

Identification of Diploid and Triploid Red Tilapia by Using Erythrocyte Indices

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Abstract — The individuals of diploid and triploid red tilapia *Oreochromis mossambicus* (Peters, 1852) X *Oreochromis niloticus* (Linnaeus, 1758) were identified by using erythrocyte indices. The major cell and minor cell axis of the erythrocyte were 27.7 and 11.5%, respectively higher in triploids as compared to diploid fishes. The increase in nucleus size of the erythrocytes of triploid was also greater by 31.7% for the major axis and 17.2% for the minor axis with respect to the erythrocytes of diploid fishes. Similarly the increase in nucleus cell surface and volume of the nucleus were also higher by 50.4 and 68.5%, respectively in the triploid fishes as compared to the diploids fishes. Moreover the erythrocyte cell surface area and cell volume of triploids were higher by 42% and 59% respectively to diploid ones. The cytoplasmic volume of erythrocyte cell was also increased by 57.9% in the triploids fishes with respect to diploid individuals. The present study clearly showed that the nuclear volume, cytoplasmic volume and nucleus surface area of the erythrocytes are significantly greater ($p < 0.005$) in the triploids as compared to the diploid fishes. These parameters thus can successfully be used in discriminating diploid and triploid red tilapia.

Key words: Chromosome; Erythrocyte; Red tilapia; Sterility; Triploidy induction.

INTRODUCTION

In recent years, the hybrid red tilapia has been opted as one of the most popular candidate species for modern aquaculture practices (WATANABE *et al.* 2002). Precocious maturation and prolific breeding activity of tilapia have often lead to the overpopulation and stunted growth, which has resulted in several serious constraints for their successful commercial production (VARADARAJ and PANDIAN 1988; HUSSAIN

et al. 1991). The production of sterile fishes following the technique of triploidy induction in tilapia is suggested as one of the best options for avoiding overcrowding during their commercial farming (PENMAN *et al.* 1987; MAIR and LITTLE 1991; MAIR 1993). The problem associated with the stunting growth in farmed tilapia can also be managed effectively by the technique of triploidization (BRAMICK *et al.* 1995). Several studies have demonstrated that the erythrocyte cells and nucleus dimensions in triploid as compared to diploid fishes varied significantly and as such these parameters can effectively be used for ploidy evaluation (KOEDPRANG and NA-NAKORN 2000; UZUNOVA 2002; WOZNICKI and KUZMINSKI 2002; JOHNSON *et al.* 2004; FOPP-BAYAT *et al.* 2006; ATKINS and BENFEY 2008). The main purpose of this study was to determine the accuracy

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of ploidy evaluation using various erythrocyte indices by directly comparing it with the karyotyping technique.

MATERIALS AND METHODS

The brooders of red tilapia weighing 200-250g were collected from a cage floated at the Lake Tasik Kenyir in Malaysia. The brooders were maintained at the Institute of Tropical Aquaculture in 5000 l rectangular cement tanks provided with adequate aeration. The fishes were given pellet feed (ASEAN Marine Fish Feed, Ltd with 43% proteins) twice in a day at a rate of 5% of their body weight. Healthy and fully matured brooders were given a dose of HCG (Pregnyl-1500) at 1500 IAU/ Kg of the body weight just below the dorsal fin. The fishes were then transferred to a glass aquarium tank (capacity: 120 liter). In the aquarium tank, a single male and a female were kept separated by a transparent Perspex glass sheet. The temperature of the aquarium tank was maintained at $28 \pm 1^\circ\text{C}$ by a digital heater (Model-D-38300, 300 W-Italy). Behavior of the fish was monitored continuously in the aquarium tank. When the female fish started showing active pre-spawning symptom such as nibbling and cleaning activity at the bottom of tank with fully protuberated urino-genital papilla, the male from same tank was taken out for spermatozoa collection. The male fish was blotted immediately with a tissue paper and its abdominal part was softly squeezed to collect spermatozoa from the genital papilla and was collected inside fine capillary tubes. Simultaneously, the female fish was also taken out from the tank and stripped softly for egg collection after wiping its body properly. The stripped eggs were collected in a cleaned spatula and transferred into a 100 ml properly dried disposable Petri dish. The dried sperm (0.6-1 ml) was spread over by gently blowing the capillary tubes followed by addition of 25 ml fresh water ($28 \pm 1^\circ\text{C}$) to initiate the activation of spermatozoa. The Petri dish was then shaken gently for proper mixing of the spermatozoa with eggs and after 2 minutes the fertilized eggs were washed with freshwater to remove the dead spermatozoa. Induction of triploidy was achieved when eggs were given a heat shock treatment (41°C , for 5 minutes duration at 4 minutes after fertilization) (PRADEEP *et al.* 2010). The eggs without heat shock treatment were treated as a control or diploid group. The fertilized eggs produced for control and triploid

groups were kept separately in an artificial recirculating type incubator for further development (PRADEEP *et al.* 2011a). After hatching, a total number of 60 larvae (30 from control group and 30 from triploid group) were reared separately under identical environmental conditions in aquarium tanks (capacity: 120 liter). Larvae of both the groups were given freshly hatched *Artemia nauplaii* (10-15 individual/ml), four times in a day for one week immediately after absorption of the yolk. This was followed by feeding the larvae up to fingerling stage with powdered pelleted feed (ASEAN Marine Fish Feed) three times in a day. Once they reached the fingerling stage, blood from 20 fingerlings of the diploid and 22 fingerlings of the triploid fishes of almost same size was collected to study variations in the erythrocyte indices. Identification of the ploidy level of each fish using well spread metaphase stages was also simultaneously conducted to compare the results. For that, each fish was injected near to the dorsal fin and just above to the lateral line, a dose of 0.01% colchicine (Sigma Ltd.) at a rate of 1 ml/100 g using a hypodermic syringe. Fishes were then kept inside an aerated aquarium (capacity: 120 l) for 4 hours.

Blood smears and cell measurement - Fishes were taken out from the aquarium tank and their body was swabbed properly using a cotton towel. The tail region was then severed, few millimeters anterior to the caudal peduncle, to cut the caudal vein by using a pair of sharp scissor. A drop of blood was then taken from the cut region and placed on a clean microscopic slide. The blood drop was gently smeared by a cover slip. Slides were allowed to dry for few seconds and then fixed in 95% methanol. The slides were then stained with Giemsa stain (10%) for 20 minutes after drying. The excess of Giemsa stain from the slides was properly cleaned by double distilled water. The slides were allowed to dry again and then mounted by Distyrene plasticizer and Xylene (DPX) and a cover slip. Length and width of the cell and nucleus were measured for 25 erythrocytes from each individual of both groups by a micrometer. As erythrocytes of tilapia were ellipsoid in shape, the cell and nucleus volumes were calculated using a formula $V = 4/3\pi AB^2$ where A and B are the major and minor semi-axis of the cell and nucleus (UZUNOVA 2002). The cytoplasmic volume was calculated by subtracting nuclear volume from the mean erythrocyte cellular volume. Surface area of the erythrocyte cells and their nucleus were calculated by a formula $S = ab\pi/4$ (DORAFSHAN *et*

al. 2008). The photographs of the erythrocytes were taken (1000X magnifications) with a Nikon research microscope (Nikon Eclipse 80i, Japan).

Chromosome preparation - After collecting the blood for erythrocyte preparation, the same fish was used for chromosome preparation to find out the ploidy level. The method of SOFY *et al.* (2008) and PRADEEP *et al.* (2011b) with some modification was followed for chromosome preparations. The fishes were killed by pithing near to the brain and the kidney was removed for chromosome preparation. The kidney was washed properly in an isotonic solution of NaCl (0.7%) to remove excess blood and debris. Excess NaCl was removed and then tissues were finely chopped by a sharp blade. The chopped tissues from the petri dishes were then transferred to small plastic vials and then homogenized for a minute. After homogenization, tissues were transferred to a bigger centrifuge tube (15 ml capacity) and the tissues were hypotonised with 8 ml KCl (0.56% kept at room temperature) that was added to each centrifuge tube. The hypotonic treatment was given for a total duration of 40 minutes and then the solution was changed twice at 15 minutes and 30 minutes intervals. The solution was then centrifuged at 100 x g for 7 minutes. Once the centrifugation was finished, the supernatant was removed carefully and tissues were immediately fixed in the same tube with 8 ml of cold Carnoy's solution (3:1) for 30 minutes. The solution was again centrifuged at 100 x g for 10 minutes after fixation. The supernatant was removed and re-fixed in Carnoy's solution for 10 minutes at 4°C. The centrifugation and re-fixation process were repeated again for the third time after one hour duration of the fixation.

After completing the fixation, the cell suspension and tissues were taken and placed on a clean

microscopic slide. This was followed by chopping the tissues by a sharp scalpel thoroughly to get a white suspension. A drop of distilled water was put onto the tissue to prevent drying and for proper dissociation during chopping. Carnoy's solution (30 µl) was put onto the chopped suspension to facilitate the proper spreading of the cells on slide. The cells were then spread by using the edge of another microscopic slide. Immediately, the slide with the spread cells was warmed under the flame using an alcohol lamp until complete evaporation of the liquid. The slide was then air dried for 10 to 15 minutes and later rinsed in acetone solution to remove the oil droplets. The slides were again air dried for 10 to 15 minutes and then stained with freshly prepared 10% Giemsa stain (prepared in 0.01 M phosphate buffer; pH 7) for 30 minutes. These slides were subsequently rinsed in distilled water, air dried and mounted with DPX after 10 minutes of xylene wash. The metaphase spreads were photographed and number of chromosomes was counted by observing slides under 400X and 1000X (oil immersion) magnifications using a Nikon research microscope (Nikon Eclipse 80i, Japan).

The data were statistically assessed by ONE WAY ANOVA ($P < 0.05$) followed by 'Tukey's test' for comparing various parameters of erythrocyte indices. All the statistical analyses were done using software, 'OriginPro 7.0'. The data were presented in arithmetic mean ($\pm$) standard deviation.

RESULTS

Among the control group, all the 20 individuals were found to be diploid whereas in the heat-shock induced group, out of 22, 20 were triploid.

TABLE 1 — Cellular and nuclear size and their combined parameters of the diploid and triploid red tilapia (mean $\pm$ SD).

Dimensions	Ploidy stage		Ratio [diploid:triploid]	Increment (%)	Significance
	Diploid (n=20)	Triploid (n=20)			
Cellular major axis (µm)	10.43 $\pm$ 0.51	13.32 $\pm$ 0.37	1:1.27	27.7	$P < 0.005$
Cellular minor axis (µm)	6.69 $\pm$ 0.36	7.46 $\pm$ 0.44	1:1.11	11.5	$P < 0.0001$
Cellular surface area (µm ²)	54.70 $\pm$ 4.05	77.70 $\pm$ 6.28	1:1.42	42.0	$P < 0.0001$
Nuclear major axis (µm)	4.47 $\pm$ 0.34	5.89 $\pm$ 0.38	1:1.31	31.7	$P < 0.0001$
Nuclear minor axis (µm)	2.50 $\pm$ 0.25	2.93 $\pm$ 0.34	1:1.17	17.2	$P < 0.0001$
Nucleus surface area (µm ²)	9.08 $\pm$ 1.45	13.66 $\pm$ 2.29	1:1.50	50.4	$P < 0.0001$

(n = number of specimens)

loid. The karyotyping analysis of diploid fishes showed the presence of 44 chromosomes (Fig. 1a). The chromosome numbers were 66 in fishes when they were induced for triploidy by giving heat-shock treatment (Fig. 1b). A significant difference ($P < 0.005$) in cellular and nuclear major axis, cellular and nuclear minor axis, cell and nuclear volume, surface area of the cell and nucleus and also the cytoplasmatic volume was observed between diploid and triploid erythrocytes ($P < 0.005$). The different measurements of erythrocyte of diploid and triploid red tilapia are summarized in Tables 1 & 2.

In diploid, the mean values of cellular major axis, cellular minor axis and cellular surface area were; $10.43 \pm 0.51 \mu\text{m}$; $6.69 \pm 0.36 \mu\text{m}$ and $54.70 \pm 4.05 \mu\text{m}^2$, respectively (Table 1). The mean values of nuclear major axis, nuclear minor axis and nuclear surface area were; $4.47 \pm 0.34 \mu\text{m}$; $2.50 \pm 0.25 \mu\text{m}$ and $9.08 \pm 1.45 \mu\text{m}^2$, respectively (Table 1). Cellular volume in diploid fishes was $245.56 \pm 29.39 \mu\text{m}^3$ whereas nuclear volume was $15.89 \pm 4.25 \mu\text{m}^3$ (Table 2). The cytoplasmatic volume in diploid was $229.68 \pm 26.95 \mu\text{m}^3$ (Table 2).

Similarly in triploid fishes, the mean values of cellular major axis, cellular minor axis and cellular surface area were; $13.32 \pm 0.37 \mu\text{m}$; $7.46 \pm 0.44 \mu\text{m}$ and $77.70 \pm 6.28 \mu\text{m}^2$, respectively (Table 1). The mean values for nuclear major axis, nuclear minor axis and nuclear surface area, were; $5.89 \pm 0.38 \mu\text{m}$; $2.93 \pm 0.34 \mu\text{m}$ and $13.66 \pm 2.29 \mu\text{m}^2$, respectively (Table 1). The cellular volume

in triploid fishes was $390.67 \pm 51.69 \mu\text{m}^3$ whereas the nuclear volume was $26.78 \pm 6.53 \mu\text{m}^3$ (Table 2). In triploids the cytoplasmatic volume was $362.81 \pm 47.60 \mu\text{m}^3$ (Table 2). The increments in cellular major axis and cellular minor axis were; 27.7 and 11.5%, respectively in the cells of triploid fishes as compared to the cells of the diploid fish ($P < 0.005$). The ellipsoidal size of erythrocytes in triploid fishes was relatively larger as compared to the diploids (Fig. 2). The increase in nuclear size in triploid was also greater by 31.7% for the major axis as compared to minor axis by 17.2%. Similarly, the increase in nuclear cell surface and nuclear volume were; 50.4 and 68.5%, respectively as compared to cellular surface area (42%) and cellular volume (59%) in triploids. The cytoplasmatic volume of the cell was increased by 57.9% in triploids as compared to diploid ($P < 0.005$) (Table 2).

DISCUSSION

In the present study the efficiency of the technique of using morphometric indices of the erythrocytes as a tool for differentiating the diploid and triploid red tilapia was confirmed by comparing it with chromosome spreads prepared from the same fish using karyotyping method. This was a novel approach compared to other studies, since in other studies only the erythrocytes mean differences of various indices were considered for differentiation between ploi-

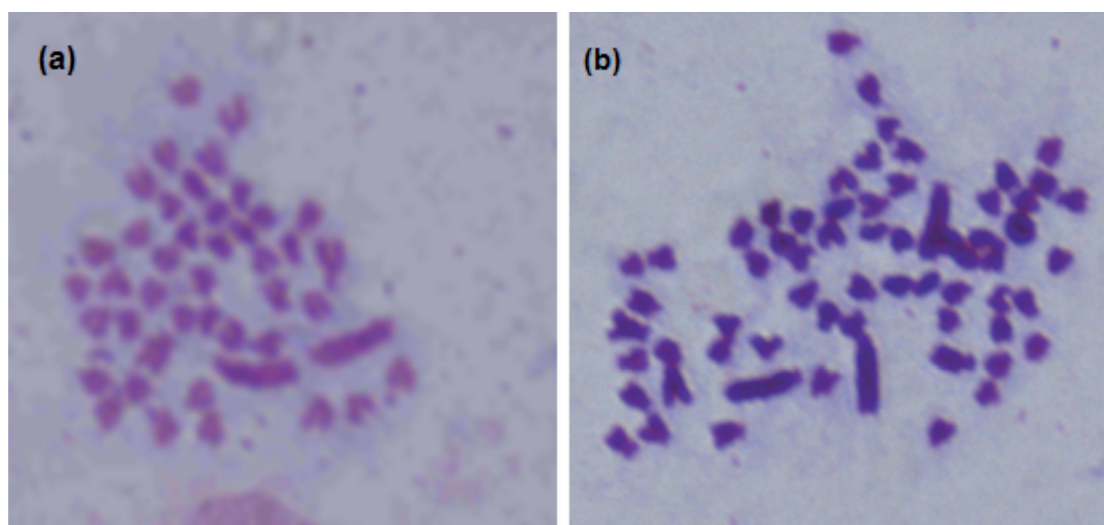


Fig. 1 — Chromosomes of diploid and triploid red tilapia (a) Diploid fish with 44 chromosomes (b) Triploid fish with 66 chromosomes.

dy levels. From the present study it has clearly been demonstrated that the parameters such as; cellular, nuclear and cytoplasmatic volumes and nuclear surface area were higher in triploid ($P < 0.0001$) as compared to the diploid fishes and as such these parameters could effectively be used for determining the diploid and triploid fishes. In Mozambique tilapia (*O. mossambicus*), VARADARAJ and PANDIAN (1990) have used RBC nuclear volume for identifying the diploid and triploid fingerlings where they found an increase in nuclear size by 1.5 times higher than that of the diploid. In other study conducted by LINCOLN and SCOTT (1983) on rainbow trout (*Oncorhynchus mykiss*), the nuclear volume of RBC was used for determining the ploidy in triploid. In Thai silver barb, *Puntius gonionotus* nuclear volume of RBC of triploid fishes was 1.63 times higher than diploid (KOEDPRANG and NA-NAKRON 2000) whereas in triploid red tilapia, it was 1.68 times higher. The nuclear volume ratio in the present study was 1:1.68 with 68.5% increase as compared to the values obtained (1:1.86) in Dojo Loach, *Misgurnus anguillicaudatus* (GAO *et al.* 2007). An increase in nuclear volume by 87% has been observed by DORAFSHAN *et al.* (2008) as compared to the theoretical expected 50% increase in the nuclear volume. The erythrocyte cellular volume of 45.9% has been reported almost reaching close to the expected theoretical 50% increase in sea bass (DORAFSHAN *et al.* 2008). However, the red tilapia showed the increment of erythrocyte cellular volume by 59% ($P < 0.0001$).

In this study, nuclear major (31.7%) and minor (17.2%) axis and cellular major axis (27.7%) were the other parameters showing relatively higher values in triploid as compared to the diploid fishes. These parameters could easily be used to differentiate the diploid and triploid fishes. PENMAN *et al.* (1987) has also recommended nuclear major axis measurements of the erythrocyte of tilapia for the identification of triploid and tetraploid fishes. RAZAK *et al.* (1999) demonstrated the ploidy status of transgenic Nile tilapia (*O. niloticus*) by using erythrocyte nuclear major and minor axis measurements. In *O. niloticus*, the measurement of erythrocyte nuclear major axis was found to be a better option for identifying the triploid and diploid individuals (HUMAYUN *et al.* 1994; HUSSAIN *et al.* 1995).

In triploid red tilapia, the cellular major axis was 1.27 times greater than the diploid whereas in triploid sea bass (*Dicentrarchus labrax*), it was 1.2–1.3 times larger (FELIP *et al.* 1999). FELIP *et al.* (1999) further reported that the cellular and nuclear major axis, nuclear and cellular volume and cellular minor axis were greatly different in diploid and triploid sea bass and hence they suggested that the most appropriate parameter to differentiate diploid and triploid is the cellular major axis and cellular volume. The cellular major axis in Caspian salmon (*Salmo trutta caspius*), has been reported to be higher by 27% than cellular minor axis as 22%, thus making the cells ellipsoidal in shape (DORAFSHAN *et al.* 2008). Similar observation was found in our study where

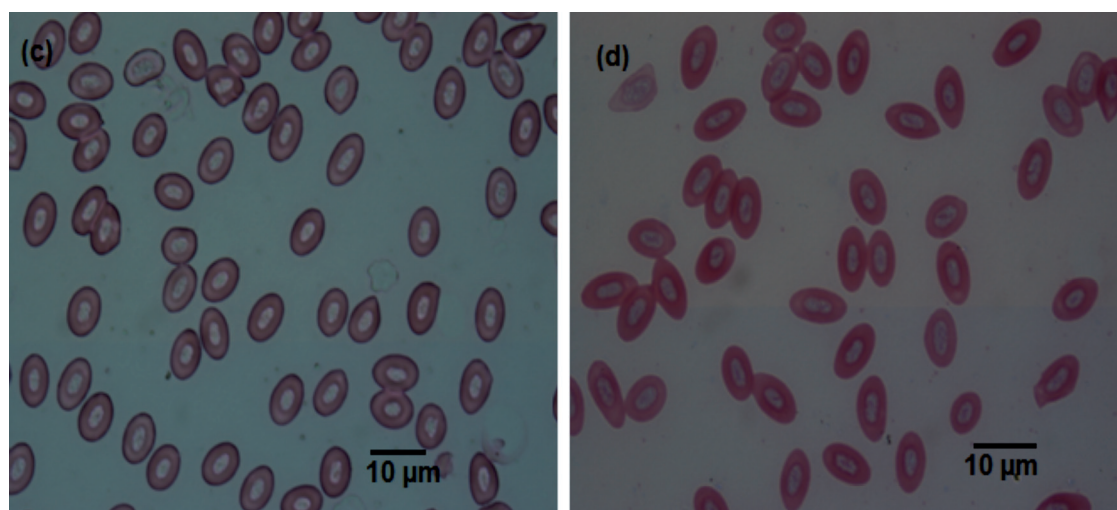


Fig. 2 — Erythrocytes of diploid and triploid red tilapia (c) Erythrocytes of diploid (d) Erythrocytes of triploid showing elongated and larger size cells.

shape of the cells was ellipsoidal in triploids. The cellular length and width have been found to be 25 and 20% larger whereas nuclear length and width was 21 and 18% greater in erythrocytes of the triploid than diploids in short-nose sturgeon, (*Acipenser brevirostrum*) (BEYEA *et al.* 2005).

The ellipsoidal size of the erythrocytes in triploid fishes was relatively larger as compared to the diploids. However, the morphology of erythrocytes and their nucleus in triploid common carps has been found to change from round to ellipsoidal as the cellular major axis of the erythrocytes was enlarged more than the minor axis (UENO 1984). In brook trout (*Salvelinus fontinalis*), the increase in nuclear size of the erythrocyte with increase in number of chromosome has been observed where the major axis was recommended as the simplest method for ploidy identification (WOZNICKI and KUZMINSKI 2002). CHERFAS *et al.* (1994) also showed a change in size and shape of the erythrocytes and their nucleus and used these parameters in determining the ploidy level in common carp (*Cyprinus carpio*) with a less than 4% error.

The erythrocytes of triploid were 1.5 times greater than diploid fishes (RAZAK *et al.* 1999). The nuclear volume in triploid red tilapia was also 68.5% higher than the diploids. The cytoplasmic volume ratio in triploid red tilapia was 1:1.57 whereas it was 1:1.48 in loach, *Misgurnus anguillicaudatus* (GAO *et al.* 2007). The cytoplasmic volume in triploid red tilapia was 57.9% higher than the diploid fish ($P < 0.005$). PERUZZI *et al.* (2005) observed that size of the erythrocyte of several indices including increase in cytoplasmic surface area (32%) and nucleus (50%) in sea bass. However, a considerable decrease in erythrocyte number (34%) in sea bass was reported. The cellular and nuclear surface area were; 1.42 and 1.50 respectively in triploids red tilapia ($P < 0.0001$) whereas in triploid common carps, these values were; 1.44 and 1.40 (UENO 1984). However, there was no significant effect of ploidy on erythrocyte nuclear minor axis (SVOBODOVA *et al.* 1998).

Among all the direct methods for determining ploidy, karyotyping has been recognized as the best and accurate one (THORGAARD 1983). In fact, a number of studies on teleostean fishes have utilized cytological karyotyping as the triploidy detection tool efficiently and successfully (DON and AVTALION 1988; HUSSAIN *et al.* 1991; MANICKAM 1991; PIFERRER *et al.* 2000). However, cytological methods are always cumbersome, time consuming, need expertise skills and have thus restricted its applicability in mass screening of the fish (HARREL *et al.* 1998). This constraint has often demanded the development of other methods for ploidy analysis. The use of erythrocyte measurements is considered as one of the indirect approaches to identify triploids, which could routinely be used under both farm as well as laboratory conditions. Moreover, this technique is easy, fast and inexpensive. However, the accuracy of this technique has been always under question for the identification of mosaic polyploids and the occurrence of misclassification when the ranges of erythrocytes overlap. Hence it becomes necessary to validate the accuracy of this technique in determining the ploidy levels for each individual species. Indeed, accuracy verification between erythrocyte indices and cytogenetically determined ploidy levels is always required before the erythrocyte measurements to be used as reliable ploidy detection tool.

The present study has clearly showed that erythrocyte measurement could be considered as a viable alternative to karyotyping for detecting triploidy in red tilapia. The study showed that the nuclear volume, cytoplasmic volume and nucleus surface area of the erythrocytes are significantly greater in the triploids as compared to the diploid fishes. These parameters thus can successfully be used in discriminating diploid and triploid red hybrid tilapia. However, considering the ease of the method for the detection of cellular major axis and nuclear major axis, this method can be opted as important parameters for distinguishing triploids and diploids of red tilapia.

TABLE 2 — Cellular, nuclear and cytoplasmic volume of the diploid and triploid red tilapia (mean $\pm$ SD).

Dimensions (μm^3)	Ploidy stage		Ratio [diploid:triploid]	Increment (%)	Significance
	Diploid (n=20)	Triploid(n=20)			
Cellular volume	245.56 $\pm$ 29.39	390.67 $\pm$ 51.69	1:1.59	59.0	$P < 0.0001$
Nuclear volume	15.89 $\pm$ 4.25	26.78 $\pm$ 6.53	1:1.68	68.5	$P < 0.0001$
Cytoplasmic volume	229.68 $\pm$ 26.95	362.81 $\pm$ 47.60	1:1.57	57.9	$P < 0.0001$

(n = number of specimens)

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