Arq.Biol.Tecnol.40(3):601-615,out.,1997

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MEASUREMENT OF CHROMOSOMES BY DIGITAL IMAGE ANALYSIS

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Recebido para publicação em 14 de março de 1997

ABSTRACT

The current availability of countless techniques for digital image analysis and pattern recognition and classification makes the use of processing tools viable in an ever widening field of applications. In the present work, chromosome measurement involving some of these tools is compared with the traditional method. The results show a close correlation.

Key words: image processing, chromosome, measurements.

INTRODUCTION

Recent advances in electronics and computing techniques allow the processing and manipulation of digital images in computers to be used in the study of cytogenetics. Thus, a significant amount of specialized software to solve cytogenic problems is commercially available. Systems to analyse chromosome banding have been developed as semi-automatic computer-aided tools (Oosterlinck et al, 1; Lloyd et al., 2) as well as automatic systems to classify and analyse human chromosomes (Piper and Lundsteen, 3). On the other hand, these packages are usable only to a limited extent in the study of other biological species, as they are dedicated to human material and the programs do not allow adaptation. Piper and Breckon (4) announced a modified system, in which an automatic tool for the analysis of human chromosomes was adapted for use with mouse cells.

In the present work, general software for processing and analysing digital images was applied to chromosome measurement, demonstrating that it is unnecessary to use dedicated systems. General-purpose packages such as this can be found at an ever decreasing cost, making viable their installation in any laboratory and their application in the study of any species.

PROCI-1997.00108
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MATERIAL AND METHODS

The measured metaphases were obtained from mouse embryos (*Mus musculus*) in morulae. Embryos were collected three days after fecundation, through wash of the oviduct with PBS (Rafferty, 6) and cultivated in M16 medium containing colcemid (16 ug/ml), in controlled atmosphere (5% of CO_2, 21% of O_2, 74% of N_2), at 38°C for six hours. After cultivation, embryos were hypotonized with sodium citrate (0,88%) at 4°C, fixed with methanol: glacial acetic acid (3:1 v/v) at 20°C overnight, and prepared on a microscopic slide (Dyban, 7).

The resulting preparation was analysed and photomicrographs taken, using a Zeiss-Axiophot photomicroscope at 250 times magnification. In choosing the metaphase, the following were taken into account: the amount of dispersion that permits individual analysis of chromosomes, the sharpness of chromosome outlines and the average degree of condensation, giving clear separation of the chromatids.

1 - DIGITAL IMAGE PROCESSING AND ANALYSIS TECHNIQUES

Basically, three stages are involved in the digital image technique: image acquisition. processing and analysis.

1.1 - Acquisition

The image was acquired by digitization. This is a critical stage in the process, as several precautions are needed to produce an image with adequate resolution and no distortion.

Two means of acquisition were tested in this work, with alternate digitizing systems: filming the negative and passing the signals through a digitizing board, and digitizing the photograph via a flatbed scanner, as in the system, belonging to EMBRAPA-CNPDIA, São Carlos - SP, shown in Fig. 1.

In the acquisition by filming, the negative was backlit and a video-camera placed perpendicularly on the central axis at a distance of 30 cm. Illumination was provided by four fluorescent lights arranged in parallel. A plate of cloudy white plastic was interposed between the light-source and the negative, so as to diffuse the light uniformly over the surface of the negative. Filming was done with a commercial portable video-camera from Sony.

Once all the negative were filmed, a digitizing board with a capacity of 512x512 pixels, each with 256 grey levels, was used to obtain digitized images.

The same negatives were then printed, and the photographs digitized by a flatbed scanner capable of resolving up to 1200 dots per inch (dpi) and 16 million colors. Despite this resolving power, so as to minimize the time taken by acquisition, the scanning was done at 150 dpi with 256 grey levels.

The images obtained by either method were processed in the same fashion. A sample digitized image of a metaphase can be seen in Fig. 2.

1.2 - Processing

A general-purpose public-domain package for digital image processing in the Macintosh (NIH Image, ver 1.23) was used. This package, like others of its kind, contains tools for the presentation, editing and processing of the digital image.

Presentation tools are those that permit the reading of images in different graphic file formats and their visualization on the screen. Files used in this work were in the format TIFF (Tagged Image File Format).

The editing tools allow the actual user, interactively, to copy, remove, shift or modify parts of the image, so as to adjust it better for subsequent analysis. The karyotypes reproduced here were mounted in this way. One by one, all the chromosomes were cut out from the image, after which they were individuallly inclined at such an angle that their principal axes were vertical. To achieve this, an approximate inclination was used, judged visually.

Next, the chromosomes were arranged on the screen, as shown in Fig. 3, ordering them in pairs by considering such aspects as the size, the presence or absence of a secondary constriction, degree of separation of the arms and morphological aspects in the region of the centromere (Bennet, 8). The identification of the sex chromosomes was based on the description given by Evans (9). In females, the two chromosomes of the second largest pair were considered to be X; while in males, the three smallest were Y.

The definitive arrangement was made after the measurement of the chromosomes, when the provisional karyotype was reorganized on the basis of the numerical data obtained.

The next stage, image-processing, followed the mounting of the provisional karyotype. Processing tools available in general-purpose packages provide a number of different operations, according to requirements. In the present case, the tools used were the thresholding filter, binarization and skeletonization.

The *thresholding filter* is used to separate objects of interest from the rest of an image; in this case, the chromosomes from the white background (Gonzalez and Wintz, 10). This separation is based on the distribution of grey levels of the image. The grey scale is searched for the value, between black and white, which enables the separation of the background level from the chromosomes. In Fig. 4 can be seen variations in grey levels along two lines placed across each chromosome.

Binarization can be applied to a whole image or within a window defined with the mouse. The area is scanned and the grey level of each pixel tested. If the level belongs to the range selected, that pixel is marked black, otherwise it is marked white, if it is the background color. In this way, a binary (black and white) image is obtained, as shown in Fig. 5. The occasional chromosomes that displayed abnormal contrast were binarized separately.

In the tool used, the table of grey levels is visualized, and by means of this the range of levels representing the chromosomes can be selected. This filter was so designed that when the option "thresholding" was chosen from the menu, the grey levels selected in the table and the pixels across the image with the same grey levels were

marked red. Thus, in an interactive way, the chromosomes were colored red and the background kept unchanged.

Once the area that is chromosome was defined and shown on the screen as red, the operation of binarization was selected.

The refinement algorithm, or *skeletonization*, was applied to the binary image. This algorithm recursively removes pixels from the edges of the chromosomes in the binary image, until the central axes or skeletons, which represent the chromosomes, are produced. This central axis has two useful characteristics: (a) it is only one pixel wide, and (b) it is approximately the same length and shape as the chromosomes (Castleman, 11). Fig. 6 shows the result of skeletonization for the image of the metaphase in Fig. 2.

1.3 - Analysis

At this stage the chromosome measurements are carried out. The most general digital image processing and analysis packages contain tools for the calculation of area and length.

Area is calculated by sweeping the binary image and counting the black pixels, that correspond to the chromosomes. In this case, the calculation is effected for each chromosome individually, multiplying the number of pixels within it by the real area of a pixel.

A digital scale was placed near the chromosome as a reference; its length corresponded to 25 mm on the negative and 10 μm on a cell. This scale was used to calculate the real area of a pixel automatically.

The length of the chromosomes was measured, using an automatic method using their skeletons. The area and perimeter of the skeleton in the image were found and converted to the average length of the chromatids by the relations:

$$C = A * N / 2 \quad \text{or} \quad C = P / 4 ,$$

where C is average length of chromatid skeleton (μm), A is skeleton area (μm^2), N the number of pixels per μm, obtained via the digital scale and P the perimeter of the skeleton (μm).

The values obtained by the above relations were called length 1 and 2 respectively.

2 - Conventional Chromosome Biometrics

The traditional methods of chromosome biometrics are extremely laborious and the work is limited by the time involved in each analysis. The techniques consist, basically, of the determination of the area and length of the chromosomes. While measuring the area affords valuable information, it is not often done, being more troublesome than the measurement of the chromosome length.

2.1 - Determination of the Chromosome Area

One commonly used method of chromosome area measurement, that served as a basis for the comparison of the results, is described by Hughes (5). In this method, the chromosome images were cut out from the photographs and weighed. Next, the areas of the photographic images of the chromosomes were calculated, using the standard weight of one square centimetre of the same photographic paper as a reference.

The true chromosome area is calculated by the following relation: $A = p*(d/D)^2/P$, where D is the length of the scale in the photograph (cm), d its length in the cell (μm), P the weight of $1cm^2$ of photographic paper (mg) and p the weight of the chromosome cutout (mg).

2.2 - Determination of Chromosome Length

In the conventional biometry of chromosome length, the karyotype is first produced, by placing in order the chromosome cutouts made in the last procedure. Next, photocopies are made, to be used with a "measuring wheel" over a sheet of carbon paper, facing upwards. The wheel, whose points are equidistant, is run along the axis of the chromatids, so that the length of each branch is determined by counting the points marked on the back of the sheet. By converting these values, in accordance with a scale of length for chromosomes in the cell, the real chromosome length can be found.

RESULTS AND DISCUSSION

In table I, the chromosome areas measured by the conventional and image-based methods are compared. The digital images results are split according to the means of acquisition of the images: video or scanner. This division was made in order to compare the degree of precision achieved in acquisition and digitization of the images. Both acquisition techniques showed good correlation with measurements obtained in the conventional way. By inspecting the angular coefficient of linear regression in each case, it can be seen that acquisition by scanner results in better agreement with the conventional method.

The chromosome lengths were also evaluated by comparison with those obtained by the conventional method (Table II). Again, with both means of acquisition, good correlations were observed, but the scanner gave better correlation coefficients and a closer fit. This is due to two factors: use of the same photograph as in the conventional technique, and the levels of distortion in the two systems of digitization.

The conventional and scanner-based methods use the photograph, rather than its negative. Although there is a small distortion in the photograph, this can be ignored since the two methods compare the same type of datum, resulting in the better fit between them. In the case of the comparison between the conventional and video-based methods, the different degrees of distortion are relevant, and may be a cause of the poor fit observed.

When the types of image-acquisition are compared, as presented in Table III, it can be seen that the difference caused by varying degrees of distortion is small, as shown by the correlation and angular coefficients.

In the current work, the processing of karyotype data by means of a digitized metaphase image took, on average, three hours.

CONCLUSION

The use of general-purpose digital image processing and analysing software for the study of chromosome biometrics has been shown to be a viable alternative, as the results were highly correlated with those obtained by conventional means.

Apart from this, the technique allowed: (1) easier construction of the karyotype, created on the computer display itself; (2) manipulation of occasionally superimposed chromosomes and (3) easier chromosome measurement process.

Moreover, these tools are equally useful in studies of chromosome banding, mainly in certain banding studies at high resolution.

Equally justified by its ease of use and by the falling price of general-purpose image digitizing and processing systems, the technique of digital image processing is becoming more viable and widely-used in cytogenetic work.

ACKNOWLEDGMENTS

The authors are grateful for the support given by FAPESP (project 90/3773-7), to EMBRAPA-CNPDIA and EMBRAPA-CPPSE, and to the Biological Sciences Centre of the Universidade Federal de São Carlos (UFSCar).

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TABLES

Table I: Values of the Correlation and Linear Regression Coefficients for different means of acquiring images, compared with the conventional method, for the measure of chromosome area.

Means of Image Acquisition	Linear Regression *	Correlation
Video-Camera	y = 0.51131 + 0.72859 x	r = 0.84743
Scanner	y = 0.42608 + 1.06095 x	r = 0.85555

Notes: Level of significance , P< 0.0001

* y = conventional method; x = image method

Table II: Values of the Correlation and Linear Regression Coefficients for different means of acquiring images, compared with the conventional method, for the measure of chromosome length.

Means of Image Acquisition	Measurement Method	Linear regresssion ***	Correlation
Video-Camera	C1 *	y = 0.17651+0.77847x	r = 0.83149
	C2 **	y = 0.26901+0.8123x	r = 0.8206
Scanner	C1 *	y = -0.16186+0.93191x	r = 0.90949
	C2 **	y = -0.13184+1.00487x	r = 0.91014

Notes: Level of significance, P<0.0001

* C1 = length determined from area of chromosome skeleton

** C2 = length determined from perimeter of chromosome skeleton

*** y = conventional method; x = image method

Table III: Values of the Correlation and Linear Regression Coefficients for Area and Length of Chromosomes, obtained from Images acquired by Video and Scanner.

	Linear Regression	Correlation
Area	y = -0.48779+1.04027x	r = 0.95953
Length 1 *	y = 0.18769+0.93546x	r = 0.86256
Length 2 **	y = 0.17298+0.96196x	r = 0.86746

Notes: Level of significance, P<0.0001

* y = video method; x = scanner method

* length 1 determined from area of skeleton

** length 2 determined from perimeter of skeleton

FIGURES

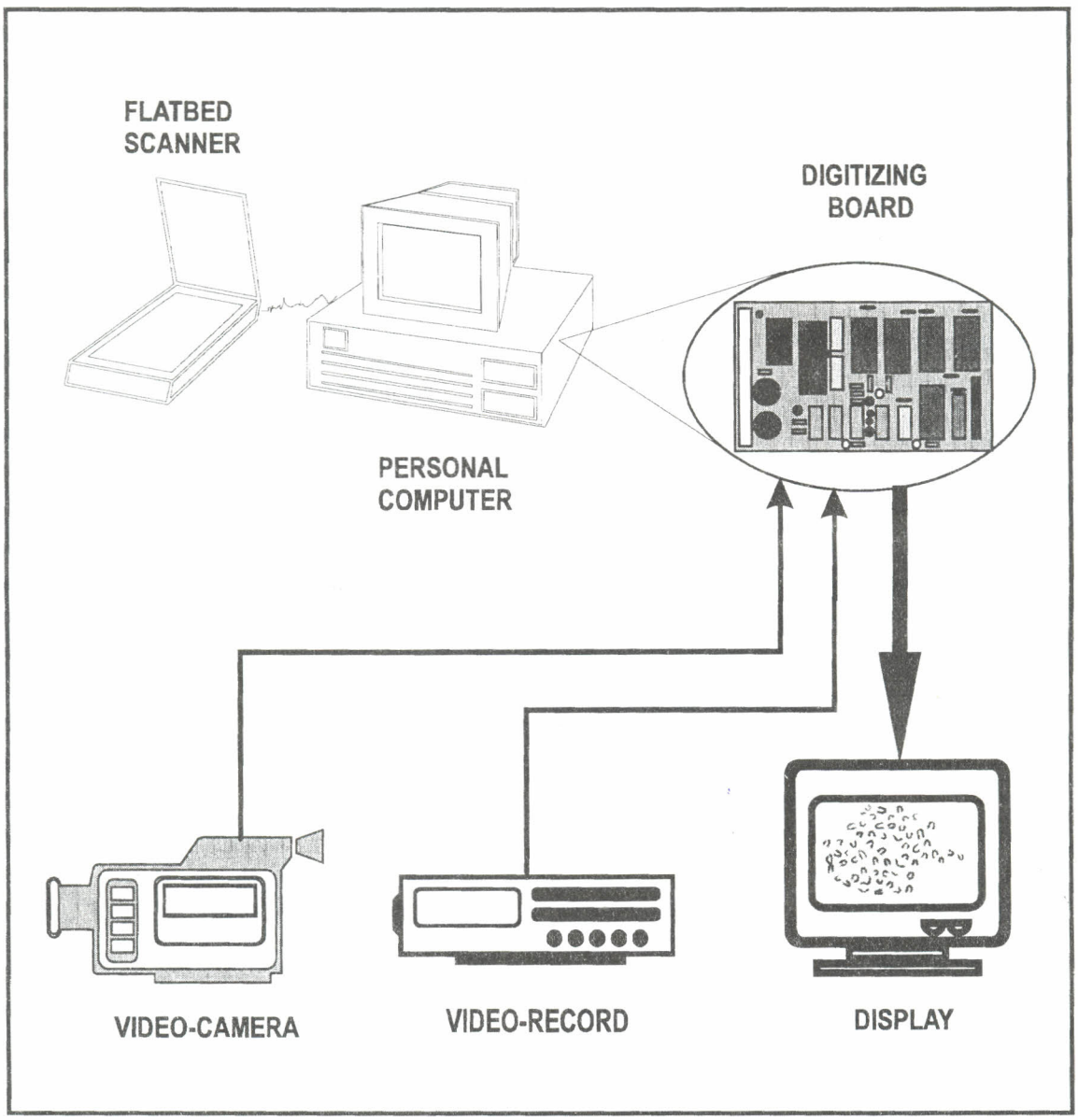

Figure 1: Digitizing system used (property of EMBRAPA-CNPDIA, São Carlos - SP)

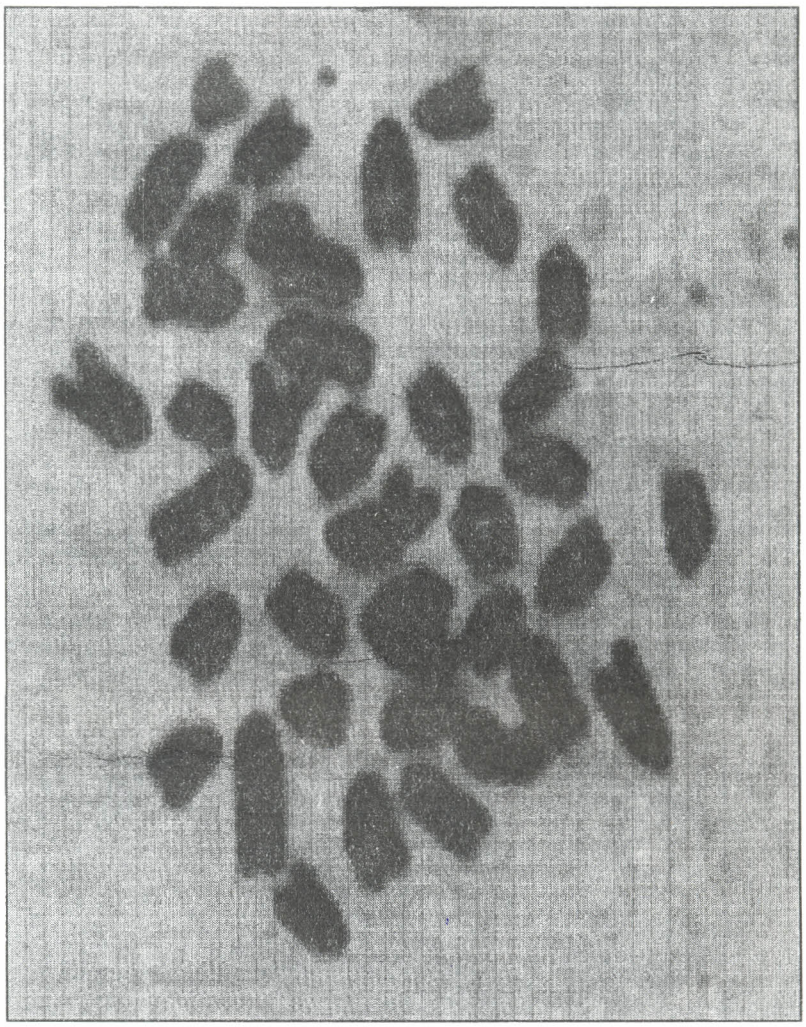

Figure 2: Digitized image of a normal metaphase (2n =40) from a embryo (*Mus musculus)*.

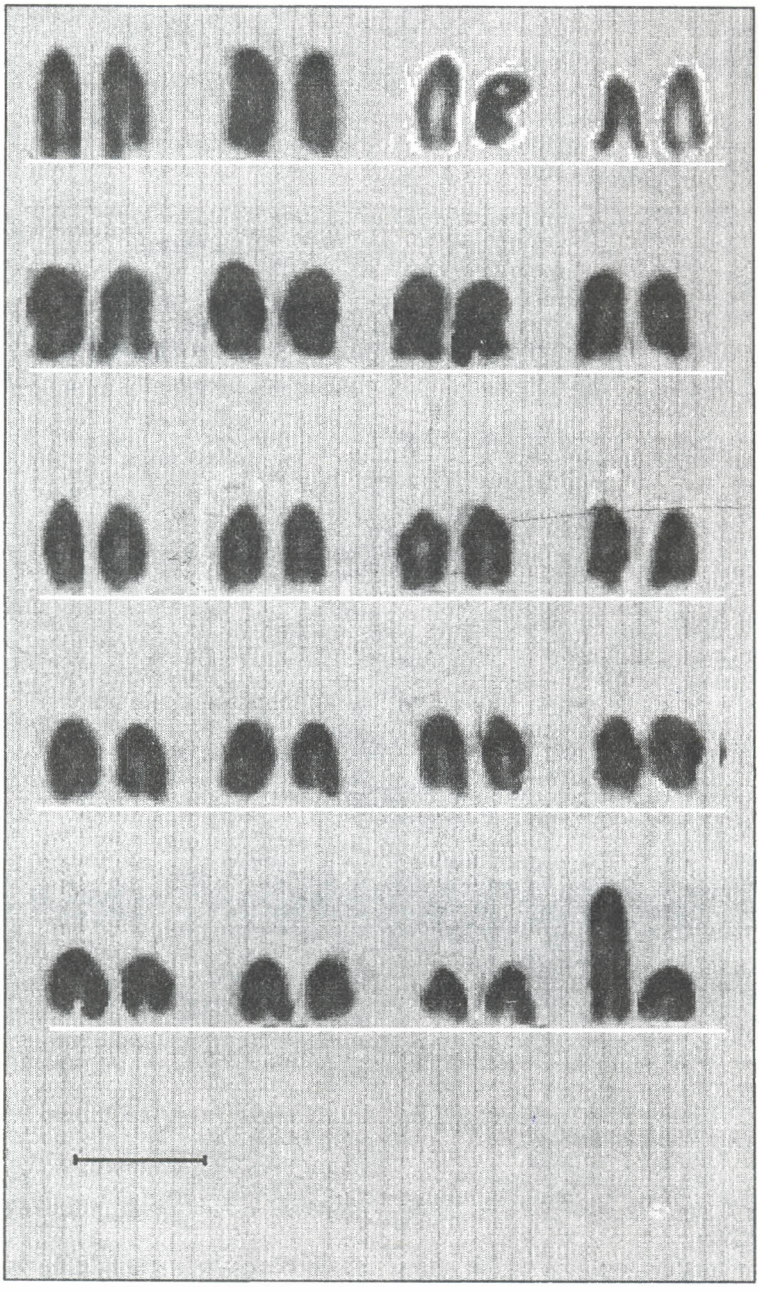

Figure 3: Mounted karyotype of the metaphase in Figure 2

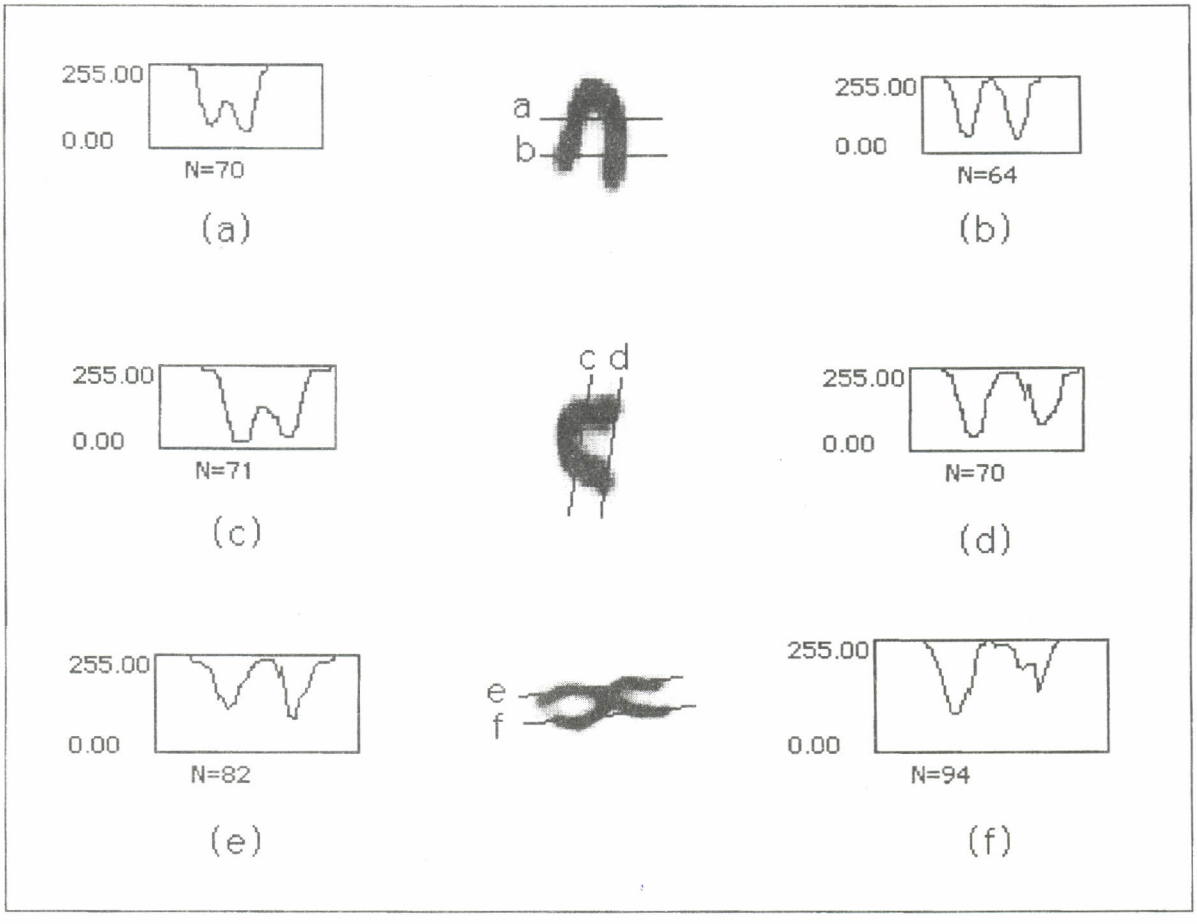

Figure 4 : Chromosomes and variations in grey levels along two lines placed across each one

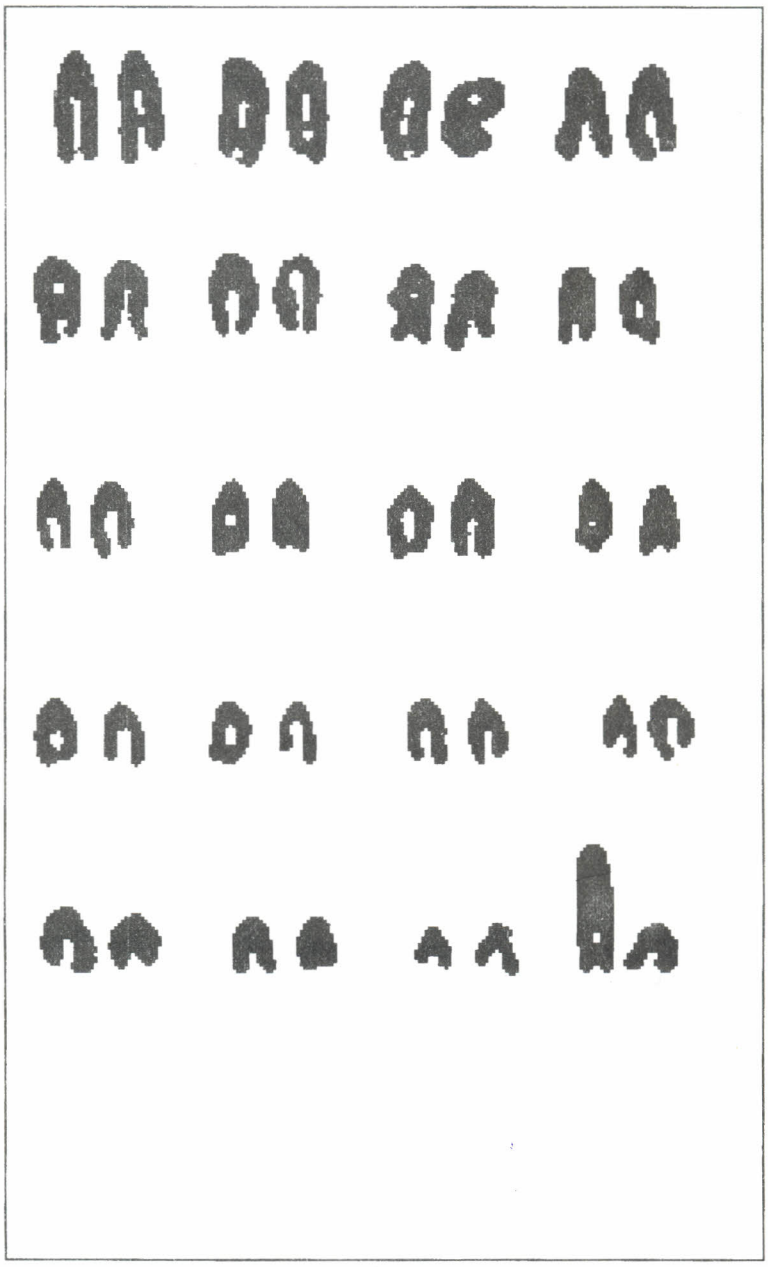

Figure 5: Binary image of the metaphase in Figure 2

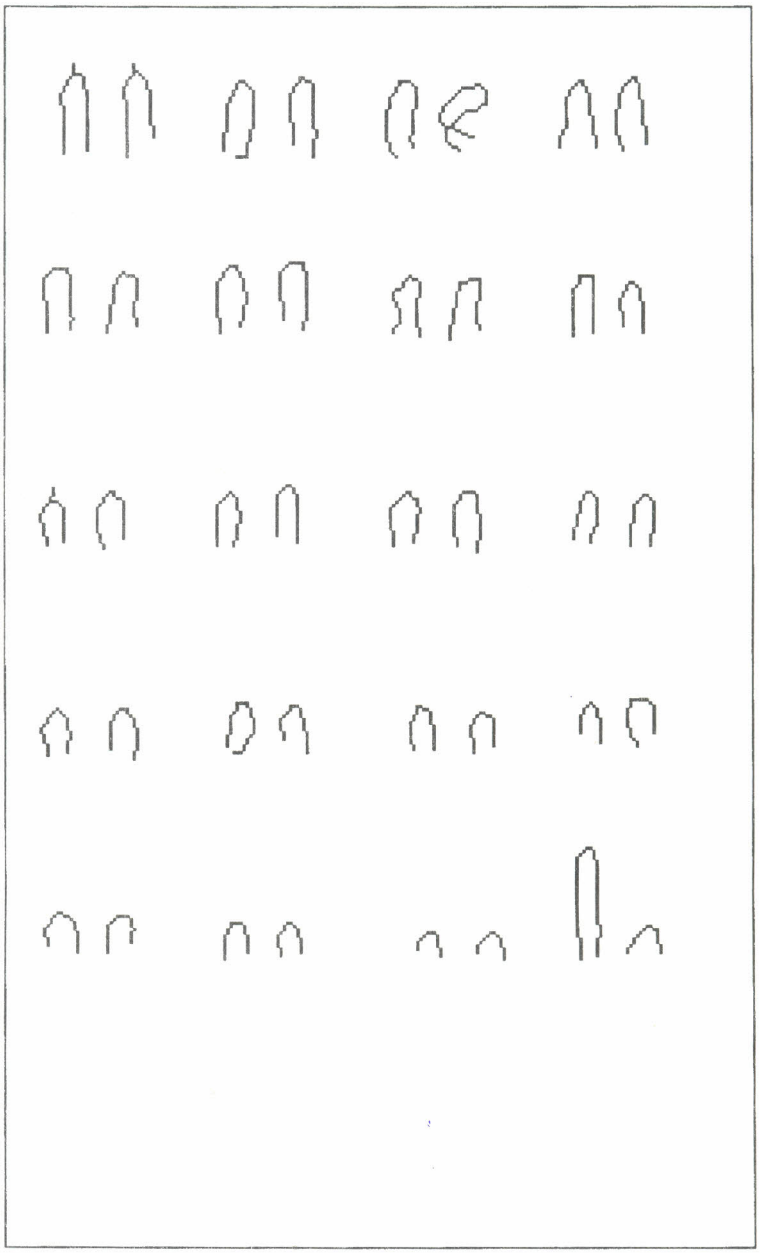

Figure 6 : Skeletonized image of the metaphase in Figure 2