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Study of the Polymorphism of ISSR Markers in Spined Loaches of the Genus *Cobitis* (Cobitidae) in Connection with Problems of Differentiation of the Species Involved in the Formation of Polyploid Forms of Hybrid Origin and Determination of Their Taxonomic Status

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Abstract—We have analyzed the polymorphism of ISSR fragments of DNA by polymerase chain reaction in spined loaches for the first time. The analysis has made it possible to successfully differentiate the genomes, which were presumably involved in the formation of polyploid forms of hybrid origin: *Cobitis taenia*; spined loaches from the Danube River basin, which are diagnosed as *C. elongatoides*; *C. tanaitica* from the Don River basin and spined loaches from Lake Sinoe, which are also assigned to the species *C. tanaitica* by a number of authors. The following conclusions have been made based on comparative morphological studies of previously undifferentiated forms: (1) spined loaches from Central Europe with karyotype 2n = 50, NF = 96 correspond to the diagnosis of the species *C. elongatoides*; this species also differs from a number of closely related species and polyploid forms in the origin of the dorsal fin in front of the base of the pelvic fins, as well as in the presence of a spot in the lower half of the caudal fin base; (2) spined loaches from Lake Sinoe and other populations of Central Europe with karyotype 2n = 50, NF = 86 are nonconspecific to *C. tanaitica* and should apparently be ranked as an independent species, *C. megaspila*.

Keywords: ISSR polymorphism, *Cobitis taenia*, *C. tanaitica* from the Don River, *C. elongatoides*, *C. megaspila*
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INTRODUCTION

The taxonomy and phylogenetic relationships of spined loaches of the genus *Cobitis* (Cobitidae) have been intensively studied by different scientific groups since the 1980s, when it was shown that spined loaches previously attributed to the same species, *C. taenia* Linnaeus, 1758 (Berg, 1949) (which was divided into separate subspecies), are actually represented by a number of independent bisexual species, as well as by polyploid unisexual forms of different origin (Vasil'ev and Vasil'eva, 1982; Vasil'ev et al., 1989, 2011; Vasil'eva et al., 1989; Vasil'ev et al., 1990, 2007; Ráb and Slavík, 1996; Boroń and Danilkiewicz, 1998; Boroń and Kotusz, 1999, 2000; Ráb et al., 2000; Šlechtová et al., 2000, 2003; Bohlen and Ráb, 2001; Bohlen et al., 2002; Mezhzherin and Chudakorova, 2002; Lusk et al., 2003; Doadrio and Perdices, 2005; Janko et al., 2005a, 2005b, 2007, 2018; Majtánová et al., 2016). These studies are of great importance not only for determining the taxonomy and phylogenetic relationships of bisexual species, but also for studying the origin of polyploid forms of fishes and mechanisms of polyploid evolution of vertebrates. Despite a significant progress over the past years, a number of issues still need to be studied. These problems include the identification of parental species of the recorded polyploid forms, the degree of their relationship, and mechanisms determining their hybridization, as well as the identification of conditions of the coexistence of polyploid forms and parental species within bisexual–clonal or diploid–polyploid complexes.

This paper presents the results of molecular genetic analysis and comparative morphological studies in a group of diploid bisexual spined loaches morphologically similar to the species *C. taenia*, which were previously suggested as possible ancestors of a number of

Fig. 1. Collection sites: (●) *Cobitis taenia*, (■) *C. tanaitica*; rivers: (*1*) Western Dvina, (*2*) Dnieper, (*3*) Don.

polyploid forms. The results of analysis of the taxonomy and nomenclature of the controversial forms are discussed.

MATERIALS AND METHODS

The molecular genetic analysis involved the materials that were collected by the authors (Fig. 1) and included two species of spined loaches, which were confirmed by karyological data and identified using diagnostic morphological characteristics (Vasil'eva, 1984; Vasil'eva and Vasil'ev, 1998). Voucher samples are stored in the collection of the Zoological Museum of the Moscow State University (ZMMU): (1) specimens of spined loach *C. taenia* from the upper reaches of the Dnieper River near the village of Bilino, Smolensk oblast, 55°13′ N, 33°29′ E (five specimens, ZMMU P-21805, collected on June 23, 2006) and from the Western Dvina River near the town of Velizh, 55°36′ N, 31°12′ E (three specimens, ZMMU P-21800, collected on June 13, 2005); (2) Don spined loach *C. tanaitica* Băcescu et Maier, 1969 from the Don River near the village of Donskoe, Lipetsk oblast, 52°37′ N, 38°59′ E (eight specimens, ZMMU P-23353, collected on June 30, 2004).

Tissues from freshly caught fish individuals (mainly pectoral fins) were fixed with 96% ethanol. In addition to our own collections, we used materials from K. Janko (Institute of Animal Physiology and Genetics, Libechov, Czech Republic). Spined loaches from which tissues were used for the genetic analysis were identified by Janko as *C. elongatoides* Băcescu et Maier, 1969 (Comana River, Romania, and Mur River, Austria) and *C. tanaitica* (Lake Sinoe, Danube River delta, Romania). These materials were also used for genetic research in the Czech Republic (Janko et al., 2003, 2007).

To analyze the polymorphism of fragments of ISSR (inter simple sequence repeats) using polymerase chain reaction (PCR) (Gupta et al., 1994; Zietkiewicz

et al., 1994), we isolated DNA by the standard method of organic extraction (Maniatis et al., 1984).

The amplification reaction was performed using three primers: CA-RT (5'-CACACACACACACA-CART-3'), T-CA (5'-TCACACACACACACACA-3'), and CTC-RA (5'-CTCCTCCTCCTCCTCRA-3'). The PCR reaction mixture with a volume of 15 μL had the following composition (to the final concentration): 1X Taq polymerase buffer, 0.75 mM of each deoxyribonucleotide triphosphate, 2 mM $MgCl_2$, 4 μM of primer, 2 units of Taq polymerase, and 200 ng of the analyzed DNA.

PCR was performed on a PTC-225 thermal cycler (MJ Research, United States) with 65 cycles according to the following scheme: (1) DNA denaturation (3 min at 94°C), (2) DNA denaturation (20 s at 94°C), (3) primer annealing (45 s at 60°C), and (4) chain elongation (80 s at 72°C). The final chain elongation lasted 3 min at 72°C and the block was cooled for 1 min at 4°C.

At the end of PCR, 7 μL was taken from each reaction mixture and loaded into wells of 6% polyacrylamide gel. Electrophoresis was performed at an electric field strength of 7.5 V/cm for 3 h. After the gel was stained with ethidium bromide, the electrophoresis results were visualized using a Typhoon 8600 gel scanner (Molecular Dynamics, United States). We used the Phoretix 1D software (Nonlinear Dynamics, Great Britain) to calculate the coefficients of similarity of individual ISSR spectra according to Dice and construct dendrograms by the method of unweighted pair-group means based on Euclidean distances.

The comparative morphological analysis involved the following spined loach samples from the ZMMU collection.

Cobitis elongatoides: P-16311, Malaya Tisza River, Transcarpathian region, 1948 (one specimen, female); P-21221, Oder River, from a karyologically studied sample (Boroń and Kotusz, 1999), collected by J. Kotusz (two specimens, a male and a female).

Cobitis elongatoides and polyploid forms (see below): P-18159, Pšovka, Elba River basin, Czech Republic, November 4, 1989, collected by P. Ráb (17 specimens); P-23067, Lake Beleu in the lower reaches of the Prut River (Danube basin), Moldova, collected by A. Moshu (five specimens); P-23068, the channel of the middle and lower reaches of the Prut River from the town of Ungheni to the city of Leova, Moldova, collected by A. Moshu (11 specimens); P-23071, channel of the Rakovets River, left tributary of the upper reaches of the Prut River (Danube basin), Moldova, collected by A. Moshu (ten specimens); and P-23073, channel of the Dragishte River, tributary of the Prut River, Moldova, collected by A. Moshu (11 specimens).

Cobitis tanaitica, P-20246, Don River near the village of Rogozhkino, May 8–27, 1989, collected by V. Vasil' ev (46 karyotyped specimens).

Cobitis taenia and polyploid forms (P-17065), Moskva River near the city of Zvenigorod (16 specimens).

Cobitis vardarensis Karaman, 1928: P-20725, Vardar River, Yugoslavia, June 22, 1972, collected by M. Povz (five specimens).

Cobitis cf. *megaspila* Nalbant, 1993: P-23078, Rakovets River, left tributary of the upper reaches of the Prut River, Moldova, collected by A. Moshu (five specimens).

In addition to these materials, we also used the previously obtained data on different spined loach species from the works cited in this article.

The correlation of qualitative morphological features was estimated based on the Spearman rank correlation coefficient (ρ) (Gubler and Genkin, 1973).

RESULTS AND DISCUSSION

Genomic polymorphism was detected in the studied spined loach samples using two informative primers: CA-RT and T-CA. The UPGMA dendrogram, which summarizes the genetic relationships between the studied species, is shown in Fig. 2. Pairwise comparison showed that all the species differed well from each other. The dendrogram constructed on the basis of the CA-RT primer shows the characteristic clusters for *C. elongatoides*, *C. taenia*, and *C. tanaitica* from the Don basin and "*C. tanaitica*" from Lake Sinoe. In another case with the use of the T-CA primer, the change in the tree topology is determined by the combining of *C. tanaitica* individuals from the Don basin and "*C. tanaitica*" from Lake Sinoe into one cluster.

The system of molecular genetic markers based on the polymorphism of ISSR DNA fragments makes it possible to clearly differentiate four studied forms of spined loaches: *C. taenia* and *C. tanaitica* s. str. from the Don River basin, spined loaches from the Danube basin that are diagnosed as *C. elongatoides*, and spined loaches from Lake Sinoe that are assigned to the species *C. tanaitica* by a number of authors (Bohlen and Ráb, 2001; Bohlen et al., 2002; Janko et al., 2003, 2007; Majtánová et al., 2016). The results demonstrate clear advantages of this method compared to previous studies based on other genetic markers (isozyme analysis and *cyt b* analysis), which did not make it possible to differentiate certain forms of spined loaches morphologically similar to the species *C. taenia* (Šlechtová et al., 2003; Janko et al., 2007). Therefore, PCR analysis of polymorphic DNA regions between microsatellites using the developed system of the two primers (CA-RT and T-CA) opens a prospect of successful analysis of the genetic structure of polyploid forms of spined loaches of different origins.

At the same time, the results provide a basis for solving a number of taxonomic problems in the genus *Cobitis*. As follows from the resulting data, although spined loaches from Lake Sinoe are closest to *C. tanaitica* from the Don River, they clearly differ

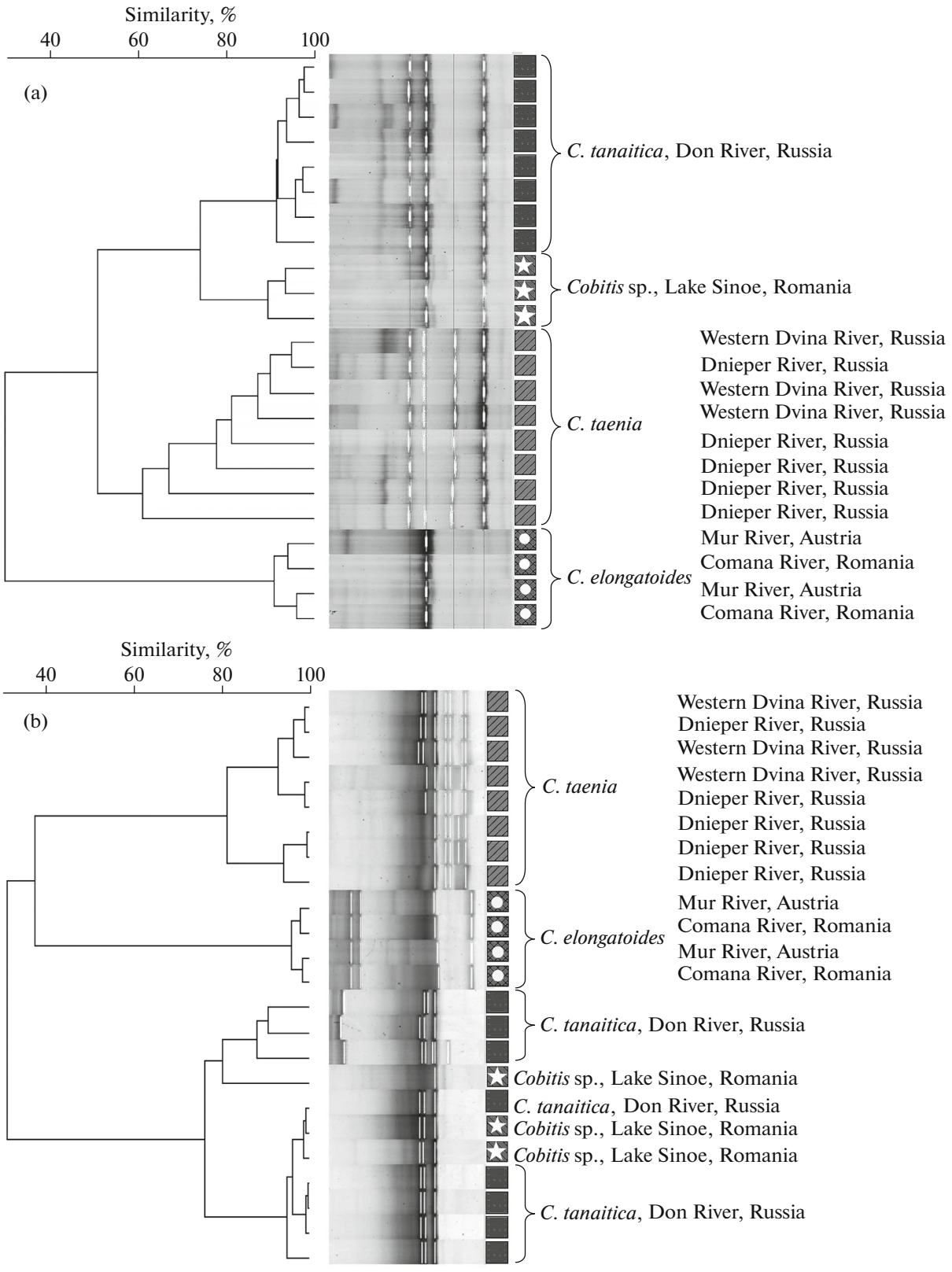

Fig. 2. UPGMA dendrogram of similarity/difference of individual ISSR spectra of different species of the genus *Cobitis* for the two informative primers: (a) CA-RT, (b) T-CA. Species-specific DNA fragments are marked with white dashes on the gel lanes.

from this form by molecular markers (primer CA-RT). It should be noted that this is not the only difference between the two forms of spined loaches. The karyotype of *C. tanaitica* from the Don River (the type locality of this species) is characterized by a fixed Y-autosomal translocation: in females, the number of chromosomes is 2n = 50 (8 meta- (m) + 28 submeta- (sm) + 14 subtelocentric (st)); in males, 2n = 49 (9m + 28sm + 12st); the number of chromosome arms (NF) is 86 (Vasil'eva and Vasil'ev, 1998: cited as *C. rossomeridionalis* Vasil'eva et Vasil'ev, 1998, which should be considered a junior synonym of *C. tanaitica*). This karyotype significantly differs from that of the spined loach *C. taenia* that was first described for the population from the Volga River basin (Vasil'eva et al., 1989; Vasil'ev et al., 1989) and later found in other diploid spined loach populations, including spined loaches from the upper reaches of the Dnieper and Western Dvina, which are presented in our study (Vasil'ev et al., 2007). The karyotype of *C. taenia* with 2n = 48 (10 m, 18 sm, and 20 subtelo-acrocentric (sta) chromosomes) and NF = 76 is currently a reliable marker of the species throughout the range of the group of closely related European diploid species and polyploid forms (Table 1). The second marker for European spined loach species is the karyotype that was first described for diploid spined loaches from the Pšovka brook in the Czech Republic: 2n = 50 (30m + 16sm + 2st + 2a), NF = 96 (Ráb and Slavík, 1996). It was later shown that spined loaches with this karyotype, which are identified by the authors as *C. elongatoides*, have a wide pan-Danubian range and are also widespread in the basins of the Baltic and North seas (Boroń and Kotusz, 1999; Ráb et al., 2000), which was also confirmed by molecular genetic studies (Janko et al., 2003, 2005a).

As for the karyotype of spined loaches from Lake Sinoe, the following should be noted here. The assumption on the presence of a karyotype similar to the karyotype of *C. tanaitica* in spined loaches living to the west of the Dnieper River basin was first made based on studying the structure of the karyotypes of polyploid forms. According to these studies, the genome of triploid spined loaches from the Vistula River basin includes a haploid set of an unknown species, whose karyotype presumably contains 50 chromosomes (12m + 24sm + 14st-a) (Boroń and Danilkiewicz, 1998) and is similar to the karyotype of *C. tanaitica* (Boroń and Kotusz, 2000). A haploid set with 8m + 13sm-st + 4a was also initially assigned to the unknown species *Cobitis* sp.; it was isolated from the genome of the triploid form of specimens from the Dyje (Middle Danube) and Morava rivers (Ráb et al., 2000). The karyotype established in the genomes of these triploids was then identified with the karyotype of *C. tanaitica* (Bohlen and Ráb, 2001; Bohlen et al., 2002). This view point was adopted in all subsequent publications on spined loaches from Central Europe (Lusk et al., 2003; Šlechtová et al., 2003; Janko et al., 2007). The above-mentioned formulas of triploid karyotypes and *C. elongatoides* suggest the following possible variants of the formula of the "*C. tanaitica*" karyotype from the Danube basin and waters of Central Europe: 16m + 26sm-st + 8a (Ráb et al., 2000); 12m + 24sm + 14st-a (Boroń and Danilkiewicz, 1998); and 10m + 22sm + 18st (Lusk et al., 2003). In these variants, NF can vary from 82 to 90.

The karyotype of diploid spined loaches from Lake Sinoe was first published in 2007 (Janko et al., 2007). According to the presented karyogram (in the text of the article, an error was made in the karyotype formula), "*C. tanaitica*" from the water bodies of Central Europe has 50 chromosomes (10m + 26sm + 14sta) with NF = 90 (Table 1). This karyotype is more different from the karyotype of *C. tanaitica* from the Don basin than the above-presented karyotypes obtained by isolating the karyotype of *C. elongatoides* from the triploid karyotype. Nevertheless, it can be assumed that all the observed differences in the ratio of bi-armed and uni-armed chromosomes in the compared karyotypes may be determined by different degrees of chromosome spiralization in the metaphases studied by the authors, as well as by discrepancies in the classification of separate chromosomes. One important aspect cannot be allowed. While presenting the karyotype of "*C. tanaitica*," Janko et al. (2007) emphasize that the biotype corresponding to this karyotype was found both in males and females of spined loaches from the lower reaches of the Danube, the Dobruja area, the upper reaches of the Oder, and the Don River. This attempt to artificially combine spined loaches from the Don and Central Europe looks more than incorrect.

The fixed Y-autosomal translocation, which is expressed in different chromosome numbers in males and females, is an essential diagnostic feature of *C. tanaitica* s. str. from the Don basin (see above). Differences in the karyotypes of males and females of *C. tanaitica* were determined based on a cytological analysis of 50 specimens from the Don, Obitochnaya, Beisug, and Kalka (basin of the Sea of Azov) rivers and the basins of the Kuban, Dnieper, Dniester, and Southern Bug rivers (Vasil'eva and Vasil'ev, 1998). The number of karyotyped spined loaches from Lake Sinoe is unknown; individuals from other localities of Central Europe were identified with them based on an allozyme analysis (Bohlen and Ráb, 2001), the results of which are very contradictory (Šlechtová et al., 2000; Janko et al., 2005b, 2007). In any case, the karyological data indicate that the spined loaches from Lake Sinoe are nonconspecific with *C. tanaitica*; they differ not only in the absence of Y-autosomal translocation but also in the karyotype formula (Table 1): they have more metacentric chromosomes and fewer submetacentric ones. The main localities of this species are confined to the lower reaches of the Danube (near the confluence of the Argeș tributary in Romania and to the southeast of the city of Vidin in Bulgaria) and to

Table 1. Karyotypes of a number of European spined loach species (genus *Cobitis*) according to different authors

Species	Aquatic system	2n	Karyotype formula	NF	Source of information
C. taenia	Volga River basin	48	10m + 18sm + 20sta	76	Vasil'ev et al., 1989; Vasil'eva et al., 1989
	Bug River basin, Poland	Same	Same	Same	Boroń, 1995
	Oder River basin	»	»	»	Boroń and Kotusz, 1999; Szlachciak and Boroń, 2003
	Vezer River, North Sea basin	»	»	»	Ráb et al., 2000
	Lake Kleiner Plöner See, Baltic Sea Basin	»	»	»	Same
	Vistula River basin	»	»	»	Boroń and Danilkiewicz, 1998; Szlachciak and Boroń, 2003
	Lakes in northern Poland	»	»	»	Ráb et al., 2000; Szlachciak and Boroń, 2003
	Water bodies of Great Britain	»	»	»	Boroń et al., 2003
C. elongatoides	Pšovka brook, Elbe River basin	50	30m + 16sm + 2st + 2a	96	Ráb and Slavík, 1996
	Lužnice River, Elbe River basin	Same	Same	Same	Ráb et al., 2000
	Dye River, Middle Danube basin	»	»	»	Same
	Area of the city of Bucharest	»	»	»	»
	Oder River basin	»	28m + 18sm + 4sta	»	Boroń and Kotusz, 1999; Szlachciak and Boroń, 2003
	Ida River, Tisza River basin	»	30m + 16sm + 2st + 2a	»	Lusk et al., 2003
	Basins of the Danube, Elbe, and Oder rivers and Kamchiya River	»	22m + 26sm + 2st	98	Janko et al., 2007
C. tanaitica	Basins of the Don, Kuban, Dnieper, Southern Bug, and Dniester rivers; water bodies of the Sea of Azov basin	50♀ 49♂	8m + 28sm + 14st 9m + 28sm + 12st	86 Same	Vasil'ev, 1995 (indicated as *Cobitis* sp.); Vasil'eva and Vasil'ev, 1998 (indicated as *C. rossomeridionalis*)
*C. tanaitica**	Danube River	50	10m + 22sm + 18a	82	Bohlen et al., 2002
	Danube, Oder, and Don rivers; Lake Sinoe	Same	10m + 26sm + 14sta	86	Janko et al., 2007
	Danube River and Lake Sinoe	»	Same	Same	Majtánová et al., 2016

* The species name is given in accordance with the cited publications. 2n, diploid set of chromosomes; m, sm, st, sta, and a are meta-, submeta-, subtelo-, subtelo-acro-, and acrocentric chromosomes, respectively; NF, number of chromosome arms, ♀, female, ♂, male.

the Black Sea coast near its estuarine zone in Romania (in addition to Lake Sinoe, it is indicated for lakes Hazarlâc, Ghiol, and Razim (= Raselm) (Janko et al., 2007, 2018). Polyploid forms with a karyotype containing the haploid set of this species are also recorded mainly in the Danube basin (Janko et al., 2007); therefore, a suitable name for this species should be sought among the nominal names of Danubian spined loaches.

Previously (Kottelat, 1997), five names were considered available for spined loaches from the Danube basin, in which males have one Canestrini's organ at the base of the pectoral fin: *taenia*, *elongatoides*, *danubialis*, *megaspila*, and *taenioides*. *C. taenia* is a valid species; as noted above, it is characterized by 2n = 48; according to the current data, it does not occur in the Danube basin. The name *elongatoides* was first proposed by Băcescu, 1962 as infrasubspecific (*Cobitis taenia* var. *elongatoides*) for spined loaches from southwestern Romania. It became available from the subsequent publication of Băcescu and Maier (1969), where it is used as a subspecific name. The problem of availability of this name and adoption of *C. taenia danubialis* Băcescu, 1993 (proposed as a replacement name) (Nalbant, 1993) as one of its junior synonyms was discussed earlier (Kottelat, 1997; Freyhof et al., 2000).

The name *elongatoides* proposed by Băcescu and Maier (1969) definitely refers to spined loaches from the Danube basin. The caption to outline drawing 3 indicates that the designation "A" refers to the image of a male of *C. taenia elongatoides* Bac. in the English version of the article (Băcescu and Maier, 1969) or *C. taenia elangotoides* Băc. in the Russian version (Băcescu and Maier, 1969, p. 54 [in Russian]). The caption also indicates a certain locality, namely, the "Argesel River," a tributary of the Argeş River in the southern Carpathians. However, two paragraphs above, another name is given for spined loaches from the Danube basin, namely, *taenioides*. On the one hand, it can be assumed that Băcescu and Maier refer this name to the same spined loaches, since they describe the pattern of relative arrangement of the dorsal and pelvic fins in *C. taenioides* Băcescu et Maier, 1969 below in their text with reference to the same figure 3A (Băcescu and Maier, 1969 [in Russian]; Băcescu and Maier, 1969); however they increase the range of its distribution to "the rest of Europe." On the other hand, this name can also be considered a synonym of *C. taenia*, as suggested by Kottelat (1997), since the cited authors characterize the structural features of Canestrini's organ of *C. taenioides* with reference to Fig. 5B, which shows the organ of a male of *C. taenia taenia* from England, judging by the figure caption (Băcescu and Maier, 1969 [in Russian]; Băcescu and Maier, 1969). Therefore, the name "*C. taenioides*" should definitely be considered nomen dubium.

In addition to the above-mentioned names, the species *C. megaspila* Nalbant, 1993 was also described based on 38 spined loach specimens (including the holotype) from the Danube delta (Caraorman, a channel from the pond) and two individuals from the Gurban valley near the village of Comana, 27 km south of the city of Bucharest. Its validity was often questioned in some way (Vasil'eva and Vasil'ev, 1998; Ráb et al., 2000; Kottelat and Freyhof, 2007). However, in the light of the current data, it seems necessary to revise the previous conclusions on the nomenclature of Danube spined loaches.

According to the publication of Băcescu and Maier (1969, p. 57), the diagnostic features of *C. taenia elongatoides*, which was ranked to the species level in further studies, can only be the shape of Canestrini's organ (ax-shaped, as in the genetic "*taenia*" series) and the pattern of arrangement of the dorsal and pelvic fins: the dorsal fin originates well in front of the bases of the pelvic fins, while it originates at their level in other species. The following description of *C. taenia danubialis* (this description is based on other material: the type series includes fish individuals from the upper reaches of the Mureş River (Harghita County), the Timiş River in Banat, and Suceava River in Moldova) is accompanied by a more extensive diagnosis. However, the number of features that make it possible to differentiate this taxon from *C. megaspila* described in the same paper is small. In addition to the position of the dorsal and pelvic fins (in *C. megaspila*, they are positioned at the same level), a reduction in the size and number of spots in fourth Gambetta's zone (spots along the midline of the flank) is indicated for *C. taenia danubialis*. This form of spined loach is considered common in the Danube basin, in contrast to *C. megaspila*, which is characterized by a narrow range (Nalbant, 1993).

The summary on European freshwater fishes (Kottelat and Freyhof, 2007) provides the following diagnosis for the species *C. elongatoides* with a range covering the entire Danube basin and the upper reaches of the Elbe and Odra: one eye-sized or pupil-sized oval or round black spot in upper part of base of caudal fin; third Gambetta's zone usually wider than second one; both zones extending beyond base of dorsal fin; one Canestrini's organ. It is indicated that this species does not differ from sympatrically occurring hybridogenic individuals in external features; similarly, it differs from *C. vardarensis* Karaman, 1928 and *C. pontica* Vasil'eva et Vasil'ev, 2006 only in the karyotype. On the photograph of *C. elongatoides* from the Nera River (Danube basin, Romania), the width of the third Gambetta's zone exceeds the depth of the spots of the fourth zone (the largest spots in the coloration of spined loaches). The diagnosis for *C. tanaitica* that is given in the cited work corresponds to the characteristics of its populations from the northern part of the Black Sea (the third Gambetta's zone usually ends under the base of the dorsal fin; this zone is usually

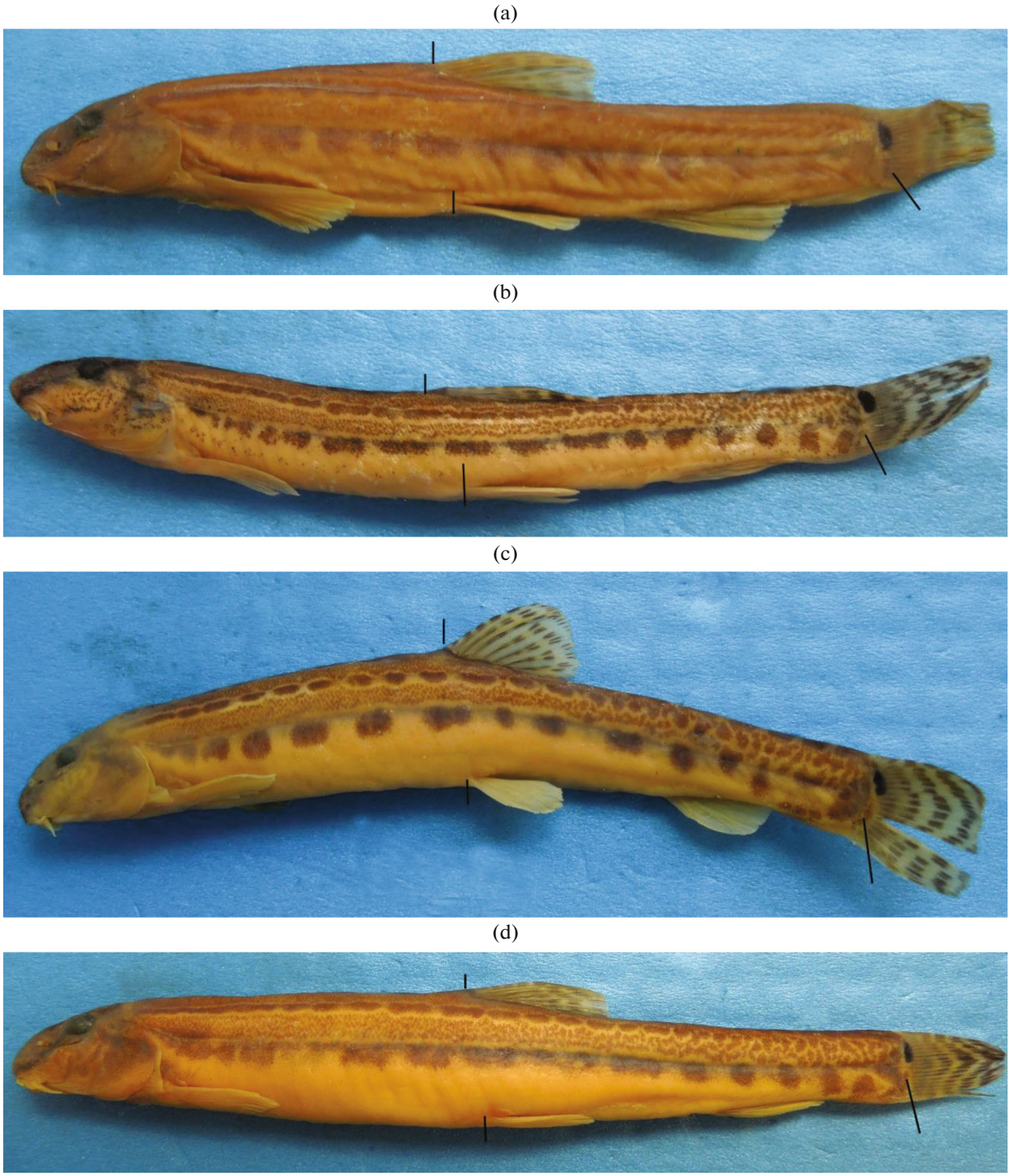

Fig. 3. External view of *Cobitis elongatoides* from the ZMMU collection (here and in Fig. 4, the origins of the dorsal and pelvic fins and the second spot at the base of the caudal fin are marked): (a) male from the Oder River, P-21221, *SL* 65.0 mm, (b) female from the Prut River, P-23068, *SL* 75.0 mm, (c) female from the Rakovets River channel, P-23071, *SL* 90.5 mm, (d) female from the Dragishte River, P-23073, *SL* 101.5 mm.

narrower than the second zone; there is one black spot at the base of the caudal fin) and the figure presents a specimen from the Kuban basin; however, the range of the species is widened up to the Danube; the species *C. megaspila* is not mentioned by the authors. The presented data give grounds for the following assumptions. Firstly, during the compilation of the keys and diagnoses, diploid spined loaches assigned to

"*C. tanaitica*" from Romanian water bodies were not available to the authors (there are no descriptions of the features of the morphology and coloration of these individuals in the literature). Secondly, the diagnosis of the species *C. elongatoides* was based on mixed material (diploid and polyploid forms of different origins) from the Danube basin. This assumption is supported not only by the diagnosis (which does not make it possible to differentiate *C. elongatoides* from other Danube spined loaches) but also by the maximum size given for the females (the standard length (*SL*) is 130 mm), since this body length can be reached only by polyploid individuals.

Therefore, comparative morphological studies of spined loaches with certain ploidy from karyotyped Polish populations (Kotusz, 2000) are of particular interest. These studies revealed the color features of *C. elongatoides* that were previously not recorded in the literature. The diploid spined loaches from two populations of the Oder River basin, where the diploid species with marker karyotype 2n = 28m + 18sm + 4sta and NF = 96, diagnosed as *C. elongatoides* (Table 1), lives together with polyploid forms of different origins (Boroń and Kotusz, 1999), had two spots at the base of the caudal fin. The second spot, located in the lower part of the fin base, was brown, less distinct, and much narrower than the bright black spot in the upper part. This spot might be strongly reduced and almost invisible; it was absent in four of the 30 studied females and 19 males, which was only 8.2%. All the cohabiting polyploid forms (a total of 98 specimens were studied), as well as the studied individuals of *C. taenia* from the populations of the Neman and Vistula river basins and sympatric polyploids (104 specimens), did not have a spot in the lower part of the caudal fin base (Kotusz, 2000). There was a second spot in both specimens of *C. elongatoides* from the Oder basin, which were transferred to the ZMMU collection by J. Kotusz (P-21221; Fig. 3a).

We studied a sample of spined loaches from the Pšovka in the Czech Republic, where *C. elongatoides* and its triploid and tetraploid forms also coexist, according to karyological analysis (Ráb and Slavík, 1996). This sample (P-18159) consisted of six males and 11 females. The second spot in the lower part of the caudal fin base (a thin spot in the form of a brownish bracket) was observed in three males (50.0%) and three females (27.3%). Taking into account that the proportion of diploid females in different samples from the Pšovka brook was 25.0 to 66.7% of all females (calculated according to: Ráb and Slavík, 1996) and fixed materials were stored for about 20 years and might be partially depigmented, the presence of two spots at the base of the caudal fin is definitely characteristic of most of the diploid individuals from the Pšovka brook. The second spot is clearly visible in the photographs of two individuals of *C. elongatoides* from other water bodies of the Czech Republic (Hanel and Lusk, 2005). A lower spot was also preserved in a spined loach female from the Malaya Tisza River (P-16311), although it was significantly discolored during the storage. Since the second spot at the caudal fin base is present in individuals from different parts of the range of *C. elongatoides*, it can be assumed that its presence (if not in all individuals from the population, then at least in most of them) is a specific feature of this spined loach.

We did not find this spot in the previously studied *C. taenia* and *C. tanaitica* and polyploid forms of spined loaches that we collected from different water bodies in the European part of the former Soviet Union (Vasil'eva, 1984; Vasil'eva et al., 1989; Vasil'ev et al., 1990; Osinov et al., 1990; Vasil'eva and Vasil'ev, 1998). There was no such spot in the type specimens of species that were also very similar to *C. taenia* in their external morphology: *C. taurica* Vasil'eva, Vasil'ev, Janko, Ráb, et Rábová, 2005 and *C. pontica* (Janko et al., 2005b; Vasil'eva and Vasil'ev, 2006). It should be emphasized that the latter species is widespread in waters of Bulgaria and (according to Kottelat and Freyhof (2007)) in Turkey; however, spined loaches from the Southern Bug River does not belong to this species, as wrongly assumed by Janko et al. (2007). In the studied sample of *C. vardarensis* from the Vardar River (P-20725), the male had a lower spot with a similar shape and all the four females had a slightly darkened spot in the lower part of the fin base, which had a shape similar to that of the spot in the upper part.

Comparison of individuals with two spots or one spot at the base of the caudal fin by the main diagnostic feature of *C. elongatoides* proposed by Băcescu and Maier (1969), i.e., the pattern of relative arrangement of the dorsal and pelvic fins, gave the following results. In all studied males from different populations (P-18159 and P-21221, seven individuals), the bases of pelvic fins are well behind the origin of the dorsal fin, namely, at the level of its first or second (57.1%) branched ray, regardless of the number of spots at the base of the caudal fin. In females with two spots at the base of the caudal fin (five individuals), the bases of the pelvic fins were more often located behind the origin of the dorsal fin, namely, at the level of its first unbranched (20%) and first (40%) or second (20%) branched ray; the bases of the pelvic fins were slightly in front of the origin of the dorsal fin only in a female from the Malaya Tisza River (P-16311). In females with one upper spot at the base of the caudal fin (nine specimens), the bases of the pelvic fins were slightly in front of the dorsal fin (11.1%), at the level of the dorsal fin (11.1%), at the level of the first (44.4%) or second (11.1%) unbranched ray, or at the level of the first branched ray (22.2%). Both features (fin position and number of spots) showed a significant correlation with a high probability: the rank correlation coefficient $\rho = 0.845$ ($n = 20$, $p < 0.001$); i.e., the bases of the pelvic fins in individuals with two spots at the base of the caudal fin are usually well behind the origin of the dorsal fin, in contrast to individuals with one upper spot.

For comparison, it can also be noted that in different samples of *C. tanaitica* from the Don basin the proportion of individuals with the bases of the pelvic fins at the level of the origin of the dorsal fin or even in front of it varies from 38.1 (Vasil'eva and Vasil'ev, 1989) to 80.4% (this paper); their proportion is 50.0% in the representative sample of *C. taenia* and polyploid specimens from the Volga basin (P-17065); such individuals prevail among the type specimens of *C. taurica* and *C. pontica* (Janko et al., 2005b; Vasil'eva and Vasil'ev, 2006), while they are only 8.3% among males and females of spined loaches with two spots from the Tisza, Oder, and Pšovka. The bases of pelvic fins are also well behind the origin of the dorsal fin (approximately at the level of its second branched ray) in the photographs of males from water bodies of the Czech Republic (Hanel and Lusk, 2005) and a male from the Nera River (Kottelat and Freyhof, 2007), as well as in a male from the Spree River (Elbe basin, Germany) with prominent darkening in the lower part of the caudal fin base and with a karyotype including 46m-sm, 2st, and 2a (Bohlen et al., 2005). In the studied individuals of *C. vardarensis*, the bases of the pelvic fins are at the level of the origin of the dorsal fin (80%) or at the level of its first unbranched ray.

Therefore, the presence of two spots at the base of the caudal fin and the dorsal fin usually shifted forward from the bases of the pelvic fins in most of the individuals of the population should be considered the diagnostic features of spined loaches with 2n = 50, an extremely low number of uni-armed chromosomes (2–4 st-a), and NF = 96–98 (Table 1). With respect to these features, they correspond to the diagnosis of the species described under the names "*elongatoides*" and "danubialis" (Băcescu and Maier, 1969 [in Russian]; Nalbant, 1993). Based on the above-listed diagnostic characteristics, the species *C. elongatoides* analyzed under field conditions can be differentiated with a high probability from the polyploid forms of hybrid origin living in the same water bodies. Accordingly, based on the data obtained from the above-mentioned sample from the Pšovka (P-18159), where *C. elongatoides* and the polyploid hybrid form were recorded by genetic methods, we distinguished 11 specimens as a separate sample (P-22187) with the diagnosis of *C. elongatoides*: all males, three females with two spots at the caudal fin base, and two females with the bases of the pelvic fins at the level of the first branched ray of the dorsal fin. Among the studied spined loaches from the basin of the Prut River (Danube system), which were primarily fixed in a formaldehyde solution, not genetically studied, and represented only by females in samples (P-23067, P-23068, P-23071, and P-23073), there were specimens in which the dorsal fin originated well in front of the base of the pelvic fins and a weak brown spot was clearly visible in the lower part of the caudal fin base (Figs. 3b–3d). We believe that these samples include *C. elongatoides* and polyploid specimens of hybrid origin.

As for spined loaches from Lake Sinoe (and other populations with the same karyotype), the only suitable name that can potentially be assigned to them as available according to this research is "*C. megaspila*." The diagnosis of this species that was given during its description (Nalbant, 1993) is very little informative compared to the set of other species and forms that are morphologically similar to *C. taenia*, as was previously noted (Vasil'eva and Vasil'ev, 1998). However, it has an important characteristic that contrasts this species with *C. elongatoides*, which also lives in the Danube basin: the dorsal fin of these spined loaches is at the same level as the bases of the pelvic fins (Nalbant, 1993). Since the description of *C. megaspila* is based on considerable material (see above), it can be assumed that the above-mentioned differences are at least frequent and the morphological features do not give grounds for assuming that *C. elongatoides* and *C. megaspila* are conspecific. In turn, the genetic data definitely indicate that, along with *C. elongatoides*, another species lives in the Danube basin, which is currently identified only by the structure of the karyotype: 2n = 50 = 10m + 26sm + 14sta, NF = 86.

Ráb et al. (2000, p. 64) reported that, according to their preliminary data, a karyotype characteristic of *C. elongatoides* "was found in the spined loach population from the vicinity of Bucharest, Romania (identified by Nalbant as *C. megaspila*)." Further publications (Bohlen and Ráb, 2001; Šlechtová et al., 2003; Janko et al., 2005a) indicate the presence of *C. elongatoides* and a triploid form with two haploid sets of *C. elongatoides* and one haploid set of "*C. tanaitica*" for the Comana River (as noted above, two spined loach specimens from the area near the village of Comana were paratypes of *C. megaspila*). However, this cannot be the reason to reject the availability and validity of the name *C. megaspila* for spined loaches from Lake Sinoe and other populations with the same karyotype, even if we assume that the paratypes of *C. megaspila* from the Comana area actually belong to the species *C. elongatoides* or represent polyploids. Although it is clear that genetic studies were carried out on other specimens and not on these paratypes, which were most likely fixed with a formaldehyde solution and were hardly suitable for genetic studies, and only six specimens were karyotyped from the population. A similar situation is also potentially possible for part of the type series from the Danube delta: thus, according to preliminary data, all the 12 karyotyped loaches from the channel in Caraorman (the type locality of *C. megaspila*), proved to be triploids with two haploid sets of *C. elongatoides* and one haploid set of "*C. tanaitica*" (Bohlen and Ráb, 2001). However, the type series of *C. megaspila* has a significant number of males (including the holotype), which, similarly to diploid females, were not found in this area by subsequent researchers.

The description of spined loaches from Lake Sinoe (and other diploid populations with the same karyo-

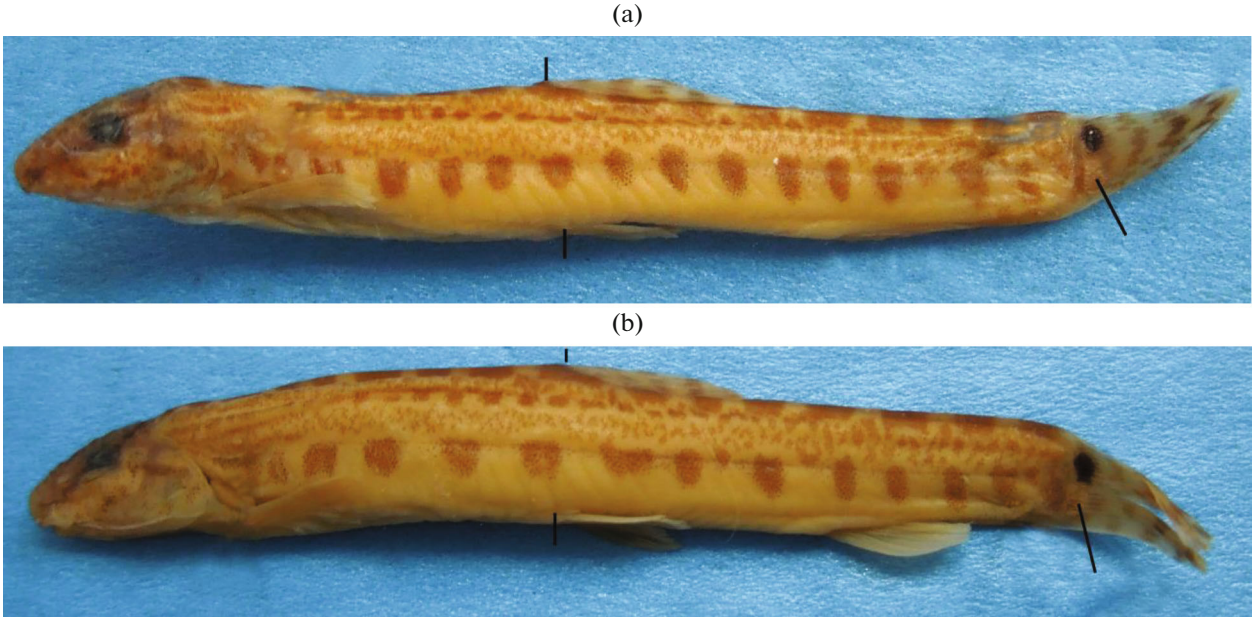

Fig. 4. External view of spined loaches from the Rakovets River, P-23078, diagnosed as *Cobitis* cf. *megaspila*: (a) *SL* 50.3 mm, (b) *SL* 54.0 mm.

type) as a new species differing from *C. megaspila* could only be proven by the fact that: (1) all the individuals of the type series of *C. megaspila* have a different karyotype (this is impossible, since the type series is unsuitable for such studies) or (2) the morphological features of individuals with the karyotype recorded in spined loaches from Lake Sinoe clearly contradict the diagnosis of *C. megaspila*, which seems to be unlikely, since this diagnosis is generally consistent for a number of species morphologically similar to *C. taenia*. In particular, we consider it necessary to note that the dorsal fin in all five small specimens (*SL* < 54 mm) from the sample of spined loaches from the Rakovets River, a tributary of the Prut River (P-23078), originated at the same level as the bases of the pelvic fins, and spots along the midline of the flank (fourth Gambetta's zone) were large and vertically elongated, which corresponds to the diagnosis of *C. megaspila*; however, all of them also had a faint spot in the lower part of the caudal fin base (Fig. 4), which is characteristic of *C. elongatoides*. Since spined loaches from the Danube are still insufficiently studied, we determine this sample as *Cobitis* cf. *megaspila*.

In conclusion, the following should be noted. The use of molecular methods is widely used in ichthyologic studies to clarify the taxonomic status of a certain fish group. At the same time, molecular methods cannot always be used without involving other methods, e.g., morphological or cytological approaches. The use of ISSR markers in this research made it possible to confirm the independent status of spined loach from Lake Sinoe, which was previously assumed based on cytogenetic data. In addition, the use of ISSR analysis is justified by the possibility of amplifying the most polymorphic part of the genome, which made it possible to reveal differences that were not detected by other molecular methods, such as sequencing of separate mitochondrial and nuclear genes. The results of our research suggest the following main conclusions.

(1) The use of molecular genetic markers of polymorphism of ISSR-DNA fragments makes it possible to successfully differentiate the genomes of different spined loach species, which were presumably involved in the formation of polyploid forms of hybrid origin. Although it is relatively difficult to reproduce DNA fingerprinting data, this method shows the necessity of further studies of genomes of diploid species and polyploids using high-throughput next generation sequencing (NGS).

(2) Spined loaches from Lake Sinoe and their other populations from Central Europe with karyotype 2n = 50, NF = 86 are differentiated from the Eastern European species *C. tanaitica* by ISSR markers; at the current stage of research, they should be ranked as an independent species, *C. megaspila*.

(3) Spined loaches from Central Europe with karyotype 2n = 50, NF = 96 correspond to the diagnosis of the species *C. elongatoides*. According to our morphological analysis, in addition to the structure of the karyotype, *C. elongatoides* also differs from a number of closely related species and polyploid forms in the following set of features: the bases of the pelvic fins are usually well behind the origin of the dorsal fin (at the level of its first branched rays); there is an additional spot in the lower half of the caudal fin base,

which is smaller and less pronounced than the large intensely black spot in the upper part.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

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