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Effect of ploidy on scale-cover pattern in linear ornamental (koi) common carp *Cyprinus carpio*

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The effect of ploidy on scale-cover pattern in linear ornamental (koi) common carp *Cyprinus carpio* was investigated. To obtain diploid and triploid linear fish, eggs taken from a leather *C. carpio* female (genotype *ssNn*) and sperm taken from a scaled *C. carpio* male (genotype *SSnn*) were used for the production of control (no shock) and heat-shocked progeny. In heat-shocked progeny, the 2 min heat shock (40° C) was applied 6 min after insemination. Diploid linear fish (genotype *SsNn*) demonstrated a scale-cover pattern typical for this category with one even row of scales along lateral line and few scales located near operculum and at bases of fins. The majority (97%) of triploid linear fish (genotype *SssNnn*) exhibited non-typical scale patterns which were characterized by the appearance of additional scales on the body. The extent of additional scales in triploid linear fish was variable; some fish had large scales, which covered almost the entire body. Apparently, the observed difference in scale-cover pattern between triploid and diploid linear fish was caused by different phenotypic expression of gene N/n. Due to incomplete dominance of allele N, triploids *Nnn* demonstrate less profound reduction of scale cover compared with diploids *Nn*.

Key words: flow cytometry; gene expression; genetics; inheritance; meiosis; phenotypic segregation.

INTRODUCTION

Induced polyploidy in fishes is usually directed for the production of sterile triploids. The common way for production of triploid fishes is suppression of the second meiotic division in eggs by application of strong physical treatments, *e.g.* temperature shocks or hydrostatic pressure (Felip *et al.*, 2001; Piferrer *et al.*, 2009). Natural triploidy in fishes is frequently observed in hybrid forms reproducing by means of natural gynogenesis or hybridogenesis (Lamatsch & Stöck, 2009; Piferrer *et al.*, 2009).

Usually, triploid fishes do not differ from diploids morphologically, especially in cases of autopolyploidy, when all three haploid sets originate from the same species. Only few cases are described when triploids differed from diploids based on some

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morphological traits (Benfey, 1999; Tiwary *et al*., 2004; Maxime, 2008). One such case was based on difference in scale-cover type in common carp *Cyprinus carpio* L. 1758 (Gomelsky *et al*., 1992).

It is known (Kirpichnikov, 1981, 1999) that scale-cover type in *C. carpio* results from the combination of two genes, each having two alleles (S/s and N/n). Four main scale phenotypes are recognized: scaled, genotype *S-nn*; mirror (or scattered), genotype *ssnn*; linear, genotype *S-Nn*; leather (or nude), genotype *ssNn*; homozygotes *NN* are unviable and perish at the time of hatching. During the past 15 years, substantial new data on molecular genetic mechanisms of scale development in fishes have been obtained, mostly through studies performed on model species [zebrafish *Danio rerio* (Hamilton 1822) and medaka *Oryzias latipes* (Temminck & Schlegel 1846)] (Monnot *et al*., 1999; Kondo *et al*., 2001; Sire & Akimenko, 2004; Tingaud-Sequeira *et al*., 2006; Rohner *et al*., 2009). Recently Rohner *et al*. (2009) have shown that gene S/s in *C. carpio*, which is involved in the appearance of mirror phenotype, was identical to one paralogue of fibroblast growth factor receptor 1 (*fgfr1*). The N/n gene product that is involved in the formation of linear and leather phenotypes has not been identified yet.

Gomelsky *et al*. (1992) have shown that diploid leather fish (genotype *ssNn*) and triploid leather fish (*sssNnn*) differed with regard to the degree of scale-cover reduction forming groups with 'strong reduction' or 'weak reduction', respectively; although no illustrations were presented in that publication. The effect of ploidy on scale cover in linear *C. carpio* has not been investigated before. This study was directed to investigate this trait in diploid and triploid linear ornamental (koi) *C. carpio*.

MATERIALS AND METHODS

The study was performed at the Aquaculture Research Center of Kentucky State University, Frankfort, KY, U.S.A. To obtain diploid and triploid linear fish, eggs collected from a leather female (*ssNn*) and sperm obtained from a scaled male (*SSnn*) were used for production control (no shock) and heat-shocked progeny. To induce ovulation and spermiation, the breeders were injected with *C. carpio* pituitary extract at 3 mg kg^{-1}. Eggs were artificially inseminated in plastic bowls and were treated with water–cow milk mixture (volumetric ratio 8:1) to remove adhesiveness. In heat-shocked progeny, the 2 min heat shock (40° C) was applied 6 min after insemination. The methodology of shock application was the same as described by Recoubratsky *et al*. (1992). Briefly, at the time of heat shock the water–milk mixture was carefully poured off from the bowl containing the eggs and heated water was added. Embryos were incubated in McDonald jars. Hatched larvae from the jars were accumulated in floating mesh cages (hapas) placed in a flow-through raceway tank.

After swimming up and transition to the external feeding, all larvae obtained from heat-shocked progeny ($n = 400$) and a sample of larvae from control progeny ($n = 600$) were stocked in 115 l flow-through aquaria and fed *Artemia* sp. nauplii for 7 days. The larvae were then stocked into two separate 20 m^3 outdoor tanks for nursing. After a 1·5 month nursery period, the tanks were drained and 270 juveniles from heat-shocked progeny and 485 juveniles from control progeny were collected and stocked into separate 0·04 ha earthen ponds for further rearing.

At 16 months of age, all surviving fish from heat-shocked progeny ($n = 105$) and a sample of fish from control progeny ($n = 118$) were individually measured (total length, L_T) and their phenotype was determined. Blood samples were collected from the control ($n = 21$) and heat-shocked ($n = 55$) progeny for ploidy determination by a Multisizer 3 Coulter counter (www.beckmancoulter.com). Digital photographs of the two lateral sides of all linear sampled

fish from heat-shocked progeny were taken using Olympus C-7070 Wide Zoom digital camera (www.olympus.co) for future fish individual identification based on scale-cover pattern. Blood samples were suspended in 10 ml of diluent (Isoton) with lysing agent (Zapoglobin). For each analysed fish, a frequency histogram of erythrocyte nuclear diameter with identified mode value (peak channel) was generated.

At 23 months of fish age, additional blood samples were collected from the control ($n = 10$) and heat-shocked ($n = 16$) progeny for ploidy determination by flow cytometry analysis using a Becton Dickinson (BD) FACSCalibur flow cytometer (www.bdbiosciences.com). The 16 sampled fish for flow cytometry analysis from heat-shocked progeny were individuals whose ploidy was previously determined by Coulter counter at 16 months of age. Instrument quality control for DNA quantitation was performed using CellQuest Pro software and DNA QC particles (BD #349523) to assess resolution and linearity. For each subject, two drops of heparinised blood from a syringe was collected in 500 µl of sheath fluid (Biosure #1019; www.biosure.com). From the blood-sheath fluid mixture, 80 µl was stained in 500 µl of propidium iodide (PI) solution (Biosure #1021) along with 40 µl of chicken red blood cells (Biosure #1005) as an internal staining control. Samples were incubated in PI solution in the dark for 10 minutes prior to analysis. For each sample analysed, 10 000 events were collected and the relative DNA content was determined as ratio of sample fluorescence peak intensity to internal standard fluorescence peak intensity (derived from chicken red blood cells).

Segregations of fish scale-cover phenotypes in progeny were compared with theoretical ratios using a χ^2 test (Zar, 1999).

RESULTS

Segregations of fish scale-cover phenotypes in control and heat-shocked progeny are presented in Fig. 1. The control progeny consisted of scaled [Fig. 2(a)] and linear [Fig. 2(b)] fish; the segregation of scaled and linear fish in control progeny did not differ significantly ($P > 0{\cdot}05$) from the expected 1:1 ratio. All linear fish in control progeny had a scale-cover pattern typical for this category with one even

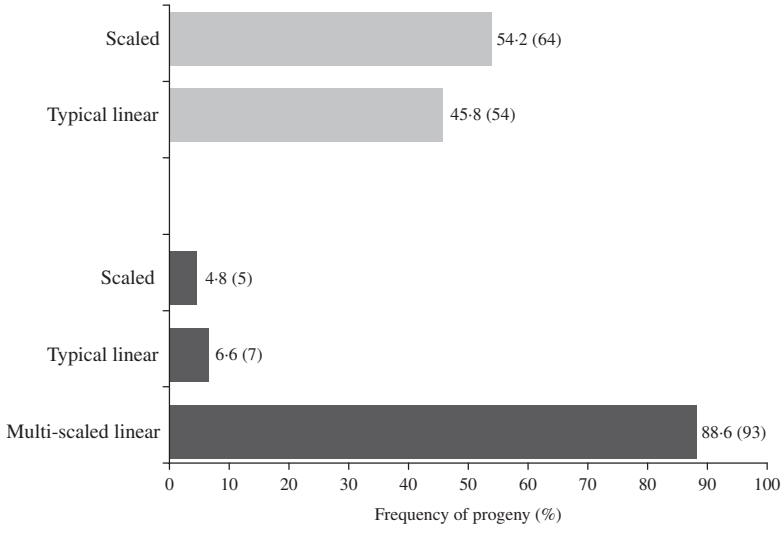

FIG. 1. Segregations of scale-cover phenotypes in control (□; $n = 118$) and heat-shocked (■; $n = 105$) *Cyprinus carpio* progeny.

FIG. 2. Scale-cover types of diploid (2*n*) *Cyprinus carpio* from control progeny: (a) scaled fish and (b) typical linear fish.

row of scales along lateral line and few scales located near the operculum, and bases of dorsal, ventral and anal fins [Fig. 2(b)]. Within the heat-shocked progeny, only five fish (4·8%) were scaled while 100 fish (95·2%) were linear (Fig. 1). Further examination of linear fish from the heat-shocked progeny showed that only seven fish had typical scale pattern for this category (the same as described for linear fish from control progeny) [Fig. 3(a)]. A majority of linear fish (93 from 100) from the heat-shocked progeny had non-typical patterns of scale cover which manifested as appearance of additional scales on the body; this type of scale cover was termed multi-scaled linear (Fig. 1). The extent of additional scales in multi-scaled linear fish was variable; some fish had additional scales only on the part of the body [Fig. 3(b)] while some fish had large scales, which covered almost the entire body [Fig. 3(c)].

Results of ploidy analysis of fish with different scale-cover phenotypes from the control and heat-shocked progeny are presented in Table I. Coulter counter and flow cytometry analyses determined diploidy of the 30 fish (16 scaled and 14 linear) from the control progeny; however, one linear triploid fish from control progeny was identified by flow cytometry analysis (Table I).

Ploidy of 55 fish from heat-shocked progeny was determined by Coulter counter analysis (Table I). All five scaled fish from the heat-shocked progeny were diploids while all multi-scaled linear fish analysed ($n = 43$) were triploids. Of seven typical linear fish from heat-shocked progeny, four individuals were diploids while three fish were triploids (Table I); the typical linear fish from heat-shocked progeny shown in Fig. 3(a) was triploid. The range of erythrocyte nuclei diameters in diploid

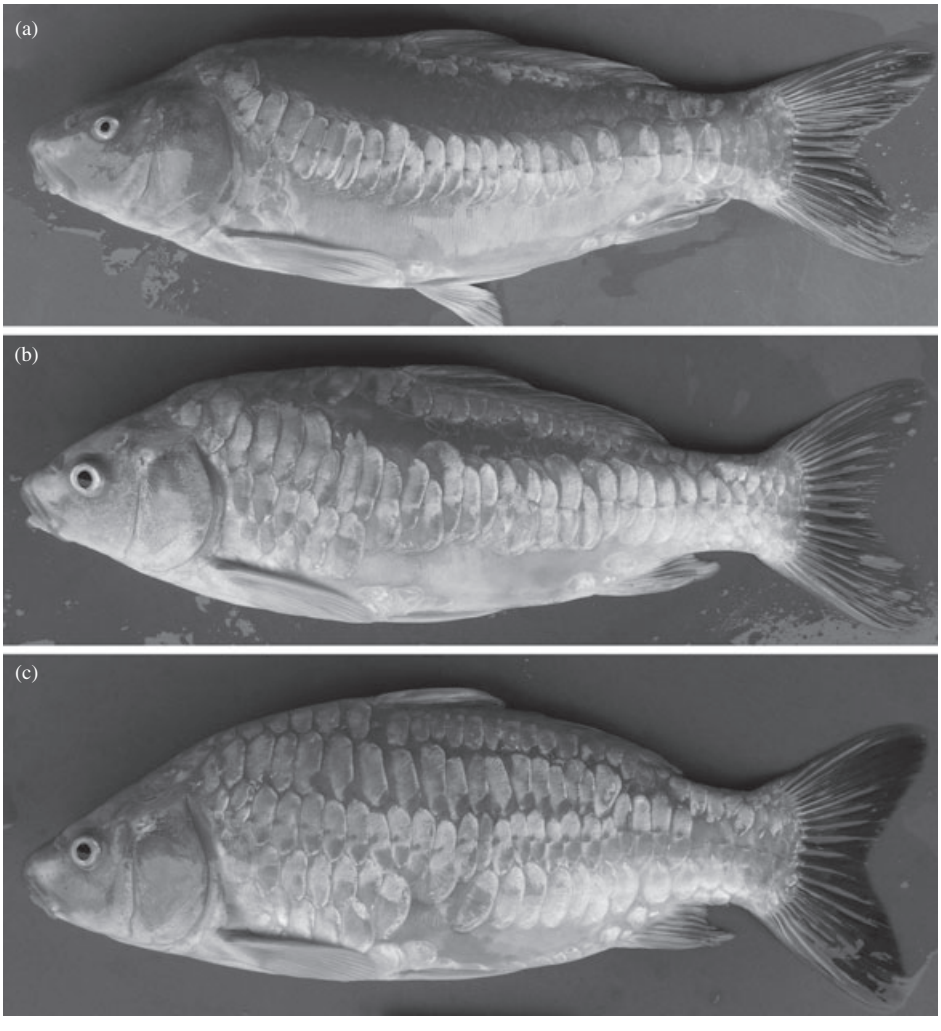

FIG. 3. Scale cover patterns of linear triploid (3*n*) *Cyprinus carpio* from heat-shocked progeny: (a) typical linear fish, (b) multi-scaled linear fish with small amount of additional scales and (c) multi-scaled linear fish with large amount of additional scales.

and triploid fish from heat-shocked progeny, as determined by Coulter counter, did not overlap (Table I).

Flow cytometry analysis of the 16 23 month-old fish from heat-shocked progeny confirmed previous results of Coulter counter analysis; 13 multi-scaled linear fish were triploids while three typical linear fish were diploids. The ratio of mean DNA content in triploid fish to mean DNA content in diploid fish (3·27:2·20; Table I) is 1·49 which is very close to a theoretical value of 1·50.

TABLE I. Results of ploidy analysis of *Cyprinus carpio* with different scale-cover phenotypes in control and heat-shocked progeny

Scale-cover phenotypic group	Fish ploidy	Number of fish	Frequency in phenotypic group (%)	Fish mean ± S.D. L_T (cm)[a]	Mean ± S.D. diameter of erythrocyte nucleus (μm) (range)	Relative DNA content Mean ± S.D. (range)
Control progeny						
Scaled	2*n*	16[b]	100	28·02 ± 1·90	3·34 ± 0·08 (3·23–3·47)	2·21 ± 0·04 (2·15–2·25)
Typical linear	2*n*	14[c]	93·3	25·66 ± 1·99	3·37 ± 0·07 (3·23–3·48)	2·18 ± 0·01 (2·17–2·19)
	3*n*	1	6·7	NR	NR	3·39
Heat-shocked progeny						
Scaled	2*n*	5	100	29·28 ± 4·99	3·36 ± 0·03 (3·32–3·41)	NR
Typical linear	2*n*	4	57·1	30·23 ± 2·46	3·41 ± 0·02 (3·39–3·43)	2·20 ± 0·03 (2·18–2·23)
	3*n*	3	42·9	31·67 ± 2·25	3·91 ± 0·01 (3·90–3·92)	NR
Multi-scaled linear	3*n*	43	100	32·81 ± 2·79	3·86 ± 0·07 (3·70–3·99)	3·27 ± 0·04 (3·22–3·34)

NR, not recorded.

[a]Mean total length (L_T) of 16 month-old fish is presented.

[b]Ploidy of 10 fish was analysed by Coulter counter and of six fish by flow cytometry.

[c]Ploidy of 11 fish was analysed by Coulter counter and of three fish by flow cytometry.

DISCUSSION

The main method for the determination of fish ploidy in this study was the application of the Coulter counter. This device is widely used by triploid grass carp *Ctenopharyngodon idella* (Valenciennes 1844) producers in the U.S.A. for verification of ploidy (Wattendorf, 1986; Allen & Wattendorf, 1987; Masser, 2002). In contrast, application of Coulter counter for determination of ploidy in *C. carpio* (koi) is not well documented. It is known that *C. carpio* have twice the number of chromosomes in karyotype and approximately twice the amount of DNA compared with most other fish from the family Cyprinidae (Ohno *et al*., 1967; David *et al*., 2003). Therefore, it was necessary to confirm the accuracy of ploidy determination in *C. carpio* by Coulter counter analysis. For this purpose, the ploidy of 16 fish (three diploid and 13 triploids) from heat-shocked progeny were checked by two methods, Coulter counter and flow cytometry analysis. For all 16 fish, the ploidy levels determined using both methods were consistent. These results have proven the accuracy of Coulter counter analysis for ploidy determination in *C. carpio*.

Theoretically, control progeny obtained by crossing of a leather female (*ssNn*) with a scaled male (*SSnn*) should consist of scaled fish (*Ssnn*) and linear fish (*SsNn*) with a ratio of 1:1. Observed segregation of scale-cover phenotypes in the control progeny did not differ significantly from the theoretical ratio. In control progeny, one triploid fish was found among 31 fish analysed. Occurrence of this triploid fish may be explained by either spontaneous suppression of the second meiotic division or possible contamination with heat-shocked progeny.

Previous experiments on induced meiotic gynogenesis in *C. carpio* revealed a very high recombination rate of gene *N* relative to the centromere with almost 100% heterozygous (*Nn*) gynogenetic offspring resulting from heterozygous (*Nn*) females (Cherfas, 1977, 1981). Gomelsky *et al*. (1992) have shown that due to the high recombination rate of gene *N*, diploid eggs *Nn* are produced predominantly by heterozygous *Nn* females when the heat shock is effective, while haploid eggs (*N* and *n*) will be formed if the applied shock is ineffective. Therefore, in heat-shocked progeny obtained in this study by crossing a leather female (*ssNn*) with a scaled male (*SSnn*), the suppression of second meiotic division should result in triploid linear fish with genotype *SssNnn*. In contrast, cases when shock fails to suppress the second meiotic division, diploid scaled (*Ssnn*) and linear fish (*SsNn*) should appear. On this basis, the percentage of scaled fish (*Ssnn*) in a progeny should decrease along with increasing shock effectiveness. In the heat-shocked progeny obtained, scale-cover phenotype segregation was shifted towards prevalence of linear fish. Only five fish (4·8%) of the progeny were scaled; all of them, as predicted, were diploids.

Among linear fish in the heat-shocked progeny only seven individuals had scale-cover pattern typical for this category; four of them were diploid while three fish were triploid. The remaining linear fish from heat-shocked progeny (93) exhibited non-typical scale patterns which were characterized by the appearance of additional scales on the body. Ploidy analysis revealed that all fish with additional scales on the body (called multi-scaled linear) were triploids. Apparently, the observed difference in scale-cover pattern between triploid linear fish (genotype *SssNnn*) and diploid linear fish (genotype *SsNn*) is caused by different phenotypic expression of gene *N*/*n*. Due to incomplete dominance of allele *N*, triploids *Nnn* demonstrate less profound reduction of scale cover compared with diploids *Nn*. Allele *S* of other

gene (S/s) is characterized by complete dominance; therefore, expression of this gene could not cause the difference between triploids *Sss* and diploids *Ss*.

The rate of development of additional scales in triploid linear fish varied to a large extent. Also, *c*. 3% of triploid linear fish did not have additional scales and demonstrated a scale-cover pattern typical for diploid fish. The cause of observed variability of linear triploid fish with regard to the scale-cover patterns needs special consideration. Recently, the existence of dosage compensation mechanisms in gene expression has been revealed in triploid fishes [*Squalius alburnoides* (Steindachner 1866): Pala *et al*., 2008; *Oncorhynchus tshawytscha* (Walbaum 1792): Ching *et al*., 2010)]. Pala *et al*. (2008) have suggested a silencing of one of the three alleles in hybrid triploid fish. Similarly, in this study, the appearance of linear triploid fish (*Nnn*) with scale-cover pattern typical for diploid fish (*Nn*) may be explained by possible silencing of one allele *n* in some triploid genotypes, although further studies for confirmation of this suggestion are needed. Potentially, the phenotypic expression of gene N/n in diploid and triploid fish can be successfully used as a model system for investigation of gene dosage effect in *C. carpio*.

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