A geometric morphometric approach to the analysis of the shape variability of the haptoral attachment structures of *Ligophorus* species (Platyhelminthes: Monogenea)

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Abstract

The taxonomy of *Ligophorus* Euzet & Suriano, 1977, like the most of monopisthocotylean monogeneans, relies heavily on the morphology of sclerites of the posterior attachment organ (haptor). Geometric morphometric approach is used to analyse variability and compare the shapes of haptoral structures of these monogeneans. We outline the shapes of the sclerites by cubic Bezier curves and store results in SVG files. Every SVG outline is reduced to a set of harmonics of Elliptic Fourier transform using ElFourier program. Harmonics are the sequence of unique numbers that describe the shape of structures and are invariant to their sizes, rotation, and orientation. They allow reconstructing source outline images, finding their average form, analyzing variability and comparing shapes in combination with other numerical data like dimensions. We use that approach to investigate intra- and interspecific variability of 400 haptoral structures of seven representatives of *Ligophorus*, parasitising four mullet species from the Black Sea, and to discriminate these monogeneans. This method is perspective for the creation of semiautomatic key for identification of helminthes, which are mainly distinguished by the shape and dimensions of the attachment organs. The obtained results and method prospects are discussed.

Key words: Monogenea, *Ligophorus*, attachment organs, haptoral structures, morphology, shape, intra- and interspecific variability, species discrimination, geometric morphometry, Elliptic Fourier transformation.

Introduction

The taxonomy of *Ligophorus* Euzet & Suriano, 1977, like the most of monopisthocotylean monogeneans (Platyhelminthes), relies heavily on the morphology of various sclerotized structures (anchors, bars, marginal hooks, etc.) of the posterior attachment organ, called haptor or opisthaptor. Their morphology and morphometry are extremely variable between monogenean species (Boeger & Kritsky 1993), which allows using them as diagnostic characters in taxonomy and correctly identifying most monogenean species (Pugachev et al. 2009). However, several studies demonstrates that some of these morphometric characters are highly intercorrelated (e.g. Mariniello et al. 2004; Dmitrieva et al. 2007) and vary intraspecific variable (e.g. Caltran et al. 1995; Mariniello et al. 2004), which reduces their usefulness to discriminate monogeneans, especially closely related species.
Genus *Ligophorus* includes 65 nominal species (Dmitrieva et al. 2012, 2013; Sarabeev et al. 2013; El Hafidi et al. 2013a, b; Kritsky et al. 2013; Soo & Lim 2015; Soo et al. 2015). Some of these species are morphologically very close. It is difficult to distinguish them, using dimensions of sclerotized structures (Dmitrieva et al. 2007; Marchiori et al., 2015).

Since dimension is a linear distance between the pair of points (landmarks), which are located in the predefined and identical positions for the same structures, the shifts between coordinates of homological landmarks, belonging to different exemplars, describes the differences in their shapes. The analysis of these displacements allows discriminating shapes of objects. This approach belongs to geometric morphometric methods (Rohlf & Marcus 1993; Adams et al. 2004, 2013) and is wide used in biology, including study of variability, functional morphology, taxonomy, and phylogeny of monogeneans (Vignon & Sasal 2010; Vignon et al. 2011; Llopis-Belenguer et al. 2015; Rodríguez-González et al. 2015, 2016, 2017; Khang et al. 2016). Advantages vs. disadvantages of traditional (based on linear distances) and geometric morphometric methods, namely actual GMM and EFA, to study monogenean haptoral structures are discussed in detail by Vignon (2011).

Elliptic Fourier analysis (EFA) is based on the analysis of the object outlines. It takes into consideration the full information about the shape of structures (Vignon 2011). The obtained data consists of digital shape descriptors, which could be analyzed using multivariate statistical methods. Because a set of descriptors contains maximal amount of data about a shape, it could be reduced to needed precision according to the specific requirements.

The latter approach is first used in the present work to discriminate the attachment structures of the haptor of the representatives of the genus *Ligophorus*.

**Material and Methods**

A total of about 400 haptoral structures of seven representatives of *Ligophorus*, parasitizing four mullet species from the Black Sea, were studied.

To compare shapes of *Ligophorus* haptoral anchors and ventral bars, outline analysis was carried out. Photos of haptoral structures were obtained using CMOS camera of Olympus CX40 light microscope. The images of anchors and bars were outlined by cubic Bezier polylines in Inkscape vector editor (inkscape.org). The resulted vector outlines were saved in SVG files (fig. 1, A).

Outlines were used to produce shape descriptors, invariant to shape position, rotation, and scaling. We describe outlines by components of Elliptic Fourier transformation, which is named elliptic Fourier descriptors (EFD) or harmonics. Each harmonic consists of four numbers. Every number is a coefficient before sinus or cosines. The sum of harmonics determines two function (for x and y coordinates) that allows restoring an original outline. This technique was firstly proposed by Giardiana & Kuhl (1977), and computational details were described in a number of works (e.g. Ferson et al. 1985).

The algorithm of harmonic normalization (Neto et al. 2006) makes harmonics invariant to rotation, translation or scaling of source object, and also to the position of the starting point of digitization. Those harmonics are normalized to the value of the first component that makes them invariant to the object dimensions. Such harmonics are named as size-invariant.

Second type of EFDs, whose components are not normalized to the value of the first harmonic component, are named as size-considered.

The sets of normalized size-invariant and not normalized to dimensions size-considered harmonics were produced using ElFourier (Lyakh, 2017). This program converts Bezier polylines stored in SVG files to the predefined number of harmonic components (fig. 1, B).

The inverted elliptic Fourier transformation converts harmonics to restored outline that differs from the source one. The difference depends on the number of used harmonics, the more harmonics are used, the less the difference. The relative value of error could be calculated (Ferson et al. 1985) or visually estimated. We find fifty harmonics for each outline, because the restored outlines visually perfectly confine with source objects (fig. 1, B).

Harmonics of different objects could be used to construct an average shape of those objects. To do so it is necessary to average harmonics values. In such manner we restore an average shape of the anchors of exemplars, belonging to one species.
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Figure 1. A Ligophorus szidati dorsal (top) and ventral (bottom) anchors were outlined by cubic Bezier polylines and stored in SVG files. B EFourier computer program converted digitized outlines into 50 EFDs. Only first four harmonics are visible at the screenshot’s bottom; the negative components of harmonics are colored in light gray. The restored outline perfectly satisfies the shape of anchor (red line around gray anchor). At the left side used anchors are shown; they differ by orientation of blades and direction of digitization; outlines oriented counterclockwise are filled.

In the following analysis we test:
- is it possible to separate dorsal and ventral anchors?
- is it possible to separate anchors and ventral bars of different species?
- is the best result for species discrimination obtained when using size-considered harmonics, rather than size-invariant ones?
- is it possible to reduce the number of harmonics and get the same results?
- is the best result for species discrimination obtained when using the combination of the shape descriptors of all haptoral structures belonging to each specimen?

To analyze data we use principal components analysis (PCA) and hierarchical cluster methods.

Figure 2. A After the automatic normalization, the outlines of anchors still have different orientation of the blades, different positions of the digitization starting point and directions of digitization; this affects the signs of the first harmonic components, which are shown in pink rectangle, and the signs of identical components differ. B, C After manual correction of the orientation of anchors (B) and bars (C), the outlines and signs of first harmonic components are identical.
Results

The calculated harmonics could not be directly compared with each other, because the signs of the calculated harmonic components depended on the orientation of the anchors and blades outlines, the direction of the digitization (clock- or counterclockwise), and the position of the digitization starting point (fig. 2, A). The ElFourier program tools applied to the outlines allow mirroring anchors so that the blades are directed to one side (left, in our case), changing the direction of all outlines to the clockwise orientation and placing the outlines starting points on the blades (fig. 2, B). Similar operations are applied to the outlines of bars (fig. 2, C).

Is it possible to separate dorsal and ventral anchors?

Size-invariant EFDs. The harmonics of dorsal and ventral anchors are well differentiated on two groups (fig. 3 A), besides of the anchors of two species, namely all dorsal anchors of *L. mediterraneus* and some dorsal anchors of *L. vanbenedenii*, belonging to the cloud of the ventral anchors (fig. 3 B). On the other hand, the dorsal and ventral anchors of *L. szidatti* well separated from each other and from the anchors of other species (fig. 3 B).

Size-considered EFDs. The full set of the size-considered harmonics shows the similar result as in the previous case (fig. 3 C vs. 3 A). However, the ventral anchors form a more compact group, slightly overlapping with the dorsal anchor of two species, viz. *L. cephalii* and *L. mediterraneus* (fig. 3 D). The dorsal anchors of other five species are well separated from the ventral anchors (fig. 3 D).

**Figure 3.** PCA of the size-invariant (A, B) and the size-considered (C, D) harmonics of all dorsal and ventral anchors of analyzed *Ligophorus* species. Left graphs (A, C) are based on fifty harmonics; right graphs (B, D) – on four ones. Keys: dots – dorsal anchors; triangles – ventral anchors.
Is it possible to separate anchors and ventral bars of different species?

**Size-invariant EFDs.** The harmonics of both dorsal (fig. 4 A) and ventral (fig. 4 C) anchors of most species form one rather compact cluster, excluding the anchors of *L. szidati*.

**Size-considered EFDs.** When considering the size, besides the anchors of *L. szidati*, both dorsal (fig. 4 B) and ventral (fig. 4 B) anchors of *L. vanbenedenii* are also separated from the anchors of other five species. The latter form overlapping groups (fig. 4 B, D).

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**Figure 4.** PCA of the size-invariant (A, C, E) and size-considered (B, D, F) harmonics of the dorsal (A, B) and ventral (C, D) anchors, and the ventral bars (E, F) of *Ligophorus* species. All graphs are based on fifty harmonics. Keys: dots – dorsal anchors; triangles – ventral anchors; rhombus – ventral bars.
Both size-invariant and size-considered EFDs of ventral bars outlines overlap in PC-plane (fig. 4 E, F). However, if consider size of bar, two species, viz. *Ligophorus pilengas* and *L. llewellyni*, form one cluster rather separated from the others (fig. 4 F).

Is the best result for species discrimination obtained when using size-considered harmonics, rather than size-invariant ones?

Thus, the size-considered harmonics (fig. 3 C, D; fig. 4 B, D, F) slightly better separate dorsal anchors from ventral ones, as well as anchors and bars, belonging to different species, than size-invariant ones (fig. 3 A, B; fig. 4 A, C, E).

Is it possible to reduce the number of harmonics and get the same results?

To test the possibility of the minimizing the number of harmonics, the same statistical analysis were applied to the 50 EFDs set (fig. 3 A, C) and to the reduced EFDs set consisting of 4 harmonics (fig. 3 B, D). Calculations show that the first four harmonic produce very similar results. Therefore, it is possible to use only four EFDs instead of fifty, at least, in relation to the studied structures of analyzed *Ligophorus* spp.

Is the best result for species discrimination obtained when using the combination of the shape descriptors of all haptoral structures belonging to each specimen?

To answer this question, the set of 59 (number of the specimens for which data for all analyzed haptoral structures are available) combinations of the EFDs, describing the shapes of the dorsal and ventral anchors, and the ventral bar (4 EFDs for each structure) of each organism was used (fig. 5).

Size-invariant EFDs. Combination of size-invariant EFDs allows separating only the specimens of *L. szidati*. Other specimens form two clusters. The first one includes specimens of two species, *L. cephalii* and *L. mediterraneus*, and the second one consists of four species, *L. llewellyni*, *L. pilengas*, *L. vanbenedenii*, and *L. acuminatus*. Inside the latter cluster, *L. acuminatus* differs from other three species (fig. 5 A, B).

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**Figure 5.** Cluster (A, C) and PC analysis (B, D) of the combinations of four harmonics for each dorsal and ventral anchors, and ventral bar obtained for each *Ligophorus* specimens. Upper graphs (A, B) are based on the size-invariant EFDs; lower graphs (C, D) – on the size-considered EFDs.
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Size-considered EFDs. Combination of size-considered EFDs changes the structures of the clusters. In that case, already two species, namely *L. szidati* and *L. vanbenednii*, are well separated from the others (fig. 5 D). In addition, the remaining five species are clearly divided into two clusters with morphologically similar pairs of species: *L. llewelyni* and *L. pilengas*, *L. cephalii* and *L. mediterraneus*. The last cluster also includes *L. acuminatus* exemplars, which, however, form a compact group that is especially evident in the dendrogram (fig. 5 C).

Thus, the combination of harmonics of all haptoral structures for each specimen better discriminates species than the harmonics of every type of structures alone; and the combination of size-considered harmonics is the best discriminator of the species, and allows separating five of the seven analyzed species of *Ligophorus*.

Discussion

The obtained results show that the shapes of the haptoral attachment structures (dorsal and ventral anchors, and ventral bar) of the seven *Ligophorus* species are rather morphologically similar, and they are not clear divided into speci groups by each set of EFDs alone (fig. 4). Similar results were previously obtained for 299 monogenean species (Vignon 2011: fig. 6).

The shapes of anchors of only two species, *L. szidati* and *L. vanbenednii*, were perfectly separated from the others with the help of size-considered EFDs. Anchors of *L. vanbenednii* are smallest; anchors of *L. szidati* have an unique shape (fig. 6). The anchors of *L. llewelyni* and *L. pilengas* have similar shapes and dimensions, as well as those of *L. cephalii* and *L. mediterraneus* (fig. 6), therefore they are difficult distinguished from each other visually and also with the help of EFDs.

![Figure 6. Combination of the outlines of all dorsal anchors of each analyzed Ligophorus species (other haptoral structures outlines see http://marineparasites.org/morphometry/](http://marineparasites.org/morphometry/)

When using the size-considered harmonics of both pair of anchors, unique anchors of *L. szidati* and *L. vanbenednii* are well separated, and groups of morphologically similar species, namely *L. llewelyni* and *L. pilengas*, *L. cephalii* and *L. mediterraneus*, are also slightly distinguished from each other (fig. 4 B, D).

The best result for species discrimination was obtained when using a combination of size-considered harmonics of all analyzed structures for each specimen (fig. 5 C, D).

The geometric morphometric approach has been used in some articles (Rodríguez-Gonzáleze et al., 2016, 2017, Khang 2016) to test the phylogenetic signal in the morphology of anchors in representatives of *Ligophorus* to determine the convergence or shared evolutionary history of anchor shape within the genus. These studies suggest that a common ancestor and a shared evolutionary history play an important role in determining the shape of anchors in *Ligophorus* spp. Other results show that the shape of monogenean anchors is determined by adaptation to the host and to specific attachment sites on the host gills (Šimková et al. 2002, Poisot et al. 2011). Our work is devoted to the search for approaches for clear discrimination of *Ligophorus* species by morphological characters, but some results can be discussed from the point of view of evolutionary morphology.

Each pair of species having morphologically undivided anchors and bars parasitizes the same host species (*Ligophorus llewelyni* and *L. pilengas* – *Liza haematocheila*; *Ligophorus cephalii* and *L. mediterraneus* – *Mugil cephalus*), what can explain the similarity of their haptoral structures by close
relationship between them and their origin from a common ancestor, which diverged within the same host. Moreover, these pairs of species form terminal clades on the phylogram of *Ligophorus* spp. obtained from the maximum likelihood analysis using ITS-1 regions (Blasko-Costa et al., 2012).

On the other hand, such species as *L. vanbenedenii* and *L. szidati*, also infecting the same host species, have completely different attachment structures. Earlier it was shown that these species differ in the sites of microlocalization on the host gills (Pronkina et al., 2010). Adaptation to different conditions of attachment, possibly, leads to differences in the haptoral structures morphology in these two species.

Despite the some analyzed *Ligophorus* species were not clearly separated, obtained results allow to consider the geometric morphometric methods, in particular the Elliptic Fourier analysis, as a perspective approach for the creation of semiautomatic key for identification of helminthes, which are mainly distinguished by the shape and dimensions of the attachment organs.

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