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SHAPE ANALYSIS OF OTOLITHS OF THE ROUND GOBY, *NEOGOBIUS MELANOSTOMUS* (GOBIIFORMES, GOBIIDAE), FROM THE BLACK SEA BASIN

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Shape Analysis of Otoliths of the Round goby, *Neogobius melanostomus* (Gobiiformes, Gobiidae), from the Black Sea Basin. Zamorov, V., Zamorova, M., Krupko, D., Matvienko, N., Leonchyk, Y., Kvach, Y. — The aim of this study was to assess the discriminability of the stocks of the round goby *Neogobius melanostomus* based on the shape of its otoliths. Recent otolith-shape-based species and stock discrimination studies were using otolith contours in sagittal plane and we are following this approach. We hypothesized the possibility of existence of several geographically separated populations of the round goby. Round gobies have been sampled from different locations of the North-Western Black Sea, otoliths were removed in course of the full biological analysis and photographed in sagittal plane. Principal components of the otolith contour were processed by linear discriminant analysis aiming to cross-validate the discriminability of round gobies placed at different geographical locations. This would allow demonstration of different stocks or populations. This research allows to conclude the limited applicability of otolith contours for discrimination of stocks or populations of round goby based on multiple annual samples. However, neither classification matrices of discriminant analysis nor cluster analysis dendrograms showed a single pattern except for the high year to year otoliths variability. This allows to hypothesise a strong response of contour formation to habitat and feeding conditions. However, this assumption needs to be verified by further studies.
Key words: morphology, ear stones, bony-fishes, Ponto-Caspian gobies.

Introduction

Fish otoliths are stone-like structures of the internal ear of a fish, reflecting the physical and chemical characteristics of the ambient water (Campana et al., 1995).

Otoliths are usually used for the stock discrimination study (Campana & Casselman, 1993), as well as in taxonomy studies, especially in paleoichthyology, because they provide a crucial evidence for potential parallel taxonomy (Schwarzhans et al., 2017). The otoliths shape and morphology has been often linked to the ecological, taxonomical phylogenetic and functional characteristics of species (Vignon & Morat, 2010; Tuset et al., 2016).

Stock discrimination is an important procedure in course of commercial fishery management and invasive species management. Attempts of commercial fish stock discrimination based on otoliths sagittal contour have been made dating back to the end of the 1980s. Publications summarizing certain success in addressing this challenge (Orlov & Afanasyev, 2013), are concomitant with critical articles pointing out the substantial otolith variability within the aggregations themselves due to fish age, gender and year of sampling (Bird et al., 1986; Castonguay et al., 1991; Campana & Casselman, 1993), impeding the discrimination of the intraspecific localities. The otolith sagittal contour analysis is generally more effective for interspecific identification than discrimination which was confirmed using Fourier analysis of the cases with gobiiform fish (Yu et al., 2014). Recent otolith-shape-based species and stock discrimination studies were using otolith contours in sagittal plane and we are following this approach.

It has to be emphasized that major part of recent otolith-shape-based stock discrimination studies which have been reporting good stock discrimination results had been based on a single-year materials and sometimes on analysis of materials collected during a single-voyage (Ladroit et al., 2017; Menezes & Rahnama, 2019; Rashidabadi et al., 2020; Reig-Bolaño et al., 2011; Vieira et al., 2014).Very few studies based on multiple annual samples reported less optimistic success, if any (Agüera & Brophy, 2011; Mahé et al., 2019). Particular single-year studies failed to discriminate geographically separated stocks as well (Yu et al., 2014; Zhang et al., 2016; Zhao et al., 2017, 2018; Song et al., 2018, 2019, 2020).

Round goby is a commercial game fish in the Black Sea and includes aggressive invasive species in Europe and North America (Brown & Stepien, 2008, 2009). Population structure of the round goby *Neogobius melanostomus* (Pallas, 1814) (Actinopterygii: Gobiiformes, Gobiidae) is important owing to fast expansion of its range in Danube, Baltic and North Sea basins (Roche et al., 2013; Ojaveer et al., 2015) and in the North American Great Lakes (Brown & Stepien, 2009). Recent research dedicated to determining the source of the Great Lakes invasion by the round goby designates as the Dnipro River near the Kherson Marine Port (Brown & Stepien, 2009). Therefore, its stock and structure of populations are currently of utmost importance in many countries. We hypothesized existence of several geographically separated populations of the round goby in Southeast part of the Black Sea and aimed to verify it using otolith contours.

Thus, this research aimed to assess the otolith-shape-based method of stock discrimination using multi-annual round goby materials, which were sampled in different locations of the Black Sea during 2008–2019.

Material and methods

The round gobies were sampled in nine locations between 2008 and 2017 (fig. 1; see supplementary table S1 for details). The standard length (SL, cm) of each sampled fish was measured first; this ranged from 7.0 to 11.9 cm. The females were sporadic in sampling and their sizes were less than 7 cm, therefore, only males were used in the study. It was also reasonable to avoid skewing caused by differences in otolith shape among genders.

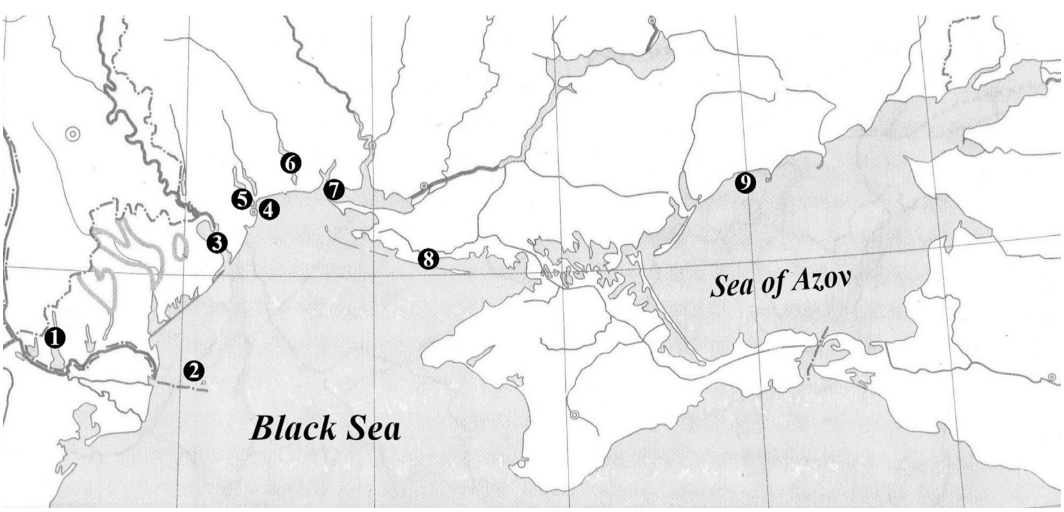

Fig. 1. Map of the study area with sampling localities: 1 — Lake Yalpuh; 2 — Snake Island; 3 — Dniester Estuary; 4 — Gulf of Odesa; 5 — Khadzhibey Estuary; 6 — Tylihul Estuary; 7 — Dnipro-Bug Estuary; 8 — Dzharylhach Bay; 9 — Obytichna Bay of the Sea of Azov.

In total, fish 784 individuals of were dissected and otoliths were isolated. Due to known asymmetry of the left and right otoliths (Mille et al., 2016; Mahé et al., 2019) only the left otoliths were used for this analysis. Otoliths were photographed with Scopetek DEM-130 (1.3 Mpix) USB 2.0 camera mounted on binocular microscope Konus Crystal-45 at magnification of 10 × 0.7 (resolution 1280 × 1024 px). The otoliths were positioned with the slide acoustic groove down (fig. 2). Square-shaped metal plate 2 × 2 mm was placed alongside of each otolith for picture scaling. Shape v. 1.3 software was used for the comparative study of the otoliths based on their digitalized pictures (Iwata & Ukai, 2002). This software package allows representing the target closed contour as elliptic Fourier descriptors (FC) for a given number of harmonics. FC obtained were normalized (by the first harmonic which is a simple ellipse), so that they were made invariant with the size of the target contour with rotation and starting point of the contour analysis. In order to estimate the harmonics number, which is necessary and sufficient for analysis, the value of contribution of each harmonic in contour description (Fourier Power, FP) (Lord et al., 2012; Bonhomme et al., 2014) was calculated based on values of FC obtained with Shape v. 1.3 software. Coefficients of the first harmonic were omitted, for this harmonic is a simple ellipse and skews the rest of coefficients. Mathematical tools for the FC computing are described in Kuhl & Giardina (1982).

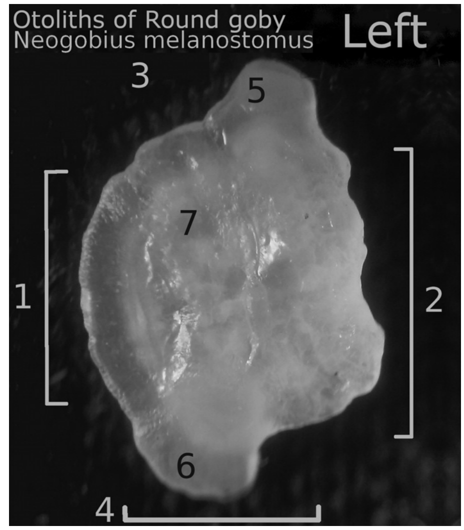

Fig. 2. Basic morphometric characters used for the shape description of the round goby otoliths. At the internal surface: 1 — dorsal part; 2 — ventral part; 3 — anterior margin; 4 — posterior margin; 5 — rostrum; 6 — pararostrum; 7 — acoustic groove.

The analysis was provided in four stages according to the accumulation of samples. After each location has been sampled one year long, acquired material has been processed. After samples were obtained at each location for the two year period of time, corresponding samples were stacked and processed using the same approach. After enough samples from each location for three years has been collected, corresponding samples have been stacked again and processed using the same approach. Stacked samples were used for cross-validation of the mathematical model the study has been verifying (discriminant analysis of otolith contours described by Fourier descriptors). It has to be verified, whether otoliths collected within one year, could be used for stock discrimination in the following years. Sedentary way of life of *N. melanostomus* and geographic isolation of our sampling sites excluded the stock mixing bias, which was affecting the samplings in Mediterranean in 2013–2016 (Mahé et al., 2019) and in Atlantic in 2008–2009 (Agüera & Brophy, 2011).

The first stage of the study included otoliths, sampled from each locality during a single year. At this stage, the otoliths of round gobies collected in certain locality in a single year were analyzed: in the Obytichna Bay in April–June 2016, in the Dzharylhach Bay in November 2017, in the Dnipro Estuary in September 2017, in the Tylihul Estuary in September 2011, in the Gulf of Odesa in August–September 2010, in the Khadzhibey Estuary in October 2011, in the Dniester Estuary in May 2008, in the Snake Island waters in August–November 2010, and in Lake Yalpuh in October–November 2015 (totally, 322 otoliths).

As mentioned previously, otolith contour can be described with Fourier descriptors (harmonics). Parsimonious number of effective harmonics has been determined according to the method described by Claude (Claude, 2008). Specifically, harmonics are considered as effective if their accumulated Fourier Power (FP) exceeds 99 % (Claude, 2008). Next, in order to determine the variation across the sampling sites, otolith contours have been transformed into the dimensions of principal components (N-dimensional space, where N is the number of principal components). Number of principal components corresponding to 9 harmonics produced by Shape v. 1.3 software amounts to 33 and has to be made parsimonious as well. Number of effective principal components has been determined by Shape v. 1.3 software based on mathematical algorithm underlying this software (Iwata, 2002). Shape v. 1.3 automatically determines the first six harmonics as effective. This is attributed to their contribution to variation exceeding 3 %. Threshold of 3 % was calculated by the Shape v. 1.3 software for this particular dataset based on the entire number of harmonics and thus cannot be voluntarily changed by the software users (Iwata, 2002). Except for their numeric contribution to the otoliths variation, we sought to assess which characters of otolith shape are attributed to principal components.

The second stage included otoliths, sampled from each locality within two years. This material was sampled in the same sampling areas, as at the first stage. Round gobies were sampled in course of two surveying seasons (and only in one sampling area — during one season) in following years: in the Obytichna Bay in September and November 2017, August and September 2018; in the Dzharylhach Bay in September–October 2012 and October 2016; the Dnipro Estuary in October 2009; the Tylihul Estuary in November 2009 and October 2012; the Gulf of Odesa in September 2009 and September 2011; the Khadzhibey Estuary in October–November 2012 and in September 2016; the Dniester Estuary in August 2016 and April 2017; the

Snake Island waters in August 2008 and May 2014; Lake Yalpuh in June 2011 and in October 2017 (totally, 496 otoliths).

The third stage included otoliths, sampled from each locality during all three years (totally 777). Entire material collected in course of all three years of study in each sampling area has been processed by means of hierarchical cluster analysis using Weighted Pair Group Method with Arithmetic Mean (WPGMA).

The fourth stage included otoliths, sampled from same locality during a year, in the case when the number of fish was not less than 14 ind. Within the fish group where individuals were of 7.0–9.0 cm size the comparison was made between two different water bodies during a year. Thereby, within the group of 9.1–12.0 cm these were compared between two or three different bodies of water. Firstly, the relation of a fish individual to the particular body of water using the data per a year was determined statistically. Then, their relation using all the dataset (all years of the study) to each particular year was determined as well.

Considering findings of previous studies which revealed considerable otoliths' variability among different fish size classes and sampling years we divided our material into size classes for further research purposes (Campana & Casselman, 1993; Mahé et al., 2019). Based on the results of previous morphologic research of round goby, individuals were divided into two groups based on their sizes. The first one contained individuals with the size from7.1 to 9.0 cm and the second one — from 9.1 to 12.0 cm in length (Diripasko & Zabroda, 2017).

Discriminant function analysis (DFA) has been performed in order to assess the accuracy of assignment of round goby individuals to certain sampling area based on the otolith shape. All Principal components (PC) and otolith surface area index (ratio of otolith surface area to the squared SL) were submitted to the DFA. Wilks' lambda value resulting from the DFA amounted to 0.02 ($p < 0.0001$, $F = 10.76$), also indicating that discrimination between groups under study is significantly high. Wilks' lambda, varying from 0 to 1, assesses the quality of discrimination. Values of Wilks' lambda, tending to 1, speak for the low discrimination level, and tending to 0 — for the high level of discrimination.

In order to get the goby otoliths visualized features attributed to six effective principal components contours of the otoliths were reconstructed. The reconstruction has visualized the contour features based on the first six principal components: first PC (accounting for the greatest percent of variance) describes the shape of the otolith ventral part and is predicated upon extent of notches in the dorsal part; second PC describes otolith shape traits related to width of the otolith and extent of irregular features (notch or prominence) in the dorsal part of the otolith; third PC describes the variability of the pararostrum and of the greater part of the contour of the dorsal part of the otolith; fourth PC describes the variability of the pararostrum contour adjacent prominence of the ventral part of the otolith; fifth PC describes the variability of the rostrum contour; sixth PC describes the variability of the dorsal part of the pararostrum.

Dendrograms were built using hierarchic clustering algorithms which are based on the same principal components used for discriminant analysis. The analyses were performed using the Statistica for Windows 10 software.

Results

First stage

In our samples first nine harmonics accumulated Fourier Power (FP) exceeds 99 % (table 1).To visualize the otolith features attributed to effective principal components contours of the otoliths were reconstructed based on the average values of normalized Fourier descriptors (fig. 3). This reconstruction has visualized the contour features predicated upon the first six principal components:

1) First PC (accounting for the greatest percent of variance) describes the shape of the otolith's ventral part and is based on extent of notches in the dorsal part;

2) Second PC describes otolith shape traits related to width of the otolith and extent of irregular features (notch or prominence) in the dorsal part of the otolith;

3) Third PC describes the variability of the pararostrum and of the greater part of the contour of the dorsal part of the otolith;

4) Fourth PC describes the variability of the pararostrum contour adjacent prominence of the ventral part of the otolith;

5) Fifth PC describes the variability of the rostrum contour;

6) Sixth PC describes the variability of the dorsal part of the pararostrum.

Following the results of descriminant function, the aposterior accuracy of assignment of round goby individuals to certain sampling area amounted to 69.3 % (table 3).

Table 1. Number of harmonics of round goby otolith contour necessary for analysis according to Fourier Power

Harmonic	Average values of Fourier coefficients					Fourier Power
i	a_i	b_i	c_i	d_i	FP_i	FP accumulated, %
			I Stage			
2	−1.82E-02	1.05E-02	1.99E-02	−4.96E-04	0.000418	10.4
3	5.61E-02	-1.93E-02	−1.17E-02	−2.19E-02	0.002068	61.8
4	5.35E-03	7.25E-03	5.95E-03	−3.06E-03	6.3E-05	63.3
5	3.76E-02	−1.15E-02	2.33E-02	2.50E-03	0.001047	89.4
6	2.18E-03	9.39E-03	4.74E-03	6.62E-03	7.96E-05	91.3
7	1.83E-02	4.19E-03	2.11E-03	1.01E-02	0.000229	97.0
8	−1.64E-03	8.34E-03	−5.36E-03	1.33E-03	5.14E-05	98.3
9	3.84E-03	9.74E-04	6.66E-04	8.04E-03	4.04E-05	99.3
10	−2.89E-03	2.23E-03	−4.47E-03	1.45E-03	1.77E-05	99.6
			II Stage			
2	−1.69E-02	1.65E-02	2.01E-02	−3.21E-03	0.000485	11.7
3	5.70E-02	−1.87E-02	−9.86E-03	−2.27E-02	0.002106	62.3
4	6.83E-03	9.86E-03	7.88E-03	−1.65E-03	0.000104	64.8
5	3.67E-02	−1.01E-02	2.39E-02	3.78E-03	0.001016	89.2
6	1.96E-03	1.17E-02	4.16E-03	7.94E-03	0.000111	91.9
7	1.73E-02	4.20E-03	2.62E-03	9.74E-03	0.00021	96.9
8	−2.30E-03	9.26E-03	−6.02E-03	9.29E-04	6.41E-05	98.4
9	3.61E-03	1.30E-03	1.35E-04	7.92E-03	3.87E-05	99.4
10	−2.80E-03	1.69E-03	−5.32E-03	1.15E-03	2.02E-05	99.9
			III Stage			
2	−1.75E-02	1.42E-02	1.93E-02	−2.19E-03	0.000443	10.9
3	5.66E-02	−1.89E-02	-1.07E-02	−2.23E-02	0.002088	62.1
4	6.27E-03	8.76E-03	7.06E-03	−2.04E-03	8.51E-05	64.1
5	3.69E-02	−1.07E-02	2.37E-02	3.23E-03	0.001025	89.3
6	2.02E-03	1.08E-02	4.33E-03	7.29E-03	9.67E-05	91.6
7	1.77E-02	4.22E-03	2.31E-03	9.80E-03	0.000216	96.9
8	−2.00E-03	8.86E-03	−5.82E-03	1.11E-03	5.88E-05	98.4
9	3.73E-03	1.11E-03	3.60E-04	7.97E-03	3.93E-05	99.4
10	−2.82E-03	1.88E-03	−4.95E-03	1.30E-03	1.88E-05	99.8

Second stage

A total of 99 % variability is predicated upon the first nine harmonics (table 1). Through similar computations the result of the first stage of research has been confirmed based on new material. Accordingly, first six PC were shown as effective components accounting for more than 3 % of variability (table 2).

Reconstruction of the otolith contours based on average values of normalized FC resulted in a graphical plot almost indistinguishable from the one based on the first stage material (fig. 3). The findings regarding the features which the first six principal components account for, remained the same.

Values of aposterior accuracy of assignment of round goby individuals to certain sampling area based on the otolith shape at the second stage of research showed some deviation from the pattern seen at the first stage. Specifically, at the second stage certain reduction of both overall assignment (classification) accuracy and assignment accuracy related to individual sampling areas was observed (table 3). Another pattern regarding

Table 2. Contribution of principal components to round goby otoliths shape variance in course of a single year period based on the samples from nine research areas at the first stage of research

Principal component	Variance percent, %	Accumulated percent of variance, %
Prin1*	38.03	38.03
Prin2*	27.74	65.77
Prin3*	8.63	74.39
Prin4*	6.07	80.47
Prin5*	4.21	84.68
Prin6*	3.06	87.74
Prin7	2.25	89.99
Prin8	1.75	91.74
Prin9	1.40	93.14
Prin10	0.94	94.08
Prin11	0.74	94.82
Prin12	0.71	95.54

Note. The asterisk denotes effective components.

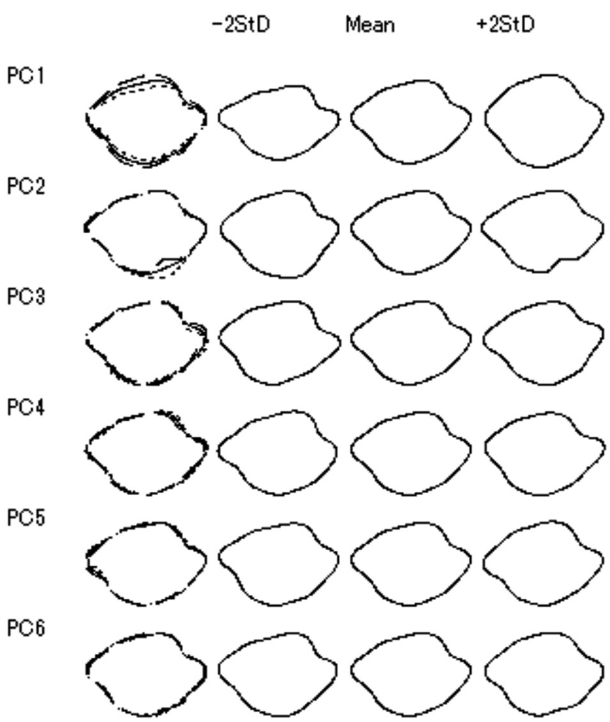

Fig. 3. The visualized reconstruction of the round goby otolith contour features predicated upon the first six Principal components (see Material and method for details).

distinctiveness of otoliths from certain sampling areas developed in new material. Fish individuals from the Dzharylhach Bay, the Khadzhibey Estuary and the Obytichna Bay (74.3 %, 66 % and 63 %, respectively) were assigned most distinctly at this stage of analysis.

Third stage

It was confirmed that 99 % of variability is on the basis of the first nine harmonics (table 1). On interpreting the dendrogram the following should be pointed out (fig. 4). Firstly, clusterisation does partially correspond to territorial contiguity of the sampling areas, although this correspondence is inconsistent. Secondly, a clusterisation pattern predicating upon environmental similarity of habitats can be observed. This dendrogram can be possibly structured into five greater clusters, which group the round goby individuals either according to the first (territorial contiguity) or to the second (attributable to the environmental similarities of habitats). In particular, the first cluster contains the Sea of Azov, Dnipro and Dniester estuaries. The second cluster comprises the Gulf of Odesa and Snake Island waters. The third cluster unites the round gobies from the Dzharylhach Bay and the Tylihul Estuary. It is conceivable that such association is attributable to environmental factors, which is subject to verification. The fourth and the fifth clusters are comprised by closed water bodies (Khadzhibey Estuary and Lake Yalpuh). Similar computations were performed on the entire material

Table 3. Values of aposterior accuracy of round gobies assignment to certain sampling locality

Sampling areas	Obytichna Bay	Dzharylh. Gulf.	Dnipro Estuary	Tylihul Estuary	Khadzhibey Estuary	Gulf of Odesa	Dniester Estuary	Lake Yalpuh	Snake Island	Fish amount from certain localities	Fish amount assigned correctly, %
					I Stage						
Obytichna Bay	36	5	7	0	1	1	2	3	0	55	65.5
Dzharylhach Bay	7	16	3	0	1	0	4	0	0	31	51.6
Dnipro Estuary	5	0	37	0	5	3	1	3	0	54	68.5
Tylihul Estuary	2	1	0	22	0	0	0	0	0	25	88.0
Khadzhibey Estuary	0	1	1	0	29	0	0	1	0	32	90.6
Gulf of Odesa	0	0	4	0	1	18	1	2	3	29	62.1
Dniester Estuary	3	2	3	0	2	0	19	1	0	30	63.3
Lake Yalpuh	1	0	6	0	2	1	0	30	1	41	73.2
Snake Island	1	0	1	0	1	2	1	3	16	25	64.0
All localities	–	–	–	–	–	–	–	–	–	**322**	**69.3**
					II Stage						
Obytichna Bay	48	6	0	5	2	5	5	4	1	76	63.1
Dzharylhach Bay	10	52	1	2	0	1	2	1	1	70	74.3
Dnipro Estuary	1	2	12	3	1	2	0	2	1	24	50.0
Tylihul Estuary	10	6	3	30	2	1	1	1	5	59	50.8
Khadzhibey Estuary	4	1	0	3	35	0	6	0	4	53	66.0
Gulf of Odesa	3	3	2	4	1	24	0	4	6	47	51.1
Dniester Estuary	7	1	3	2	7	1	20	1	2	44	45.4
Lake Yalpuh	8	2	1	5	1	3	4	43	1	68	63.2
Snake Island	4	1	1	1	3	10	5	2	28	55	50.9
All localities	–	–	–	–	–	–	–	–	–	**496**	**58.9**
					III Stage						
Obytichna Bay	74	12	11	6	5	5	6	6	6	131	56.5
Dzharylhach Bay	21	54	0	6	0	0	8	1	1	91	59.3
Dnipro Estuary	13	0	40	2	8	4	4	3	4	78	51.3
Tylihul Estuary	15	4	3	37	3	5	4	1	3	75	49.3
Khadzhibey Estuary	6	0	9	3	55	0	8	0	4	85	64.7
Gulf of Odesa	6	3	6	1	4	29	1	10	10	70	41.4
Dniester Estuary	15	5	6	4	11	3	22	5	3	74	29.7
Lake Yalpuh	10	3	3	2	3	6	6	63	4	100	63.0
Snake Island	4	1	5	3	6	17	5	5	34	80	42.5
All localities	–	–	–	–	–	–	–	–	–	**784**	**52.0**

of long-term research confirmed that the first six principal components are the effective components accounting for more than 3 % of variability (table 2). Reconstruction of otolith contours based on average values of normalized FC resulted in the plot, which was almost undistinguishable from the one based on the first stage material (fig. 3). These findings confirmed our initial suggestions regarding the otoliths morphologic features which are described by the first six effective principal components.

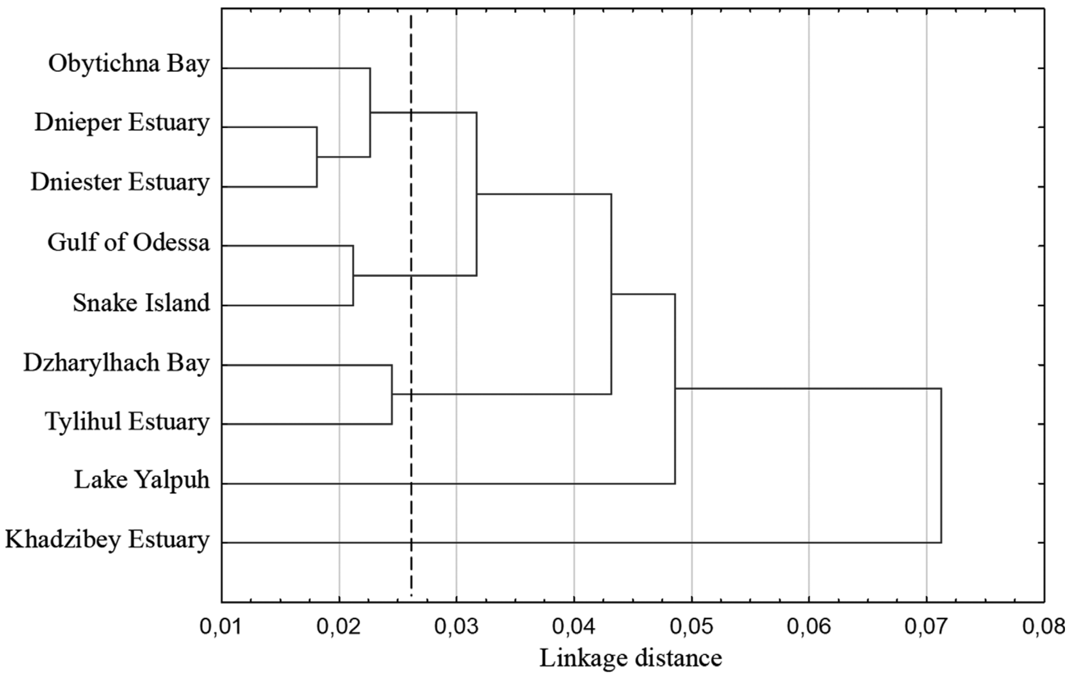

Fig. 4. Dendrogram for Euclidian distances between otolith contours of round goby (n = 786) from nine sampling areas, sampled during three years of study. Stippled line represents five clusters representing pairs of similar sampling sites. It shows that similarity of the sampling sites does not follow the pattern of their allocation chain along the coast of the Black sea visible in fig. 1.

Table 4. Values of aposterior accuracy of round gobies (grouped by size) assignment to one of the nine sampling areas by otoliths morphology

Standard length, cm	Sampling areas	Fish amount from certain sampling area, ind.	Fish amount assigned correctly, %
7.0–9.0	Obytichna Bay	55	69.1
	Dzharylhach Bay	23	65.2
	Dnipro Estuary	44	65.9
	Tylihul Estuary	11	90.9
	Khadzhibey Estuary	48	85.4
	Gulf of Odesa	30	60.0
	Dniester Estuary	26	42.3
	Lake Yalpuh	25	68.0
	Snake Island	30	53.3
	All sampling areas	292	66.8
9.1–11.9	Obytichna Bay	85	52.9
	Dzharylhach Bay	73	72.6
	Dnipro Estuary	37	40.5
	Tylihul Estuary	68	51.5
	Khadzhibey Estuary	42	61.9
	Gulf of Odesa	43	48.8
	Dniester Estuary	52	46.2
	Lake Yalpuh	79	69.6
	Snake Island	56	53.6
	All sampling areas	535	56.8

According to the classification matrix comprising the entire collected material, the pattern of further evenning of differences between samples from different water bodies had been observed, apparently due to year-to-year variability of the otoliths in fishes sampled in these water bodies (tables 3–4; see also supplement tables S2, S3, S4). In the Dniester Estuary the assignment accuracy decreased to 29.7 %. Nevertheless, the pattern of relatively higher assignment accuracy persisted in individuals from the Khadzhibey Estuary (64.7 %), Dzharylhach (59.3 %) and Obytichna bays (56.5 %). Higher accuracy of assignment of individuals from Lake Yalpuh (63 %) developed in the integrated material.

Fourth stage

In the 7.0–9.0 cm size group, the relative fish amount assigned correctly by water bodies varied between 77.5–83.8 % (table 5). The fish amount assigned correctly in relation to one of two compared years varied in 69.6–77.8 % (table 6). Average fish amount assigned correctly caught within the same year in relation to one of two water

Table 5. Assignment of fish individuals caught in same year to certain water body

Standard length, cm	Year of sampling	Compared sampling areas (fish number, ind.)			Relative fish amount assigned correctly, %	Average fish amount assigned correctly, %
7.0–9.0	2016	Sea of Azov (23)	Dnipro-Bug Estuary (27)	–	81.6	80.9
	2017	Sea of Azov (23)	Dnipro-Bug Estuary (17)	–	77.5	
	2017	Sea of Azov (23)	Lake Yalpuh (14)	–	83.8	
	2017	Dnipro-Bug Estuary (17)	Lake Yalpuh (14)	–	80.6	
9.1–12.0	2008	Dniester Estuary (28)	Snake Island (18)	–	86.9	83.2
	2010	Gulf of Odesa (15)	Snake Island (20)	–	77.1	
	2011	Tylihul Estuary (14)	Gulf of Odesa (16)	–	96.7	
	2011	Tylihul Estuary (14)	Lake Yalpuh (31)	–	97.8	
	2011	Gulf of Odesa (16)	Lake Yalpuh (31)	–	76.6	
	2012	Dzharylhach Bay (31)	Tylihul Estuary (29)	–	85.0	
	2016	Sea of Azov (32)	Dzharylhach Bay (19)	–	72.5	
	2016	Sea of Azov (32)	Dniester Estuary (19)	–	74.5	
	2016	Dzharylhach Bay (32)	Dniester Estuary (19)	–	82.4	
	2017	Sea of Azov (26)	Dzharylhach Bay (18)	–	72.7	
	2017	Sea of Azov (26)	Lake Yalpuh (14)	–	85.0	
	2017	Dzharylhach Bay (18)	Lake Yalpuh (14)	–	90.6	
	2011	Tylihul Estuary (14)	Gulf of Odesa (16)	Lake Yalpuh (31)	80.3	68.6
	2016	Sea of Azov (32)	Dzharylhach Bay (19)	Dniester Estuary (19)	60.0	
	2017	Sea of Azov (26)	Dzharylhach Bay (18)	Lake Yalpuh (14)	65.5	

Table 6. Assignment of fish individuals caught in same water body to certain year

Fish standard length, cm	Sampling areas	Year of comparing (fish number, ind.)			Relative fish amount assigned correctly, %	Average fish amount assigned correctly, %
7.0–9.0	Obytichna Bay	2016 (23)	2017 (23)	–	69.6	73.4
	Dnipro-Bug Estuary	2016 (27)	2017 (17)	–	72.7	
	Lake Yalpuh	2015 (14)	2017 (14)	–	77.8	
9.1–12.0	Obytichna Bay	2016 (32)	2017 (26)	–	70.7	78.4
	Obytichna Bay	2016 (32)	2018 (18)	–	70.0	
	Obytichna Bay	2017 (26)	2018 (18)	–	75.0	
	Dzharylhach Bay	2012 (31)	2016 (19)	–	64.0	
	Dzharylhach Bay	2012 (31)	2017 (18)	–	79.6	
	Dzharylhach Bay	2016 (19)	2017 (18)	–	83.8	
	Tylihul Estuary	2009 (21)	2011 (14)	–	97.1	
	Tylihul Estuary	2009 (21)	2012 (29)	–	74.0	
	Tylihul Estuary	2011 (21)	2012 (29)	–	97.7	
	Gulf of Odesa	2010 (15)	2011 (16)	–	70.9	
	Dniester Estuary	2008 (28)	2016 (19)	–	87.2	
	Snake Island	2008 (18)	2010 (20)	–	63.1	
	Lake Yalpuh	2011 (31)	2015 (28)	–	81.4	
	Lake Yalpuh	2011 (31)	2017 (14)	–	82.2	
	Lake Yalpuh	2015 (28)	2017 (14)	–	83.3	
	Obytichna Bay	2016 (32)	2017 (26)	2018 (18)	59.2	67.1
	Dzharylhach Bay	2012 (31)	2016 (19)	2017 (18)	58.8	
	Tylihul Estuary	2009 (21)	2011 (14)	2012 (29)	84.4	
	Lake Yalpuh	2011 (31)	2015 (28)	2017 (14)	65.8	

bodies was slightly higher (80.9 %) than the fish individuals in same water body, caught in different years (73.4 %).

In the 9.1–12.0 cm size group, the fish amount assigned correctly in two water bodies was higher (83.2 %) than in three water bodies (68.6 %). The correctly determined fish amount in relation to one of two years in the same water body was higher (78.4 %) than the number of individuals compared during three years (67.1 %). Increasing the number of the studied water bodies, as well as study years, decreases the number of correctly assigned fish.

Also, the fish amount assigned correctly from two different water bodies in one year was just slightly higher than the same parameter of fish individuals from the same body of water, caught within two years (the difference between them was 4.8 %). There are almost no differences in this parameter in fish, studied within one year in three different water bodies (68.6 %), and in one water body in fish caught within three years (67.1 %). The fish of the certain size group have almost no differences in the values of their correct assignment in relation to certain water body by the shape of their otoliths, compared with the same parameter for fish caught during one year in different waters.

Size differences

Results of DFA (table 3; see also supplement tables S2, S3, S4) indicate much higher accuracy of assignment of individuals to sampling area than when gobies size class was not taken into account (table 3 (III Stage)). In number of cases this accuracy reached 100 % (Dzharylhach Bay in 2017 and Khadzhibey Estuary in 2012). This result suggests the feasibility of otolith contour for verification of the affiliation of an individual to certain geographic location, provided that these fishes belong to the same sex and size class and caught during the same year.

However, further analysis questions the practical feasibility of this method when applied to round goby. Likewise, attempt has been made to perform this analysis on entire yearly material without separating it into size classes. Table 4 represents results of discriminant analysis of contours of otoliths of the entire range of fish individuals (standard length from 7 to 12 cm). On comparison with previous results obtained on more extensive samples a pattern of assignment accuracy decline can be observed as the extent of material increases. Table 4 represents comparison of results of DFA based on the 7.1–9.0 cm and 9.1–12.0 cm samples encompassing all sampling years.

According to the results of triennial samples discriminant analysis, a significantly worse assignment accuracy of 9.1–13.0 cm long round gobies (mean aposterior assignment accuracy 56.8 %), comparing to 7.1–9.0 cm long individuals (66.8 %) becomes noticeable. In spite of relatively high aposterior assignment accuracy values of the individuals from the Dzharylhach Bay (72.6 %) and Lake Yalpuh (69.6 %) within the triennial sample of individuals 9.1–13 cm long, mean aposterior assignment accuracy value in this sample (56.8 %) only insignificantly differs from the figure obtained without size classes segregation, which equaled to 52.0 % (table 3, III Stage). This suggests high variability within individual groups of the otolith contours within 9.1–13 cm size class depending upon the sampling year.

Discussion

The results obtained in this study suggest the limited applicability of otolith contours for discrimination of territorial aggregations of round goby based on multiple annual samples. Findings presented in this study are based on material collected in nine sampling areas.

Overall classification accuracy (69.3 %) is sufficiently high, taken into consideration that fishes were subject to be territorially discriminated between nine sampling areas. A value amounting to 11 % (1 of 9 possible cases) could be considered as a low value of correct classification, as it would speak for random assignment to one of the 9 sampling areas. Values exceeding the random 11 % six- to eightfold should be considered a good discrimination value. According to the classification matrix given in the table 3, significant distinctiveness of round gobies otoliths from Khadzhibey and Tylihul estuaries can be pointed out (90.6 % and 88.0 %, respectively). Accuracy of assignment of individuals from other sampling areas was tending to the average value (about 70 %).

When comparing these data with those from a similar study, in which materials were collected from 12 sampling areas in a three-year course, the same pattern can be noticed. This is decline of accuracy of assignment of fishes to certain sampling area. Accuracy of assignment of bogue (*Boops boops* L., 1758) individuals to certain sampling area in Mediterranean Sea based on triennial material amounted to 39 % (Mahé et al., 2019). Results of the bogue assignment are coherent to ours and speak for the low efficiency of otolith shape as a marker of geographical assignment of individuals.

The increase of material amount approximately 1.5-fold and material collection period to two surveying seasons resulted in a decline of overall accuracy of individuals assignment to certain sampling area approximately by 10 %. Such assignment accuracy decline coheres with findings of Canadian ichthyologists, who also pointed out significant year-to-year variability of otoliths of Atlantic cod (*Gadus morhua* L., 1758) sampled in 19 areas of the Northwestern Atlantic in its spawning seasons, as well as of Atlantic herring (*Clupea harengus* L., 1758), Atlantic salmon (*Salmo salar* L., 1758) and Atlantic mackerel (*Scomber scombrus* L., 1758) (Bird et al., 1986; Castonguay et al., 1991; Campana & Casselman, 1993).

It is not inconceivable that such results of fish classification using otolith contours are based on habitat conditions rather than geographic proximity of sampling sites. Otolith sagittal contour and morphology has been often linked to the ecological, taxonomical phylogenetic characteristics of species (Vignon & Morat, 2010; Tuset et al., 2016).

This suggests that clusterisation based on the otolith contours characterizes the sampling habitat rather than genetic affiliation of populations. This was also hypothesized by previous studies (González-Salas & Lenfant, 2007). It is known from literary sources, that otolith shape depends on food abundance and composition (Molony & Choat, 1990; Fernandez-Jover & Sanchez-Jerez, 2015), as well as on concentration of Ba^{2+}, Sr^{2+} and Mn^{2+} ions in water and food (Mille et al., 2016). The diet influences the general otolith shape indirectly and influences the edge indentation directly (Cardinale et al., 2004; Hüssy, 2008).

The most extensively studied sagittal otoliths of fishes are composed mainly (by 90.0–99.9 %) of calcium carbonate, and approximately 0.1 to 10 % of their chemical composition is organic matrix. It is this organic matrix that plays the crucial part in otolith biomineralisation (Nagasawa, 2013). Otoliths grow in course of accretion and precipitation of organic and ion components contained in saccular endolymph. Therefore, the otolith biomineralization completely depends on the endolymph contents and on substances synthesized or transported by saccular epithelium (Payan et al., 2004). Otolith biomineralization is predicated upon a variety of factors: both extrinsic (environmental factors) and intrinsic (physiological) (Allemand et al., 2007). In particular, the otolith shape variability is influenced by such environmental factors as water temperature (Cardinale et al., 2004), Ba^{2+} and Sr^{2+} concentrations in water and food (Walther & Thorrold, 2006), as well as Mn^{2+} (Sanchez-Jerez et al, 2002). Otolith morphology is also predicated upon physiologic factors: fish size (Hüssy, 2008), sexual maturation (Mérigot et al., 2007), sex by itself (Castonguay et al., 1991).

Our clusterization findings are partially consistent with those obtained by American researchers using genetic methods. Previously Brown & Stepien (2008) discriminated five populations within the range of Black Sea and Sea of Azov. According to their conclusions, samples from the Western part of the Black Sea, the marine waters of the Crimean peninsula, the Dnipro River and the Sea of Azov comprise distinguishable aggregations at population level, and invasive ranges of this goby species in the Danube river were assigned to the samples from the northwestern part of the Black Sea in the Odesa area (Brown & Stepien, 2008). However, neither classification matrices of discriminant function analysis nor cluster analysis dendrograms present some single pattern apart from high year to year otolith variations, which can be explained by the strong response of contour formation to habitat and feeding conditions. This assumption will require verification by further research.

The evidence has clearly demonstrated, that if we researched data only for a single year, then significant differences could be found between samples taken from different regions. However, if research is carried out over a higher number of years, then such differences fade away. In other words, the magnitude of differences between samples from different years for the same region is comparable to the one between samples from different regions in the same year. This fact does not allow identification and stabilization of the values of features, by which the samples from different regions could be distinguished in the future. Finally, we have considered that the use of otoliths (which is time consuming and costs a lot of effort) is not the right method to distinguish round goby populations from different localities.

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