*Article*

# Status of cell division, cytogenetical index, and chromosome details on the immature stage of *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae)

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**Abstract**

Investigation of various tissues of immature stages *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) from Iran was examined in an attempt to determine its cell division. In addition, the Metaphase plate of the wasp is evaluated to elucidate its chromosome feature and asymmetry/ symmetry karyotype. We found that: 1) Brain tissue of prepupa and early hours of the pupa stage are the highest mitotic index (%) and metaphase index, 2) The number of haploid chromosomes of the wasp is n = 5, and 3) The karyotype of the wasp is almost symmetrical.

**Keywords** chromosome; mitotic index; *T. brassicae*; brain tissue.

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## 1 Introduction

The status of cell division varies in different tissues of the insect body and during various stages. Almost immediately after fertilization, cell divisions begin and more cells are formed. For more than two decades now, the study of the chromosome behaviour in the newly laid egg of Trichogramma and the formation of polar bodies has been passed (Stouthamer and Kazmer, 1994). Gokhman (2005) stated that one of the requirements of a chromosome study is having tissue by high cell division. Various tissues which have been selected and studied by cytogeneticists for the preparation of karyotypes were included: gonads, embryonic cells, brain tissue, midgut and hindgut cells, salivary glands, Malpighian tubes, rectum, fat bodies, and hemolymph (Kocak and Okutaner, 2017). The main reason for selecting these tissues was their high mitotic index (MI). The cells were been located at each stage of interphase, prophase, metaphase, anaphase, and telophase but to achieve a suitable karyotype, the metaphase and prometaphase are perfect and ideal steps. During the cell cycle, the metaphase in which the chromosomes are compressed takes place for a short time.

Although Trichogramma has 239 described species (Khan and Yousuf, 2017), only about 5% of them are

cytogenetically evaluated (Table 1). *Trichogramma brassicae* which is known as a biological control agent for lots of lepidopteran pests has the widest dispersion in Iran. At 25±1°C, the length of the immature developmental period of the waspis10 days, in which the larval, prepupa, and pupa stages respectively last more than 2 days, 2.5 days, and 3.5-4 days (Farsi et al., 2020). The karyotype study of *T. brassicae* showed the haploid number of chromosomes is n=5 (Laurent et al., 1998). In all Trichogramma species studied, this number has been constant, except *T. kaykai* which the first B chromosome was identified (Stouthamer et al., 2001), as 5+0+1B. In the karyotype published of Trichogramma species, the focus has been on different stages and tissues of the wasps. Most cells were provided from cerebral ganglia, brain, testes, and egg (Hung, 1982; Vavre et al., 2004; Gokhman et al., 2017) (Table 1).

**Table 1** Detail of karyotypic studies of different species of Trichogramma.

| Species of Trichogramma | Reference | Chromosome number (n) | Stage | Tissue |
|---|---|---|---|---|
| *T. nubilale* | Hung (1982) | 5 | Pre- pupa, and pupa | Testes and ovaries(pupa)/ Brain (pre- pupa) |
| *T. evanescens* | Hung (1982) | 5 | Pre- pupa and pupa | Testes and ovaries(pupa) Brain (pre- pupa) |
| *T. chilonis* | Hung (1982) | 5 | Pre- pupa and pupa | Testes and ovaries(pupa)/ Brain (pre- pupa) |
| | Amutha Murugan and Manickavasagam (2003) | 5 | - | - |
| *T. pretiosum* | Hung (1982) | 5 | Pre- pupa and pupa | Testes and ovaries(pupa)/ Brain (pre- pupa) |
| | Stouthamer and Kazmer (1994) | 5 | Embryonic | Egg |
| | Gokhman et al. (2017) | 5 | Pre- pupa | Cerebral ganglia |
| *T. deion* | Stouthamer and Kazmer (1994) | 5 | embryonic | Egg |
| *T. brassicae* | Laurent et al. (1998) | 5 | Embryonic cell | Egg (Twenty- four hours later egg laid) |
| *T. dendrolimi* | Liu and Xiong (1988) | 5 | Pre- pupa | |
| *T. kaykai* | Stouthamer et al. (2001) | 5+0+1B | Larvae | |
| *T. japonicum* | Amutha Murugan and Manickavasagam (2003) | 5 | - | - |
| *T. cacoeciae* | Vavre et al. (2004) | 5 | Embryonic cell | Egg |
| *T. embryophagum* | Farsi et al. (2020) | 5 | Pre- pupa | Brain cell |

The logic of the present study is the evaluation of cell division and cytogenetical index in different tissues during various stages of immature growth to reach the optimal tissue and stage. Over that, it should be noted that these types of studies, in addition to the theoretical value, can provide basic information for future investigations in other sciences, including higher quality karyotypic studies. Thereupon, the karyomorphological characteristics of *T. brassicae* were researched again.

## 2 Materials and Methods

### 2.1 Insect

The samples in our study were collected from Iran (Golestan Region, 36.8223° N, 54.4255° E) in 2018. The specimen was sent to Dr. Kahn (Forest Research Institute Dehradun, India) for confirmation of identity. What results from mating a single female with a male which is originated from the same field-collected host is an isofemale line. The wasps were reared on daily laboratory host eggs *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). The culture was conducted in a growth chamber (25±1°C, 70±10% RH, and 16: 8 L: D photoperiod).

### 2.2 Examination of cell division in different tissues and stages

A single wasp was placed in a glass vial (10×2.5 cm) with a strip of paper containing 25 host-eggs using non-toxic and water-soluble glue and allowed to parasitize all of the eggs. The parasitized egg transmitted at the climate-controlled chamber (25±1°C, 70±5% RH, 16L: 8D photoperiod) until the considering time. According to Farsi et al. (2020), the duration of each developmental stage of *T. brassicae* has been known; larval stage (more than two days), prepupa stage (2.5 days), and pupa stage (3.5-4 days). Abdominal and brain tissues of each developmental stage were examined to specify both optimal stages and determining the tissue which has a large number of cell division and cytogenetic index. For evaluation of stages; the different stages of wasp was dissected in Saline solution (NaCl 0.9%+KCl 0.042%+$CaCl_2$ 0.025% in distilled water). Tissues were stained in 2% aceto-orcein for 30-45 minutes, and then squashed on the cover glass.

50 slides were prepared from the developmental stages; those had the highest cell division. In each slide randomly detected and measured 5 optical fields 40×lenses light microscope. In each field of view, the total number of cells, the mitotic index (number of mitotic cells / total number of cells ×100), and metaphase index (number of cells in metaphase stage/total number of cells×100) were calculated. The data were analyzed using Excel 2013 (Microsoft Excel [computer software]).

For the investigation of cell division status and cytogenetical index in different tissues, brain cell from 50 larvae, prepupa, and pupa stages (50 slides per each stage, 50×3=150), in additional of 150 slides of abdominal were evaluated cytogenetically.

### 2.3 Chromosome analysis

Karyotype studies were carried out on the brain cell. Preparations of metaphase chromosomes were following as described by Farsi et al. (2020). Dissect out of sample from egg host in physiological solution (for Ephestia According to Glaser, 1917). Transfer samples into a drop of 0.075 M KCl for 10 minutes. After wise, the samples were excised and pretreated in solution mixture of 8-hydroxyquinoline (0.002 w.v): colchicine (0.05 w.v) at about 4°C for 30 minutes, and were washed in hypotonic solution (NaCl 0.9%+KCl 0.042%+$CaCl_2$ 0.025% in distilled water) for 3 times and followed by fixation in Carnoy`ssolution (v/v) (6:3:1 - ethanol: chloroform: acetic acid) for 20-30 minutes. Transfer the samples on a clean slide into a drop of 60% acetic acid using a tungsten needle and cut their head off from another part of the body with tungsten needles; Put the slides on a heating plate at 45°C until the acetic acid almost evaporates. Pass the slides through an ethanol series to remove water and let the samples air-dried. The slides were stained immediately with 5% Giemsa in phosphate buffer (pH 6.8).

Chromosome complements were taken using HD-Ultra camera VC 3036 attached to the Zeiss Axiophot microscope. Measurement of characteristics was performed on a 10 metaphase plate using MicroMeasure 3.3 software (Reeves, 2001). We calculated Arm rations, relative lengths (%) which was obtained by the ratio of chromosome length to the total length of a haploid set of chromosomes in percent. Also, the centromere index CI=S/S+l (in percent) was assessed that used for the chromosomal type. The formula S/AI=(1×M)+(2×SM)+(3×A)+(4×T)/2n (Eroğlu, 2015) was used for karyotype asymmetry/ symmetry. DRAWID software was used to draw ideogram.

## 3 Results

Cell division of *T. brassicae* is various in each stage of development from embryo to adult. The stage and tissue with the highest mitotic index (MI), which shows the intensity of cell to divide, is the main condition for achieving an ideal karyotype.

### 3.1 Brain and abdomen cell division in the larval stage

At the beginning of the larval stage, separation of the head section from the other sections of the body was not possible due to the lack of specific sections in the body. At this stage, the interphase nuclei were very much and after that, prophase had the highest number, and dividing cells (prometaphase and metaphase stage) were rarely seen (Fig. 1). In our study, we considered the larvae's primary body section equivalent to the head. Accordingly, during the larval stage of the wasp up to the end of the stage, we found the rate of these cells in the head is more than in another part of the body.

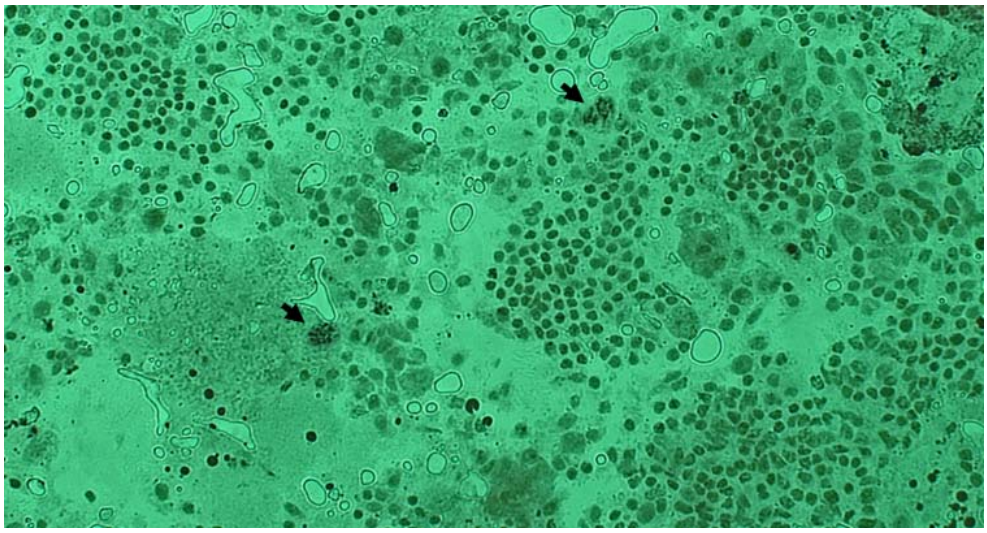

**Fig. 1** Brain cells in the larval stage of *T. brassicae* (Aceto-orcein staining. Magnification 40×).

Again, at the late hours of the larval stage, the number of interphase cells increase sharply in both head and abdominal part of the body and the number of cell in other steps of cell cycle becomes down. The larval stage showed the lowest number of cells in the Metaphase stage (Fig. 2, Table 2).

**Table 2** Data, the mean and standard deviation of the brain and abdomen cells on different stages of *T. brassicae*.

| Stages | Total number of cells | Mitotic index (%) | Metaphase index (%) |
|---|---|---|---|
| Larvae (Brain) | 79408 | 0.23±1.18 | 14.28±0.33 |
| Larvae (Abdomen) | 79982 | 0.07±1.14 | 3.62±6.82 |
| Pre- pupa (Brain) | 80868 | 2.52±5.64 | 58.03±3.81 |
| Pre- pupa (Abdomen) | 71435 | 0.21±1.14 | 5.11±5.47 |
| Pupa (Brain) | 78977 | 2.22±5.10 | 49.74±2.95 |
| Pupa (Abdomen) | 76514 | 0.18±1.57 | 6.06±5.29 |

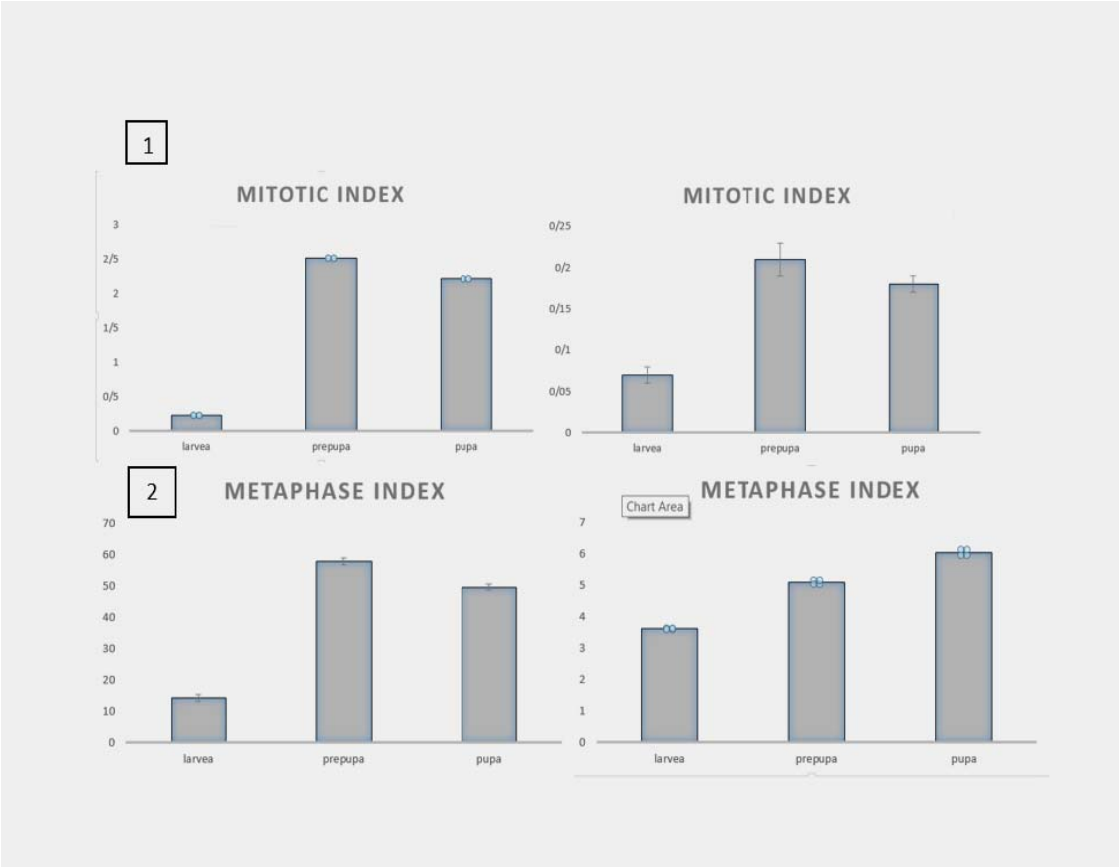


**Fig. 2** Changes in metaphase and mitotic index in a different stage of immature growth of *T. brassicae*. A) abdomen B) brain.

### 3.2 Brain and abdomen cell division in the prepupa stage

At the prepupa stage, the number of cell divisions was increased, and this stage is one of the best times for the preparation of karyotype due to the highest number of cell divisions. Over time, cell differentiation increases, this increases MI (%). Investigating showed that in head tissue, the amount of cell division is higher than the other sections of the body. The stained preparations released that the mitotic index is the highest in this stage (Fig. 2, Table 2). A comparison of brain and abdomen cells showed that the quality of the chromosome acquires from brain cells is higher. Because of the formation of a urate disk in the abdomen, this part of the body was not appropriate for chromosome studies.

### 3.3 Brain and abdomen cell division in the pupa stage

In the pupae stage, the metaphase plate is still visible in the head section but their number decline as pupae age increase and approaching the emergence of adult (Fig. 2). The quality of the chromosome preparations obtained from the abdomen and brain is not the same, the quality of stained preparation from the abdominal is not enough suitable for karyotype, location of centromere and length of arms is not clear. The total number of cells counted at each stage, the sum of cells present in the microscopic field of view of that stage, equally decreases with increasing insect age (Table 2). The downside trend of indicators at the pupa stage is shown in Fig. 2, which reveals although both MI and metaphase index are still higher than the larval stage has a decrease in comparison with the previous stage.

### 3.4 Karyotype

We confirm that the number of chromosomes was 2n= 10. As regards the centromere position and dimensions of all chromosomes, we arranged chromosome complement in 5 pairs, according to the terminology of Levan et al. (1964). The karyotype showed 2 pairs of submetacentric (1, 2 pairs, centromere was located submedian position), one pair of somewhat metacentric (3 pairs, centromere was located in median position), and two smaller pairs of acrocentric (4, 5 pairs) chromosomes (Table 3, Fig. 3). The ideogram was drawn using chromosomal indicators (Fig. 4). The karyotype formula is n=2sm+1m+2a.

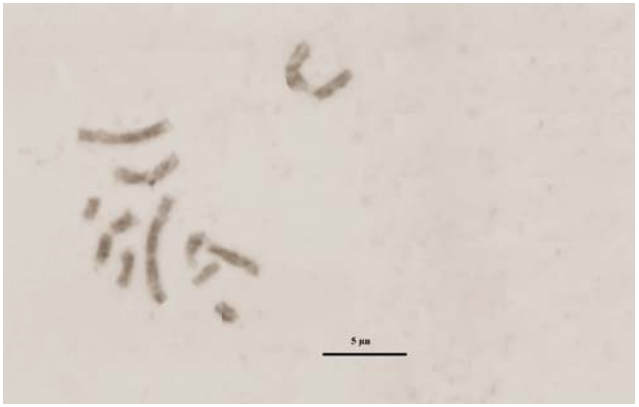

**Fig. 3** Mitotic metaphase plate of *T. brassicae*. Bar= 5µm.

**Table 3** Chromosome details of *T. brassicae*.

| Pair | L (µm) | S (µm) | L + S (µm) | RL (%) | L/S | CI (%) | Type |
|---|---|---|---|---|---|---|---|
| 1 | 4.476995 | 2.342442 | 6.819437 | 24.68837 | 1.958353 | 34.21467 | Sm |
| 2 | 4.045035 | 1.959144 | 6.004179 | 20.88075 | 2.076639 | 32.84084 | Sm |
| 3 | 2.59157 | 2.526231 | 5.117801 | 20.52975 | 1.022607 | 49.45021 | M |
| 4 | 1.043051 | 0.457777 | 1.500828 | 5.118819 | 4.762001 | 28.23427 | A |
| 5 | 0.840964 | 0.221776 | 1.062739 | 3.810254 | 4.248135 | 21.67529 | A |

Abbreviations; long arm length (L), short arm length (S), total chromosome length (L + S), relative length (RL), arm ratio (L/S), centromeric index (CI), metacentric (M), acrocentric (A), submetacentric (Sm)

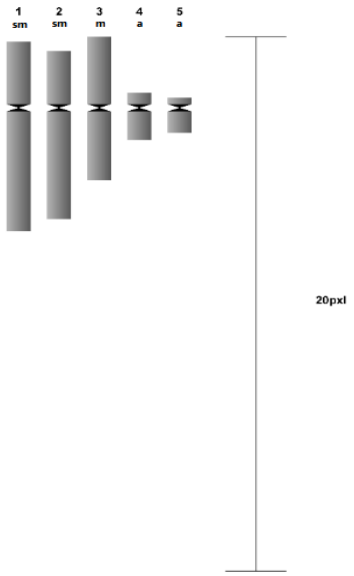


**Fig. 4** Haploid ideogram of *Trichogramma brassicae*.

## 4 Discussion

Two main features emerge from these results: First, different stages and tissues had distinct cell division, MI and metaphase index; Second, the present study clearly showed that the brain cell of the prepupa stage is an efficient tissue and stage for preparation karyotype of *T. brassicae*. Using the cell of brain tissue provides several advantages that make the tissue ideal for karyotype studies not only in *T. brassicae*, but also in whole different Trichogramma species. The advantages are as follows:

1. The brain has more dividing cells than other tissues.

2. Characteristics of the chromosomes, including the length of the arms, location of the centromere were clearer in the metaphase plate of the pre- pupa's brain cells compared to the other.

3. Accessing brain tissue cells, especially in small-sized species, including Trichogramma, is easier than many other tissues, such as embryo cells.

4. The cells of brain tissue are available during a larger time. Chromosome preparation of gonads and embryo cells for example are limited to a special time.

Unfortunately, information from the development of the Trichogramma brain is not available, and our knowledge about this is limited to some insects such as Drosophila as a model insect. Formation of the Drosophila brain occurs in three developmental phases including embryonic, larval, and pupation: in the embryonic stage, populations of neuroblasts produce a lineage of cells, and then go to mitotic dormancy that lasts until the end of the first larval instar. After this neuroblast is reactivated again, they produce a stereotyped set of secondary lineage. During the pupal stage, the neurons become mature and form the brain (Hartenstein et al., 2008). Gatenby (1917) stated that at the Trichogramma larval stage, the body and head are sak-like and interconnected. The larvae did not need a developed nervous system, nerves and ganglia were absent but we could observe a nerve cord (Flanders, 1930). Britton and Edgar (1998) stated that the larval stages of Drosophila are specialized to grow and eating. Growth was described through an increase in cell size at this stage. Specific tissues of larvae continue the endoreplication cycle included successive rounds of DNA

synthesis without intervening mitoses and led to an increase in DNA ploidy, this request for growth (Royzman et al., 1997). This cycle includes the larval tissues gut, salivary gland, fat body, trachea, malpighian tubules, and epidermis (Britton and Edgar, 1998).

According to Britton and Edgar (1998), while the increase in DNA ploidy and cell size taking place in some cells, some others are setting aside for generation adult-specific tissues including germ cells, abdominal gistoblast nest, imaginal disk, and larval neuroblast. Cevallos and Nation (2004) expressed that sigmoid not linear growth of brain happening *Anastrepha suspensa* (Diptera: Tephritida) from hatching to prepupa. Our results showed based on changes in MI and metaphase index, the brain growth of *T. brassicae* is sigmoid too.

A brain cell is used to preparation of karyotype some Trichogramma including *T. chilonis*, *T. evanescens*, *T. nubilale*, and *T. pretiosumin* many of the previous studies, mainly in the prepupa and pupa stage (Hung, 1982). Gokhman et al. (2017) also used cerebral ganglia of prepupa for karyotype study in *T. pretiosum*.

Due to the formation of urate cells in the abdomen at being prepupa stage, this section is full of it (Flanders, 1937; Farsi et al., 2020). Cells in this section, from the 6th day, were mainly at the interphase, and as days pass the number of them increase, while the cell division was hardly visible.

Laurent et al., (1998) previously studied the karyotype of *T. brassicae* and counted n=5. The haploid chromosome number (n=5) is reported for other species of Trichogramma (Wei and Pinging, 1988; Hung, 1982; Gokhman et al., 2017). Laurent et al. (1998) did not explain chromosome types and only described the size of chromosomes in the range of 4.8-2.4 µm. our metaphase images showed a different size of chromosomes before paper, of course, should be considered that the length of the chromosome is dependent on the stage of the cell, level of chromatin condensation, and technique used for making the preparations. Gokhman et al. (2017) reported a different metaphase plate from Hung (1982) in the *T. pretiosum*. The difference in the chromosome structure of members of Trichogramma can be observed.

The total length of chromosomes ranged from 6.81 to 1.06 µm which are longer than those reported by Laurent et al. (1998). The karyotype of *T. brassicae* is almost symmetrical. According to Eroğlu (2015) karyotype can be classified from full symmetric to full asymmetric. Our result showed that the karyotype of *T. brassicae* is symmetrical and asymmetrical (S/AI value=2.2). Farsi et al. (2020) calculated this parameter for the first time in the *T. embryophagum*. They stated that karyotype is between symmetric and asymmetric type. Karyotype dissymmetrisation is one of the trends that have occurred in the evolution of karyotype in parasitoids (Gokhman, 2009). The existence of acrocentric chromosomes in one chromosome set is related to dissymmetrisation (Gokhman, 2006). The presence of one or two acrocentric chromosomes has been observed almost as a general condition in all examined Trichogramma species until now; *T. pretiosum*, *T. embryophagum*, *T. chilonis*, *T. evanescens*, *T. nubilale* (Hung, 1982; Gokhman et al., 2017; Farsi et al., 2020).

In this study, it was shown that different tissues and stages have a clear variation in a cytogenetical index. By entering the prepupa stage, MI and metaphase index had a significant increase. Following these results can understand the brain cells of prepupa are the best for the preparation of karyotype but the pupa stage is also usable.

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