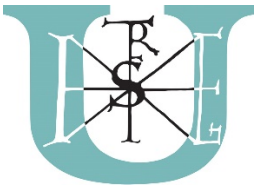

**Szent István University**

# FISH SCALE SHAPE STUDIES USING GEOMETRIC MORPHOMETRICS

**Theses**

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**GÖDÖLLŐ**

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**Doctoral School**

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# 1. INTRODUCTION AND AIMS

## 1.1 Preliminaries

Taxonomic classification of organisms was abled by morphometry (shape science). Later on, morhological characteristics were the only base of phenetic classification and numeric taxonomy in the whole discipline of biology.

However phenetics based numeric taxonommy was recently replaced by evolutionary – genetic based cladistics (new methodology of modern taxonomy), morphometry is still important methodology during taxonomical research.

Numerous processes could cause alterations in the shape of a given individual, or in a given structure of it, such as diseases, ontogenetics, adaptations or long-term evolutionary changes.

The landmark based geometric morphometry, a new trend in morphomertrics is inecraseingly used in fish biology, mainly aimed to analyze the shape of fish to distinguish between species or populations. These protocols have one main disadvantage: the stress caused to the sampled specimen is lethal in most of the cases. Approximately ten years ago this disadvantade was faded in the need of a new, analyzable structure, variable enough to distinguish fish populations and paralelly its sampling does not cause permanent damage to the fish. In case of fishes, scales shows matches with these criteria, while this structure is easy to regenerate and sampling is much less stressful, than the other methods.

Although several studies have studied the scale shape, these papers only pointed out the successful disrimination. Therefore in these theses the potential other possibilities of scale-shape studies and the potential factors behind the acale shape variability would be addressed.

## 1.2 Aims

1. To compare the scale-shape of chosen Hungarian fish species, study their separability, and compare their patterns with phylogenetic relationships. Reveal the connection between the similarity of the species' scale-shape and their genetic relationships.
2. To study the population level separatability of four freshwater species (gibel carp *Carassius gibelio* BLOCH 1782, carp *Cyprinus carpio* L. 1758, roach *Rutilus rutilus* L. 1758, perch *Perca fluviatilis* L. 1758) based on their scale-shape. Is the fish scale-shape suitable for population level discrimination?
3. A laboratory experiment was performed, to find out whether the intraspecific genetic differences may influence scale-shape. Does the genetic background play a role in the differences between the scale-shape of populations?
4. To perform a laboratory experiment, in which the effect of food availability, as environmental impact on the scale-shape of zebrafish (*Danio rerio* HAMILTON 1822) would be studied. Are the environmental differences manifested in scale-shape?
5. Examination the potential sexual dimorphism of the scale-shape in case of zebrafish and gibel carp. Is there any perceptible sexual dimorphism in scale-shape? Is it possible that the sex ratio of the sample affects the results?
6. Study the effect of ontogeny on scale-shape of gibel carp. Can ontogenetic changes be detected in scale-shape? Do the age distribution of the sample affect the result?

## 2. MATERIAL AND METHODS

### 2.1 Scale sampling and data analysis

Fish specimen were anesthetised in every case using one drop/litre concentrated clove oil. Scales of Cyprinids (razorfish (*Pelecus cultratus* L. 1758) excluded) were removed from the area defined as follows: horizontally the flank anterior of the dorsal fin and vertically the middle area between the dorsal and lateral line. In case of Percids and razorfish scales were removed from the area defined by the end of the longitudinally positioned pectoral fin. All scale were removed from the feft side of the fish to avoid fluctual asymmetry. One scale per specimen were involved to the analyses in all cases. Scales were then placed between two glass slides and scanned with an HP ScanJet 5300C XPA scanner at 2400 dpi.

After creating the file (at this stage its empty, therefore LM=0), the seven landmarks were placed to the scale in tpsDig2 software. The first and third landmarks were palced to the dorsal and ventral edge on the cranial border. The second landmark is at the midpoint of the line, defined by the first and third. The fourth and sixth landmarks were placed to the dorsal and ventral border of the free and other scale covered areas. The fifth landmark was placed to the focus and the seventh to the caudal border to the midpoint of line, defined by landmark four and six.

Further analyses were made using the MorphoJ software. The first step was a full Procrustes-fit on the landmark-series to fit, resize and rotate them into maximum coverage. Thereafter the group ID was added to each specimen and a multiple linear regression was made on the Procrustes-coordinates (dependent vaiable) and log10 of centroid size (independent variable). The reliability of the regression s were tested usind a permutation test (10.000 permutations). Further analyses were made using the regression residuals (the non-size effected part of

the variance). The group level separatability was tested usind Canonical Variance Analysis and Discriminance Function Analysis (DFA).

### 2.2 Studies on fish species separatability

To assess the species separatability 25 specimen of thirteen species (roach, chub, gibel carp, razorfish, bleak, mudminnow, pumpkinseed, common carp, topmouth gudgeoon, perch, pikeperch, rudd and zebrafish) were studied. The taxonomical status of the studied species was asessed using cytochrom-oxydase (COI) gathered from the database of NCBI GeneBank. Sequence alignments were made using the ClustalW software and a distance matrix have been created. The genetical distances were compared with Procrustes distances using Mantel-tests. These analyses were made in PAST v3.01 software.

### 2.3 Studies on population level separatebility

Suitability of the scale geometric morphometric analyses for population level discrimination were studied in case of four species (gibel carp, common carp and perch). Gibel carp samples were caught in five loacalities: Lake Fenéki, Lake Hídvegi, Lake Balaton, Nagyberek and the thermal ake of Héviz. Roach samples were collected from Balaton and he two stages of Kis-Balaton (Lake Fenéki and Hídvégi). Perch samples were available from Ráckevei-Danube-Sidearm, Kemecse (Northeast Hungary) and Vodňany, Poland.

### 2.4 Effect of genetic factors on scale shape

To assess the role of genetic determination in the scale shape zebrafish was used as model organism. The registered AB (wild) line and an other common line

(LF BASKA were used for our experiments. The fishes were kept under constant laboratory circumstances in a Tecniplast (Zebtec) recirculation system (water temperature: 525 ± 50 µS; 14h light/10h dark fotoperiod; 30ind/3.5l). Fishes were fed twice a day with SDS Small Gran food (Special Diets Services Limited International Dietex GB) and fresh hatched *Artemia nauplii*.

### 2.5 Effects of environmental factors on scale shape

For assessing the potential effects of environmental factors the AB line of zebrafish was used. Fishes were kept inthe above described system. Food supply was chosed as the studied environmental factor. The brood of four AB line females were grouped to 2-2 groups, therefore eight groups (N1-H1, N2-H2 etc.) were formed, where the genetical distance amongst the pairs was minimal. 'N' groups were treated as controls, where the food supply was normal (twice a day), while 'H' groups get the quantity of food only for survive (once in two days). Groups were maintained for three months under the above described conditions. After that, two 'H' groups were reared for another three months under normal (control) feeding regime to assess the effects of the potential compensatory growth.

### 2.6 The possible effect of sex on scale shape

The potential sexual dimorphism of scale shape was studied on zebrafish and gibel carp. The zebrafish (AB line) were kept under the above described conditions, while the gipel carp samples were caught in the Kis-Balaton I.-II. reservoirs

### 2.7 The effect of age on scale shape

For this issue the scales of previoiusly aged gibel carp scales, sampled from the two reservoirs of Kis- Balaton were used.

## 3. RESULTS

### 3.1 Separability of fish species

Significant allometric effect were found in every cases. The 5.5% (mean) of the within-species shape variance was explained by the size ($p<0.001$). All species pairs were significantly distinguishesd using the Hotelling t-test ($p<0.001$), except the case of common carp, gibel carp, roach and rudd). For detailed analysis of non-significantly separated species, another CVA has been performed with the exclusion of all other species. This analysis showed significant differences, which suggest the scale dependence of the method. No crrelation were found between the genetic distances and scale shapes (Mantel-test: $R = 0.178$; $p = 0.124$). The reason of the difference was most possible the status of the small bodied Cyprinids (bleak, topmouth gudgeon), threfore another Mantel-est was performed with the exclusion of this three species. This analysis revealed significant correlation between the genetic distances and scale shape ($R = 0.354$; $p = 0.037$).

### 3.2 Separatability of fish populations

Four freshwater species (gibel carp, common carp, roach, perch) were used to assess the scale shape based separartability in population level. Significant allometric effect were found in every cases.

Gibel carps of four habitats, formed three groups in the analysis based on their scale shape. No significant difference was found between the two reservoirs of Kis-Balaton. In case of common carp the difference was significant in all cases an additionally the shape of specimen inhabiting Lake Hévíz showed extremely high differences from the others.

Bodorka esetén mindhárom csoport szignifikánsan elkülönült egymástól, a csoportok közötti átfedések ellenére ($p < 0,01$). A három sügér populáció szignifikánsan elkülönül egymástól pikkelyalakjuk alapján, a jelentős átfedések ellenére is ($p < 0,01$).

### 3.3 The effect of genetic differences on the scale shape

The effect of size was significant (mean: 7.7% variance) in tis study. The scale shape of the two studied groups could be separated significantly, however the reliability of the classification was moderate (reliability: 81.3%, cross-validation: 78,79%).

### 3.4 Effect of environmental factors on scale shape

Size affected the scale shape significantly (mean 24.27%) in this analysis. Significant differences were found almost in every cases (except N1 – REH2 comparison). In case of the groups originated from common parents the differences were signifiant in all cases ($p<0.0001$). Results of the compensatory growth study showed intermediate scale shape between the normal (control) and lowered feed groups.

### 3.5 The effect of sex on scale shape

The potential effect of sex on scale shape was studied on zebrfish and gibel carp.

Scale shape was affected significantly by the size in case of zebrafish (7.41%; $p < 0.001$). Although the scale shape showed differences ($T^2 = 35,02$; $p < 0,001$), the classification reliability and the cross validation results not supported this finding. Therefore the scale shape of the two sexes considered to be not different.

The size effect was marginally, but sigificant in case of gibel carp (3.69%; $p = 0.04$). The two sexes poroved to be not different regadring their scale shape ($T^2=7.22$; $p=0.79$). The classifying reliabilities and cross validations) showed therefore low values (68.18 and 48.48%), which means random classifications.

### 3.6 The impact of age on scale shape

As in all previous studies, the scale size affected the shape significantly ($p = 0.001$), the size was responsible for the 3.47% of the total shape variance. The groups were differed significantly each other at 95% confidence level, except two cases (3+-5+ and 4+-5+). The validation level was high, the mean rate was 91.6%.

### 3.7 Theses – new scientific results

1. Correlations were found between the scale-shape and taxonomic position by the studied freshwater fish species (roach, chub, pumpkinseed, pikeperch, perch, mudminnow, gibel carp, rudd, razorfish, carp), for the first time.

2. The scale shape based separatability at species- and the population level were proved first time in case of several species.
3. Myexperiments showed that the low-level, intraspecific genetic differences are detectable in the scale-shape too.
4. The effect of an environmental factor (food supply) on scale shape was first time demonstrated in a laboratory experiment. The effect of compenstory growth was also observed.
5. No sexual dimorphism was detected in scale-shape of zebrafish and gibel carp, therefore the sex ratio would not affect the resuls of morphometric analyses.
6. -Ontogenetic changes of scale shape were first time described in case of gibel carp. The age structure of the sample may therefore affect the result of scale morphometric analyses.

## 4. CONCLUSIONS AND PROPOSALS

### 4.1 Conclusions regarding the separability of fish species

The applicability of the scale shape based geometric morphometryy ws proved in wide taxonomic spectrum, even in case of closely related cyprnid species. Scale shape based classification of the species overlapped with genetic based classifications, with some exceptions. One of these excptions was the case of ’small bodied cyprinids’, namely bleak, topmouth gudgeon and zebrafish, which were classified closely based on their scale shape, but not closely related based on the genetics data. Common traits of these species are the small size, the bentho-pelagic habitat and the omnivor feeding, which are probably in the background of the similar scale shape. Another conflict between the genetic relations and scale shape was found in case of pumpkinseed and razofish. This consideret to be strange, while the scale types of these species are different:

Pumpkinseed have ctenoid, razorfish have cycloid scales. Common point of species traits could also be found (invertivore/planktivore feeding; pelagic habitat at least as juvenile), which could be drivers towards convergent scale shape. This could be supported wtih the results of the experiment on food supply, where the environmental stress, which was suffered in juvenile age have been still accoutable after compensatroy growth. These results also point out of the sale dependence of the scale based geometric morphommetric analyses. In case of large number of groups, characterized with harsh between-group shape differences, the closely related shpe differences might be faded. In such cases, the re-grouping or a re-structured analysis could be considered.

### 4.2 Conclusions about the examination of fish populations severability

The applicability of the method was proved at population level, as the separation was succesful in studied cases. No significant scale shape differences were found in case of gibel carp populations of Kis-Balaton I. and II., which is well explained with the close connectance and similar environmental conditions of this two reservoirs. The connectance between the Kis – Balaton and Lake Balaton is still given, but the environmental factors are different. The benthic omnivore gibel carp is strongly affected by the nutrient load of the water. Therefore the hyperthropic water of Kis-Balaton, the euthropic circumstances in Nagyberek and the oligo-mesothropic environmenti in Lake Balaton may have strong effect on condition factor and the scale shape as well.

In case of carp, Lake Hévíz stock was characterized by the most different scale shape, which might have several reasons. The thermal lake is characterized with extreme environmental conditions, compared to the surrounded areas, where the annual mean of water temperature is 11-12 °C, in Lake Hévíz the mean of winter water temperature is 22 °C, while in summer 38 °C. Its effects appears in

the dwarf size of the carps in Lake Hévíz, which is most likely due to these extreme conditions. The extreme conditionsare indirectly affects the reproductive cycle, growth, the sensitivity to diseases and food base as well. On the other hand, the preliminary results of genetic analysis showed that the carps in Lake Hévíz are genetically distinct from the populations inhabiting the surrounding waters, which can also affect the scales shape. In case of roach, the reliability of the group separation was significantly lower than carp and gibel carp, due to the high within-group standard deviations. Since the three areas are directly connected to each other, migration may sustain the the polymorphism in catchment level.. It should also be noted, that the roach inhabits mainly the littoral zone, in Lake Balaton this is the most constant and dominant species of the reed belt, and this asscociation with the microhabitat can mask the environmental differences between the areas. In case of perch, the differences between the groups were below the former species, despite the very large geographical distances. However, if we consider that the specimen from Szabolcsi Halászati Ltd. farm in Kemecse cannot considered as natural (there was artificial feeding), and that the population from Poland was transported to Kemecse at least one growing season before the sampling, then the small differences between groups are no longer seem inexplicable. As there is no former reference study regarding the scale shape of perch, the kow level differences might also be due to the phenotypic plasticity, which was proved regarding other traits of the species.

### 4.3 Conclusions regarding the impact of genetic background on scale shape

The experiment on zebrafish stocks demonstrated that genetic differences can be detected in the scale shape. This result supports that intraspecific variability of scale shape in fishes has a strong genetic component and genetically isolated populations of fish might have different scale shape patterns in the wild.

### 4.4 Conclusions regarding the impact of environmental differences

This experimment proved that scale shape is affected by the environmental factors, such as food supply. The scales of zebrafish became dorso-ventrally plained due to the minimal food supply, and this was not normalized after the compensatory phase.

Amongst the potential environmental factors, which may affect the whole body morphology, the role of temperature and food supply are the best known. The composition and the quantity of the consumed food, can influence the condition, and especially the extent of the fat reserve of the specimen and finally the body shape. Condition of fish may also change dynamically during the life-span due to the variations in individual feeding strategies, diseases, ontogenetic stages, and even seasonally according to the reproductive and wintering cycle. These fomer studies shown consistent changes in body parameters related to the condition and fat metabolism of the examined individual, like body depth, and the largest fat depots in the caudal and trunk region. Body shape parameters which are influenced by the conditional state of fish might therefore limitedly be applicable for intraspecific stock discriminations. Scale shape is potentially less sensitive to short-term environmental effects and instantaneous processes, therefore less dependent upon the conditional state of fish. Present results showed that although scale shape might also recover partly during the compensatory growth (i.e. with the normalization of feeding conditions), this process is much slower and presumably is not as complete as it is in condition related body shape parameters. My experiments with zebrafish proved that intraspecific scale shape variations are generated by the interactions of genetic and environmental factors and reflect phenotypic plasticity.

### 4.5 Conclusions regarding the impact of sex on scale shape

Sexual dimorphism is a typical phenomena in fishes. In case of zebrafish, where sexing of specimen do not cause problem for an experienced technician, the sexes separated significantly based on their scale shape. However, this difference proved to be minimal that the method cannot be considered as reliable. In case of gibel carp no sexual dimorphism is known, there was no significant difference between the sexes based on their scale shape as well. These results suggest that the scale-morphometric studies can not generally applied for fish sexing.

### 4.6 Conclusions regarding the impact of age on scale shape

The results of the investigation on impact of age on scale shape supports the observation, that scale might need years to reach the species-typical shape, although it can appear on the larvae of the species in a very early life-stage. In case of gibel carp, this species-characterized shape reached at the age 3+. Therefore it can be said, that in scale shape there is a kind of ontogenetic development. This observation is important, because if different age groups are compared (a very young group and an adult group) the age-distribution itself can cause significant separation between the groups, without any environmental or remarkable genetic differences.

### 4.7 General conclusions

As scale based geometric morphometry proved to be an appropriate method for species level classifications, it might be useful to identify archaeological scale samples or by analyzing the gut cotents of piscivore predators, such as otter (*Lutra lutra*) or cormorant species (*Phalacrocorax* sp.). The applicability of this method sufferes from the same weaknesses like whole-body studies, namely the

role of genetic and environmental factor is quite unpredictable. Morphometric analyses are not able to separate the effect of genetic and environmental factors, however some patterns could be observed in some cases. Despite the above mentioned weakness, the method have some advantages compared to the whole-body studies:

1. The scale-sampling cause considerably less stress to the specimen, than the whole body investigation, therefore the introduction of the method is highly recommended in case of protected or endangered fish species.
2. Much easier, time and cost efficient, than the traditional whole body methods, as in many cases the scale- sampling and preparation already done due to the measurement of other population dynamic parameters.
3. Scale shape is presumably less sensitive to short-term environmental effects and instantaneous processes, as well as it is less dependent upon the conditional state of fish, therefore much more suitable for examining the long-term environmental effects and genetic separation.
4. Due to the ring structure of the scales, with detailed examination the environmental changes can be traced.

**4.8 Suggestions**

- It would be worthwhile to examine the scale-morphometric method from the aspect of how it fit with the other morphometric methods in terms of repeatability and reliability.
- This study shows that the scale shape, as other phenotypic characteristics, is under the influence of the genetic background, and environmental impacts as well. However, these investigations could not reveal to what extent these are released or whether there is a possibility to separate the

two factors. In case of the separation of gibel carp and roach populations, some signs were shown about it.

- In connection with it, it might be worth to consider whether there is a relationship between the genetic distances and the distances based on the scales shape, or not.
- In case of gibel carp, there was no significant differences between the scale shape of the two sexes. In case of zebrafish, the separation was significant, however the reliability of the separation was not sufficiently robust based on the validation tables. Further examinations needed on other species to clarify this issue.
- In case of scale-morphometric studies, it is essential to pay attention to the age-distribution of the samples. It is worth to complement the scale-shape analysis with age estimation, or exclude those specimens, that do not reach the appropriate body size.
- As the ring structure of the scales can preserve the individual life-history of the fish, with a detailed analysis of the scale rings, the environmental changes and the life-history of the fish can be explored.
- The detection of the toxicological effects of different materials among the environmental factors plays an important role in recent researches. Therefore, it would be important examine whether the scale-shape morphometric methods can be incorporated to the system of toxicological tests.

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