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COMPARATIVE GENE MAPPING FOR EQUUS PRZEWALSKII AND E. HEMIONUS ONAGER WITH INVESTIGATION OF A HOMOLOGOUS CHROMOSOME POLYMORPHISM IN EQUIDAE

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ABSTRACT OF DISSERTATION

Jennifer Leigh Myka

The Graduate School
University of Kentucky
2003
COMPARATIVE GENE MAPPING FOR *EQUUS PRZEWALSKI* II AND *E. HEMIONUS ONAGER* WITH INVESTIGATION OF A HOMOLOGOUS CHROMOSOME POLYMORPHISM IN EQUIDAE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
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Lexington, Kentucky
2003

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ABSTRACT OF DISSERTATION

COMPARATIVE GENE MAPPING FOR EQUUS PRZEWALSKII AND E. HEMIONUS ONAGER WITH INVESTIGATION OF A HOMOLOGOUS CHROMOSOME POLYMORPHISM IN EQUIDAE

The ten extant species in the genus Equus are separated by less than 3.7 million years of evolution. Three lines of investigation were pursued to further characterize equid genome organization. 1.) The Przewalski’s wild horse (E. przewalskii, EPR) has a diploid chromosome number of 2n=66, while the domestic horse (E. caballus, ECA) has 2n=64. A comparative gene map for E. przewalskii was constructed using 46 bacterial artificial chromosome (BAC) probes previously mapped to 38 of 44 E. caballus chromosome arms and ECAX. BAC clones were hybridized to metaphase spreads of E. przewalskii and localized by fluorescent in situ hybridization (FISH). No exceptions to homology between E. przewalskii and E. caballus were identified, except for ECA5, a metacentric chromosome with homology to two acrocentric chromosome pairs, EPR23 and EPR24. 2.) The onager (E. hemionus onager, EHO) has a modal diploid chromosome number 2n=56 and a documented chromosome number polymorphism within its population, resulting in individuals with 2n=55. Construction of a comparative gene map of a 2n=55 onager by FISH using 52 BAC probes previously mapped to 40 of 44 E. caballus chromosome arms and ECAX identified multiple chromosome rearrangements between E. caballus and E. h. onager. 3.) A centric fission
Robertsonian translocation polymorphism has been documented in 5 of the ten extant equid species, namely, *E. h. onager*, *E. h. kulan*, *E. kiang*, *E. africanus somaliensis*, and *E. quagga burchelli*. BAC clones containing equine (*E. caballus*, ECA) genes SMARCA5 (ECA2q21 homologue to human (HSA) chromosome 4p) and UCHL1 (ECA3q22 homologue to HSA4q) were FISH mapped to metaphase spreads for individuals possessing the chromosome number polymorphism. These probes mapped to a single metacentric chromosome and two unpaired acrocentrics showing that the centric fission polymorphism involves the same homologous chromosome segments in each species and has homology to HSA4. These data suggest the polymorphism is either ancient and conserved within the genus or has occurred recently and independently within each species. Since these species are separated by 1-3 million years of evolution, the persistence of this polymorphism would be remarkable and worthy of further investigations.

**KEYWORDS:** Comparative Gene Map, Chromosome Number Polymorphism, *Equus*, Chromosome Rearrangement, Robertsonian Translocation

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23 July 2003
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DISSERTATION

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DISSertation

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

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Co-Director: Dr. Teri L. Lear, Research Assistant Professor;
Co-Director: Dr. Ernest Bailey, Professor;
Department of Veterinary Science

Lexington, Kentucky
2003

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This dissertation is dedicated to my husband, Thomas B Brackman.
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Chapter One: Introduction to Equid Chromosome Evolution

I. Introduction

A. The family Equidae

1. Introduction to Equidae

The phylogenetic order Perissodactyla, or odd-toed ungulates, was very diverse and species-rich during the late Paleocene into the Eocene, but extinctions reduced the order to three families, Tapiridae, Rhinocerotidae, and Equidae (Nowak, 1999). Equidae, once a worldwide and diverse family, is now composed of a single genus, Equus, with 10 extant species (Bowling and Ruvinsky, 2000). Equus first appeared in the fossil record 3.7 million years (MY) ago, and diverged in as little as 1.7 MY to form four related groups: horses, true asses, hemiones, and zebras (Bowling and Ruvinsky, 2000). These extant equid species are listed in Table 1.1, along with their common names and modal and polymorphic diploid chromosome numbers.

Despite their relatively recent evolution, equids have widely varying chromosome numbers, ranging from 2n=66 in Przewalski’s wild horse (E. przewalskii, EPR, a.k.a. E. ferus przewalskii) to 2n=32 in Hartmann’s mountain zebra (E. zebra hartmannae, EZH) (refer to Table 1.1), suggesting that the Equidae have undergone rapid chromosome evolution concurrent with speciation (Bush et al, 1977; Wichman et al, 1991). While equids may congregate in large herds under particular environmental conditions (Nowak, 1999), they typically separate into smaller herds or bands with horses specifically forming harems and bachelor herds (Houpt and Boyd, 1994). Separation of equids into small groups supports the hypothesis that small populations are necessary for rapid speciation (Bush et al, 1977). Indeed, the genus Equus shows a high rate of speciation correlated with a high rate of chromosomal evolution (Bush et al, 1977). Also, several classes of repeated DNA sequences, or tandem repeat elements, were shown to be in an evolutionarily dynamic state in six equid species (Wichman et al, 1991). This suggests a molecular basis for the rapid chromosomal evolution in that tandem repeats provide sites for breaks within chromosomes which may have minimal impact on the functional genome (Wichman et al, 1991). Since chromosome number is one of the distinguishing characteristics of a species, it is noteworthy that these species
Table 1.1. Equidae species. Species nomenclature, common names, and chromosome numbers for *Equus* species (Benirschke and Malouf, 1967; Ryder *et al.*, 1978; Nowak, 1999; Bowling and Ruvinsky, 2000). This table excludes chromosome number polymorphisms that are rare and thought associated with pathology.

<table>
<thead>
<tr>
<th>Species</th>
<th>Species Abbreviation: Common Name(s)</th>
<th>Modal 2N</th>
<th>Observed Polymorphic 2N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Horses:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equus przewalskii</em></td>
<td>EPR: Przewalski’s wild horse; Mongolian wild horse; <em>E. ferus przewalskii</em></td>
<td>66</td>
<td>-</td>
</tr>
<tr>
<td><em>Equus caballus</em> L.</td>
<td>ECA: domestic horse; <em>E. ferus</em> (domestic)</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td><strong>True asses:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equus asinus</em></td>
<td>EAS: donkey; ass; <em>E. africanus</em> (domestic)</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td><em>Equus africanus somaliensis</em></td>
<td>EAF: Somali wild ass</td>
<td>62</td>
<td>63, 64</td>
</tr>
<tr>
<td><strong>Hemiones:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equus hemionus onager</em></td>
<td>EHO: onager; Persian wild ass</td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td><em>Equus hemionus kulan</em></td>
<td>EHK: kulan; Transcaspian wild ass</td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td><em>Equus kiang</em></td>
<td>EKI: kiang; Tibetan wild ass</td>
<td>52</td>
<td>51</td>
</tr>
<tr>
<td><strong>Zebras:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Equus grevyi</em></td>
<td>EGR: Grevy’s zebra</td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td><em>Equus quagga burchelli</em></td>
<td>EQB: Burchell’s zebra; plains zebra</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td><em>Equus zebra hartmannae</em></td>
<td>EZH: Hartmann’s mountain zebra</td>
<td>32</td>
<td>-</td>
</tr>
</tbody>
</table>
have diverged so greatly in their respective chromosome numbers in such an evolutionarily short time.

2. Brief history of Equidae

The earliest members of the family Equidae date from the early Eocene, with a distribution in Holarctica, which includes the global regions of Europe, Asia, the Canadian Arctic and western North America (MacFadden, 1992). However, by the middle Eocene, equids were found only in North America (MacFadden, 1992). During the Miocene, equids dispersed by the Bering route back to Holarctica and Africa (MacFadden, 1992). Finally, in the Pliocene and Pleistocene, equids attained their maximum geographic distribution and were found in Holarctica, Africa, and South America (MacFadden, 1992). The genus *Equus* dates to 3.7 MY with fossils found in the New World, and within 1-1.5 MY had dispersed to every continent but Australia and Antarctica (MacFadden, 1992). Horses became extinct in the New World at the end of the Pleistocene about 11,000 years ago, but were reintroduced by Spanish explorers in the 1500s, while wild equids persisted in the Old World into the Holocene (MacFadden, 1992).

3. Extant equid taxa

There are ten extant species in the genus *Equus*, as listed in Table 1.1. The domestic equids, the horse and donkey, have spread worldwide with humans. The Przewalski’s wild horse is now found either in zoos or in Mongolia, China, Kazakhstan and Ukraine where it has been reintroduced (van Dierendonck and Wallis de Vries, 1996). Kulans, onagers, and kiangs are found in Asia, while the Somali wild ass, Grevy’s zebra, Burchell’s zebra, and the Hartmann’s mountain zebra are found in Africa (MacFadden, 1992; Oakenfull et al, 2000).

B. *Equus* phylogenetics

Various scientific approaches have been used to study the phylogenetics of the genus *Equus*. The relative timing of equid speciation events and the precise taxonomic relationships of the extant equids are still the subject of debate, as referenced by Oakenfull and Clegg in a review of eight published phylogenetic trees for this genus (Oakenfull and Clegg, 1998). As illustrated in Figure 1.1, the results of current
Figure 1.1. Simple phylogenetic tree for *Equus*.
molecular analyses suggest that the ancestor of the modern horse and of the zebra and ass diverged first, with subsequent speciation of the zebra/ass clade (Oakenfull and Clegg, 1998; Oakenfull et al, 2000). Where hemiones fit in this tree is unclear, with some reports suggesting that hemiones are more closely related to asses, but others suggesting that they are closer to zebras or horses (Oakenfull and Clegg, 1998).

Classical taxonomic studies based on physical features and morphological characters have revealed certain differences between equid species. For example, a study of the shoulder and leg skeletons of two adult Przewalski’s wild horses and three adult domestic horses showed remarkable differences in the structure of the scapula, with a more curved and rounded caudal border in the Przewalski’s wild horses than in the domestic horses (Sasaki et al, 1999). The domestic horse scapula has a sharp border due to the outer muscular line shifting to this border, while the more curved Przewalski’s scapular border is the result of the muscular line lying beside the border (Sasaki et al, 1999). This difference in scapular architecture suggests that these horses differ in scapular movement due to changes in the attachments of the triceps muscle (Sasaki et al, 1999). In addition, the Przewalski’s wild horse leg skeleton is shorter and thicker than that in the domestic horse, indicating a lower center of gravity and shorter stride in the wild horse (Sasaki et al, 1999). Skull measurements, including basal, greatest, palatal, and tooththrow length, incisor and nasal breadth, of 154 wild ass skulls, and characterization of the colouration and markings of 67 skins led Groves and Mazák to place the Asiatic wild asses in the historical genus *Asinus* (Groves and Mazák, 1967). Additionally, they proposed a total of six subspecies of *Asinus hemionus* and three subspecies of *Asinus klang* (Groves and Mazák, 1967). Later work on the hemiones confirmed differences in the Transcaspian hemione, *E. hemionus kulan* (EHK), and the Iranian hemione, *E. hemionus onager* (EHO) (Eisenmann and Shah, 1996). Examination of a total of twenty-five kulan and thirty onager skulls revealed that the kulans had narrower supra-occipital crests than the onagers, suggesting that the animals may carry their heads in a different manner because this is the site of insertion for the ligaments suspending the skull (Eisenmann and Shah, 1996). While there is overlap in the basilar length and occipital width measurements between these groups, the regression lines for occipital widths are different between kulans and onagers since
onagers tend to have a wider occiput (Eisenmann and Shah, 1996). More recent analysis of a total of 332 skulls from seven equid species plus four quagga and four fossil skulls produced conflicting information that did not resolve questions of phylogeny within the genus, but did confirm that the extinct quagga was not equivalent to the Hartmann’s mountain zebra (Klein and Cruz-Uribe, 1999). The study involved determination of the means of skull measurements (Klein and Cruz-Uribe, 1999). Additional analysis was conducted to determine the residual shape, or the shape aside from size (Klein and Cruz-Uribe, 1999). The cluster diagrams of cranial size-and-shape variables linked equid species primarily by skull size (Klein and Cruz-Uribe, 1999). They were considered to produce a less meaningful result from a biological point of view than the residual-shape clusters (Klein and Cruz-Uribe, 1999). However, the residual-shape clusters contrasted sharply with published mtDNA results (see below). The authors believed the differences were likely reflective of actual evolutionary relationships while the residual-shape clusters were reflective of a mix of shared descent and shared adaptation or parallelism (Klein and Cruz-Uribe, 1999). The fact that cranial size and shape did not distinguish between equid species is supported by work showing that the relatively long cranial length character is common to all extant Equus (Eisenmann and Baylac, 2000). Eisenmann and Baylac compared 225 equid skulls from seven species, including skulls from animals ranging from a donkey with a basilar length of only 323 mm to a gigantic draft horse with a basilar length of 662 mm, with twenty-eight fossils (2000). Extant and fossil skulls were distinguished by plots involving skull measurements, and all fossil skull plots that were close to extant skull plots were younger than 1.5 MY. The authors propose that the modern pattern may be related to a larger brain case, and this pattern is seen on all extant equid skulls, regardless of size or species (Eisenmann and Baylac, 2000). In summary, classical studies of physical features and morphological characters have discerned distinguishing characteristics such as scapular morphology differences between EPR and ECA, and skull measurement differences between EHO and EHk, as well as features in common within Equus such as the modern larger brain case found in extant equids but not in fossil horses. Overall these studies agree with the phylogenetic tree presented in Figure 1.1.
**C. Gene mapping and chromosome evolution**

The genome of an organism is composed of all of the DNA in the nucleus of the cell, and that DNA is found in discrete pieces called chromosomes. A karyotype is a depiction of the pairs of chromosomes of a diploid organism in metaphase of mitosis, when the chromosomes are condensed and visible by light and fluorescent microscopy. Interestingly, the genome size is highly conserved among mammals, with the human genome containing about 40,000 genes (Venter *et al.*, 2001; International Human Genome Sequencing Consortium, 2001), even though the karyotypes of different mammalian species may differ considerably in diploid chromosome number and morphology, with a range from \(2n=6\) to \(2n=134\), in the Indian muntjac, *Muntiacus muntjak*, and the black rhinoceros, *Diceros bicornis*, respectively (reviewed in O'Brien *et al.*, 1999).

The genes of an organism are found in the DNA sequences composing the chromosomes, and the order of genes on a particular chromosome is often conserved in homologous chromosomes of closely related species. Rearrangements can occur in which chromosome segments are inverted within a chromosome or moved to another location in the genome. Common rearrangements include inversions, fusions, fissions, and translocations of segments. These rearrangements can be detected both by examination of chromosome morphology and by determining gene location and/or gene order on a particular chromosome segment. For example, Figure 1.2 illustrates the appearance of fusion and fission chromosomes and their effect on diploid chromosome number. Acrocentric chromosomes, or chromosomes with a terminal centromere and one chromosome arm, may fuse at the centromere to form metacentric chromosomes, or chromosomes with two chromosome arms. Chromosome number is characteristic for a species, as well as the appearance of the karyotype with regard to chromosome morphology. Changes in karyotype between similar animals often suggests events that have or may lead to speciation, particularly if such changes have contributed to reproductive isolation between populations (Searle, 1998).

Comparative gene mapping has confirmed that not only has the mammalian genome organization been highly conserved, gene order has been highly conserved in mammals throughout their 200 MY of evolution (Comparative Genome Organization:
**Figure 1.2.** Schematic of Robertsonian fusion/fission rearrangements. a) Fusion Robertsonian translocation involving two acrocentric chromosome pairs. b) Fission Robertsonian translocation from one metacentric chromosome pair. X and Y represent the diploid chromosome number for a particular species.
First International Workshop, 1996). Several methods of determining genome organization are available, including physical and genetic mapping of markers, as well as fluorescent in situ hybridization (FISH) and cross-species FISH (Zoo-FISH) (Comparative Genome Organization: First International Workshop, 1996). Three categories of markers are used in gene mapping: Type I, Type II, and Type III (reviewed in O’Brien et al, 1999). Type I markers are coding genes which are often used to identify gene orthologs that derive from a common ancestor in distantly-related species (O’Brien et al, 1999), and include expressed sequences tags (ESTs) or partial gene sequences derived from cDNAs. Type II markers are hypervariable microsatellites, or short tandem repeats (STRs), which are non-coding short sequences of DNA repeated in tandem (O’Brien et al, 1999). Type III markers are common single-nucleotide polymorphisms (SNPs) which are found once every 500-1000 base pairs in the human genome (O’Brien et al, 1999).

FISH mapping is a very useful technique in comparative gene mapping. FISH mapping can identify areas of homology between the chromosomes of animals of different species. Essentially DNA probes, often including large sections of DNA in vectors such as bacterial artificial chromosomes (BACs), are labeled with biotin or digoxigenin. The labeled probes are applied, or hybridized, to metaphase spreads on glass slides. After incubation, the slides are developed by the application of antibodies which are tagged with fluorescent labels such as FITC or rhodamine, and the chromosomes themselves are counterstained with DAPI or other DNA binding stains. Under a fluorescent microscope the DAPI-stained chromosomes appear blue and the areas of homology appear as localized spots (Figure 1.3). The advantage of FISH mapping is that sexually reproducing animals contain homologous pairs of autosomes, which are duplicated in mitosis, therefore specifically localized probes are located on the two homologues (paired chromosomes) per metaphase spread. Each metaphase spread has this feature which can be used as an internal control for probe specificity. Additionally, the bound probe often has a distinctive appearance, with two parallel spots per chromosome, each bound to one of the sister chromatids in the same relative location.
**Figure 1.3.** Schematic of the FISH mapping technique. The images at the top represent the view seen in a microscopic field when viewed with a fluorescent microscope. The chromosomes are counterstained with DAPI, producing the blue colour under fluorescent activation, while the probe is labeled with rhodamine red. The bottom images show the sister chromatids of the metaphase chromosome pair.
Comparative gene mapping is commonly used to discern relationships between related species. Genes and markers that have been FISH mapped to specific loci on chromosomes of one species are used to probe the chromosomes of another species. When hybridized to chromosomes of related species, the location of the probe is taken as the presence of a homologous DNA sequence to the original species. This approach assumes that specific hybridization occurs in the presence of sequence homology. A successful probe is one which consistently hybridizes to the same location on a pair of homologues in the target species on many metaphase spreads. If a probe hybridizes to more than one locus consistently on the chromosomes of the target species, then it is assumed that there is either a gene duplication in that species or a repetitive element in the probe sequence which is being recognized in more than one location in the target genome.

D. Equid chromosomes

Chromosomes can be banded by several methods, each of which differentiates between chromosomes by producing specific banding patterns. G-banding appears to alter the protein coverage of DNA which allows the methylene blue in Giemsa stain to bind to certain previously inaccessible areas (Holmquist and Motara, 1987). C-banding preferentially removes DNA from euchromatin over heterochromatin (Holmquist and Motara, 1987). The DAPI counterstain used in FISH has a selective affinity for DNA of a certain base composition, and fluoresces when bound to DNA (Holmquist and Motara, 1987). Earlier work relied on the specific pattern of bands to suggest homologous chromosomes between equid species. Later work has used FISH to confirm the homology suggested by banding.

In addition to providing karyotypes for the several equid species, namely: the domestic horse, Przewalski's wild horse, the donkey, the onager, Hartmann’s mountain zebra, Burchell’s zebra, as well as for *E. burchelli antiquorum*, a hybrid between a Burchell’s zebra and a donkey, a pygmy mule and a hinny; Benirschke and Malouf summarized earlier studies of equid chromosomes dating from as early as 1914 (Benirschke and Malouf, 1967). Later work by Ryder and co-workers established both G- and C-banded karyotypes for the following extant equids: the domestic horse,
Przewalski’s wild horse, the donkey, the onager, Grevy’s zebra, Burchell’s zebra, and Hartmann’s mountain zebra, plus a hybrid between a Grevy’s zebra and *E. burchelli antiquorum* (Ryder *et al.*, 1978). These karyotypes also identified several differences between the extant equids. For example, the *E. caballus* (ECA) karyotype has one metacentric pair not seen in EPR, but the Przewalski’s wild horse karyotype contains two acrocentric pairs of chromosomes not seen in the domestic horse (see Figures 2.2 and 2.3) (Ryder *et al.*, 1978). Also, the donkey and the Hartmann’s mountain zebra each have inversions detected by G-banding indicating that these specific autosomes differ from those of other equids (Ryder *et al.*, 1978). Other autosomes were determined to be homologous, also based on chromosome banding patterns.

**E. Equid gene mapping**

The gene map for the domestic horse has developed rapidly, from early synteny maps constructed using somatic cell hybrids (Williams *et al.*, 1993; Bailey *et al.*, 1995; Shiue *et al.*, 1999; Caetano *et al.*, 1999a; Caetano *et al.*, 1999b), to microsatellite-based linkage maps (Breen *et al.*, 1997; Lindgren *et al.*, 1998; Guérin *et al.*, 1999), to a comparative gene map of the horse using universal primers to mammalian genes (Caetano *et al.*, 1999c). In addition, many of these gene markers have been physically mapped to equine chromosomes by FISH (Lear *et al.*, 1998a; Lear *et al.*, 1998b; Lear *et al.*, 1998c; Marklund *et al.*, 1999; Lear *et al.*, 1999; Caetano *et al.*, 1999a; Caetano *et al.*, 1999b; Godard *et al.*, 2000; Lear *et al.*, 2000; Mariat *et al.*, 2001; Lindgren *et al.*, 2001; Lear *et al.*, 2001; Raudsepp *et al.*, 2002; Milenkovic *et al.*, 2002; Hanzawa *et al.*, 2002), culminating in the recent publication of a composite horse gene map incorporating current radiation hybrid panel, linkage, and FISH mapping results (Chowdhary *et al.*, 2003). In addition, the technique of chromosome painting is useful in detecting large blocks of homology on chromosomes between species. Chromosome painting studies have established homologies between horse and human chromosomes (Raudsepp *et al.*, 1996; Rettenberger *et al.*, 1996), as well as between horse and donkey (Raudsepp *et al.*, 1999; Raudsepp and Chowdhary, 1999), and human and Hartmann’s mountain zebra (Richard *et al.*, 2001). These studies have provided a basis of comparison to the human karyotype, a standard which can be used to predict chromosome homologies for
more distantly related species. Currently, 38 loci have been FISH mapped to donkey chromosomes, with a total of twenty chromosome arms and one centromere region being identified in the donkey from a total of 49 autosomal arms (Raudsepp et al, 1999; Raudsepp et al, 2001). Other extant equids have not been explored by comparative gene mapping to date, leaving a dearth of knowledge regarding chromosomal evolution between most equid species. This research was undertaken to gain further knowledge on equid chromosomal evolution and a better understanding of the role genome organization has played in the evolution and speciation of extant equids. It used molecular cytogenetics to study chromosome differences among the equids. This research comprised three separate but related studies with the following objectives:

1. Compare the genome organization of EPR with ECA.
2. Compare the genome organization of EHO with ECA.
3. Investigate a unique chromosome fission/fusion polymorphism found in five of the ten extant equid species.

**F. Research objective one**

The first research objective was to cytogenetically characterize the two horses, EPR and ECA. The hypothesis tested was that the EPR and ECA karyotypes differ at multiple sites, leading to the prediction that if there are no differences, chromosome banding patterns and morphology will predict gene position.

The diploid chromosome number of these two horses differs by one pair of autosomes, with EPR having two more pairs of acrocentric chromosomes and one fewer pair of metacentric chromosomes than ECA (Benirschke et al, 1965; Benirschke and Malouf, 1967). Based on chromosome banding patterns, ECA5 was predicted to be the ECA chromosome resulting from a Robertsonian fusion of two acrocentric EPR chromosomes (Ryder et al, 1978). Preliminary data using a human HSA13 whole chromosome paint suggested an additional difference between the ECA and EPR karyotypes because chromosomes with different morphologies were identified, i.e. the acrocentric chromosome ECA17 in ECA and a metacentric chromosome in EPR (T. Lear, personal communication). Based on these differences, the hypothesis that the EPR and ECA karyotypes differ from each other at multiple sites was tested. Probes
that had been physically mapped to ECA chromosomes were applied to EPR chromosomes and analyzed by FISH, resulting in a comparative gene map of the Przewalski’s wild horse.

**G. Research objective two**

The second research objective was a.) to construct a comparative gene map for EHO by FISH using the domestic horse as the reference point and b.) to identify the polymorphic chromosomes in the EHO karyotype. The hypothesis tested was that EHO genome organization would be similar to ECA genome organization with the exception of fusions and fissions to explain the different number of diploid chromosomes in EHO (2n=56) as compared to ECA (2n=64). The prediction is that differences in genome organization will be reflected as differences in gene position.

As noted in Table 1.1, the onager has a modal diploid chromosome number of 56, but animals have been observed with 2n=55 (Ryder, 1978). Along with the chromosome number changes, the karyotypes of the animals in question exhibited an unpaired metacentric chromosome and two unpaired acrocentric chromosomes with a morphology resembling that illustrated in the “heterozygote” images in Figure 1.2. G-banding pattern homology predicted ECA16 and ECA18 as the ECA chromosomes homologous to those involved in the polymorphism. Therefore, probes containing genes mapping to ECA16 and ECA18 were selected to try and identify the chromosomes involved in the polymorphism. Because these initial attempts were unsuccessful, a revised goal of creating a comparative gene map of the onager was undertaken to identify the polymorphic chromosomes and other presumed differences in genome organization. The hypothesis tested was that the EHO genome organization would be similar to that of ECA, with the exception of chromosome fusions and fissions that would explain the different number of diploid chromosomes in EHO (2n=56) as compared to ECA (2n=64).

**H. Research objective three**

The third research objective was to identify the fission-fusion chromosomes in hemiones with chromosome number polymorphisms, using probes containing ECA
genes. The hypothesis tested was that homologous chromosomes are responsible for the chromosome number polymorphisms in all equid species.

Some EHO individuals have chromosome number polymorphisms leading to a diploid chromosome number of 2n=55 in contrast to the modal 2n=56 for this species (Ryder, 1978). These polymorphic chromosomes were identified with ECA probes during the creation of the EHO comparative gene map. Interestingly, EHK also had a similar range of diploid chromosome numbers (Ryder, 1978). Houck and co-workers identified a similar situation in *E. africanus somaliensis* (EAF) (Houck *et al.*, 1998), and they proposed that these polymorphisms (refer to Table 1.1), as well as those seen in *E. kiang* (EKI) (Ryder and Chemnick, 1990) and *E. quagga burchelli* (EQB) (Whitehouse *et al.*, 1984), were homologous based on banding pattern similarities. The hypothesis tested was that the EHO polymorphic chromosomes were homologous to chromosomes involved in the polymorphisms seen in the other equid species. To confirm the homology, the same probes that identified the polymorphic chromosomes in the EHO were hybridized to EHK, EAF, EKI, and EZH chromosomes and analyzed by FISH.
Chapter Two: Confirmation of chromosomal homology between the domestic horse, *Equus caballus*, and Przewalski’s wild horse, *Equus przewalskii*, by FISH

I. Introduction

The Przewalski’s wild horse (*Equus przewalskii*, EPR, a.k.a. *E. ferus przewalskii*) is the only extant wild horse. Historically, it lived in an area that is now comprised of areas of Mongolia, Khazakstan, and the Xinjiang-Uygur Autonomous Region of China (Ryder, 1993). All living Przewalski’s horses are descendants of 13 individuals (Ryder, 1994) and are now found only in captive settings such as zoos and where reintroduced to preserves. The current population is estimated at over 1500 individuals world-wide (Kolbas, 2002).

The domestic horse (*Equus caballus*, ECA, a.k.a. *E. ferus ferus*) and EPR are thought to have diverged from other equid species as recently as 0.32-0.62 MY ago, as estimated by the genetic divergence of the mitochondrial control region and 12S rRNA sequences (Oakenfull *et al.*, 2000). A close relationship between ECA and EPR has been shown by many researchers. Based on protein electrophoretic polymorphisms, ECA and EPR are the most closely related among the extant equid species (Kaminski, 1979). This finding was supported by immunological systematics (Lowenstein and Ryder, 1985), genetic analysis of blood markers (Bowling and Ryder, 1987), and molecular DNA studies of α and θ globin genes (Oakenfull and Clegg, 1998). Indeed, the amino acid sequences of protamine P1, a protein known to evolve very rapidly, are identical for these two equid species (Pirhonen *et al.*, 2002). Analysis of mitochondrial DNA sequences suggested that earliest divergence in the genus *Equus* occurred between the horses and the zebra/ass ancestor (Oakenfull *et al.*, 2000). Additionally, ECA/EPR hybrids are viable and can produce fertile offspring (Short *et al.*, 1974), while hybrids of horses with other equids are usually viable but almost always infertile.

Chromosome number is a characteristic of a species, therefore, analysis of chromosome configuration is often of use in characterizing a species. EPR has a complement of 2n=66, in contrast to the 2n=64 in ECA (Benirschke *et al.*, 1965; Benirschke and Malouf, 1967). Examination of the karyotypes of both horses revealed that the difference in diploid chromosome number could be explained by two additional
pairs of acrocentric chromosomes and one fewer metacentric pair of chromosomes in the EPR karyotype than in the horse karyotype, possibly the result of Robertsonian fusion (Ryder et al, 1978). Ryder compared the G-banded karyotypes of the two horses, and suggested that the metacentric chromosome ECA5 was homologous to two pairs of acrocentric chromosomes in EPR (Ryder et al, 1978). The current study was undertaken to a) specifically determine if ECA5 homologues were involved in the Robertsonian rearrangements associated with the two populations, and to b) investigate homology between EPR and ECA chromosomes by FISH mapping. Large insert equine probes have been successfully used to identify horse chromosome homology with donkey chromosomes (Raudsepp et al, 1999). This approach was selected for comparative mapping since ECA and EPR are closely related. Probes were readily available due to the recent increase in genes and chromosome markers on the ECA gene map.

II. Materials and Methods

A. Chromosome preparations

Metaphase chromosome spreads were prepared by the Center for the Reproduction of Endangered Species (CRES) at the San Diego Zoo. *E. przewalskii* studbook #7413 and #12925 metaphase spreads were prepared from fibroblast cell cultures as previously described (Kumamoto et al, 1996). Briefly, skin biopsies were processed using a collagenase disaggregation technique. Fibroblasts were then cultured in a 1:1 mixture of fibroblast growth medium 2 (FGM2) (Clonetics) and minimal essential medium (MEM) alpha (Gibco) plus 10% fetal bovine serum, 1% antibiotic-antimycotic, 1% L-glutamine at 37°C in 6% CO₂. Cells at peak mitotic activity were exposed to colcemid (Gibco) at a concentration of 0.25 µg/mL, followed by incubation for 105 minutes (min.). In the final minutes of incubation, cells were trypsinized, pelleted by centrifugation, and subsequently exposed to pre-warmed 0.067 M potassium chloride for 30 min. Cells were pre-fixed by adding 1 mL 3:1 methanol: acetic acid, mixing, then pelleting cells at 1000 rpm for 10 min. at room temperature (RT). Pellets were washed 3 times with 3:1 methanol: acetic acid. Cells were dropped onto wet, pre-cleaned glass slides and allowed to air dry. Dried slides were washed 3
times in 1X PBS for 5 min., then dehydrated by 5 min. incubations in 70%, 85%, and 100% ethanol, air dried and stored at -70°C in a desiccant chamber.

**B. Giemsa staining**

Representative slides from each batch were either examined by phase contrast microscopy or Giemsa stained to ensure a sufficient number of metaphase spreads for FISH. Slides were removed from storage at -70°C and immediately dehydrated by 5 min. incubation in a RT ethanol series: 70%, 85%, and 100% ethanol. Air-dried slides were stained for 2.5 min. in Giemsa stain (2.5% Gurr’s Improved R66 (BDH Limited) and 1.25% Wright’s stain (0.25% stock in methanol) (Fisher) in Gurr’s phosphate buffer, (pH 6.8 made with Gurr’s tablets) (BDH Limited)), at RT, followed by washing in distilled deionized water, blotting and air-drying. Slides were viewed without coverslips under a Zeiss Axioplan2 microscope.

**C. BAC DNA preparation**

Equine BAC clones (Table 2.1) were selected for use for comparative gene mapping based on published reports of their location on ECA chromosomes. In connection with gene mapping to ECA, the investigators cited in Table 2.1 verified the identity of each clone by DNA sequencing. The original Institut National de la Recherche Agronomique (INRA) BAC library has about 40,000 clones and a mean insert size of 110 kb with a 1.5 genome equivalent (Godard et al, 1998), and the complemented INRA BAC library has a total of 108,288 clones, a mean insert size of 100 kb, and 3.4 genome equivalent (Milenkovic et al, 2002). The USDA CHORI-241 Equine BAC library, prepared by Pieter DeJong at the Children’s Hospital Oakland Research Institute, has 190,652 clones with a mean insert size of 171 kb with an 11.8-fold total genomic representation (http://www.chori.org/bacpac/equine241.htm).

The 46 ECA BAC clones were selected for FISH mapping on EPR chromosomes to compare genome organization between the domestic horse and Przewalski’s horse karyotypes. BACs were selected from 38 out of 44 autosomal chromosome arms in the ECA karyotype. However, probes were not available for ECA9p, ECA9q, ECA11q, ECA12p, ECA24, and ECA27. Of the 46 BACs used in this study, 5 were mapped for
Table 2.1. Equine BAC clones used for comparative gene mapping of Przewalski’s wild horse.

<table>
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<tr>
<th>BAC clone</th>
<th>ECA map position</th>
<th>HSA map position</th>
<th>Locus name</th>
<th>Reference paper</th>
<th>BAC Source and ID</th>
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<td>A4</td>
<td>ECA1p</td>
<td></td>
<td>anonymous BAC</td>
<td>(Lear, personal comm.)</td>
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<td>solute carrier family 7, member 10</td>
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<td>ALOX5AP</td>
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<td>3q12</td>
<td>5-lipoxygenase-activating protein</td>
<td>(Mariat et al, 2001), (Milenkovic et al, 2002)</td>
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<td>ALPL</td>
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<td>1p36.1-p34</td>
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<td>AMD1</td>
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<td>6q21-q22</td>
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<td>ECA15q12</td>
<td></td>
<td>septin 2-like cell division control protein</td>
<td>(Lear, personal comm.)</td>
<td>INRA266E6</td>
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<tr>
<td>SMARCA5</td>
<td>ECA2q21</td>
<td>4q31.1-q31.2</td>
<td>SW1/SNF related, matrix associated</td>
<td>(Lear et al, 2001)</td>
<td>INRA281E7</td>
</tr>
<tr>
<td>SOD1</td>
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<td>21q22.1</td>
<td>superoxide dismutase</td>
<td>(Godard et al, 2000)</td>
<td>INRA389A2</td>
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<tr>
<td>TCRG</td>
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<td>7p15-p14</td>
<td>T cell receptor gamma</td>
<td>(Lear et al, 2001)</td>
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<tr>
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<td>ECA30q14 and ECA6q21</td>
<td>1q41</td>
<td>transforming growth factor, beta 2</td>
<td>(Milenkovic et al, 2002), *(Lear, personal comm.)</td>
<td></td>
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<tr>
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<td>ECAXp15-p14</td>
<td>Xp11.4-p11.2</td>
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<td>(Raudsepp et al, 2002a)</td>
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<td>(Lear et al, 2000)</td>
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<td>(Lear et al, 2001)</td>
<td>INRA208G12</td>
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<td>urate oxidase</td>
<td>(Godard et al, 2000)</td>
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<td>INRA53D7</td>
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<td>1</td>
<td>vitamin D up-regulated protein 1</td>
<td>(Lear et al, 2001)</td>
<td>INRA12G4</td>
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the first time in the horse (T.L. Lear, unpublished data) with the remaining 41 mapped to ECA in previously published studies (Shiue et al., 1999; Marklund et al., 1999; Godard et al., 2000; Lear et al., 2000; Mariat et al., 2001; Lindgren et al., 2001; Lear et al., 2001; Raudsepp et al., 2002; Milenkovic et al., 2002; Hanzawa et al., 2002; Chowdhary et al., 2003). Of the total loci mapped, 44 were specific equine genes, one contained equine DNA in the form of an anonymous BAC, and one was an expressed sequence tag (EST). A summary of information for the BACs used, including horse and human genome location, gene products, and references can be found in Table 2.1.

To prepare BAC clones, LB agar plates (2% Lennox L Broth Base, 1.5% Select Agar (Gibco)) containing 12.5 µg/mL chloramphenicol were inoculated with *Escherichia coli* containing BACs and incubated overnight at 37°C. Single colonies were used to inoculate 5 mL LB/chloramphenicol broth (2% Lennox L Broth Base (Gibco), 12.5 µg/mL chloramphenicol) followed by incubation at 37°C with continuous shaking at 230 rpm. One-twentieth volume of overnight culture was used to inoculate 50 mL of LB/chloramphenicol broth. Cells were pelleted for 20 min. at 5500 x g (6400 rpm using a JA-17 rotor in a Beckman J2-21M centrifuge) and resuspended in 10 mL TGE (25 mM Tris-HCl, pH 8.0, 50 mM dextrose, 10 mM EDTA, pH 8.0) plus 10 µL RNase-IT (Stratagene). 10 mL SDS/NaOH solution (1% SDS, 0.2 N sodium hydroxide) was added, followed by a 5 min. incubation at RT. 10 mL potassium acetate solution (0.6 M potassium acetate, 0.52 N glacial acetic acid, pH 4.8) at 4°C was added, followed by a 15 min. incubation at 4°C and centrifugation for 30 min. at 20,000 x g at 4°C (12,500 rpm in JA-17 rotor in Beckman J2-21M). The supernatant was clarified for 15 min. at 20,000 x g at 4°C. A Qiagen 100 column was equilibrated with 4 mL QBT buffer (Qiagen) and the supernatant applied to the column to bind the DNA. The column was washed twice with 10 mL QC buffer (Qiagen), and DNA was eluted using five 1 mL washes of QF buffer (Qiagen) at 65°C. DNA was precipitated using 0.6 volumes of cold isopropanol followed by centrifugation at 15,000 x g for 30 min. at 4°C (10,500 rpm in JA-18.1 rotor in Beckman J2-21M). Pellets were washed with 70% ethanol, centrifuged for 15000 x g for 10 min. at 4°C, and air-dried for 5-10 min. BAC DNA was resuspended in 40 µL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and quantitated by
spotting 1 µL onto 1-2% agarose with ethidium bromide followed by comparison to known standards. Agarose gel electrophoresis was performed on BAC DNA samples to confirm the lack of RNA contamination.

D. BAC probe preparation

One µg of BAC DNA of each probe was nick translated and labelled either with biotin-14-dATP (Life Technologies BioNick Labeling System) or with digoxigenin-11-UTP (Roche DIG-Nick Translation Mix) according to manufacturer’s directions.

Briefly, biotin-labelled probes were prepared by incubating 1 µg of BAC DNA in 1X Enzyme Mix and 1X dNTP in a 45 µL volume with incubation at 16°C for 105-270 min. When the probe was between 200-500 base pairs (bp), reactions were stopped by the addition of 5 µL of 0.5 M EDTA, pH 8.0. The probe was precipitated using 0.1 volume 3M sodium acetate and 2 volumes 100% ethanol. After washing with 70% ethanol, pellets were vacuum dried for 20 min. in a Savant vacuum centrifuge or air-dried, and resuspended in 50 µL TE.

DIG-labelled probes were prepared by mixing 1 µg of BAC DNA with 4 µL of DIG-Nick translation mix in a 16 µL reaction volume followed by incubation at 15°C for 60-155 min. Three µL of this reaction was analyzed by denaturing at 95°C for 5 min. followed by a 2 min. incubation at 4°C. When the probe was cut to 200-500 bp fragments, 1 µL of 0.5 M EDTA, pH 8.0 was added per 20 µL of reaction and the reaction was heated at 65°C for 10 min. and precipitated using 0.1 volume 4M lithium chloride and 2.5 volumes 100% ethanol. After washing with 70% ethanol, pellets were vacuum dried for 20 min. in a Savant vacuum centrifuge or air-dried, and resuspended in 50 µL TE.

E. Competitor DNA preparation

To obtain 1 mg of DNA, available genomic DNA samples for EPR were combined. One-half mL of 10X NT buffer (0.5 M Tris-HCl, pH 7.8-8.0, 50 mM magnesium chloride, 0.5 mg/mL BSA) was added and sterile DNase-free water was added up to a final volume of 5 mL. DNA-buffer solution was sheared using a 10 cc
syringe twice through each of 18g, 22g, and 30g needles. The DNA-buffer solution was vortexed to remove bubbles and placed in a water bath at 15°C. The DNase working solution was prepared on ice by adding 1 µL DNase I stock to 1 mL water (DNase stock: 1 µg/mL DNase I in 50% glycerol, 0.15 M sodium chloride), and 10-30 µL of working solution was added to the DNA-buffer solution, followed by incubation at 15°C until DNA reached 300-500 bp fragment size. The reaction was terminated by adding 0.5 M EDTA and 10% SDS to a final concentration of 10-15 mM EDTA and 0.1% SDS, followed by heating at 65°C for 20 min. to inactivate the DNase I. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. After washing in 70% ethanol, the pellet was dried in a Savant vacuum centrifuge, resuspended in 0.2-0.4 mL TE, and quantitated using a spectrophotometer.

F. FISH mapping and analysis

Approximately 100 ng of labeled probe was mixed with 4 µg of domestic horse competitor DNA and 4 µg of competitor DNA from the species being probed, along with 6 µg of salmon sperm DNA carrier. The probe mixture was ethanol precipitated using 0.1 volume of 4M lithium chloride for DIG-labeled probes or 0.1 volume of 3M sodium acetate for biotin-labeled probes. The precipitated probe was dried, and resuspended in 5.5 µL of 100% formamide, followed by the addition of 5.5 µL of 20% dextran sulfate in 2X SSC. After denaturing at 75°C for 10 min., the probe was pre-annealed at 37°C for a minimum of 1 hour to allow repeat elements to anneal together and reduce background hybridization. Prepared slides with metaphase spreads were dehydrated in an ethanol series at RT and air-dried, followed by aging at 65°C for 30 min. to 1 hour. Slides were then denatured for 2 min. at 70°C in denaturation solution (60% formamide, 2X SSC, pH 7.0). The probe was applied to the slides, and glass coverslips sealed with rubber cement, followed by incubation in a hydrated chamber at 37°C for 2-5 days. After incubation, coverslips were removed and slides washed for 3-5 min. in three changes in 50% formamide/2X SSC, pH 7.3 at 37°C, followed by 3-5 min. washes in three changes of 2X SSC at 37°C, with a final 2 min. wash in 1X PBD (Ventana) at RT.
For all subsequent blocking and antibody application steps, plastic coverslips were used. After removing slides from 1X PBD (Ventana), slides were blocked for 15-30 min. Biotin-labelled slides were blocked with 5% casein in maleic acid solution (0.1M maleic acid, 0.15M sodium chloride, pH 7.5) and DIG or dual-labelled slides were blocked with 1% casein in maleic acid solution. Primary antibodies, reconstituted as per manufacturer’s instructions and diluted in maleic acid solution, were applied as a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated IgG fraction monoclonal mouse anti-biotin (Jackson ImmunoResearch Laboratories, Inc.) or 1:200 sheep anti-digoxigenin-rhodamine Fab fragments (Roche). After 30 min. incubation, slides were washed in 1X PBD three times for 2 min. at RT. If necessary, FITC amplification was performed using a 1:100 dilution of rabbit anti-mouse Fab fragments IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) followed by FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) with 15 min. incubations preceded by 5-15 min. blocking and followed by 1X PBD washes as above. Rhodamine amplification was performed using 1:100 dilution of rhodamine red-X-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories, Inc.). Chromosomes were counterstained with either 50 ng/mL 4’,6’-diamidino-2-phenylindole hydrochloride (DAPI) or 31.5 ng/mL DAPI III counterstain: Antifade solution (Vysis, Inc.), glass coverslips applied, and slides were stored at -20°C protected from light. Hybridization results were examined and analyzed using a Zeiss Axioplan2 fluorescent microscope and Cytovision©/Genus™ Application Software Version 2.7 (Applied Imaging).

G. FISH mapping controls

Several important controls were included to test the validity of the comparative gene mapping data obtained by FISH mapping. 1.) BAC probe hybridization to specific chromosomes must be repeatable to be valid. To control for repeatability, a minimum of 20 metaphase spreads were examined visually for each experiment, as described above, and up to ten images were digitally captured. For each metaphase spread with visible specific probe, the location on the chromosome pair needed to be consistent for a successful experiment. Experiments that did not yield consistent specific hybridization were repeated. 2.) To control for repeatability, each time a dual or multiple probe
combination was hybridized to metaphase spreads, the location of each probe was compared to results obtained in individually hybridized experiments for that probe. In all such cases, the probes hybridized consistently on their target chromosomes in each experiment. 3.) Specific probe hybridization has a classic appearance, with one signal per sister chromatid seen on each chromosome of a pair of chromosomes in metaphase as illustrated in Figure 1.3. However, occasionally one or both chromosomes would exhibit a single signal due to folding of chromatids during slide preparation. To control for altered probe appearance, again the location of signal on the chromosome pair was consistent on each metaphase spread with visible specific signal for each successful experiment. Experiments that did not yield consistent placement of probe hybridization on chromosome pairs were repeated.

Background signal is often seen, both in the microscopic field and on chromosomes. This background signal could be confused with specific signal. 4.) To control for misidentification of map location due to background signal, multiple metaphase spreads were examined, as described above. Signal that did not consistently appear on a specific site of the same chromosome in numerous metaphase spreads was considered background signal. Experiments that did not yield clear hybridization to specific chromosome loci due to excessive background were repeated.

5.) To control for increased background signal due to hybridization of repeated elements to metaphase spreads, the optimum amount of competitor DNA was determined, resulting in the 2-fold increase to 4 µg of equid competitor DNA being used per reaction. 6.) To control for increased background signal due to cross-reactivity of antibodies, the optimum order of antibody application was determined. 7.) To control for increased background signal due to amplification of antibody signal, this procedure was avoided when possible. Typically, primary antibodies were applied and slides processed as described above. Finally, each slide was examined by fluorescent microscopy. Only slides with those probes which required amplification were washed and secondary antibodies applied. 8.) The locations of genes and chromosome markers on the ECA gene map were used to predict locations on EPR chromosomes. If results conflicted with predictions, more metaphase spreads were examined, or hybridizations were repeated. 9.) To control for problems due to a weak probe signal, experiments were
repeated. Also, when necessary, new BAC DNA was prepared and labeled or a new labeling of BAC DNA was performed for the repeat experiments. Overall, experiments rarely needed to be repeated, when using freshly prepared BAC DNA and labeled probe.

III. Results

All of the 46 equine BACs hybridized to EPR chromosomes. The 46 BACs used included at least one probe from 38 of the 44 autosomal chromosome arms in ECA, and both arms of ECAX. A summary of BAC localizations on ECA, EPR and human genomes can be found in Table 2.2.

BAC probes located on ECA5 were hybridized to Przewalski’s horse metaphase chromosome spreads and examined by FISH. BAC clones containing the genes DIA1 (ECA5q17), LAMC2 (ECA5p17-p16), LAMB3 (ECA5p15), UOX (ECA5q15-q16), VCAM1 (ECA5q14), and VDUP1 (ECA5p12) were FISH mapped to Przewalski’s horse chromosomes. BAC probes containing genes from ECA5p and ECA5q hybridized to two separate acrocentric chromosome pairs, EPR23 and EPR24, respectively. For example, VDUP1 and VCAM1 identified two separate acrocentric chromosome pairs, as shown in Figure 2.1. Identification of the ECA5 homologues as EPR23 and EPR24 was based on G-banding patterns. Figure 2.2 depicts EPR G-banded chromosomes labeled with the equine BACs that were localized to each position by FISH mapping. No other rearrangements were found. Except for the differences involving ECA5 and its homologues EPR23 and EPR24, the distribution and order of the genes used in the study were the same for both species. As shown in Table 2.2, each ECA chromosome has one EPR homologue, with the exception of ECA5, which was shown to have two homologues, as discussed above.

IV. Discussion

The domestic horse and Przewalski’s wild horse diverged from a common ancestor within the past 1 MY (Oakenfull et al., 2000), and possibly as recently as a half MY (Ishida et al., 1995), based on the diversity of mitochondrial DNA sequences. This study showed that the genetic material from the metacentric ECA5 is contained in two
Table 2.2. ECA, EPR, and *Homo sapiens* map positions of equine BAC clones. References for ECA and HSA map positions can be found in Table 2.1.

<table>
<thead>
<tr>
<th>BAC clone</th>
<th>ECA map position</th>
<th>EPR map position</th>
<th>HSA map position</th>
</tr>
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<td>anonymous BAC</td>
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<td>2p</td>
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Table 2.2 (continued).

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<th>EPR map position</th>
<th>HSA map position</th>
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Figure 2.1. Fluorescently labeled BAC clones containing **VDUP1** (ECA5p12) and **VCAM1** (ECA5q14) hybridized to *E. przewalskii* chromosomes. **VDUP1** was labeled with FITC (green), and **VCAM1** was labeled with Rhodamine Red-X (red). Chromosomes were counterstained with DAPI III.
Figure 2.2.

- EPR1
- ECA1
- EPR2
- ECA2
- EPR3
- ECA3
- EPR4
- ECA4
- EPR5
- ECA6
- EPR6
- ECA8
- EPR7
- ECA10
- EPR8
- ECA7
- EPR9
- ECA9
- EPR10
- ECA11
- EPR11
- ECA12
- EPR12
- ECA13
- EPR13
- ECA14
- EPR14
- ECA15
- EPR15
- ECA16

- A4
- FES
- PKM
- ALPL
- SMARCA5
- GLG1
- UCHL1
- TCRG
- EN2
- INHA
- KRAS
- SART3
- TYMS
- AAT10
- AMD1
- LDHA
- LYVE-1
- HLR1, GH
- CHRM1
- POR
- PRM1
- LOX
- Septin2-like
- GLB1
Figure 2.2 cont..
Figure 2.2. Comparative gene map of *E. przewalskii*. G-banded EPR chromosomes (provided by CRES) and published ECA standard G-banded ideogram images are labeled with the equine BAC probes used in FISH mapping.
acrocentric chromosome pairs in EPR, namely EPR23 and EPR24. While these data do not distinguish between a fusion of ancestral acrocentric chromosomes to form ECA5 or a fission of the ancestral ECA5 homologue, the most parsimonious explanation for this phenomenon favors the second scenario. ECA5 contains a large segment of genetic material homologous to HSA1 (Raudsepp et al., 1996). Proposed ancestral mammalian karyotypes, based on molecular phylogenetics using DNA sequence variations and on reciprocal chromosome painting, suggest that the majority of HSA1 homologous genetic material was originally found on one ancestral mammalian chromosome (Murphy et al., 2001; Yang et al., 2003). Therefore, since the genetic material homologous to HSA1 was originally found on one ancestral chromosome, parsimony suggests that the ancestral HSA1 homologue subsequently fissioned and formed the two pairs of chromosomes found in the Przewalski’s horse karyotype.

This study did not identify any exceptions to homology between the domestic horse and Przewalski’s horse, except for ECA5. However, other rearrangements may have occurred and testing a single genetic marker on each chromosome does not provide high resolution. Therefore, it is possible that other rearrangements exist that would distinguish the organization of genes in these two species.

Because of the chromosome rearrangement and because chromosome identification in a karyotype is originally based on size, the nomenclature of chromosomes for the two species are different even though the chromosomes appear homologous. The ECA5 homologues in EPR are EPR23 and EPR24, while EPR5 is homologous to ECA6. Indeed, the G-banding patterns of EPR23 and EPR24 have similarities to the G-banding pattern of ECA5, as shown in Figure 2.3. The G-banding homologies between ECA and EPR are identified in Figure 2.2.

Chromosome painting using human chromosome specific libraries hybridized to horse chromosomes revealed that the human homologue for ECA5 includes part of HSA1, but HSA1 also had homology to ECA2p and ECA30 (Raudsepp et al., 1996). The HSA1 homology data for both the horse and the donkey is summarized in Figure 2.4. Microdissected horse chromosomes were hybridized to donkey chromosomes, leading Raudsepp and Chowdhary to deduce indirectly that ECA5 homologues include both the metacentric EAS16 and the acrocentric EAS25 (Raudsepp and Chowdhary,
Figure 2.3. EPR23 and EPR24 are the ECA5 homologues. EPR23 and EPR24 are arranged next to ECA5, illustrating the similarities in the G-banding patterns.
Figure 2.4. HSA1 has predicted homology to three *E. asinus* (EAS) chromosomes (EAS16, EAS25, and EAS5) (Raudsepp *et al*, 2000) and homology to three ECA chromosomes (ECA5, ECA2p, and ECA30) (Raudsepp *et al*, 1996). The bars to the sides of the chromosomes indicate regions of homology. ECA5 has homology to both EAS16 and EAS25, and ECA2p has homology to EAS5p, as shown by horse chromosome painting (Raudsepp and Chowdhary, 1999). Based on FISH mapping, ECA5p has homology to EPR23 and ECA5q has homology to EPR24.
The corresponding genes for laminin beta 3 (LAMB3) are located on ECA5p15 and HSA1q32, and LAMB3 was also FISH mapped to EAS25 (Raudsepp et al, 2001). However, to date no ECA5q markers have been mapped to donkey chromosomes.

Human chromosome painting probes were also applied to E. zebra hartmannae (EZH) metaphase spreads. These revealed that HSA1 has homology to 4 separate EZH chromosomes: EZH4, EZH7, EZH8, and EZH11 with deduced homology to ECA2, ECA5 and ECA30 (Richard et al, 2001). Indeed, the genetic material homologous to HSA1 is found in 4 homologous segments in EZH, and in 3 homologous segments in the horse, with the presumed ancestral eutherian karyotype containing two autosomes (Richard et al, 2001).

In conclusion, the only difference detected between ECA and EPR was a single rearrangement, with ECA5 having homology to both EPR23 and EPR24. However, the resolution provided by the FISH mapping done in this study is not sufficient to detect putative additional rearrangements such as inversions. The resolution of this comparative gene map would be enhanced by FISH mapping of additional markers.

A method used specifically for detection of inversions and other gross chromosomal rearrangements is the analysis of synaptonemal complexes in germinal tissue from hybrids. Synaptonemal complexes are found in meiotic cells, and consist of proteinaceous structures along homologous chromosomes. Inversions can be detected in synaptonemal complexes because the affected homologous chromosome pair will form an inversion loop that is visible by microscopy (Figure 2.5). Translocations may also be detected as an interaction of homologous sequences on nonhomologous chromosomes. Interspecies hybrids can be tested for areas of chromosomal homology using this technology (reviewed in Switonski and Stranzinger, 1998). A study of the synaptonemal complexes of an ECA/EPR hybrid would identify putative chromosomal inversions. The chromosomes containing such putative inversions could then be targeted for more precise molecular mapping to determine the extent of any rearrangement.
Figure 2.5. Hypothetical inversion loop seen during meiosis between a normal chromosome and the inverted homologous chromosome.
Chapter Three: Comparative gene mapping of the onager, *Equus hemionus onager*, compared to *E. caballus* by FISH

I. Introduction

The onager (*E. hemionus onager*, EHO), also known as the Persian wild ass, belongs to a group of three species in the genus *Equus*, collectively referred to as the Asiatic wild asses. The onager and the Transcaspian wild ass, *E. hemionus kulan* (EHK) or kulan, are sometimes considered subspecies of *E. hemionus* (Groves and Mazák, 1967). It is not clear if the differences between these two wild asses are the result of genetic drift over the last 100-200 years or a more ancient divergence (Eisenmann and Shah, 1996). Mitochondrial DNA sequence information suggests a very recent divergence (Oakenfull *et al*, 2000). The onager is an endangered species with its original range in Iran. A few hundred wild onagers may now be found in Iran and also in Israel where they were introduced in 1991 (Duncan, 1992).

As noted in Table 1.1, EHO has a modal diploid chromosome number of 56 (Ryder *et al*, 1978), but some individuals have been identified with only 55 chromosomes (Ryder, 1978). The difference in chromosome number in the karyotypes of such individuals occurs as an unpaired metacentric and two unpaired acrocentric chromosomes that morphologically resemble the “heterozygote” images in Figure 1.2. EHO23 and EHO24 were identified as the EHO chromosomes involved in the Robertsonian translocations in animals with the polymorphic number of diploid chromosomes (Ryder *et al*, 1978).

The present study was undertaken a.) to use FISH to construct a comparative gene map for EHO using the domestic horse, *E. caballus*, (ECA, a.k.a. *E. ferus*) as the reference point, and b.) to identify the polymorphic chromosomes in the EHO karyotype. The hypothesis tested was that the EHO genome organization would be similar to that of ECA, with the exception of fusions and fissions to explain the different number of diploid chromosomes in EHO (2n=56) as compared to ECA (2n=64). BAC probes have been used successfully to identify chromosome homology between ECA and *E. asinus* (EAS) by FISH mapping (Raudsepp *et al*, 1999). This approach was selected for
comparative mapping using an EHO individual with the chromosome number polymorphism (2n=55).

II. Materials and Methods

A. Cell culture and chromosome preparation

Metaphase chromosome spreads were prepared by CRES at the San Diego Zoo as previously described in the Materials and Methods section of Chapter Two for *E. h. onager* studbook #4791 with the following change. Cells at peak mitotic activity were harvested without colcemid exposure.

B. Giemsa staining

Slides were examined or Giemsa stained as previously described in the Materials and Methods section of Chapter Two.

C. BAC DNA preparation

The 52 equine BAC clones (Table 3.1) from 40 of 44 ECA autosome chromosome arms and both ECAX chromosome arms were selected for use for comparative gene mapping based on published reports of their location on ECA chromosomes. Refer to the Materials and Methods section of Chapter Two for details.

Of the 52 BACs used here, 6 were mapped for the first time in the horse (T.L. Lear, unpublished data) with the remaining 46 mapped to ECA in previously published studies (Shiue *et al.*, 1999; Marklund *et al.*, 1999; Godard *et al.*, 2000; Lear *et al.*, 2000; Mariat *et al.*, 2001; Lindgren *et al.*, 2001; Lear *et al.*, 2001; Milenkovic *et al.*, 2002; Hanzawa *et al.*, 2002; Chowdhary *et al.*, 2003). Of the total loci mapped, 49 were specific equine genes, two contained equine DNA in the form of anonymous BACs, and one was an expressed sequence tag (EST). A summary of information for the BACs used, including horse and human genome location, gene products, and references can be found in Table 3.1.

BAC clones were prepared as previously described in the Materials and Methods section of Chapter Two.
Table 3.1. Equine BAC clones used for comparative gene mapping of the onager.

<table>
<thead>
<tr>
<th>BAC clone</th>
<th>ECA map position</th>
<th>HSA map position</th>
<th>Locus name</th>
<th>Reference paper</th>
<th>BAC Source and ID</th>
</tr>
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<tr>
<td>A4</td>
<td>ECA1p</td>
<td></td>
<td>anonymous BAC</td>
<td>(Lear, personal comm.)</td>
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<tr>
<td>AAT10</td>
<td>ECA10p15</td>
<td>19q13.1</td>
<td>solute carrier family 7, member 10</td>
<td>(Hanzawa et al, 2002)</td>
<td>INRA638E6</td>
</tr>
<tr>
<td>ADD1</td>
<td>ECA3q26</td>
<td>4p16.3</td>
<td>adducin 1, alpha</td>
<td>(Lear et al, 2001)</td>
<td>INRA199C6</td>
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<tr>
<td>ALOX5AP</td>
<td>ECA17q14-q15</td>
<td>3q12</td>
<td>5-lipoxygenase-activating protein</td>
<td>(Mariat et al, 2001), (Milenkovic et al, 2002)</td>
<td>INRA170C8</td>
</tr>
<tr>
<td>ALPL</td>
<td>ECA2p14</td>
<td>1p36.1-p34</td>
<td>Alkaline phosphatase, liver/bone/kidney</td>
<td>(Mariat et al, 2001)</td>
<td>INRA20D8</td>
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<tr>
<td>AMD1</td>
<td>ECA10q21</td>
<td>6q21-q22</td>
<td>s-adenosylmethionine decarboxylase</td>
<td>(Lear et al, 2001)</td>
<td>INRA51A3</td>
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<td>BACD7</td>
<td>ECA14q12-q14</td>
<td></td>
<td>anonymous BAC</td>
<td>(Lear, personal comm.)</td>
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<td>CHRM1</td>
<td>ECA12q14</td>
<td>11q13</td>
<td>Muscarin acetylcholine receptor 1</td>
<td>(Milenkovic et al, 2002)</td>
<td>INRA105H6</td>
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<td>CHRNA</td>
<td>ECA18q24-q25</td>
<td>2q24-q32</td>
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<td>(Shiue et al, 1999)</td>
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<td>CTLA3</td>
<td>ECA21q13-q14</td>
<td>5q11-q12</td>
<td>cytotoxic T-lymphocyte associated serine esterase 3</td>
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<td>USDA151J1</td>
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<td>DIA1</td>
<td>ECA5q17</td>
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<td>diaphorase</td>
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<td>EN2</td>
<td>ECA4q27</td>
<td>7q36</td>
<td>engrailed 2</td>
<td>(Lear et al, 2001)</td>
<td>INRA175B2</td>
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<td>FES</td>
<td>ECA1q</td>
<td>15q26.1</td>
<td>v-fes feline sarcoma viral oncogene homolog</td>
<td>(Lear et al, 2000)</td>
<td>INRA52B7</td>
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<td>FN1</td>
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<td>2q34</td>
<td>fibronectin 1</td>
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<td>GGTA1</td>
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<td>9q33-q34</td>
<td>glycoprotein, alpha-galactosyltransferase 1</td>
<td>(Milenkovic et al, 2002)</td>
<td>INRA15C12</td>
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<td>GH</td>
<td>*ECA11p13</td>
<td>17q22-q24</td>
<td>growth hormone</td>
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<td>GLB1</td>
<td>ECA16q22</td>
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<td>galactosidase, beta-1</td>
<td>(Shiue et al, 1999)</td>
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<td>GLG1</td>
<td>ECA3p13-p12</td>
<td>16q22-q23</td>
<td>golgi apparatus protein</td>
<td>(Lear et al, 2001)</td>
<td>INRA50H12</td>
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<td>GNMT</td>
<td>ECA24q16</td>
<td>6p12</td>
<td>gysline N-methyltransferase</td>
<td>(Lear, personal comm.)</td>
<td>INRA226G6</td>
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<td>GPR3</td>
<td>ECA2p14</td>
<td>1p36.1-p35</td>
<td>G-protein-coupled receptor 3</td>
<td>(Mariat et al, 2001)</td>
<td>INRA91C10</td>
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<td>HESTG05</td>
<td>ECA29qter</td>
<td>EST</td>
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<td>HLR1</td>
<td>ECA11p13</td>
<td>17q23-q25</td>
<td>helicase RNA nuclear 1</td>
<td>(Lear et al, 2001)</td>
<td>INRA237E12</td>
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<td>IFNB1</td>
<td>ECA23q16-q17</td>
<td>9p21</td>
<td>interferon, beta 1, fibroblast</td>
<td>(Lear et al, 2001)</td>
<td>INRA238F2</td>
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<td>IGL@</td>
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<td>22q11.1-q11.2</td>
<td>immunoglobulin lambda</td>
<td>(Mariat et al, 2001), *(Lear, personal comm.)</td>
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<td>IL1B</td>
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<td>interleukin-1 beta</td>
<td>(Milenkovic et al, 2002)</td>
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<td>KRAS</td>
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<td>12p12.1</td>
<td>v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog</td>
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<td>LAMC2</td>
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<td>1q25-q31</td>
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<td>LDHA</td>
<td>ECA7p14.1-p13</td>
<td>11p15.4</td>
<td>lactate dehydrogenase A</td>
<td>(Milenkovic et al, 2002)</td>
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<td>LOX</td>
<td>ECA14q22</td>
<td>5q23-q31</td>
<td>lysi oxidase</td>
<td>(Lear et al, 2001)</td>
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<td>LYVE-1</td>
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<td>lymphatic vessel endothelial hyaluronen receptor 1</td>
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<td>MGF</td>
<td>ECA28q13</td>
<td>3p14.1-p12.3</td>
<td>mast cell growth factor</td>
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<td>MUT</td>
<td>ECA20q21</td>
<td>6p21</td>
<td>methylmalonyl CoA mutase</td>
<td>(Lear et al, 2001)</td>
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<td>OMG</td>
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<td>17q11.2</td>
<td>oligodendrocyte myelin glycoprotein</td>
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<td>PGK</td>
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<td>phosphoglycerate kinase 1 (PGK1)</td>
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<td>PKM</td>
<td>ECA1q21</td>
<td>15q22</td>
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<td>PLG</td>
<td>ECA31q12-q14</td>
<td>6q26</td>
<td>plasminogen</td>
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Table 3.1 (continued).

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<th>BAC clone</th>
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<td>POR</td>
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<td>(Milenkovic et al, 2002)</td>
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<td>PRM1</td>
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<td>16p13.2</td>
<td>protamine 1</td>
<td>(Lindgren et al, 2001)</td>
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<td>PROS1</td>
<td>ECA19q21</td>
<td>3p11-q11.2</td>
<td>Vitamin K dependent protein S</td>
<td>(Milenkovic et al, 2002)</td>
<td>INRA60B6</td>
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<td>RPN2</td>
<td>ECA22q17</td>
<td>20q12-q13.1</td>
<td>ribophorin II</td>
<td>(Chowdhary et al, 2003)</td>
<td>INRA0350D10</td>
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<td>SART3</td>
<td>ECA8p16-p15</td>
<td>12q24.1</td>
<td>squamous cell carcinoma antigen 3</td>
<td>(Lear et al, 2001)</td>
<td>INRA326B4</td>
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<td>Septin2-like</td>
<td>ECA15q12</td>
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<td>septin 2-like cell division control protein</td>
<td>unpublished data</td>
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<td>SMARCA5</td>
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<td>4q31.1-q31.2</td>
<td>SW1/SNF related, matrix associated</td>
<td>(Lear et al, 2001)</td>
<td>INRA281E7</td>
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<td>SOD1</td>
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<td>superoxide dismutase</td>
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<td>spectrin, beta, non-erythrocytic-1</td>
<td>(Lear et al, 2000)</td>
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<td>TCRG</td>
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<td>7p15-p14</td>
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<td>TGFB2</td>
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<td>transforming growth factor, beta 2</td>
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<td>TRAP170</td>
<td>ECAXp15-p14</td>
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<td>TYMS</td>
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<td>18p11.32</td>
<td>thymidine synthase</td>
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<td>UCHL1</td>
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<td>4p14</td>
<td>ubiquitin carboxyl-terminal hydrolase L1</td>
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<td>UGT1</td>
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<td>2q37</td>
<td>UDP glycosyltransferase 1</td>
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<td>VCAM1</td>
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<td>1p32-p31</td>
<td>vascular adhesion molecule 1</td>
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</table>
D. BAC probe preparation
BAC probes were prepared as previously described in the Materials and Methods section of Chapter Two.

E. Competitor DNA preparation
Competitor DNA was prepared from EHO genomic DNA samples as previously described in the Materials and Methods section of Chapter Two.

F. FISH mapping and analysis
FISH mapping and analysis were performed as previously described in the Materials and Methods section of Chapter Two.

G. FISH mapping controls
FISH mapping controls were previously described in the Materials and Methods section of Chapter Two.

H. Construction of a composite EHO karyotype
Chromosomes stained with DAPI have a morphology similar to but less distinct than that of G-banded chromosomes. To facilitate identification of DAPI-stained chromosomes, a composite G-banded EHO was constructed from published data (Figure 3.1). Two EHO karyotypes were scanned from the original publication (Ryder et al., 1978; Ryder, 1978). Two additional karyotypes were constructed from G-banded metaphase spread images from CRES.

I. Grouping of EHO chromosomes
Ryder and co-workers numbered the EHO chromosomes as shown in Figure 3.1 (Ryder et al., 1978; Ryder, 1978). However, the original numbering relied on G-banded chromosomes. It was difficult to identify all of the EHO chromosomes because of the reduced resolution seen with the DAPI staining that is essential for FISH mapping. In these experiments, groups of chromosomes could be reproducibly identified, but not necessarily distinguished individually. Due to limitations in the resolution of DAPI-
Figure 3.1 (continued).
Figure 3.1. Composite *E. hemionus onager* karyotypes. The first pair of chromosomes in each grouping is taken from Ryder et. al (Ryder, 1978), while the second pair of chromosomes in each grouping is taken from Ryder et. al (Ryder *et al.*, 1978). The final two pairs of chromosomes were taken from current G-banded onager karyotypes provided by CRES.
labeled chromosomes, the EHO chromosomes were placed into several groupings based on both size and distinctive morphology.

There were eleven EHO chromosomes which were unambiguously identified (Figure 3.2). The first five EHO chromosomes presented include large metacentric chromosomes, namely EHO1, EHO2, EHO3, EHO4, and EHO5. The unpublished data on EHO1 is included to facilitate discussion of comparisons between the horse and the onager (T. Lear, unpublished data). EHO5 was not distinctive when stained with DAPI, however, EHO4 was larger and EHO6 had a distinctive dark centromere, enabling definitive identification of this chromosome by size, allowing unambiguous assignment of these chromosomes. The next three EHO chromosomes (EHO19, EHO20, EHO21) are large telocentric chromosomes with very short p arms. EHO19 is the telocentric chromosome with a characteristic large central band. The large telocentric chromosome EHO20 was not distinctive when stained with DAPI, however both EHO19 and EHO21 were unambiguously identifiable enabling identification of this chromosome by size. EHO21 was a telocentric chromosome with a distinguishing q arm banding pattern. EHO23 and EHO24 were identified as the acrocentric chromosomes involved in the diploid chromosome number polymorphism (see Chapter 4). These two acrocentrics were distinguishable both by the probes identifying them on the EHO23;EHO24 chromosome and the fact that they were clearly two different sizes when compared directly in each metaphase spread. The single large metacentric chromosome EHOX was easily recognizable in this male onager.

‘Group A’: The EHO karyotype contains 4 medium metacentric chromosomes with a distinctive dark centromere when stained with DAPI. These four ‘Group A’ chromosomes, which are depicted in Figure 3.3b, include EHO6, EHO7, EHO8, and EHO9.

‘Group B’: There are seven medium to small metacentric chromosomes in the EHO karyotype without distinctive morphology when stained with DAPI. These seven ‘Group B’ chromosomes, which are depicted in Figure 3.4b, include EHO10, EHO11, EHO12, EHO13, EHO14, EHO15 and EHO16.

‘Group C’: The EHO karyotype contains three tiny metacentric chromosomes. Because of their much smaller size, the three tiny metacentric chromosomes, EHO17,
Figure 3.2. Individually identified *E. hemionus onager* chromosomes on the right with their *E. caballus* homologues as the ideogram on the left. Locations of BAC clone hybridization are identified with bars.
Figure 3.3.

Probes hybridizing to 'Group A' p arms: FN1/UGT1, GNMT, MGF
Probes hybridizing to 'Group A' q arms: AAT10, IFNB1

Probes:
- FN1/UGT1
- GNMT
- MGF
- AAT10
- IFNB1

Genes:
- LAMC2
- DIA1
- VCAM1
- AAT10
- IFNB1
- GNMT
- MGF
- DIA1
- VCAM1
- AAT10
- IFNB1
- LAMC2
- DIA1
- VCAM1
- AAT10
- IFNB1
- LAMC2
- DIA1
- VCAM1
- AAT10
- IFNB1

Chromosomes:
- EHO6
- EHO7
- EHO8
- EHO9
- ECA6
- ECA5
- ECA24
- ECA28
- ECA10
- ECA23

'A' p arm

'A' q arm
**Figure 3.3.** a.) Summary of results for ‘Group A’ hybridizations. b.) G-banded *E. hemionus onager* ‘Group A’ metacentric chromosomes with dark centromeres when DAPI-stained. c.-h.) Cartoon of EHO ‘Group A’ chromosomes on the right with *E. caballus* homologues as the ideograms on the left. Locations of BAC clone hybridization are identified with bars.
Probes hybridizing to ‘Group B’ p arms: GLG1, CHRM1, PRM1/POR
Probes hybridizing to ‘Group B’ q arms: MUT, ALPL/GPR3, ALOX5AP, CTLA3, TGFB2
Other ‘Group B’ probes: GLB1
Figure 3.4. a.) Summary of results for ‘Group B’ hybridizations. b.) G-banded *E. hemionus onager* ‘Group B’ metacentric chromosomes. c.-l. Cartoon of EHO ‘Group B’ chromosomes on the right with *E. caballus* homologues as the ideograms on the left. Locations of BAC clone hybridization are identified with bars.
EHO18, and EHO22, were distinguishable from the metacentric chromosomes in ‘Group B’ and have been placed in the separate ‘Group C’. These tiny metacentric chromosomes are depicted in Figure 3.5b as GTG-banded chromosomes.

‘Group D’: There are three tiny acrocentric chromosomes in the EHO karyotype. These three ‘Group D’ acrocentric chromosomes, which are depicted in Figure 3.6b, include EHO25, EHO26 and EHO27.

III. Results

All of the 52 equine BACs hybridized to EHO chromosomes. The 52 BACs used included at least one probe from 40 of the 44 autosomal chromosome arms of ECA, and both arms of ECAX. A summary of the BAC localizations in the horse, onager, and human genomes can be found in Table 3.2. To present a broad comparative mapping perspective on the FISH localizations, the results are presented as a comparison with domestic horse chromosomes as the standard.

A. Individually identified EHO chromosomes

Figure 3.2 shows the map positions of genes and chromosome markers to 11 EHO chromosomes that could be unambiguously identified based on chromosome morphology, banding pattern and size: EHO1, EHO2, EHO3, EHO4, EHO5, EHO19, EHO20, EHO21, EHO23, EHO24, and EHOX.

**EHO1** EHO1 was shown to be homologous to ECA1 (Lear et al., unpublished data). A4 (ECA1p) mapped to EHO1p, FES (ECA1q) mapped to EHO1q, and PKM (ECA1q21) mapped to EHO1q as depicted in Figure 3.2a.

**EHO2** As shown in Figure 3.2b, CHRNA (ECA18q24-q25) mapped to the terminal third of EHO2p. BACD7 (ECA14q12-q14) and LOX (ECA14q22) both mapped to the q arm of EHO2. BACD7 hybridized near the centromere of EHO2q while LOX mapped to the central third of EHO2q.

**EHO3** Figure 3.2c shows EHO3 homology to both ECA4 and ECA31. Two probes containing genes from ECA4, TCRG (ECA4p15-p14) and EN2 (ECA4q27), mapped to EHO3p and EHO3q, respectively. PLG (ECA31q12-q14) also mapped to EHO3p, but more terminally than TCRG.
Figure 3.5. a.) Summary of results for ‘Group C’ hybridizations. b.) G-banded *E. hemionus onager* ‘Group C’ tiny metacentric chromosomes. c.-g.) Cartoon of EHO ‘Group C’ tiny metacentric chromosomes on the right with *E. caballus* homologues as the ideograms on the left. Locations of BAC clone hybridization are identified with bars.
Figure 3.6. a.) Summary of results for ‘Group D’ hybridizations. b.) G-banded ‘Group D’ acrocentric chromosomes. c.) Cartoon of *E. hemionus onager* ‘Group D’ acrocentric chromosomes on the right with *E. caballus* homologues as the ideograms on the left. Locations of BAC clone hybridization are identified with bars.
Table 3.2. *E. caballus, E. hemionus onager,* and *H. sapiens* map positions of equine BAC clones. References for ECA and HSA map positions can be found in Table 3.1.

<table>
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<th>BAC clone</th>
<th>ECA map position</th>
<th>EHO map position</th>
<th>HSA map position</th>
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EH04) As shown in Figure 3.2d, *KRAS* (ECA6q21) mapped to EHO4p and *PROS1* (ECA19q21) mapped to EHO4qter.

EH05) *SART3* (ECA8p16-p15) hybridized to the very small p arm of the large metacentric chromosome EHO5, as shown in Figure 3.2e.

EH019) As shown in Figure 3.2f, *IGL@* (ECA7p13), *LDHA* (ECA7p14.1-p13), and *LYVE-1* (ECA7q16-q18) mapped to the q arm of EHO19. When hybridized in the same experiment, *LDHA* was located near the centromere of EHO19 while *LYVE-1* was located in the terminal quarter of EHO19. *IGL@* hybridized to EHO19pter.

EH020) As depicted in Figure 3.2g, *TYMS* (ECA8q12) hybridized to EHO20, while in the same experiment, *SART3* (ECA8p16-p15) hybridized to a separate large metacentric chromosome (EHO5).

EH021) Figure 3.2h shows the location of ECA11 probes mapped to the q arm of EHO21. *HLR1* (ECA11p13) and *GH* (ECA11p13) mapped to the terminal third of the EHO21 q arm, while *OMG* (ECA11q14) mapped near the centromere of EHO21q.

EH023, EH024) EHO23 and EHO24 were identified as the acrocentric chromosomes involved in the diploid chromosome number polymorphism (complete data and results are presented in Chapter 4). *SMARCA5* (ECA2q21) mapped to EHO24 as shown in Figure 3.2k, while *UCHL1* (ECA3q22) and *ADD1* (ECA3q26) both mapped to EHO23 as shown in Figure 3.2j. Additionally, *SMARCA5* also mapped to the p arm of the EHO23;EHO24 chromosome and *UCHL1* and *ADD1* mapped to the q arm of the EHO23;EHO24 chromosome (Figure 4.1).

EH0X) EHOX was easily recognizable in this male onager using the probes *TRAP170* (ECAXp) and *PGK* (ECAXq13-q14), with map locations for these probes shown in Figure 3.2i.

B. ‘Group A’ EHO chromosomes with dark centromeres

Seven experiments, including both single and dual probe, resulted in hybridization to the dark centromere ‘Group A’ chromosomes (Figure 3.3b).

1.) As depicted in Figure 3.3c, *UGT1* (ECA6p12) and *FN1* (ECA6p15) mapped to the same small p arm of a dark centromere metacentric chromosome.
2.) Figure 3.3e shows that GNMT (ECA24q16) mapped to the terminal end of the q arm of a dark centromere metacentric chromosome.

3.) As shown in Figure 3.3g, MGF (ECA28q13) mapped to the p arm of a dark centromere metacentric with distinctive terminal morphology.

4.) Figure 3.3d shows that VCAM1 (5q14) and DIA1 (5q17) mapped to the same q arm of a dark centromere metacentric chromosome with distinctive p arm morphology, with DIA1 located at qter and VCAM1 mapping to the center of the q arm. This chromosome resembles the metacentric with homology to ECA28. This observation could be confirmed with a dual probe experiment.

5.) While LAMC2 (ECA5p17-p16) also mapped to the q arm of a dark centromere metacentric chromosome, a dual probe experiment of both LAMC2 and VCAM1 showed that these probes map to separate onager chromosomes (Figure 3.3d).

6.) Figure 3.3f shows that AAT10 (ECA10p15) mapped to the p arm of a dark centromere metacentric chromosome while in the same experiment AMD1 (ECA10q21) mapped to a separate small metacentric chromosome in ‘Group C’.

7.) As shown in Figure 3.3h, IFNB1 (ECA23q16-q17) mapped to the q arm of a dark centromere metacentric chromosome, while in the same experiment CTLA3 (ECA21q13-q14) and GGTA1 (ECA25q17-q18) mapped to separate chromosomes in ‘Group B’ and ‘Group D’, respectively.

Figure 3.3a. summarizes the results of experiments for all the chromosomes in ‘Group A’.

C. ‘Group B’ EHO medium to small metacentric chromosomes

Ten experiments resulted in hybridization to ‘Group B’ chromosomes (Figure 3.4b).

1.) As shown in Figure 3.4c, GLG1 (ECA3p13-p12) mapped to the p arm of a ‘Group B’ metacentric chromosome.

2.) CHRM1 (ECA12q14) mapped to two different metacentric chromosomes in the onager. Figure 3.4d depicts CHRM1 hybridization on the p arm of the larger metacentric chromosome. Additionally, CHRM1 hybridized to a much smaller metacentric in ‘Group C’.
3.) In the dual probe experiment illustrated in Figure 3.4e, \textit{PRM1} (ECA13q14-q16) and \textit{POR} (ECA13p13) both hybridized to the p arm of the same metacentric chromosome. \textit{PRM1} was located more p-terminally than \textit{POR}.

4.) \textit{GLB1} (16q22) mapped to a metacentric chromosome, but the resolution of the chromosomes did not allow for assignment of a specific chromosome arm. These results are depicted in Figure 3.4f.

5.) Three probes with genes from the acrocentric ECA15 hybridized to a metacentric onager chromosome, as shown in Figure 3.4g. Both \textit{IL1B} (ECA15q13) and \textit{SPTBN1} (ECA15q22) were hybridized in the same experiment, while \textit{Septin2-like} (ECA15q12) was hybridized alone. \textit{Septin2-like} hybridized to the terminal end of the p arm of the metacentric chromosome, with \textit{IL1B} hybridizing closer to the centromere on the p arm. \textit{SPTBN1} hybridized to the central area of the q arm of the same metacentric chromosome.

6.) \textit{MUT} (ECA20q21) hybridized to the q arm of a ‘Group B’ metacentric chromosome (Figure 3.4h).

7.) \textit{ALPL} (ECA2p14) and \textit{GPR3} (ECA2p14) are both mapped to the same location in ECA. While separate experiments were performed, these two probes mapped to the q arm of a medium metacentric EHO chromosome, as shown in Figure 3.4i).

8.) \textit{ALOX5AP} (ECA17q14-q15) mapped to the q arm of a metacentric chromosome (Figure 3.4j).

9.) \textit{CTLA3} (ECA21q13-q14) mapped to the q arm of a metacentric chromosome (Figure 3.4k).

10.) \textit{TGFB2} (ECA30q14) mapped to two different metacentric chromosomes in the onager. The larger of the two metacentric chromosomes showed \textit{TGFB2} hybridization on the q arm (Figure 3.4L). The smaller metacentric chromosome was placed in ‘Group C’ (see below).

Figure 3.4a. summarizes the results of experiments for all the chromosomes in ‘Group B’.
D. ‘Group C’ EHO tiny metacentric chromosomes

Five experiments yielded hybridizations to chromosomes in ‘Group C’ (Figure 3.5b).

1.) In one experiment, **AMD1** (ECA10q21) mapped to the p arm of a tiny metacentric chromosome, in contrast to **AAT10** (ECA10p15) which mapped to the q arm of a ‘Group A’ metacentric chromosome with a dark centromere. The results of the AMD1 hybridization are shown in Figure 3.5c.

2.) **CHRMR1** (ECA12q14) mapped to the p arm of a tiny metacentric chromosome (Figure 3.5e).

3.) As shown in Figure 3.5g, **SOD1** (ECA26q15) mapped to the p arm of a tiny metacentric chromosome.

4.) Figure 3.5d shows that **RPN2** (ECA22q17) mapped to the q arm of a tiny metacentric chromosome.

5.) **TGFB2** (ECA30q14) mapped to two metacentric chromosomes. **TGFB2** mapped to the q arm of the smaller of the two chromosomes (Figure 3.5f), and also to the q arm of the larger chromosome in ‘Group B’.

Figure 3.5a. summarizes the results of experiments for all the chromosomes in ‘Group C’.

E. ‘Group D’ EHO tiny acrocentric chromosomes

Two experiments yielded hybridizations to one of the three ‘Group D’ chromosomes (Figure 3.6b). **HESTG05** (ECA29qter) and **GGTA1** (ECA25q17-q18) each mapped to one of these three largely indistinguishable tiny acrocentric chromosomes, as shown in Figure 3.6c.

Figure 3.6a. summarizes the results of experiments for the chromosomes in ‘Group D’.

F. Comparison of ECA and EHO gene and chromosome marker locations

ECA and EHO gene and chromosome marker locations are compared in Figure 3.7, providing a direct comparison of chromosome homology between ECA and EHO.
Figure 3.7.
Figure 3.7 (continued).

ECA7  EHO19

ECA8  EHO20

ECA9  ECA10

ECA12  ECA11  EHO21

EHO5

CHRM1

OMG  HLR1, GH

SART3  TYMS

LYVE-1  LDHA

AAT10

AMD1

IgL@  p arm

p arm

q arm
Figure 3.7. (continued).

ECA13

'B'

p arm

ECA14

BACD7

LOX

EHO2

ECA18

CHRNA

ECA15

IL1B

SPTBN1

ECA16

GLB1

ECA17

ALOX5AP

ECA18

CHRNA

BACD7

LOX

EHO2

ECA14
Figure 3.7. (continued).

ECA19

EHO4

ential.

ECA6

ECA20

'B'

ECA19

EHO4

ential.

ECA6

ECA20

'B'

q arm

ECA21

CTLA3

'ECA21

' B'

q arm

ECA22

RPN2

'ECA22

'C'

q arm

ECA23

IFNB1

'ECA23

'A'

q arm

ECA24

GNMT

'ECA24

'A'

p arm

ECA25

GGTA1

'ECA25

'D'

ECA26

SOD1

'ECA26

'C'

p arm
Figure 3.7 (continued).

ECA27

ECA28

ECA29

ECA30

ECA31

EH03

ECAX

EH0X
Figure 3.7. Published FISH results for *E. caballus* genes and chromosome markers are shown for 31 autosomes and the X chromosome along with FISH mapping results for *E. h. onager* from this study. For each horse chromosome, the ECA ideogram is shown on the left, with the genes and chromosome markers identified by their acronyms in the center (Table 3.1), and the EHO homologue shown on the right. Where the specific EHO chromosome has been unambiguously identified, this information is provided. Where the EHO chromosome has not been identified specifically, the chromosome group is identified. To the left of the EHO chromosomes ‘(ECA?)’ indicates that the homology to that EHO chromosome segment is not known with certainty. Where two ECA chromosomes have homology to one EHO chromosome, the second ECA chromosome is shown to the right to preserve the numerical progression for ECA chromosomes. [EHO chromosome groups: ‘A’ = EHO6–EHO9; ‘B’ = EHO10–EHO16; ‘C’ = EHO17, EHO18, EHO22; ‘D’ = EHO25–EHO27]
G. Corrected ECA map positions

The ECA map positions for three BAC probes were corrected by FISH mapping to ECA metaphase spreads (data not shown). GH mapped to ECA11p13, IGL@ mapped to ECA7p13, and TCRG mapped to both ECA30q14 and ECA6q21, as noted in Table 3.1.

IV. Discussion

This is the first time that a low density comprehensive comparative gene map has been prepared for EHO. Overall, 52 equine BAC clone probes were successfully hybridized to EHO metaphase spreads. Of these probes, 50 hybridized to a specific segment of chromosomal DNA on one pair of EHO chromosomes, while two probes hybridized to a specific segment of chromosomal DNA on two separate pairs of EHO chromosomes (Figure 3.7).

A. Chromosomes with conserved gene order and chromosome morphology

The gene order and chromosome morphology of four ECA chromosomes (ECA1, ECA25, ECA29, ECAX) have been conserved in four EHO chromosomes. For example, TRAP170 mapped to EHOXp and PGK mapped to EHOXq, as illustrated in Figure 3.7. Therefore, EHOX has the same relative gene placement as ECAX and EZHX, in contrast to EASX which has a rearrangement which results in TRAP170 mapping to EASXq (Raudsepp et al., 2002b).

B.) Chromosomes with altered centromere positioning

ECA7, ECA8q, and ECA11 are metacentric chromosomes in ECA. Pericentric inversions resulted in the three telocentric EHO chromosomes with minimal p arms, EHO19, EHO21, and EHO20, respectively. Probes from both ECA13p and ECA13q mapped to the p arm of a single metacentric ‘Group B’ EHO chromosome, suggesting that an additional fusion to form the q arm must also have occurred. ECA15 is an acrocentric chromosome in ECA but a pericentric inversion resulted in a metacentric ‘Group B’ chromosome in EHO with conserved gene order.

For example, GH and HLR1 hybridized to the same location on EHO21, prompting a review of the equine locus for GH (Caetano et al., 1999a) by FISH mapping
(data not shown) resulting in the corrected location of ECA11p13 listed in Tables 3.1 and 3.2. Since HLR1 and GH map to ECA11p while OMG map to ECA11q, the telocentric EHO21 may be the result of an inversion in the ECA11 homologue which changed the positioning of the centromere, resulting in very tiny p arms and a reversal of gene placement with HLR1 and GH on the terminal end of the q arm and OMG near the centromere, also on the q arm (Figure 3.7).

C. Fusions involving whole chromosomes

ECA4 and ECA31 appear to have fused to form the metacentric chromosome EHO3, while ECA14 and ECA18 appear to have fused to form the metacentric chromosome EHO2.

For example, TCRG (ECA4p) and PLG (ECA31) both hybridized to EHO3p, with PLG more terminal. EN2 (ECA4q) hybridized to EHO3q. FISH mapping showed that EAS1q has homology to ECA31, and EAS1q has homology to ECA4 (Raudsepp et al., 2001), suggesting that the gene order on EHO3 is similar to EAS and not to ECA, while the centromere position may be altered.

D. Chromosome fissions

Six ECA chromosomes appear to have fissioned and formed twelve EHO chromosome arms. Fission of two of the chromosome arms appeared to result in acrocentric chromosomes (ECA2q and ECA3q). One of the chromosome arms, ECA8q, appeared to have undergone a pericentric inversion that resulted in the telocentric chromosome EHO20. The other nine chromosome arms, ECA2p, ECA3p, ECA5p, ECA5q, ECA6p, ECA6q, ECA8p, ECA10p, and ECA10q, appeared to have undergone subsequent fusion to form metacentric EHO chromosomes.

For example, LAMC2, a BAC probe containing a gene from ECA5p, mapped to the q arm of one EHO metacentric chromosome, while both VCAM1 and DIA1, probes containing genes from ECA5q, mapped to the q arm of a different chromosome (Figure 3.7).
E. Chromosome fusions

Eleven ECA acrocentric chromosomes (ECA16, ECA17, ECA19, ECA20, ECA21, ECA22, ECA23, ECA24, ECA26, ECA28, ECA30) appear to have fused with other chromosome arms to form EHO metacentric chromosomes. It is also possible that some of these ECA acrocentric chromosomes have sustained a pericentric inversion which created the EHO metacentric chromosomes. A higher density of FISH mapping would be required to distinguish between these two alternatives.

For example, IFNB1 (ECA23q16-q17) hybridized to the q arm of a ‘Group A’ metacentric EHO chromosome. The location of this probe near the terminal end of the q arm is similar to the location of the IFNB1 gene on ECA23.

Additionally, ECA2q and ECA3q appear to have fused to form the metacentric chromosome involved in the chromosome number polymorphism seen in some onager individuals (refer to Chapter 4). The configuration of the EHO23;EHO24 metacentric chromosome is homologous to that of EAS3 as shown by homology to ECA2q and ECA3q (Raudsepp et al, 1999), and one donkey mother:daughter pair has exhibited a fissioned EAS3 (Bowling and Millon, 1988).

F. Gene duplication

One BAC probe containing a gene from ECA12q hybridized to the p arms of a ‘Group B’ and a ‘Group C’ metacentric chromosome in EHO. The consistent hybridization of the CHRM1 probe to two loci on EHO chromosomes may be due to a gene duplication event followed by a translocation event. It is also possible that there is a duplication of a repeat element, because each BAC clone contains intronic and exonic DNA in addition to the specific gene noted.

G. Correspondence of FISH mapping data with banding pattern predictions

Chromosome banding patterns are relied upon for chromosome identification (Burkholder, 1993). This research has shown agreement with one published prediction (Ryder et al, 1978) based on banding pattern homology, but is in contradiction with four published predictions (Ryder et al, 1978) also based on banding pattern homology (Table 3.3).
Table 3.3. Correlation of predicted homology to \textit{E. h. onager} chromosomes based on chromosome banding patterns. The first two columns report the predicted homology between EHO and \textit{E. asinus} chromosomes based on banding patterns (Ryder \textit{et al}, 1978). The third column presents published homology between \textit{E. caballus} and EAS (Raudsepp \textit{et al}, 2000; Raudsepp \textit{et al}, 2001). The fourth column presents the results of homology between ECA and EHO based on FISH (present study). The final column presents predicted homology between EHO and EAS based on deduction (this study). The deduced homology was obtained by comparing the ECA homology for EHO chromosomes to the reported ECA homology to EAS chromosomes. The homology supported by FISH mapping in EHO is shown in bold type. \textsuperscript{a} (Raudsepp \textit{et al}, 2001). \textsuperscript{b} (Raudsepp and Chowdhary, 1999). \textsuperscript{c} (Raudsepp \textit{et al}, 2000).

<table>
<thead>
<tr>
<th>\textit{E. h. onager} chromosome</th>
<th>\textit{E. asinus} homology predicted by banding</th>
<th>\textit{E. caballus} homology to \textit{E. asinus}</th>
<th>\textit{E. h. onager} homology to \textit{E. caballus}</th>
<th>Predicted \textit{E. h. onager} homology to \textit{E. asinus}</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHO4</td>
<td>EAS4</td>
<td>ECA18, ECA28 \textsuperscript{a}</td>
<td>ECA6p, ECA19</td>
<td>EAS19\textsuperscript{a}, EAS5\textsuperscript{a}</td>
</tr>
<tr>
<td>EHO19</td>
<td>EAS20</td>
<td>ECA7\textsuperscript{b}</td>
<td>ECA7</td>
<td>EAS20\textsuperscript{b}</td>
</tr>
<tr>
<td>EHO20</td>
<td>EAS22</td>
<td>ECA6q \textsuperscript{c}</td>
<td>ECA8q</td>
<td>EAS8q/EAS5p\textsuperscript{b}</td>
</tr>
<tr>
<td>EHO21</td>
<td>EAS23</td>
<td>ECA23 \textsuperscript{c}</td>
<td>ECA11</td>
<td>EAS13\textsuperscript{a,b}</td>
</tr>
<tr>
<td>EHO24</td>
<td>EAS25</td>
<td>ECA5p \textsuperscript{c}</td>
<td>ECA2p</td>
<td>EAS3q\textsuperscript{a}</td>
</tr>
</tbody>
</table>
For example, EHO4 was predicted to have homology to EAS4 (Ryder et al., 1978), an EAS chromosome with homology to ECA18 and ECA28 (Raudsepp et al., 2001). Instead, EHO4 had homology to ECA6p and ECA19, suggesting a deduced homology to EAS19 and EAS5. And EHO21 was predicted to have homology with EAS25 (Ryder et al., 1978), an EAS chromosome with homology to ECA5p (Raudsepp et al., 2001). Instead, EHO21 had homology to ECA11, suggesting a deduced homology to EAS13. However, the prediction that EHO19 would have homology to EAS20 was supported by FISH mapping, in that both chromosomes have homology to ECA7 (Raudsepp and Chowdhary, 1999, present study). While banding patterns led to predictions of homology, FISH mapping provided definitive identification of homology between EHO and ECA chromosomes.

H. Conclusions

In conclusion, the comparative gene map of EHO has revealed some similarities with the ECA genome, but also with EAS. Additionally, EHO has some chromosome configurations that are not seen within the other equids for which comparative mapping data are available. Further work is needed to detect homology to the remaining five ECA chromosome arms and this would help to expand this comparative gene map. In addition, selected experiments with dual or multiple probes should be able to distinguish between the chromosomes currently grouped by morphology. Three markers published for the ECA gene map have been corrected as a result of the findings of this work, adding to current knowledge in this area. Finally, this work significantly adds to comparative gene mapping in the Equidae, which previously was based on data on ECA, EAS, and *E. zebra hartmannae* exclusively.
Chapter Four: Homologous fission event(s) implicated for chromosomal polymorphisms among 5 species in the genus Equus

I. Introduction

Each of the equid species has a unique, modal number of chromosomes. However, polymorphisms for chromosome number have been found among normal, healthy members of the hemiones: the onager (*E. hemionus onager*, EHO) (Ryder, 1978), the kulan (*E. hemionus kulan*, EHK) (Ryder, 1978), and the kiang (*E. kiang*, EKI) (Ryder and Chemnick, 1990); as well as in the Somali wild ass (*E. africanus somaliensis*, EAF) (Houck *et al*, 1998) and Burchell’s zebra (*E. quagga burchelli*, EQB) (Whitehouse *et al*, 1984) (Table 1.1).

The karyotypes of individuals heterozygous for a centric fission have an unpaired large metacentric chromosome and two unpaired acrocentric chromosomes. This study was initiated to determine if the chromosome number polymorphisms seen in *E. hemionus onager*, *E. hemionus kulan*, *E. kiang*, *E. africanus somaliensis*, and *E. quagga burchelli* involved homologous or nonhomologous chromosomes. Fluorescently labeled DNA probes based on domestic horse sequences have been shown to work well for gene mapping in other equids (Raudsepp *et al*, 2002). Therefore, horse BAC clones, each containing a horse gene previously mapped to a specific horse chromosome, were mapped by FISH to the chromosomes of polymorphic individuals with an odd number of chromosomes, and to some non-polymorphic individuals.

In connection with this dissertation study, and reported in Chapter Three, two horse BAC clones were mapped to the polymorphic chromosomes of an onager with 2n=55. One of these horse BAC clones contained the SW1/SNF related, matrix associated gene, SMARCA5 (ECA2q21 homologue of HSA4q31.21; (Lear *et al*, 2001) and the other contained the ubiquitin carboxyl-terminal esterase L1 gene, UCHL1 (ECA3q22 homologue of HSA4p13; (Lear *et al*, 2001). These same probes were used to investigate the chromosome polymorphisms in other equids and to determine whether or not they were homologous to those found in the onager.
II. Materials and methods

A. Chromosome preparations

Metaphase chromosome spreads were prepared by CRES at the San Diego Zoo for *E. hemionus onager* #4791 (2n=55), *E. hemionus kulan* #3939 (2n=55), *E. kiang* #12336 (2n=51) and #9630 (2n=52), *E. africanus somaliensis* #11061 (2n=63) and #4634 (2n=64), *E. quagga burchelli* #10652 (2n=45), *E. zebra hartmannae* #6482 (2n=32) and #10833 (2n=32), and *E. grevyi* #4931 (2n=44) as previously described in the Materials and Methods section of Chapter Two with the following change. Cells at peak mitotic activity were exposed to colcemid (Gibco) at 0.25 $\mu$g/mL, followed by incubation from 0-105 min.

B. BAC probes

DNA was prepared from two equine BAC clones, obtained from Institut National de la Recherche Agronomique (INRA). Refer to the Materials and Methods section of Chapter Two for BAC library details. The SMARCA5 BAC (INRA281E7) was previously mapped to ECA2q21, and the UCHL1 BAC (INRA208G12) was previously mapped to ECA3q22 (Lear *et al.*, 2001).

C. FISH mapping and analysis

BAC probe labeling, FISH mapping, and analysis were performed as previously described in the Materials and Methods section of Chapter Two.

III. Results

BACs containing the genes SMARCA5 and UCHL1 hybridized to three different chromosomes in individual equids known to exhibit the polymorphism: *E. hemionus onager* with 2n=55, *E. kiang* with 2n=51, *E. hemionus kulan* with 2n=55, *E. africanus somaliensis* with 2n=63, and *E. quagga burchelli* with 2n=45 (Figure 4.1). SMARCA5 hybridized to the p arm of a single metacentric and its acrocentric homologue while UCHL1 hybridized to the q arm of the same metacentric and its acrocentric homologue. The position of each probe on the metacentric chromosome appeared to correspond to a similar position on the acrocentric chromosome. In addition, SMARCA5 and UCHL1
Figure 4.1. Homologous chromosome polymorphism in a) *E. hemionus onager*, 2n=55; b) *E. kiang*, 2n=51; c) *E. hemionus kulan*, 2n=55; d) *E. africanus somaliensis*, 2n=63; and e) *E. quagga burchelli*, 2n=45. The SMARCA5 probe is labeled with FITC while the UCHL1 probe is labeled with rhodamine red-X.
were FISH mapped in *E. przewalskii* (EPR), *E. grevyi* (EGR) and *E. zebra hartmannae* (EZH). In EPR, SMARCA5 and UCHL1 hybridized to the q arms of two metacentric chromosome pairs (see Chapter Two), while in both EGR and EZH, SMARCA5 and UCHL1 hybridized to opposite arms of one metacentric pair (Figure 4.2).

**IV. Discussion**

The chromosome configuration of the HSA4 homologue is known for all extant equid species, following the results in this study. Raudsepp and co-workers demonstrated that HSA4 in domestic horses is split between ECA2q and ECA3q (1996), as shown in this study for EPR. Meanwhile, HSA4 homologous DNA is present as a single metacentric chromosome in *E. asinus* (EAS) (Raudsepp and Chowdhary, 1999), EZH (Richard *et al.*, 2001) and EGR (this study). HSA4 homologues have been conserved as single chromosomes or in large segments in many species. For example, large portions of HSA4 have been conserved on chromosome 4 of the domestic chicken (*Gallus gallus domesticus*) (Chowdhary and Raudsepp, 2000), and on chromosome 8 of the domestic pig (*Sus scrofa*) (Larsen *et al.*, 1999). However, HSA4 homologous DNA is divided amongst BTA6, BTA17, and BTA27 in domestic cattle (Fisher *et al.*, 1997; Sonstegard *et al.*, 2000).

The results described above (Figure 4.1) demonstrated that homologous chromosomes were involved in the chromosome polymorphism of five extant equid species. The results for EGR and EZH (two probes mapped to opposite arms of a single pair of metacentric chromosomes) were homologous to those found in EAS by both cross-species painting and FISH mapping (Raudsepp *et al.*, 1999; Raudsepp *et al.*, 2001) and with human chromosome paints to EZH (Richard *et al.*, 2001), while EPR results were homologous to those found in ECA, as reported in Chapter Two.

Balanced chromosome polymorphisms are relatively uncommon but have been described in other species. A common example of a balanced chromosome polymorphism is the 1;29 Robertsonian translocation found in domestic cattle (*Bos taurus*, BTA). In some individuals, the smallest bovine chromosome, BTA29, has fused with the largest bovine chromosome, BTA1 (Gustavsson and Rockborn, 1964). Daughters of bulls with rob(1;29) experience lowered fertility, but the polymorphism
Figure 4.2. Homologous chromosomes in a) *E. grevyi*, 2n=46; and b) *E. zebra hartmannae*, 2n=32. The SMARCA5 probe is labeled with FITC while the UCHL1 probe is labeled with rhodamine red-X.
persists in some herds of domestic cattle (Weber et al., 1989). Balanced chromosome polymorphisms have been identified in other mammalian species as well, such as oryx (Oryx dammah and O. leucocryx) (Kumamoto et al., 1999), gazelle (Gazella subgutturosa marica, G. bennetti, and G. saudiya) (Vassart et al., 1993; Kumamoto et al., 1995), the rock wallaby (Petrogale lateralis pearsoni) (Eldridge and Pearson, 1997), domestic sheep (Ovis aries) (Koop et al., 1983), and the owl monkey (Aotus) (Ma et al., 1976), but in all cases are relatively uncommon. One out of four species in the waterbuck, genus Kobus, (K. ellipsiprymnus) exhibited two polymorphic centric fusions (Kingswood et al., 2000). A balanced chromosomal polymorphism has been reported in another Perissodactyl, the northern white rhinoceros (Ceratotherium simum) (Houck et al., 1994), and efforts to determine if the rhinoceros polymorphism is homologous to the equids is in progress (Lear et al., personal communication). To our knowledge, Equus is the only genus with a confirmed homologous centric fission polymorphism in several species.

The discovery of the same chromosome polymorphism in five closely related equid species separated by as many as 3 MY of evolution is remarkable. The polymorphisms could be the result(s) of fission of a single metacentric chromosome resulting in two acrocentric chromosomes or of fusion of two acrocentric chromosomes forming a single metacentric chromosome. The two possible events are depicted in Figures 4.3a and 4.3b. The premise for the following hypotheses is that the ancestral karyotype contained the genetic material of the HSA4 homologue in a metacentric chromosome (Chowdhary et al., 1998; Murphy et al., 2001; Yang et al., 2003). If the ancestral HSA4 homologue was metacentric, then parsimony favors fission of that metacentric chromosome, an event that resulted in the two acrocentric chromosomes with homology to HSA4. With respect to fission, two opposing hypotheses may account for the existence of these polymorphic chromosomes: 1) a single ancestral fission or 2) multiple, independent fissions. The ancestral fission hypothesis, illustrated in Figure 4.3a, suggests that the polymorphism occurred once in an ancestral equid species. Essentially, one metacentric chromosome from a pair homologous to HSA4 in the ancestral equid could have undergone a fission event, resulting in the polymorphism seen as a single metacentric and two acrocentric chromosomes. Also, this hypothesis
Figure 4.3a. Model for ancestral fission event hypothesis.

Figure 4.3b. Model for ancestral fusion event hypothesis.
Figure 4.3. Fission/fusion hypothesis models. a.) Model for ancestral fission event hypothesis. In this model, the polymorphism arose by a fission event in the HSA4 homologue in the ancestral equid species. Subsequently, the polymorphism was fixed and maintained in EHO, EHK, EKI, EQB, and EAF. However, the fission event was followed by a fusion event with different chromosome segments, leading to the configuration in ECA and EPR with the HSA4 homologous DNA found in two different arms in two metacentric chromosome pairs. The metacentric condition seen in EAS, EGR, and EZH may represent the ancestral condition prior to the fission event, or a fixation of the metacentric chromosome following the fission event. b.) Model for ancestral fusion event hypothesis. In this model, the polymorphism arose by a fusion event which fused two acrocentric chromosomes with homology to HSA4 in the ancestral equid species. Subsequently, the polymorphism was fixed and maintained in EHO, EHK, EKI, EQB, and EAF. However, the fusion event was followed by a second fusion event with different chromosome segments, leading to the configuration in ECA and EPR with the HSA4 homologous DNA found in two different arms in two metacentric chromosome pairs. The metacentric condition seen in EAS, EGR, and EZH may represent the fixation of the metacentric chromosome following the fusion event.
suggests that the polymorphism would have been maintained throughout speciation of EHO, EHK, EKI, EQB, and EAF, and that these extant species carry the legacy of the ancestral fission event. EAS, EGR, and EZH have metacentric pairs of chromosomes homologous to the ancestral HSA4 homologue. Finally, before the speciation events leading to ECA and EPR, a fusion event could have occurred resulting in the current situation in the horses, namely that the HSA4 homologous arms are found in two separate chromosomes, ECA2 and ECA3.

The independent fission hypothesis would have involved multiple, and possibly as many as 5, independent fission events in the extant equid species or their ancestors. Furthermore, independent fissions of this chromosome would suggest that some characteristic of the HSA4 homologue in the equids renders it susceptible to fission. This hypothesis is supported by the occurrence of de novo fissions of the HSA4 homologue found in a donkey foal (EAS) (Bowling and Millon, 1988) and in a Somali wild ass (Houck et al, 1998).

Determining which of these historical events occurred may be difficult. Studies of DNA sequences in mitochondria are useful in suggesting a sequence of events and times of divergence for the different equid species (Oakenfull et al, 2000). However, chromosomal genes can participate in genetic recombination which destroys haplotype associations. Bailey et al. (2002) reported that chromosome rearrangements associated with the evolution of primates resulted in segmental duplications of genomic DNA sequences. If the fusions or fissions in equid evolution produced similar complex features then discovery of these features may suggest which chain of events occurred.
Chapter Five: Conclusions

The extant species in the genus *Equus* have evolved within the past 3.7 MY (Bowling and Ruvinsky, 2000). Their evolution was associated with a particularly rapid rate of chromosome evolution. While the estimated rate of chromosome changes for the horse (0.2 changes per MY) is well within the estimated rates of chromosome changes for vertebrates (0.1-2.3 changes per MY), these estimates compare rates between vertebrate lineages and not within specific lineages (Burt *et al*., 1999). Another estimate, based on the number of living species, the fossil record for the genus, and information on extinction rates, puts the rate of chromosome evolution within the genus *Equus* at a much higher 0.6-0.8 changes in chromosome and chromosome arm numbers per MY, (Bush *et al*., 1977).

To find similar wide ranges of chromosome number differences within a family, we need to look at lemurs where the diploid chromosome number ranges from 2n=20 to 2n=70 (Kolnicki, 1999), or the canids with diploid chromosome numbers ranging from 2n=34 to 2n=78 (Todd, 1970). However, those species are separated by 15 MY or more. In contrast, humans and great apes have highly conserved chromosome numbers with diploid chromosome numbers ranging from 2n=46 to 2n=48 (Dutrillaux, 1979; De Grouchy, 1987) and almost all the felids, from domestic cats to Siberian tigers, have 38 chromosomes (Vinogradov, 1998; Gregory, 2001). What are the events or evolutionary changes that caused such rapid chromosome change among the equids?

The equids have diploid chromosome numbers ranging from 2n=32 to 2n=66 (Benirschke and Malouf, 1967; Ryder *et al*., 1978). Since the natural modern range of equids extends from northwest Asia to southern Africa, and the number of chromosomes in those species generally shows a steady decline as they approach the equator, Ryder asked whether there could be some influence of environment on chromosome number (O.A. Ryder, personal communication)? These broad questions cannot be answered at present, but identification of the comparative genome organization of these equids may shed light on their patterns of evolution.

The research presented in this study has posed three specific questions concerning the evolution of equids, namely:
1.) Are the differences between *E. caballus* (ECA) and *E. przewalskii* (EPR) sufficient to consider them as separate species?

2.) What is the extent of chromosome evolution seen when comparing the genome organizations of ECA and *E. h. onager* (EHO), as detected as changes in gene position?

3.) To what extent are the chromosome number polymorphisms identified in *Equus* representing the same or different events?

The present studies did not completely answer those questions. However, these data added to the current understanding of genome organization and chromosome evolution in *Equus*, allowing us to ask more focused questions.

For example, results of this research demonstrate that the chromosome number polymorphisms found in five equid species (Ryder, 1978; Whitehouse *et al*, 1984; Ryder and Chemnick, 1990; Houck *et al*, 1998) all contain homologous DNA segments (see Chapter Four). These findings raise interesting evolutionary questions such as: Are the polymorphic chromosomes ancient and conserved throughout the rapid speciation of the equid species or are they the result of multiple independent fissions in five equid species? If the polymorphism is a conserved feature, one would predict that there must be some benefit to having a balanced chromosome polymorphism in the population to offset the decrease in fertility expected due to nondisjunction during meiosis. If the polymorphism is due to multiple independent fissions, one would predict that there was some aspect of the HSA4 homologue or its centromere which is prone to fission.

Comparison of the results for *E. przewalskii* and *E. caballus* raises the issue of the definition of a species. Some researchers have claimed that the two horses are subspecies of *Equus ferus*, while others have maintained that they are separate species. The protamine P1 amino acid sequences are identical in both horses (Pirhonen *et al*, 2002). There is substantial overlap of mitochondrial DNA sequences between the two horses (Ishida *et al*, 1995) (Oakenfull and Ryder, 1998), and the 12S rRNA (Oakenfull and Ryder, 1998) and α2 globin DNA (Oakenfull and Clegg, 1998) sequences are identical. However, there are some mitochondrial D-loop DNA sequences specific to *E. przewalskii* (Jansen *et al*, 2002) and studies of cranial morphology (Eisenmann and Baylac, 2000) and limb and scapular morphology (Sasaki
et al, 1999) have shown differences between *E. caballus* and *E. przewalskii* suggesting distinct morphological characters for the two horses.

In some respects, the issue of determining a species is a human decision, and there are many different limits which can be set to define a species. For example, *E. przewalskii* and *E. caballus* can produce viable and fertile offspring (Chandley et al, 1975). Some species definitions argue that two organisms are a single species if they can reproduce and produce fertile offspring (Mayr, 1963). In this respect, the two horses could be considered one species with two subspecies. Another aspect of species determination is related to the number of chromosomes which characterize two closely related groups. In this respect, with differing diploid chromosome numbers (Benirschke et al, 1965), the two horses would remain two separate species. A final aspect to this debate is that fact that humans have eliminated all wild populations of *E. przewalskii*. Perhaps *E. przewalskii* should be conserved solely on the basis of their value to humans as examples of the last wild horse.

The findings of these studies tend to support the hypothesis that *E. caballus* and *E. przewalskii* are very similar and could be defined as the same species or at least two subspecies. However, the resolution of the comparative gene map does not rule out additional chromosome rearrangements which would lend support to arguments that the two horses are separate species. The density of this comparative gene map must be increased by mapping more genes and chromosome markers on *E. przewalskii*.

One way to look at genome organization in a quantitative manner is by constructing a karyograph. Figure 5.1 presents a karyograph for the genus *Equus*. The karyograph method is used to correlate chromosome number and the number of chromosome arms in a genus, resulting in a quantitative analysis of karyotypes. The karyograph method was originally proposed by Imai and Crozier for analysis of mammalian karyotype evolution (Imai and Crozier, 1980). Essentially, the karyotype is represented on a karyograph as the point (2AN, 2n) where 2AN represents the total arm number per diploid karyotype for a species and 2n represents the diploid chromosome number for that species. 2AN is plotted on the X-axis and 2n is plotted on the Y-axis for each species. The point will move vertically on the graph as 2n increases by centric fission or decreases by centric fusion, or Robertsonian rearrangements. The point will
Figure 5.1. Karyograph for equid species where diploid chromosome arm number (2AN) is plotted on the X-axis and diploid chromosome number (2n) is plotted on the Y-axis.
move to the right on the graph as 2AN increases due to pericentric inversions, or inversions which change acrocentric chromosomes into metacentric chromosomes. Imai proposes that the direction of pericentric inversions is strongly towards the formation of metacentrics (Imai et al, 2001).

The karyograph for the ten members of the genus Equus indicates several chromosome rearrangement events between the species (Figure 5.1). Analysis as described above suggests that E. caballus and E. przewalskii, E. grevyi and E. quagga burchelli, and E. h. onager and E. h. kulan karyotypes differ by single Robertsonian rearrangements. Additionally, the analysis suggests that E. africanus somaliensis and E. asinus differ by a pericentric inversion, and indeed E. asinus has two additional metacentric chromosomes and two fewer acrocentric chromosomes than E. africanus somaliensis.

Larger differences in chromosome numbers between the species are also evident in the karyograph. These differences could be explained by a karyotypic fissioning event during meiosis if each metacentric chromosome fissions into two acrocentric chromosomes, with the resulting karyotype containing exclusively acrocentric chromosomes (Todd, 1970; Kolnicki, 2000). Karyotypic fissioning may provide one explanation for rapid chromosome evolution because the resulting karyotype would have a greatly increased chromosome number as compared to the ancestral genome (Godfrey and Masters, 2000). Indeed, Kolnicki has proposed karyotypic fissioning to explain the wide range of diploid chromosome numbers in lemurs (Kolnicki, 1999).

Equus has experienced rapid karyotype evolution in its 3.7 MY history (Bush et al, 1977). However, there are insufficient data to test the hypothesis that karyotypic fissioning may have occurred during equid karyotype evolution. Comparative gene mapping for all equid species could provide sufficient data to test this hypothesis more fully. To characterize the genome organization for all equids, the limitations of chromosome banding must be considered and care must be taken to provide a FISH map with sufficient resolution to be able to draw conclusions concerning karyotypic fissioning.
Chromosome banding is a powerful cytogenetic technology which can help researchers characterize the chromosomes in a genome (Burkholder, 1993). For example, similarities in chromosome banding patterns suggested that the chromosomes involved in the chromosome number polymorphism were homologous in five equid species (M. Houck, personal communication), and FISH confirmed this prediction. However, this research has demonstrated many differences in homology from that predicted by banding patterns. For example, EHO24 was predicted to have homology to EAS25 by banding patterns (Ryder et al., 1978). This prediction was not supported because EAS25 has homology to ECA5p (Raudsepp et al., 2001) while EHO24 showed homology to ECA2 by FISH. While chromosome banding remains critical for chromosome identification, the presence of similar banding patterns on chromosomes in different species cannot be relied upon to predict homology, and therefore cannot be solely relied upon to detect changes in chromosome organization between species, as had been thought in the past.

This research also utilized FISH technology, a technology which can be used to determine the homology of DNA segments in related species. FISH has a resolution of 1-10 Mb depending on the contraction of the metaphase chromosomes. A minimum of 1 Mb between sites on a metaphase chromosome is required to distinguish between adjacent probes hybridized to the target metaphase spreads (Heiskanen et al., 1996). In these studies, the resolution was low because only one or two markers were used per chromosome arm. At this resolution, only gross morphological changes in chromosome organization can be detected. Intrachromosomal rearrangements can only be detected if the region of chromosome involved has many markers available. Therefore, at the resolution used here, both *E. przewalskii* and *E. hemionus onager* genomes may contain internal rearrangements as compared to *E. caballus* which further comparative gene mapping may bring to light. Higher resolution may be required to rigorously test the karyotypic fissioning hypothesis for the equids.

Future research goals include:

a.) Extension of the *E. przewalskii* comparative gene map by investigating internal chromosomal rearrangements and to address the unmapped *E. caballus* chromosome arms, ECA9p, ECA9q, ECA11q, ECA12p, and ECA27.
b.) Extension of the *E. hemionus onager* comparative gene map to address the unmapped *E. caballus* chromosome arms, ECA9p, ECA9q, ECA12p, and ECA27, and to identify unambiguously the grouped *E. h. onager* chromosomes in dual and multiple probe FISH experiments.

c.) Further investigations to help determine if the chromosome number polymorphism is ancient and conserved or recent and independent in each of the five equid species. If the polymorphism is ancient and conserved, sequencing genes near the centromere of the metacentric HSA4 homologues would be predicted to yield diversity between the equid species. If the polymorphism is recent and independent in each of the five species, gene sequences near the centromere would be predicted to yield little or no diversity in the metacentric HSA4 homologues. Recombination is reduced near the centromere, so considerable time would need to have passed for significant change in gene sequences near the centromere.

d.) Extension of comparative gene maps for all remaining equid species to gain sufficient information both to propose an ancestral equid genome organization and to test the hypothesis that karyotypic fissioning contributed to the rapid karyotypic evolution in *Equidae*. This research has provided the first comprehensive comparative gene maps for both *E. przewalskii* and *E. h. onager*. Some gene mapping data are available for *E. asinus* (Raudsepp *et al*, 1999; Raudsepp *et al*, 2001), and *E. caballus* chromosome paints have been applied to *E. zebra hartmannae* chromosomes (Richard *et al*, 2001). To date, no comparative mapping data has been published for *E. h. kulan*, *E. kiang*, *E. africanus somaliensis*, *E. grevyi*, or *E. quagga burchelli*. 

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APPENDIX A

Appendix A lists the names and addresses of collaborators who contributed to this dissertation.

<table>
<thead>
<tr>
<th>Collaborators</th>
<th>Address</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>
### APPENDIX B

Appendix B lists the names and addresses of companies cited in this dissertation.

<table>
<thead>
<tr>
<th>Company</th>
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<tbody>
<tr>
<td>Applied Imaging Corporation</td>
<td>Santa Clara, California</td>
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<tr>
<td>BDH Limited</td>
<td>New York, New York</td>
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<td>Beckman Coulter, Inc.</td>
<td>Fullerton, California</td>
</tr>
<tr>
<td>Clonetics (Cambrex Corporation)</td>
<td>East Rutherford, New Jersey</td>
</tr>
<tr>
<td>Gibco (Invitrogen)</td>
<td>Carlsbad, California</td>
</tr>
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<td>Fisher Scientific</td>
<td>Pittsburgh, Pennsylvania</td>
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<td>Jackson ImmunoResearch Laboratories Inc.</td>
<td>West Grove, Pennsylvania</td>
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<tr>
<td>Life Technologies (Invitrogen)</td>
<td>Carlsbad, California</td>
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<tr>
<td>QIAGEN Inc.</td>
<td>Valencia, California</td>
</tr>
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<td>Roche (F. Hoffman – La Roche Ltd.)</td>
<td>Basel, Switzerland</td>
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<td>Savant (Thermo Savant)</td>
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<td>Sigma Chemical Company</td>
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<td>Ventana Medical Systems, Inc.</td>
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<td>Vysis, Inc.</td>
<td>Downer’s Grove, Illinois</td>
</tr>
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<td>Zeiss</td>
<td>Thornwood, New York</td>
</tr>
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</table>
APPENDIX C

Appendix C lists abbreviations used in this dissertation.

2AN .............................................................................................................................. diploid chromosome arm number
2n ................................................................................................................................ diploid chromosome number
BAC .............................................................................................................................. bacterial artificial chromosome
bp ................................................................................................................................. base pairs
CRES .......................................................... Center for the Reproduction of Endangered Species
DAPI ................................................................. 4',6'-diamidino-2-phenylindole hydrochloride
DIG ................................................................................................................................ digoxigenin
EAF .............................................................. Equus africanus somaliensis
EAS ............................................................................................................................. Equus asinus
ECA ............................................................................................................................. Equus caballus
EGR ............................................................................................................................. Equus grevyi
EHK ............................................................................................................................. Equus hemionus kulan
EHO ............................................................................................................................. Equus hemionus onager
EKI ................................................................................................................................ Equus kiang
EPR ......................................................................................................................... Equus przewalskii
EQB ............................................................................................................................. Equus quagga burchelli
EST ................................................................................................................................ expressed sequence tag
EZH ............................................................................................................................. Equus zebra hartmannae
FGM2 ........................................................................................................................ fibroblast growth medium 2
FISH ............................................................................................................................ fluorescent in situ hybridization
FITC .............................................................................................................................. fluorescein isothiocyanate
HSA .............................................................................................................................. Homo sapiens
MEM .......................................................................................................................... minimal essential medium
min ................................................................................................................................. minute(s)
MY .............................................................................................................................. million years
personal comm. ......................................................................................................... personal communication
RT ................................................................................................................................. room temperature
SNP .............................................................................................................................. single nucleotide polymorphism
STR .............................................................................................................................. short tandem repeats
Appendix C (continued).

TE................................................................................................................................. Tris-EDTA buffer
Zoo-FISH...........................................................................................................cross species fluorescent \textit{in situ} hybridization
Appendix D includes representative FISH images for many of the experiments performed in this dissertation.

Figure D1 depicts representative hybridizations of genes and chromosome markers to *E. przewalskii* (EPR) chromosomes. Figure D2 depicts representative hybridizations of genes and chromosome markers to *E. hemionus onager* (EHO) chromosomes.
Figure D1. Representative FISH images on EPR chromosomes. White text indicates the species acronym, red text and arrows indicate genes or chromosome markers labeled with Rhodamine Red-X, and green text and arrows indicate genes or chromosome markers labeled with FITC. See Chapter Two for additional information.
Figure D.2. Representative FISH images on EHO chromosomes. White text indicates the species acronym, red text and arrows indicate genes or chromosome markers labeled with Rhodamine Red-X, and green text and arrows indicate genes or chromosome markers labeled with FITC. See Chapter Three for additional information.
References


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