

Chromosome count improvement and digitalization of *Neolamarckia cadamba*

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Methodology

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Abstract

Background: Chromosome count is the only direct way to determine the number of chromosome of a species. This study is often considered trivial that seldom described and discussed in detail. Therefore, it is inevitable that chromosome count protocol should be revised and revisited before it becomes obliterated. Our chromosome counts in *N. cadamba* root tips have encountered challenges that prevent us from obtaining clear micrograph with the correct chromosome number. Several obstacles were determined through micrograph observation such as existing unwanted particles in cells, poor chromosome staining and chromosome crumpling.

Results: The chromosome counts of *Neolamarckia cadamba* under optimized procedure yielded 44 chromosomes per nucleus. To overcome these, root tip types, stain types, squashing methods were among factors assessed to obtain clear micrograph. We also apply digital technology in term of online database and graphic software that are open source and freely accessible to the public.

Conclusions: Throughout the study, we used a few basic laboratory equipment and chemicals, thus making this study economical and applicable in a basic laboratory. The availability of online digital software and databases provide open-source platforms that will ease the efforts in chromosome count.

Introduction

Chromosome count has been developed for more than 100 years. It is still a direct, unambiguous and routine way of determining chromosome number. This method is vital in plant systematics, taxonomy and breeding studies. In classical plant systematic study, chromosome count data are used to categorize plants and to identify relatedness between species in a family [1, 2]. In plant breeding, chromosome count is also used to identify the production of polyploids [3], double haploids [4] and interploids [5]. Although chromosome count protocol has been developed for a long time ago, it is still primarily conducted manually through sample preparation and microscopic observation. This protocol is often criticized as labour intensive, time-consuming and therefore challenging to obtain desirable micrographs [6]. At the moment, total automation in chromosome count to make chromosome count faster is still unavailable. Chromosome count study is getting lesser attention lately due to the invention of flow cytometry (FCM) analysis. FCM has somehow eclipsed the importance of chromosome count in plant science as FCM is conducted digitally with minimum sample preparation. However, FCM is still unavailable in many laboratories due to its cost.

Multiple online sources of data available allow researchers to access the databases and counter reference chromosome counts from different other studies to ascertain the validity of the assessments. Two recent articles [7, 8], discuss a list of online resources regarding chromosome count data of various plants. Nevertheless, chromosome count data obtained a long time could be different from the present chromosome count data due to natural causes such as geolocation of the samples obtained [7, 9], polyploidy, amphiploidy, aneuploidy, haploidy and monosomics [10, 11]. Erroneous chromosome counts

can occur when the number of chromosomes is numerous and small [12]. Some old chromosome count data were disputable in family Rubiaceae [13] because the researches were conducted many decades ago when microscopic technology was not advanced.

Chromosome count procedure is often simplified and generalized as a three-step procedure, namely pre-treatment, fixation and staining [6, 14, 15, 16]. This procedure provides generalized basic steps in sample preparation for chromosome count. However, the vast number of diverse plants in planta kingdom are varied in terms of cell size, the composition of cytoplasm, chromosome size, cell wall thickness and so forth. These variations render modification in steps difficult to undertake, such as the concentration of reagents, treatment durations and squashing methods to obtain an accurate result [5, 17]. Most reagents and stains in chromosome count study were developed many decades ago. Therefore, information on the origin, characteristics and reactions of these chemicals is not readily available in recent publications. Two reference books have provided some details of chromosome count chemical repertoire [18, 19]. Based on the details of the chemical reaction, chemical treatment can be modified accordingly to obtain the desired result.

In this article, we demonstrate the chromosome number, chromosome morphology and the mitosis mechanism of *N. cadamba* through chromosome count. Optimization of chromosome count procedure was conducted to obtain a satisfactory result in chromosome count. The semi-automated approach was adopted to determine the number of chromosomes in *N. cadamba* by using open-source software. Several databases of plant taxonomy and number of chromosomes were identified, that could help the study of chromosome count. The flow of this article was demonstrated in a reverse manner from the result obtained, challenges, solutions and application of information technology in chromosome count.

Materials And Methods

Procurement of roots

Root tips of a cm in length each were excised from the plantlets between 9.00 to 10.00 am and then cleaned with running tap water. Two types of root-tips were evaluated for suitability in chromosome count, namely: (a) young roots, 5-day-old root tips and (b) older root, 20-day-old root tips before proceeding to the next process.

Pre-treatment and fixation

The cleaned root tips were dipped in 0.0002 M 8-hydroxyquinoline for 2 h at 4°C then for another 2 h at room temperature (25°C) and washed the pre-treated root tips three times with distilled water before fixation procedure.

After the pre-treatment, the root tips were fixed in freshly prepared cold Carnoy's solution (Ethanol 95% : Acetic Acid; 3:1) for 24 H at 4 °C. The fixed root tips were then washed three times with distilled water before proceeding to the next process.

Staining and squashing

The pre-treated and fixed root tips were first digested using 1N HCl for 15 min at 60°C and then washed three times with distilled water. All the staining was conducted at room temperature, but the Feulgen stain was applied to the root tips under dark condition. The digested young root tips were stained and squashed in the following five ways:

- (a) aceto-orcein 1% for 15 min then squashed with a metal bar in a drop of aceto-orcein 1% before the thumb squash;
- (b) aceto-carmin 1% for 15 min then squashed with a metal bar in a drop of aceto-carmin 1% before the thumb squash;
- (c) Feulgen stain for 30 min then squashed with a metal bar in a drop of Feulgen before the thumb squash;
- (d) Feulgen stain for 30 min before squashed with a metal bar in a drop of aceto-orcein 1% before the thumb squash; and
- (e) Feulgen stain for 30 min before squashed with a metal bar in a drop of aceto-carmin 1% before the thumb squash.

Observation under microscope

LEICA DM500 compound light microscope was used to observe the chromosomes with 10x, 40x and 100x objective lens. Root cells were observed initially using the 10x objective lens to determine the location of the root tip where the young cells are located. The cells are round in shape with bigger nuclei occupying most of the cytoplasm while the older cells are elongated with smaller nuclei. After the location of young cells was determined, the objective lens was changed to 40x to determine the location of the actively dividing cells with the nuclei in the pro-metaphase stage. Next, increase the power of the objective lens to 100x to select an ideal nucleus at the pro-metaphase stage with well-spread and visible chromosomes. Micrographs were captured using LEICA Application Suite (LAS EZ) and LEICA ICC50 HD camera.

Counting chromosome using ImageJ

ImageJ 1.52 software (open source) was used to crop and magnify the micrographs captured. This software can be downloaded for free from <http://imagej.nih.gov/ij/>. On the toolbar, choose “Multi-point” tool, and it was used as a counter to mark and label the stained chromosomes. The number of chromosomes will be shown at the window of “Multi-point” tool.

Results And Discussion

Chromosome number, chromosome morphology and the mechanism of mitosis of *Neolamarckia cadamba*

Under the optimized procedure in chromosome count of somatic cells of *N. cadamba* root tips, the micrographs show that both the tetraploids and the controls contain 44 chromosomes per nucleus (Figure 1). The micrograph captured in this study also showed well-spread and visibly distinct chromosomes with a clear background in the nucleus. The micrographs captured in this study also showed well-spread and visibly distinct chromosomes with a clear background in the nuclei. The stained chromosomes are small and without any discernible chromosome constrictions. Therefore, the shapes of the chromosomes appear roundish but cannot be determined in greater details.

The requirements to obtain well-spread and visibly distinct chromosomes with a clear background in the nucleus can be more favourably achieved through the use of 5-day-old root tips, double staining and double squashing as described in Materials and Methods section. The double staining and squashing procedure used in this study comprises treatment with Feulgen stain for 30 min before squashing the root tip sample with a metal bar on a slide with an additional drop of aceto-orcein 1%. After that, crushing the root tip again using thumb squash with a coverslip against the slide.

Our chromosome count is in agreement with the chromosome count conducted previously using mitotic cells, that is $2n = 4x = 44$ [20]. Other studies that are in agreement with our chromosome count were using pollen – meiotic cells which showed 22 chromosomes or $n = 2x = 22$ [2, 21, 22]. All the previous chromosome counts in *N. cadamba* stated that *N. cadamba* is a tetraploid plant. There was no dispute or deviation between chromosome counts published in between decades and researches carried out on different locations across vast geological distances. Chromosome counts were identical for samples from Thailand [2], China [20], India [21, 22] and Malaysia. Our finding is that there is no variation in chromosome counts from different publications. According to Kiehn [13, 23], chromosomes number counts in family Rubiaceae that deviates from published records should be treated with caution as it was counted long ago. Errors in chromosome count might cause the deviation.

The size of *N. cadamba* chromosome is small (less than 1 μm) and its shape is roundish (Figure 1). The chromosomes are so small and compact. Hence, the chromosome constrictions are not visible using the current method of sample preparation and the compound microscope. Without visible chromosome constriction, the shape of the chromosomes cannot be determined accurately, which makes karyotypical studies a challenge. Chromosomes without particular shape and pattern in all dimensions are termed as metacentric and symmetrical chromosomes, where the short chromosome's arms are equal in length and median kinetochore [24]. According to a study on karyo-morphology of Rubiaceae [25], chromosome less than 1 μm in length is exceptionally small. The higher number of somatic chromosomes the smaller the chromosomes, for example, bigger chromosomes in *Coffea robusta* ($2n = 22$) versus smaller chromosomes in *Coffea arabica* ($2n = 44$). Chromosomes of woody angiosperms are usually small, and there is little size difference between related species and genera [24]. However, chromosome size and morphology are of little taxonomic use in family Rubiaceae [26].

Several phases in mitosis of *N. cadamba* root tip cells can be observed under the microscope for the tetraploids (Figure 4.2a-f) during the process of searching for cells in the pro-metaphase stage when the chromosomes can be observed and counted. The micrographs captured also showed different phases of mitosis, namely interphase, pro-metaphase, metaphase, anaphase and telophase. The phases of mitosis captured in these micrographs were described based on stages of mitosis [24].

During interphase, no chromosome is visible and only the stained nuclei are visible (Figure 2a). In prophase, the chromosome threads will be progressively coiled or spiralled to form condensed thicker chromosomes that are visible under the microscope (Figure 2b). When the cell enters the early metaphase, maximum condensation in chromosomes occurs and the disappearance of the nucleus membrane are observed. This stage enables chromosome counting to be conducted as the condensed chromosomes are spaced evenly and do not crowd together (Figure 4.2c and Figure 4.3c). In the metaphase, chromosomes arrange themselves along the equator of the cell (Figure 4.2d and Figure 4.3d). In anaphase, the chromosome pair splits and moves to the opposite poles (Figure 4.2e and Figure 4.3e). In the final phase, telophase, the chromosome pair will split and go to the opposite pole of the cell and starting to form two daughter nuclei each with a separate nucleus membrane. The collection of micrographs provides an overview of the mitosis cycle of *N. cadamba*, thus helping the researcher to identify these phases of mitosis and understand the mechanism of mitosis. Of all the phases of mitosis observed, only pro-metaphase or early metaphase can provide distinctly clear and separately individual chromosomes, an ideal micrograph for counting chromosomes.

It is generally accepted that the majority of species in the Rubiaceae family which *N. cadamba* belongs have a basic chromosome number 11 ($x = 11$). This basic chromosome number is still valid, in agreement with our study and many other studies in decades [1, 13, 21, 23, 26, 27, 28, 29]. Based on the most comprehensive chromosome number data compiled in 1979; 730 species in the Rubiaceae family were examined; 493 species have chromosome base number $x = 11$; 200 species are polyploids at different levels of ploidy and 155 species are tetraploids with 44 chromosomes ($2n = 4x = 44$) [1]. Furthermore, tribe Naucleae which *N. cadamba* also belongs has chromosome base number $x = 11$ which is either diploid ($2x = 22$) or tetraploid ($4x = 44$) [1, 30].

Chromosome count optimization

Chromosome count is the conventional technique of ploidy level determination which is laborious and requires much time and effort to produce the desired result. This procedure has been practised since 1921, firstly by Belling J. but it is still widely used to determine the exact chromosome number of a sample. The main part of this chromosome count procedure practised nowadays has been derived from the old literature [31]. In our opinion, the entire chromosome count procedure should consist of six main steps, online data searching, pre-treatment, fixation, staining, squashing and counting. However, to obtain the desired result, further modification of the steps may be required to suit the particular species as each species is different from the other in many respects such as chromosome size, chromosome number, cell characteristics and cytoplasm composition. Experience and skill of chromosome count can only be built

up by practicing the method frequently over a period of time. The procedure of chromosome count narrative in the current literature is often summarized, shortened and simplified, which makes the procedure difficult to master. Apart from knowing the textbook procedure, the technique gained and developed from the hands-on experience is utterly essential.

An initial study has to be undertaken to evaluate which is the more suitable age of the root tips to be used for chromosome count, namely 5-day-old root tip (Figure 3a) or 20-day-old root tip (Figure 3b). It was found that the 5-day-old root tip was better than the 20-day-old root tip. The younger root tip is thicker, softer and without root hairs. These characteristics make the chromosome count easier. Younger root tip, which is thicker, has more actively dividing cells, thereby increasing the chances of observing cells in pro-metaphase stage with good chromosomes spread.

Furthermore, younger root tip, which is softer, makes squashing using a coverslip easier. After squashing the younger root tip cells would spread more evenly, thus avoiding the problem of overlapping cells (Figure 3c). Older root tip aged 20 days old and onwards is slender and tends to produce root hairs (Figure 3d) which not only reduce the visibility of the chromosomes but also produce very little cell spread. In the root section further away from the older root tip, the cell walls are thick and the nuclei are much smaller in relation to the size of cytoplasm (Figure 3e). This indicates the cells are not actively dividing.

In term of cellular characteristics, cells in older roots tips tend to have more particles than the younger cells. These particles are suspected to be starch grains (Figure 3f). The morphology of starch grains is species-specific [32]. In the present study, the suspected starch grains are similar to starch grains of sweet potato and rice (Figure 3f) which are compound starch grains.

Often compound starch grains will break up when squashed leading to their spreading across the whole cell and making chromosome count impossible. To reduce the chance of obtaining cells with accumulated starch grains, younger root tips should be used. In *N. cadamba*, older cells accumulate starch grains that often create unwanted noises among chromosomes, thus making chromosome count the impossible (Figure 3f). Newly developed root cells have less accumulation of starch grains as time is needed to transport newly synthesized photosynthate from leaves to roots.

Starch grains in the root are insoluble, energy-dense and osmotically inert. Starch grains also serve as the storage and will be remobilized when photosynthesis process is inadequate to support the functions of the plant. [33]. Both root tips and shoot tips are often used in chromosome count. In the case of *N. cadamba*, root tips are preferred because root tips are more non-destructive as roots are easily generable. Under *in vitro* condition, roots can be formed easily without inflicting much harm to the root system of the plant.

The solution of 0.002 mM of 8-hydroxyquinoline has been used effectively to pre-treat root tips of plants with small chromosomes [16]. The purpose of pre-treatment is to arrest dividing cells at pro-metaphase by stopping the formation of spindles that are important to chromosomes mobility in the nucleus. It also

reduces the chromosome length by increasing coiling of chromosomes and thereby making clearer cytoplasm. It increases the cell plasma viscosity that will avoid chromosome from clumping together [16, 34]. Furthermore, this reagent serves as a fixative to a certain degree by coagulating proteins in the cell's organelles [34].

Three commonly used fixatives for chromosome study are, Carnoy's Solution I (3:1; ethanol : glacial acetic acid), Carnoy's Solution II (1:3:6; glacial acetic acid : Chloroform : ethanol) and Propionic Acid Alcohol (3:1; ethanol : propionic acid) [16]. Carnoy solution should be prepared immediately after pre-treatment of root tips to avoid acetic alcohol esterifying as the acid will lose its effectiveness over time. The fixative used in chromosome study is to kill the cells rapidly, particularly the cells in their pro-metaphase stage. The process of fixation should avoid distortion, swelling, or shrinkage to chromosomes as the occurrence of any of these will change the morphology of the chromosomes. Good fixatives will improve chromosome visibility. Ethanol in the fixatives can penetrate the cells rapidly before the cells dehydrate and their proteins denature. The glacial acetic acid, in combination with ethanol, can preserve the chromosome morphology and avoid distortion, swelling or shrinkage [16].

Three types of stains were evaluated to assess which is the most suitable stain or combination of stains for use in this study, and these stains are Feulgen, aceto-orcein 1% and aceto-carmine 1%. These stains were used either singly or in combination to obtain vividly stained chromosomes. During the staining process, it is essential to observe that at the end of the staining, root tips must be visible in magenta colour, especially at the tips before proceeding to squash. If the root tips remained white even though the staining duration was extended, the sample should be discarded as no staining has taken place. This is vital to create a clear micrograph that shows vividly stained chromosomes in distinct contrast against the background. A clear micrograph will enable chromosome count easier. The choice of suitable stain types will be the result of several trial and error process.

The micrographs of *N. cadamba* taken on root tip cells stained with either aceto-orcein 1% (4a) or aceto-carmine 1% (4b) individually was grainy and not clear. These two stains could have caused the blurry background among the chromosomes. The cytoplasm around the chromosomes appeared to be partially stained especially when aceto-orcein 1% was used, leading to a blurry background. Feulgen stain produces clearer micrograph with more countable chromosomes (4c). All the three stains if used singly are not able to stain every chromosome intensely and uniformly. They stain and also stain the chromosomes at a different intensity.

Furthermore, the chromosome number obtained is always less than the established count that is 44 chromosomes for the tetraploid cells of *N. cadamba*. When a combination of two types of stains was used, that is either Feulgen + aceto-orcein 1% (Figure 4d) or Feulgen + aceto-carmine 1% (Figure 4e), 44 clear and vividly stained chromosomes were visible in each *N. cadamba* tetraploid cell and likewise 88 chromosomes in each of the octoploid cells. However, a combination of Feulgen + aceto-orcein 1% of stains produced better colour intensity and clarity of stained chromosomes. Therefore, stain combination

of Feulgen + aceto-orcein 1% is recommended for application in the staining chromosome of *N. cadamba* for this chromosome count.

Different stains will stain the chromosomes differently. Some stains require a chemical reaction while others do not. For Feulgen stain, Schiff's reaction will take place in order to get the chromosomes stained. It starts with hydrolyzing the root tip using HCl to separate purine from sugar in the DNA and exposing the aldehyde group. Then, the Fuchsin sulphurous acid from Feulgen will react with the aldehyde to form the magenta colour in the chromosomes [18]. Feulgen stain is a better stain than aceto-carmine 1% and aceto-orcein 1% for staining chromosomes of *N. cadamba*. This stain produces fairly vivid chromosomes but does not stain the cytoplasm around the chromosomes.

Carmine is a stain used in chromosome count that produces crimson colour in the chromosomes. Carmine is extracted from a type of insect of Homoptera family found in tropical America. Commercial carmine powders are variable in quality due to different sources of materials used to produce the carmine powder [35]. In the present study, aceto-carmine 1% is the less favourable stain compared to Feulgen and aceto-orcein 1% as the former produces faintly stained *N. cadamba* chromosomes. Orcein, a deep purple-coloured dye is obtained from orcinol extracted from lichens, *Rocella tinctoria* and *Lecanora oarella*. When orcinol reacts with hydrogen peroxide and ammonia, orcein stain is produced. Orcein is soluble in water and ethanol. However, it is prepared in the same way as aceto-carmine [18]. In our study on *N. cadamba*, aceto-orcein 1% produce intense colour to the chromosomes that lead to partially stained cytoplasm. Aceto-orcein and aceto carmine are often used interchangeably if one of them does not stain effectively. Acetic acid 45% is used to dissolve the carmine and orcein powder. Acetic acid (45%) also ensures fast penetrative effects for stains, and it acts as a fixative to the chromosomes [18].

There are many stains available for chromosome count study. To achieve good results, evaluation of these stains should be conducted either by single stain application or combination of stains application. Apart from selecting the type(s) of stains used, other parameters can also be assessed, such as the stain concentration and the duration of staining.

A well-stained root tip was mounted on a slide and squashed to release a monolayer of root cells revealing stained chromosomes before observation under the microscope. A double squashing method is employed to obtain well-spread chromosomes. Root tip was squashed with a metal bar before thumb squash using a coverslip (Figure 5a). Initially, we used the single squashing method, that is thumb squash by using a coverslip, but this method prevented the chromosomes from distributing evenly. As a result, most of the chromosomes clump together (Figure 5b) and some cells overlapped (Figure 5c). This is caused by inadequate exertion of force onto the root tip to spread the chromosomes and cells evenly. Deformity in cell shape (Figure 5d) can also be avoided by holding the coverslip firmly against the thumb when squashing the root tip.

Thumb squash using a coverslip is a common practice to spread the cells and chromosomes on the slide. In our experience, this method does not exert sufficient force to spread the cells and chromosomes evenly for viewing under the microscope. Inadequate force of thumb squash method on the root tip of *N.*

cadamba, causes cells to overlap each other and creates multiple layers of cells on the slide. The chromosomes in *N. cadamba* also tend to clump together and overlap with each other. These factors make the counting of chromosome impossible. In order to overcome these obstacles, a metal bar was introduced to initially squash the root tip directly on a slide with a drop of selected stain before the thumb squash with a coverslip. By double squashing the root tip as mentioned, the chromosomes in the cell can be more evenly spread to make chromosome count easier. The root tip cells will be well-spread so that there are many choices to select the best cell with perfectly spread chromosomes for viewing and counting under the microscope. Squashing techniques are rarely described and discussed in the published articles that mention chromosome count. To achieve a well-spread chromosome scenario, the researchers may need to modify the existing methods or invent new squashing strategies to suit their samples.

Automation in chromosome count

Micrograph with well-spread cells and vividly stained chromosomes will be used to determine the number of chromosomes per nucleus using the ImageJ program (Figure 6a). There are many tools available in ImageJ program that is useful in scientific image processing. "Multipoint tool" can be used as a marker and counter to the chromosomes on micrograph. Chromosomes can be marked by selecting "Multipoint tool" and positioning the cursor on the chromosomes followed by right-clicking (Figure 6b). Then, by choosing "label", all marked chromosomes will be labelled with a number each that will add up to the total chromosome number (Figure 6c). The labelled micrograph can be saved by using "Plugins" - "Utilities" - "Capture image". This process prevents the researcher from wrong counting of the chromosomes, especially when the chromosomes are numerous and small like *N. cadamba*. Counting chromosomes manually can be laborious and prone to error.

In this study, ImageJ was used to analyze micrographs obtained during chromosome count study. These include micrograph cropping and chromosomes counting in the selected cell. We used a semi-automated approach in chromosome count with the help of ImageJ. Selection of stained chromosomes was made manually with the assistance of ImageJ by clicking on the stained chromosomes with the purpose to mark and count the chromosomes on the micrograph captured by the digital camera. ImageJ was used to select stained chromosomes manually on the monitor screen. By such selecting, the number of chromosomes will be summed up automatically at the end of chromosome selection. Selecting manually by the researcher will prevent the inclusion of wrong particles that are not chromosomes in the counting. *N. cadamba* cells are rich in starch grains, as evident in this study. Studies on members of the Rubiaceae family also showed similar evident [13]. These Rubiaceae plant cells produce a large amount of particles that are trapped in the plant cell walls. A good sample preparation for chromosome count should have clear cytoplasm and free from particles other than the chromosomes. The presence of these particles is a major challenge when using ImageJ as a total automated procedure without human intervention to count the chromosomes in *N. cadamba*. Total automation in chromosome count is still far from perfect as errors and variation in chromosome number exist even within the same species [36, 37].

Two chromosome count studies conducted, utilized computer software to count the chromosomes automatically on micrographs captured by an electronic camera [36, 37]. This method uses an image thresholding technique to calibrate the micrograph to identify individual chromosomes, separate overlapped or clumped chromosome and sharpen the images. ImageJ software was used to automatically determine chromosome number yielding a range of different chromosome numbers of a species [37]. However, the mean number of chromosomes was used to determine the chromosome number of the species. In another study, automation of the chromosome count of human cells resulted in 6% error rate as known somatic human cell that has 46 chromosomes [36]. Automation in chromosome count requires optimization in slide preparation which is tedious, especially when plant material is to be examined such as *N. cadamba*. The success and accuracy of the automation techniques used would largely depend on the quality of sample preparation that should minimize the occurrence objects other than chromosomes [36, 37].

Online database for chromosome count

Current online database, although not exhaustive, help the researcher to cross-refer and obtain data regarding taxonomy, ploidy level and chromosome count of plants. There is no single database that provides all the required data. However, a combination of databases may be helpful. These databases are built serially over time based on researches done by different researchers. The database eases the researcher's effort to obtain the data as some of the researches were done decades ago and their publications are not readily available online now. To obtain these data, knowing the taxonomy and phylogeny of the species and related species is essential (Table 1). Furthermore, many species of plants have more than one name either Latin name or synonym and they also have older names. Knowing these names will increase the success rate of online data searching for chromosome count (Table 2).

Online databases for taxonomic classification in plants

The detailed taxonomy of *N. cadamba* has rarely been described in recent studies. *N. cadamba* belongs to subfamily-Cinchonoideae, tribes- Naucleae and Subtribes- Neolamarckiiinae (Figure 4.8). Comprehensive data of chromosome counts and taxonomy were combined to form cytotaxonomy study of the family. Usually, members of tribes have the same basic chromosome number (x) and ploidy level (Table 4.1). This relationship helps to provide some clue on chromosome number or ploidy level of the species which are yet to be determined.

They are many synonymous names of *Neolamarckia cadamba* (Roxb.) Bosser, (1984), [38], namely, *Cephalanthus chinensis* Lam. Encycl. (1785); *Nauclea cadamba* Roxb. (1824); *Anthocephalus indicus* A. Rich. (1834); *Anthocephalus moridaefolius* Korth. (1842); *Anthocephalus chinensis* (Lam.) A. Rich ex. Walp. Repert (1843); *Anthocephalus cadamba* (Roxb.) Miq. (1856) and *Sarcopcephalus cadamba* (Roxb.) Kurz. (1877). Many old references are difficult to find in libraries and online databases. The origin of these name can be traced using online resources provided by the Biodiversity Heritage Library (Table 1). These references are often scanned and transformed into digital books for the public. Knowing

synonymous names of a species would make taxonomic and cytogenetics data searching less laborious and painstaking.

Online databases for chromosome number in plants

When searching for the chromosome count of a species, different synonymous names should be searched to obtain chromosome counts from different sources. This will make data obtained more reliable. Different articles may use different names in *N. cadamba* chromosome count study, such as *Neolamarckia cadamba* [2]; *Anthocephalus cadamba* [21, 22] and *Anthocephalus chinensis* [20]. *N. cadamba* should be used universally until a new name is given. *N. cadamba* is the newest name given to this plant since 1984. Older names should be avoided as it will reduce the visibility of the findings to the community who are conducting the researches.

Conclusion

This study has verified that the chromosome number of *N. cadamba* is 44. The chromosomes are small and roundish. This study also in agreement with the basic number of chromosome ($x = 11$) in the family Rubiaceae. Different phases of mitosis from somatic cells of the root tip of *N. cadamba* were captured and compared. Only cells at early metaphase can be used for chromosome count study due to chromosome structure and its location. In *N. cadamba* chromosome count optimization study, several factors were found to affect the result of micrograph used for chromosome count. These include very small chromosomes, chromosome clumping, poor chromosome staining, existent of starch granules and hairy roots. Younger root tips (5-day old), double stains (Feulgen then aceto-orcein) and double squashes (metal bar then thumb) were found to be the key to well-stained, spread and clear micrograph. We also utilized the tools in ImageJ to help in facilitating the counting of chromosomes that were numerous. Several databases were found to be useful to the study of polyploidization and cytogenetics.

Declarations

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Authors' contributions

Eng, WH and Ho, WS designed the research project. Eng, WH performed the experiments and analyzed data. Eng, WH and Ho, WS wrote the manuscript. Ho, WS and Ling, KH modified the manuscript. All authors read and endorsed the final manuscript.

Availability of data and materials

The datasets used for the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: Online databases for taxonomic classifications in plants

Organisation	Website	Information provided
Integrated Taxonomic Information System	https://www.itis.gov	Taxonomy hierarchy and nomenclature.
Plant Genera, Illustration	http://www.plantgenera.org/taxonomy	Taxonomy hierarchy, nomenclature and Illustration.
United States Department of agriculture (USDA)	https://plants.usda.gov/classification.html	Taxonomy hierarchy, nomenclature and distribution in USA.
The Plant List	http://www.theplantlist.org	Nomenclatural and scientific names.
International Plant Names Index (IPNI)	https://www.ipni.org	Nomenclature and scientific names.
Biodiversity Heritage Library (BHL)	https://www.biodiversitylibrary.org	Digital library for classical biodiversity literature and archives.

Table 2: Online databases for chromosome numbers in plants

Organisation	Website	Information provided
Chromosome Counts Database	https://www.ccdb.tau.ac.il	Plants from different families.
Index to plant CNs	http://www.tropicos.org/Project/PCN	Plant from worldwide
Prota4U	www.prota4u.org/	Tropical African plants.
CN database – PLANTS	http://chromosomes.binoz.uj.edu.pl//chromosomes/	Polish vascular flora.
Chromosome counts for Malvaceae	www.malvaceae.info/Biology/Chromosomes.php	Plants in family Malvaceae.
Online plant CN databases	www.bio.uni-bayreuth.de	Plants in Asclepiadoideae, Periplocoideae, and Apocynaceae.
Karyological database of the genus Cardamine	http://www.cardamine.sav.sk/	Plants in genus Cardamine (Brassicaceae, eudicots)

Figures

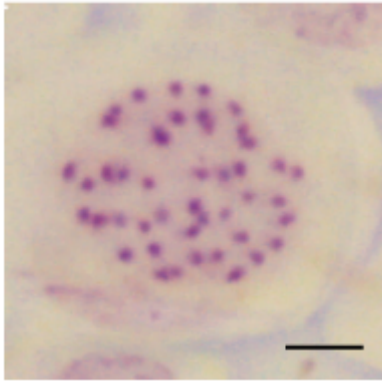


Figure 1

Chromosome spread observed under magnification of 100x, showed *N. cadamba* with 44 chromosomes.
(Bar = 5 μ m)

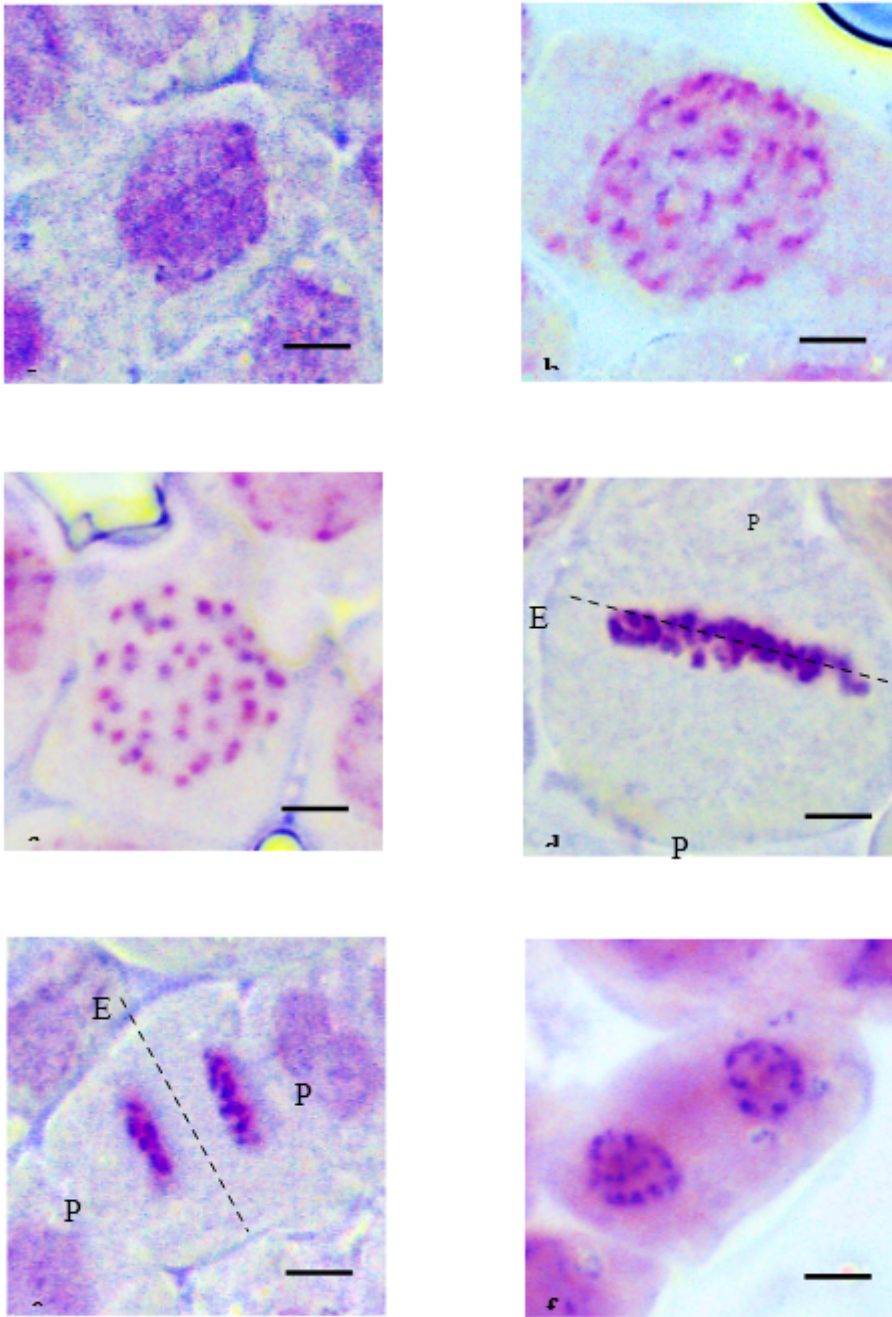


Figure 2

Different stages of division of tetraploid cell of *N. cadamba*, (a) interphase, (b) prophase, (c) pro-metaphase, (d) metaphase, (e) anaphase and (f) telophase. (Bar = 5µm)

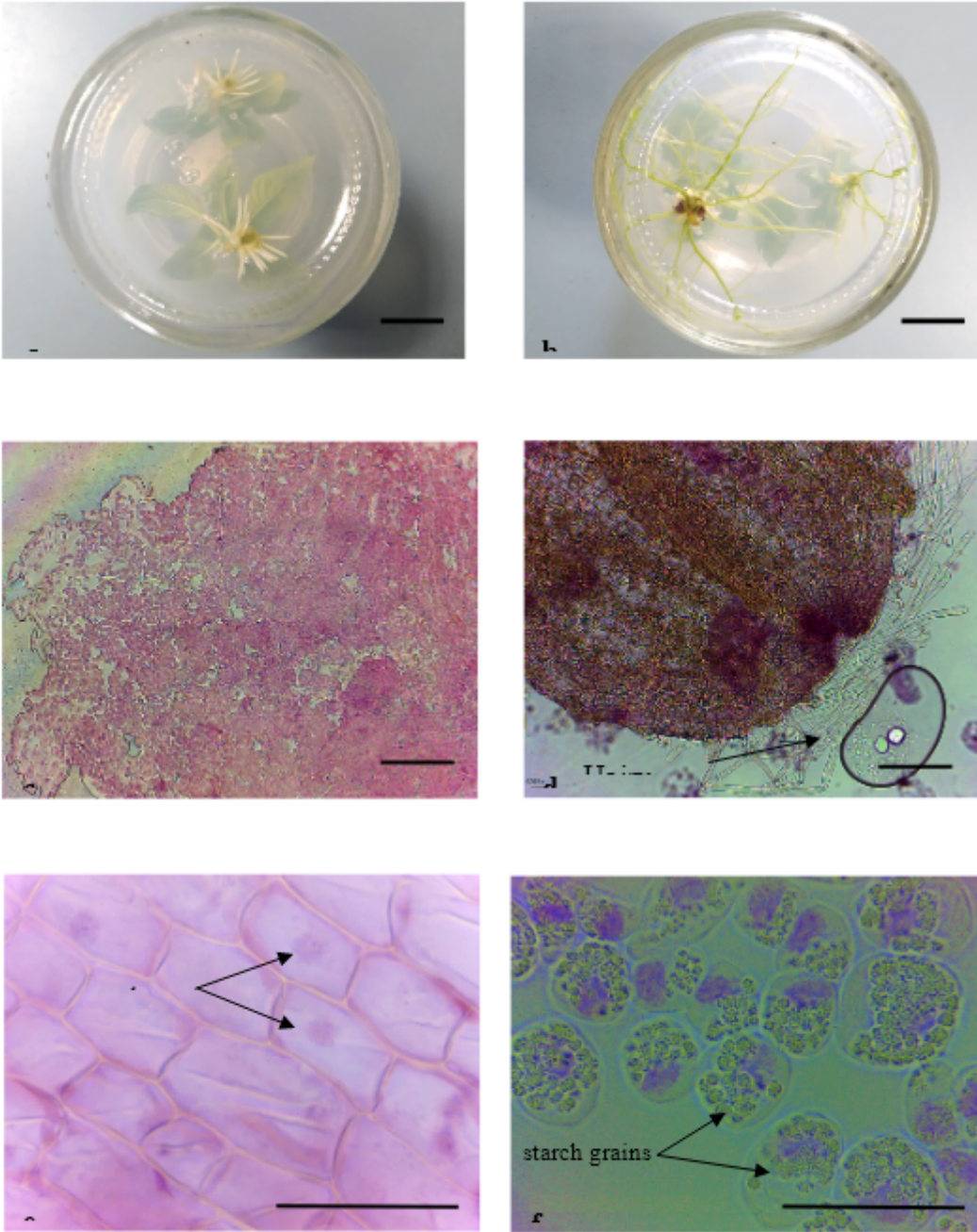


Figure 3

In vitro roots and root cells of *N. cadamba*. (a) 5-day-old roots (Bar = 1 cm), (b) 20-day-old roots (Bar = 1 cm), (c) root tip with well-spread cells (Bar = 0.2 mm), (d) root tip of hairy root without cell spread (Bar = 0.2 mm), (e) older cells with very small nuclei with thick cell walls and in angular shape (Bar = 50 μm) and (f) cells with starch granules. (Bar = 50 μm)

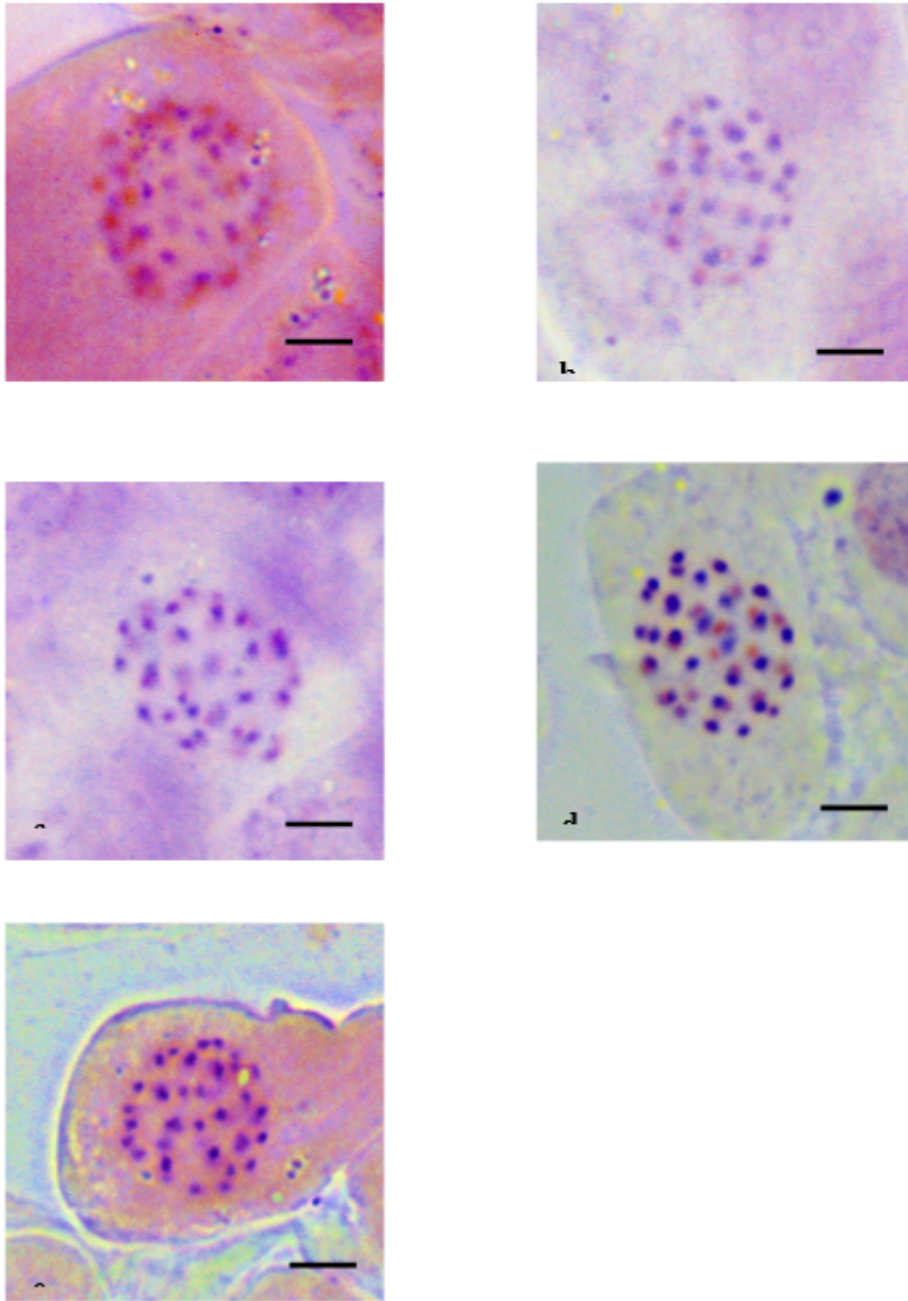


Figure 4

Micrograph of stained chromosomes of *N. cadamba* using single stain or combination of stains. (a) aceto-orcein 1%, (b) aceto-carmin 1%, (c) Feulgen, (d) Feulgen + aceto-orcein 1% and (e) Feulgen + aceto-carmin 1% (Bar = 5 µm)

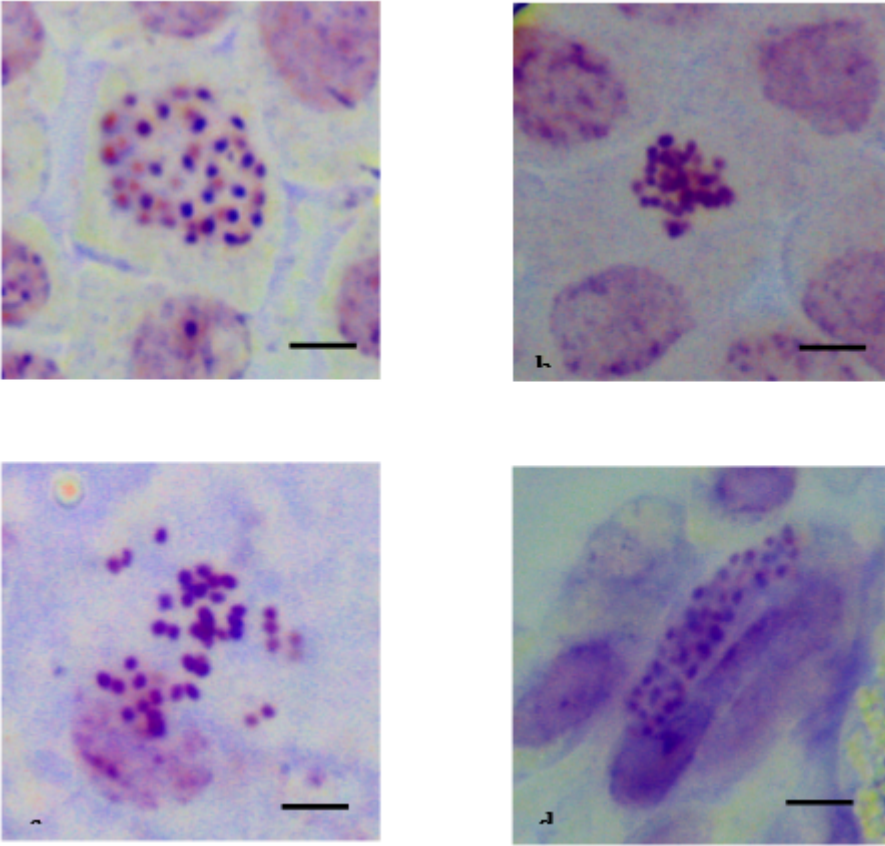


Figure 5

Squashed root tip cells of *N. cadamba*. (a) Well-squashed cell, (b) poorly squashed cells - chromosome clumping, (c) poorly squashed cells – overlapped cells and (d) poorly squashed cells – skidded squash. (5 μ m).

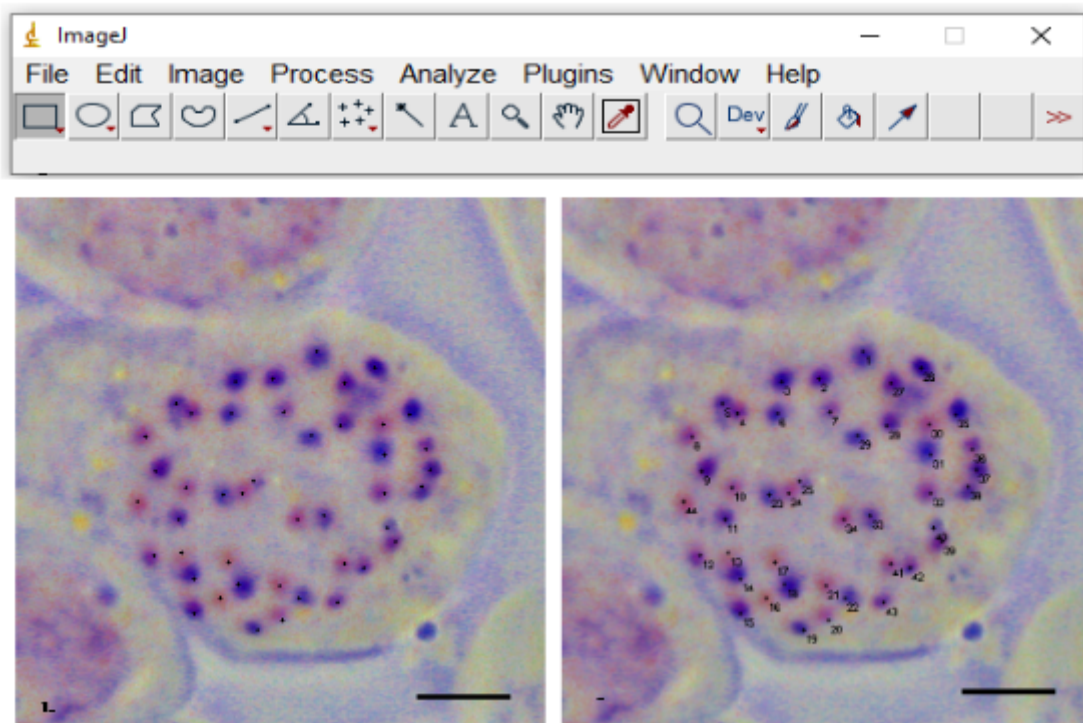


Figure 6

Digital counting. (a) ImageJ software toolbar, (b) marked chromosomes of octoploid of *N. cadamba* cell (Bar = 1 μm) and (c) numbered chromosomes of octoploid of *N. cadamba* cell (Bar = 5 μm).