CHAPTER 3

Reciprocal Zoo-FISH analysis of DNA homologous to human chromosome 22 in the domestic dog and Siamang gibbon

3.4.5 Hybridisation of human chromosome 22, 16, 5 and 2 paints onto Siamang metaphase chromosomes

3.5 Discussion

3.1 Introduction

The aim of the work reported in this chapter was to carry out reciprocal zoo-FISH analyses of the metaphase chromosomes of the dog and gibbon to identify regions corresponding to evolutionarily conserved chromosome segments (ECCS) in material homologous to human chromosome 22.

For the dog, the first step towards a reciprocal zoo-FISH study was the production of a bivariate flow karyotype, a panel of canine chromosome-specific paints and the establishment of a standard karyotype of the dog, which are described in this chapter. At the time of this work I was involved in a collaboration to standardise the dog karyotype, and the information generated by that collaboration is bound into this thesis (Breen, *et al.,* 1999).

For the gibbon study, it was necessary to generate metaphase chromosome spreads from cultured cells, a bivariate flow karyotype and a chromosome 18-specific paint from flow-sorted chromosomes, which are described here.

RESULTS

3.2 Establishing the Standard Karyotype of the Domestic Dog

3.2.1 Establishing the canine flow karyotype

Before staining for flow cytometric analysis, chromosomes may be isolated from a range of cell types, which must be healthy and growing optimally. A healthy, dividing population of cells is arrested in metaphase before chromosome isolation, by the addition of a spindle inhibitor (Colcemid). For the chromosome preparation, arrested cells are swollen in a hypotonic buffer, then the cell membrane is removed using a combination of

detergent and mechanical disruption to release the chromosomes into a stabilising buffer (Sillar and Young, 1981, Ross and Langford, 1997).

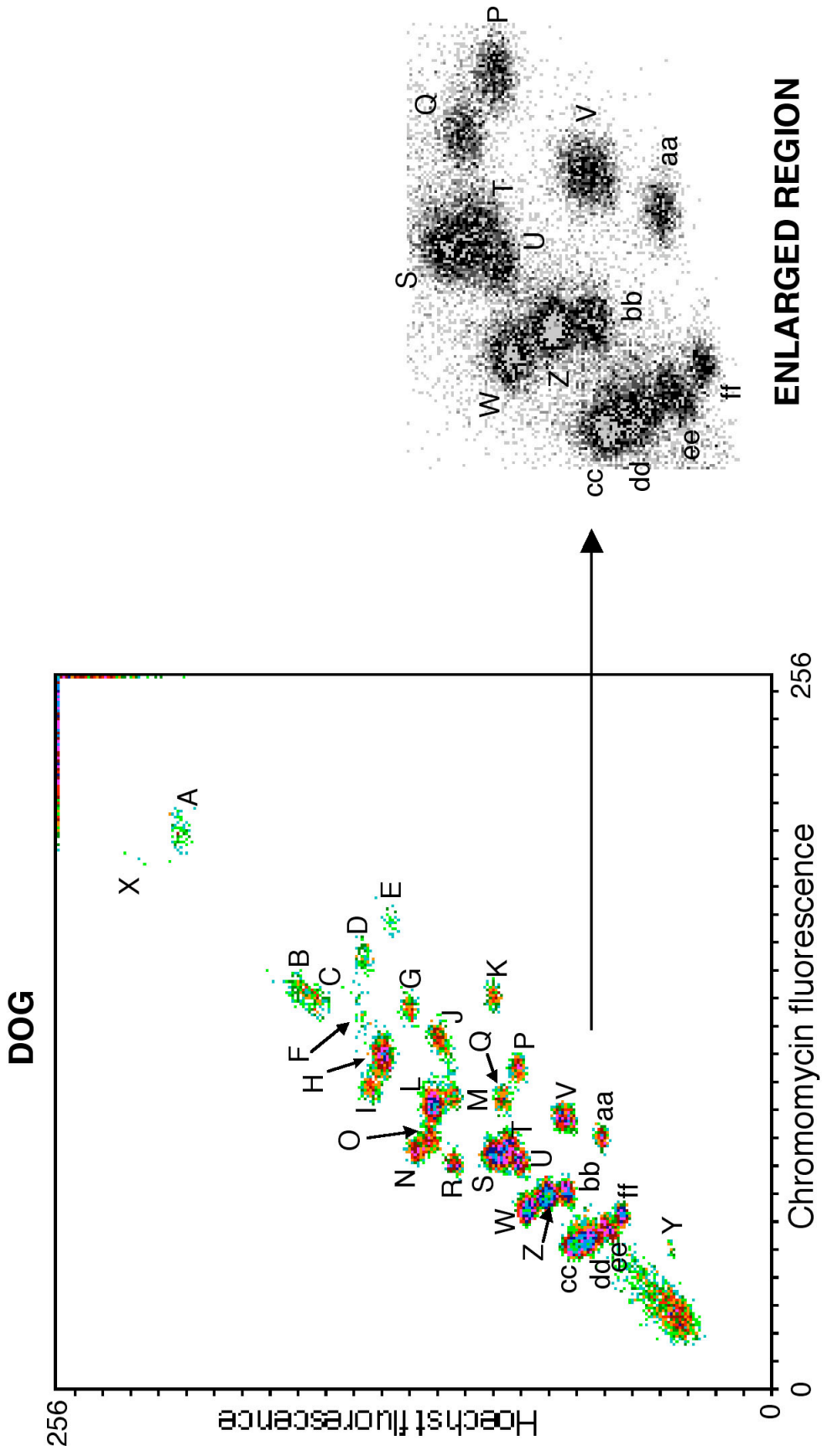
Peripheral blood lymphocytes from karyotypically normal Red Setter dogs were stimulated to divide in short-term cultures with phytohaemagglutinin and pokeweed mitogen. Previous protocols for the *in vitro* stimulation of isolated lymphocytes had often relied on the use of only one mitogen. The two mitogens used in this study had a synergistic effect on each other. After incubation with Colcemid the mitotic indexes of the cultures were high (up to 35%) which led to good chromosome preparations.

Chromosome suspensions were prepared and stained with Hoecsht 33258 and Chromomycin A3 and analysed on a dual-laser Elite ESP flow cytometer (Coulter Electronics). The chromosomes were resolved by DNA content and AT to GC base pair composition. Despite the complexity of the canine karyotype, the good quality chromosome preparations coupled with the high calibre instrumentation available resulted in the first-reported production of reproducible high-resolution bivariate flow karyoytpes of the dog from short-term cultures of peripheral blood lymphocytes (Langford, *et al* 1996, bound into this thesis).

The bivariate flow karyotype of a male red setter dog is shown in Figure 3.1. In the male dog, the 76 autosomes and two sex chromosomes were resolved into 32 peaks. These were labelled arbitrarily from A to Z, followed by aa to ff.

The number of homologues represented by a flow karyotype peak is indicated by the relative number of chromosome events in that peak. Using the sorter workstation software it is possible to display the number of events as a count frequency. The count frequency of a normal chromosome peak representing two homologues of the same size will be double that of the chromosome X and Y peaks in a male flow karyotype, which represent only one homologue each.

Figure 3.1 (next page) Bivariate flow karyotype of the male dog. The expanded panel shows the karyotype for the smaller chromosome peaks (Q to ff)


DOG
Hoechst fluorescence
Chromomycin fluorescence
256
0
256
0
A
X
E
D
B
C
G
K
F
H
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Q
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ENLARGED REGION
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The sum of the count frequencies for all the peaks in the male flow karyotype was divided by the total number of chromosomes (78) to calculate the count frequency of one chromosome homologue. The number of homologues represented by each peak in the male flow karyotype was then estimated (to the nearest integer) from the relative count frequencies of each peak and are presented in Table 3.1 (see over).

Eight of the 32 peaks contained four chromosome homologues, which was not surprising considering the similarity in size of many of the smaller autosomes. The peaks representing chromosomes X and Y (peaks X and Y, respectively) were identified by their position in the flow karyotype (size) and their relative count frequencies, which were each equivalent to a single chromosome homologue.

In the male flow karyotype, all the chromosomes were accounted for in the peaks (39 pairs).

Table 3.1 The number of chromosome homologues represented by each peak in the male dog flow karyotype.

Peak	Number of homologues	Peak	Number of homologues
A	2	Q	2
B	2	R	2
C	2	S	4
D	2	T	2
E	2	U	2
F	2	V	4
G	2	W	4
H	4	X	1
I	2	Y	1
J	4	Z	4
K	2	aa	2
L	4	bb	2
M	2	cc	4
N	2	dd	2
O	2	ee	2
P	2	ff	2

In the female flow karyotype, an additional peak (labelled V') was observed close to peak V with a slightly reduced DNA content. (In the male flow karyotype peak V contained 4 chromosome homologues). The female peak V' contained a count frequency equivalent to a single chromosome homologue, and the concomitant count frequency for female peak V was equivalent to only three chromosome homologues. It is most likely that the

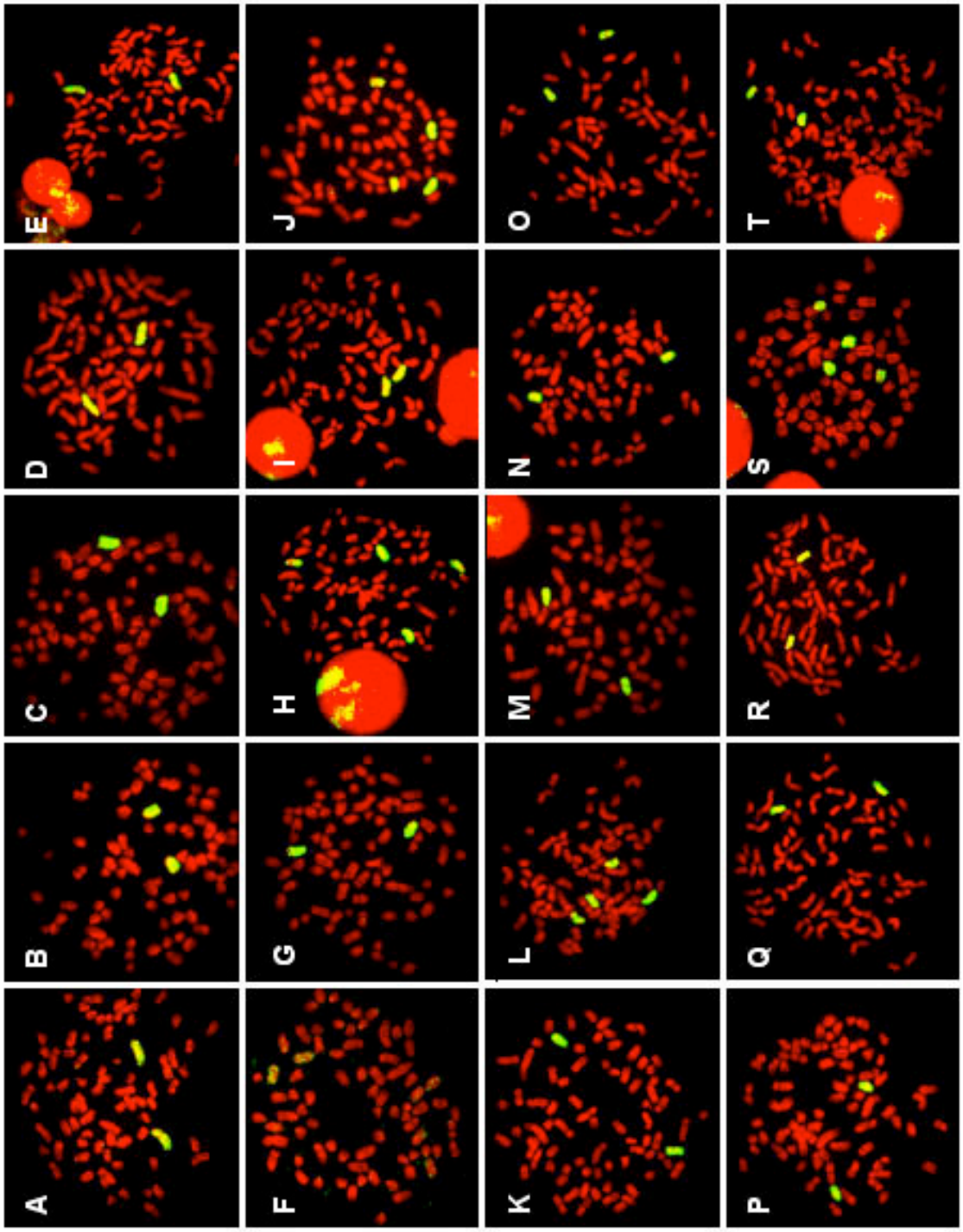
homologue in peak V' was a smaller size to the other peak V chromosomes due to a heterochromatic polymorphism (heteromorphism).

3.2.2 Characterisation of canine chromosome specific paint probes

Five hundred chromosomes were sorted from each resolved peak from the male and female flow karyotypes into sterile 0.5 ml PCR tubes for the generation of biotinylated chromosome specific paint probes by DOP-PCR.

Figure 3.2 (next page) Chromosome painting pattern for bivariated peaks A-T. The DAPI counterstain is pseudocoloured in red and the hybridisation signal is pseudocoloured in green.

Figure 3.3 (subsequent page) Chromosome painting pattern for bivariated peaks U-ff. The DAPI counterstain is pseudocoloured in red and the hybridisation signal is pseudocoloured in green.


A
B
C
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E
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G
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K
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Q
R
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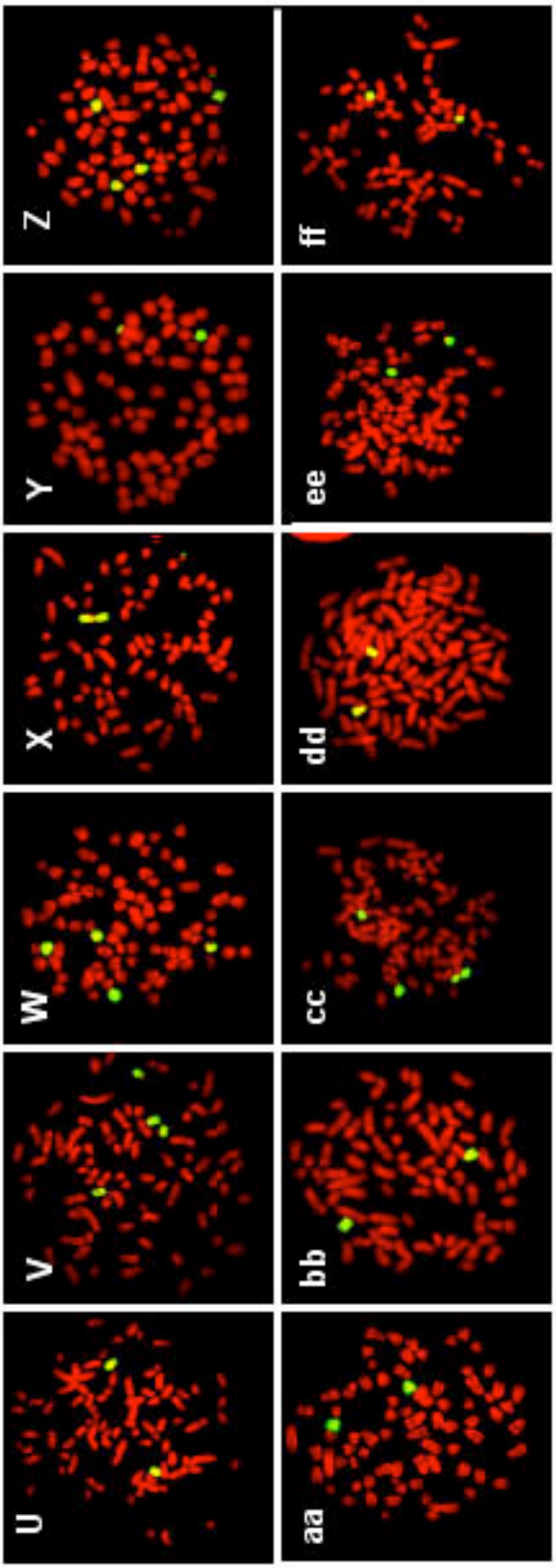
T


Z
ff
Y
ee
X
dd
W
cc
V
bb
U
aa

The canine chromosome specific paints were assessed by hybridisation to the metaphase spreads of a normal male red setter dog (Figures 3.2 and 3.3). Images from 20 metaphase spreads were acquired and processed using a FISH workstation comprising a fluorescence microscope (Axiophot, Carl Zeiss) equipped with an 83000 triple dichroic mirror block and separate excitation filter set (Chroma Technologies), a cooled CCD camera (KAF1400, Photometrics, Tucson, AZ, USA) driven by a Macintosh PowerMac 8100 computer and dedicated software (SmartCapture, Digital Scientific, Cambridge, UK).

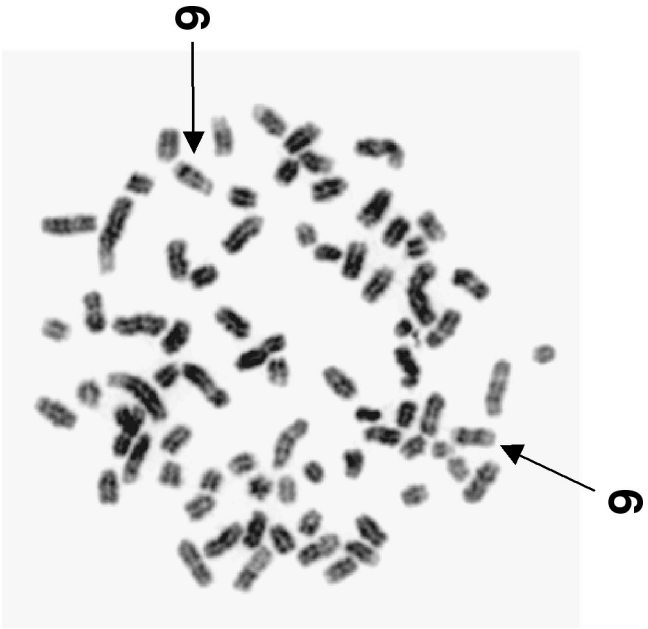
The estimated number of homologues contained in each peak was confirmed in each case by chromosome painting. Twenty-two of the 32 paints (generated from the male flow karyotype) each hybridised to individual pairs of chromosomes. Eight of the paints each hybridised to two pairs of chromosomes and two of the paints represented one of the sex chromosomes each (Figure 3.2 and 3.3). The paint generated from the female peak V' hybridised to one homologous pair, which was also one of the pairs hybridised by the paint from peak V.

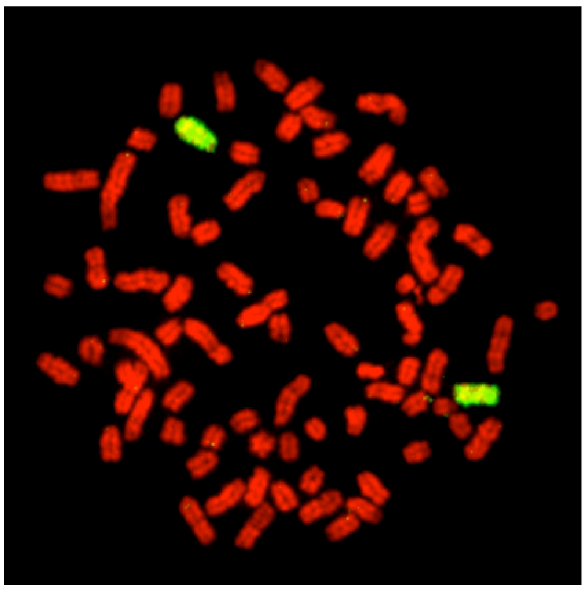
In most cases, hybridisation to the metaphase chromosomes was strong and specific although weak labelling just above background was found on six additional homologues for peak F and two additional homologues each for peaks J, O, R, T, U, cc and dd. This can be explained by the proximity of other flow karyotype peaks to F, J, O, R, T, U, cc and dd. It is quite possible that in this situation, co-sorting of a small proportion of chromosomes from the nearby peak(s) occurred, and were represented to a low degree in the paint.

The chromosome X-specific paint also hybridised to a region of Yp and, similarly, the Y-specific paint hybridised to a region of Xp. A similar pattern of hybridisation is seen with human X and Y paints on metaphase spreads. It is assumed that these Xp and Yp regions that show homology are the short arm pairing segments (pseudoautosomal regions), which pair and recombine during male meiosis.

The image of each metaphase spread was processed using a high-pass digital filter (SmartCapture, Digital Scientific, Cambridge, UK) to reveal enhanced DAPI bands (Figure 3.4).

Figure 3.4 (next page) Hybridisation image of the paint from flow karyotype peak K and the corresponding enhanced DAPI banded metaphase image are shown. Analysis of the enhanced DAPI band image, revealed that peak K contains dog chromosome 9.


6
6



An initial study of our enhanced DAPI-banded images enabled the identification (P. Fischer (The Animal Health Trust, Newmarket, UK and N. Reiman (Heidelberg, Germany)) of canine metaphase chromosomes 1-21 plus X and Y and their corresponding flow karyotype peaks (Langford *et al*. 1996, bound into this thesis).

In a subsequent analysis the identification of the remaining 17 undesignated chromosomes and their corresponding flow karyotype peaks, was carried out by M. Breen (Newmarket, UK) (Breen *et al.* 1999a, bound into this thesis).

Although eight of the flow karyotype peaks each contained two similarly-sized pairs of homologous chromosomes (H = 8 + 11, J = 10 + 17, L = 13 + 15, S = 21 + 23, V = 24 + 28, W = 29 + 32, Z = 31 + 34, cc = 33 + 36), the enhanced DAPI banding patterns were sufficiently different to distinguish each homologous pair.

The assignment of each dog chromosome to its corresponding flow karyotype peak is presented in Table 3.2.

Table 3.3 (Next page) Chromosome assignment of 33 canine flow karyotype peaks

Peak	Number of homologues	Chromosome assignment	Peak	Number of homologues	Chromosome assignment
A	2	1	R	2	19
B	2	3	S	4	21,23
C	2	4	T	2	25
D	2	2	U	2	27
E	2	5	V	4 (3)	24, 28
F	2	7	V'	1	28
G	2	6	W	4	29, 32
H	4	8, 11	X	1	X
I	2	12	Y	1	Y
J	4	10, 17	Z	4	31, 34
K	2	9	aa	2	26
L	4	13, 15	bb	2	30
M	2	16	cc	4	33, 36
N	2	22	dd	2	37
O	2	14	ee	2	38
P	2	20	ff	2	35
Q	2	18			

3.2.3 Standardisation of the canine DAPI-banded karyotype

In a collaborative study with Matthew Breen (The Animal Health Trust, Newmarket, UK) the panel of 33 canine chromosome-specific paints were used to identify unequivocally each chromosome type in a normal canine metaphase spread. After hybridising the first paint to be tested and following image capture from 30 metaphases, the same metaphases were re-used for repeat hybridisations for all paints (following successive re-denaturation steps for 15 to 60 seconds at 65˚C). Using this process all chromosome

pairs were conclusively identified in each metaphase spread. Accurate karyotypes were produced from 30 mid-metaphase spreads and used to derive a 460-band DAPI ideogram, which has been incorporated into the karyotyper software of Vysis Quips™ Image Analysis Software (Breen *et al.,* 1999a). We were able to produce the first complete DAPI-banded karyotype of the dog in which each chromosome was accurately placed, together with a 460-band ideogram. The data formed the basis for a proposed standard for the dog karyotype (Breen *et al.* 1999a).

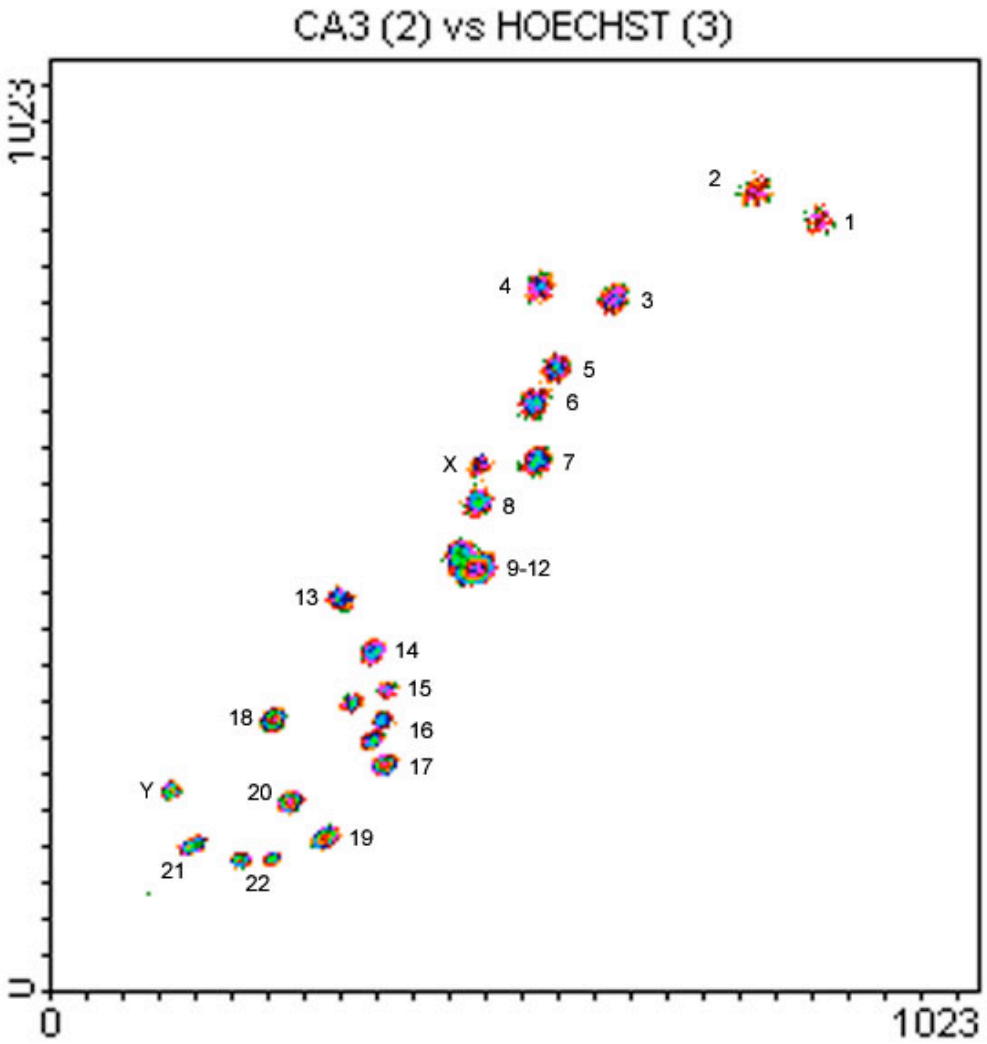
3.3 Reciprocal chromosome painting analysis between human and the dog

3.3.1 Generation of human chromosome-specific paints

A chromosome suspension from a normal anonymous female lymphoblastoid cell line (HRC 160) was prepared and analysed as described in 2.*. In the resulting bivariate flow karyotype (Figure 3.5), all the human chromosomes except 9, 10, 11 and 12 were resolved into individual peaks. Human chromosomes 9, 10 11 and 12 share a similar size and base pair ratio and, thus, usually remain as one large peak in the centre of the flow karyotype. Three chromosome types (15, 16 and 22) were each represented by two small adjacent peaks, which each contained a count frequency equivalent to a single chromosome homologue. The separation of the individual homologue peaks were due to resolvable size differences caused by heterochromatic polymorphisms, as described earlier.

Five hundred copies of each resolved human chromosome type were isolated into sterile 0.5 ml PCR tubes for DOP-PCR. The chromosome paint probes were generated and labelled as described in section 2.8, with either biotin-16-dUTP (for use in reciprocal zoo-FISH experiments between human and dog) or FITC- or Cy3-dUTP (for use in the reciprocal chromosome painting experiments between human and gibbon, described later).

Figure 3.5 Bivariate flow karyotype of a normal male human



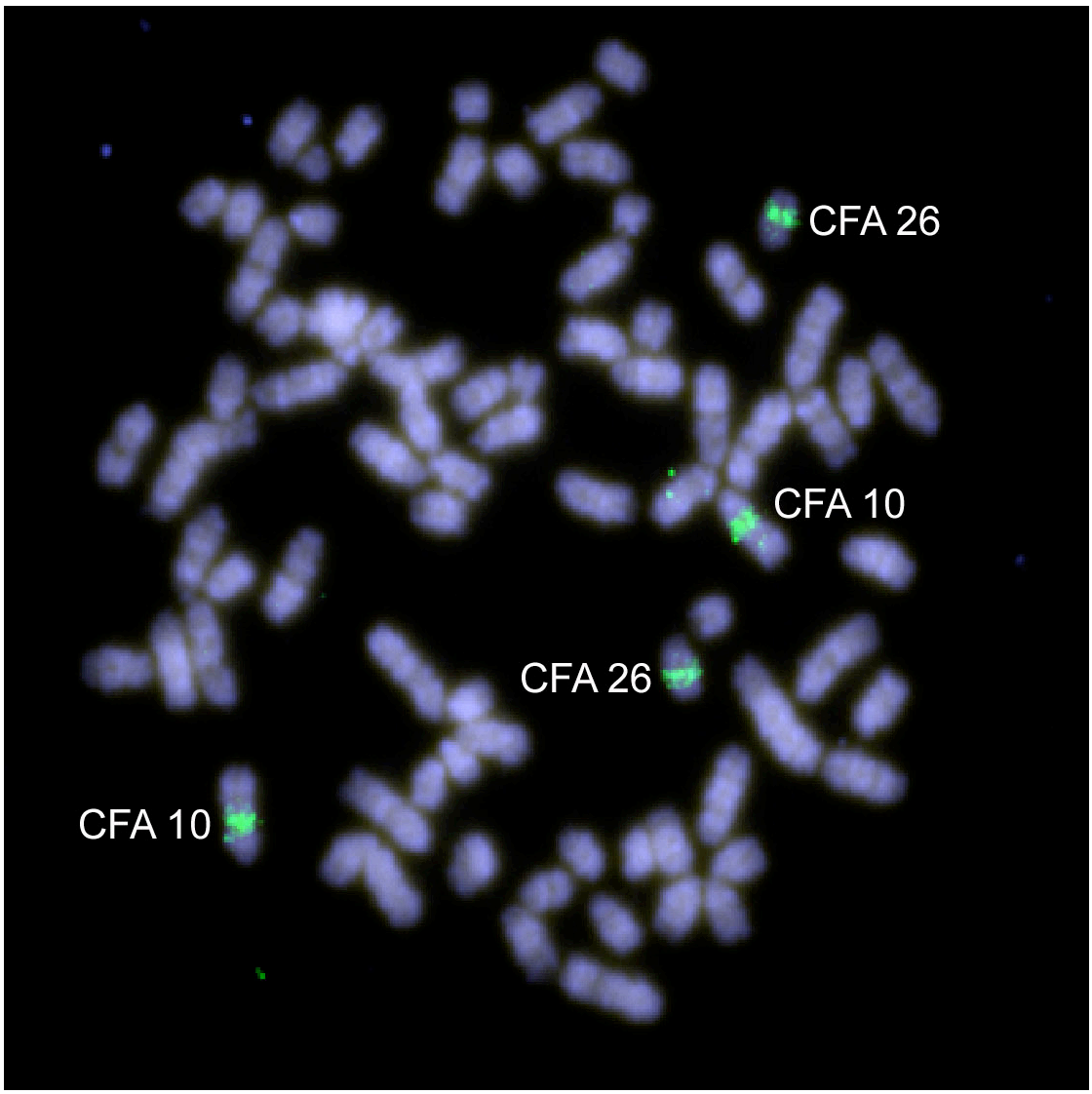
As the dog is distantly related to the human it was deemed likely that there would be considerable sequence divergence leading to weak hybridisation signals after zoo-FISH. The human chromosome paint probes that were to be hybridised onto canine metaphase spreads were, therefore, labelled with biotin-16-dUTP because the biotin-avidin detection system allowed for signal amplification after hybridisation.

Each human chromosome-specific paint was checked for specificity on normal human metaphase spreads by FISH. At least 10 metaphases were analysed for each test-paint. Each of the human chromosome-specific paints hybridised only to the two chromosome homologues of origin in each human metaphase spread studied.

3.3.2 Hybridisation of human chromosome 22 specific paint onto dog metaphase chromosomes

The biotinylated human chromosome-22 paint was hybridised onto normal dog metaphase spreads, and data from 20 metaphases were analysed. The human chromosome 22 paint hybridised onto four blocks per canine metaphase, representing segments in two different chromosome types (Figure 3.6, next page). Paint hybridisation was detected on both homologues of canine chromosome 10q21-23.1 and both homologues of canine 26q21-24, identified according to the nomenclature by Breen *et al*. (1999).

Figure 3.6 Hybridisation pattern of human chromosome 22 paint on dog metaphase spread. The paint is pseudocoloured green, the chromosomes blue. Canine chromosomes 10 and 26 are indicated.

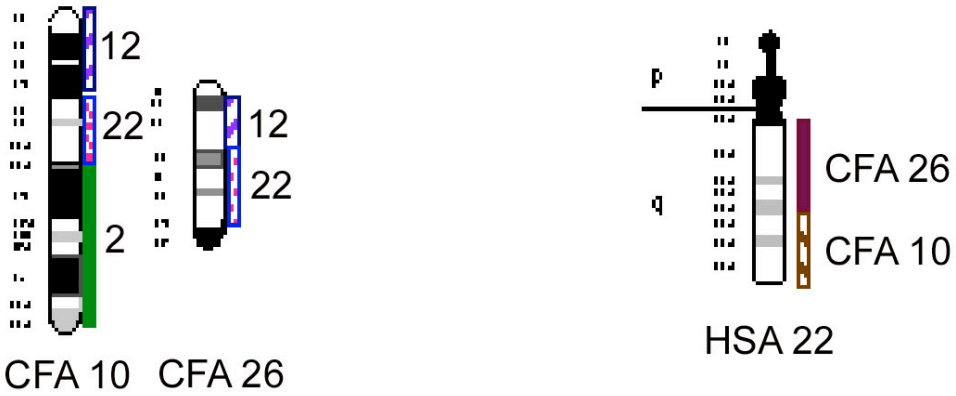


3.3.3 Reciprocal hybridisation of dog chromosome paints onto human metaphase chromosomes

In order to establish which part of each canine chromosome was syntenic with which part of human chromosome 22 a reciprocal painting study was carried out. Canine chromosome 10 and 26 paints were hybridised onto normal human metaphase spreads, and data from 20 metaphases were analysed for each paint probe.

The canine chromosome 10 paint hybridised onto 6 blocks per human metaphase, both homologues of 2pter-q21.1, 12p15-21.2 and 22q13.1-qter. The canine chromosome 26 paint hybridised to 4 blocks per human metaphase, both homologues of 12p24.1-pter and 22cen-q13.1 (Figure 3.7, and Breen *et al.* 1999).

Figure 3.7 Ideogram illustrating hybridisation patterns of canine chromosome 10 and 26 paints on human metaphase chromosomes. From Breen *et al.* 1999.



3.4 Reciprocal chromosome painting analysis between human and gibbon

3.4.1 Hybridisation of human chromosome 22 paint onto gibbon metaphase chromosomes

Various different protocols for producing good quality fixed metaphase spread preparations were investigated. These involved variations in the starting cultured cell concentration, duration of incubation with the spindle inhibitor Colcemid and duration in the hypotonic potassium chloride swelling solution. The most satisfactory fixed metaphase preparations were achieved following the protocol described in chapter 2.

Standard hybridisation and signal detection techniques carried out routinely in the FISH laboratory use biotinylated single-copy probes or chromosome paints. The biotinylated probes are hybridised and the signal is amplified and detected using the three-layer technique described in chapter 2. Although the probe is pre-annealed with C_0t = 1 DNA prior to hybridisation, the period of hybridisation will facilitate the non-specific binding of probe to repetitive elements in the metaphase chromosomes as well as to the surface of the microscope slide. Any binding will be amplified by a three-layer detection step. In certain systems it is possible to minimise the occurrence and subsequent amplification of background hybridisation by using probes or paints which have been directly labelled with a fluorescent molecule, rather than with biotin. The directly labelled probes do not usually need signal amplification as long as the binding is quite specific. As well as reducing the appearance of background non-specific hybridisation signals, the directly labelled probes also use fewer reagents per experiment compared to biotinylated probes that need a three-layer detection.

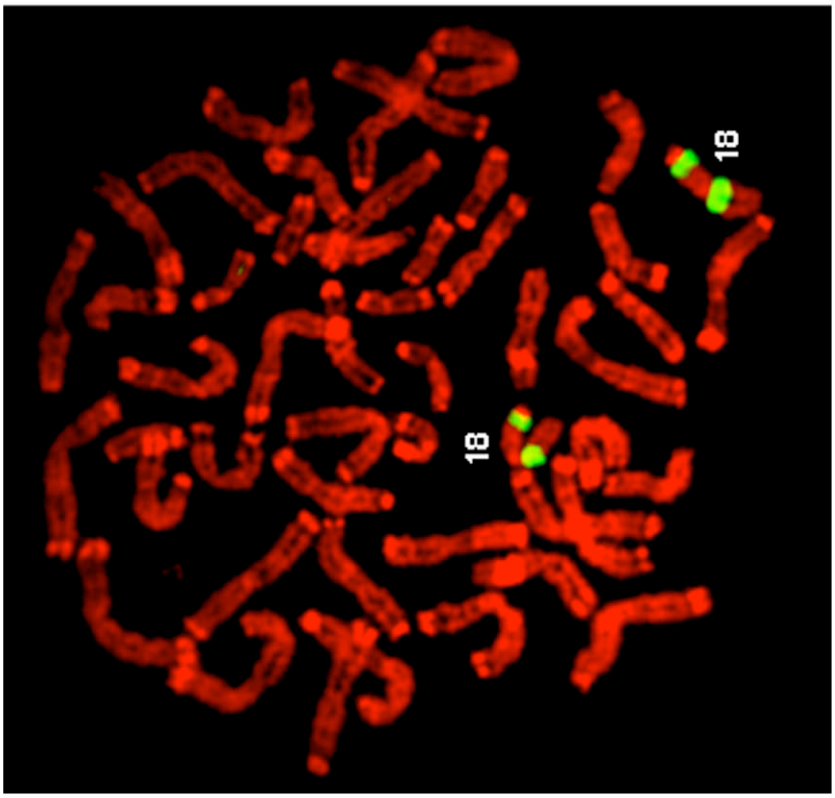
Because the Siamang gibbon is so closely related to the human, it was believed that a direct labelling of the paints would be sufficient to visualise the hybridisation signals, and would minimise the occurrence of background non-specific hybridisation signals.

Therefore, the gibbon metaphase spreads were analysed using a human chromosome 22-specific paint directly labelled with the fluorescent molecule FluoroGreen™-dUTP.

The FluoroGreen™-labelled human chromosome 22-specific paint was hybridised to gibbon metaphase chromosomes, and data from 20 metaphases were analysed. In each metaphase spread the human chromosome 22 paint hybridised onto four blocks, two on each homologue, one on each p arm and one on each q arm (Figure 3.8). The Siamang chromosome 18 was identified according to Van Tuinen and Ledbetter (1983).

Figure 3.8 (next page) Hybridisation signals obtained by hybridising human chromosome 22-specific paint to Siamang metaphase spreads. The metaphase chromosomes have been pseudo-coloured red and the paint signals pseudo-coloured green. The corresponding digital DAPI-banded metaphase image is in black and white.


18
18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18

There were clearly regions on both arms of Siamang chromosome 18, which were not painted by the human chromosome 22 probe, implying that those regions were syntenic with one or more other human chromosomes. Previously published painting experiments had also indicated that those regions were not homologous to human chromosome 22, but shared homology with (unspecified) regions of human chromosomes 5, 2 and 16 (Koehler *et al.* 1995).

3.4.2 Generation of the Siamang flow karyotype and chromosome isolation

Confirmation of the human chromosome homology map of Siamang chromosome 18 by a reciprocal chromosome painting study required the generation of a Siamang chromosome 18-specific paint and its hybridisation to human metaphase chromosomes.

To establish a Siamang bivariate flow karyotype, a suspension of chromosomes was isolated, stained with Hoechst 33258 and Chromomycin A3 and analysed on a dual laser flow cytometer. In the resulting bivariate flow karyotype, twenty-one peaks representing the 25 homologous chromosome pairs were resolved (Figure 3.9). The peaks were labelled alphabetically from A to U. Sorting regions were set up and 500 to 1000 chromosomes from each resolvable peak were isolated directly into sterile 0.5 ml PCR tubes for the generation of chromosome-specific paints labelled with FluoroGreen™-dUTP by DOP-PCR.

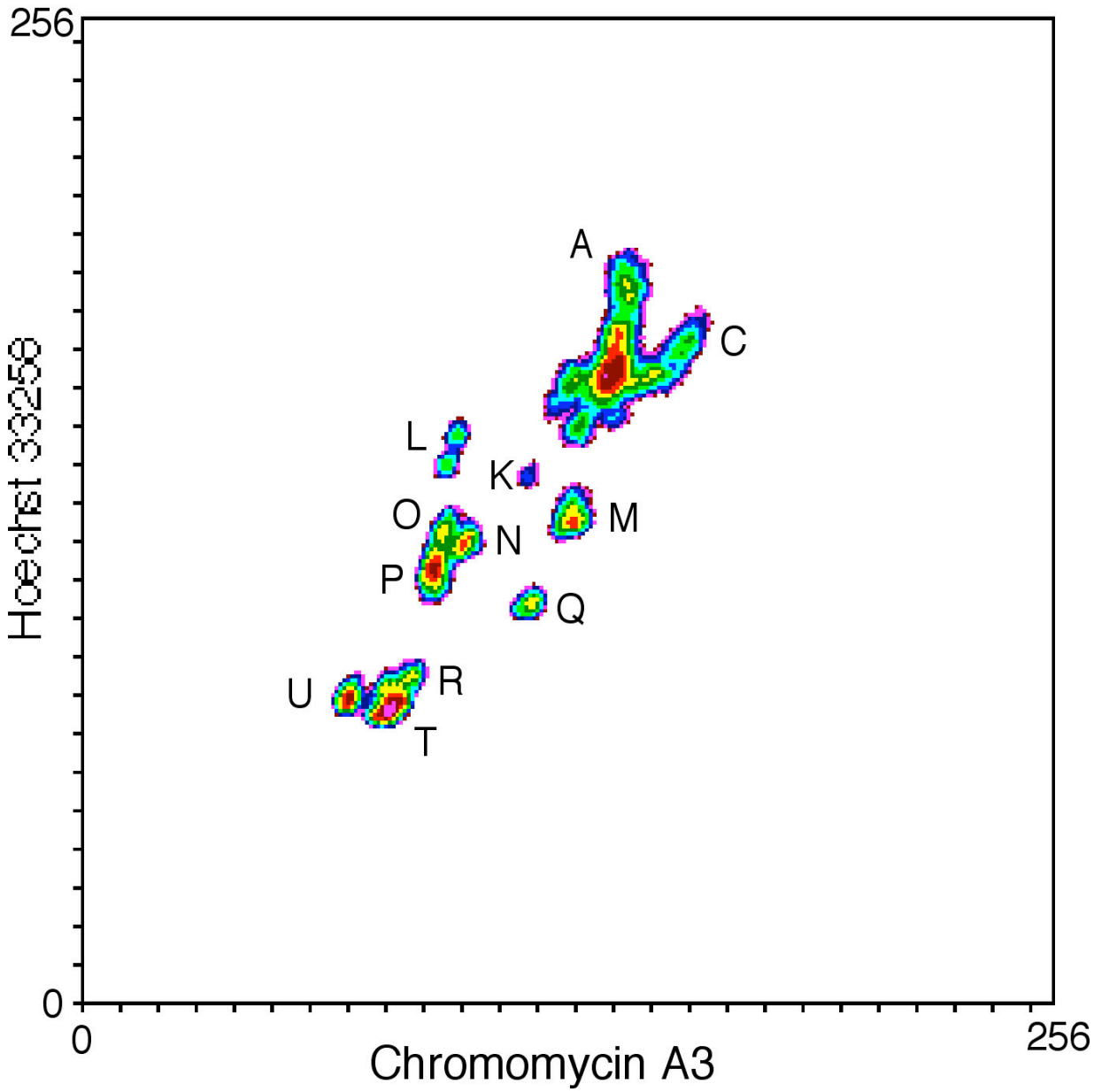


Figure 3.9 Bivariate flow karyotype of the gibbon.

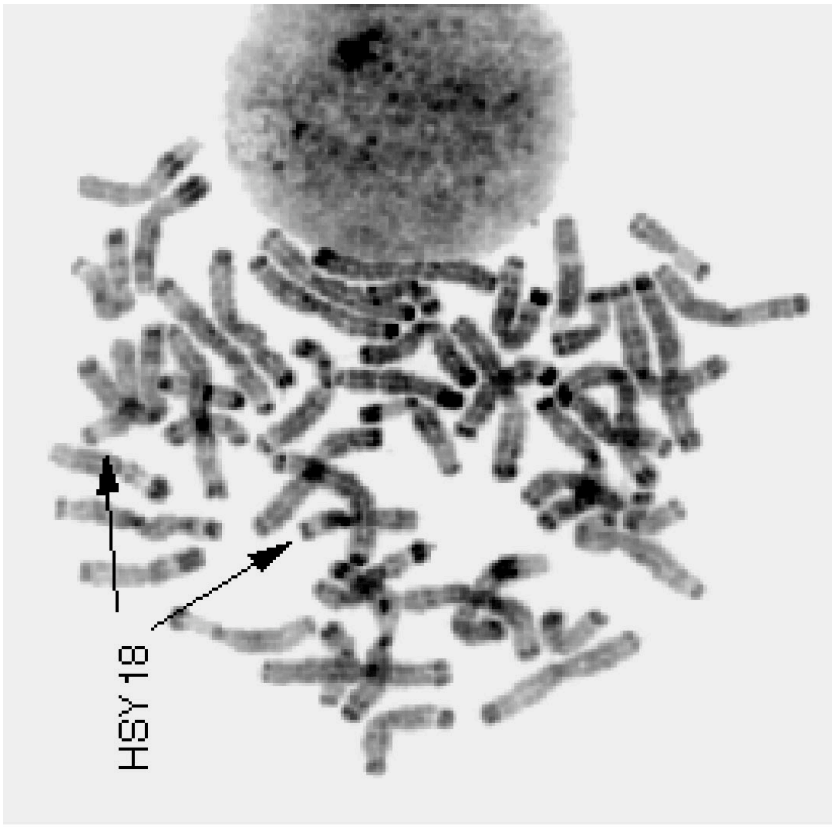
3.4.3 Identification of the Siamang chromosome 18 specific paint

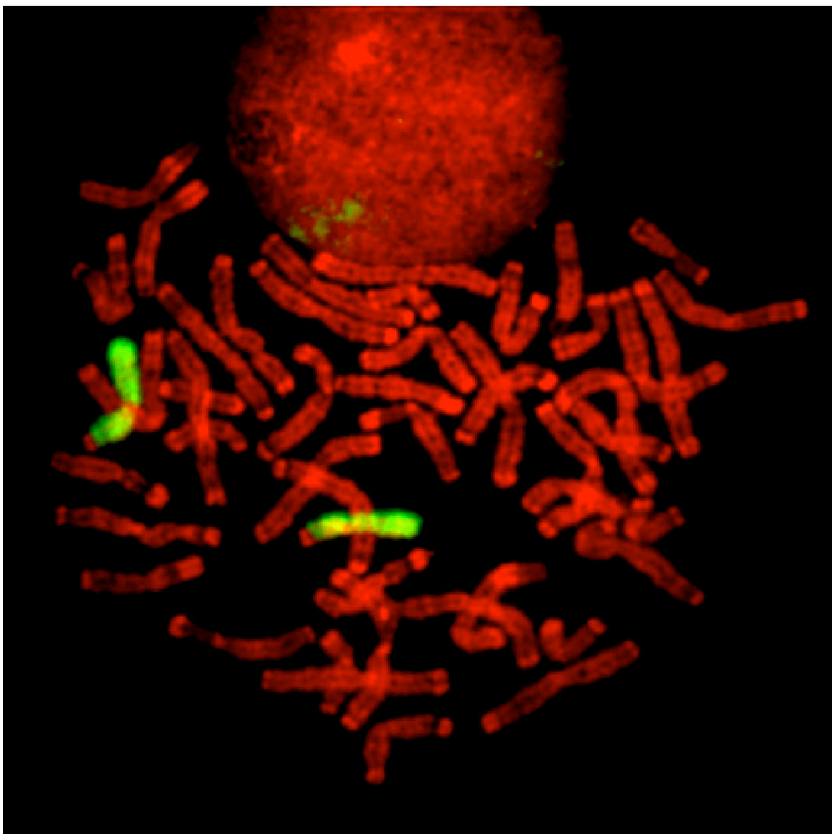
To identify which flow karyotype peak represented Siamang chromosome 18 each of the 21 paints was hybridised onto Siamang metaphase chromosomes. For each paint probe, data from 20 metaphases were analysed. The paint generated from GC base pair rich flow karyotype peak "Q" hybridised to both Siamang chromosome 18 homologues,

according to Van Tuinen and Ledbetter, (1983) indicating that flow karyotype peak Q contained Siamang chromosome 18 alone (Figure 3.10).

A narrow region at the telomere of the short arm of Siamang chromosome 18 was unlabelled by FISH with paint Q. Previous Q-banding studies, which highlighted heterochromatin in chromosomes, indicated that there is a block of heterochromatin at the telomeres of nearly every Siamang chromosome, including chromosome 18 (Koehler *et al.* 1995). It is likely that the heterochromatic sequences were either not amplified by DOP-PCR and were not represented by the chromosome 18 paint, or the repetitive heterochromatic sequences were present in the paint but were self-annealed by the human C_ot=1 DNA during the pre-annealing step and thus, did not hybridise to that region.

Figure 3.10 (next page) Hybridisation pattern of the paint from peak Q. In the left panel, the paint signals are pseudocoloured green and the chromosomes red Siamang chromosome 18 is marked on the DAPI image in the right panel.


HSY 18



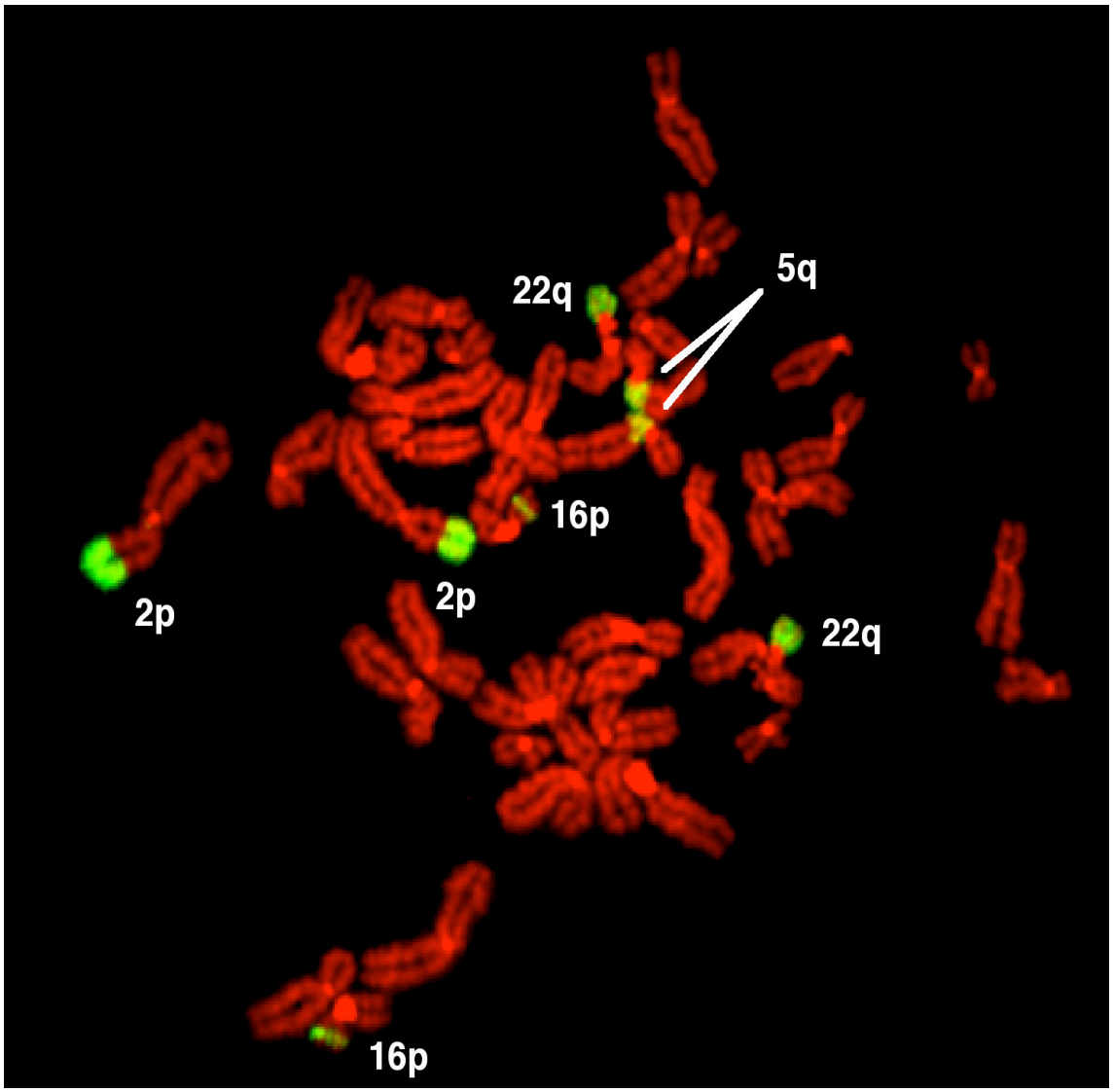
3.4.4 Hybridisation of Siamang chromosome 18 paint onto human metaphase chromosomes

The FluoroGreen™-labelled Siamang chromosome 18 paint was hybridised onto human metaphase spreads, and data from 20 metaphases were analysed. As well as human chromosome 22, regions of the other human chromosomes sharing homology with Siamang chromosome 18 were highlighted. The Siamang chromosome 18 paint hybridised to parts of four different human chromosome types: the whole of 22q, a narrow band in 16p12-13.2, a block in 5q11.2-13 close to the centromere and a terminal block in 2p22-pter (Figure 3.11).

3.4.5 Hybridisation of human chromosome 22, 16, 5 and 2 paints onto Siamang metaphase chromosomes

In order to establish the precise arrangement in Siamang chromosome 18 of the various human-homologous blocks, FluoroGreen™-labelled human chromosome 16-, 5- and 2-specific paints were hybridised individually to Siamang metaphase spreads. Data from 20 metaphases were analysed for each paint. The homologous regions corresponding to each of the human chromosomes 16, 5 and 2 were highlighted on other Siamang chromosomes in the metaphase spread, as well as on chromosome 18.

Figure 3.11 (next page) Hybridisation pattern of gibbon chromosome 18 paint on human metaphases. The paint signal is pseudocoloured green and the chromosomes red. The human chromosomes with hybridisation signal are indicated with arrows.


22q
5q
16p
2p
2p
22q
16p

The human chromosome 16 paint hybridised to two blocks on Siamang 11 (p and q), one block on 14q and to two narrow blocks on Siamang 18, one in the p arm adjacent and centromeric to the human chromosome 22-homologous block, and one on the q arm, adjacent and telomeric to the human chromosome 22-homologous block.

The human chromosome 5 paint hybridised to one block in 7p, one block on 11q, one block on 16q and a single block on Siamang 18p, between the centromere and the human chromosome 16-homologous block.

The human chromosome 2 paint hybridised to one block on 8q, one block on 9p, two blocks on 14 (p and q), one block on 22q and a single block on Siamang 18q between the human chromosome 16-homologous block and the telomere.

A homology map was constructed indicating the regions of homology between Siamang chromosome 18 and human chromosomes 22, 16, 5 and 2. (Figure-3.12)

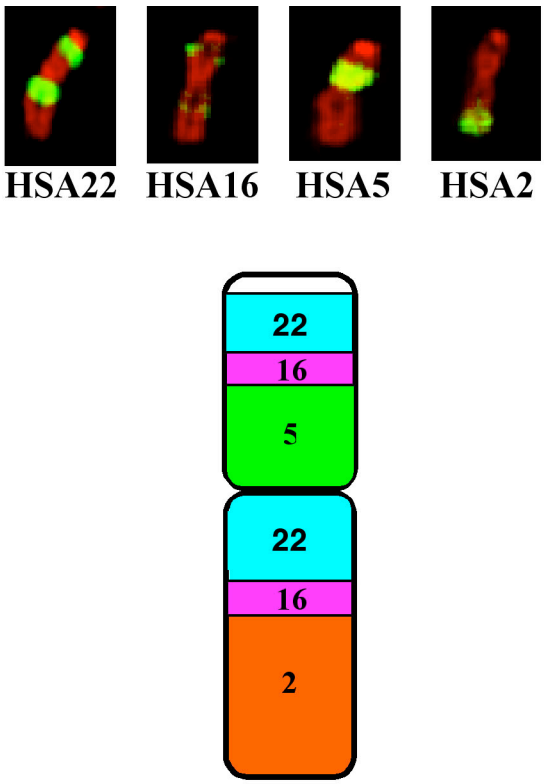


Figure 3.12 Above, hybridisation patterns of paints for human chromosomes 22, 16, 5 and 2, respcetively, on gibbon chromsome 18. The hybridisation pattern is pseudocoloured green and the chromosome red. Below, Ideogram of Siamang chromosome 18 and the corresponding mapped human homology.

3.5 Discussion

The aim of this work was to conduct a reciprocal zoo-FISH analysis of DNA homologous to human chromosome 22 in the domestic dog as well as a study of human homologies with Siamang chromosome 18. Work towards this aim has involved the production of dog and gibbon flow karyotypes and the generation of chromosome specific paints by DOP-PCR. Reciprocal zoo-FISH analyses have led to the construction of homology maps for human chromosome 22, dog chromosomes 10 and 26, and Siamang chromosome 18.

The Canine Study

Flow-karyotyping the dog presented particular challenges. Several pairs of canine chromosome types, which shared size and base pair similarities, did not resolve into individual flow karyotype peaks. But despite the complexity of the canine karyotype, good quality chromosome preparations resulted in the generation of reproducible high-resolution flow karyotypes of the male and female dog.

The ability to assess the number of chromosome events represented by each dog flow karyotype peak assisted in the identification of the peaks containing chromosomes X and Y in the male. It also allowed an explanation of the additional peak V' in the female flow karyotype, as it was most likely caused by a heterochromatic polymorphism (heteromorphism) in one chromosome 28 homologue (as determined by chromosome painting). In the male, flow karyotype peak V contains 4 homologues: 2 of chromosome 24 and 2 of chromosome 28. All four homologues are in the same peak because the size and AT to GC base pair ratios of the two chromosome types are similar. A heteromorphism of one chromosome homologue may affect its size, and, because heterochromatin is AT-rich, its AT to GC ratio. In the case of peak V', the heteromorphic chromosome 28 homologue is reduced in size enough to be resolved as an extra peak on the flow karyotype.

To identify the dog chromosome types in each peak, chromosomes were flow sorted, amplified and labelled by DOP-PCR and painted onto metaphase spreads of a normal male dog. Twenty-two of the peaks from the male flow karyotype each hybridised to single homologous pairs, and eight of the peaks hybridised to two pairs. Paints from the remaining two male flow karyotype peaks hybridised to only one homologue each in male metaphase spreads, thus corresponding to the sex chromosomes X and Y. All of the 38 pairs of autosomes and the two sex chromosomes of the dog were accounted for in the flow karyotype.

Prior to the work towards this thesis, there was no reliable method of identifying every chromosome in a canine metaphase spread. The complex nature of the dog karyoytpe, coupled with difficulties in stimulating lymphocytes and optically aligning a flow cytometer for chromosome analysis, had also previously frustrated attempts to flow sort dog chromosomes. Work carried out by VanDevanter *et al.* (1994), to generate a limited number of canine chromosome paints, avoided the need to obtain good resolution of flow karyotype peaks by sorting only single chromosomes. Ligation-adapter PCR was then used to make paint probes of limited representation from these single chromosomes.

The canine chromosome paints generated by DOP-PCR for this study were of good quality. The eight pairs of chromosome types, which were represented by paints from peaks H, J, L, S, V, W, Z and cc, had sufficiently different banding patterns to enable the distinction between each comprising pair.

Microscopy and flow karyotyping used alone could not easily distinguish each individual canine chromosome. However, using the two technologies together has resolved the problem and has enabled the unequivocal identification of each chromosome pair in a metaphase spread, and the proposal of a DAPI banded standard karyotype for the domestic dog (Breen *et al.*, 1999).

The characterisation of the canine chromosome paint probes, as well as the proposed DAPI-banded karyotype permitted the reciprocal zoo-FISH analysis between human and the dog. The study revealed detailed regions of conserved synteny between human chromosome 22 and canine chromosomes 10 and 26.

One of the most striking widely conserved ancestral neighbouring chromosome segment combinations across a number of mammalian orders is that of human chromosome 12 and chromosome 22 (Chowdhary, *et al.,* 1998). Regions homologous to human chromosomes 12q15-21.3 and 22q13.1-qter are associated on canine chromosome 10, and regions homologous to human 12q24.1-qter and 22q11.1-13.1 are associated on canine chromosome 26. This provides more evidence that these combinations probably represent ancestral chromosome arrangements.

Segments of human chromosomes 12 and 22 appear as neighbours on two chromosomes in cattle (BTA 5 and BTA 17), pig (SSU 5 and SSU 14), dolphin (TTR 8 and TTR 9), cat (FCA B4 and FCA D3), horse (ECA 8 and ECA 26), lemur (EMA 10 and EMA 19), American mink (MVI 3 and MVI 12) (summarised in Glas *et al.*, 1998), and giant panda (AME 12 and AME 15) (Nash *et al.*, 1998). It has also been observed on just one chromosome of the harbour seal (PVI m3) (Frönicke *et al.*, 1997).

Unidirectional chromosome painting involving the dog was first reported by Werner *et al.* (1997), using a human chromosome 17 paint probe to identify homologous segments on two dog chromosomes. The only previous report of reciprocal chromosome painting involving human and the dog was by Thomas *et al.* (1999), who used the paints generated by this study to identify conserved segments of synteny between human chromosomes 1p31.2-p32.3, 11q23-q25, 16q21-q24 and 17p12-p13 and dog chromosome 5.

A unidirectional approach to the work for this thesis, would only have yielded the information that human chromosome 22 has shared homology with dog chromosomes 10

and 26. It was only due to the reciprocal nature of the study, that it was possible to identify which segment of human chromosome 22 is homologous to which dog chromosome. For this thesis, a detailed reciprocal zoo-FISH analysis between the domestic dog and the human has generated metaphase chromosome homology maps for human chromosome 22 and canine chromosomes 10 and 26. (For full canine/human chromosome homology maps, see Breen *et al.*, 1999b). Canine chromosome 10 shares homology with human 22q13.1-qter and canine chromosome 26 shares homology with human 22cen-q13.1.

These results strongly suggest that a site of evolutionary rearrangement (fusion) is present in human chromosome 22q13.1. At the low level of resolution afforded by these and other zoo-FISH studies, it might be suggested that the arrangement of human chromosome 22 homologous material in the canine karyotype represented the ancestral state. During the karyotype evolution of the primates a rearrangement event probably lead to the fusion of the human chromosome 22 homologous material at a point now identified within 22q13.1.

The Siamang Study

In the second part of this chapter, Siamang metaphase chromosomes were analysed with a human chromosome 22 specific paint probe. In each spread analysed Siamang chromosome 18 was identified by its DAPI-banding pattern. Two distinct blocks of synteny were identified on Siamang chromosome 18 p and q, as described by Koehler *et al.* (1995).

For further analysis, a high-resolution flow karyotype of the gibbon was produced and a paint probe for Siamang chromosome 18 was generated. Reciprocal chromosome painting between the human and Siamang was used to generate a homology map for Siamang chromosome 18 in relation to human chromosomes 22, 16, 5 and 2.

A previous FISH study had been carried out which involved the unidirectional hybridisation of human paints onto Siamang metaphase chromosomes (Koehler *et al.* 1995). The homologies between Siamang 18 and human chromosomes 22, 16, 5 and 2 were reported in different orientations to the arrangement I found. However, the metaphase spreads for the two studies had been prepared from the same Siamang individual. After discussion, the authors commented that their hybridisations had been difficult to interpret due to the fact that they had been using paints generated from chromosome specific libraries. The authors agreed that their results, if repeated using DOP-PCR paints, could in fact have looked the same as mine (Johannes Weinberg, personal communication).

From the information in the homology map between Siamang chromosome 18 and human chromosomes, a proposed homology map of the chromosome ancestral to Siamang chromosome 18 could be suggested (Figure 3.13).

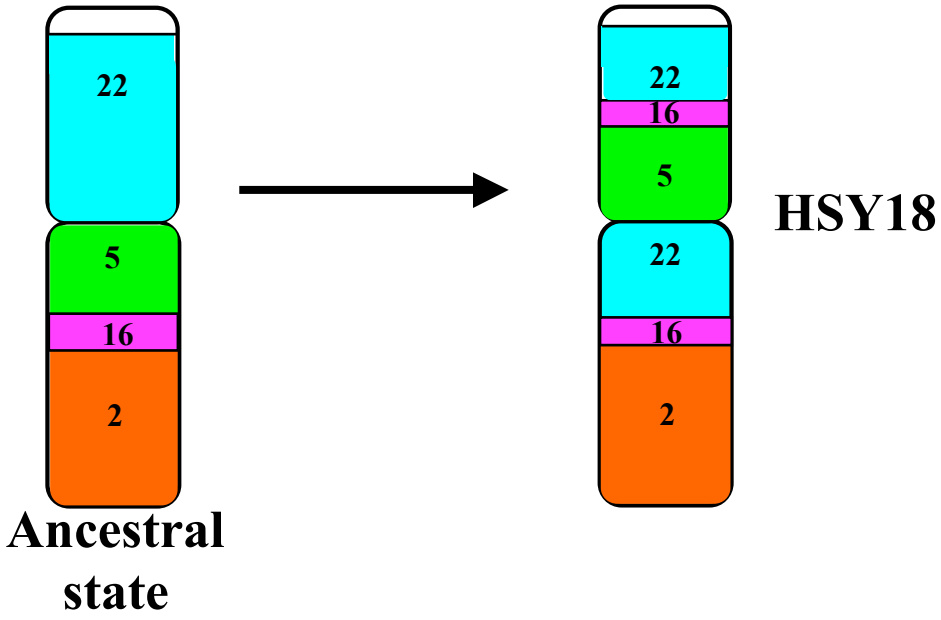


Figure 3.13 Homology map of the current Siamang chromosome 18 (right) and a proposed homology map of the ancestral chromosome (left). The human chromosome 22-homologous material is intact in one block on 18p, the human chromosome 5-homologous block is in the proximal q arm, the human chromosome 16-homologous material is intact in one block on 18q adjacent to the human chromosome 5-homologous block and the human chromosome 2-homologous block is on 18qter. Fission in the blocks homologous to human chromosomes 22 and 16 and a pericentric inversion in the ancestral chromosome could have lead to the current state of Siamang chromosome 18.

Summary

A detailed reciprocal zoo-FISH analysis between the human and the dog was used to identify a region corresponding to an evolutionary chromosome fusion point in human chromosome 22q13.1. At the low level of resolution afforded by these and other zoo-FISH studies, it might be suggested that the arrangement of human chromosome 22 homologous material in the canine karyotype represented the ancestral state. During the

karyotype evolution of the primates a rearrangement event probably lead to the fusion of the human chromosome 22 homologous material at a point now identified within 22q13.1.

The reciprocal zoo-FISH study between human and Siamang did not lead to the identification of a chromosome rearrangement site in human chromosome 22 because both 22q-homologous blocks reside in Siamang chromosome 18. It was possible to suggest that a pericentric inversion might have lead to the fission of human chromosome 22 homologous material in the ancestor to Siamang chromosome 18.

To further refine the analysis of the 22q rearrangement point, a higher resolution zoo-FISH study was carried out. This work is described in the following chapter.