Cytogenetic Analysis:
A New Era of Procedures and Precision

Diones Krinski[1], Anderson Fernandes[2,3]
and Marla Piumbini Rocha[4]
[1]Federal University of Paraná,
Department of Zoology/Curitiba
[2]University of Mato Grosso State,
Department of Biology/Tangará da Serra
[3]Federal University of Viçosa,
Department of General Biology/Viçosa
[4]Federal University of Pelotas,
Department of Morphology/Pelotas
Brazil

1. Introduction

The origin of cytogenetics is linked to technological advances. Cytogenetics differs from other sciences in which the observation of reality is sufficient to construct theories without the help of specialized equipment, as cytogenetics requires the use of imaging technology.

Theses technologies began with the discovery of the magnifying lenses, starting with microscopy and continuing into the era of informatics, allowing researchers to obtain more collaborative data to construct new hypotheses and substantiate theories. Novel ideas are constantly being developed, and science is continuously changing and reconstructing previous theories with new knowledge. In 1866, Gregor Mendel made his experiments with pea plants, using the existing technology of the time. He described the principles of genetics, but the technology did not permit the great scientist to infer the physical mechanisms involved in heredity. The function of chromosomes and hereditary was only understood half a century later, with the development of new technologies, improved microscopy for the study of cells, and new techniques to obtain chromosomes, which in turn led to the discovery of new methods of coloration and chromosomal banding. Cytogenetics arose from this fusion of knowledge.

Concurrent to these advances in cytogenetics, the field of molecular biology was expanding as well. Cytogenetics took advantage of this new knowledge and technology developed for molecular biology, thus forming a new area called molecular cytogenetics that is mainly represented by the techniques of FISH (*fluorescent in situ hybridization*) and microdissection.

The described improvements in the areas of genetics, cellular biology, cytogenetics and molecular biology were also accompanied by advances in the field of informatics. The

creation of image analysis software permitted a refined analysis of banding patterns, making it easier to measure chromosomes and differentiate varying levels of coloration. With these new data, cytogenetics is now able to produce more specific responses about the composition of chromatin, the behavior of the chromosomes, karyotypic evolution, species phylogeny, as well as other aspects of molecular genetics.

1.1 A model of the advances in cytogenetics

Human cytogenetics has always had the greatest appeal in science, as is expected since man has always searched for his own self-knowledge. However, cytogenetics has also contributed to the generation of knowledge for other groups, one of which is the tribe of Meliponini bees. These bees are distributed in tropical climate regions where they are of great ecological and economic importance, due to their ability to pollinate cultivated native plants (Michener, 2000, Kerr *et al.*, 1996; Heard, 1999).

Cytogenetic studies of native bees of the tribe Meliponini in Brazil began with the work of Kerr (1948), and until the 1980s the majority of the technical and applied works consisted of spread out metaphases and determining the number of chromosomes of each species. In 1988, Imai *et al.* published an article that used a new technique, called air drying, to obtain chromosomes with mitotic metaphases. That technique is now used in Meliponini cytogenetics and has shown itself to be the most efficient method of numbering and differentiating the chromosomes, in comparison to older spreading out techniques (Hoshiba & Imai, 1993; Brito *et al.*, 2003; Mampumbu & Pompolo, 2000, Maffei *et al.*, 2001, Rocha *et al.*, 2003, 2007, Krinski *et al.*, 2010, Barth *et al.*, 2011, Lopes *et al.*, 2011). For example, for the species *Melipona quinquefasciata,* individuals with chromosome numbers of n=9, 10 and 18 have been observed (Kerr, 1972; Tarelho, 1973). In 2002, Rocha *et al*. used the air drying technique to prove that the numerical difference was due to the presences of B chromosomes.

After the adaptation of the air drying technique, different methodologies were applied to the chromosomes of Meliponini. The most common of these techniques is C-Banding, following the BSG (Barium Hydroxide/Saline Solution/Giemsa) method of Sumner (1972), with modifications for different generas. This technique permits the differentiation of the euchromatin from the heterochromatin, as dark bands form in places with heterochromatin. These bands facilitated the identification of heteromorphism among homologues, the pairing of the chromosomes (Hoshiba & Imai, 1993) and assisted in the study of heterochromatin behavior during the mitotic cycle (Rocha *et al.,* 2002).

Another banding technique is NOR (nucleolar organizer region) banding. Although the NOR is defined by a sequence of DNA, the NOR band is based not on the properties of DNA, but rather the acidic protein produced during interphase and accumulated in and around the NOR (Schwarzacher *et al.* 1978). In Meliponini, this technique was used according to Howell and Black (1980), making it possible to locate the NOR regions and use this data to study species evolution. Within molecular cytogenetics, various works were done using FISH and Restriction Enzymes (RE).

The use of the REs *in situ* is part of a new generation of cytogenetic techniques that employ the tools of molecular biology to provide responses about the molecular composition of chromatin (Pieczarka *et al*., 1998). As the name Restriction Enzymes implies, the DNA is

cleaved in specific sequences. For example, *Hae*III cleaves the sequence GC↓GC and *Dra*I cleaves AAA↓TTT. According to Torre *et al*. (1993), the first work proposing a methodology for the use of the REs *in situ* for chromosomes fixed on slides was done by Mezzanotte *et al*., in 1983. It showed that certain REs were capable of cleaving the fixed chromatin, removing the DNA in different dominions of the chromosome. The removal of the DNA from certain regions can be visualized by subsequent coloration with Giemsa or with fluorochromes, since the removal promotes the emergence of one or more transverse bands in the chromosomes or a simple reduction in the coloration of the regions (Verma & Babu, 1995). Rocha *et al.,* (2003a), using REs, showed that regions that appeared to be homogeneous in the C-Banding technique actually contained regions with different color intensities. The FISH technique uses dyes which are specifically connected to certain regions of the chromosomes and emit fluorescence. For example, Quinacrine Mustard (QM) and 4-6-diamidino2-phenylindole (DAPI) are connected to the regions rich in AT base pairs, while chromatin A_3 (CMA$_3$) is connected to the regions rich in GC. With the use of this technique, it is possible to infer the nature of the heterochromatin present in the karyotypes of various species, the origin of supernumery chromosomes and to study the nucleolar organizer region (Brito-Ribon *et al.,* 1998; Mampumbu & Pompolo, 2002, Brito *et al*., 2003, 2005; Rocha *et al*., 2003; Krinski *et al*., 2010; Barth *et al*., 2011).

More recently, Fernandes *et al.,* (2011) standardized a microdissection protocol for cytogenetic studies of bees. This new technique will permit the generation of extremely specific markings of the genome -- markings such as centromeric, telomeric, heterochromatic regions of supernumerary chromosomes, or even the entire chromosome itself. This technique has opened a window for the study of chromosomal evolution, and by consequence the genetic diversity of the species using a new method of analysis.

1.2 Image analysis in cytogenetics

Over the years, digital imaging technology has improved considerably, especially in studies related to microscopic analyses (such as cytogenetics) that essentially study the characteristics of the macro and micro chromosomal structures of the cells of diverse organisms. In recent decades cytogenetics has been transformed principally by developments in two areas of science, molecular biology and bioinformatics. The manipulation of DNA has been improved by the PCR (Polymerase Chain Reaction) technique, and advances in the FISH technique for chromosomal microdissection have taken chromosomal analysis to a new level of specificity and reliability in terms of studying karyotypical evolution, composition and rearrangements of species. The creation of image analysis software developed for use in bioinformatics has greatly enhanced the study of banding patterns, generating extremely reliable information with respect to chromosomal measurements and, with the use of coloration intensity, has enabled a comparison between these parameters in statistical form. The majority of the cytogenetic research currently done, whether focused on animals or plants, uses the various available software packages only to organize the chromosomes in order by size. The use of software for the study of images obtained by chromosomal banding techniques, and images of chromosomal and nuclear markings, is still not well known in cytogenetics. This is probably simply because most researchers do not know how these tools function. On the other hand, the increase in research using a wide variety of software makes these instruments become more sophisticated and precise each day.

In this chapter, we will discuss the basic use of the Image-Pro Plus™ (IPP) version 6.3 software program (Media Cybernetics, 2009) in the grouping and measurement of chromosomes, along with other methods of analyzing specific areas of interest, such as the macro and microstructures of chromosomes, which may be used in any cytogenetic study. As a model, we will use research done with karyotypes of stingless bees (Meliponini) in Brazil.

2. Using Image-Pro Plus™ for the study of chromosomes

2.1 Chromosome pairing and mounting of the karyotype

The majority of chromosomal studies start with the most basic technique of pairing the homologous chromosomes, as a pre-requisite for all other karyotype analysis. If this is done in a subjective manner, using only the visual perception of the researcher, then the pairing of the homologues may be inconsistent or doubtful, reaching a result that is not consistent with reality. Thus, to promote a more secure pairing of the chromosomes, the use of image analysis programs, such as IPP, is recommended. In this section we will show how these programs can act as powerful tools to be used in the most diverse studies of cytogenetics, so that the researcher can make a more detailed analysis in order to pair and mount the chromosomes in the correct manner.

Depending on the group of organisms being analyzed cytogenetically, some characteristics, such as the size of the chromosomes, the position of the centromeres and the distribution of the euchromatin/heterochromatin may be used to assist in the organization of the karyotype. To obtain a satisfactory result, this information must be taken into consideration when used in an image analysis program.

Not so long ago, the pairing of chromosomes meant that they had to be photographed, developed on photographic paper, cut out by hand with scissors, measured with tools (a caliper rule, for example), and then paired manually. This process was time-intensive and, as written above, gave subjective results most of the time.

With the use of the IPP program, we will demonstrate how technology can improve the accuracy of pairing chromosomes. Model chromosome images obtained from some species of wingless bees (Meliponini) will be used to perform the counting, separation and pairing of homologous chromosomes. However, before using these specific procedures, we emphasize that some precautions must be taken into consideration, including:

a. The slides must be prepared in accordance with the standard procedures for cytogenetic analysis (conventional and technical specifications);
b. The slides must be analyzed, and the best metaphases captured using a 100X objective and stored in a computer database. Remember that the higher the resolution of the image, the better the quality of the photographed metaphases;
c. Your computer must have the IPP software (or similar) to analyze the images of chromosomes.

2.1.1 Counting the chromosomes

The IPP program has some tools which can be used to automatically determine the number of chromosomes of a given species. After the captured metaphase images are

saved in a database on your computer, open the image that you wish to analyze and follow these instructions: go to the *Menu* bar and click *Measure* > *Count/Size* > *Select Colors*, and in the window marked *Segmentation*, click the *Color Cube Based* tab and select the *eyedropper* icon, then position the cursor over a chromosome and left-click to select the objects that have the same color. Note that the chromosomes will be partially selected (marked in red), repeat the procedure until all of the chromosomes are selected (Fig. 1 – step 1 and 2). After selecting all the chromosomes of the metaphase, close the *Segmentation*
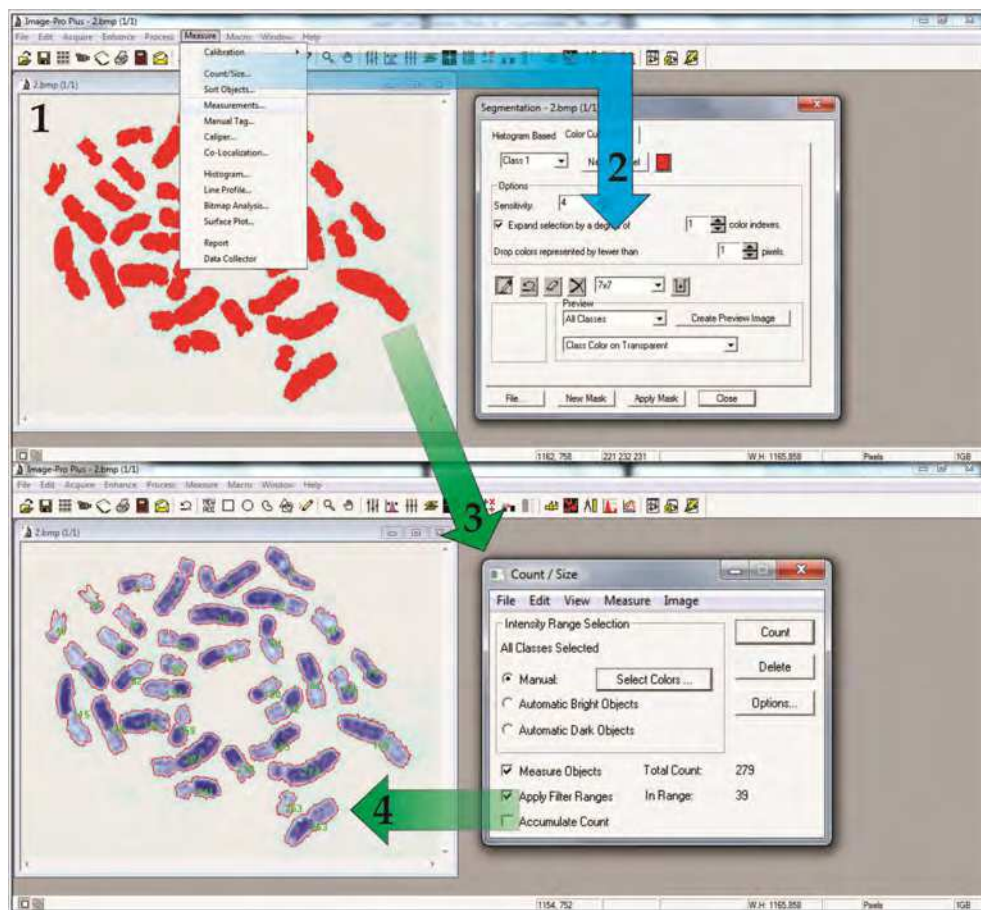
1- Chromosomes selected in red; 2 –*Segmentation* window in the *Color Cube Based* tab where the tools used to select the chromosomes that you wish to count are located; Automatic count of the chromosomes of the metaphase of *T. angustula fiebrigi*. 3- chromosomes numbered in green; 4–*Count/Size* window where the tools used to count the chromosomes will be available.

Fig. 1. Image-Pro Plus™ program with metaphase of *Tetragonisca angustula fiebrigi* (Schwarz, 1938) (Hymenoptera, Apidae, Meliponinae).

window and return to the *Count/Size* window. This time click on the button with the option to *Count*. Note that the chromosomes that were previously selected will now be numbered (Fig. 1 – step 3 and 4).

In the case of artifacts, such as nuclei or other structures with the same tonality as the chromosomes, these should be removed (deleted) from the figure with an image editing program first, otherwise they will also be selected and counted by IPP. In some cases, chromosomes may be very close, or even superimposed over one another. If this happens, the program may interpret these chromosomes as a single structure. To separate them, you should use another image editing program to copy the superimposed region to an area away from the other chromosomes.

2.1.2 Pairing homologous chromosomes

The pairing of the chromosomes is another important step for the mounting of the karyotype, because it allows the detection of homologues and possible alterations in the karyotype. In this way, chromosomal banding techniques have broadened the horizons of cytogenetics by showing specific regions, since each pair of chromosomes presents a distinct pattern and well-characterized bands. Therefore, the Q, G and C bandings are especially important in cytogenetics, because they permit the identification of small structural variations such as deletions, duplications and inversions, among other characteristics. This occurs because the bands show differences in the distribution of chromatin components or differences in the chemical composition of the chromatin along the chromosome, thus enabling the better understanding of chromosomal alterations established in each karyotype (Guerra, 1998). Here we will show the pairing done for the same metaphase used for the chromosomal count, using the C-banding technique.

After the previous steps, with the chromosomes still selected and already counted, return to the *Menu* bar and click *Measure > Sort Objects*. A new window and a dialog box of the same name will now be open. Select *Sort by: Area* and click on the box for *Sort Objects*. De-select the *Labels* option (the numbers that appear to the side of the chromosomes). The chromosomes will now be shown in a sequence, one that probably does not place the homologues side by side (Fig. 2).

Nevertheless, you can re-position them by clicking and dragging them. If you need to rotate a chromosome, just double-click on it to select it. Thus, you can organize them in what you think is the best possible way. After the chromosomes are organized in pairs, save the figure, preferably in "bmp" format. If any edits need to be made in the figure, such as inserting text and legends, you must be able to open the file in another image editing program (Corel Photo-Paint, Photoshop, etc) and make the necessary text insertions. If the chromosomes of the analyzed metaphase are very similar, we can use other tools to help in the pairing of the homologues, as shown in the next section.

2.1.3 Measuring chromosomes

The use of imaging software in the study of chromosomes generally makes the results more reliable, because through their use it possible to measure a curved chromosome simply by finding the two extremities of that chromosome (Barret & Carvalho, 2003). Here we will use

1 – Organized by area size. 2 - *Sort Objects* window where the options to choose the placement of the chromosomes are located.

Fig. 2. Chromosomes of *T. angustula fiebrigi* shown side by side.

IPP, as well as CorelDraw® and Microsoft Excel®, (similar programs may also be used) as tools to measure the chromosomes.

Thus, after capturing and processing the metaphases as explained in items 2.1.1 and 2.1.2, follow the following steps:

a. Save the image of IPP's automatic chromosomal pairings (Fig. 2). Next, import or open the figure in CorelDraw®;
b. In CorelDraw®, use the text tool to insert numbers below each chromosome and save the figure;
c. Open the numbered image in IPP to start the measurement of the chromosomes (Fig. 3 – step 1);
d. In the *Menu* bar, click *Measure* > *Measurements* and use the available tools to make measurements of the total size, the size of the arms, or the specific bands along the chromosomes (Fig. 3 – step 2);
e. Note that each measurement made will be numbered in the *Features* window. The measurements will be saved in IPP and can be used for calculating the Centromeric Index. These values should be exported to Microsoft Excel® with the *Input/Output* option (Fig. 3 – step 3). The image in IPP should also be saved;
f. The obtained values can be used to create graphs to organize the chromosomes by arm length or any other specific characteristic, such as the size of the euchromatin (Fig. 3 – step 4).

In figure 3, it is clear that IPP helped in the study of Lazaroto (2010), as this author used the obtained values in the graphs, especially the lengths of the euchromatic arms, to mount a karyotype (Fig. 3 – step 4) that presented more harmonious pairings among homologues. Note that the numbering in the final pairing does not correspond with the original numbering of the chromosomes. The problems presented in the first pairing were solved with the measure of each chromosome, and we can see the heteromorphism in homologous
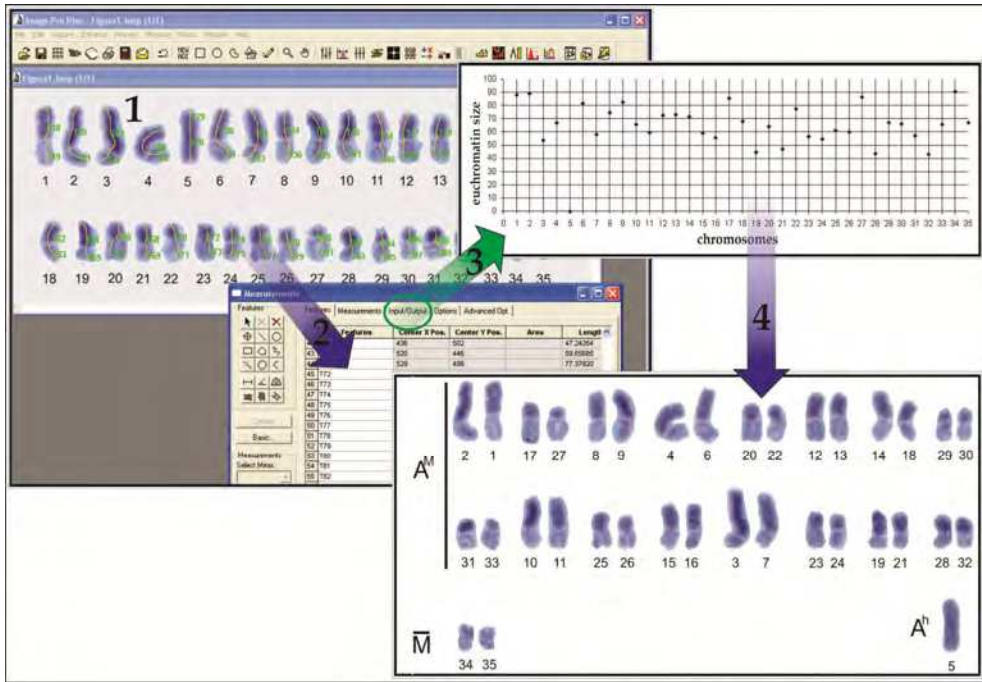
1 – Measurement and arrangement of chromosomes done without the use of IPP; 2 – Use of IPP to measure chromosomes and help in the grouping of homologues; 3 – Graphic made using data exported (*Input/Output*) from IPP to Microsoft Excel®. The points of each line indicate the size of the euchromatin; and 4 – Re-structured karyotype after the use of IPP. Observe the difference between 1 and 4.

Fig. 3. Karyotype of stingless bee *T. angustula fiebrigi*, previously numbered in CorelDraw®.

pairs among the heterochromatic arms of chromosomes 14 and 18, a feature that had not yet been described in the literature.

2.1.4 Important information in the mounting and pairing of chromosomes

1. Metaphase selection – The greater the difference between the tone of the background and the metaphase, the greater the quality of the karyotype. There are ways to amplify these differences both before and after image capture;
2. Follow the protocols precisely to obtain the best results with metaphases. Upon metaphase capture, the contrast between the chromosomes and the background can be increased by increasing the quantity of light emitted. After capture, images of metaphases can also be altered in image editing programs to achieve this contrast;
3. *Avoid metaphases containing overlapping*. There are ways to remove superimposed chromosomes, but this procedure requires time, ability, and the manipulation of reality. If you do not have the option of avoiding metaphases of this type, use programs such as CorelPhoto, Photoshop, etc.;
4. *NEVER alter your original figure*. It is not possible to restore the original figure after image-altering procedures. Always save a backup copy of your figures.

IMPORTANT

Procedures 3 and 4 imply alteration of the images, but the use of this method may cause the researcher to cross the line that separates research from scientific fraud.

2.2 Analyzing specific areas of chromosomes and nuclei

After knowing the karyotype of a species, the study of specific areas of the chromosomes is one of the principal objectives of cytogenetics, because these areas can reveal much information about the genetic evolution of the species or population studied. Thus, there are innumerable techniques to examine specific areas, such as Ag-NOR banding which shows the active nucleolar organizer regions (NORs), C-banding which shows the regions rich in heterochromatin, diverse types of fluorochromes that highlight the regions in specific base pairs, plus the techniques of FISH and Restriction Enzymes (REs). Here we will show how the IPP program can assist in the analysis of specific areas of nuclei and chromosomes.

Before showing the steps that must be followed to analyze specific areas of nuclei and chromosomes, we emphasize that the precautions described at the beginning of topic 2.1 must be followed. When the images are saved in the database on your computer and ready for analysis, follow the following steps.

2.2.1 Measuring areas of interest in nuclei and/or chromosomes

When you already have the images of the nuclei or chromosomes saved in a database on your computer, open the image that you wish to analyze by clicking *Menu > Measure*, and choose the *Measurements* option. A tab of the same name will open, so select the polygon icon and circle the region of which you wish to measure the area size (Figure 4 – step 3). Do not forget to select the area option, located on the left side of the *Measurements* window, below the options buttons (Select Meas:). Here, images of nuclei and nucleoli of the stingless bee species *Frieseomelitta trichocerata* Moure, 1990 (Fernandes *et al.,* 2008) and *Tetragonisca fiebrigi* Schwarz, 1938 are used to exemplify this process.

To see the values, and edit the name of each measured region, open the *Features* tab. To use the values obtained in other program just go to the *Input/Output* tab and choose the desired option (Fig. 4).

In cytogenetics, studies involving Ag-NOR banding, and more precisely those that refer to the relative size of the nucleoli found in interphasic nuclei, are scarce. Thus, the use of the tools of IPP by Fernandes *et al.*, (2008) provided information with respect to possible differences in the metabolic activities of cells of this species of bee. For both *F. trichocerata* and *T. fiebrigi*, the nuclei with three nucleoli (group III) presented a total area of nucleoli larger than that of the nuclei that presented only one nucleolus (group I), with this being a characteristic conserved among these species (Fig. 5). Therefore, these authors concluded that the nucleoli are cellular components which can offer important information about the metabolic activities and evolution of different organisms also indicate the technique of Ag-NOR banding as a possible cytological marker for distinct cellular types. We note that this tool can also be used to measure specific areas of chromosomes.
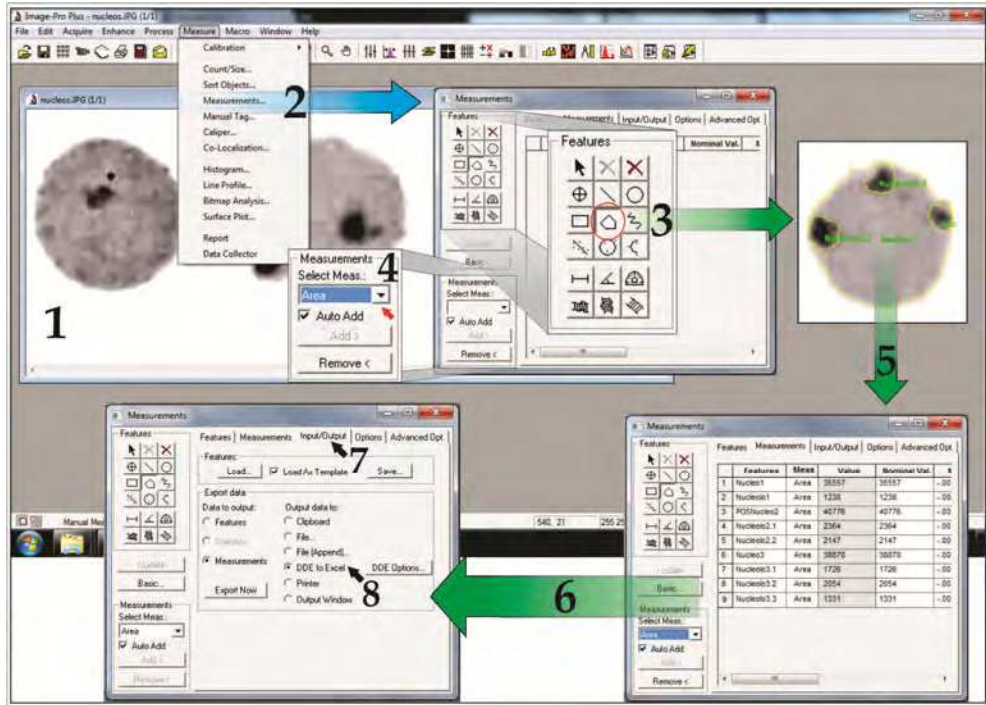
1 – Image open to start the area measurements by IPP; 2 – *Menu > Measure* with the *Measurements* option; 3 – Tools of the *Measurements* window, with the polygon (red circle), used to make measurements of the highlighted areas; 4 – Select the area option; 5 – Perform the measurements with the values shown in the *Measurements* tab; 6,7,8 –*Input/Output* tab where the values obtained in IPP can be exported to Microsoft Excel®.

Fig. 4. The IPP program open with nuclei and nucleoli of the stingless bee *T. fiebrigi* shown by the technique of Ag-NOR and previously numbered in CorelDraw®.
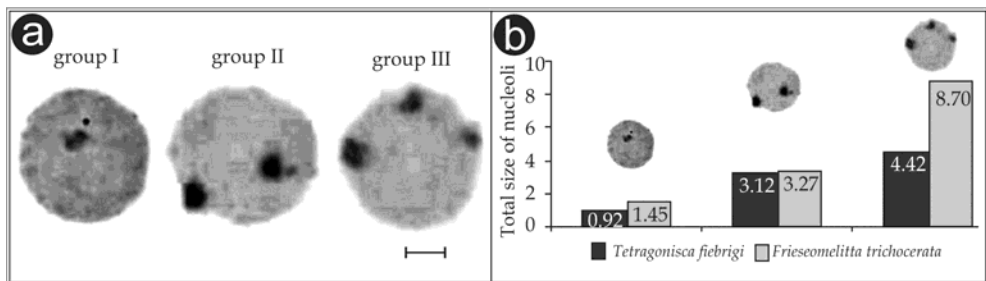
a) Three groups of nucleoli found in the nuclei of two species; and b) Differences in size (area) of the nucleoli of the nuclei of the analyzed species. Bar: 5 µm.

Fig. 5. Analysis of an area of nuclei and nucleoli of stingless bees *F. trichocerata* and *T. fiebrigi*.

2.3 Understanding chromosomal differentiation through the computational analysis of images

Historically, chromosomal banding techniques have assisted in trivial cytogenetic practices (such as the pairing of homologues in a more reliable manner) and more advanced analysis including understanding of patterns associated with chromosomal evolution of the species, the speciation processes, and the generation of genetic variability. Thus, we will discuss some research done with chromosomes of stingless bees that used the tools of IPP to assist in the cytogenetic analysis of this group of insects.

We will next show a re-analysis of the data obtained from the chromosomes of three species of the genus *Melipona* (*M. bicolor*, *M. quadrifasciata* and *M. subnitida*) which were subjected to digestion by two Restriction Enzymes (*Hae*III and *Dra*I) and colored with two fluorochromes (DAPI and CMA_3) (Fernandes, 2004). But first we need to understand what the Restriction Enzymes (REs) are and how they act on the chromosomes.

Thus, in this re-analysis we will use the results found after the degradation of the chromosomes of the three species of bees of the genus *Melipona* with the Restriction Enzymes (*Hae*III and *Dra*I), followed by coloration with the two fluorochromes (DAPI and CMA_3). In the first analysis done by Fernandes (2004), it was possible to verify the emergence of a complex banding pattern along of the chromosomes where a total of 135 were counted using the traditional visual counting method. Based on the similar and divergent banding patterns among these species, the author concluded that *M. quadrifasciata* and *M. bicolor* share a large number of bands in common when compared to the species *M. subnitida*.

However, the analysis of among-species band similarity caused some errors, since band equivalence was determined using only visual perception. A new proposal of band equivalency may be done using image analysis tools, which increases the spatial reliability (location) of the bands. In this analysis, IPP verified the exact locations of decreasing luminescence, generating a graph that indicates exactly where enzyme digestion occurs. Also, the overlap of the graphs allows precise comparisons between the bands, providing solid criteria for the conclusion about whether or not the bands within the chromosomes of the species are equivalent.

As an example of this analysis proposal, we used only the first chromosome of each species and treated it with *Hae*III, followed by coloring with DAPI (Fig. 6a). An extremely powerful tool of IPP involves the quantification of the light intensity of each pixel below a specific target (as seen in item 2.2.2). We then trace a line along the chromosome and the program quantifies the scale of shades of gray from 0 to 255 (from black to white), recording all of the pixels below this line (Fig. 6a). After this step, a histogram (graphic of pixel intensity by unit of length) can be made for each chromosome (Fig. 6b). In this way we will have the exact location of each chromosome degradation, and the overlap of the graphs provides the possibility of an extremely precise comparison between the patterns of digestion (Fig. 6c). Note that the valleys are highlighted by different geometric figures (triangles, stars and circles) for each chromosome.

Using this new analysis proposal, we note that there are three points at which there are high proximity between the chromosomal digestion of the three species (grouped by the pink elliptical circles). These locations of digestion have extreme proximity, and are the only unique points where we could consider that there is band similarity among the species.
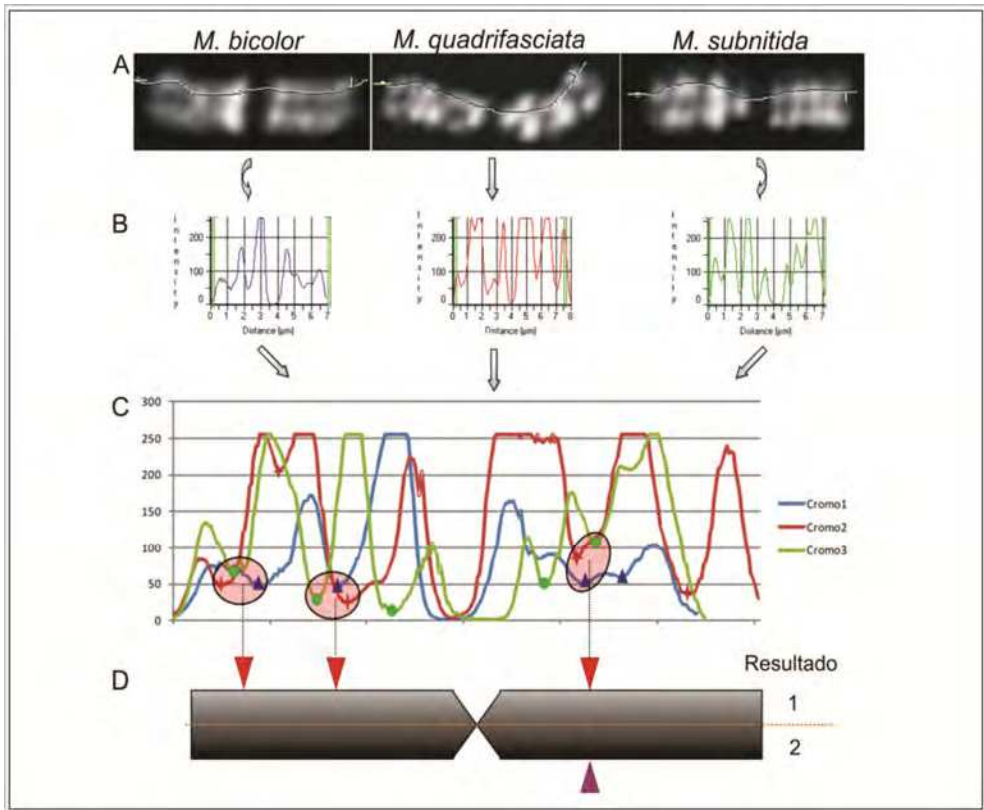
a) Arrangement of one chromosome of the first pairs from *Melipona bicolor*, *M. quadrifasciata* and *M. subnitida*, with the line above each chromosome indicating which pixels will be quantified to generate the histogram. b) Individual histograms of each chromosome. Note that in the graphic, the peaks represent the fluorescent regions and the valleys represent the regions of digestion caused by Restriction Enzymes. c) Overlap of the individual histograms. The regions of enzyme digestion are highlighted by different geometric figures (triangles, stars and circles) for each chromosome. The pink elliptical circle indicates locations of digestion present in the three chromosomes. d) The ideogram in which the exact locations of enzyme digestion in the three chromosomes are highlighted in accordance with the current proposal of diagnostic image analysis and by the previous proposal of Fernandes (2004) in which only traditional visual resources were used.

Fig. 6. Scheme of the procedures for the validation of the resulting bands of the restriction enzyme digestion (*Hae*III) followed by coloration with the fluorochrome DAPI.

Figure 1D represents the ideogram of a chromosome. This ideogram highlights the degradations of the enzymes, which we present as a new proposal of band similarity after image analysis in IPP in Result 1. The original proposal of Fernandes (2004) is shown in Result 2. We found that a more refined analysis allowed the visualization of two other digestion locations shared between the three species that, by the methods of traditional visualization, could not be distinguished, so it is possible that the conclusions of Fernandes (2004) can be modified.

Another example of the use of software analysis was recently presented by Krinski *et al.*, (2010). In this work, the authors described the karyotype of a stingless bee, *Oxytrigona* cf. *flaveola*. With the advent of image analysis, any of the various characteristics presented in karyotype may be evaluated. The first pair of chromosomes of this bee presents a heteromorphism of size, and in accordance with the application of fluorochrome CMA_3, this heteromorphism is due to a block of heterochromatin rich in CG bases. A thorough analysis with the help of IPP enabled the visualization of a subtle secondary constriction along the heterochromatic arm of the largest chromosome (Fig. 7).
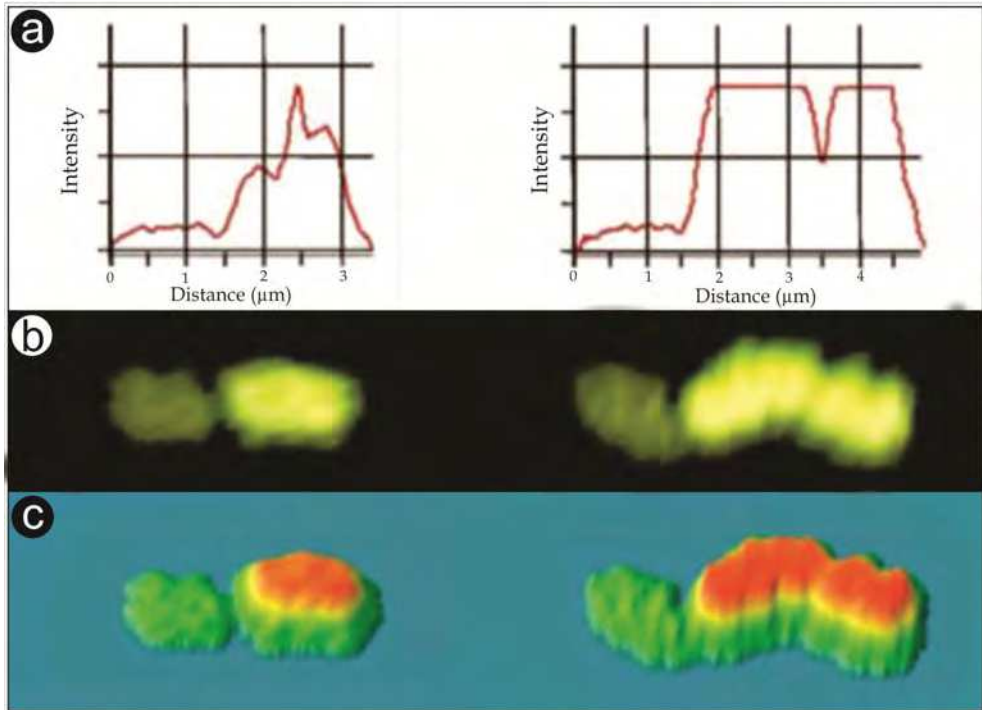
a) Histogram obtained from the fluorescence emitted from the chromosomes treated by fluorochrome CMA_3. b) Chromosomes treated with CMA_3. c) High relief image of the fluorescent chromosomes. Note that in a) and c) it is easy to see the secondary constriction present in the larger arm of the second chromosome, which is not possible in B). Figure courtesy of magazine Genetics and Molecular Biology (2010, Vol.33, No.3).

Fig. 7. Highlights the size heteromorphism presented by the first pair of chromosomes of the stingless bee *Oxytrigona* cf. *flaveola* (Krinski *et al.*, 2010,).

From the image obtained by the use of fluorochrome CMA_3, a graph was constructed which presented the fluorescence intensity of the pixels along the chromosomes. In this graph, secondary constriction was evident in the chromosome of the greatest length, represented by the valley in the graph. This constriction is located 3.5 micrometers from the start of the chromosome (see graphic in Fig. 7a) and causes a decrease of approximately 46% of the fluorescence in this location. It is here that image analysis shows its strength, since even a

decrease of this magnitude could not be perceived using conventional optical analysis, as shown in the image of the chromosome.

In the same mounting, another resource that brought a refinement to the approach of the problem was the use of a topographic image of the chromosomes (Fig. 7c). This technique is based on the different light intensities emitted by those portions of the image (in this case by the chromosomes). Note that the smaller arms of both chromosomes present less fluorescence (reflection of the small quantities of the CG bases) which translates to a region of some increase in relief representation (Fig. 7c). The path of the long arm shows that the intensity of the fluorescence increases, and this increase is reflected in the rise of the image topology. Interestingly, the secondary constriction is visible using this resource, reinforcing the information obtained from the histogram. Either the relief or the graph may be analyzed to identify the presence of the secondary constriction, but only with the help of image analysis with IPP.

2.4 Linearization of chromosomes

Other resources may be suggested for better organization of cytogenetic data. In this way, the tool of image linearization enables a better visualization technique, which ultimately extends and refines the information that can be extracted from the chromosomes. Two commercial software packages can be used for this, *BandView®* (developed by Applied Spectral Imaging[Ltda]) or *Lucia Karyo®* (developed by Laboratory Imaging s.r.o. [Ltda]), both of which contain linearization tools . There are also free programs which can do chromosome linearization as well, such as *ImageJ* (Abràmoff *et al.,* 2004) and *Image SXM* (Barrett & Carvalho 2003).

In 2003, the karyotype of a species of bee (*Euglossa townsendi*) was presented by Fernandes *et al.* using various banding techniques. A suggested chromosomal pairing was presented by coloration with Giemsa. As an example, we submitted one of the suggested chromosomal pairs to the process of chromosomal linearization (Figure 8a). This process yielded new
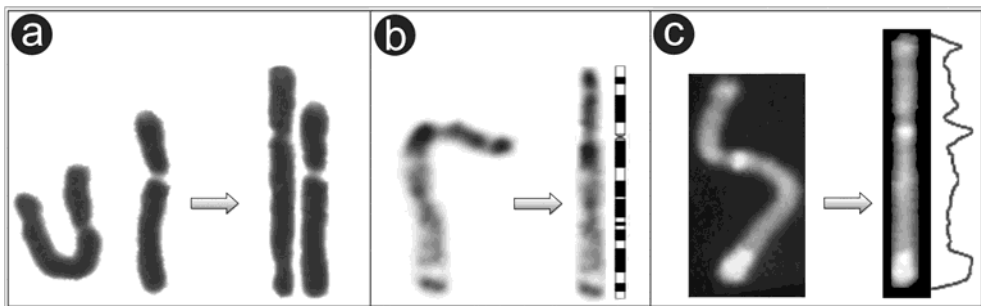
a) A pair of chromosomes colored with Giemsa that showed heteromorphism after the process. b) A chromosome subjected to the G-Banding technique, after image linearization it was possible to construct an ideogram of the banding patterns. c) Chromosome treated with fluorochrome CMA_3 the linearization enabled the construction of an extremely reliable graph of the luminescent intensity.

Fig. 8. Chromosome of bee *Euglossa townsendi* before and after the linearization of the images.

information, showing that the chromosomes were not of similar sizes, an observation that could not be made initially since one of the chromosomes was extremely curved. Now the karyotype of this species can be subjected to further analysis for us to discover if there is a size heteromorphism between the chromosomes of this pair, or if we have only a pairing error. The linearization of images can also be used for the construction of more precise ideograms (Figure 8b), and their use is not restricted to bright field microscopy, as it can also be used when the chromosomes are treated with fluorochromes. Note that independent of which proposal is used, the information obtained from straight chromosomes is larger in quantity and quality.

3. Conclusion

Note that in the most diverse areas of biological applications, and consequently in cytogenetic studies, image analysis through the use of current tools of bioinformatics, arises to overcome a series of limitations of common methods in the study of chromosomes. Considering this, and with the current technological developments in the fields of lenses, molecular biology and informatics, we can infer with more certainty about the mechanisms involved in karyotypic evolution. In a few years, other technologies are certain to be developed and new data will be obtained. Within this constant evolution of technology, the past cannot be ignored nor the current technology negated, because science is constructed on a base of pre-acquired knowledge, projecting itself to the future with the tools of the present moment

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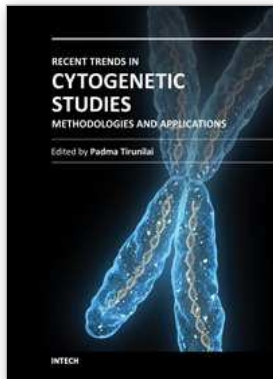
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