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Karyotype analysis of *Trichogramma embryophagum* Htg. (Hymenoptera: Trichogrammatidae) using a new method and estimate its karyotype symmetry

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Abstract. Different methods of chromosome preparation for insect are now being used across the world. Well-spread chromosomes with explicit morphology, in addition to no cell wall debris are required for karyotype investigations. Cytogenetic knowledge in *Trichogramma* is extremely limited. In the article, chromosome characters, karyotype and monoploid ideogram of *Trichogramma embryophagum* Htg. (Hymenoptera: Trichogrammatidae) were evaluated using a new method. Also, karyotype symmetry/asymmetry of this species was calculated for the first time as one of the *Trichogramma* species. Our results showed that the diploid chromosome number of the wasp was $2n = 10$. The karyotype formula was 6m + 4a. The symmetry/asymmetry index value was 1.8. The new method resulted in higher quality metaphase plates spread and provided an ideal karyomorphology for this parasitoid which has small chromosomes.

Keywords: ideogram, karyotype, 8-hydroxyquinoline, symmetric karyotype.

INTRODUCTION

A high-quality chromosome preparation is critical for cytogenetic studies of Hymenoptera parasitoids. In the majority of parasitoid species, complications are caused by the minute size of the insect (Baldanza *et al.* 1993), the small size of their chromosomes (Paladino *et al.* 2013), and low number of chromosomes, $2n = 6$ found in *Brachymeria intermedia* (Chalcididae) and *Encarsia protransvena* (Aphelinidae) (Hung 1986; Baldanza *et al.* 1999) which make their cytogenetic studies limited. Although modern molecular techniques such as Fluorescent *in situ* Hybridization (FISH) have already taken been on an important role (Yalcin and Kulduk 2018), they have never been able to be a complement replacement for classical methods. Some disadvantages including being costly and difficult (due to requiring probes and fluo-

rescent microscope), requiring highly-skilled cytogeneticists, and the inability to reveal chromosome inversions (Di- Nizo *et al.* 2017) have caused cytogenetics to show a significant tendency towards using classical methods.

Squash is a well-known and oldest method for flattening metaphase plate in insects (Kocak and Okutaner 2017). However, difficulty in achieving well-spread, distorted, and stretched chromosome are the main disadvantages of this method (Chirino *et al.* 2014; Kocak and Okutaner 2017). During squashing, producing well-spread plates are often not desirable due to the concentration of cells in a small area. Over time, spread (hot-plate spread) method conducted by various researchers flourished (Bressa *et al.* 2009; Sadilek *et al.* 2016). Its major disadvantage is the attendance of cellular debris around the chromosomes (Chirino *et al.* 2014), making their morphology unpleasant. Considering the mentioned constraints, there is always a need for a simple and efficient method for the preparation of metaphase plates in parasitoids.

Despite the vast amount of research on insect karyotype, only about 500 species of parasitoid wasps have available data (Gokhman 2009). Among these, only more than 10 are related to *Trichogramma*. So far, 239 species of *Trichogramma* have been reported throughout the world (Khan and Yousuf 2017). There are eleven species of this parasitoid in Iran (Nazeri *et al.* 2015) that *Trichogramma embryophagum* Htg. is one of them. *T. embryophagum* distributed in some of regions of Iran, including the provinces of West and East Azerbaijan, Yazd, Khorasan (Poorjavad *et al.* 2011). Cytogenetic studies and karyotype investigation have not yet been done on this species. Because of the substantial position that *Trichogramma* occupy in the biological control programs (Parra 2009), investigations on their chromosome details have provided precious information for the understanding various perspectives such as mechanisms of sex determination, evaluation of sex chromosome, existence of accessory chromosomes and phylogenetic relationships.

An evaluation of existing literature shows that different methods was used in karyotype studies of *Trichogramma*. Studies about *Trichogramma* were derived from routine chromosomal preparation methods, including the use of various percentages of colchicine as pretreatment, staining by different colors, and then squashed (Hung 1982; Liu and Xiong 1998). Interestingly, no banding staining on the *Trichogramma* species has ever been reported. Molecular cytogenetic including Florescence *in situ* hybridization (FISH) has been applied for only the two species of *T. kaykai* Pinto & Stouthamer (van Vugt *et al.* 2005) and *T. pretiosum* Riley (Gokhman *et al.* 2017). It should be noted that the problems described for each of these methods also exist for *Trichogramma* karyotype studies. The minute size and lifestyle of *Trichogramma* species also intensify these problems (Manickavasagam 1991; Hung 1982). In most studies, only the haploid number of these species has been raised, and the chromosomal details including the length of the arms that could provide many comparisons were not possible (Hung 1982; Laurent *et al.* 1998). One parameter that can be obtained from the chromosomal information is karyotype symmetry/asymmetry (Peruzzi and Eroğlu 2013). Karyotype symmetry/asymmetry is obtained based on the availability of chromosome detail, including relative chromosome size and position of centromere. Until now, this parameter which can help in the evaluation of enter- species relationship seldom has calculated in class of insect. In the cytogenetic studies, species that are more similar in the terms of chromosomal parameters such as karyotype symmetry/asymmetry will have more affinity.

In this research, the karyotype, ideogram, and chromosomal detail of *T. embryophagum* were investigated for the first time with the introduction of a new method considering all steps of Hymenoptera parasitoids chromosome preparation. These chromosome analyses were complemented by an estimation of its karyotype symmetry as one of the species of Trichogrammatidae.

MATERIAL AND METHOD

Insect

T. embryophagum Htg. individuals originated from parasitized eggs of carob moth, *Ectomyelois ceratoniae*, (Zeller) (Lepidoptera: Pyralidae), were collected on pomegranate in the center of Iran (Yazd Region, 32.1006° N, 54.4342° E). The wasps were reared on *Ephestia kuehniella* Zeller (Lepidoptera; Pyralidae) eggs in a climate-controlled chamber at 25 ± 1 °C, 70 ± 5% relative humidity, L16: D8 photoperiod. *E. kuehniella* eggs were obtained from a culture maintained in the Biosystematics Laboratory at The University of Tehran, Karaj, Iran.

Preparation chromosome

Sample from egg host were Dissected out in physiological solution for *Ephestia* (Glaser, 1917, cited by Lockwood 1961). Two kinds of pre-treatment were used, including hypotonic and other is combined it. First, samples were transferred into a drop of 0.075 M KCl for 8 minutes [0.075 M KCl: 0.5592 g KCl/100 ml redistill.

H_2O] on a shaker at 20 °C and 30 rpm. After wise, the samples were excised and pretreated in solution mixture of 8-hydroxiquinoline (0.002 w.v): colchicine (0.05 w.v) contains low concentration of dimethyl sulfoxide (DMSO) at about 4° C for 30 minutes on shaker 30 rpm; and were washed in hypotonic solution (NaCl 0.9% + KCl 0.042% + $CaCl_2$ 0.025% in distilled water) for 3 times. Then they were Fix in freshly prepared Carnoy`s fixative (6:3:1 - ethanol: chloroform: acetic acid) for 20-30 minutes. They were Transferred on a clean slide into a drop (5-10µl) of 60% acetic acid and their head was cut off from other parts of the body with tungsten needles, after a while, 5-10µl of 60 % acetic acid was added again. The slides were put on a heating plate at 45 °C until the acetic acid almost evaporates. Water was removed bypassing the slides through an ethanol series (70 %, 80 %, 96 %; for 30 seconds each of it, respectively) and the samples were let air-dried. The slides were stained immediately with 5 % Giemsa in phosphate buffer (pH 6.8).

Microscopic photograph and analysis

The chromosome slides were examined under Olympus BX53 microscope and chromosomes were photographed using an Olympus DP72 camera. Chromosome measurements were made on 10 metaphase plates by application of KaryoType software (Altınordu *et al.* 2016). Arm rations, average lengths, and centromeric index were calculated and then were classified according to Levan *et al.* (1964) considering their centromere position.

The karyotype symmetry/asymmetry was calculated. The formula for symmetry/asymmetry index is given below.

$S/A_I = (1 \times M) + (2 \times SM) + (3 \times A) + (4 \times T) / 2n$ (Eroğlu 2015).

Eroğlu (2015) reported the new classification model from full symmetric to full asymmetric with five different types. The chromosomes, are all metacentric, form full symmetric karyotype; unlike those, are all telocentric, form full asymmetric karyotype.

RESULT

The karyotype of *T. embryophagum* Htg. contained three pairs of large metacentric and two considerably smaller pairs of acrocentric chromosomes. Two of the metacentric chromosomes were of similar size whereas the third pair was close to submetacentric. Thus, the diploid chromosome number was $2n = 10$, and the karyotype formula was 6m + 4a (Figure 1a, b). The detailed chromosomal data are given in Table 1, and monoploid ideogram is given in Figure 2.

In *T. embryophagum*, haploid chromosome length and mean chromosome length are 6.38 and 1.28 µm. The rates of relative length and centromeric index range from 9.40 to 28.06 and from 18.33 to 48.04, respectively. The symmetry/asymmetry index value is 1.8.

DISCUSSION

In this research, the main issues were to have a regular cell layer without overlapping and the absence of qualitative damages to the morphology of chromosomes while allowing the possible spread of chromo-

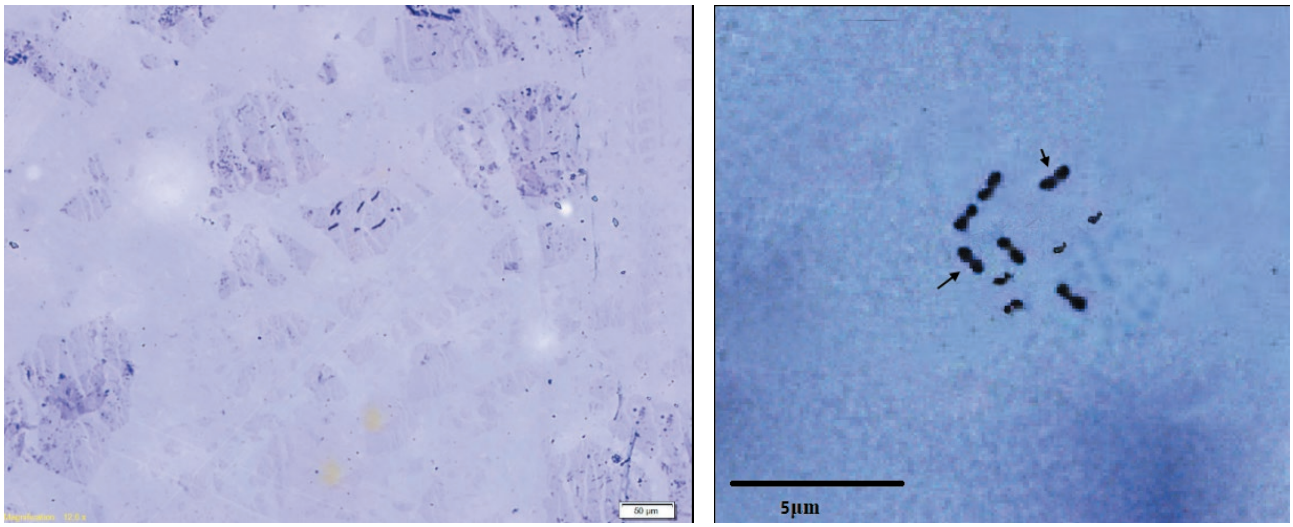

Figure 1. a, b) Mitotic metaphase plate of *T. embryophagum*. Giemsa staining. Magnification 100x.

Table 1. The detailed measurement data of chromosome pairs of *Trichogramma embryophagum*.

Pair	L (µm)	S (µm)	L + S (µm)	RL (%)	L / S	CI (%)	Type
1	0.93	0.86	1.79	28.06	1.08	48.04	m
2	0.91	0.73	1.64	25.71	1.25	44.51	m
3	1.02	0.61	1.63	25.55	1.67	37.42	m
4	0.58	0.14	0.72	11.29	4.14	19.44	a
5	0.49	0.11	0.60	9.40	4.45	18.33	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).

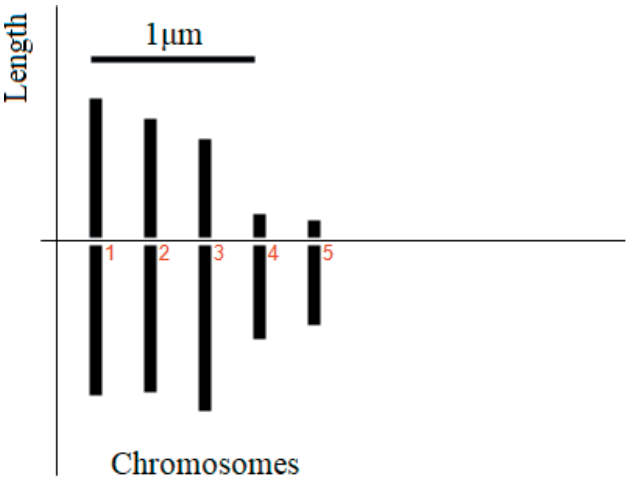

Figure 2. Ideogram of *T. embryophagum*. Chromosomes are numbered from the longest (1) to shortest (5).

somes within the cell. The method explained here has aimed at achieving both goals. The chromosome structure had a high resolution, which is related to how the chromosomes was prepared. The process of karyotyping is completed through some steps such as pretreatment, fixation and staining. On both sides of the cell, outside and inside, the concentration of water is the same, and Potassium and sodium ions are the most osmotically solutions inside and outside of the cell, respectively (Leaf 1973). In this case, the cell is in an isotonic state. The KCl changed the osmosis of the cell. Pre-treatment by KCl affects both cellular swelling and better chromosomes spread. About KCl, hypotonic rate and duration of treatment are important. Rupturing the cell membrane and the damages of chromosome (Earley 1975) can be caused by the lack of attention to these cases. Our result indicated that the hypotonic rate and duration of KCl reported here overcome protoplast damage. Sadilek *et al.* (2016) stated that KCl causes the osmosis of the cell due to receiving additional water that resulted in a larger cell. This process effects more identified of the chromosome. The next step after KCl was using combined pretreatment. Colchicine is a substance that affects the metaphase stage of the dividing cell. Guo *et al.* (2018) stated that colchicine increased the yield of the metaphase plate. The 8-hydroxyquinoline is effective in prolonging the metaphase and further compression of the chromosomes. It is noteworthy that, until the present time, this substance has not been used as pre-treatment in insect karyotype studies but has been used extensively in plant karyotype. This substance can maintain the shape of the chromosome during the preparation process. This feature has been used in some plant studies (Fernandez *et al.* 2009; Zarifi and Güloğlu 2016). We found that 8-hydroxyquinoline prevented the distortion and stretch of the chromosome, which are the main drawbacks of squash and spreading methods. Use of colchicine alone showed each chromosome clumped exceedingly and their centromeres were not distinctly in our pre-tests while using 8-hydroxyquinoline alone, the metaphase plates were few. As a result, a combination of two substances as pretreatment improved chromosome spreading. Ma *et al.* (1996) reported that the effects of these materials together make this as improvement whereas colchicine depolymerized microtubules, 8-hydroxyquinoline decreased the rate of progression among mitotic stages and also resulted in a disorderliness in chromosome movement. In combined pretreatment, dimethyl sulfoxide (DMSO) affected the better penetration of compounds of pretreatment into the cell. DMSO has been reported as an effective penetration enhancer (Gurtovenko and Anwar 2007; Williams and Barry 2012). Notman *et al.* (2006) stated that DMSO induces pores in the membrane. Another study indicated that DMSO concentration is critical (Gurtovenko and Anwar 2007). We used a low concentration of DMSO in our method. In this concentration, DMSO induces membrane thinning (Gurtovenko and Anwar 2007).The approach used in the present study focused on providing a quick and easy method for those insects which have small chromosomes, especially *Trichogramma* wasps. The advantages of the new method are as follows:

1. In the present method, the chromosomes are readily accessible through morphological features, including the centromere position. According to this, the evaluation of all of the parameters such as karyotype symmetry/asymmetry can be available.

2. Higher quality metaphase plates spread which is achieved through the use of a combined pre-treatment which its results are an ideal karyomorphology.

3. Providing a single layer of cell and decreasing the overlapping cells, therefore, a better selection of metaphase plates is possible.

The Trichogrammatidae species were less likely to be karyotypically studies in comparison with other parasitoid families. The karyotype *T. embryophagum* Htg. consists of 10 chromosomes (n= 5, $2n$ = 10). Members of this family showed 10 chromosomes previously published (Hung 1982; Van Vugt *et al.* 2003; Gokhman *et al.* 2017), although there is an exception, *T. kaykai*, which has been diagnosed with one B chromosome that is termed *psr* (from Paternal Sex Ratio) (Stouthamer *et al.* 2001). The metaphase images indicated that chromosomes are metacentric (first, second and third chromosome pairs) and acrocentric (fourth and fifth chromosome pairs). According to these chromosome types, the S/A_I value is 1.8, and the karyotype is symmetric type in *T. embryophagum*. Karyotype symmetry/asymmetry is one of the cheapest and most popular parameters that can be obtained from cytogenetic studies (Peruzzi and Eroğlu 2013). The symmetric karyotype is characterized by an excess of metacentric, submetacentric chromosomes. In *T. embryophagum* due to two pairs of acrocentric chromosomes, S/A_I value is close to 2.0 (between symmetric and asymmetric). Gokhman (2009) expressed that two trends have occurred in evolution of karyotype in parasite Hymenoptera. First one is decreasing in chromosome number, and the other one is karyotype dissymmetrisation, which happened as a result of the proportion acrocentric in a chromosome set. Despite the widespread use of various methods that are adopted to calculate the symmetry/asymmetry karyotype in plants, this parameter has not been taken into consideration in other organisms (Eroğlu 2015). So far, karyotype symmetry/asymmetry has been seldom calculated in the class of insects and other species of parasitoids. Our result showed for the first time that in one of the species of *Trichogramma* wasps, the karyotype is symmetric type according to a new method. Karyotype symmetry/asymmetry is applied on one hand to determination evolutionary relationship, and on the other hand, to compare different levels of the taxonomy (Eroğlu 2015). In the case of Trichogrammatidae, information obtained due to the lack of other information's in this case is not possible to assess the issue mentioned above in this time but our results can be used as a basis for future studies in the above fields.

In conclusion, the described method is cost-effective and technically easier to make for the resolution of chromosome details. The morphological characteristics of each chromosome are better observed. Also, no special equipment is required as compared to molecular methods. According to the described method preparation of chromosome detail, karyotype and also ideogram of *T. embryophagum* were provided for the first time. We adduce as other species of *Trichogramma* are also evaluated in which the diploid number is $2n$ = 10. The karyotype is a symmetric type.

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