UNIVERSITY OF PÉCS

Biological and Sportbiological Doctoral School

Scale analysis of Mediterranean Teleosts

PhD thesis

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1. Introduction

Fish scales have been used for systematic ichthyology for many years. Besides gills and fins, they are one of the major characteristics of Teleostean. Scales are present in all major fish groups and show a huge variety in their morphology, histology and ontogeny (SIRE *et al.*, 2009). This variability over all the fish groups attests fish scales to be of high value to contribute in various ichthyological issues. LOUIS AGASSIZ (1833-1843) was the first to recognize this potential and to classify fishes into four groups according to their scale types. Although his classification was unnatural, the nomenclature introduced by AGASSIZ has been fully incorporated into ichthyology. The use of scale morphology in teleost systematics generally has been confined to notations of scales being either simply "cycloid" or "ctenoid". ROBERTS' work (1993), however, revealed that this is an oversimplification of the situation. Some scale characteristics in teleost lineage contain a wealth of potentially valuable information that would make a significant contribution towards a better understanding of teleost systematics ROBERTS (1993). Others, such as COBURN & GAGLIONE (1992) and KHEMIRI *et al.* (2001) have confirmed the utility of scale characters while acknowledging the difficulties encountered in defining character states and in dealing with problems such as variation among scales from different areas of the body. Well-elaborated scale catalogues demonstrating a wide range of scale types and species-specific descriptions of pre-defined scale characteristics are needed to provide more reliable determination. Scale atlases are demanded not only by ichthyologists but also by ecologists, palaeontologists, and archaeologists. Some studies already took advantage of the valuable information preserved by scales in lacustrine (e.g., PENNINGTON & FROST, 1961; DAVIDSON *et al.*, 2003) or marine (e.g., SOUTAR & ISAACS, 1969, 1974; HOLMGREN-URBA & BAUMGARTNER, 1993; PATTERSON *et al.*, 2005; VALDÉS *et al.*, 2008; DÍAZ-OCHOA *et al.*, 2009) sediments to determine ancient or historic species compositions and environmental changes. Ever since VAN OOSTEN (1957) stated that scales had "limited use in fish systematics", it has been proven many times that scales indeed bear valuable information on different levels of systematics, as well as they provide knowledge in different aspects of ecology. However, the lack of an integrated system for scale identification and the small number of reference materials still limit the utilization of scales in many scientific fields. Therefore, well-elaborated scale atlases are needed to provide references for scale morphology and variability, as well as, methodological guidelines are demanded to facilitate the practical utilization of scale analysis in ecology and fishery science.

2. Aims and objectives

The main focus of my research is to investigate the morphological characteristics and morphometric parameters of the scales of teleost species and to reveal the key characteristic features that facilitate identification at different taxonomic levels. The primary aim is (1) to establish a system that aids scale identification of common Mediterranean teleost species. Due to the high variability and plasticity of some scales, especially within the family Clupeidae, the secondary aim (2) is to provide methodological approaches that allow a reliable differentiation among morphologically similar scales using morphometry and geometric morphometry. Last but not least, (3) a study was conducted to test practical aspects of scale geometric morphometry and to facilitate its application in ecology.

With this research I attempt to address the following questions:

Q1: What are the most relevant scale characteristics that aid species identification among common Mediterranean teleosts?

Q2: Does scale morphology provide identification at species level?

Q3: How can scale characteristics be transformed into numerical data that facilitate the investigation of phenotypic relations among different types of teleost scales?

Q4: Does scale morphometry allow the differentiation between the morphologically similar scales of clupeid species?

Q5: Is the landmark-based geometric morphometric approach suitable for the discrimination of sympatric clupeid species and even among their local populations?

To answer these five questions, I addressed the following specific objectives in my studies:

2.1. Scale morphology of Mediterranean Teleost species

- Introduce categories of scale types and shapes to aid objective classification;
- Define the characters of scale morphology that enable the discrimination among a large number of species;
- Describe the discriminatory features of the scale characters in 80 species of Mediterranean teleosts;
- Code characteristic features of scale morphology and utilize the numerical data for a cluster analysis to create a dendrogram showing the phenotypic relations among different types of teleost scales;
- Illustrate the inter-specific and intra-individual variability of scales among 80 Mediterranean teleosts.

Clupeiform species such as herrings, shads, sardines, and their relatives possess derived cycloid scales that are easily distinguishable from other taxa by the presence of distinctive grooves and a membranous posterior field with a crenulated margin. However, the high degree of similarity in scale morphology between species and the plasticity within single individuals hinder species identification at least within families. To exemplarily reveal the inter-specific variability of scale shape in two clupeid species the following objectives were addressed:

2.2. Scale morphometry of sympatric clupeids

- Demonstrate the variability of scale shape within the European sardine (*Sardina pilchardus* Walbaum 1792) and the round sardinella (*Sardinella aurita* Valenciennes 1847);
- Describe the morphometric parameters of clupeid scales using relative size and shape indices;
- Apply multivariate statistical analyses in order to investigate the usefulness of scale shape indices to separate the two sympatric clupeids, as well as the scale sampling areas along the fish body.

2.3. Scale geometric morphometry of sympatric clupeids and their populations

- Apply landmark-based geometric morphometric analysis to investigate, whether the differences in clupeid scale shape are sufficient to allow a reliable discrimination between *S. pilchardus* and *S. aurita* as well as among their local populations;
- Propose a rapid stock assessment method for clupeid species to shed light on their population segregation thus providing valuable demographic information from an ecological and fishery management perspective.

3. Material and methods

3.1. Scale morphology

Scale samples for morphological description were obtained from 80 species, belonging to 50 families and 16 orders of teleost fishes occurring in the Mediterranean Sea. All scale samples were derived from the museum collection of the Deutsches Meeresmuseum in Stralsund, Germany. Each individual fish was measured and rinsed with distilled water before scale removal. Scales were taken from specifically pre-defined 10 body areas on the left side of each specimen. Scale material was stored in 70% ethanol, gently brushed to remove loose tissue remains and stained with Alizarin Red S (ROTH, Germany). Images were taken from

mounted scales (5 scales/ body area). For mounting, scales were transferred into 100% ethanol, followed by acetone and embedded into MOUNTEX (MEDITE, Germany) on object slides. Imaging was performed using a LEICA MZ75 microscope with a mounted camera DFC-425 and dedicated software (LAS, LEICA, Germany). The imaging software provided all pictures with correct scale bars. The Photoshop CS6 (ADOBE, United States) photo-editing program was used for composing the plates for each species. Definitions of scale types follow ROBERTS (1993) with some improvements. In this study, two main scale types (cycloid and ctenoid) and six subtypes (i.e., true cycloid, crenate, spinoid; and peripheral ctenoid, transforming ctenoid, whole ctenoid) are distinguished. In total, 56 scale characteristics were defined as main discriminative features to aid scale identification. The definitions of scale characteristics are based on LAGLER (1947) to promote congruence with the criteria used in previous works by other scientists. The observed scale shapes were categorized into five main types with a total of 18 subtypes. Each scale shape subtype was defined using geometric definitions. The characteristic features of scale morphology were coded in the form of a number and served as input into a dendrogram based on the Euclidean distances as a measure of dissimilarity. Due to the large variety of species and the small sample size (number of studied species) within some orders, only those orders were selected for the analysis that consisted of more than five studied species (i.e., four orders with 15 families and 37 species). Five scales per species were selected from a pre-defined sampling area (i.e., positioned in the anterior, dorsal region of the fish body above the lateral line). The predominant features of the scale characteristics were used to create the data matrix of coded characters. The between-groups-linkage method was applied as the clustering algorithm to gather the phenotypic relationships by using the software programme PAST v3.01.

3.2. Scale morphometry

To investigate whether scale morphometry allows differentiation between morphologically similar scales of clupeid species, techniques of traditional morphometry were applied. Scale samples were derived from two sympatric clupeid species, namely *Sardina pilchardus* and *Sardinella aurita*, from the Gulf of Ambracia, north-western Greece. In July and August 2014, 487 clupeid scales were investigated. In total, 219 individuals of *S. pilchardus* (mean SL ± SD: 81.65 ± 4.68 mm) and 268 individuals of *S. aurita* (mean SL ± SD: 155.35 ± 8.51 mm) were sampled (i.e., one scale per individual per body area). Scales were removed from 10 pre-defined body areas from the left side of the fish to avoid fluctuation asymmetry and auto-correlation problems among scales. The scale preparation

was followed in the same way as described above. Five morphometric parameters (i.e., the maximal longitudinal, vertical, and transverse diameter, perimeter, area) were measured to the nearest 0.1 mm on the digital images of scales using Photoshop CS6. Relative scale sizes (J-indices) were calculated following ESMAEILI (2001). Scale shape indices, including circularity, rectangularity, roundness, form-factor, and aspect ratio, were then calculated following TUSET *et al.* (2003). In scale shape analysis, to remove the effect of the fish size on the morphometric data (i.e., shape indices), standardized morphometric measurements were calculated following the method of THORPE (1975) and LLEONART *et al.* (2000) for each fish. Correlations between transformed variables and standard length were calculated to inspect whether the data transformation was effective in removing the size effect from the data. A non-parametric discriminant analysis using the normal kernel density estimation was applied to investigate the usefulness of scale shape in separating the two clupeid species. Cross validation was used to estimate the accuracy of classification rules. Comparisons of scale shapes between species and among their scale sampling areas were conducted using a permutation multivariate analysis of variance (PERMANOVA). The PERMANOVA was based on the Bray-Curtis dissimilarity measure (4999 random permutations). To present the major trends in shape differences among the sampling areas in both species graphically, cluster analysis of the adjusted shape indices was used. Similarity matrices were constructed based on Bray-Curtis' similarity. Analyses and tests were carried out using SPSS and PAST.

3.3. Scale geometric morphometry

A landmark-based geometric morphometric analysis was used to determine, whether the differences in clupeid scale shape are sufficient to enable a reliable discrimination between *S. pilchardus* and *S. aurita* as well as among their local populations. Six populations were sampled from four geographically different areas of the central and eastern Mediterranean Sea, i.e., northern Adriatic Sea, Inner Ionian Sea Archipelago, Gulf of Ambracia, and Gulf of Kavala. The scale samples were obtained from commercially caught specimens. Scales of *S. pilchardus* were sampled from all four study sites (i.e., 50 individuals from each site), whereas the same number of scales of *S. aurita* could be collected only from two study sites (i.e., Gulf of Ambracia and Gulf of Kavala). Scales were removed only from the left flank, below the dorsal fin above the lateral line following STASZNY *et al*. (2012) and stored in vials with 70 % ethanol. Only one randomly selected scale per vial was used for the analysis to evaluate a rapid stock discrimination methodology. Seven landmarks were recorded on each scale using "tpsDig2" v.2.17 utility program to identify the key features as suggested by

STASZNY *et al*. (2013). Shape data of the scales were processed with the software program "MorphoJ" v. 1.06d. First, group identities (by species or by sampling sites) were assigned to raw landmark coordinates. The centroid size (CS) was used as the size metric of the scales as the only mathematically shape-free size variable. To rotate, scale and align the raw coordinates into new shape variables, a Generalized Procrustes Analysis (GPA) was performed. Furthermore, a multivariate linear regression of shape (i.e., Procrustes coordinates) was performed on size (logCS) for each group to remove possible allometric effects. The significance of the relationship (i.e., the presence of an allometric effect) was evaluated by using a permutation test against the null hypothesis of independence (10,000 iterations). As data were free of allometric effects associated with growth, residuals of this regression provided the basis of further analyses. Finally, the differences between groups were assessed with a canonical variate analysis (CVA) and a discriminant function analysis (DFA) and examined the reliability of the results with permutation tests with 10,000 iterations.

4. Results

4.1. Scale morphology

The categorization of scale types, shape categories, and the scale characteristics were applied to 80 common species of Mediterranean teleosts. The description of scale morphology according to these criteria allowed the identification of a large variety of scales at species-level, which satisfies the postulated questions Q1 and Q2. To facilitate scale identification and the differentiation of closely related species, images of the scales from ten body areas were illustrated in the Appendix. The postulated Question 3 was approached by coding characteristic features of the scale morphology. In the depicted dendrogram, the scale characteristics of the 37 species cluster into two major groups (i.e., cycloid scales, including crenate and spinoid scales clustered separately from ctenoid scales). In the first cluster (ctenoid scales), scale characteristics were found to be similar among the majority of studied species that belong to the series Percomorpha of the superorder Acanthopterygii (i.e., Perciformes and Pleurinectiformes). In the second cluster (true cycloid, crenate, spinoid scales), similarities were found among basal teleosts, i.e., Clupeiformes and some representatives of the superorder Paracanthopterygii (i.e., Gadiformes). The scale characters of Macrouridae (spinoid scales) showed stronger similarity with Clupeidae (crenate scales) than with other representatives of Gadiformes. This further supports the existence of shared plesiomorphic scale characteristics among basal teleost species.

4.2. Scale morphometry

Traditional morphometry and shape indices in *S. pilchardus* és *S. aurita* proved to be effective tool for discrimination morphologically similar scales from each other. The usefulness of scale shape indices to separate the two sympatric clupeids from each other was tested. The effect of fish size on scale shape was eliminated successfully. The size correction greatly reduced the correlation between scale shape and fish lengths (P>0.05). The discriminant analysis between *S. pilchardus* and *S. aurita* resulted an overall percentage of correct classification of 76%. The comparisons of shape indices using a PERMANOVA test yielded significant differences among sampling areas in both species; F = 15.84, P = 0.0002 in *S. pilchardus*, and F = 26.55, P = 0.0002 in *S. aurita*. The two-way PERMANOVA test performed on combined shape indices yielded significant differences among the sampling areas of both species (F = 35.16, P = 0.0002) and between the two species (F = 17.70, P = 0.0002). The cluster analysis outputs depicting the linkage dendrogram (Bray-Curtis' similarity) computed on scale shape indices among the sampling areas in both species demonstrated similarities among specific body regions. In *S. pilchardus*, sampling areas with similar scale shape values were found to segregate along the antero-posterior axis. In *S. aurita*, on the other hand, the segregation of the sampling areas according to their scale shape similarities occurs along the dorso-ventral axis.

4.3. Scale geometric morphometry

In this study, four populations of *S. pilchardus* and *S. aurita* were tested whether the mean scale shape is applicable in separating species and their populations by using landmark-based geometric morphometry. The mean scale shape of the two species showed no overlap in a CVA, and it resulted significant (P < 0.001) differences among all four populations of *S. pilchardus* (i.e., Adriatic, Ionian, Ambracian, and Kavalan). The two populations of *S. aurita* could also be distinguished from each other with high reliability (P < 0.0001). The validity of the results was also confirmed by the large proportion of correct classifications with an average discrimination rate as high as 98.6 %. The results further indicated that the scale shape of the local population of *S. pilchardus* from the Gulf of Kavala (i.e., eastern Mediterranean Sea) is easily distinguishable from the mean scale shapes of those conspecifics sampled in the central Mediterranean Sea.

5. Summary

Fish scales possess valuable information on many aspects of the bearers' biology which can be equally useful in systematics and ecology. However, a suitable approach of retrieving this information is essential.

The primary aim of this study to establish a system that facilitates scale identification in the common Mediterranean teleost species has been successfully achieved with the creation of a scale atlas. The novel classification system of scale morphology, introduced in this study, not only provides an orientation among the vast diversity of scales, but also aids the specific identification. Furthermore, the well-defined categories provide an opportunity for integration of scale descriptions and for transformation of scale characteristics for numerical analysis. Among the characteristic features, scale type and the shape of the scale, as well as the distribution of grooves or ridges on the scale's surface are most useful to determine a taxon. Type, shape and ornamentation of single scales allow the designation to certain taxa; in many cases to species level. In some cases, however, scale features show a high degree of plasticity and vary significantly within a single specimen depending on the body area sampled. Therefore, the representation of scales from several body areas is important when producing a scale catalogue for species identification.

In a phylogenetic context, the classification of scale types found in this study differs from traditional views by assigning crenate and spinoid scales as subtypes of cycloid instead of ctenoid scales. Evidence from previous ontogenetic studies of scale development and juvenile structures retained in adult scales had suggested already that crenate, spinoid and ctenoid scales each develop from a generalized cycloid state. Ctenoid spines in teleosts were already shown to be derived structures rather than plesiomorphic characters, which further supports their separation from other forms of spined scales (i.e., crenate and spinoid). The classification of the scales of 80 Mediterranean teleosts revealed some phylogenetic relationships among the major teleost groups. The phenotypic relationships among scale characteristics showed that ctenoid scales with transforming cteni clustered among representatives of the orders Perciformes and Pleuronectiformes. In contrast, the rather basal forms of scale types, e.g., crenate scales of the Clupeidae and spinoid scales found among the Macrouridae, segregated in their morphological characteristics from the scales of phylogenetically advanced taxa.

Although the morphological analysis is the primary step towards successful identification, in some cases scale morphology alone does not allow a reliable species determination. Therefore, the second aim of this study was to apply additional methodological approaches to enable a successful differentiation in a model system of morphologically similar scales of sympatric clupeid species. On the basis of the morphometric shape analysis, the scales of European sardine *Sardina pilchardus* and round sardinella *Sardinella aurita* from the eastern Mediterranean Sea were successfully separated by discriminant analysis based on the standardized scale shape indices. Although there are body areas with higher degrees of similarity in scale shape within the same species, the multivariate analysis on combined shape indices yielded significant differences between the two species. The application of morphometric analysis using scale shape indices helps to overcome the limitations of morphological analysis, thus aiding more reliable species identification. Furthermore, the morphometric analysis revealed size and shape differences of scales along the fish body. Changes in scale size and shape along the body appear to be related to the curvature and the swimming mode in different taxa. Consequently, the similarity among different species in patterns of scale shape variation across the fish body might become the object of further analyses to shed light on the differences in morphology and phylogenetic relationships.

Beyond species determination, the separation of the local populations of *S. pilchardus* and *S. aurita* based on scale shape differences was attempted. Landmark-based geometric morphometric analysis was applied to reveal differences in scale shape between the two clupeids as well as among the local populations of each species. The findings indicated that scale shape of *S. pilchardus* and *S. aurita* from the central and eastern Mediterranean Sea form separate morphometric groups, thus supporting previous genetic studies that suggested a genetic differentiation due to isolation by distance. The geometric morphometric method allows rapid differentiation between two clupeid species providing an insight into the segregation of their populations.

In conclusion, fish scale analysis has proven to be a useful tool in species identification and many other research fields. The utilization of the established classification system of scale morphology has the potential to unify scale descriptions among studies which could not only integrate scale identification but would also facilitate the exploitation of the phylogenetic information stored in scale morphology.