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Re-visiting the inheritance of scale pattern of common carp
(Cyprinus carpio L.).

DOCTOR OF PHILOSOHY (PHD) THESIS POINTS

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1. Introduction and objectives of the study

Some of the genes controlling the formation and growth of fish scales have been studied, very little is known about the genetic mechanisms regulating scale pattern formation. Although the existence of two genes with two pairs of alleles (S; s and N; n) regulating scale coverage in cyprinids has been predicted by Kirpichnikov and colleagues nearly eighty years ago, their identity was unknown until recently. In 2009, the ‘S’ gene was found to be a paralog of fibroblast growth factor receptor 1, \(fgfr1a1\), while the second gene called ‘N’ has not yet been identified. The thesis is about the genetics of the scale pattern inheritance of the common carp. We are re-visiting the original model of Kirpichnikov, that proposed four major scale pattern types connected to the genetics of common carp scale pattern inheritance and two genes (s and N) regulating scale coverage in cyprinids. We generated a large number of crosses between different carp phenotypes involving loss-of-scale mutants of European and Asian origin and worked out a new model for the objective classifying the phenotypes of F1; F2 offspring generations. In this thesis, the author describes the ratio of scale pattern phenotypes in offspring groups. The experiments show that varieties of the so-called scattered phenotype with a larger number of non-overlapping scales often appear in offspring. Therefore, we divided the scattered type into three sub-types: irregular, incomplete scaled and classical mirror. We also analyzed the
survival rates of offspring groups potentially inheriting two N alleles and found distinct differences between Asian and European crosses, indicative of the presence of a strong N allele with homozygous lethality in the former and a weaker, non-lethal one in the latter. We further extended Kirpichnikov’s work by correlating changes in phenotype (scale pattern) to the deformations of fins and losses of pharyngeal teeth.

Crosses were performed parallely in Hungary and in Singapore. The current study describes the first steps of our continuing search towards the identification of the second gene, called “N”.

**Objectives of the study**

- The aim of the present work is to search for the „N” gene through systematic analysis with a larger set of crosses. (As part of an international group.)
- To propose a new model that could explain the ‘deviating phenotypes’ observed in some of the crosses and the lack of the expected 25% lethality in Hungarian crosses.
- Further extending Kirpichnikov’s work by correlating changes in phenotype (scale pattern) to the deformations of fins and losses of pharyngeal teeth, gill raker numbers.
- Analyzing the survival rates of offspring groups potentially inheriting two N alleles. Examine if the European nude carp is suitable for breeding.
2. Materials and methods

The brooders were prepared for artificial propagation by hypophysation. For the crosses performed in Hungary (Table 1.), two minutes after fertilization, the eggs were stacked onto a tulle netting (in 2009) that was stretched onto a metal frame. This provided easy and accurate tracking of embryonic development, as fertilization rate and hatching percentage were calculated by counting the live or dead eggs using digital photos of the eggs stacked to the net. We have determined the survival rates of the offspring either by i) counting fertilized eggs with (viable embryos) or without eye spots (dead eggs) stuck onto nets; or ii) sorting a few hundred embryos randomly removed from the hatching jar under a dissecting scope. For the crosses performed in 2008, 2010 the stickiness of fertilized eggs was first removed through a treatment with Woynarovich solution and later they were placed into Zuger jars and they were hatched there.

<table>
<thead>
<tr>
<th>2008 TEHAG – Százhalombatta</th>
<th>2009 HAKI - Szarvas</th>
<th>2010 HAKI - Szarvas</th>
</tr>
</thead>
<tbody>
<tr>
<td>♀ ♂</td>
<td>♀ ♂</td>
<td>♀ ♂</td>
</tr>
<tr>
<td>1) Li1 x Li1</td>
<td>1) Nu x Nu</td>
<td>1) Sc x Sc</td>
</tr>
<tr>
<td>2) Li2 x Nu</td>
<td>2) Li x Nu</td>
<td>2) Mi x Sc</td>
</tr>
<tr>
<td>3) Li2 x Mi</td>
<td>3) Mi x Nu</td>
<td>3) Sc x Li</td>
</tr>
<tr>
<td>4) Li2 x Li2</td>
<td>4) Nu x Mi</td>
<td>4) Nu x Sc</td>
</tr>
<tr>
<td>5) Mi x Nu (failed)</td>
<td>5) Nu x Li</td>
<td></td>
</tr>
<tr>
<td>6) Li x Mi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7) Li x Li</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. : Crosses in Hungary; Li= linear; Nu= nude; Mi= mirror; Sc= scaled
Carps were maintained under standard conditions of fish husbandry. For the crosses performed in Hungary, common carp brooders (males and females) were selected from the following sources: scaled carp – Amur wild type carp, and Tata common carp from the live cyprinid collection of HAKI (Szarvas, Hungary; offspring used for the comparative analysis of fin and tooth losses only); mirror carps - Line No2 from HAKI; linear carps from Tiszaker fish farm (Kőröstarcsa, Hungary) and nude carps from Béke Fish Farm (Hajdúböszörmény, Hungary). For the crosses performed in Singapore, a nude male carp originating from the Béke Fish Farm was shipped from Hungary to Singapore and used as a father for a large number of crosses. In addition to that, koi carps of the four major and some minor scale pattern types were purchased from Qian Hu Fish Farm (Singapore), and used as brooders.

The families were reared for four months so that the scale pattern could be clearly identified. At this time point classification was performed directly through visual observation of the fish, whereas fingerlings were individually photographed from both sides and scalation was assessed based on the photos. Phenotypic analysis was performed by assessing the scale patterns based on a classification that is a modified version of Kirpichnikov’s, as our classification contains a total of five (six*) categories instead of the four used earlier. We have retained three of the four major scale patterns,
namely, scaled, linear, and nude. In addition, we have divided Kirpichnikov’s ‘scattered’ category into two (three*) sub-categories: irregular (irregular+ incomplete scaled) (mirror with additional scales that are typically larger in size and cover most of the body surface often without partial overlaps) and mirror. Phenotype frequencies within the families as percentage were compared to the expected values calculated from the Kirpichnikov model.

*: Kirpichnikov’s scattered category was divided first time into three sub-categories: incomplete scaled, irregular and classical mirror. Later the incomplete scaled’ and ‘irregular’ categories have been merged into one, for better understanding, as there was no statistically significant difference between their fin and tooth phenotypes. Now categories are: Scaled (Sc); Irregular (Ir); Linear (Li); Mirror (Mi); Nude (Nu)

For the isolation of pharyngeal teeth, individuals were culled by placing them into MS222 for 15 minutes. Then, their head portion was cut off at the distal end of the operculum and: 1.) In Hungary cooked in water for 15 minutes 2.) in Singapore submerged in 4% potassium hydroxide to dissolve the soft parts. After 2-3 days, the pharyngeal teeth were picked from the remaining mass of tissue and thoroughly washed in water and dried. The number of teeth was counted under stereomicroscope and the photographs were taken. So happened in case of the gill rakers number.
In Hungary the fins were observed directly through visual observation of the fish. In Singapore fin defects (including loss) were quantified on a per-fish basis using an arbitrary cumulative scale (absent fin: 1 point; stunt fin: 0.75 point; reduced fin: 0.5 point and slightly reduced fin: 0.25 point).

The survival rate was tested by One-Way Analysis of Variance (ANOVA). The specific growth rate (SGR) and feed conversion rate (FCR) were compared by Student’s t-test. For the examination of pharyngeal teeth (regard to the relatively low number of data) non-parametric Kruskal-Wallis ANOVA and post-hoc Mann-Whitney U-probe were used. For statistical analysis Statistica for Windows statistical package (8.0. version, StatSoft, Inc., 2007) was applied.

Fin clips of fish were collected, placed into 95% ethanol and stored at 4°C until the use for DNA isolation. Genomic DNA was isolated using the standard phenol-chloroform method.

For RNA isolation, the ends of caudal fins were cut using a sharp scalpel, the fins were then allowed to regenerate for three days. Following this period, the regenerated part of the fin was collected, immediately immersed in Trizol and stored at -80°C until further analysis.
Total RNA was extracted from regenerating fins samples collected on the 3rd day following the cut by using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. The quality and concentration of nucleic acids was tested by spectrophotometry using a Nanodrop Spectrophotometer ND-1000 UV/Vis (Nanodrop Technologies, Wilmington, DE, USA), followed by agarose gel electrophoresis.
3. Results

We generated in Hungary 16 different crosses. Analysis of the survival rates didn’t show the expected 25% lethality among the offspring of Hungarian linear x linear, linear x nude or nude x nude crosses. The combined mean survival rate for these latter three offspring groups was 89.16+/-3.76%, not significantly different from that of those crosses, where no NN offspring individuals could be theoretically produced (88.20+/-2.77%; p=0.63; Student’s t-test).

Analysis of the survival rates in Singapore showed the expected 25% lethality (due to the inviability of NN individuals) in all nude x nude crosses performed. The crosses involving parents with Hungarian origin have shown unexpected ratios of scale pattern phenotypes. They were significantly different from those expected by the Kirpichnikov values. (Table 2.)

Scaled crosses (Sc x Sc; Mi x Sc; Sc x Li, Nu x Sc (2010)) according to the literature have shown the dominance of the scaled phenotype. Our preliminary experiments and breeding crosses showed that crosses the classic MixMi give 100% mirror phenotype, so these crosses were not repeated.
Table 2. Chi-square test for the inheritance rate. The expected (according to Kirpichnikov’s model) values were compared to those experienced by us. In this table, only the p-values are shown. For the yellow cells, p <0.05, significantly different from those expected by the Kirpichnikov values.

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Scaled</th>
<th>Linear</th>
<th>„Scattered”</th>
<th>Nude</th>
<th>Lethal</th>
<th>F1_No</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiLi7 (2008)</td>
<td>0.0017</td>
<td>0.5419</td>
<td>0.0000</td>
<td>0.0115</td>
<td>0.0002</td>
<td>50</td>
</tr>
<tr>
<td>LiLi8 (2008)</td>
<td>0.035</td>
<td>0.0406</td>
<td>0.0375</td>
<td>0.0723</td>
<td>0.0168</td>
<td>20</td>
</tr>
<tr>
<td>LiMi9 (2008)</td>
<td>0.2733</td>
<td>0.4142</td>
<td>0.2733</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiNu10 (2008)</td>
<td>0.0223</td>
<td>0.0013</td>
<td>0.0000</td>
<td>0.0013</td>
<td>0.0013</td>
<td>36</td>
</tr>
<tr>
<td>MiNu21 (2009)</td>
<td></td>
<td>0.0002</td>
<td>0.0002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiNu22 (2009)</td>
<td>0.0115</td>
<td>0.0001</td>
<td>0.0000</td>
<td>0.0021</td>
<td>0.0002</td>
<td>50</td>
</tr>
<tr>
<td>LiMi23 (2009)</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiLi24 (2009)</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>175</td>
</tr>
<tr>
<td>NuMi25 (2009)</td>
<td></td>
<td>0.0000</td>
<td>0.0000</td>
<td></td>
<td></td>
<td>233</td>
</tr>
<tr>
<td>NuNu26 (2009)</td>
<td>0.4086</td>
<td>0.3135</td>
<td>0.0024</td>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>NuLi27 (2009)</td>
<td>0.1449</td>
<td>0.0332</td>
<td>0.0000</td>
<td>0.1463</td>
<td>0.0332</td>
<td>17</td>
</tr>
</tbody>
</table>

Analysis of the survival rates didn’t show the expected 25% lethality among the offspring of Hungarian linear x linear, linear x nude or nude x nude crosses.

The most extreme deviation was shown by the two Hungarian nude x mirror crosses that yielded only 2.7% and 0% nudes.

In the two linear x nude type crosses maximum 12.5% scattered were expected and 88.1-98.6% were found. On the other hand, no fully scaled offspring were produced in the crosses, despite the predicted range being 12.5-50%, depending on the parental genotypes.

The ratio of the nude offspring also did not meet those expected based on the Kirpichnikov system in three of the four crosses: 0.3-9.3% (detected) vs. 0% or 25%.
When two Hungarian brooders carrying the proposed ’N’ allele were crossed, no lethality was observed among the offspring. Also, the distortions and losses of fins as well as severely reduced pharyngeal teeth counts often detected in Asian nudes, were not observed in most of their Hungarian counterparts:

The association between the scale pattern and the number of pharyngeal teeth was also tested and compared across crosses involving Hungarian and Asian brooders.

There was a progressive loss of pharyngeal teeth in parallel with decreasing scale coverage across both groups, but was much more drastic in the latter.

For crosses performed in Hungary, the average teeth numbers for scaled and irregular individuals were similar (10 and 9.75, respectively) while the mirrors and nudes had on an average, ~8 and 6 teeth (Table 3. B).

In addition to the loss of teeth, the nudes also displayed a distinct weakening and thinning of the 5-ceratobranchial arch, resulting in its reduction from a three dimensional structure observed in most phenotypes into a thin, boomerang-shaped object.
Table 3. A) Comparison of the scaled and nude phenotype- data from European carp. B) The average number of teeth is shown for the four scale-pattern phenotypes (scaled, irregular, mirror and nude) from individuals sampled in Hungary. Numbers in white at the bottom of the bars indicate the number of individuals analyzed from each scale pattern category. Different letters on top of the bars indicate statistically significant differences (P-value: <0.01, Student’s t-test).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Gill rakers No.</th>
<th>Phar. teeth</th>
<th>body lenght</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude</td>
<td>21,83</td>
<td>6</td>
<td>12,02</td>
</tr>
<tr>
<td>Scaled</td>
<td>25,4</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

The gill rakers number varied between 18-27, and the number of pharyngeal teeth counts 4-10 (Table 3. A). Our observations happened in 4-5th months. According to this observations the fish have not yet developed the maximum number of gill rakers and pharyngeal teeth, so errors may have occurred during the preparation. Only in the width of the studied pharyngeal arches were significant differences according to the Kruskal-Wallis ANOVA (p = 0.0096).

In addition, several crosses involving parents with full or partial Hungarian origin showed unexpected ratios of scale pattern
phenotypes, including i) severely reduced proportion (or even complete absence) of nudes from nude x mirror and nude x irregular crosses; ii) complete absence of scaled and iii) unexpected proportion of nudes in linear x nude crosses. These observations seem to indicate that the Hungarian brooders tested migh contain a new mutant ’s’ allele with stronger effects not described earlier and/or a mutant ’N’ allele with a milder effect on scale pattern, pharyngeal teeth and fins than the ones described earlier by Kirpichnikov. The (near) complete lack of nude offspring from the two nude x mirror crosses could be produced by a cross between an ordinary mirror and one that carries two hitherto unknown, strong ’s’ alleles that cause a complete loss-of-function of the gene product resulting in the disappearance of all scales and as such a nude-like phenotype. The other possibility is the presence of a weak ’N’ allele in the Hungarian nudes that causes the loss of scales, but not the lethality in homozygotes, and it has limited, if any, effect on teeth and fin formation. If we were to assume that the Hungarian nudes all carry the NN genotype, this could potentially explain the vastly reduced proportion of nude offspring produced by the Hungarian nude x mirrors and linear x nude crosses.

Feed conversion rate (FCR) and SGR (specific growth rate) values were examined (Figure 1., 2.). On the 50th day of the experiment the average weight increased according to groups: LiLi → LiNu→
LiMi→MiNu→NuMi→ NuLi→ NuNu. Growth ability of offspring from the linear female brooders were slightly lower than those derived from the nude phenotype females.

![FCR50 diagram](image1)

Figure 1.: Feed conversion rate (FCR)

![SGR50 diagram](image2)

Figure 2.: SGR (specific growth rate) values.

In Singapore the average fin defects were the highest for nudes (4.5 points) and the lowest for irregulars (<1 point) with mirrors showing 1.2 points of the loss/distortions on an average (Fig. 3). The pairwise
differences were significant between each of the pairs tested (P<0.01; Student’s t-test).

The Hungarian nude offsprings have a strong vigorous growth, they have a normal body shape, while the Asian subspecies have become non-viable offsprings, cattle runted into possibly due to the effects of different levels of N, causing the entire pikkelyvesztést mutant allele. The European pikkelyvesztést causing allele, while the Asian allele markedly affect the garatfogakra, swimmers as well, so that the offspring may have survived almost can not swim (Fig. 3).

<table>
<thead>
<tr>
<th><strong>Nude carp in Hungary</strong></th>
<th><strong>Nude carp in Singapore</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Fish image" /></td>
<td><img src="image2" alt="Fish image" /></td>
</tr>
<tr>
<td><img src="image3" alt="Fish image" /></td>
<td><img src="image4" alt="Fish image" /></td>
</tr>
</tbody>
</table>

Figure 3.: Differences between the nude offsprings of European and Asian carps. (Photo by Laura Cassas; Szucs Reka)
4. Additional studies and results in Singapore

Genetic studies were performed in Singapore. There are certain pathways which could be potentially involved in finding the „N” gene. These include the Eda, Wnt, FGF and RA signalling pathways. In order to begin this search, they compared the expression level of few genes belonging to the above mentioned pathways between mirrors and nude fishes. Unfortunately, they could not see a significant expression difference between the nudes and mirrors-for the genes they tested. In order to take this study forward, these are some of the steps they are planning to take:

1. Do a comprehensive comparison of the expression levels between the nudes and mirrors so as to get a clearer picture of all the pathways which maybe potentially involved.

2. To collect RNA samples from typical nudes and mirrors- they plan to sequence at least three from each group on Hiseq to get a global view of the transcriptome changes between the nudes and mirrors.

3. Sequence some potential candidate genes to check for truncations / variations in the open reading frame of nudes.

Important result is, that in Singapore the research group isolated and structurally characterized a hitherto missing members of the Fgfr1 receptor family, *fgfr1b1* and *fgfr1b2*.
5. Conclusions and suggestions

The research group proposes a new model that could explain the ‘deviating phenotypes’ observed in some of the crosses described above, and proposes that the new sub-types of scattered were formed due to increased levels of Fgf signals compared to mirrors and especially nudes, either due to an additional mutation in one of the FGF signaling pathway genes or, that in an upstream pathway, it functionally connected the Fgf signaling.

![Figure 4.](image)

Figure 4.: The proposed rheostat-like new model.

Our working hypothesis showing the rheostat-like action (Figure ) of mutations to the level of signals probably from multiple pathways. An uneven signal level at various locations combined with a gradually decreasing signal threshold in different allelic combinations (plus potential effect from additional modifier genes) might result in the scale pattern phenotypes. An imaginary dorsoventral cross-section shown in the top panel in each case (D: dorsal,
L: lateral and V: ventral) shows the typical locations of preferential scale formation on the body surface while the green line indicates the threshold for variants of scaled (A), irregular (B), and mirror carps (C). (An alternate scenario for this hypothesis; stable threshold with decreasing signal intensity from scaled to nude would produce the same outcome.)

We would like to introduce this linear grass carp (Fig. 5.). It belongs also to the Cyprinidae family. We hope, it will help us to find the missing „N” gene through it’s easier genetics (2n=48).

Figure 5.: János Sári and Géza Simonics have found in Hortobágy Fish Farm Co. a linear grass carp (2n=48) (Bercsényi et al., 2011).

In summary, we revisited the classical model of scale pattern inheritance proposed by Kirpichnikov and his colleagues in the 1930s. We began by performing a systematic analysis of crosses involving carps of varying scale patterns. On doing this, we found a new scattered phenotype, called irregular (irregular +incomplete scaled), that can be regarded as a variation of mirror with additional scales providing an incomplete coverage of the body surface. As the
irregular phenotype was found consistently in many crosses, we incorporated it into the model by dividing the scattered category into mirror and irregular, instead of regarding it as an aberration as Kirpchnikov did. We also addressed the lack of 25% lethality expected based on Kirpichnikov’s original genetic model that was observed in nude x nude and nude x linear crosses performed in Hungary. Further, we studied the correlation between the number of scales with fin defects (absence as well as distortions) and teeth loss. We could observe in Asian crosses a clear correlation between fin/teeth loss and scale number with such defects being the strongest in nudes and weakest in irregular.
6. Thesis Points

1. The model of Kirpichnikov was re-visited for the first time by performing a systematic analysis with a larger set of crosses.

2. In order to analyze the ratio of scale pattern phenotypes and the survival rates of offspring groups potentially inheriting two N alleles 16 crosses were performed. There was no lethality (expected by Kirpichnikov) observed among the Hungarian crosses.

3. The phenotypic classification were extended into 6 categories by taking into consideration the two new classification types (incomplete scaled and irregular categories).

4. We found distinct differences between Asian and European crosses: different degrees of deformity and stuntedness appeared in offspring groups.

5. We found that the European nude carp is suitable for breeding.
7. Publication list: Publications related to the PhD thesis

Refereed International Journals

IF: 4,07

National Publications (In Hungarian)


Conferences (In Hungarian)


8. Major publications not related to the PhD thesis

Refereed International Journals


National Publications (In Hungarian)


Total Impact Factor: 4,574