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First data on the structure of the vertebral column in *Gobio* and *Romanogobio* species (Actinopterygii, Cypriniformes, Gobionidae) from Ohrid Lake and the Vardar River basin

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Abstract

The validity of four gudgeon species known for the ichthyofauna of North Macedonia has been a matter of different taxonomic disputes (except for *Gobio ohridanus* Karaman, 1924). Recently, a restoration of the species status was proposed for *Gobio balcanicus* Dimovski et Grupče, 1977 and *Romanogobio banarescui* (Dimovski et Grupče, 1974). So far, *Romanogobio stankoi* (Karaman, 1974), has not been a part of any recent investigation and it is still considered as a junior synonym of *Romanogobio elimeius* (Kattoulas, Stephanidis et Economidis, 1973) or *R. banarescui* due to the lack of distinctive morphological characters. The osteological characters in the structure of the vertebral column, have significance to the systematics of the gudgeons, especially in elevation of *Romanogobio* at the level of genus. To contribute to resolving the taxonomic status in these gudgeon species, the goal of this paper is to identify distinctive characters by presenting and analyzing the structure of their vertebral column. Samples were inspected from the fish collections in the Institute of Biology (*G. ohridanus*), the Institute of Animal Science (*G. balcanicus*), and the Macedonian Museum of Natural History (both *Romanogobio* species). Fish samples were X-rayed as well as cleared and double-stained. Obtained data on the number of vertebrae were presented through the vertebral formula following Naseka (1996). Multiple Correspondence Analysis (MCA) was used to check the interrelation between taxa and the number of vertebrae in all units of the vertebral column. *G. balcanicus* is distinguished from *G. ohridanus* mainly in the predorsal and preanal subregion (10 vs. 11 vertebrae and 1 vs. 0 vertebrae, respectively), while *R. stankoi* distinguishes from *R. banarescui* by the number of vertebrae in the preanal and postanal subregion (2 vs. 4 vertebrae and 19 vs. 17 vertebrae, respectively). The structure of vertebral column offers distinctive morphological characters for the four gudgeon species from Ohird Lake and the Vardar River basin.

Keywords

Gudgeons, MCA analysis, vertebrae, vertebral column, vertebral formula

Introduction

Although the monophyly of the family Gobionidae (formerly in the family Cyprinidae) is well supported (Yang et al. 2006; Tang et al. 2011), its systematics still contains unresolved issues that deem taxonomic challenge due to ongoing speciation (Takács et al. 2014) and the application of different species concepts (Kullander 1999; Lusk and Šlechta 2005; Nowak et al. 2008b, 2009). New data to make an advance in the taxonomy, systematics, and phylogeny of Gobionidae is still required, Naseka (1996) analyzed the structure of the Gobionine vertebral column, proposing a vertebral formula for each genus and species. Genus *Gobio* Cuvier, 1816 (type species *G. gobio*) formerly included subgenera *Romanogobio* Bănărescu, 196—type species *R. kessleri*—and *Rheogobio* Bănărescu, 1961—type species *Romanogobio uranoscopus* (Agassiz, 1828). The structure of the vertebral column, among other morphological characters, gave recognition to *Romanogobio* as a distinct genus (Naseka 1996) based on analysis of *Romanogobio kesslerii* (Dybowski, 1862), *Romanogobio albipinnatus* (Lukasch, 1933), *Romanogobio ciscaucasicus* (Berg, 1932), and *Romanogobio persus* (Günther, 1899). Later, Naseka and Freyhof (2004) synonymized subgenus *Rheogobio* with the accepted *Romanogobio* reevaluating the status of 15 species (see Naseka 1996; Kottelat 1997) in *Romanogobio* based on the external morphology, as well as vertebral column's features. Then, the presence of two native genera in Europe, *Gobio* and *Romanogobio,* became widely accepted (Lusk and Šlechta 2005; Kottelat and Freyhof 2007; Naseka and Bogutskaya 2009; Nowak et al. 2008a, 2011). On the other hand, some authors, based on osteological analyses including the vertebral column, proposed restoration of *Rheogobio* and required revision of all gudgeon subspecies that were previously under the species name *uranoscopus* (Talabishka 2014).

Four native gudgeon species that currently belong to genera *Gobio* and *Romanogobio* are known for the ichthyofauna of North Macedonia. Extensively studied in the past, one gudgeon species–*Gobio ohridanus* Karaman, 1924–was described for the old tectonic Ohrid Lake, while the other three were described for the Vardar River drainage. *Gobio balcanicus* Dimovski et Grupče, 1977 is widely distributed throughout the Vardar River and its tributaries. *Romanogobio banarescui* (Dimovski et Grupče, 1974) was described for the middle and lower course of the Vardar River, and *Romanogobio stankoi* (Karaman, 1974) (as interpreted by Dimovski and Grupče 1976a) for fast-flowing upper courses of the Vardar River, and its tributaries Lepenec, Bregalnica, Crna, and Treska rivers.

The validity of these species (except that of the *G. ohridanus*) has been a matter of different taxonomic disputes in which the need for contemporary morphological description was often emphasized. Dimovski and Grupče (1976b) showed differences between *R. stankoi* and *Romanogobio elimeius* (Kattoulas, Stephanidis et Economidis, 1973) from the Aliakmon River. Later, both *R. stankoi* and *R. banarescui* were suggested to be junior synonyms to *R. elimeius* (see Economidis et al. 1981; Kottelat and Freyhof 2007). Owing to the lack of comparative morphological data, Kottelat and Freyhof (2007) suggested that *G. balcanicus* should be a synonym of *Gobio bulgaricus* Drensky, 1926. Conversely, recent studies based on molecular data (Geiger et al. 2014; Jelič et al. 2018; Friedrich et al. 2018) proposed restoring the taxonomic status of *G. balcanicus* and *R. banarescui* as clearly distant from *G. bulgaricus* and *R. elimeius*, respectively. So far, *R. stankoi* from the type locality has not been subject to recent studies and is still considered either a synonym of *R. banarescui*, or of *R. elimeius* (see Banarescu 1992; Kottelat and Freyhof 2007). In this paper, the name *R. stankoi* is used for material from the type locality aiming to contribute to the clarification of the taxonomic status of this taxon.

Having in mind that ambiguities in species delineations seriously hamper the conservation measures (Kottelat 1998, 2013; Economou et al. 2007; Kottelat and Freyhof 2009) and that gudgeon species are very important to both conservation and ecology (Lusk and Šlechta 2005; Telcean and Cupşa 2012; Curtean-Bănăduc et al. 2019), it is of urgent matter to clarify the taxonomic status of all gudgeon taxa from the Balkan Peninsula. In this context, the goal of this paper is to analyze and present in detail, for the first time, the vertebral formula in gudgeons from North Macedonia following Naseka's (1996) methodology to shed light on the taxonomic potential of the structure of the vertebral column.

Material and methods

For this study, the material from the collections of the Macedonian Museum of Natural History, the Institute of Biology, and the Institute of Animal Science from Skopje was used. *Romanogobio banarescui* and *R. stankoi* were analyzed from the type material, collected by Dimovski and Grupče from 1961 to 1975 and deposited at the Macedonian Museum of Natural History. The material of *G. balcanicus* was collected in 2007 from the Vardar River and deposited at the Institute of Animal Science, while the material of *G. ohridanus* was collected in 2015 from Ohrid Lake and stored at the Institute of Biology. The specimens of *G. ohridanus* (n = 22), *G. balcanicus* (n = 16), *R. banarescui* (n = 36), and *R. stankoi* (n = 36) were X-rayed as well as cleared and double-stained according to the protocol of Hanken and Wassersug (1981).

The structure of the vertebral column was analyzed following Naseka (1996). Namely, to present the vertebral formula, seven characters of the vertebral column were analyzed: total number of vertebrae (T), abdominal number of vertebrae (A), caudal number of vertebrae (C), predorsal number of vertebrae (a_1), intermediate number of vertebrae (i), preanal number of vertebrae (c_1) and postanal number of vertebrae (c_2). The vertebral formula is composed of the mean values of these characters. Additionally, the number of vertebrae was counted in the distance between the origin of the dorsal fin and the origin of the anal fin (D–A distance), as well as in the distance between the origin of the dorsal fin and first caudal vertebrae (D–C1 distance). The last D–A and D–C1 distances are not part of the vertebral formula, but were counted as additional explorative characters.

The Multiple Correspondence Analysis (MCA) is a multivariate analysis that is used to examine the relations between more than two categorical variables (Sourial et al. 2010; Di Franco 2016). In this paper, MCA was applied to explore the interrelations between taxa and a certain number of vertebrae in different units of the vertebral column. As each unit of the vertebral column was considered as a character, the number of vertebrae observed in it was considered as a character state. The characters of the vertebral column (T, A, a_1, i, C, c_1, and c_2) and taxa (genera *Gobio* and *Romanogobio* and species *G. balcanicus, G. ohridanus, R. banarescui*, and *R. stankoi*) were analyzed as active variables. The distances D–A and D–C1 were plotted as supplementary variables (they do not contribute to forming of the dimensions in MCA) to enhance the interpretation of the analysis. MCA was performed using Statistical Software for Excel–XLSTAT 2014 5.03.

Results

Vertebral formula

The total number of vertebrae in *G. ohridanus* ranges from 37 to 39 (Table 1). The abdominal vertebrae (20 or 21) dominate over the caudal vertebrae (17 or 18) in all specimens. There was no variation in the predorsal vertebrae number (11) in all examined specimens. The number of intermediate vertebrae ranged from 3 to 5. An absence of preanal caudal vertebrae was noted in 18% of individuals in the sample. The number of postanal caudal vertebrae ranged from 16 to 18, with a mode of 17 in 59% of the specimens. The D–A distance had a mode of 10 vertebrae, the same as the mode of the A–C1 distance. The vertebral formula of *G. ohridanus* was: 38.23: (11) 20.45 (4.36) + (0.95) 17.73 (16.77).

The total number of vertebrae in *G. balcanicus* ranged from 37 to 39 (Table 1). All specimens had a greater number of abdominal vertebrae (20 or 21) compared to the number of caudal vertebrae (17–19). The predorsal subregion most often included 10 vertebrae. The range of intermediate vertebrae was from 3 to 5. The number of preanal vertebrae was 0–2, and the absence was observed in only 5% of the specimens. The number of postanal vertebrae ranged from 15 to 18 vertebrae. The D–A distance had a mode of 11 vertebrae, while D–C1 distance had a mode of 10 vertebrae. The vertebral formula of *G. balcanicus* was: 38.67: (10.33) 20.27 (4.40) + (1.33) 18.40 (17.07).

The examined specimens of *R. stankoi* had a total number of 39 or 40 vertebrae (Table 1). The abdominal and caudal vertebrae had the identical range (19 or 20), but the mean value of the specimens showed a dominance of the caudal vertebrae. A higher number of caudal than abdominal vertebrae (19:20 and 19:21) was observed in the majority of the examined specimens. The number of abdominal vertebrae was higher in 7 specimens (20:19) while an equal number between the abdominal and the caudal vertebrae (20:20) was observed in 11 specimens. The number of predorsal vertebrae most often was 11, while the intermediate vertebrae most often were 4. There is a mode of 2 preanal caudal vertebrae, while the number of postanal caudal vertebrae ranged from 17 to 19. The D–A distance had a mode of 10 vertebrae, while the D–C1 distance had a mode of 8 vertebrae. The vertebral formula of *R. stankoi* was: 39.42: (11.08) 19.50 (4.08) + (1.69) 19.92 (18.22).

The total number of vertebrae in *R. banarescui* ranged from 38 to 40 (Table 1). The number of abdominal vertebrae ranged from 18 to 20, while that of the caudal vertebrae ranged from 19 to 21. A higher number of caudal vertebrae, over the abdominal ones, was recorded in the majority of the examined specimens, 18:21 (2), 19:20 (20), and 19:21 (11). The number of abdominal vertebrae prevailed (20:19) in one specimen, while an equal number of the abdominal and the caudal vertebrae (19:19 and 20:20) was observed in 2 specimens. There were 10–12 predorsal vertebrae, while the number of intermediate vertebrae ranged from 3 to 5. There were 2–4 preanal caudal vertebrae, while postanal caudal vertebrae were most often 17. The most common value of D–A distance was 11 vertebrae and a mode of 8 vertebrae was counted for the D–C1 distance. The vertebral formula of *R. banarescui* was: 39.31: (10.97) 19.00 (4.06) + (2.92) 20.31 (17.42).

Multiple Correspondence Analysis

MCA was used to investigate the taxa as a function of the vertebral column structure. The overall inertia of the sample was 2.8889. The first two dimensions accounted for the largest part of the total variability, explaining 70.93% of the inertia (Fig. 1). The first dimension (F_1) accounted 62.75% of the total inertia and grouped specimens according to their

Table 1. Frequency table of the vertebral column of *Gobio* and *Romanogobio* species.

	T				*A*				a_1			*i*			
Species	**37**	**38**	**39**	**40**	**18**	**19**	**20**	**21**	**10**	**11**	**12**	**3**	**4**	**5**	
G. ohridanus	1	15	6				12	10		22		2	10	10	
G. balcanicus	2	6	12			1	15	4	12	8		2	9	9	
R. stankoi			21	15		18	18			33	3	5	23	8	
R. banarescui		1	23	12	2	32	2		3	31	2	4	26	6	
			C					c_1					c_2		
Species	**17**	**18**	**19**	**20**	**21**	**0**	**1**	**2**	**3**	**4**	**15**	**16**	**17**	**18**	**19**
G. ohridanus	6	16				4	15	3				7	13	2	
G. balcanicus	1	11	8			1	11	8			1	3	11	5	
R. stankoi			7	25	4		12	22	2				3	22	11
R. banarescui			2	21	13			7	25	4		1	19	16	

Abbreviations are given in Material and methods.

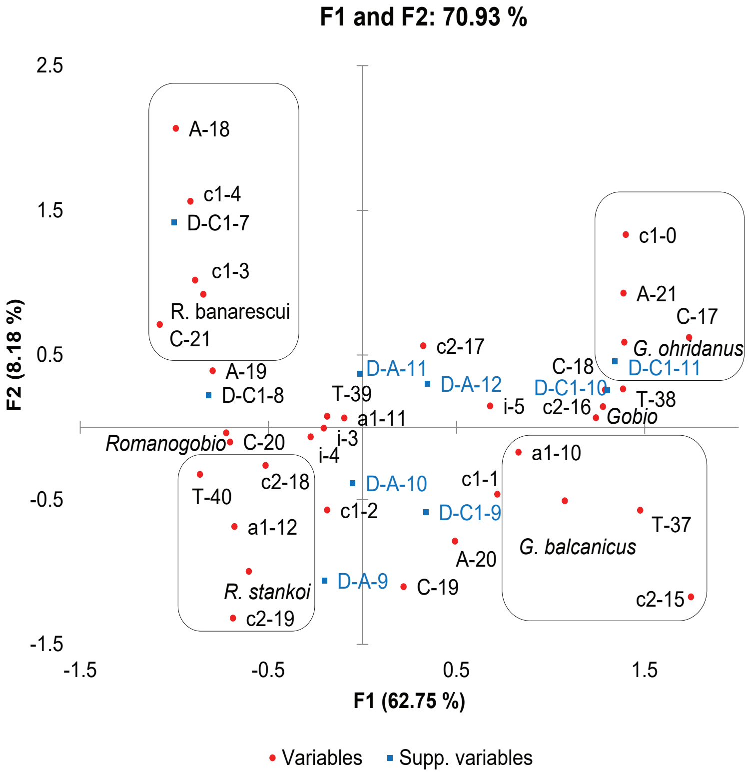

Figure 1. Results of Multiple Correspondence Analysis (MCA). Squares represent closely associated character states of the vertebral column with the analyzed species. The numeric suffixes in the active variables represent the actual number of vertebrae (the character state). Abbreviations: T – total vertebrae, A – abdominal vertebrae, C – caudal vertebrae, a1 – predorsal vertebrae, i – intermediate vertebrae, c1 – preanal vertebrae, c2 – postanal vertebrae.

genera. Thus, it can be called the generic dimension. Positive loadings on the F_1 featured genus *Gobio* with a lower total number of vertebrae, a higher number of abdominal vertebrae, a lower number of caudal vertebrae, a lower number of preanal vertebrae, a lower number of postanal vertebrae, and a lower number of predorsal vertebrae (Table 2, Fig. 1). Negative loadings of the first dimension (F_1) represented genus *Romanogobio* with a higher total number of vertebrae, a lower number of abdominal vertebrae, a higher number of caudal vertebrae, a higher number of preanal vertebrae, and a higher number of postanal vertebrae (Table 2, Fig. 1).

The second dimension (F_2) accounted 8.18% of the total inertia and grouped each species with certain character states of the vertebral column. Thus, this dimension can be called the species dimension (Fig. 1). Positive loadings associated *G. ohridanus* with 21 vertebrae in abdominal region and with the absence of preanal vertebrae; and *R. banarescui* with 18 abdominal vertebrae, 3 and 4 preanal vertebrae, as well as 21 caudal vertebrae. Negative loadings of F_2 associated *R. stankoi* with the highest number (19) of postanal vertebrae observed in all taxa (Fig. 1). *Gobio balcanicus* was associated with the lowest number of total vertebrae (37), the lowest number of postanal vertebrae (15), and the presence of 10 predorsal vertebrae. These character states did not contribute significantly to the forming of the second dimension, but to the third dimension (F_2) (Table 2).

The D–A distance was not closely associated with any of the genera, since it significantly contributed to the creation of the second, species dimension (Fig. 1; Table 2). This was not the case with D–C1 distance, where lower values were associated with *Romanogobio* and higher values with *Gobio* (Fig. 1; Table 1).

Discussion

The great variability in European gudgeon species that makes their identification difficult perpetuates the scientific interest for resolving of their taxonomy, morphological and genetic diversity, and phylogenetic relations between them (Freyhof et al. 2000; Kottelat and Persat 2005; Lusk et al. 2005; Mendel et al. 2008, 2012; Takács 2012; Nowak et al. 2013, 2014; Szlachciak and Nowak 2015; Zangl et al. 2020). The structure of the vertebral column offers a powerful tool in resolving taxonomic distinction between gudgeon species (Naseka 1996, 2001; Talabishka 2014). The total number of vertebrae in gudgeon species is usually provided as a meristic count, merely a character used in either description or diagnosis of the species (Karaman 1924; Drensky 1926; Kattoulas et al. 1973; Grupče and Dimovski 1975; Dimovski and Grupče 1974, 1976a, 1977). Species within *Gobio* can have both low (e.g., 38–39 in *Gobio maeandricus* Naseka, Erk'akan

Table 2. MCA Test values of active and supplementary variables of the first three dimensions (F_1–F_3) of *Gobio* and *Romanogobio* species.

Variables/dimensions	F_1	F_2	F_3
G. balcanicus	**5.2747**	**–2.4917**	**–6.0344**
G. ohridanus	**7.2362**	**3.0602**	**5.0598**
R. banarescui	**–6.1045**	**6.6366**	**–2.1616**
R. stankoi	**–4.3550**	**–7.1959**	**2.8035**
Gobio	**10.0791**	0.5389	–0.6185
Romanogobio	**–10.0791**	–0.5389	0.6185
T–37	**2.5814**	–1.0021	**–1.9610**
T–38	**7.2024**	1.3744	**3.3163**
T–39	**–2.1713**	0.8810	**–6.6659**
T–40	**–5.1138**	–1.9306	**5.4694**
A–18	–1.4083	**2.9341**	0.0652
A–19	**–7.6140**	**3.7337**	–1.5859
A–20	**4.3842**	**–7.0118**	0.6810
A–21	**5.5227**	**3.6861**	1.3550
a_1–10	**3.4333**	–0.7142	**–6.1559**
a_1–11	**–2.2183**	1.4762	**5.4949**
a_1–12	–1.5473	–1.5629	–0.0443
i–3	–0.7498	–0.0191	1.2853
i–4	**–3.6924**	–0.8966	0.5179
i–5	**4.5125**	0.9844	–1.4389
C–17	**4.7232**	1.6875	**2.2329**
C–18	**7.6394**	1.5278	0.9230
C–19	0.9732	**–4.9035**	**–4.6068**
C–20	**–6.1563**	–0.8856	–0.6382
C–21	**–4.7953**	**3.1625**	**2.8794**
c_1–0	**3.1875**	**3.0305**	1.3755
c_1–1	**5.4977**	**–3.5517**	**2.2954**
c_1–2	–1.4358	**–4.3843**	–1.2417
c_1–3	**–5.2658**	**6.0253**	**–2.2559**
c_1–4	–1.8533	**3.1669**	0.9649
c_2–15	1.7473	–1.1717	**–2.8202**
c_2–16	**4.4425**	0.4983	**2.2279**
c_2–17	**2.8343**	**4.9311**	**–2.6849**
c_2–18	**–4.4188**	**–2.2599**	–1.5547
c_2–19	**–2.3886**	**–4.5805**	**5.6978**
D–A–9	–0.3529	–1.8554	–0.8431
D–A–10	–0.4792	**–3.5798**	**3.7303**
D–A–11	–0.1206	**3.5399**	–1.0774
D–A–12	1.1978	1.0468	**–3.9830**
D–C1–7	**–2.0285**	**2.8691**	0.2018
D–C1–8	**–7.4095**	**2.0236**	–0.7995
D–C1–9	**2.5828**	**–4.4943**	**2.1404**
D–C1–10	**6.3905**	1.2563	–0.7728
D–C1–11	**2.3469**	0.7953	**–2.2733**

Abbreviations and grouping are given in Material and methods. Values displayed in bold are significant at the level alpha = 0.05.

et Küçük, 2006, 38–40 in *G. bulgaricus*) and a high total number of vertebrae (e.g., 39–41 in *Gobio battalgilae* Naseka, Erk'akan et Küçük, 2006 and *G. gobio*, 40–42 in *Gobio microlepidotus* Battalgil, 1942) (see Naseka et al. 2006; Szlachciak and Ząbkiewicz 2008). The ancestral vertebral structure of Gobionidae has a low total number of vertebrae (Naseka, 1996). *Romanogobio* has undergone a specialization by increasing the total number of vertebrae, however, this count (36–39) is low within Gobionidae, yet higher than *Gobio* (Naseka, 1996). The results in this study showed that the total number of vertebrae in *R. stankoi* (39–40) and *R. banarescui* (38–40) is higher than in *G. balcanicus* (37–39) and *G. ohridanus* (37–39, thus confirming their intergeneric difference suggested by Naseka (1996) (Fig. 1).

As the total number of vertebrae does not reflect the internal structure of the vertebral column (Ford 1937), only recently it has been analyzed for systematic purposes in Gobionidae, revealing that three states of abdominal–caudal ratio (A:C) can be noticed within *Romanogobio* and *Gobio*: an equal number of abdominal and caudal vertebrae (A = C), the prevalence of the abdominal over the caudal vertebrae (A > C) and prevalence of the caudal over the abdominal vertebrae (C > A) (Naseka 1996). The latter is the synapomorphy of *Romanogobio* that supported its re-evaluation as a distinct genus (Naseka 1996). The literature data from other studies mainly follow this distribution pattern of A: C ratio with few exceptions (see Table 3). *G. balcanicus* and *G. ohridanus* in this study show only prevalence of the abdominal vertebrae (A > C) (Table 3), with the abdominal region of *G. ohridanus* being longer than in *G. balcanicus* (Fig. 1, Table 4). This is due to the high number of abdominal vertebrae (21) in *G. ohridanus* (Fig. 1), as well as the lower number of predorsal vertebrae (10) in *G. balcanicus* (Tables 2, 4). On the other hand, even though both *R. stankoi* and *R. banarescui* have all three states of abdominal–caudal ratio, the caudal region is relatively longer than the abdominal in both taxa (Fig. 1, Tables 3, 4), but longest, up to 21 vertebrae, in *R. banarescui* (Fig. 1, Tables 3, 4). The results of these studies, once again confirmed the usefulness of the A:C ratio in determining the intergeneric difference and should be further pursued to unlock its taxonomic potential within *Gobio* and especially *Romanogobio* species where different A:C ratios are present within one sample of the population (Table 3).

Table 3. Relative frequency of abdominal-caudal ratio in different species of *Romanogobio* and *Gobio* including results of this study.

Species	A = C	A > C	C > A
G. gobio [1]	7%	81.4%	1.6%
G. soldatovi [1]		100%	
G. delyamurei [3]		100%	
R. uranoscopus [1]	45%	30%	25%
R. kesslerii [1]	46%	4%	50%
R. ciscaucasicus [1]	31%	22%	47%
R. pentatrichus [4]	39%	28%	33%
R. albipinnatus [2]		2%	98%
R. belingi [2]	10%	3%	87%
R. vladykovi [2]	55%	9%	36%
R. tanaiticus [2]			98%
R. parvus [5]	28%		72%
R. benacensis [6]		100%	
G. ohridanus (this study)		100%	
G. balcanicus (this study)		100%	
R. banarescui (this study)	5.5%	2.7%	91.6%
R. stankoi (this study)	30.6%	19.4%	50%

Abbreviations are given in Material and methods. Superscript explanation: [1] Naseka (1996); [2] Naseka (2001); [3] Freyhof and Naseka (2005); [4] Naseka et al. (2002); [5] Naseka and Freyhof (2004); [6] Jelič et al. (2018).

Table 4. Relative frequency of modal numbers in vertebral column in different species of *Gobio* and *Romanogobio* including results from this study.

Characters/ Species	A% of T	a_1% of T	a_1% of A	i% of T	i% of A	C% of T	c_1% of T	c_1% of C	c_2% of T	c_2% of C
G. gobio [1]	52	28	53	12	22	48	5	10	43	90
G. soldatovi [1]	53	28	53	11	21	47	3	6	44	94
G. coriparoides [1]	54	28	52	13	24	46	3	6	44	94
R. uranoscopus [1]	50	30	59	10	20	50	5	9	45	91
R. kessleri [1]	49	28	56	12	25	51	8	15	44	85
R. ciscaucasicus [1]	50	26	53	12	24	50	5	11	45	89
R. persus [1]	49	27	55	11	23	51	5	9	47	91
R. albipinnatus [2]	47	—	—	—	—	53	11	21	42	79
R. tanaiticus [2]	46	—	—	—	—	54	12	22	42	78
R. belingi [2]	48	—	—	—	—	52	10	19	42	81
R. vladykovi [2]	50	—	—	—	—	50	7	14	43	86
G. bulgaricus [3]	54	26	48	—	—	46	4	8	—	—
G. carpathicus [4]	51	25	49	8	16	49	5	11	44	89
R. uranoscopus [4]	49	25	52	9	18	51	4	7	47	93
R. kessleri [4]	44	25	57	9	21	54	9	16	46	84
R. vladykovi [4]	48	25	52	9	19	52	7	13	45	86
G. ohridanus [5]	54	29	54	11	21	46	2	5	44	95
G. balcanicus [5]	52	27	52	11	22	48	4	7	44	93
R. stankoi [5]	49	28	57	10	21	51	4	9	46	91
R. banarescui [5]	48	28	58	10	21	52	7	14	44	86

Abbreviations are given in Material and Methods. Superscript explanation: [1] Naseka (1996); [2] Naseka (2001); [3] Naseka et al. (2006); [4] Talabishka (2014); [5] this study.

In addition to the abdominal and caudal regions, the number of vertebrae in their respective subunits plays an important role in the taxonomic distinction between *Gobio* and *Romanogobio* species (Naseka 2001; Naseka et al. 2006; Bogutskaya et al. 2013). The increased number of preanal vertebrae (with consequent increase of the caudal vertebrae) is another synapomorphic character that distinguishes *Romanogobio* from *Gobio* (see: Naseka 1996). The preanal subregion has its external morphological reflection in the position of the anus, being closer to the anal fin in *Gobio* and distant from the anal fin in *Romanogobio*, favoring it as an important taxonomic key character (Naseka 1996; Naseka and Freyhof 2004; Kottelat and Freyhof 2007; Bogutskaya et al. 2013; Friedrich et al. 2018). Preanal vertebrae in some *Romanogobio* species (e.g., *R. kessleri*) comprise 8%–9% of the total number of vertebrae, while in others (e.g., *Romanogobio tanaiticus* Naseka 2001, *Romanogobio belingi* (Slastenenko, 1934), and *R. albipinnatus*) they are high in number up to 10%–12% of the total number of vertebrae (Table 4). It is hypothesized that the low value of preanal vertebrae (plesiomorphic characteristic) in *R. uranoscopus* (4%–5% of T) (Table 4) is due to its specialization towards rapid waters that moves the anal fin forward, elongating the caudal peduncle at the expanse of preanal vertebrae (Talabishka 2014). The elongation of the postanal subregion (up to 47% of the total number of vertebrae) (Table 4), which was seen as ecological adaptation, is one of the osteological peculiarities that Talabishka (2014) used to propose a revision of all gudgeon species previously assigned under the species name *uranoscopus*.

The presently reported study of *Romanogobio* species showed that *R. banarescui* has up to 4 preanal vertebrae that comprise 7% of the total number of vertebrae. On the other side, *R. stankoi* has a lower number of preanal vertebrae (4% of T), contributing to a significantly higher number of postanal vertebrae (19) that comprise 46% of the total number of vertebrae (Tables 1, 4; Fig. 1). Concerning *Gobio* species examined in this study, the preanal subregion in *G. ohridanus* is one of the shortest (2% of the total number of vertebrae) (Table 4) due to the frequent character state of absence of preanal vertebrae (Fig. 1). Therefore, the preanal subregion is shorter in *G. ohridanus* than in *G. balcanicus*, even though both species have identical values of range (0–2 vertebrae) and mode (1 vertebra) (Table 1). Moreover, this study showed that the D–C1 distance in *R. banarescui* was the shortest (7 vertebrae), while in *G. ohridanus* it was the longest (10–11 vertebrae) (Fig. 1), which is in line with the high number of preanal vertebrae in the first species and their absence in the second one (Fig. 1; Table 1), strongly confirming again the importance of the number of preanal caudal vertebrae as a key taxonomic character in Gobionidae.

Conclusions

In this publication, for the first time, the vertebral formula of 4 gudgeon species is presented, and also for the first time, the structure of the vertebral column is analyzed through MCA analysis, which enables defining the closely associated character states with the analyzed taxa. The genera herein fall in line with the already known conditions of low total vertebrae and dominance of the abdominal region in *Gobio*, and the high total number of vertebrae with the dominance of the caudal region in *Romanogobio*.

Within genus *Gobio*, the character state of 10 predorsal vertebrae in *G. balcanicus* contributes to this subregion being shorter than in *G. ohridanus*, where 11 vertebrae without variation are present. The abdominal region of *G. ohridanus*, closely associated with 21 vertebrae, is longer than that of *G. balcanicus* (20 vertebrae). Due to the character state of 0 preanal vertebrae, more frequently noted in *G. ohridanus*, the preanal subregion is shorter than in *G. balcanicus*. Within *Romanogobio* the preanal subregion is significantly longer in *R. banarescui* (closely associated with 3 and 4 vertebrae) than in *R. stankoi*. And finally, *R. stankoi* is associated with 19 postanal vertebrae displaying elongation of the postanal subregion compared to *R. banarescui*. So, based on the structure of the vertebral column, *R. stankoi* and *R. banarescui* considerably differ and we offer a hypothesis that they are not conspecific–an opinion that should be further investigated using morphological, osteological, and molecular markers.

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