissection has found a broad range of applications. Microdissected whole chromosomal or partial probes can be used as paints for i) detection of tiny structural rearrangements undetectable with conventional cytogenetic techniques, ii) isolating marker chromosomes from malignant cells (Johnson et al. 1992), iii) identifying the origin of double minutes (Rajcan-Separovic et al. 1995), iv) detecting translocation breakpoints (Rubtsov et al. 1996), v) prenatal diagnostics (Müller-Navia et al. 1995), etc. (see for review Ried et al. 1998). Further, microdissected DNA has been used for generating libraries from regions deleted or amplified in malignancies (Guan et al. 1992; Guan et al. 1996) and from translocation breakpoints in cancer (Zhang et al. 1995). Such microlibraries have been screened for isolating microsatellites, sequence tagged sites (STSs) and genes (Gingrich et al. 1996; Meltzer et al. 1997; Yu et al. 1997). Lastly, regional microlibraries have also been used for screening genomic libraries (cosmid, BAC or YAC) and cDNA libraries to isolate clones

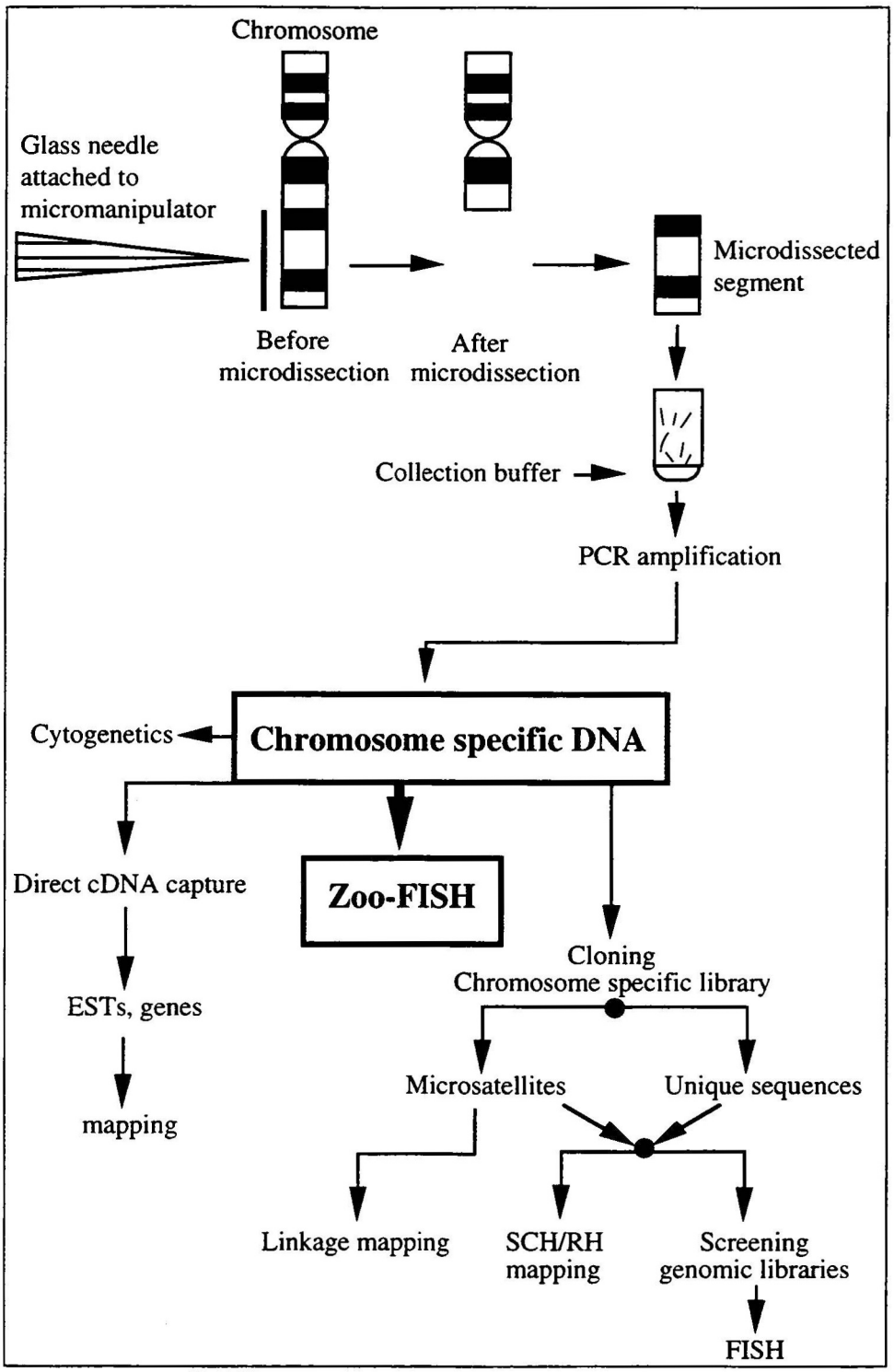

Figure 1. Schematic representation of chromosome microdissection showing possible applications in genome analysis.

specific to the region of interest (Karakawa et al. 1993; Chen-Liu et al. 1995; Choi et al. 1996; Gracia et al. 1997).

Contrary to this, chromosome microdissection has, as yet, found limited use in domestic animal gene mapping. There are only a few reports about microcloning and marker development from defined chromosomal regions in cattle (Schmutz et al. 1994; Goldammer et al. 1996; Weikard et al. 1997), pigs (Ambady et al. 1997; Chaudhary et al. 1998), horse (Bowling et al. 1998; Chowdhary et al. 1998) and chicken (Shaw et al. 1996; Zimmer et al. 1997). However, during the recent past, this technique has found some use in detecting cross species chromosomal homology, and has partially helped in resolving questions about comparative genome organization (discussed later).

Comparative chromosome painting - Zoo-FISH: Mapping of individual genes for comparative purposes is a time consuming endeavour which gives only patchy information on chromosome homology between species. Cross species conservation of synteny/linkage between genes is generally taken as a reflection of homology for segments lying between these genes. However, this extrapolation of information may or may not be true and might therefore need validation. Comparative chromosome painting, also referred to as Zoo-FISH, helps in overcoming this problem and fairly precisely delineates homologous chromosomal regions between species (see Chowdhary 1998b). As evident from the term, this is a FISH-based approach, where whole or partial chromosome specific paints from one species are used as probes on metaphase chromosomes of another species (Fig. 2 b). Depending on the evolutionary distance between the species involved, Zoo-FISH can be divided into two major categories:

1. *Painting between closely related species belonging to the same mammalian order or family:* Initial success with cross species chromosome painting was first reported between human and great apes (Wienberg et al. 1990; Jauch et al. 1992). Presently, *all* human chromosome specific paints have been applied to metaphase chromosomes of 10 different primate species (see Table 1. for references). As a step further in refining some of these homologies, chromosome specific paints from gibbon and two lemur species were painted back to human metaphase chromosomes (Arnold et al. 1996; Müller et al. 1997) - a process referred to as reversed painting (Fig. 2 c). With the availability of flow sorted or microdissected chromosome specific paints for more species, comparative painting studies have now been carried out also within suids, cervids, rodents, marsupials and bovids (see Table 1 for details). The findings of all these investigations have mainly helped in understanding karyotype evolution within these orders. Further, in some cases, it has also helped in direct transfer of genetic information from the developed/partially-developed maps of one species to those which are less developed.

2. *Comparative chromosome painting between distantly related species:* Closely related species share a high degree of sequence homology which makes the use of chromosome specific paints straightforward within the group. However, extension of this concept to distantly related species was

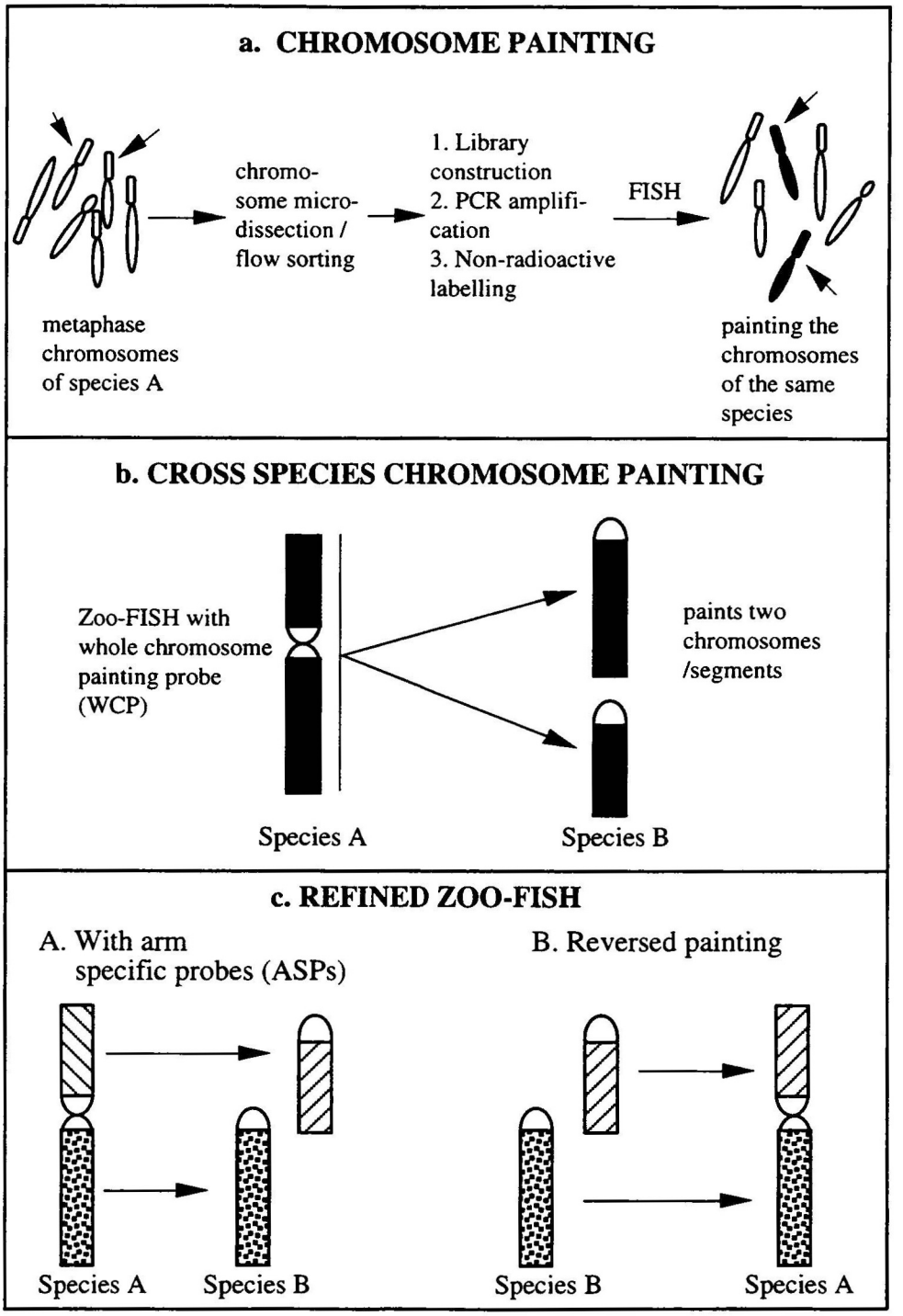

Figure 2. Schematic representation of (a) main principles of chromosome painting, (b) Zoo-FISH with WCP and (c) refined Zoo-FISH using ASPs or reverse painting.

Table 1. Zoo-FISH between closely related species within a mammalian order or family. Arrow indicates "species of origin of the paints→species investigated".

Human→Primates	
Macaque, *Macaca fuscata*	Wienberg et al. 1992
Gibbon, *Concolor gibbon*	Koehler et al. 1995b
Siamang, *Hylobates syndactylus*	Koehler et al. 1995a
Marmoset, *Callithrix jacchus*	Sherlock et al. 1996
Red howler monkey, *Alouatta seniculus sara; A. s. arctoidea*	Consigliere et al. 1996
Capuchin monkey, *Cebus capucinus*	Richard et al. 1996
Black-handed spider monkey, *Ateles geoffroyi*	Morescalchi et al. 1997
Silvered leaf monkey *Presbytis cristata*	Bigoni et al. 1997
Hylobates hoolock	Yu et al. 1997
Eulemur macaco macaco and *E. fulvus mayottensis*	Müller et al. 1997
Gorilla, *Gorilla gorilla*	Stanyon et al. 1992
Six lemur species	Apiou et al. 1996; Vezuli et al. 1997
Bovids	
Cattle→sheep, goat, buffalo (X-chromosome)	Ponce De Leon et al. 1996; Hassanane et al. 1998
Cervids	
Indian muntjac→Chinese muntjac, Gongshan, Brown brocket deer	Yang et al. 1995
Bovids→cervids	
Sheep→Indian muntjac	Burkin et al. 1997b
Suids	
Domestic pig→babirusa	Bosma et al. 1996
Suids→bovids	
Pig→cattle	Schmitz et al. 1998
Equids	
Horse→donkey	paper VI
Rodents	
Akodon cursor → A. montensis	Fagundes et al. 1997
Mouse→rat	Scalzi and Hozier 1998
Marsupials	
Tammar wallaby→Swamp wallaby, *Wallabia bicolor, Macropus eugenii*	Toder et al. 1997

initially considered less likely. This was primarily attributed to sequence divergence between the species, which in turn was expected to affect hybridization efficiency.

However, in 1994, some methodological changes in the FISH protocol were suggested (e.g., increasing probe concentration and hybridization time, changing washing stringency) which, for the first time, made it is possible to hybridize human chromosome specific paints to chromosomes of such

distantly related mammals like mouse, fin whale and Indian muntjak (Scherthan et al. 1994). This also resulted in the coining of the term Zoo-FISH, which has now become synonymous to comparative or cross-species painting. After this success, human CSLs have been extensively used on the chromosomes of a wide range of mammals (15 species belonging to seven orders), among which several are domesticated/farm animals (see Table 2 for details). The results thus in several cases decipher complete homology of the human karyotype with that of the species compared.

In the majority of the Zoo-FISH studies, experiments have been uni-directional, i.e., human chromosome paints were probes while chromosomes of another species were targets. There are two reasons for this: i) human chromosome specific paints have since long been readily

Table 2. An overview of uni-directional Zoo-FISH with ***human*** WCPs across distantly related species.

Species	No of human WCPs	Reference
PERISSODACTYLA		
Horse, *Equus caballus*	all	paper I
Donkey, *E. asinus*	HSA4, 8, 9, 16, 21, 16p/q, 19q	Lear & Bailey,1997a; paper V; Raudsepp & Chowdhary, unpubl.
Hartmann's zebra, *E. zebra hartmannae*	HSA4, 8, 9, 16, 21	Lear & Bailey,1997a
ARTIODACTYLA		
Cattle, *Bos taurus*	all	Hayes 1995; Solinas-Toldo et al. 1995; Chowdhary et al. 1996
Indian muntjac, *Muntiacus muntjak vaginalis*	all	Frönicke & Scherthan 1997; Yang et al. 1997
Pig, *Sus scrofa*	all	Rettenberger et al. 1995a; Frönicke et al. 1996
Sheep, *Ovis aries*	HSA2, 12, 17, X	Chowdhary et al. 1996
CARNIVORA	all	Rettenberger et al. 1995b
Cat, *Felis catus*		
Harbor seal, *Phoca vitulina*	all	Frönicke et al. 1997
American mink, *Mustela vison*	all	Hameister et al. 1997
CETACEA		
Fin whale, *Balaenoptera physalus*	HSA1, 16, 17, X	Scherthan et al. 1994
Atlantic bottlenose dolphin, *Tursiops truncatus*	all	Bielec et al. 1998
INSECTIVORA		
Common shrew, *Sorex araneus*	all	Dixkens et al. 1998
LAGOMORPHA		
Rabbit, *Oryctolagus cuniculus*	all	Korstanje et al. 1998
RODENTIA		
Mouse, *Mus musculus*	HSA1, 16, 17, X	Scherthan et al. 1994

available (commercially or as kind gifts from colleagues involved in creating them) and ii) because human is the best mapped mammal.

In addition to uni-directional painting, in some cases (pig and cat), reciprocal or reverse painting results are also available (Goureau et al. 1996; Milan et al. 1996; Wienberg et al. 1997). These findings provide a refined overview on segmental homologies between the chromosomes of the compared species. Refinement in homology can also be attained by using microdissected chromosomal arm and band specific paints (Fig. 2 c), or even large insert BAC/PAC/YAC clones across species. Success in mapping human Mega-YAC probe in cat (Wienberg and Stanyon 1995), three YAC clones from HSA2 on common shrew (Dixkens et al. 1998) and pooled human PAC clones on pig chromosomes (Frengen et al. 1997) encourages the use of this type of Zoo-FISH in mapping single copy genes.

Since the inception, Zoo-FISH has emerged as an important tool to detect comparative homology at the chromosomal level. It is considered that over 90% of the Zoo-FISH data hitherto available is in agreement with the gene mapping results (see Chowdhary 1998b). However, it also needs to be stressed that there are certain limitations of Zoo-FISH. One of these is the failure to detect small cross hybridizing segments on the chromosomes. The limit of signal detection through Zoo-FISH is suggested as 5 Mb or more (Scherthan et al. 1994), i.e., any target sequence smaller than this would be difficult to detect. Rapid expansion of gene maps in farm animals has exposed this drawback. Secondly, though Zoo-FISH shows gross chromosomal homology between species, it gives no information on intrachromosomal evolutionary rearrangements. This is best exemplified in farm animals by analysing Zoo-FISH and comparative gene mapping data between HSA3-SSC13 (Sjöberg et al. 1997b), HSA4-SSC8 (Johansson et al. 1995) and HSA17-BTA19 (Yang and Womack 1998; Yang et al. 1998). Very recent whole genome comparison between humans and ruminants has further highlighted this fact (Schibler et al. 1998).

There are several advantages of Zoo-FISH. First, the gross homology between defined chromosomal segments of two species is useful in transferring genetic information from "map rich" genomes to "map poor" genomes. This enables rapid development of gene maps in species which have fewer than needed markers on their maps. The comparative information also helps in the development of gene maps in targeted regions of a less mapped genome. The latter becomes easier if refined information on the comparative status of the segments involved in the two species is available. Such information facilitates search for candidate genes in, for example, homologous regions of the human genome, if map location of the comparable condition is known in pig or cattle. Thus Zoo-FISH maps can act as a "reference" for comparative data. Lastly, the interspecies chromosomal correspondence provided by Zoo-FISH in different mammalian species gives an insight into how these genomes are organized in relation to each other. This in turn significantly contributes in predicting the likely karyotype of their immediate ancestor and gives some clues about the probable constitution of the ancestral mammalian karyotype (paper IV, this thesis). In other words Zoo-FISH gives us an opportunity to take a look into the "black hole" of genome evolution.

3. Genome analysis in the horse

Selection in horses (*Equus caballus*, ECA) for strength, size, speed, gait, colour and conformation has been carried out ever since their domestication over 6,000 years ago (Bailey 1998). Stud books for several horse breeds of the present era go back to the late 1600s and are probably the oldest recorded pedigree for any animal population (Marti and Binns 1998; Bailey 1998). The long standing breeding interest coupled with high commercial value should have triggered genome analysis in horse, just like in other farm animal species. However, as compared to cattle, pig, sheep, goat and chicken, organized efforts to develop a gene map in the horse began only 3-4 years ago (see Marti and Binns 1998).

One of the explanations to this late start could be that horses are primarily luxury and recreation objects, and are not looked upon as basic production animals. Further, because of relatively late sexual maturity, long gestation period, seasonal breeding and large body size (Bowling 1996) they do not fulfill the ideals of a classical organism for genetic studies. Nevertheless, convincing arguments over the past few years have generated a general consensus for the need to construct a gene map for this hitherto ignored species.

3.1 Main objectives of equine gene mapping

Basic genetic linkage and physical maps are necessary to study the underlying genetics of numerous congenital disorders known in horses and find means to control them. It has also been realized that information coming out from the maps can be useful in addressing questions related to enhancement of performance traits (Bailey 1998). Further, mapping genes associated with phenotypic traits of interest like the coat colour, is of equal importance because they serve commercial interests. A skeletal horse gene map is also essential for comparative purposes.

3.2 Present status of the equine gene map

The equine karyotype comprises 31 pairs of autosomes, and the X and Y chromosomes. Thus the diploid chromosome number in horse is 2n=64. Of the autosomes, 13 pairs are metacentric/submetacentric and 18 pairs acrocentric. Recently, a report of the Third International Committee for the Standardization of the Domestic Horse Karyotype was published (ISCNH 1997) where an improved standard karyotype of G- and R-banded chromosomes is presented along with schematic drawings and nomenclature. The standard is recommended for use by all groups involved in equine cytogenetics, and gene mapping. This would permit a consensus for physical location of markers and will also allow accurate description of chromosomal breakpoints and rearrangements.

3.2.1 Genetic linkage map

Like in several other species, linkage mapping in horses started with the X-chromosome (Trujillo et al. 1965; Mathai et al. 1966). The first equine autosomal linkage group (*EAK-PGD*) was detected almost 20 years ago by Sandberg (1974). Since then, linkage mapping in horses developed at a remarkably slow speed though some significant contributions (Andersson and Sandberg 1984; Sandberg and Andersson 1984) were made during early '80s. Until 1995 five autosomal linkage groups were established while only three of them were chromosomally assigned (see Chowdhary and Gustavsson 1992). Recently, an International Horse Reference Family Panel comprising 12 families based on 12 stallions and 448 halfsib offspring, was established (see Guérin et al. 1998). Additionally, a Swedish panel involving eight half-sib families with 263 offspring was reported (Lindgren et al. 1998). As a result, there are now over 200 linked markers assigned to 28 equine autosomes and the X chromosome. Further, a new resource (full-sib family) with 5 grandparents, 5 parents and 41 progeny was recently generated (Swinburne et al. 1998). Until now, linkage groups were numbered according to the order of their establishment. However, it is probably the right time to rename the groups according to chromosome number.

In spite of these developments within a short span of time, the equine genetic map needs more markers for efficient search of genes affecting traits of interest. A look at the distribution of markers on different chromosomes shows that they are not evenly spaced along the whole genome. Some chromosomes, e.g., ECA8, 27, 28 and 31 have no genetically mapped loci at all, while others like ECA3 has over 10 linked markers. Of the different markers, only 30 are coding sequences. These are spread on ten chromosomes (Sandberg and Andersson 1993; Godard et al. 1998; Lindgren et al. 1998). Comparative linkage analysis between horse and other species is limited because there are very few equine linkage groups comprising two or more Type I loci.

3 2.2 SCH syntenic map

The first syntenic group in horse can be traced back to indirect analysis carried out in a mule x mouse hybrid cell panel (Deys 1972). Three genes (*G6PD, HGPRT* and *PGK*) were then assigned to the X chromosome. However, the first direct synteny study was based on biochemical detection methods using a SCH panel obtained from horse x mouse heterohybridoma cells (Williams et al. 1993). The analysis resulted in identification of three syntenic groups with eight enzyme genes. Later three more mouse x horse SCH panels were constructed (Bailey et al. 1995; Raney et al. 1998; Shiue et al. 1998). These panels have hitherto not been characterized for the equine chromosomes/segments they contain. However, attempts have been made to see numerically how many whole chromosomes or parts are present in individual clones of some of the panels (Lear et al. 1992; Bailey et al. 1995; Shiue et al. 1998). The authors also report about the preferential loss of large

submetacentric equine chromosomes from the hybrids. This makes the panels unsuitable for excluding syntenic groups.

Using the panel described by Lear et al. (1992), six syntenic groups including two genes and fifteen microsatellites were established after PCR analysis (Bailey et al. 1995). At that time, none of the syntenic groups were assigned to specific chromosomes. Recently, 33 syntenic groups with 182 microsatellites and 58 random amplified polymorphic DNA (RAPD) markers were synteny mapped (Shiue et al. 1998). Based on FISH mapping data for some of the markers, 23 syntenic groups were chromosomally located. Further, using a trisomic individual, two microsatellite markers were assigned to ECA30 and it was also possible to reveal the maternal origin of the extra chromosome (Bowling et al. 1997). Thus the overall status of the syntenic map in horse looks encouraging.

Despite significant progress made in generating a syntenic map in horse, the data is insufficient for comparative analysis with other species because the number of mapped Type I markers is very low. Although attempts are being made to map specific genes within the available panels, alternative approaches like the use of CATS primers is also in progress (Lyons et al. 1997). Preliminary results showed that of the 52 CATS primer sets used, 34 gave a single PCR product making them potentially useful for mapping in the panel. Recently, eight CATS primer pairs (from HSA5) were mapped into two equine syntenic groups, which were later indirectly assigned to ECA14 and 21 (Caetano et al. 1998). The latter are known to be HSA5 homologues (papers I and III from this thesis).

3.2.3 Cytogenetic map

Direct physical assignment of loci to horse chromosomes by *in situ* hybridization started a decade ago using the radioactive approach. Due to lack of horse specific probes, human and pig genomic or cDNA clones were used. The first assignments were for the equine major histocompatibility complex (*ELA*) to ECA20q14-q22 (Ansari et al. 1988; Mäkinen et al. 1989) and glucosephosphate isomerase (*GPI*) to ECA10pter (Harbitz et al. 1990). During the initial 5 years, not more than 6-8 genes were ISH mapped in horse. Later, when the trend shifted to the application of the FISH technique, Oakenfull et al. (1993) mapped hemoglobin alpha (*HBA*) to ECA13q. From here onwards, and with the beginning of international collaboration for developing a gene map in horse, the number of ISH (rather FISH) mapped loci has soared.

During recent years, significant progress has been made to construct equine genomic libraries and to isolate genes and microsatellites from large insert clones ranging from phage and cosmid to BACs (Breen et al. 1997; Godard et al. 1997; Godard et al. 1998). Recently, 41 sequence tagged sites (STSs) were isolated from a cosmid library and FISH mapped to equine chromosomes (Hirota et al. 1997). By now 83 DNA markers and 34 genes (Table 3) have been localized to equine chromosomes using the *in situ* techniques.

Table 3. Genes mapped by *in situ* hybridization in the horse (human mapping data are retrieved from GDB).

Gene symbol	Gene name	Location	Human homology	Reference & method
ALB	albumin	3q14.3	4q11-q13	Godard et al. 1998 (F)
ASIP	agouti (mouse)-signaling protein	22q15-16	20q11.2-q12	Godard et al. 1998 (F)
BLG1	beta lactoglobulin 1	28q18-qter		Lear et al. 1998d (F)
BLG2	beta lactoglobulin 2	28q18-qter		Lear et al. 1998d (F)
C3	complement component 3	7pter	19p13.3	Millon et al. 1993 (F)
CAD	carbamoylphosphate synthetase 2, asparate transcarbamylase, and dihydroorotase	15q25	2p22-p21	Godard et al. 1998 (F)
COL9A1	collagen, type IX, alpha 1	20q24	6q12-q14	Godard et al. 1998 (F)
ELA	major histocompatibility complex	20q14-q22	6p21.3	Ansari et al. 1988 (R); Mäkinen et al. 1989 (R)
ESR	estrogen receptor	31q15-q17	6q25.1	Lear et al. 1998a (F)
ETS2	v-ets avian erythroblastosis virus E2 oncogene homolog	26q17	21q22.3	Lear et al. 1998b (F)
F13A	coagulation factor XIII, A1 polypeptide	20q13	6p25.1-p24.3	Godard et al. 1998 (F)
F18	?	Xq29	Xq28	Tozaki et al. 1998 (F)
GOT2	glutamic-oxaloacetic transaminase 2	3p15	16q13 16q21	Lear et al. 1998c (F)
GPI	glycose phosphate isomerase	10pter	19q13.1	Harbitz et al. 1990 (R)
HBA	alpha-globin gene complex	13qter	16p13.3	Oakenfull et al. 1993 (F)
IGF2	insulin-like growth factor II	12q14	11p15	Paper II (F)
KIT	tyrosine kinase transmembrane receptor for mast/stem cell growth factor	3q21	4q12	Lear et al. 1998c (F); paper V (F)
LCT	lactase	15q21	2q21	Godard et al. 1998 (F)
LTF3	lactotransferrin	16q23	3q21-q23; 3p21.3-p21.2	Lear et al. 1998d (F)
MC1R	melanocortin 1 receptor	3p12	16q24.3	Paper V (F)
ME1	malic enzyme 1, soluble	10q12-q13	6q12	Godard et al. 1998 (F)
MPI	mannose phosphate isomerase	1	15q22-qter	Godard et al. 1998 (F)
MX1	myxovirus (influenza) resistance 1	26q17	21q22.3	Lear et al. 1998b (F)

NP	nucleoside phosphorylase	1q26-q27	14q11.2 14q13.1	Godard et al. 1998 (F)
ODC1	ornithine decarboxylase 1	15q27	2p25	Godard et al. 1998 (F)
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	3q21	4q11-q12	Paper V
PGD	6-phosphogluconate dehydrogenase	2p	1p36.3-p36.13	Gu et al. 1992 (R)
PGR	progesterone receptor	7p16-p15	11q22.1-q22.3	Lear et al. 1998a (F)
PI (AAT)	protease inhibitor 1 (anti-elastase, alpha-1-antitrypsin)	24q15-q16	14q32.1	Godard et al. 1998 (F); Lear et al. 1998d (F)
PIM1	pim-1 oncogene	20q24	6p21.2	Godard et al. 1998 (F)
PRKDC, DNA-PK	DNA protein kinase catalytic subunit, candidate gene for CID	9p12	8q11	Bailey et al. 1997 (F)
RNR	ribosomal RNA (rDNA)	1pter 27cen 28cen 31cen		Deryusheva et al. 1997 (F)
RYR1 (CRC)	ryanodine receptor 1 (calcium release channel gene)	10p15; 10pter	19q13.1	Chowdhary et al. 1992 (R); Godard et al. 1998 (F)
TF4	transferrin	16q23	3q21	Lear et al. 1998d (F)

(F) - fluorescent in situ hybridization
(R) - radioactive in situ hybridization

In situ hybridization, like in other species, has enabled to anchor linkage and synteny groups to specific horse chromosomes. Precise physical localization of markers has on the other hand been instrumental in integrating genetic linkage and physical maps (Breen et al. 1997; Godard et al. 1997). ISH based physical order and relative distances between loci has allowed to draw comparisons between linkage and FISH mapping data (paper V, this thesis). A summary of all genes mapped in horse, irrespective of the technique used, is presented in Fig. 3.

3.3 Karyotype evolution in the equids

The family Equidae consists of one genus (Equus) with twelve extant species - two horses, seven asses and three zebras (see Bowling 1996). Analysis of abundant fossil records date the divergence of these species from a common ancestor about 3-5 Myrs ago (Lindsay et al. 1980). Recent comparison of horse and donkey mtDNA sequences predicts the evolutionary distance between the two species to be 8-10 Myrs ago (Xu et al. 1996). How this distance compares with other equids is not clear. Nevertheless, on an evolutionary time-scale the period is short and it is

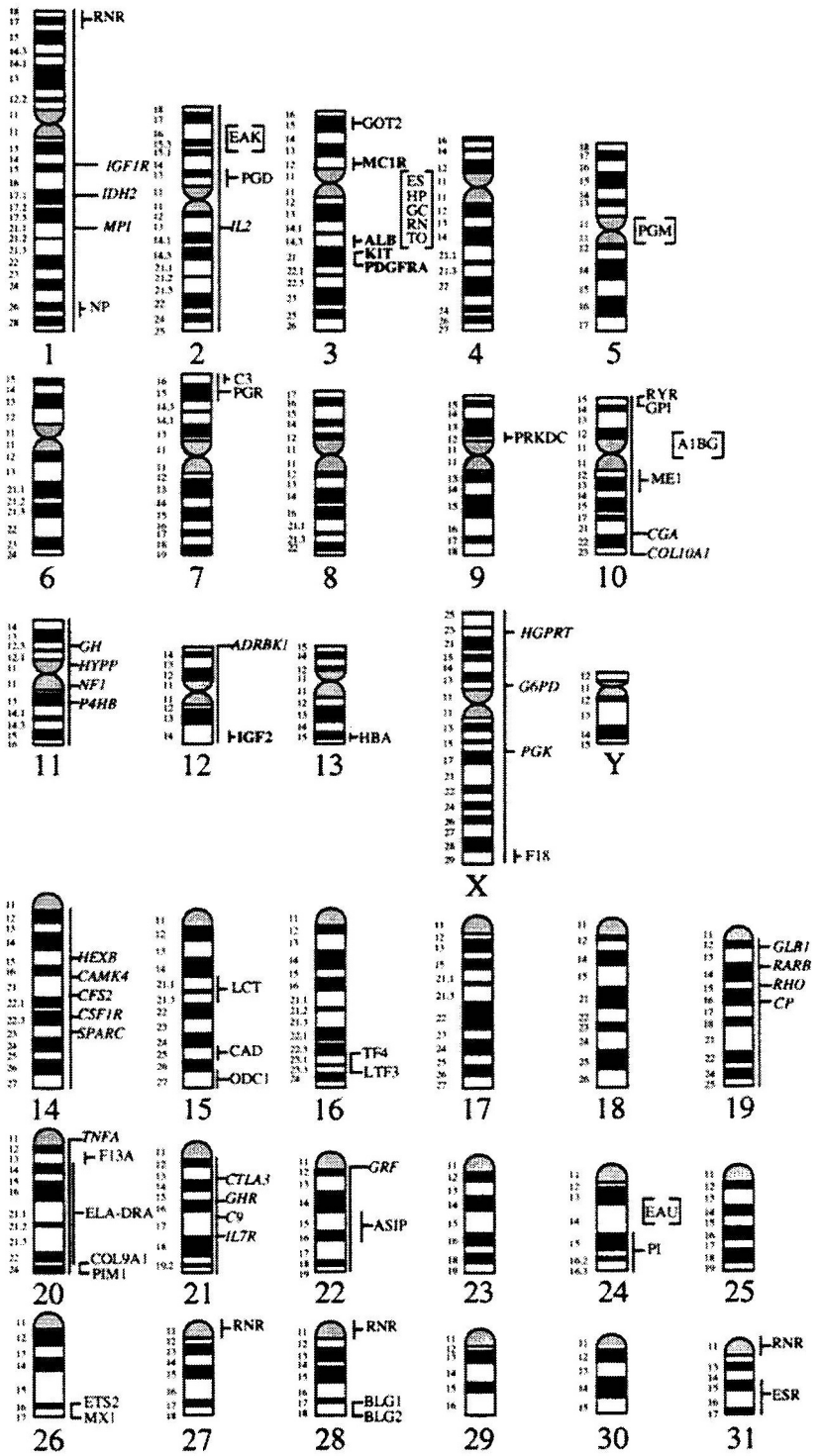

Figure 3. A karyogram of G-banded horse chromosomes showing all the genes hitherto mapped. *In situ* mapped - normal font; synteny mapped - in italics; linkage mapped - in brackets; gene assignments presented in this thesis - bold. Synteny data for ECA11, 14, 19 and 21 were kindly provided by Dr. A.T. Bowling.

expected that the genome organization of such related species should be fairly similar. For example, among bovids (Allard et al. 1992) and majority of the primates (Dutrillaux 1979; see Comparative Genome organization...1996; Wienberg and Stanyon 1997), the comparative karyotype structure is fairly preserved, though species within each of the groups diverged from their common ancestor about 16-17 and 5-15 Myrs ago, respectively (Allard et al. 1992; Arnason et al. 1996b).

Support to the expectation that the karyotypes of equids should not differ much from each other comes from the fact that all equids can freely interbreed and give viable, though usually infertile, offspring (Allen and Short 1997). However, chromosome number between equids varies considerably. It fluctuates in a broad range, from 2n=32 in Hartmann's mountain zebra to 2n=66 in the Przewalski horse (see Bowling 1996). Cytogenetic studies have shown significant morphological differences in the chromosomes of different equid species (Ryder et al. 1978; Power 1984). However, there is a reasonable number of chromosomes in some equids which do show banding homology. The paradox between short evolutionary distance and surviving healthy hybrids on the one hand, and extensive chromosomal rearrangements on the other, makes this mammalian group interesting for comparative genomics/cytogenetics. Until now horse is the only equid with the prospects of having a reasonably developed gene map in the future. Hence possibilities of drawing conclusions on comparative organization of different equid genomes seems difficult - at least for the moment. The present thesis will try to address this aspect and suggest possible ways to partly overcome the problem.

Aims of this thesis

The objectives of this study were to:

- compare horse and human karyotypes using Zoo-FISH
- refine some of the above results using arm specific paints from microdissected human chromosomes 2, 5, 6, 16 and 19
- carry out "heterologous FISH" for mapping new loci to equine and asine chromosomes
- generate whole chromosome painting probes for all meta- and submeta-centric horse autosomes (ECA1-13), and X and Y chromosomes using microdissection
- compare the donkey genome primarily with the horse and partly with the human genomes using different physical mapping approaches
- develop an overview of the mammalian genome organization using available Zoo-FISH and gene mapping data

An overview of the salient findings of the thesis

1. Zoo-FISH analysis between human and horse (papers I, II, III)

Whole chromosome paints (WCPs) for individual human chromosomes (22 autosomes and sex chromosomes) were used separately on horse metaphase chromosomes in a Zoo-FISH experiment. All human probes, except the Y, hybridized to one or more chromosomes in the horse. Altogether 43 conserved segments were detected. This is the first study which delineated gross chromosomal homology between the human and horse karyotypes. Immediately thereafter, two more studies reported use of HSA3, 4, 14 and 16, paints on horse chromosomes (Rettenberger et al. 1996; Lear and Bailey 1997b). The findings of these studies are in full accordance with those reported by us.

The Zoo-FISH results showed for seven chromosomes of the two species one-to-one homology (HSA7/ECA4, HSA8/ECA9, HSA13/ECA17, HSA17/ECA11, HSA20/ECA22, HSA21/ECA28 and HSAX/ECAX). For other chromosomes, variable degree of synteny conservation ranging from one human chromosome corresponding to 1-4 segments on different equine chromosomes was observed. However, the majority of human chromosomes were homologous to whole chromosome arms or large segments in the horse karyotype (Fig. 1, paper I). This reflects a fairly high degree of synteny conservation between the two genomes.

Some equine chromosomes/regions viz., ECA6p, 12, 13p, 27 and 31, however, did not hybridize with any human WCP. This was attributed to a possible variation in the representation of coding sequences between the human chromosome specific libraries - a phenomenon which can occur while libraries are grown. It is thus very likely that the DNA obtained from some of the library cultures were under-represented for conserved coding sequences. Due to this, the hybridization signals produced by them were probably too weak or insufficient for detection. A good example of this can be observed from the fact that in paper I the HSA11 WCP painted only ECA7. However, HSA11 probe from a different source (microdissected) used in paper II, painted ECA7 and ECA12. Thus it seems that the library in the latter case had a better representation of HSA11 than that in the former.

Comparison of human-horse Zoo-FISH results with the genes hitherto mapped in both species shows that, in most cases, the two data sets agree very closely with each other. However, as is evident in other species, it is expected that with the expansion of the equine gene map some discordance with the Zoo-FISH data will surface. The reasons for this have been pointed out earlier while discussing limitations of Zoo-FISH (see Introduction). Until now, ECA26 and 28 are the two horse chromosomes where Zoo-FISH results are not in accordance with gene mapping data. In paper I homology is shown between ECA26-HSA12/22 and ECA28-HSA21. However, recently two HSA21 genes (*ETS2* and *MX1*) were FISH mapped to ECA26 (Lear et al. 1998b). The likely explanation for this discrepancy could be similar size and G-banding patterns of the two equine chromosomes, due to which the chromosomes might have been incorrectly identified either by us during

Zoo-FISH, or by Lear et al. (1998b) during the FISH localizations. Of course, there is also a very slight possibility that ECA26 shares a small homologous region with HSA21. However, the former explanation appears more reasonable. Mapping of more genes to the two equine chromosome will conclusively solve this disparity.

In Zoo-FISH, when a WCP from one species paints two or more chromosomes/segments in a distantly related species, it is difficult to ascertain precise homology of the painted segments in relation to the probe chromosome. For example, if a human metacentric chromosome paints two whole chromosomes or segments in the horse, it will be difficult to predict which of the two equine chromosomes/segments correspond to the short or the long arm of the human chromosome. In such instances, preliminary refinement of conserved syntenies can be derived through available comparative gene mapping data.

Other alternatives for refinement would be either *reverse painting* whereby horse individual chromosome paints could be used as probes on human metaphase chromosomes, or Zoo-FISH with *sub-chromosomal* probes. The latter approach was used in paper III where some human chromosome arm specific paints (ASPs) were generated via microdissection, and applied to horse and pig (*Sus scrofa*, SSC) chromosomes. The two species were chosen to widen the scope of analysis, specially considering that horse, on the one hand had very few mapped loci, while the pig, on the other hand, had enough information to countercheck the results.

ASPs from HSA2, 5, 6, 16 and 19 were chosen because accumulated comparative data shows that each of them correspond to two homologous segments in a number of mammalian species (paper IV, Fig. 1). HSA2 paints two segments in pig. In horses, the human chromosome shows homology with two entire chromosomes (ECA15 and ECA18) and a small weakly hybridizing block on ECA1q. Our arm painting results showed that individual arms of HSA2 are not conserved as separate segments both in horse and pig. In each of the species, one of the homologous blocks corresponded to the complete short arm and a contiguous small part of the long arm of the human chromosome (HSA2p + proximal part of 2q). However, the other equine and porcine blocks showed homology only with the remaining part of the long arm (HSA2q). Very recent gene assignments in horses (Godard et al. 1998) and the available mapping data from pigs (PigBase) confirm this observation. No homology with either human arms was seen for the weak hybridizing segment on ECA1q (paper I). The latter needs further verification with the help of new gene mapping data.

The findings, together with the observations in cattle and primates, support the concept that evolutionary synteny disruption of HSA2 is on band q13 (Avarello et al. 1992; Ijdo et al. 1992; Wienberg et al. 1994). However, very recent assignment of lactase (*LCT*; HSA2q21), carbamoylphosphate synthetase (*CAD* ; HSA2p) and ornithine decarboxylase 1 (*ODC1*; HSA2p) to ECA15 (Godard et al. 1998) indicates that, at least in horses, the HSA2 synteny is either disrupted at another site, or the two proposed ancestral chromosomes have followed a slightly different evolutionary path. Additional gene mapping data on ECA15 can provide a solution to this problem.

Similar to HSA2, the HSA5 arm painting results clearly indicated that the two arms are not conserved as individual blocks in the equine and porcine genomes (Fig. 2 d, paper III). Available gene mapping data help in suggesting that HSA5 synteny is disrupted at q13. Contrary to these observations, Zoo-FISH with ASPs from HSA6, 16 and 19 on horse and pig metaphase spreads was in agreement with our hypothesis that individual arms of the human chromosomes are conserved as separate blocks (Fig. 2 b, c, paper III). Comparative gene mapping data in horse and pig support this view. Recently HSA16 and 19 ASPs were also used on donkey and cat chromosomes (T. Raudsepp and B.P. Chowdhary, unpublished) with similar observations.

When human-horse Zoo-FISH results (paper I) were first reported, very little was known with regards to horse vs. other mammalian genomes. The findings thus provided a first hand insight into comparative organization of horse and human genomes but also indirectly helped to relate the horse genome to other mammalian genomes. The results are of significance in horse because, in the absence of a proper gene map, the comparative information has served as a framework to extrapolate information from the developed gene maps. Further, the findings have also acted as a reference point to compare all new gene assignments in horse.

The Zoo-FISH observations have also contributed in assigning linkage or syntenic groups to specific horse chromosomes. In paper I, we predicted that equine linkage group 2 (LG2) is most likely located on ECA3. Recent gene mapping data (Lear et al. 1998c; Godard et al. 1998; paper V) strongly support our prediction. Similarly, we also proposed that an equine syntenic group (*NP, MPI, IDH2*; Williams et al. 1993) maps to ECA1q. Recent FISH mapping of *NP* locus to ECA1q26-q27 (Godard et al. 1998) confirm our observations. Presently, only one linkage group containing Type I markers (*APOA1* and *APOA4*) is not anchored to a specific horse chromosome (Kakoi and Gawahara 1997). Because both these genes are mapped to HSA11, it is expected that the linkage group is located either on ECA7 or ECA12 (see Fig. 1, paper I; paper II).

The arm specific painting results, the first of it's kind in farm animals, have successfully refined previously known homologies between the human and horse (+pig) chromosomes. Though the findings cover a chosen set of human chromosomes for comparison (chosen specifically to check whether these chromosomes each represent two ancestral segments), they provide interesting pieces of information both supporting and modifying our hypothesis about synteny conservation of the studied human arms.

2. Comparative mapping in horse and donkey using homologous and heterologous FISH (papers II, V)

Described in papers II and V are studies where four genes were localized to specific chromosomal bands in horse and five in donkey. Insulin-like growth factor II (*IGF2*), melanocortin 1 receptor (*MC1R*), mast/stem cell growth factor receptor (*KIT*) and platelet-derived growth factor receptor α (*PDGFRA*) were mapped in both species. An additional gene – albumin

(*ALB*) - was also chromosomally assigned in donkey to compare it's location with that in horse. Further, two equine microsatellite-containing cosmid clones (*SGCV18* and *SGCV33*) were also FISH mapped in donkey. A summary of the FISH results obtained in the two species is presented in Table 4 below.

Of these, *IGF2* (paper II) was the only equine probe used on horse chromosomes. For all other localizations, heterologous probes were used. Application of equine probes on donkey chromosomes can also be considered as "homologous FISH" because most probes give distinct hybridization signals across closely related species (e.g., bovids: cattle, sheep, goat and buffalos) (Hayes et al. 1996; Prakash et al. 1997; Xiao et al. 1998).

Porcine genomic clones were used for the localization of *MC1R*, *KIT* and *PDGFRA* genes. Pig is considered to have diverged over 65 Myrs ago from the horse and donkey (Graur and Higgins 1994). Except for the use of a human Mega-YAC clone on cat (Weinberg and Stanyon, 1997) and three HSA2 YAC clones on common shrew chromosomes (Dixkens et al. 1998), there is no published data showing application of individual large sized genomic clones for FISH across distantly related species (a method termed as *heterologous FISH*). Paper V shows that this was successfully carried out. Earlier, pooled rather than individual human PAC clones were used on pig chromosomes to map the *LCAT* gene on SSC6 (Frengen et al. 1997), and three bovine X chromosome specific cosmid clones on reindeer chromosomes (Prakash et al. 1996). The latter two species belong to the same order.

Subsequent to the FISH localization of *IGF2* in horse and donkey, the gene has been physically mapped in three more species: kangaroo (Toder et al. 1996), mouse (Beechey et al. 1997) and pig (A. Törnsten, pers. communication). In all these species the gene maps on the terminal part of the chromosome. Thus there are now eight species where the gene is *in situ* mapped and interestingly maintains a similar distal position. The observations support our earlier proposition that the location represents the ancestral condition (Fig. 4, paper II). Interestingly, Human *IGF2* is situated on HSA11p15.5, which harbours several disease loci and a cluster of imprinted genes (Junien and van Heyningen 1991; Feil et al. 1994; Higgins et al. 1994; Banerjee and Smallwood 1995). In this context, the physical localization of this gene in horse and donkey, and detected homology between HSA11, ECA12 and EAS17 (Zoo-FISH based – discussed in part 1 and 3 of this section) could be of importance in studying similar phenomena in equids.

Mapping of *MC1R*, *KIT* and *PDGFRA* to horse chromosome 3 is of significance because i) the results confirm an earlier preliminary assignment of equine LG2 to ECA3 (paper I; Lear and Bailey 1997b; Lear et al. 1998c) and ii) the former two loci determine the inheritance of some coat colour patterns in horse. These gene assignments make LG2 the best studied

linkage group in horse, thus increasing it's utility for cross species comparisons. The alignment of the physical and genetic linkage maps involving these loci shows that the physical distance between *ALB-MC1R* and *MC1R-GOT2* is almost the same, but the genetic distances differ by more than four-fold (Andersson and Sandberg 1982; Andersson et al. 1983a). This disparity is most likely attributed to the presence of the centromere between *ALB* and *MC1R*, which is known to be a "crossover-suppressant" (Johansson et al. 1994).

This study shows that the short (p) and the long (q) arms of horse chromosome 3 are homologous to donkey chromosomes EAS28 and EAS3q, respectively (see Fig. 2, paper V). When these localizations are compared with the available human mapping data, it emerges that ECA3q and EAS3q correspond to HSA4q, while ECA3p and EAS28 to HSA16q. The observations for HSA16q are in complete agreement with the arm painting results in horse and donkey (Paper III; T. Raudsepp and B.P. Chowdhary, unpublished). The findings will be further discussed together with comparative painting data in donkey (part 3 of this section).

Table 4. Detailed information on all loci FISH mapped in this study.

Gene name	Gene symbol	Probe origin	Location in		
			horse	donkey	human*
Insulin-like growth factor 2	*IGF2*	equine λ phage	12q14**	17qter	11p15
Mast/stem cell growth factor receptor	*KIT*	porcine BAC	3q21	3q #	4q12
Platelet-derived growth factor receptor α	*PDGFRA*	porcine BAC	3q21	3q #	4q11-q12
Albumin	*ALB*	equine BAC	3q14.3***	3q #	4q11-q13
Melanocortin 1 receptor	*MC1R*	porcine BAC	3p12	28qter #	16q24.3
Microsatellite	*SGCV33*	equine cosmid	3p12	28qter #	-
Microsatellite	*SGCV18*	equine cosmid	3p13-p14	28q #	-

* Human gene locations are retrieved from GDB.

** The published location for *IGF2* is ECA12q13 (paper II), because at that time the horse standard karyotype (ISCNH 1997) was not finalized.

*** In horses *ALB* is FISH mapped by Godard et al. (1998).

\# See Fig. 2 in paper V for precise locations.

3. Comparative chromosome painting in donkey (papers V, VI)

Increasing success of Zoo-FISH studies between distantly related mammalian species, encourages the use of this approach also for addressing questions related to comparative karyotype organization within an order or family. The application is most interesting where the rate of chromosome evolution has been rapid and cytogenetic comparisons do not give sufficient information. However, the main limitation of this approach up to now has been that chromosome specific probes are available only for few species.

This problem was overcome in the horse by generating microdissected chromosome specific paints for all equine meta-and submetacentric autosomes, and the X-and Y-chromosomes. After the paints were individually tested on equine metaphase chromosomes for their hybridization efficiency, they were applied in separate experiments on donkey metaphase spreads. Thus, from a total of 15 equine chromosomes, a total of 21 homologous chromosomes/segments were detected in the donkey karyotype (Fig. 2, paper VI). Nine of the equine paints used showed correspondence with only a single donkey chromosome/segment (Fig. 3 A-I, paper VI). Except for ECA4, which painted the long arm of EAS1 (Fig. 3 F, paper VI), each of these equine chromosomes showed one-to-one homology with the asine chromosomes. However, each of the remaining six equine chromosomes showed homology with *two* segments on different chromosomes in the donkey karyotype (Fig. 3 J-O, paper VI).

In order to cytogenetically compare the homologous segments detected between the two species, a G-banded karyotype was prepared in the donkey (see Fig. 4) using metaphase spreads from two female and one male donkey. The chromosomes were arranged according to their centromeric position and size. The arrangement was slightly different from that reported by Ryder et al. (1978), partly because the banding patterns were not optimal in that publication. A new donkey karyotype with distinct banding patterns was therefore needed.

Although eight of the 15 equine chromosome specific paints corresponded to a single chromosome each, only four (ECA1, 9, 12 and 13; see Fig. 3 A-D, paper VI) showed moderate to high degree of banding pattern homology with the respectively painted chromosomes. Among the equine chromosomes which painted two arms/segments on different chromosomes, banding pattern homology was evident only between ECA2p and ECA2q, and the corresponding asine segments (EAS5q and EAS3p, respectively) (see Fig. 3 J, paper VI). In general, about 60% of the segments homologous between the two species did not show clear correspondence in their banding patterns. This comparative painting work has thus for the first time disclosed molecular homology between a large part of the horse and donkey karyotypes. This is significant because other approaches, mainly cytogenetic, were hitherto unable to precisely define homology between their

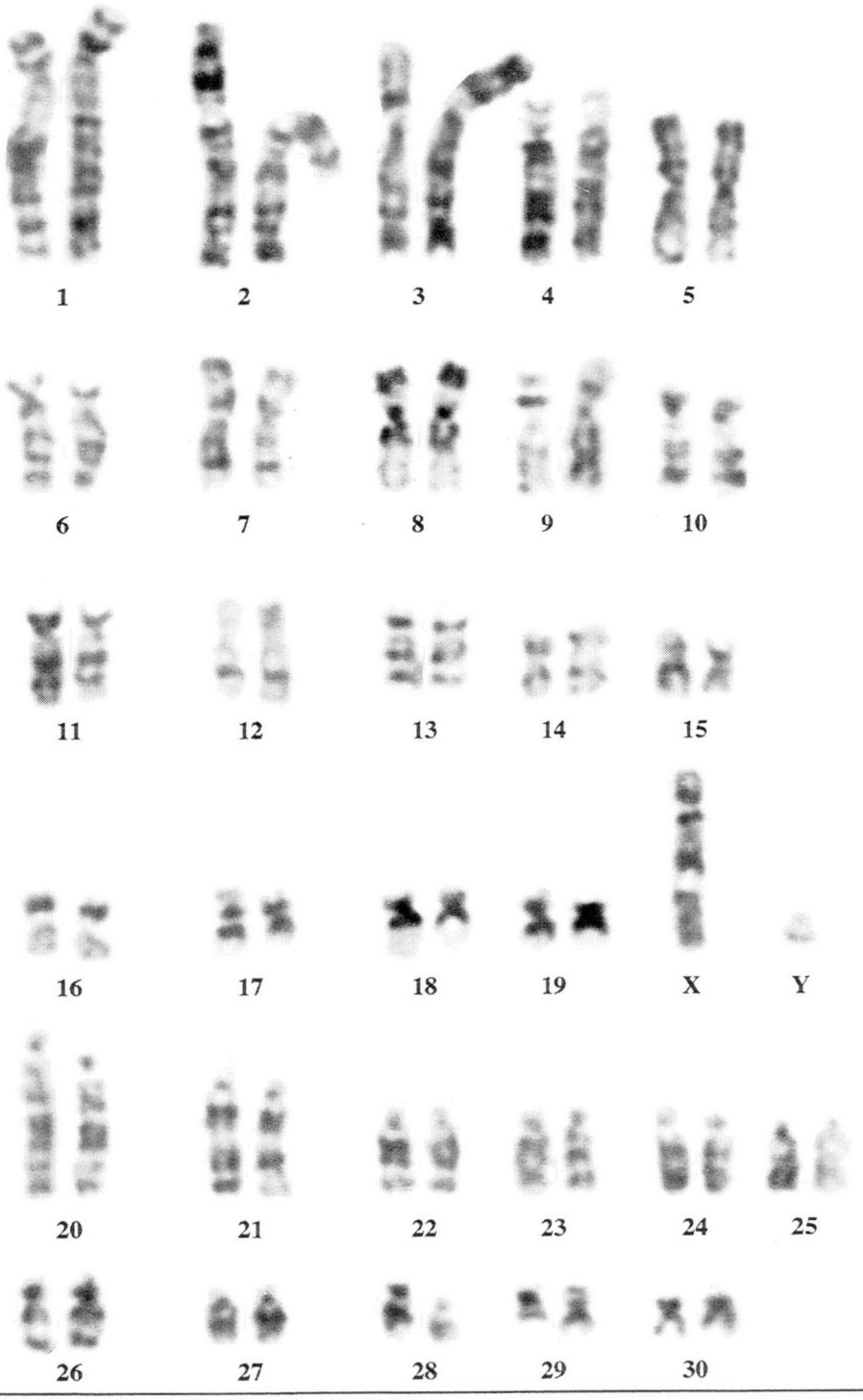

Figure 4. G-banded karyotype of a male donkey (*Equus asinus*).

chromosomes, even though the two species are evolutionarily closely related.

One of the interesting facts which emerged from the horse→donkey comparative painting is that 29 equine chromosomal arms corresponded to 29 asine arms. Although two bi-armed chromosomes ECA4 and ECA7 show homology with a single arm each in donkey (EAS1q and EAS20, respectively; Fig. 3 F and G, paper VI), a balance in the numbers remained because the two arms of ECA5 and ECA6 are homologous to a total of three arms each in the donkey karyotype (see Fig. 3 K and N, paper VI).

Based on earlier defined homology between human and horse karyotypes (paper I), the present use of horse WCPs on donkey chromosomes enables drawing indirect conclusions about homology between the human and donkey chromosomes. Some of these homologies are supported by human→donkey Zoo-FISH results using HSA4, 16p, 16q and 19q specific paints, while some are supported through comparative gene mapping data (see papers II and V; T. Raudsepp and B. P. Chowdhary, unpublished). A general overview of the available correspondence between donkey - horse - human chromosomes is summarized in Table 1, paper VI).

Normally, attempts to use Y-chromosome specific paints among distantly related species has not produced any results (Hayes 1995; Solinas-Toldo et al. 1995; Rettenberger et al. 1995a,b; Chowdhary et al. 1996; Frönicke et al. 1996; paper I). Attempts to use bovine Y-specific microdissected paint on goat, sheep and buffalo did not give satisfactory cross hybridization (B. P. Chowdhary, unpublished), though the probe for *ZFY* gene has been successfully used in other bovids (Xiao et al. 1998). Similar observations have been made for the Y-chromosome among the primates where, in some cases, the human Y-specific paint shows clear hybridization signal (Wienberg et al. 1992; Koehler et al. 1995a, b; Bigoni et al. 1997; Müller et al. 1997), while in others no hybridization (Consigliere et al. 1996; Richard et al. 1996; Sherlock et al. 1996; Morescalchi et al. 1997). In the present study, ECAY clearly painted EASY. However, additional signals on the centromeres and/or telomeres of some asine chromosomes were also observed. The signal was fairly prominent on the heterochromatic region of EASXq, which corresponds to similar region on ECAXq. The results indicate that some of the ECAY specific sequences are common to telomeric/centromeric/intercalary sequences in the donkey.

4. Emerging patterns of comparative genome organization in some mammals as revealed by Zoo-FISH (paper IV)

During recent years, Zoo-FISH studies between humans and a variety of non-primate mammalian species have been conducted (see Table. 2, Introduction). This information, together with constantly increasing comparative gene mapping data, helps to understand how these genomes are organized in relation to each other. The observations are of greater

significance when the species are evolutionarily distantly related because the data enable identification of ancestral genomic segments. Hence, we assimilated our Zoo-FISH results with those published during recent years to find chromosomal segments which are similar in different species. Where possible, the comparative chromosome painting results were integrated with the available gene mapping information. The analysis spans eight species (human, pig, cattle, Indian muntjac, horse, cat, American mink, and harbor seal) representing four mammalian orders (Primates, Artiodactyla, Perissodactyla and Carnivora). In all discussions, the human chromosomes serve as reference point, merely because all the species were probed with human WCPs. Although very limited human→mouse Zoo-FISH results are available (Scherthan et al. 1994), mouse was included for comparison due to it's well developed gene map. Depending on the degree and pattern of chromosome conservation between species, with respect to individual human chromosomes, three distinct classes of conserved syntenies are pointed out:

i) Conservation of whole chromosome synteny: These include chromosomes corresponding to HSA13, 17, 20 and X (Fig. 1 A-C, paper IV). Recent Zoo-FISH results in rabbit (Korstanje et al. 1998), dolphin (Bielec et al. 1998) and common shrew (Dixkens et al. 1998) also demonstrate similar observations. Despite whole chromosome synteny conservation, several comparisons of available gene mapping data between the species, e.g., HSA17 and BTA19 (Yang and Womack 1998; Schibler et al. 1998), suggest extensive intrachromosomal rearrangements during karyotype evolution.

ii) Conservation of chromosomal arms or large segments: This category includes homologues related to HSA2, 4, 5, 6, 9, 11, 16, 19 and 21 (Fig. 1D-K, paper IV). The observation that arms or large parts of these chromosomes are evolutionarily conserved is further reiterated by very recent comparative painting results in rabbit (Korstanje et al. 1998), common shrew (Dixkens et al. 1998) and dolphin (Bielec et al. 1998) using human WCPs. It is interesting to note that although Dixkens et al. (1998) found only one segment corresponding to HSA2 in common shrew, the authors were able to point out very clearly the proposed evolutionary breakpoint and an inversion, as compared to the human chromosome.

Conserved synteny of large chromosomal segments was refined by using arm specific paints for HSA2, 5, 6, 16 and 19 on horse and pig chromosomes (discussed in the section above; paper III). Further refinement also comes from accumulating gene mapping data and reverse painting information in e.g., cat (Wienberg et al. 1997), pig (Goureau et al. 1996; Milan et al. 1996) and from recent pig→cattle Zoo-FISH results (Schmitz et al. 1998).

As compared to the human karyotype, the equids, particularly the horse, do not show any specific variation for the conserved chromosomal arms/segments, as against other mammalian species. However, it needs to

be mentioned that for some chromosomes, for example HSA9, horse does appear to be an exception. While all (or most) studied species show a single chromosome or segment corresponding to this human chromosome, horse shows two complete acrocentric chromosomes as homologues (Fig. 1D, paper IV). Similarly for HSA4, horse is the only species (except the common shrew and bovids) which has two homologous segments (Fig. 1H, paper IV). This result is of even greater significance when the same human chromosome paints only a single chromosome in donkey and Hartmann's zebra (paper V; Lear and Bailey 1997a), suggesting that the proposed fission of the equine homologues to HSA4 occurred only in horse and not in other equids. It would, therefore, be interesting to check more Equidae karyotypes to further understand how the homologue of HSA4 evolved in this mammalian group.

iii) Neighbouring or contiguous segment combinations: Analysis of different Zoo-FISH results show that some genomic regions, which are present as separate chromosomes in human, are syntenic in a wide range of evolutionarily distantly related species. This synteny is not reflected simply as presence of corresponding human segments on the same chromosome, but also their association as contiguous or neighbouring regions - i.e. segments lying next to each other on the chromosome. Such persistent association is a strong indication that each of the syntenies represent an ancestral condition. It is therefore evident that what we see in the human karyotype is a result of a series of fission events in the ancestral karyotype, which separated these combinations into independent units (chromosomes).

In the majority of the species analysed, segments homologous to HSA3/21, HSA14/15, HSA12/22 and HSA16/19 have been found to be syntenic. Even in a fairly rearranged genome of mouse (as compared to other mammalian species) traces of most of these syntenic combinations are present. Novel Zoo-FISH data show that the same synteny combinations are present also in dolphin (Bielec et al. 1998), while only HSA14/15, HSA12/22 and HSA16/19 in rabbit (Korstanje et al. 1998) and HSA3/21, HSA16/19 and HSA14/15 in common shrew (Dixkens et al. 1998). Among the different contiguous combinations, the consistent telomeric/centromeric location of HSA21 homologous segments (Fig. J, paper IV) and the refinement of synteny of HSA16/19 to that concerning only their long arms (Fig. K, paper IV; see also paper III), are worth mentioning.

However, it needs to be pointed out that horse is an exception where two of the neighbouring syntenies (HSA3/21 and HSA16/19) are disrupted (paper I). The former is also disrupted in rabbit (Korstanje et al. 1998), and the latter in donkey (paper VI; T. Raudsepp and B.P. Chowdhary, unpublished).

Overview: Comparative analysis of conserved blocks observed in evolutionarily diverged species gives an idea about the constitution of the ancestral eutherian karyotype (Fig. 2, paper IV). The constitution is

expressed in terms of human homologues. Sequence of events which *probably* led to the formation of the studied karyotypes are depicted. It needs to be emphasized that these observations are merely a *small contribution* to the constant efforts carried out by a large number of laboratories to find out what the mammalian ancestral karyotype looked like. It is expected that with the accumulation of more Zoo-FISH data from other mammalian orders, and with the refinement of available Zoo-FISH maps through reversed painting, arm painting and gene mapping, the view about ancestral genome organization will sharpen.

Conclusions

The work presented in the thesis was undertaken with the primary aim to study the equine genome. Because of the very limited mapping resources (e.g., equine specific probes etc.) available in the horse, indirect approaches like Zoo-FISH and heterologous FISH mapping were employed. The work was extended to another equid - the donkey - to see how the karyotypes of two very closely related species are organized in relation to each other. Comparison with contemporary Zoo-FISH information in other species provided some clues on genome organization among the mammals. However, with horse at the centre of all investigations, a broader view of the horse genome was realized with the following specific observations:

- A Zoo-FISH based comparative chromosome map between human and horse was established, which provided comparative information on the majority of the equine chromosomes. The findings significantly contribute towards a) anchoring equine linkage/synteny groups to specific chromosomes, b) predicting likely map location of equine genes on the basis of the location of their homologues in humans, c) and providing possibilities to transfer gene mapping data from human to horse, which in turn could open the prospects for rapid development of the horse gene map.

- Zoo-FISH with microdissected ASPs from selected human chromosomes refined the overall comparative status between the horse and human genomes. This enabled more accurate demarcation of the boundaries of conserved syntenies between the two species. The information can be of importance in focused development of equine gene map using the comparative information from human and other developed gene maps. Microdissected sub-chromosomal probes emerged as a viable alternative to refine gross chromosome homology between distantly related species.

- FISH assignment of four genes to equine chromosomes is a small contribution to the list of cytogenetically mapped Type I markers in the horse. The map location of *IGF2* emphasizes the evolutionarily conserved terminal location of the gene, while other localizations provide detailed information on LG2 in horse.

- Successful use of heterologous porcine probes for FISH mapping in horse (+donkey) represents the first such work where *individual* large sized genomic clones have been used across distantly related species. The results illustrate the potential for accelerating physical gene mapping in horses and other mammals for creating refined comparative maps.

- Comparative analysis between horse and donkey chromosomes using microdissected equine meta-/submetacentric and sex chromosome specific paints provided the first molecular cytogenetic evidence for homology between the two karyotypes. An important derivative of this work was indirect deduction of homology between human and donkey karyotypes. A large part of the donkey genome can now be compared with other mammalian genomes.

- For the first time, physical gene mapping was carried out in the donkey. Though FISH mapping of seven loci (five genes and two microsatellite markers) provides very little information, together with the comparative painting data, it serves as a starting point to study the donkey genome.

- Zoo-FISH data between human and a variety of non-primate species enabled the detection of evolutionarily conserved whole chromosomes, large chromosomal segments and contiguous syntenies in mammalian genomes. Interestingly, horse provided several exceptions as compared to other species. The results allowed us to identify a set of human chromosome segments that very likely comprised the karyotype of the eutherian ancestor. Possible fusion/fission events leading to the karyotypes of studied species are proposed.

Future prospects

The last few years represent a period of significant breakthroughs in equine genome analysis. After having awaited fairly long for the attention of animal geneticists, gene mapping in horse has now received a good start. The statement of this fact in no way means that the work is even *partly* done. Uneven distribution of markers throughout the equine genome and scarcity of mapped specific genes, severely restricts the utility of the map for approaching genes responsible for various traits of interest. The status also limits exchange of map information with other species. Although human/horse Zoo-FISH map now serves as a framework for comparison with other species - directly with human, and indirectly with others - there is still a strong need to have a broad coverage of the chromosomes with both type I and polymorphic markers.

Development of a fundamental genetic linkage and physical map with reasonably uniform coverage of the equine genome is presently one of the top priorities of horse geneticists. The international consortium of equine gene mappers are concentrating their efforts in this direction. However, lessons have to be learned from the pattern of development of gene maps in other livestock species, specially the cattle and pigs, where the efforts got too much concentrated on developing maps enriched mainly with Type II markers. It is only during recent years, when the search for genes responsible for traits of interest started, that the urgent need of a well developed map with coding sequences was realized (primarily for comparative purposes). Hence, a *balanced* development of the equine gene map, with reasonable number of loci of both types, is something which will have to be kept in mind in horse.

This thesis presents a comprehensive picture of homology between the human and horse karyotypes. Though all human chromosome specific paints were used, it was not possible to get complete coverage of the equine chromosomes. This does not immediately imply that the unpainted regions of the equine genome do not share homology with the human genome. Additional Zoo-FISH experiments reported in this thesis, together with recent gene mapping data show that two (viz., ECA12 and 31; paper II; Lear et al. 1998a) of the unpainted segments now show homology with parts of human genome. Hence, some efforts will have to be diverted for studying the remaining "blank" regions of the equine genome.

Heterologous FISH mapping has been introduced in this thesis as one of the ways to rapidly develop the gene map of one species using large sized probes from evolutionarily distantly related species. It is expected that this alternative will gain more momentum and will be of use to map specific genes in horse, even without horse specific gene probes. The approach will also be of specific use in cases where a dense comparative map will be needed in a small region of the equine genome. The availability of PACs, BACs, YACs etc. in a number of species will make this task possible.

Microdissection of equine chromosomes is a relatively new addition to the tools for studying the horse genome. Though it is one of the fairly well established and useful method in the humans to approach the gene of interest (Guan et al. 1996; see for review Cannizzaro 1996), it is too early to say whether the equine geneticists are prepared to further invest and embark on this approach. By now, there are two preliminary reports about construction of horse microlibraries from ECA1, 6 and 12 (Bowling et al. 1998; Chowdhary et al. 1998). However, for the moment it appears that the use of microdissected chromosome or arm specific material will be restricted mainly to comparative analysis within the equids/Perissodactyls or even across other orders.

The present resolution of the physical gene map in horse is very low. Under these circumstances it may seem that there are no reasons why fine mapping techniques like interphase-mapping and fiber-FISH etc. will be applied in the near future. However, with studies already concentrating on traits of specific interest, it will not be long when the need of these approaches will be felt. Among other physical gene mapping techniques, somatic hybrid cell genetics will continue to be at the forefront, primarily due to the significant contributions it is making in the expansion of the equine gene map. Nevertheless, construction of a radiation hybrid panel in horses will be a timely step in the right direction, specially considering that such developments have significantly contributed in making the cattle and pig gene maps more informative and integrated than before.

On the genetic linkage front, generating more family material will continue to remain a priority. Recently a new reference pedigree, involving eight half-sib families was constructed in Sweden (Lindgren et al. 1998), while the first full-sib family material was created in England (Swinburne et al. 1998). It is anticipated that linkage mapping will be intensified to achieve a reasonably good and even coverage of the equine genome. With the availability of an international panel of markers for mapping in various family materials, it will be possible to align different maps in the coming years.

Gene mapping in the horse is also expected to benefit from recent advances in tools and methodologies introduced to analyse e.g., human and mouse genomes. Recently introduced sets of primer pairs for orthologous genes (CATS or TOASTs) are new tools for cross species comparisons. The former have already been used in some of the very recent studies in horses (Caetano et al. 1998). Application of microarray (Schena et al. 1998) or DNA-chip technology (Ramsay 1998) cannot be left out as a distant probability in horses. With some methodological advancements, the technology may soon become a part of equine genome analysis. Further, studies of gene expression related to immune response, growth, development and genomic imprinting in horses (Otte and Engström 1994; Otte et al. 1996) are receiving growing attention. It may not be long when new approaches, such as microarray technology, will be of use also in

horse. Establishment of cDNA libraries in the horse is, therefore, another aspect which might gain significance in coming years.

"What will be the immediate use of a gene map in horse", is a question which was present much before organized gene mapping started in horses. One of the fields where equine gene map will find it's first use is "disease genetics". There is a good probability that, like in other species, DNA based tests will be available for some of the genetic disorders. Advances in this direction already made in horses involving hyperkalaemic periodic paralysis (Rudolph et al. 1992), severe combined immunodeficiency (Shin et al. 1997) and the Overo lethal white foal syndrome (Santschi et al. 1998) are very encouraging. With regards to traits related to performance, all which can be said for the moment is that, though it may appear difficult to find genes directly influencing these traits, it will not be impossible to approach them as the equine gene map develops further.

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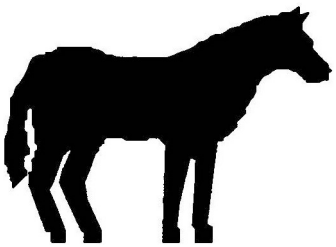

Acknowledgements

The studies summarized in this thesis were carried out in the Department of Animal Breeding and Genetics of the Swedish University of Agricultural Sciences. Financial support received from the Swedish Institute, Erik Philip-Sörensen Foundation and Swedish Council for Forestry and Agricultural Research is gratefully acknowledged.

First of all I would like to express my sincere, infinite thanks to *Dr. Bhanu P. Chowdhary,* my main supervisor, for his guidance, brilliant ideas, all possible support, endless patience and friendship. Bhanu, you are the best supervisor one could ever have! Besides scientific guidance you managed also to brush my Finno-Ugric English so, that some things became understandable even to other people.

I am deeply indebted to *Prof. Leif Andersson,* my co-supervisor, for sharing his bright ideas and for continuous support during all these years.

I am very grateful to *Prof. Birgitta Danell* for her constant encouragement, help and support whenever needed.

During these study years I have been lucky to collaborate with many nice people from different laboratories - in Ultuna, BMC, Pathology Department, Genetic Centre and University of Kaiserslautern, Germany. My sincere thanks to all of you!

In particular I would like to express my deep gratitude to:

- all the "fishermen" in our small *in situ* group, especially to the closest FISHing friends *Anna Törnsten* and *Renuka Chaudhary.* Without your friendship and humor no good fish could have ever been caught. Dear Anna and Renu, you showed me that Sweden is not just the lab and continuous pipetting, but something much, much more - warm people, interesting places, beautiful nature! Your homes helped to cure home-sickness and to fill long lonely evenings far from my family.

- all the co-authors: *Harry Scherthan* and *Lutz Frönicke* from Kaiserslautern for constructive co-Zoo-FISHing, *Kerstin Otte* and *Björn Rozell* from the Pathology Department for their friendly lab atmosphere and creative collaboration, *Gérard Guérin* and *Sophie Godard* from Jouy-en-Josas for sharing their valuable experience and probes with us. Special thanks to *James Kijas* who has been not only a colleague and co-author, but also a patient and enthusiastic teacher. Your evergreen optimism, James, is just great! Thank you also for critical comments on the manuscript of this thesis.

- *Lena Andersson-Eklund* for always finding time in her busy work schedule to help with good advice and just the right paper from the complicated labyrinth of Ph.D. network.

- all the "Husdjursgenetik" people in BMC. It was a great pleasure to work in your lab. In particular I would like to thank *Jin-Tae Jeon* for always having good ideas and helpful hand to solve even impossible problems, *Gabiella Lindgren* with whom we have tried to "talk to horses", and *Anna-Karin Fridolfsson* whose active mind and open eyes have helped to bring fresh air into everyday lab routine.

- *Gudrun Wieslander* for never failing help, support and warm sense of humor. Dear Gudrun, whenever I get lost in a foreign place, I think about you!

- all the neighbours in our department in Ultuna. Specially I'd like to mention *Anne Lundén, Kaj Sandberg, Stefan Marklund, Lieuwe Appel* and *Birgitta Malmfors* for giving company during long and dark evening hours and the weekends. It is really encouraging to realize that there are some more people who have no watch or calendar. Thanks are also due to *Kumar Juneja* for always sharing his knowledge and experience and for friendly company, to *Annika Dahlén* who patiently shared the PCR machine with us and to *Thomas Johansson* for kind help in isolating horse DNA.

- the secretaries, *Britt-Marie Gillberg* and *Monica Jansson,* for everyday friendly smile and help with all possible papers.

- *Håkan Hellder.* Even dark and gloomy winter mornings become sunny after hearing your "Hej!".

- *Mari Wallbring* and *Heléne Gille* from "OG Stallet" for helping with horse blood samples whenever needed. I am also grateful to all the Klinikcentrum horses for never saying a bad word when they saw me coming with vacutainers.

- all the dogs of our department for unconditional tolerance and friendly attitude.

- colleagues and friends from my Estonian lab. Your moral support and e-mail greetings have been a great help during these years. And sure, sooner or later I will join your friendly company again!

- my Estonian friends in Uppsala, *Ivar Puura* and *Signe Kask* for just being as you are - bright, full of jokes and innate ethics.

Words are too poor to express my deep and warm gratitude to my family in Estonia, who have been supporting my stay away from home and encouraging to overcome all difficulties. Without your love and help this thesis never became a reality! It is dedicated to you!

.....

And finally I would like to express my sincere admiration to Gary Larson whose creative mind from the "far side" has helped to make at least some things clear here around: "horse is a horse, of course, of course...".

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ISSN 1401-6257
ISBN 91-576-5405-0