ANALYSIS OF POLYMORPHIC CHROMOSOME BANDING PATTERNS IN *BOS TAURUS*

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Table of Contents

Tables

Table of Figures

Table of Figures (cont'd)

I. Introduction

Chromosome banding has become a very important tool in cytogenetics and gene mapping, especially in the area of human chromosomes. Such chromosome banding has been adapted to studies of *Bos taurus* where the diploid number is 60 (Strickberger, 1976). Bovine chromosome studies have dealt primarily with sister chromatid exchanges and Robertsonian translocations, but banding has opened an entire new field of research.

II. Early chromosome studies

For many years scientists have attempted to explore genetics by studying chromosome structure and function. One of the most significant chromosome studies determined that the human diploid number was 46 (Tizo and LeVan, 1956, cited by Yunis, 1974). In 1957 the first human chromosomal disease was identified (Lejeune, *et. al.*, 1957, cited by Lejeune, 1973). During the late 1950's and early 1960's the number of chromosomal studies increased at a rapid rate. Due to the large number of chromosomal studies, it was necessary to construct a chromosome classification system. In 1960 in Denver, Colorado a conference was held to standardize chromosomal nomenclature. Additional conferences were held in the following years to further clarify chromosomal nomenclature (e.g. London Conference of the Normal Human Karyotype, 1963; Chicago Conference of Standardization in Human Cytogenetics, 1966; and Paris Conference of Standardization in Human Cytogenetics, 1971). In the mid-60's discoveries concerning human chromosomes started to plateau.

The reason for such a moderation in discoveries stemmed from the fact that primitive techniques in chromosomal studies were still being used.

A revolution in human cytogenetics occurred in 1968. Caspersson et. al. (1969, cited by Blazak, 1976) discovered that quinacrine mustard, a fluorescent compound, would bind to the chromosomes of Vicia faba and Trillium erectum. Such binding of fluorochromes to chromosomes produced characteristic banding patterns of varying intensity and width. The fluorochrome technique was immediately applied to human chromosomes and produced differentiating banding patterns (Caspersson et. al., 1970, cited by Blazak, 1976). Eventually, the identification of individual chromosomes of the human karyotype was developed. Such chromosomal bands resulting from fluorochrome banding were termed Q-bands.

Following the discovery of Q-banding patterns, other chromosomal banding techniques were developed. More than twenty banding techniques have been developed (Bartalos, 1967). Two of the more widely used banding procedures have been C-banding and G-banding. C-banding (constitutive banding) produced banding patterns in the heterochromatic regions of chromosomes; G-banding (Giemsa banding) produced banding patterns in intercalary heterochromatin regions of the chromosomes.

Each banding technique has its attributes. Q-banding has been designated as very accurate for chromosome differentiation. G-banding has been very important as a diagnostic tool in detecting chromosomal anomalies and individual chromosomes. G-banding does not require fluorescence microscopy while a more defined banding pattern is still possible.

Chromosomal banding studies have produced a great impact on medical cytogenetics. Briefly, chromosome studies have confirmed established syndromes, improved the analysis of chromosomal rearrangements, uncovered new syndromes, and analyzed abnormal cells (e.g. cancerous and leukemic cells) (Lejeune, 1978). Chromosomal studies have affected the theories of chromosome mechanics and organization.

Chromosomal studies have also had a very important impact on non-human cytogenetics, especially bovine cytogenetics. Prior to the discovery of banding studies scientists had analyzed and identified chromosome pairs in animals such as the pig and cat. Due to the fact that ruminants such as cattle possess telocentric chromosomes, identification of individual chromosomes was virtually impossible without banding studies. Before banding studies the only morphological identification was chromosomal size which was very innaccurate. Banding studies in cattle chromosomes have demonstrated chromosomal abnormalities indicative of phenotypic anomalies. Banding techniques have been developed at a rapid rate.

III. Chromosome preparation

The techniques of preparing human, bovine, or other animal chromosomes are basically the same. The search for an efficient culturing technique of chromosomes was an arduous task. Originally, skin tissue produced chromosomes when analyzed after two to three weeks of culturing (Winchester, 1977). It was later found that cultures of cells from red bone marrow produced chromosomes in relatively short periods of time

(Winchester, 1977). However, the procedure for obtaining red bone marrow cells was extremely painful for the subject.

Eventually, the most efficient culturing medium was discovered to be a mixture of kidney bean extract, nutrients, antibiotics, and blood. The most important component of the mixture was the kidney bean extract which was commonly called phytohemagglutinin (PHA). Phytohemagglutinin stimulated the lymphocytes to continue to grow and to divide in the culture medium. This culturing procedure, which produces chromosomes in two to three days is currently being used in chromosome studies (GIBCO, 1978).

The techniques of chromosomal preparation from human blood have been adapted to chromosomal preparations from cattle blood (Crossley and Clarke, 1962; Nichols et. al., 1962, cited by Halnan, 1977). Numerous modifications of the original technique have been developed. Techniques now exist which allow chromosomes to be cultured from heparinized cattle whole blood which has been transported by car for up to two hours, train for up to six hours, or plane for up to fourteen hours (Halnan, 1977). Improved chromosome preparations were obtained when the cattle whole blood was in the culture medium during extended transportation periods. The temperature for transportation ranged from 21°C to 32°C.

Halnan (1977) formulated a culture medium which proved successful in obtaining chromosomes from cattle whole blood. Table 1 (page 20) contains a list of all the ingredients of the culture medium. The major constituent of the culture medium is Medium 199 which is a

mixture of various amino acids, vitamins, inorganic salts, and carbohydrates (Table 2; page 21). Additional quantities of glutamine in the culture medium improved the preservative quality of the medium (Halnan, 1977). As a result of the additional glutamine the culture medium could be stored for as long as fourteen days when frozen. Excess L-cystine increased the number of mitotic cells in the preparation (Halnan, 1977). The bovine medium also contained non-preserved heparin at a concentration twice that necessary for human blood chromosomal preparations. Sodium bicarbonate was added to the solution in order to obtain the optimum pH of 7.3.

According to Halnan's (1977) technique bovine whole blood was cultured in bovine media for 48 to 72 hours between 37.5° and 39.5°C. The cultures were gently agitated twice a day in order to increase the mitotic index (Brown, 1973, cited by Halnan, 1977). After the incubation period, the cells were treated with colchicine for two hours and then processed.

Colchicine, an alkaloid derivative of Colchicum autmnale Linn., prevents spindle fiber formation thus arresting the cells in the metaphase stage of mitosis. Colchicine is tricyclic. Ring A is a benzonoid ring; ring B is a seven-membered ring. Ring C, a six-membered ring, gives enolone properties to the alkaloid.(Eigsti and Dustin, 1955).

The exact mechanism of colchicine-mitosis (colchicine-arrested metaphase) is not completely understood. Several experiments have indicated that colchicine acts upon the cellular spindle fiber mechanisms similar to the reactions of enzymes and substrates.

Additional studies have shown the importance of the chemical structure of colchicine in colchicine-mitosis. Structural and activity analysis of colchicine and similar spindle fiber poisons reveal that for high colchicine activity ring A of the molecule nust have at least one methoxy group. The activity of colchicine is increased if the amino group of ring B is esterified. Ring C must be seven-membered with an esterified hydroxyl group for increased activity (Figure 1).

The chromosomes were harvested by centrifugation after a treatment with a hypotonic solution. The cells were then fixed with a mixture of glacial acetic acid and methyl alcohol. Fixed cells were stained on microscope slides.

IV. Mechanisms of chromosome banding

There are numerous staining procedures which have produced banding patterns on chromosomes. Among the more important banding techniques are quinacrine banding (Q-banding), Giemsa banding (G-banding), constitutive banding (C-banding), and reverse banding (R-banding). Various mechanisms are involved in each of the different banding techniques. It was once believed that histone proteins were major factors involved in chromosome banding. However, recent research has shown that it is improbable that histones are the critical molecules responsible for chromosome banding. Chromosomes treated with 0.2 N hydrochloric acid for up to four hours produced uniform staining with Giemsa. Sodium dodecyl sulfate (SDS) gel electrophoresis showed that the acid treatment removed all of the chromosomal histones. After

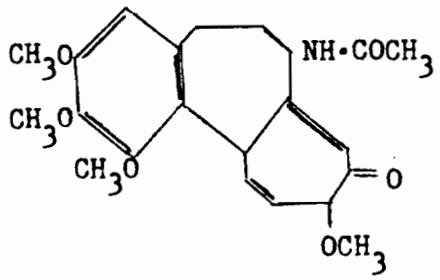


Figure 1. Colchicine (Eigsti and Dustin, Jr., 1955).

a trypsin treatment, such chromosomes without any histones produced a typical banding pattern (Comings et. al., 1974, cited by Comings, 1978).

A. Q-banding

Q-banding or quinacrine-banding utilzes an acridine dye to produce a banding pattern on the chromosomes when viewed with a fluorescent light microscope (Winchester, 1979). Chromosomes with Q-banding patterns have dark bands in areas of high DNA content (euchromatic) and light bands in areas of low DNA content (heterochromatic).

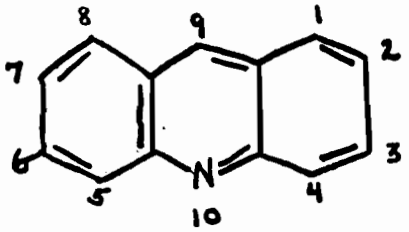
A common quinacrine fluorescence procedure involves quinacrine mustard or quinacrine dihydrochloride staining of fixed chromosome preparations. The stained metaphase chromosomes possess characteristic banding patterns when observed with a fluorescent light microscope. The quality of the quinacrine fluorescence patterns is affected by pH, temperature, and hydration state (Lin et. al., 1975, cited by Blazak, 1976).

The quality of Q-banding is also dependent upon the type of fluorescent dye compound utilized. The most common groups of dyes used in

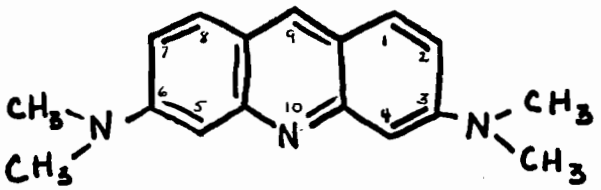
Q-banding are acridine derivatives. A few of the most popular acridine derivatives which interact with DNA include acridine orange, proflavine, acriflavine, quinacrine, and quinacrine mustard. Figure 2 shows the fundamental structure of an acridine compound as well as specific structures of acridine derivatives.

Acridine dye compounds interact with nucleic acids either by alkylation of particular purine bases in DNA (quinacrine mustard) or by interaction of the negatively charged DNA phosphate backbone with the positive charges of the dye compounds (acridine orange, proflavine, and acriflavine) (Comings, 1978). Some of the acridine derivatives when allowed to interact with DNA do not produce characteristic chromosomal banding patterns. For instance, acridine orange molecules are not capable of intermolecular interations in the double-stranded DNA helix and fluoresce green. In contrast, acridine orange molecules interact with single-stranded DNA and fluoresce red. As a result of such properties acridine orange is considered an indicator of the secondary structure of DNA.

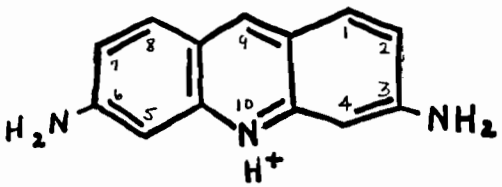
Other acridine derivatives such as quinacrine, proflavine, and acriflavine are important in chromosome identification (Rigler, 1973). Proflavine and acriflavine produce distinct bands on plant chromosomes, but faded and unclear bands appear on animal chromosomes (Zech, 1973). The intensity of Q-bands is dependent upon the structures of the acridine dye compound and the DNA molecule. Early studies proposed that good Q-banding was attributed to the presence of a large side group on position 9 of an acridine derivative (Comings et. al., 1975,



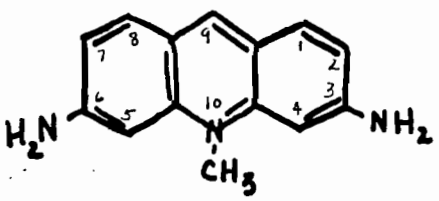
FUNDAMENTAL STRUCTURE OF ACRIDINE COMPOUND



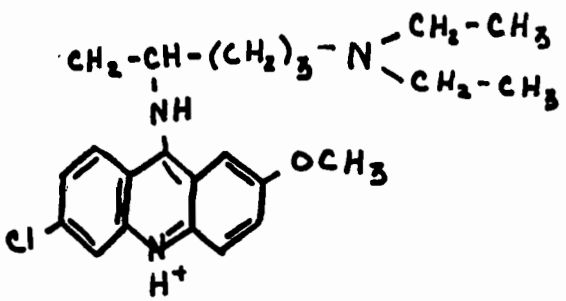
ACRIDINE ORANGE



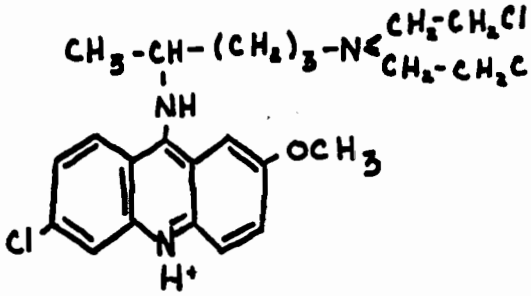
PROFLAVINE



ACRIFLAVINE



QUINACRINE



QUINACRINE-MUSTARD

Figure 2. Structures of acridine compounds. (Rigler, 1973).

cited by Comings et. al., 1978). However, recent investigations have shown that it is not necessary to have large groups at position 9 of an acridine compound to produce good Q-banding (Comings et. al., 1978). For example, 6-chloro-9-amino-2-methoxyacridine produces excellent quality Q-bands. Acriflavine without a group at position 9 produces fair bands. Comings et. al. (1978) state that good Q-banding is produced when an acridine compound has a 6-chloro, or 6-bromo, and an amino group or larger side group at position 9. Acridine derivatives with a 2-methoxy group produce high quality Q-banded chromosomes (Comings et. al., 1978).

The most important factor involved in the quality of Q-banding is the DNA base content found in the chromosome. It is well-documented that quinacrine fluorescence is increased in A-T rich sequences and decreased in G-C rich sequences of DNA (Ellison and Barr, 1972; Wessblum and deHasseth, 1972, cited by Mayfield and McKenna, 1978). Fluorescence occurs only when acridine molecules are not exposed to a G-C base pair (Rigler, 1973).

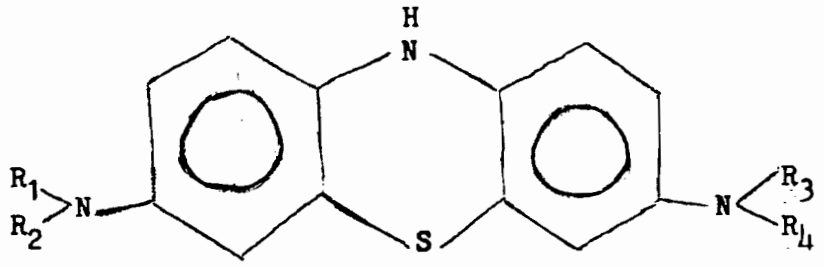
B. G-banding

G-banding or Giemsa banding has become one of the most useful chromosome identification techniques for clinical and research purposes. The primary reason for such widespread use of G-banding is that banding patterns are produced without expensive equipment such as a fluorescent light microscope. Arrighi and Hsu (1970, cited by Schnedl, 1973) developed the original G-banding methods involving

heat and alkali pretreatments. Dutrillaux et. al. (1971, cited by Schnedl, 1973) modified G-banding techniques with the introduction of a proteolytic enzyme such as trypsin. Currently, G-bands are produced either by an acid-saline-Giemsa (ASG) treatment or a proteolytic enzyme treatment (Winchester, 1979). Variations of G-banding include Giemsa 9 and Giemsa 11 banding which produce similar banding patterns. Giemsa 9 and Giemsa 11 banding patterns are produced with a G-banding technique in an environment of pH 9 and pH 11, respectively.

One of the most important elements of the Giemsa-banding techniques is the Giemsa stain. Giemsa is a complex mixture of dyes of various oxidation levels which include methylene blue (4-methylthiazin), Azure B (3-methylthiazin), Azure A (2-methylthiazin), Azure C (1-methylthiazin), thionin (0-methylthiazin), and eosin (Comings, 1978). Eosin is not necessary for the production of Giemsa bands but when the eosin is present the quality of the G-banded chromosomes is enhanced. Neither pure eosin nor methylene blue produces characteristic G-banded chromosomes. Most of the Giemsa dye constituents are derivatives of the aminophenothiazine molecule (Figure 3).

Ionic interation occurs with the DNA phosphate groups and the positively charged planar thiazin derivatives which causes side stacking along the DNA molecule (Comings, 1978). Maximum binding occurs in the presence of an equimolar ratio of DNA phosphate groups and thiazin dye molecules. Studies have shown that an interaction of excess Giemsa and pure DNA have a dye/phosphate molar ratio of 1.0 when all the phosphate groups are bound to the dye. Under similar



COMPOUND	R_1	R_2	R_3	R_4
Methylene blue	Me	Me	Me	Me
Azure B	Me	Me	Me	H
Thionine	H	H	H	H
Toluidine blue (2-methyl substituted)	Me	Me	H	H

Figure 3. Aminophenothiazine chromophores (shown in reduced form)(Modest and Sengupta, 1973).

conditions unfixed chromatin has a dye/phosphate molar ratio of 0.5 - 0.6 when half the phosphates are bound. In methanol-acetic acid fixed chromosomes the ratio is 0.8 where some of the protein is removed and, more interaction occurs between the dye and the phosphate groups (Comings, 1978).

The exact mechanisms of G-banding are not completely understood. Basically, Giemsa-banding is an "enhancement of basic chromomere pattern" (Comings, 1973). Electron microscopy has been an invaluable tool in analyzing chromosomes before and after various treatments. With the use of electron microscopy it has been shown that methanol-acetic acid fixed chromosomes are uniformly electron dense and have no banding patterns (Burkholder, 1974 and 1976, cited by Comings, 1978).

However, after a trypsin treatment of the fixed chromosomes, electron dense areas and banding areas are observable. From these observations it was concluded that either there is a rearrangement of chromatin fibers or a loss of chromatin in the interband regions (Bath, 1976, cited by Comings, 1978).

Several experiments have been conducted utilizing labeled ^{3}H-thymidine chromatin (Comings et. al., 1973; Pathak et. al., 1973, cited by Comings, 1978). In such studies there was little loss of DNA with G-banding. Therefore, a major factor of G-banding was a rearrangement of chromatin fibers primarily aided by a removal of calcium ions during the G-banding procedure.

Positive G-bands are produced where the thiazin dye molecules are capable of binding with chromatin which has free DNA phosphate groups. Negative G-banding occurs because metaphase chromosomes have chromatin-dense areas as well as chromatin-sparse interband areas. Also, DNA phosphate groups which are covered and unable to bind with the Giemsa dye produce negative G-banding. It is also believed that trypsin and hot salt treatments extract some of the interband DNA and, thus, contributing to the production of negative G-banding.

C. C-banding

Constitutive-banding or C-banding techniques stain the constitutive heterochromatin in the areas of the centromere, satellites, and secondary constrictions (Winchester, 1977). The C-banding techniques are actually modifications of Giemsa staining. Pardue

and Gall (1970) discovered that centromeric heterochromatin stained deeply with Giemsa after chromosomal pretreatment. Fixed chromosomes were pretreated with a basic solution of NaCl followed by 2 X SSC (0.3 M NaCl, 0.03 M trisodium citrate) solution at 66°C for overnight (Comings, 1978). Arrighi and Hsu (1971, cited by Comings, 1978) modified the Pardue and Gall (1970) technique so as to produce C-banding patterns on the chromosomes. Originally, it was believed that the NaOH treatment denatured the DNA while the SSC incubation allowed for renaturation of highly repetitive DNA sequences (heterochromatin areas). However, the original G-banding theory was questioned when C-bands appeared as G-bands disappeared on the same chromosome after extended periods of trypsin treatment (Merrick et. al., 1973; Oskinay et. al., 1979).

The fundamental mechanism of C-banding is the extraction of non-C-band DNA and the retention of C-band DNA (Comings, 1973, cited by Comings, 1978). Comings (1978) notes that C-banding techniques may remove as much as 60% of the chromosomal DNA, primarily from the non-C-band areas. From such observations it is concluded that the centromeric heterochromatin has a more tightly bound coat of proteins than the non-centromeric heterochromatic areas. Only with extensive chemical treatments was the centromeric heterochromatic protein coat removed which allowed for the appearance of C-bands.

D. R-banding

R-banding or reverse banding occurs when chromosomes are Giemsa

stained after a high temperature buffer treatment for ten minutes. R-banding stains chromosomes between the G-bands (interband regions). R-banding is useful in chromosome analysis since the telomeres stain well. T-banding, a modification of R-banding, stains only the telomeres (Comings, 1978).

The major factor in the R-banding mechanism is the G-C richness of the R-bands. Several observations substantiate the theory of G-C richness in R-bands. R-banding procedures selectively denature A-T rich DNA sequences while at the same time do not affect the G-C rich DNA sequences. Studies with fluorescent compounds such as chromomycin A_3, olivomycin, and mithramycin (which bind with G-C rich DNA sequences) reveal that the fluorescent G-C rich areas are darkly stained in R-banded chromosomes (Comings, 1978).

The various chromosome banding techniques enable categorization of three different types of chromatin: euchromatin, intercalary or G-band heterochromatin, and constitutive or C-band heterochromatin. Euchromatin, areas of high DNA content, is dispersed in interphase, potentially genetically active, and early replicating. Euchromatin is stained deeply in R-banding techniques. Intercalary heterochromatin is late replicating, A-T rich, and located in G-bands. Centromeric or constitutive heterochromatin is detected by C-banding. The three major types of chromatin and specific chracteristics are summarized in Table 3.

V. Applications of bovine chromosome studies

Table 3. Three major types of chromatin in chromosome bands.(Comings, 1978).

	CENTROMERIC CONSTITUTIVE HETEROCHROMATIN	INTERCALARY HETEROCHROMATIN	EUCHROMATIN
Relation to bands	In C-bands	In G-bands	In R-bands
Location	Usually centromeric	Chromosome arms	Chromosome arms
Condition during interphase	Condensed	Condensed	Usually dispersed
Genetic activity	Inactive	Probably Inactive	Usually active
Timing of DNA replication	Late S	Late S	Early S
AT content of DNA	GC-rich, neutral or AT-rich depending on satellite DNA	AT-rich	GC-rich
Repetitiousness of DNA	Usually satellite DNA	Moderately repetitious and unique	Moderately repetitious and unique
DNA methylation	Frequently highly methylated	±	±
Relation to pachytene chromomeres	Centromeric chromomere	Intercalary chromomeres	Interchromeric

Analysis of banding patterns are useful in animal cytogenetics, gene mapping, and chromosome structural studies. The various banding techniques are especially useful in studying bovine chromosomes. Generally, G-banded chromosomes of mammals such as humans and rodents have a darkly stained centromere region (Arrighi and Hsu, 1971, cited by Schnedl, 1973). Exceptions to such a general observation exist in cattle chromosomes. G-banded bovine chromosomes have an unstained centromeric region of G-banded chromosomes which is also observable in sheep and pigs (Schnedl, 1973). The Giemsa banding technique produces banding patterns on cattle chromosomes as well as sheep chromosomes. The G-banded metaphase chromosomes of cattle and other mammals have major bands or stained regions.

The approximate number of bands on a single chromosome is relatively constant within a species. Table 4 shows that cattle chromosomes have 81 major bands, human chromosomes have 82 major bands and mouse chromosomes have 56 major bands. Such major chromosomal bands contain smaller bands (minor bands). It is assumed that the minor bands have even more minute structures. It is estimated that one of the minor bands of the chromosome consists of approximately three million nucleotide pairs (Schnedl, 1973).

Bovine chromosome studies utilizing G-banding and R-banding techniques have been involved with the analysis of sister chromatid exchanges (Logue, 1978). Chromosome banding patterns are extensively utilized in the analysis of Robertsonian translocations. Such translocations are the most frequent chromosomal structural abnormalities

Table 4. Approximate number of major chromosome bands in various mammalian species (Schnedl, 1973).

SPECIES	NUMBER OF MAJOR CHROMOSOME BANDS
Human	82
Cattle	81
Sheep	83
Pig	72
Mouse	56
Rat	66

that occur in the superfamily Bovoidea which includes Bos taurus. Such structural abnormalities of the chromosome increases the rate of non-disjunction during meiosis. A 14/20 Robertsonian translocation is identified in Swiss Simmental cattle (Logue et. al., 1978). Utilizing G-, Q-, and T-staining techniques Gustavsson et. al. (1976) noted the widespread occurrence of a 1/29 Robertsonian translocation in the Swedish Red and White cattle breed. The 1/29 Robertsonian translocation is widespread in all of the cattle breeds. Other Robertsonian translocations in cattle as well as other structural chromosomal abnormalities are widely documented (Popescu, 1977; Popescu, 1978; Genest et. al., 1978).

Methods and Materials

Culturing of bovine chromosomes:

Blood samples were collected from the coccygeal artery or vein of registered Holstein-Friesian dairy cattle using a heparinized (without preservative) 3 cc - 22 G1 disposable syringe. A quantity of 1.5 ml of heparinized whole blood was immediately added to 9.0 ml of bovine culture medium which was contained in a 30 ml sterile bottle. After innoculation, 0.5 ml of phytohemagglutinin (PHA) (Gibco) was added to the blood-culture medium (Halnan, 1977). The culture was then transported for approximately thirty minutes by car to Lycoming College. Additional blood samples were obtained from bulls at Sire Power, Inc. (Tunkhannock, Pennsylvania). Blood samples of 1.5 ml were collected from three bulls at Sire Power, Inc.. As each blood sample was collected it was immediately added to the bovine media. A quantity of 0.5 ml of phytohemagglutinin was also added to each blood-culture medium. The cultures were transported to Lycoming College by car for approximately one and one-half hours. After arriving at Lycoming College the blood cultures were incubated at 37°- 39°C for approximately 72 hours. The cultures were gently agitated twice a day in order to increase the mitotic index (Brown, 1973, cited by Halnan, 1977).

The bovine medium was formulated to induce leukocyte mitosis. The medium with a primary ingredient of Medium 199 was prepared according to Halnan's (1977) method (see Table 1 for the formula of Medium 199). The recipe of the bovine culture medium is located in Table 2. The media was prepared and stored in a freezer at 0°C for as long as 14

Table 1. Ingredients of Medium 199 (GIBCO, 1978).

MEDIUM 199[1]

	Hanks' Salts Cat. #	Earle's Modified Salts Cat. #	Earle's Unmodified Salts Cat. #
LIQUID MEDIUM ▸	320-1151	320-1153	320-1150
POWDERED MEDIUM ▸	400-1200		400-1100
Component	*mg/L*	*mg/L*	*mg/L*
INORGANIC SALTS			
$CaCl_2$ (anhyd.)	140.00	200.00	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.72	0.72	0.72
KCl	400.00	400.00	400.00
KH_2PO_4	60.00		
$MgSO_4 \cdot 7H_2O$	200.00	200.00	200.00
(anhyd.)[a]	97.67	97.67	97.67
NaCl	8000.00	6800.00	6800.00
$NaHCO_3$[b]	350.00	1250.00	2200.00
$NaH_2PO_4 \cdot H_2O$		125.00	140.00
$Na_2HPO_4 \cdot 7H_2O$	90.00		
(anhyd.)[a]	47.70		
OTHER COMPONENTS			
Adenine sulfate			10.000
Adenosinetriphosphate (Disodium salt)			1.000
Adenylic acid			0.200
Cholesterol			0.200
Deoxyribose			0.500
Glucose			1000.000
Glutathione			0.050
Guanine HCl (Free base)			0.300
Hypoxanthine (.354 Na salt)[a]			0.300
Phenol red			20.000
Ribose			0.500
Sodium acetate			50.000
Thymine			0.300
Tween 80®			20.000
Uracil			0.300
Xanthine (.344 Na salt)[a]			0.300

MEDIUM 199[1] (Cont'd.)

Component	*mg/L*
AMINO ACIDS	
DL-Alpha-Alanine	50.000
L-Arginine HCl	70.000
DL-Aspartic acid	60.000
L-Cysteine HCl·H_2O	0.110
L-Cystine 26.00 (2 HCl)[a]	20.000
DL-Glutamic acid·H_2O	150.000
L-Glutamine	100.000
Glycine	50.000
L-Histidine HCl·H_2O	21.880
L-Hydroxyproline	10.000
DL-Isoleucine	40.000
DL-Leucine	120.000
L-Lysine HCl	70.000
DL-Methionine	30.000
DL-Phenylalanine	50.000
L-Proline	40.000
DL-Serine	50.000
DL-Threonine	60.000
DL-Tryptophan	20.000
L-Tyrosine 57.88 (2 Na)[a]	40.000
DL-Valine	50.000
VITAMINS	
Ascorbic acid	0.050
alpha tocopherol phosphate (disodium salt)	0.010
d-Biotin	0.010
Calciferol	0.100
Ca pantothenate	0.010
Choline chloride	0.500
Folic acid	0.010
i-Inositol	0.050
Menadione	0.010
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.050
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.010
Thiamine HCl	0.010
Vitamin A (acetate)[c]	0.140

References:

(1) Proc. Soc. Exp. Biol. Med., 73.1 (1950). Morgan, Morton and Parker.

Remarks:

(a) As supplied in powdered media

(b) Omitted from 10X preparations and powdered media

(c) Values established by the Tissue Culture Standards Committee.

Table 2. Recipe for bovine medium (Halnan, 1977).

Medium 199	65%
(Glutamine 0.1 gm/ 100 ml)	
(L-cystine 0.2 g/ 100 ml)	
Bovine serum or calf serum	25%
Heparin without preservative	750 iu %
Glutamine	0.01 g %
L-cystine	0.02 g %
L-tyrosine	0.03 g %
Penicillin	6 mg %
Streptomycin	10 mg %
Sodium bicarbonate 2.8% to correct pH to 7.3	approx. 5%

Table 2A. Preparation of 100 ml of bovine medium as used in this project.

Medium 199 (GIBCO)	65 ml
(Glutamine 0.1 gm/ 100 ml)	
(L-cystine 0.2 g/ 100 ml)	
Bovine serum (GIBCO)	25 ml
Heparin without preservative (Sherwood)	750 iu
Glutamine	0.01 g
L-cystine	0.02 g
L-tyrosine	0.03 g
Streptomycin (740 mg - gm)	10 mg
Penicillin (5,000,000 iu)	6 mg
Sodium bicarbonate (2.8%)	corrected to pH 7.3

days.

The cells of the blood cultures were arrested in the metaphase stage of mitosis with the addition of colchicine one hour before the cells were to be processed. Colchicine was added to the blood culture so as to obtain a final concentration of 0.05 ug per ml.

Preparation of Bovine Chromosomes

The chromosomes were harvested after an incubation period of approximately 72 hours as outlined by Supik (1973). The blood-culture medium was transferred to a 15 ml centrifuge tube, and centrifuged at 109 g (740 RPM) for one-half hour in an IEC - HN - SII model centrifuge. A thin, buffy coat of leukocytes was pipetted from the top of a sedimented mass of red blood cells. The leukocytes were then transferred to another 15 ml centrifuge tube. After gentle agitation the cells were treated with a hypotonic solution so as to attain a final volume of 7.0 ml. The hypotonic solution was a mixture of three parts distilled water and one part calf serum (Gibco) with a trace of sodium citrate. After the solution was centrifuged at 109 g (740 RPM) for seven minutes the supernatant fluid was discarded. The remaining cells were resuspended and 4.0 ml of freshly prepared fixative solution (3 parts methyl alcohol to one part glacial acetic acid) was added. The fixed mixture was centrifuged at 109 g (740 RPM) for seven minutes. The supernatant was removed after centrifugation. The fixation and centrifugation procedures were repeated two times. The cells were then resuspended in the fixative after discarding the supernatant so as to

leave a cell volume of 1.25 ml. The resuspended cells were dropped on chilled, clean microscope slides and air-dried.

G-banding

The majority of the harvested chromosomes were subjected to a Giemsa staining procedure. Slides were immersed in a 0.025% trypsin (DIFCO trypsin 1: 250) solution at pH 7.0 - 7.1 for 60 to 70 seconds at room temperature. The trypsin solution was prepared in Hank's balanced salt solution free of calcium and magnesium. Slides were gently rinsed with distilled water. They were then stained with a Giemsa solution for three minutes. The Giemsa solution was composed of one part Giemsa stain and 15 parts phosphate buffer, pH 7.0. The phosphate buffer was a mixture of 39 ml 0.2 M monobasic sodium phosphate, pH 5.5, and 61 ml 0.2 M dibasic sodium phosphate, pH 8.5, diluted with 100 ml of distilled water. Table 5 summarizes the Giemsa staining procedure.

Microscopic Analysis:

The stained chromosomes were preserved on the microscope slides by cover slipping with "perma-mount". Chromosome spreads in metaphase were identified with a microscope at 400X and 1000X magnification powers. Such metaphase cells were identified by their coordinate locations so that they could be easily found later for photomicrography.

Table 5. Giemsa-banding technique (Supik, 1973).

1.	Trypsin treatment	60 - 70 seconds
2.	Distilled water rinse	
3.	Giemsa stain	3 minutes
4.	Distilled water rinse	

Karyotyping:

The identified chromosome spreads were photographed with Kodak High Contrast Copy Film (HC- 135) using a Biostar Inverted Biological Microscope equipped with an AO Model 1053 35mm camera and an AO Expostar Shutter Control. The film was processed with D-19 developer. The pictures of the chromosomes were printed on Agfa 5 paper. Later, enlarged pictures of the chromosomes were karyotyped. The karyotypes of several animals were compared and analyzed.

Results

By utilizing the chromosome harvesting techniques as described in the methods and materials section, chromosome spreads were processed. Figure 4 is a photograph of a chromosome spread which was stained with a Giemsa solution without any proteolytic enzyme pretreatment. Giemsa- banded chromosome spreads of seven animals within the same pedigree (Figure 5) were obtained. Each spread was photographed and enlarged. From each chromosome spread a karyotype was produced (Figures 7, 10, 13, 16, 19, 22, and 25). A karyotype consists of 29 homologous pairs of autosomes arranged from the largest to the smallest chromosome. The thirtieth pair of chromosomes are the sex chromosomes. A female has two X chromosomes; a male has an X and a Y chromosome.

For each animal a metaphase chromosome spread, karyotype, and idiogram are presented (Figures 6-26). Each idiogram is my subjective interpretation of specific banding patterns found on each pair of chromosomes in a karyotype. Preparation of each idiogram was completed independent of any previous analysis. This was particularly important since it is the purpose of this project to make an analysis of chromosome banding patterns found in a lineage of cattle.

In order to compare the banding patterns between animals several chromosomes were selected to make a critical analysis of Giemsa banding patterns. The first chromosome and the X chromosome of each karyotype were compared. Chromosome #1 was chosen because it is the largest autosome. The X chromosome was compared because it is easily identified in both female and male animals. Chromosomes #8, #17, and #22 of each

karyotype were also compared. These three chromosome numbers were chosen randomly using a random numbers table.

Comparison and analysis of specific chromosomes are presented in Figure #27. The purpose of such a presentation is to provide an easy way of visualizing the banding patterns in the chromosomes.

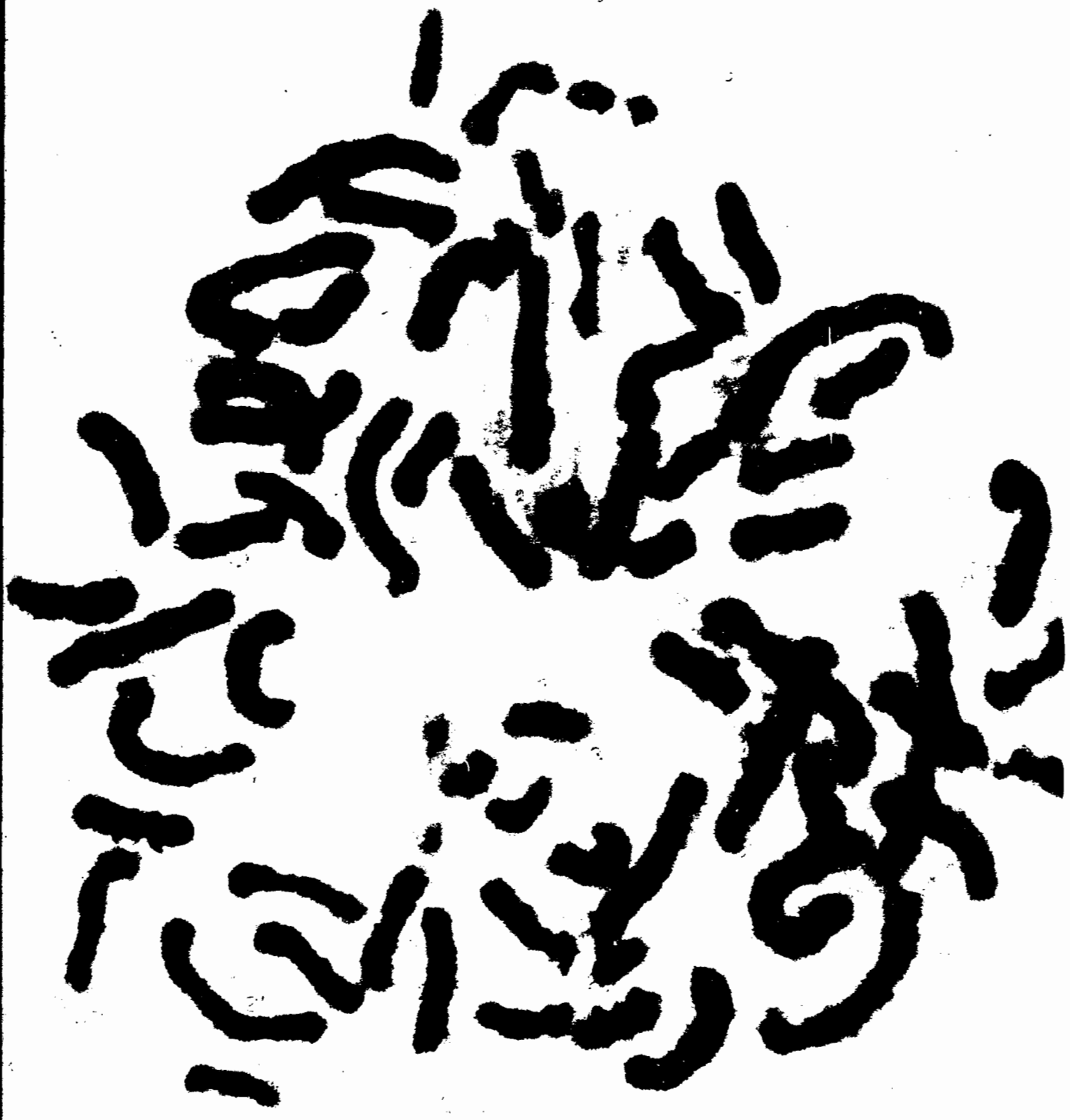
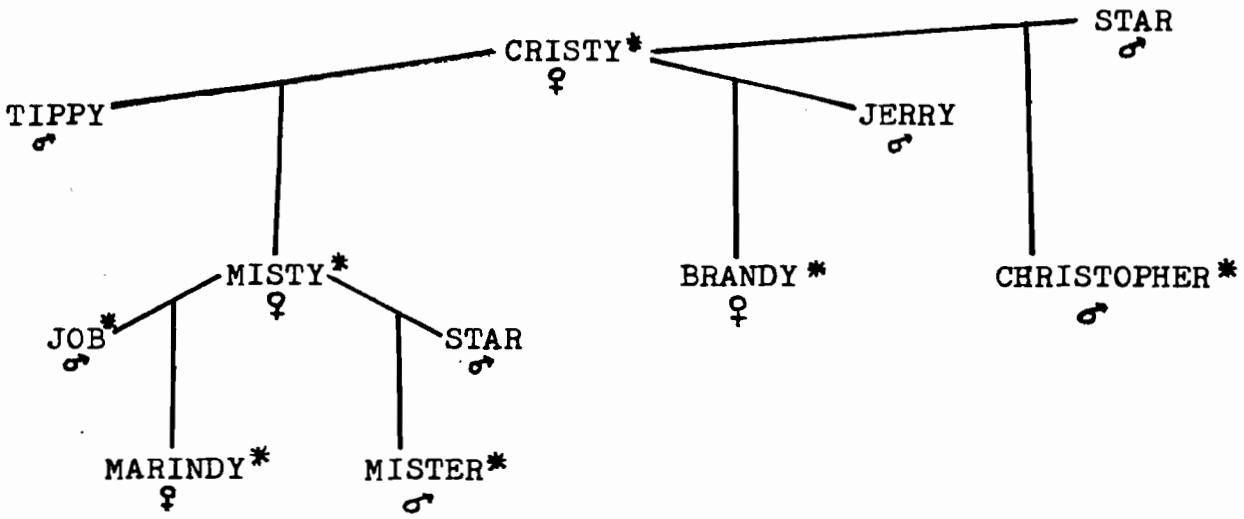


Figure 4. Giemsa stained (no proteolytic enzyme pre-treatment) chromosome spread of Bos taurus.

PEDIGREE OF CRISSONS ELEVATION CRISTY



* Blood available (chromosome analysis completed)

NOTE: Blood is not available from Tippy or Jerry because they are deceased.

Blood is not readily available from Star due to transportation problems. Star is a bull in Carnation Genetics, Inc. which is located in California.

Figure 5. Pedigree of Crissons Elevation Cristy.

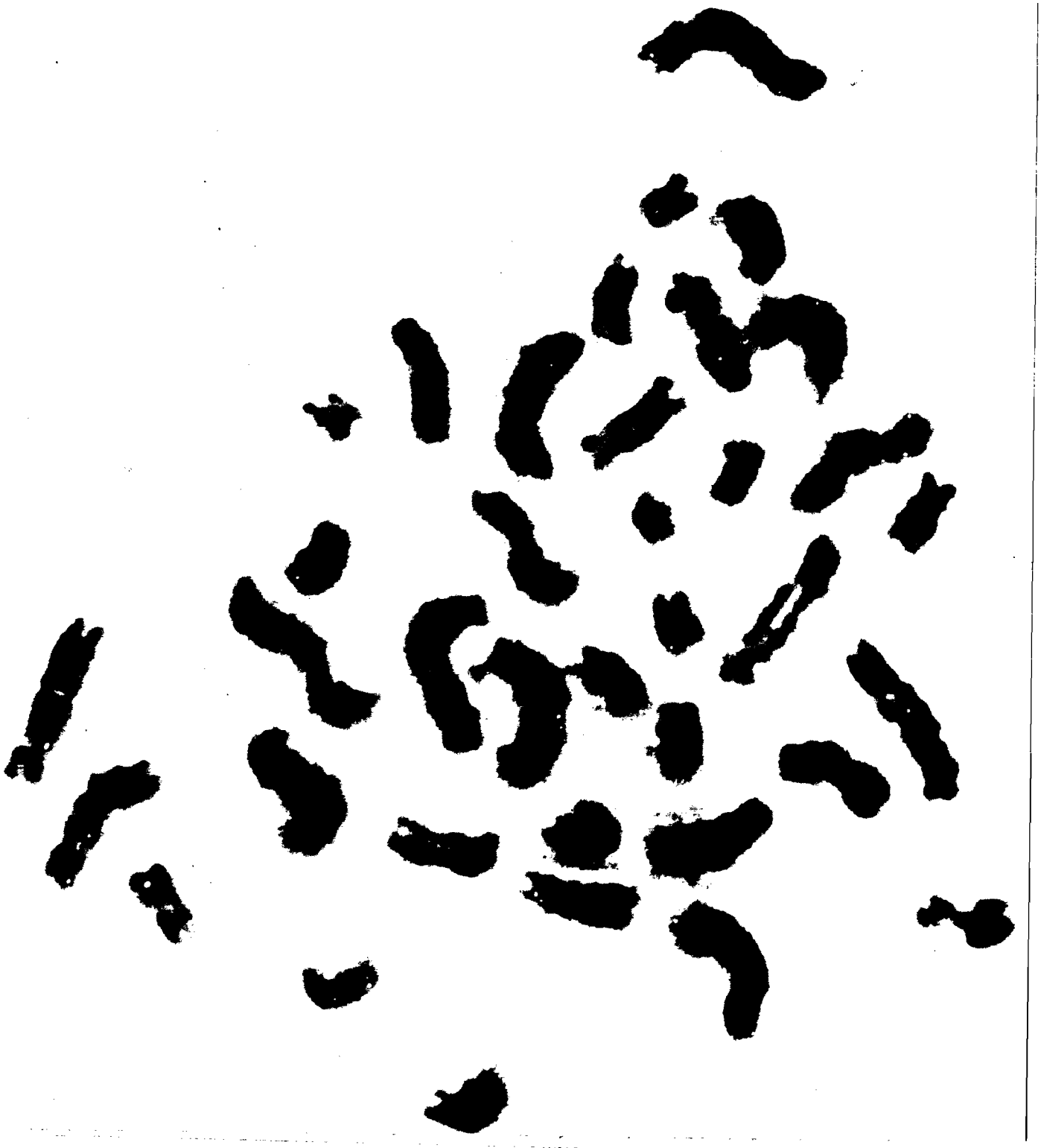


Figure 6. Giemsa-banded chromosome spread of a Holstein female (Cristy).

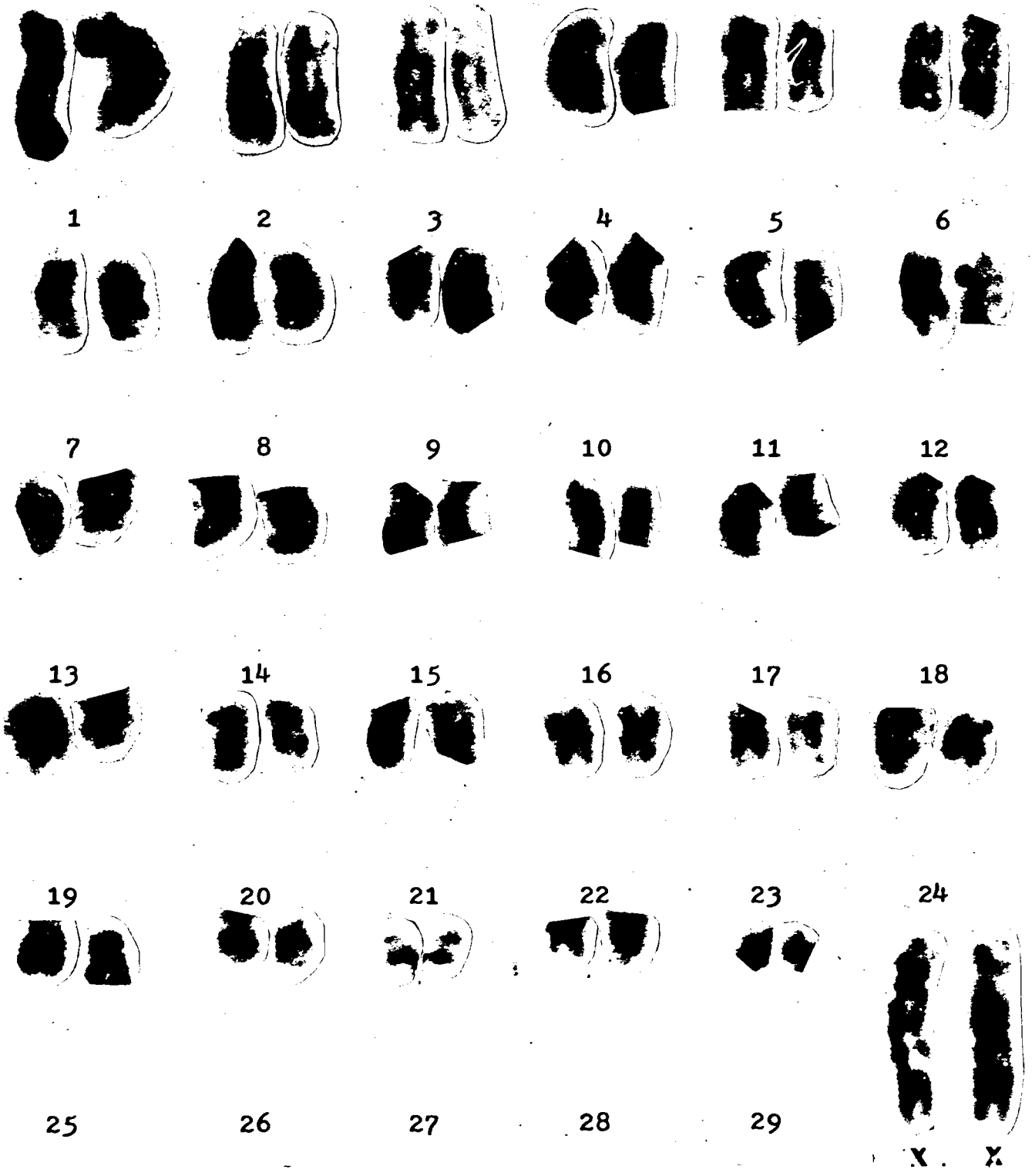


Figure 7. Karyotype of a Holstein female (Cristy).

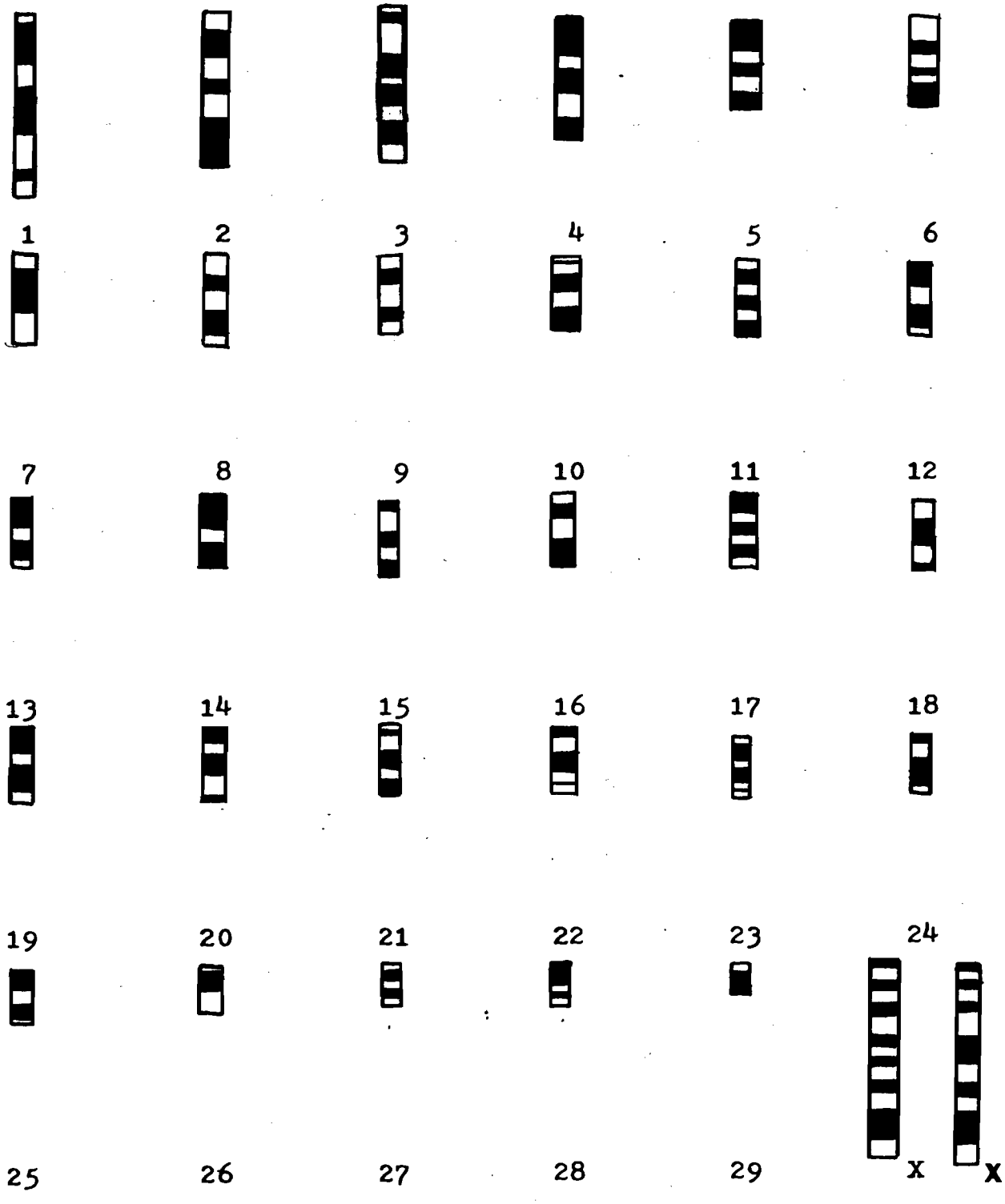


Figure 8. Idiogram of a Holstein female (Cristy).

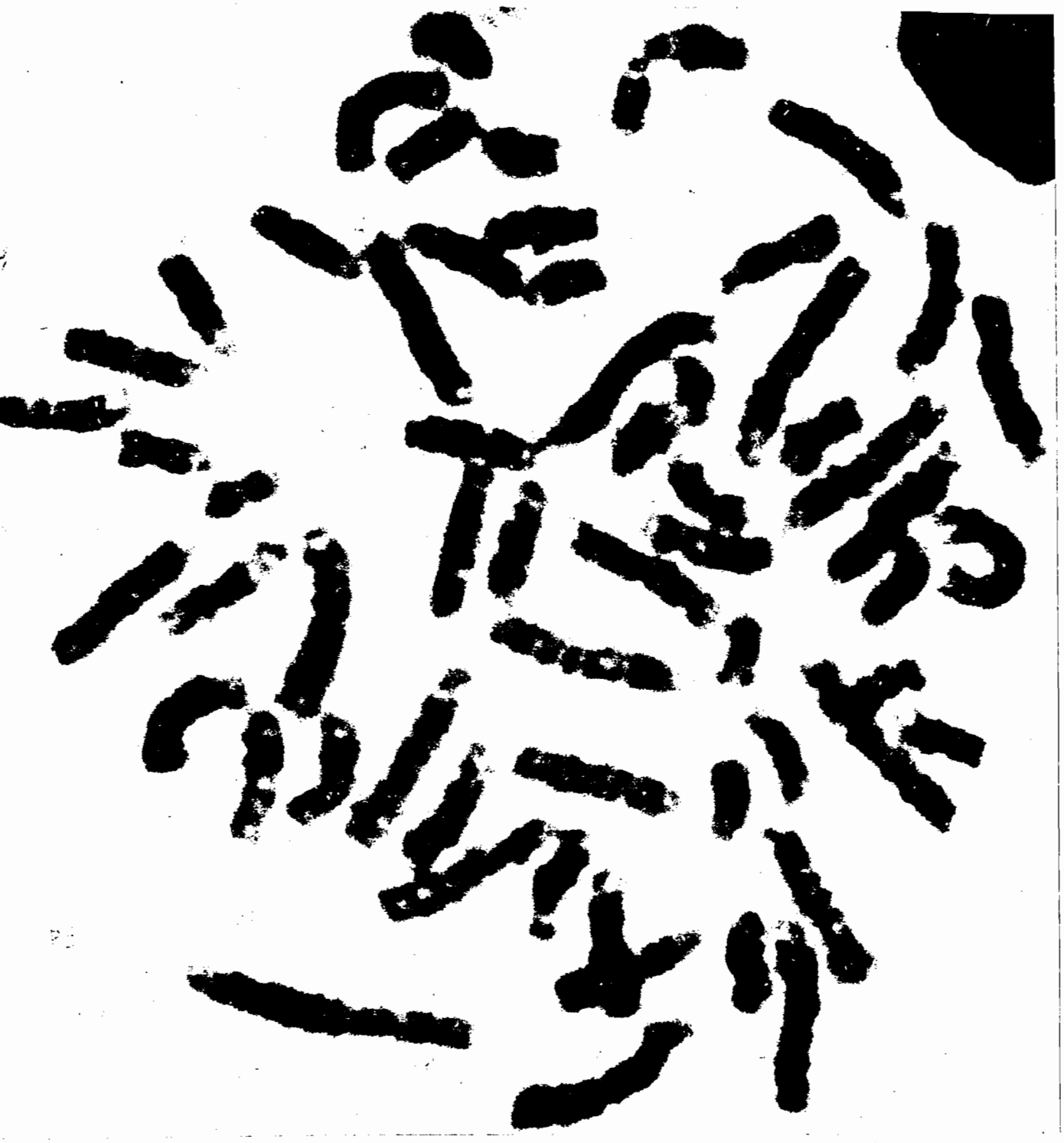


Figure 9. Giemsa-banded chromosome spread of a Holstein male (Christopher).

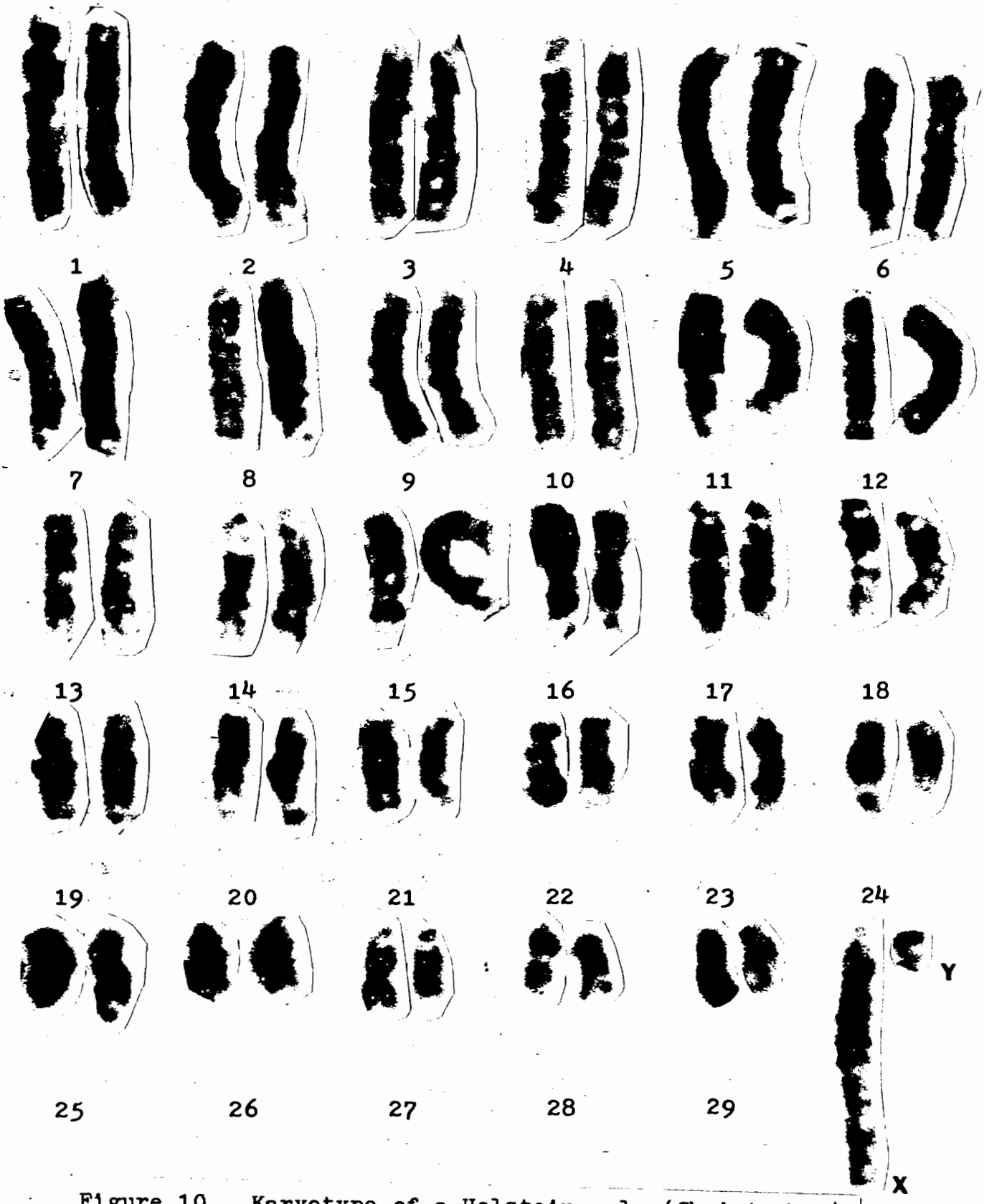


Figure 10. Karyotype of a Holstein male (Christopher).

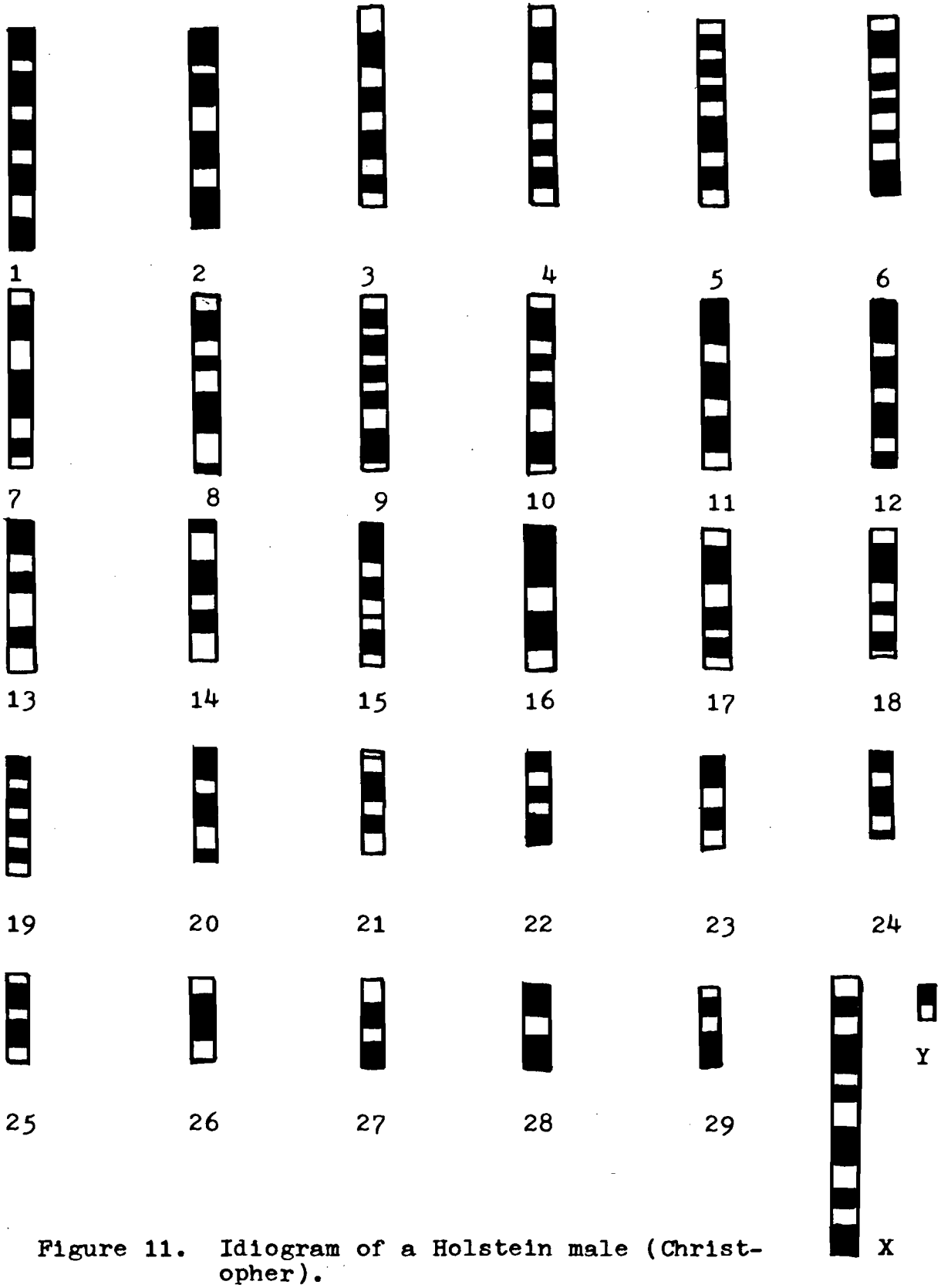


Figure 11. Idiogram of a Holstein male (Christopher).

Figure 12. Giemsa-banded chromosome spread of a Holstein female (Brandy).

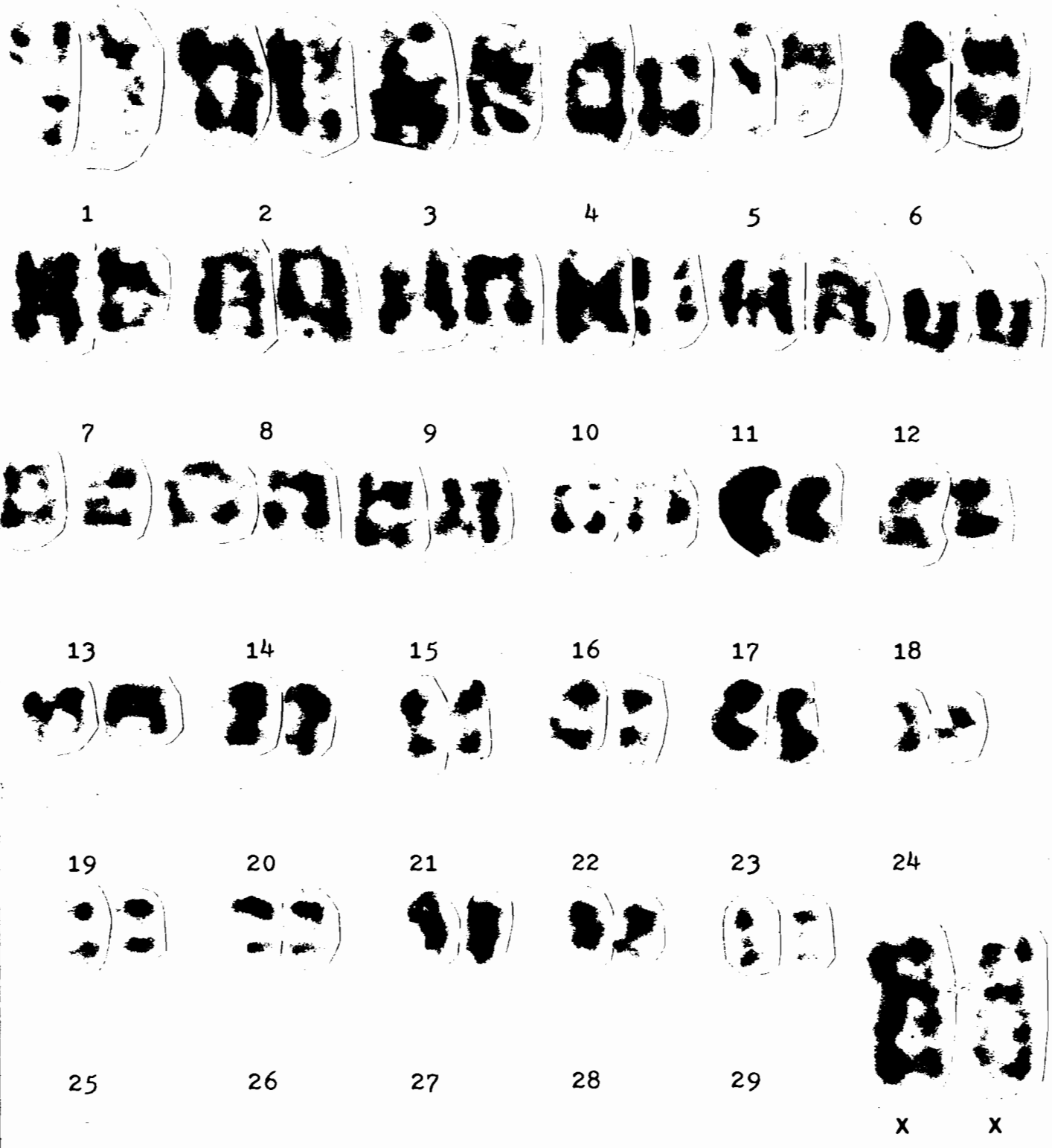


Figure 13. Karyotype of a Holstein female (Brandy).

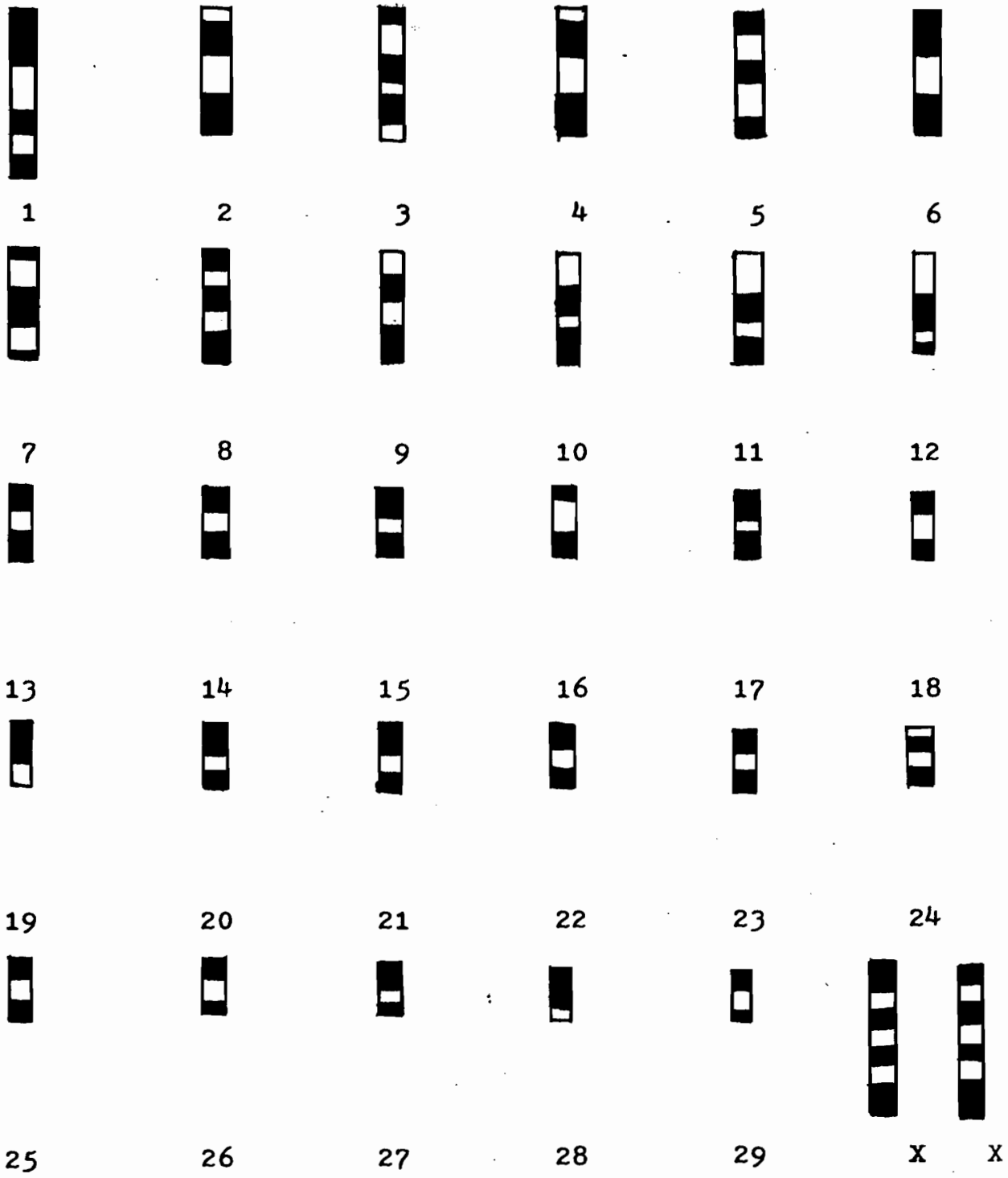


Figure 14. Idiogram of a Holstein female (Brandy).

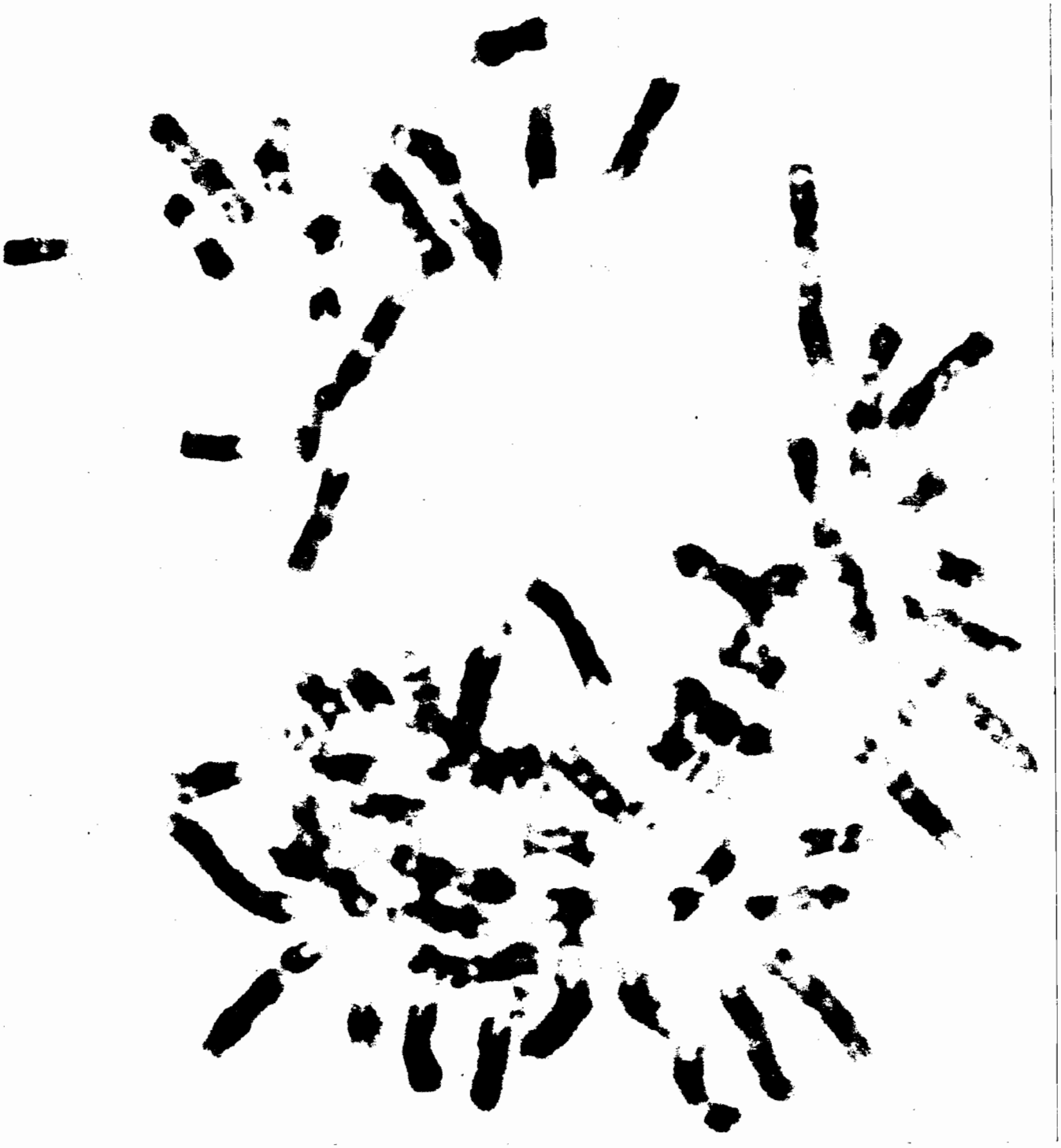


Figure 15. Giemsa-banded chromosome spread of a Holstein female (Misty).

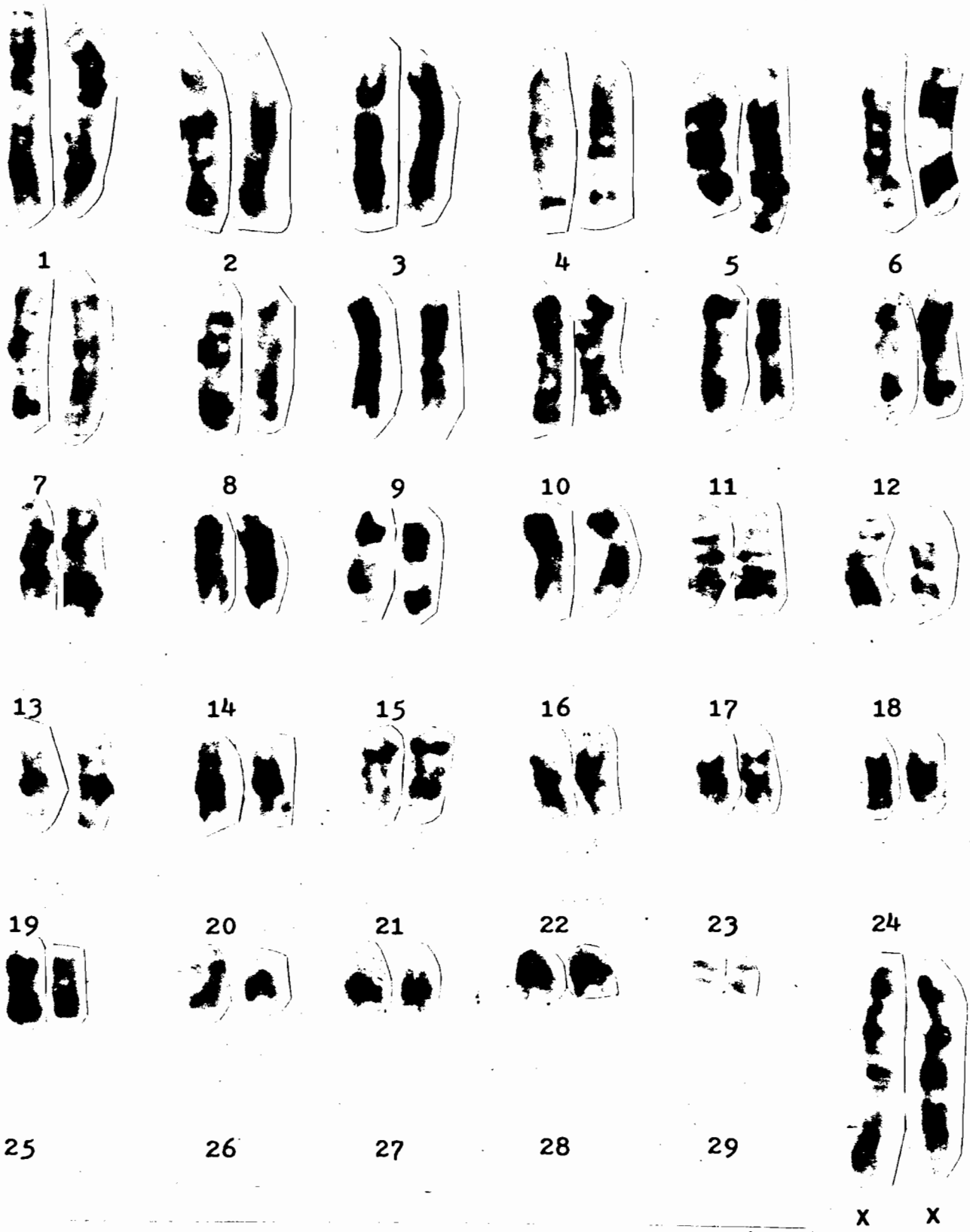


Figure 16. Karyotype of a Holstein female (Misty).

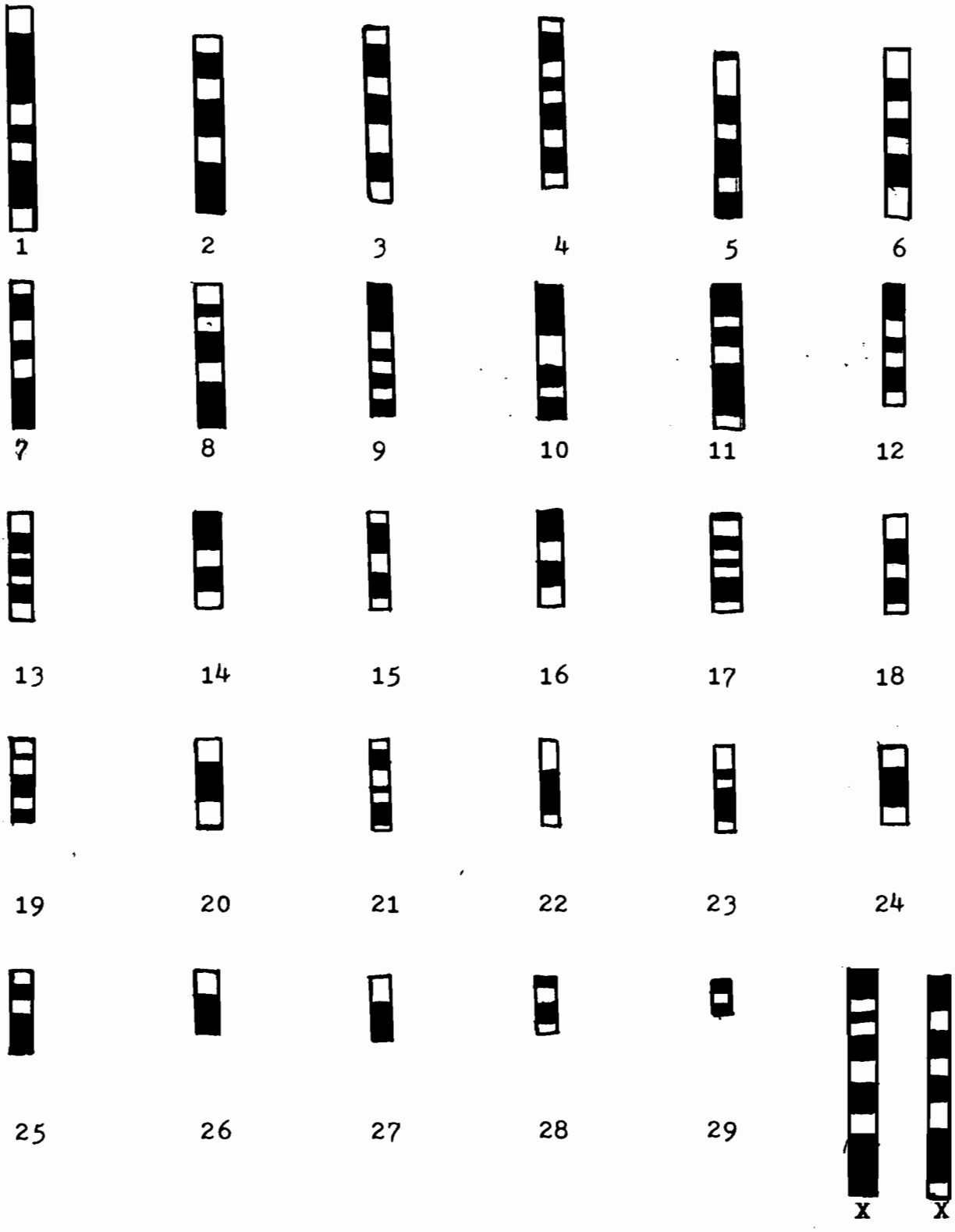


Figure 17. Idiogram of a Holstein female (Misty).

Figure 18. Giemsa-banded chromosome spread of a Holstein male (Mister).

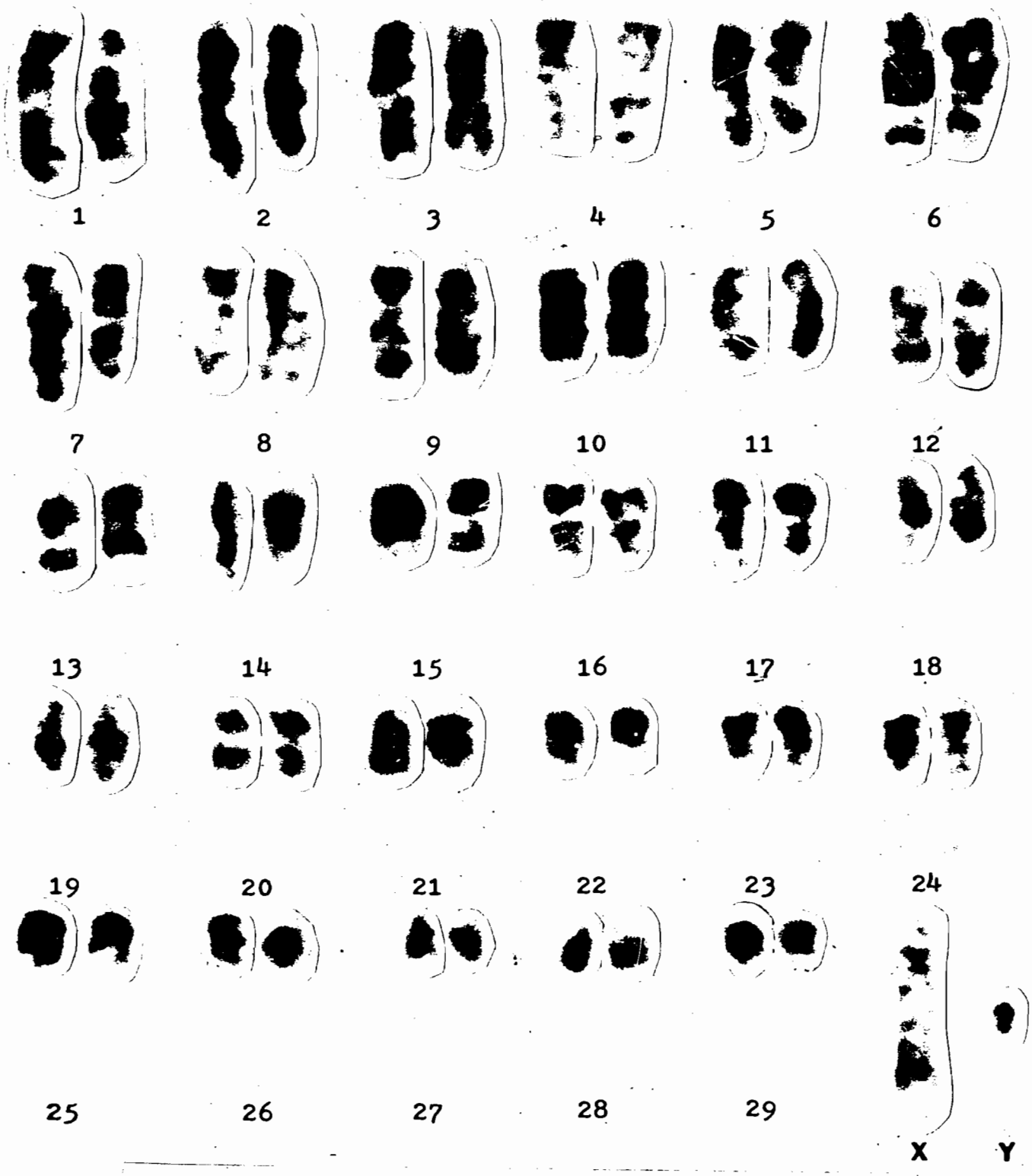


Figure 16. Karyotype of a Holstein male (Mister).

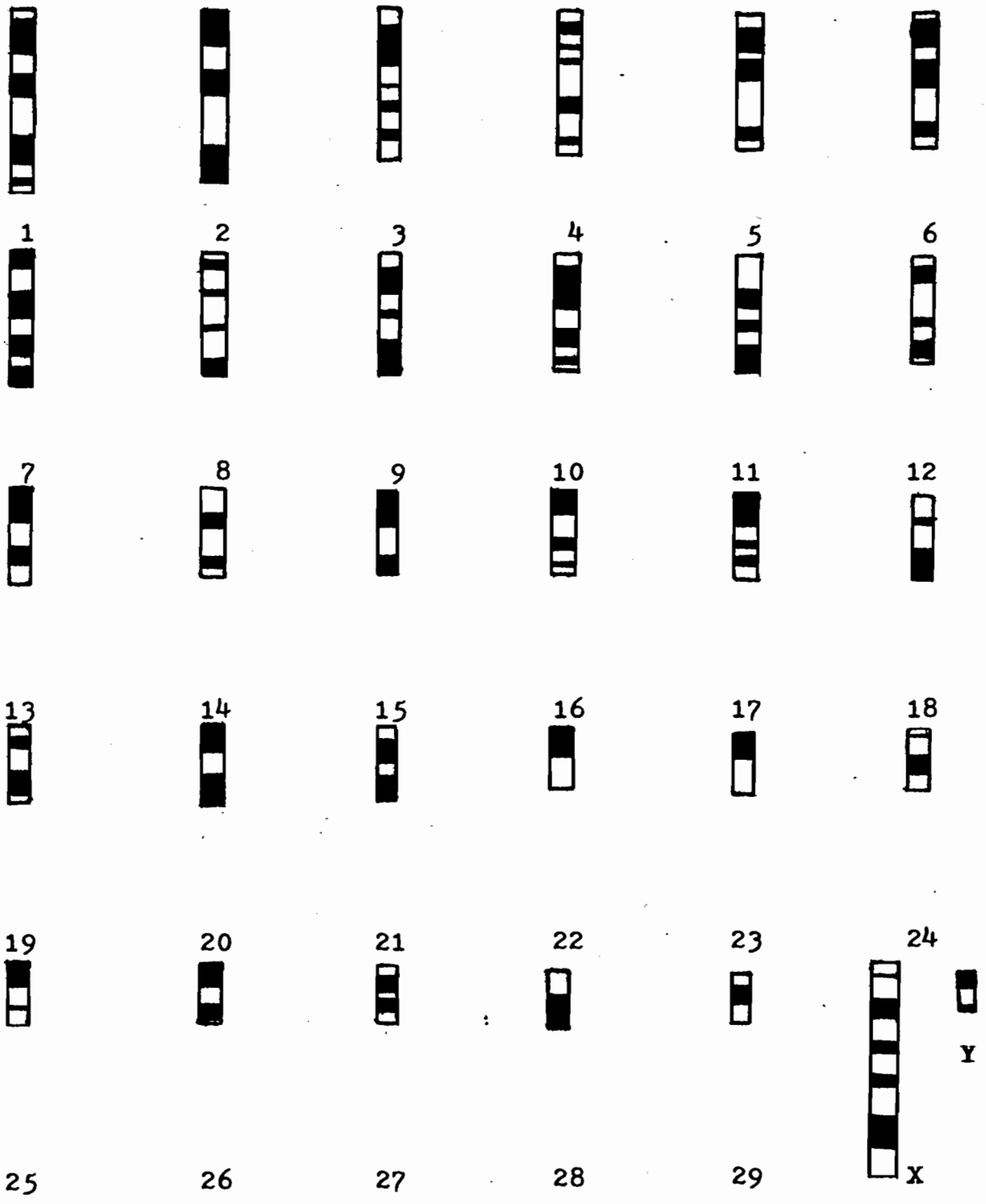


Figure 20. Idiogram of a Holstein male (Mister).

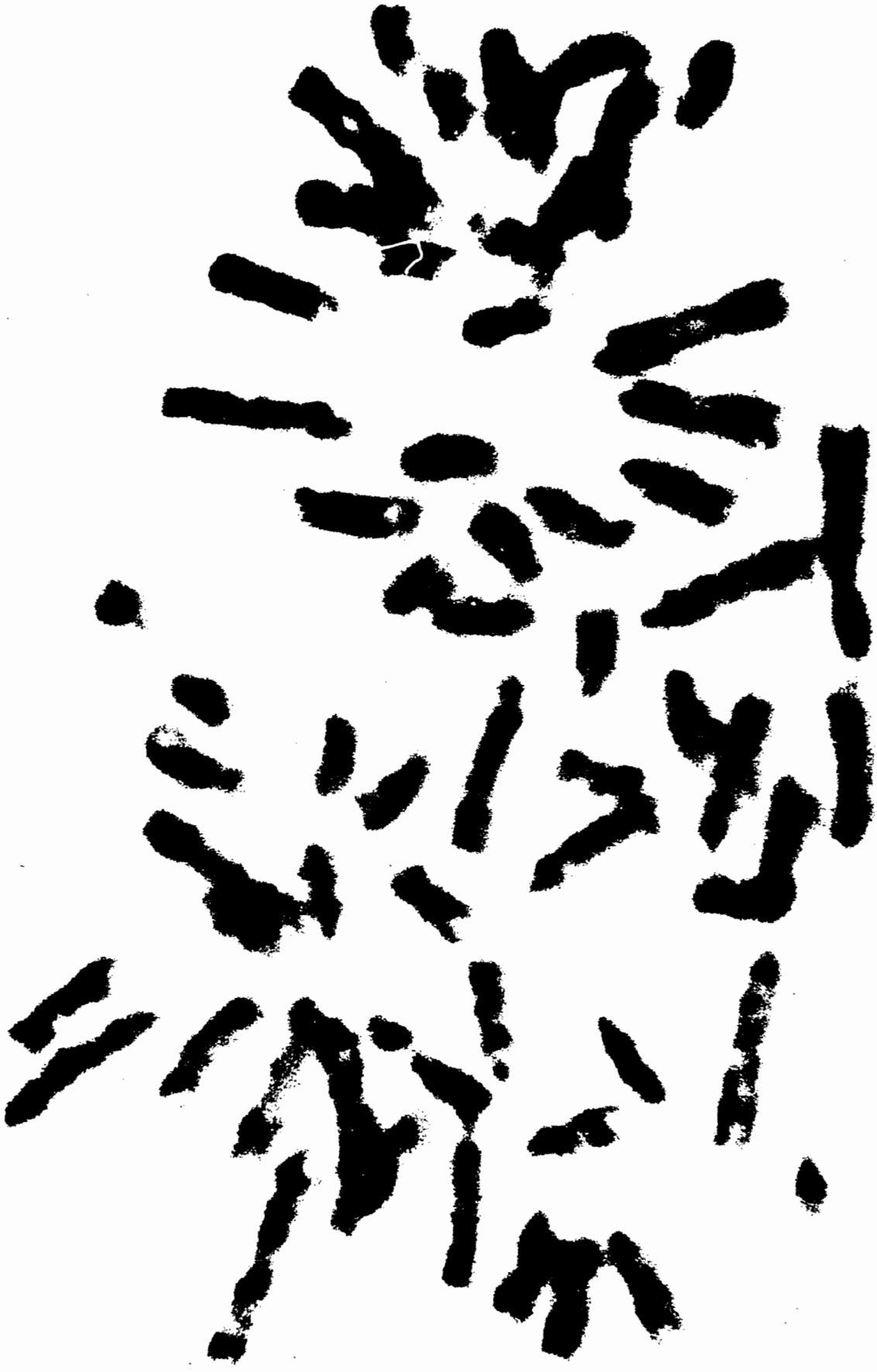


Figure 21. Giemsa-banded chromosome spread of a Holstein female (Marindy).

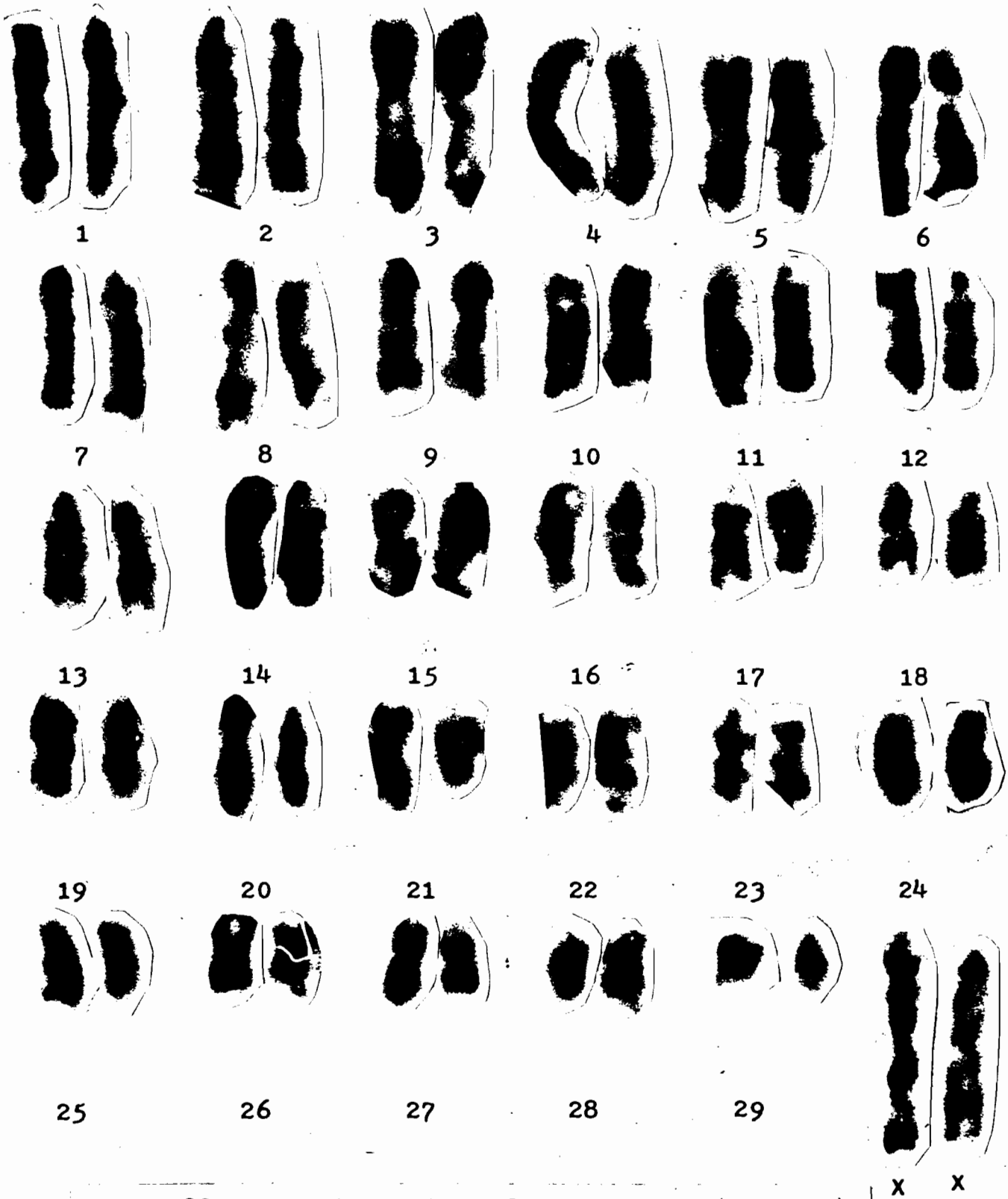


Figure 22. Karyotype of a Holstein female (Marindy).

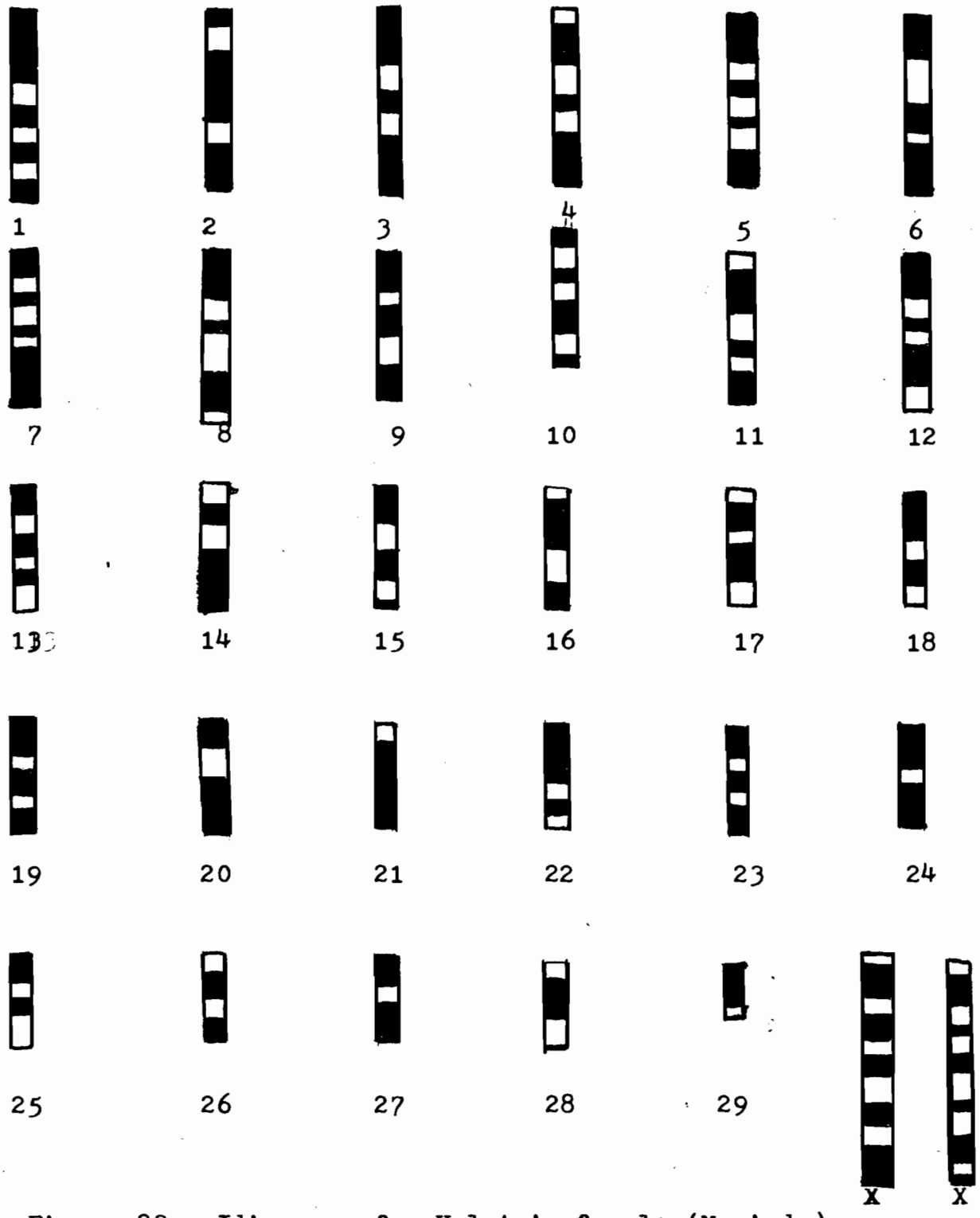


Figure 23. Idiogram of a Holstein female (Marindy).

Figure 24. Giemsa-banded chromosome spread of a Holstein male (Job).

Figure 25. Karyotype of a Holstein male (Job).

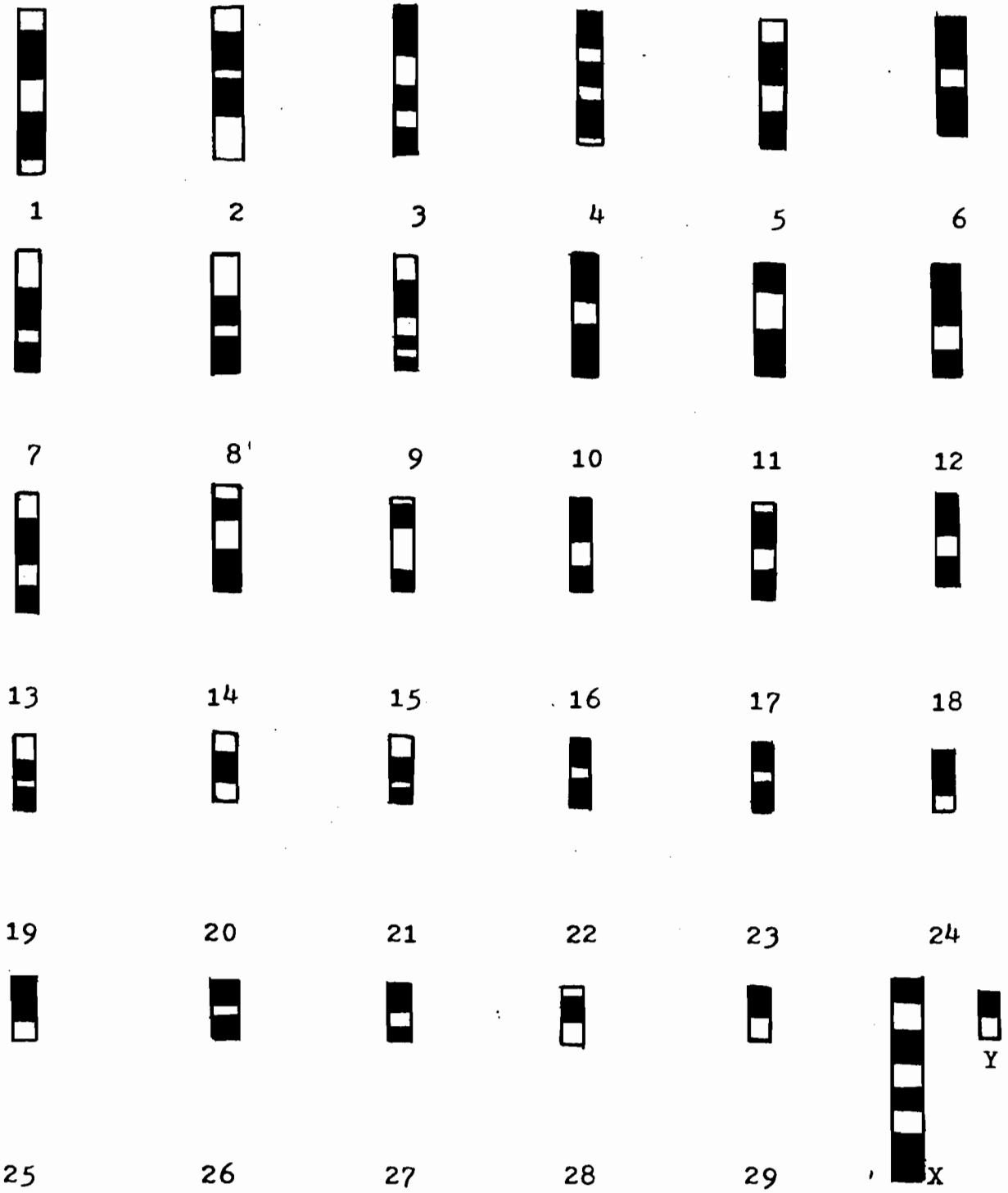


Figure 26. Idiogram of a Holstein male (Job).

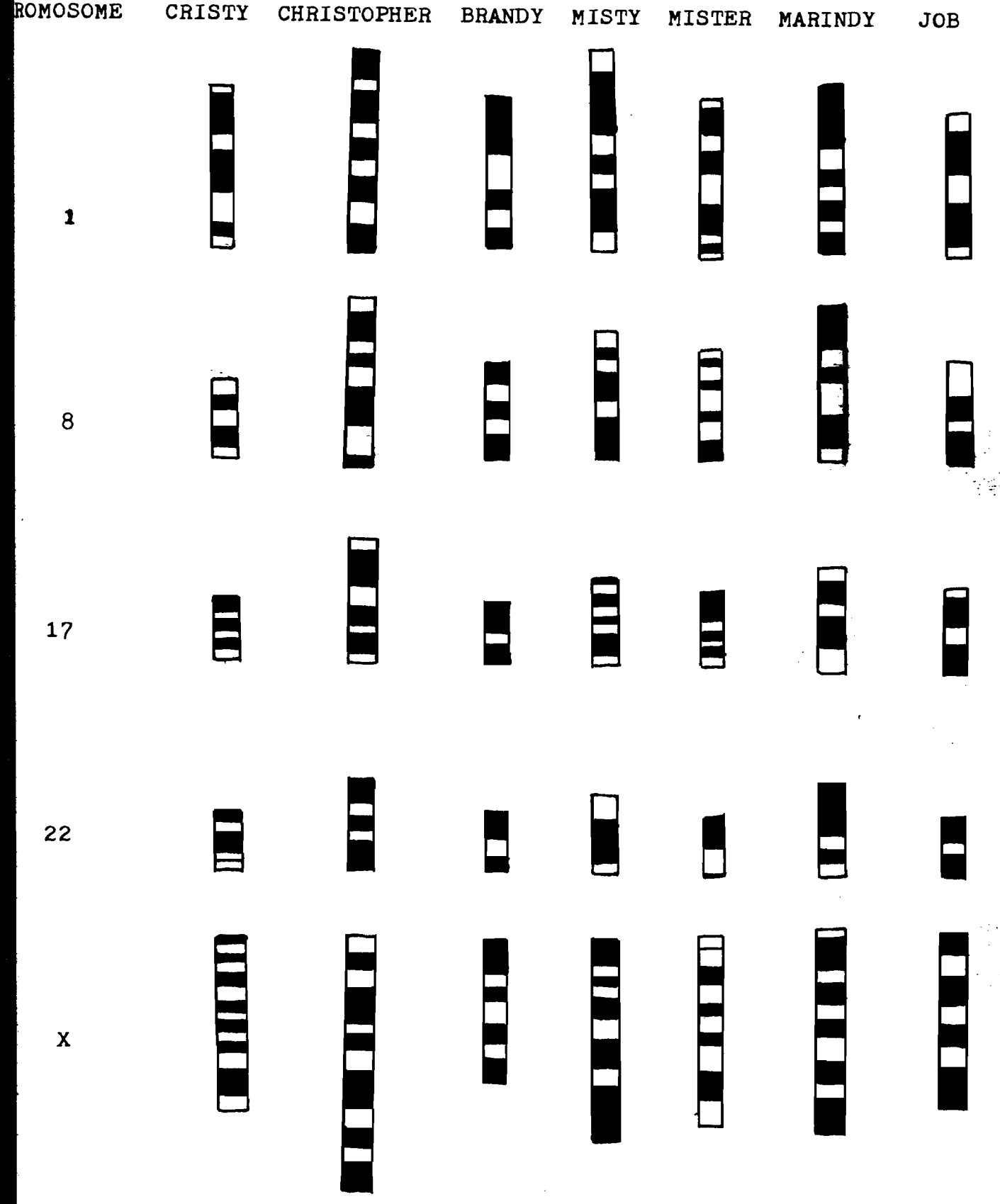


Figure 27. Comparison of idiograms of chromosomes in closely related cattle.

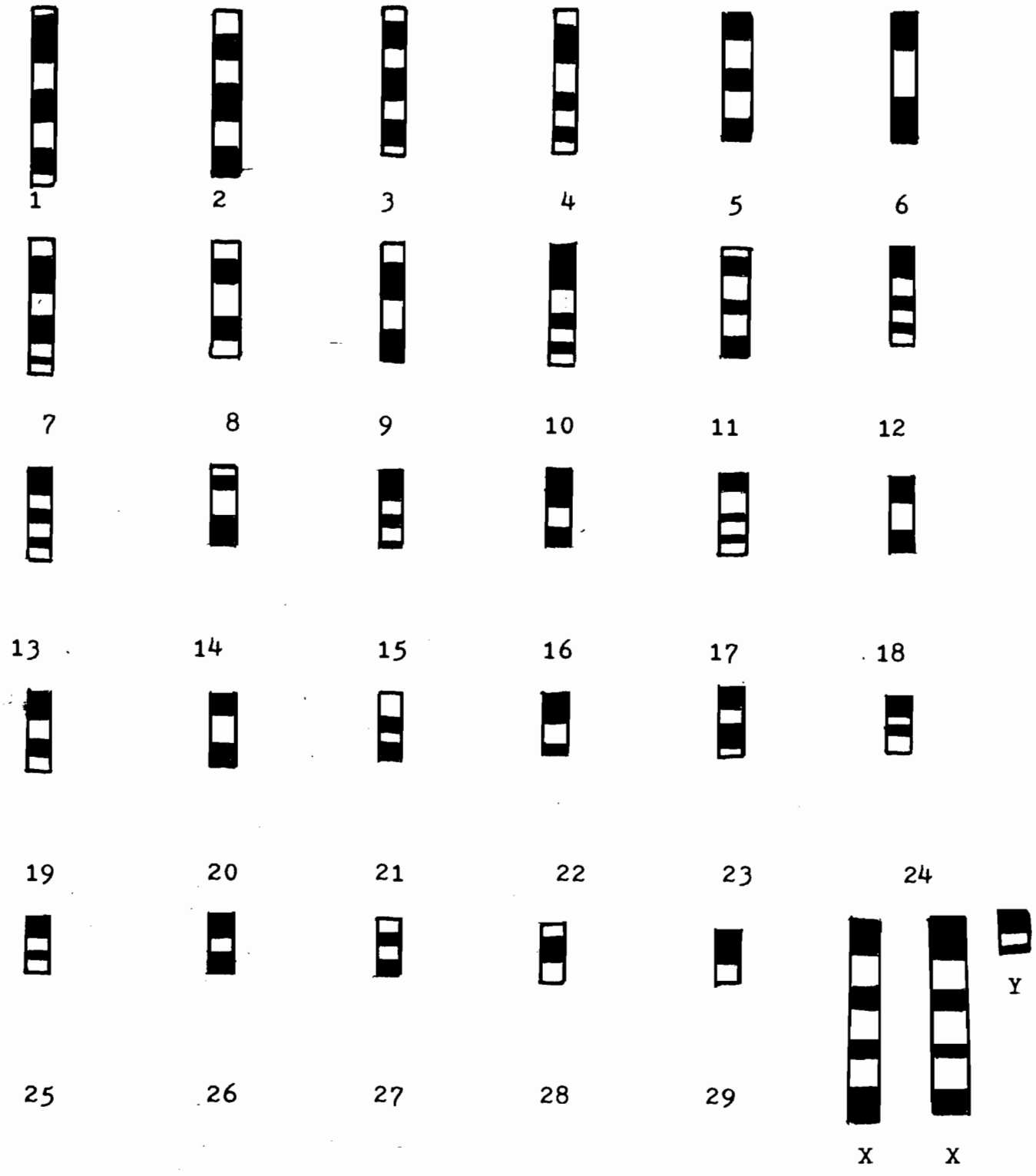


Figure 28. Standardized idiogram.

Discussion

One of the most important aspects of a chromosome study is a good cell culture technique. In order to culture bovine blood it is necessary to stimulate growth of the lymphocytes in a special bovine medium (Halnan, 1977). Such a bovine medium contains various vitamins, inorganic salts, amino acids, and carbohydrates. The medium consists of Medium 199 (GIBCO), bovine calf serum (GIBCO), and small amounts of various amino acids and antibiotics (Table 2). It is important that each culture medium be properly prepared. A poor culture medium is produced when the ingredients are not completely dissolved. Crushing the dry ingredients into small particles aids in the production of a good and uniform bovine medium.

An important ingredient of the bovine medium is non-preserved heparin. Heparin with a preservative prevents cell growth in the bovine medium. Heparin without a preservative prevents blood clotting and allows for cell growth and proliferation.

Another important aspect of a chromosome study is the process of karyotyping. The preparation of karyotypes in this project involved some subjective judgements. The karyotypes were arranged in order from the largest autosomal pair of chromosomes to the smallest autosomal pair of chromosomes. The sex chromosomes were dealt with separately. The Giemsa-banding patterns on each chromosome aided a great deal in the determination of homologous pairs. However, some problems existed in determining the correct order of the chromosomes. Several of the smaller chromosomes appeared to be of equal length. Such chromosomes were placed in order

by size as best as possible.

The quality of individual karyotypes is variable. A karyotype is only as good as the original cell culture chromosome spread. Good karyotypes (Figures 10 and 16) are possible when the chromosome spread has a minimum of overlap. It is also important that the chromosomes not be contracted. Chromosome length is primarily dependent upon the duration and concentration of the colchicine treatment . Small concentrations of colchicine prevents spindle fiber formation and, thereby, arrests the cell in metaphase (Eigsti and Dustin, Jr., 1955). A one-hour colchicine treatment was utilized in this project. Even though the colchicine concentration (0.05 ug per ml) and the duration of colchicine treatment were constant, there were variables at the cellular level of the blood-culture medium. Such cellular interations caused different lengths of chromosomes. For instance, the chromosomes of Figure 10 are extremely long. The chromosomes of Figure 13 are contracted and have little variation in length.

Variations of chromosome length produce different morphological banding patterns. As previously mentioned, dark Giemsa bands represent areas of high DNA concentration. Light Giemsa bands represent areas of low DNA concentration. An extended chromosome with distinct areas of high and low DNA concentration will produce detailed banding patterns. A contracted chromosome with non-distinct DNA concentration areas will only demonstrate major Giemsa banding patterns. Difficulties arise when the chromosome banding patterns of extended and contracted chromosomes are compared and analyzed. For instance, a banding pattern on a long chromosome may consist of a small light band between two dark bands. If the same banded

chromosome were to be contracted the banding pattern may appear as one large dark band.

Another aspect in chromosome studies is the production of idiograms. Idiograms are subjective interpretations of the banding patterns on the chromosomes of specific karyotypes.

With such thoughts in mind the comparison and analysis of banding patterns in chromosomes can be conducted. The banding pattern analysis will be conducted on the karyotypes of seven animals. Each of the animals are a part of a single pedigree (Figure 5). Gross analysis of individual karyotypes indicate that there are no structural chromosomal abnormalities.

An intense comparison and analysis of chromosome banding patterns was conducted on chromosomes #1, #8, #17, #22, and X. Figure 27 is a visual comparison of the banding patterns on the five chromosomes of each animal. Differences and similarities exist in individual chromosomes of each animal. Such differences may be due to actual variations of banding patterns in individual bovine chromosomes. However, different banding patterns may exist due to subjective judgements made in the chromosome order in the individual karyotypes. Variations in the banding patterns of a specific chromosome in different animals may also result from misinterpretations of the Giemsa banding patterns in contracted chromosomes. For instance, the banding pattern of chromosome #17 in Brandy and Misty may be exactly the same. However, more detailed bands are visible in the longer chromosome (Misty). The contracted chromosome (Brandy) only shows the major Giemsa-bands. The X chromosome of Cristy also shows a more detailed Giemsa-banding pattern than the banding pattern of the X chromosome of

Brandy. In both cases the banding patterns may be exactly the same. This explanation of differences in banding patterns may also be applied to chromosome #1 of Christopher and Marindy.

Another possible explanation for different banding patterns of the same chromosome of different animals may be due to age variation. A young animal such as Christopher (3 months old) may express different genes and, thereby, produce variable banding patterns than an older animal such as Cristy (6.5 years old).

Similarities do exist in the chromosome banding patterns of different animals. For instance, the banding patterns of chromosome #17 in Mister and Cristy are almost identical. The Giemsa-banding pattern of chromosome #22 of Brandy and Job are similar; the X chromosome banding pattern of Brandy and Job are also similar. Basically, the X chromosome of all seven animals are similar. Also, the G-banding pattern of chromosome #8 of Brandy and Marindy are similar.

Several similarities do exist in the banding patterns of specific chromosomes of different animals. An extensive literature search did not reveal any standard karyotype for cattle. Since a standard karyotype has been produced for human chromosomes it is reasonable to assume that a bovine standard karyotype will be established by researchers in the area of bovine cytogenetics. From the karyotypes I analyzed, a standard karyotype has been produced. Figure 28 is a representation of the standard karyotype for the pedigree of cattle which was studied. The standard karyotype which was produced contains 74 major chromosome bands. The number of bands compares relatively well with the literature value of 81 major bands in cattle(Schnedl, 1973).

Conclusion

More than one hundred bovine blood samples were collected in order to establish leucocyte cultures for chromosome analysis. The cell cultures were arrested in metaphase by a colchicine treatment. The cultures were treated with a hypotonic, fixed, and then prepared on microscope slides. The chromosomes were subjected to a banding procedure which produced Giemsa banding patterns. The banded chromosomes were photographed and karyotyped. Comparison and analysis of the chromosomal banding patterns on seven animals within one pedigree of cattle were conducted. Gross analysis of the karyotypes of individual animals showed no structural chromosomal abnormalities. Differences and similarities existed in the chromosomal banding patterns of different animals.

Differences in the chromosome banding patterns may be primarily due to subjective judgements made in the karyotyping process. There are definite similarities in individual chromosomes of different animals. From such similarities of the animals of one pedigree a standard karyotype was produced.

Much more work needs to be completed in this area of chromosome study. An additional study might involve repeated chromosome banding research of the same pedigree of cattle in order to validate the results of this study. Other studies might include analysis of chromosome banding of different cattle within the same pedigree or cattle of different breeds. It seems appropriate that bovine karyotypes should be standardized as has been human karyotypes. Such standardized karyotypes would aid in future karyotyping of cattle chromosomes and facilitate the ability of different researchers in the

discussions of karyotypic phenomena. As bovine chromosome studies are perfected they will become very important. Future chromosome studies may allow gene mapping to be completed, banding patterns to be associated with disease, and comparison of bovine chromosome structure with other mammals.

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