Phylogenetic meta-analysis of the Cyprinidae (Teleostei: Cypriniformes) family using mitochondrial cytochrome *b* region

Filogenetikai metaanalízis a *Cyprinidae (Teleostei: Cypriniformes)* család tagjai között a mitokondriális citokróm *b* régió alapján

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SUMMARY

In this study, phylogenetic relationships were investigated within the Cyprinidae family. This is the largest taxonomic group of freshwater fishes, which therefore has ecological and economic importance. Sequences of the mitochondrial cytochrome *b* (cyt *b*) region were collected from the NCBI (National Center for Biotechnology Information) database, which was screened to identify a total of 1,025 sequences belonging to 219 species of the Cyprinidae family, which were contained to 684 haplotypes. The results of the meta-analyses showed that species of fish belonging to different subfamilies were well separated in their haplotypes (apart from 1), while those belonging to the same or closely related species have shared identical haplotypes. In some cases (n=12), haplotypes belonging to more than one species were found. In this sense, although the exact relationship between subfamilies and other groups is controversial in several instances, this study has addressed several phylogenetic issues as well as discussed and supported theories proposed in the literature. However, it confirmed that taxonomic classifications within the Cyprinidae family are still generally correct. To the best of our knowledge, this study is the first attempt to analyse the mitochondrial cytochrome *b* region in sequences of the Cyprinidae family available from the NCBI database by phylogenetic analysis.

Keywords: Cyprinidae, cytochrome *b*, haplotype, phylogenetic, taxonomy

ÖSSZEFOGLALÁS

Célkitűzés: A *Cyprinidae* család az édesvízi halak legnagyobb taxonómiai csoportja, így mind az ökológia mind a gazdaság fontos szereplője (pl. modell-, táplálék- és díszhalak). Jelen tanulmányban a *Cyprinidae* családon belüli filogenetikai kapcsolatokat vizsgáltuk.
Módszertan: Az elemzés során a mitokondriális citokróm *b* (cyt *b*) régió szekvenciáit gyűjtöttük össze az NCBI (National Center for Biotechnology Information) adatbázisából, amelyet a fajok és nemzetségek szintjén végzett filogenetikai vizsgálatokban általánosan használnak. Az adatbázis szűrése után 1025 darab, 684 haplotípust képviselő, összesen 219 *Cyprinidae* fajhoz tartozó szekvenciát vontunk be az analízisbe. A szekvenciákat Kalign szoftverrel igazítottuk egymáshoz, majd a MEGA-X 10.2.2 programmal vágtuk meg, a haplotípusok kinyeréséhez és kapcsolatuk megismeréséhez a DNA Sequence Polymorphism v.06 (DnaSP) és a NETWORK 10 szoftvereket használtuk. A haplotípusok populációgenetikai struktúrájának kiszámításához az ARLEQUIN 3.5.2.2 programot alkalmaztuk, míg filogenetikai kapcsolatukat a MEGA-X 10.2.2 szoftverrel térképeztük fel.
Eredmények: A metaanalízis alapján a különböző alcsaládokba tartozó halfajok haplotípusai jól elkülönültek egymástól, míg az azonos vagy közeli rokon fajokhoz tartozó egyedek szekvenciái azonos haplotípusokba sorolódtak. Több esetben találtunk egynél több fajhoz tartozó haplotípust, sőt egy esetben 2 különböző alcsalád is azonos haplotípuson osztozott. További elemzések során pedig felmerült annak a lehetősége, hogy ezek alapján a *Barbus petitjeani* fajt célszerű lehet átsorolni a *Torinae* alcsaládba. Azonosítottunk 5 darab egymással szoros kapcsolatban álló haplotípust, melyek egyaránt tartoztak a *Carassius auratus* és a *Carassius gibelio* fajokhoz, míg a *Poropuntius bolovenensis*, *Poropuntius lobocheiloides* és *Poropuntius solitus* fajok 6 közös haplotípussal rendelkeztek. Ezek a fajok a szakirodalomban sem különülnek el egyértelműen egymástól.

Következtetések: Jelenleg az alcsaládok és fajok közötti pontos taxonómiai kapcsolatok több esetben vitatottak. Így ez a tanulmány számos aktuális filogenetikai kérdést tárgyalt, továbbá megvizsgált, illetve egyes esetekben alátámasztott szakirodalomban található elméleteket. Mindezek mellett azonban megerősítette, hogy a *Cyprinidae* családon belüli rendszertani osztályozások a filogenetikai metaanalízis alapján többnyire helytállóak. Tudomásunk szerint ez a tanulmány az első kísérlet arra, hogy az NCBI adatbázisában rendelkezésre álló *Cyprinidae* család cyt *b* régiójának teljes szekvenciakészletét felhasználva egy, a teljes taxonómiai családot átfogó filogenetikai elemzést végezzen.

Kulcsszavak: *Cyprinidae*, citokróm *b*, haplotípus, filogenetika, taxonómia

1. Introduction

As the oldest and largest group of vertebrates, the fish hold a prominent place among the vertebrates. Thus, it is not surprising that their karyotype and genome size are more diverse and complex than those of other vertebrate taxonomic groups. Throughout their evolution, they have undergone whole genome duplication (WGD) and re-diploidization several times (*Volff*, 2005; *Shao et al*., 2019; *Xu et al.*, 2019). These events are linked to the appearance of the wide variation in genetic and phenotypic features during their development, which has contributed to their adaptability and widespread distribution around the world (*Kuang et al.*, 2016; *Moriyama and Koshiba-Takeuchi*, 2018; *Tian et al.*, 2022). An example of their genetic complexity is their difference in chromosome numbers caused by the WGD, as in the case of the goldfish *(Carassius auratus)* and common carp *(Cyprinus carpio).* Their chromosome numbers (n=100) are twice those of the other diploid (n=50) species from the same Cyprininae subfamily, suggesting that they are tetraploids (*Zhou and Gui*, 2002; *Xu et al.*, 2019; *Chen et al.*, 2020). Fish species from some other families also have polyploidy (e.g. *Salmo trutta*), and there are natural occurrences of some normally diploid specimens found to be polyploidy (*Gharbi et al.*, 2006; *Zhou and Gui*, 2017). This genetic variance helps all fish species to be able to be model animals for different biotechnological reproductive techniques, and to produce different kinds of hybrids that will be able to breed (*Piferrer et al.*, 2009; *Atsumi et al.*, 2018; *Müller et al.*, 2020; *Marinović et al.*, 2022). So, this high degree of diversity makes fish suitable as subjects for many biological (e.g. for the improvement of market fish quality, and quantity) and evolutional (e.g. for conservation) investigations in general but makes it more difficult for researchers to study them (*Volff*, 2005; *Horváth et al.*, 2015; *Xu et al.*, 2019; *Tóth et al.*, 2020; *Berrebi et al.*, 2021; *Balog et al.*, 2022; *Ren et al.*, 2022; *Tian et al.*, 2022; *Tóth et al.*, 2022).

With more than 3000 species and 300 genera, the Cyprinidae (Teleostei) family is the largest family of vertebrates and thus of freshwater fishes with a significant role in the market and nature (*Kuang et al.*, 2016). To put it another way, it is a family of soft-finned fishes within the Cypriniformes order which belongs to the superorder Ostariophysi (*Tan and Armbruster*, 2018). Due to the large number of species, the study of this family is often challenging because of the multiple occurrences of WGD. After the teleost-specific third-round (3R) WGD, the previously mentioned allopolyploidization event (4R) in the family involved goldfish and common carp, however, the origin of the other Cyprinidae polyploid lineages is poorly understood (*Xu et al.*, 2019; *Wen et al.*, 2020; *Tóth et al.*, 2022). So due to

the high diversity of the Cyprinidae family, the majority of phylogenetic investigations concentrated on smaller groups of the family. Some of these studies despite being very carefully researched, indicate that there are still unsettled taxonomic and phylogenetic questions that need answers (*Vasil'eva et al.*, 2022), because in many areas of biological sciences, understanding phylogenetic relationships between species is essential (*Kapli et al.*, 2020).

This is the reason why it is clear that there must be more and new approaches to the phylogenetics of this very important group of fish (*Wang et al.*, 2012; *Stout et al.*, 2016; *Tan and Armbruster*, 2018; *Alrefaei et al.*, 2023; *Sudasinghe et al.*, 2023). Hence the application of phylogenetics – based on molecular data – is commonly used to gain a better understanding of the evolution of genes and genomes associated with the origin and divergence of species (*Kapli et al.*, 2020). Nowadays several different genes are used for phylogenetic analysis, such as mitochondrial (e.g., cytochrome c oxidase subunit I- COI, cytochrome *b* – cyt *b*) and nuclear (e.g.: recombination-activating gene - RAG, adenosine triphosphate synthase subunit alpha - ATPS-α) in order to study the evolutionary development of species, resulting in different phylogenetic classifications (*Parhi et al.*, 2019; *Raguž et al.*, 2021; *Alrefaei et al.*, 2023). With these genes, we can find different haplotypes in a group of specimens, which can help to understand the phylogenetic associations between individuals and populations. A haplotype is a combination of alleles (gene variants), SNPs (single nucleotide polymorphisms), or other mutations of polymorphic loci on the same chromosome, which are usually inherited together from both parental lines and can affect the characteristics of the specimens. This means, that each new mutation in a haplotype sequence increases the number of haplotypes of that DNA region within a population. For these reasons, haplotypes can reveal valuable information about individual quantitative traits, hereditary diseases, genetic diversity, evolutionary history as well as population structure of species (*Miller et al.,* 2012; *Garg,* 2021; *Liu et al.,* 2022; *Sun et al.,* 2023). An example of a wider range of phylogenetic research was when *Wen et al.* (2020) based on nuclear and mitochondrial genomes, found that Schizothoracinae and Cyprininae subfamilies share the same maternal common ancestor, while Schizothoracinae and Schizopygopsinae subfamilies can be related on the paternal side. For a more specific phylogenetic study, the research made by *Alrefaei et al.* (2023) is a good example. This was possibly the first time when the genetic diversity was studied through the cytochrome *b* gene, by using phylogenetic analysis of *Garra tibanica* and other endemic species from the same genus, sampled in Saudi Arabia. As the other studies show, the mitochondrial cytochrome *b* region is often used for evolutionary studies, and one reason can be that the resolution of that gene is better than its mitochondrial counterparts. An article included the analysis of 185 individuals from 11 species for COI, and 264 individuals from 23 species for the cyt *b* regions to see, which gene is more suitable in the case of the *Schizothorax* genus for phylogenetic analysis. There they found that the cyt *b* worked better in that case, but they warned, that it can change in different conditions (e.g.: cryptic species) (*Ma et al.*, 2020).

The aim of this study was a phylogenetic meta-analysis of the Cyprinidae family via the mitochondrial cyt *b* gene region from the National Center for Biotechnology Information (NCBI) database.

2. Material and methods

At the beginning of this study, only whole cyt *b* sequences from the NCBI international database were selected, where the first 15377 hits were narrowed down to 1080 by filtering out everything that was not a complete cyt *b* sequence from the Cyprinidae family. A database was created with all the interested species, including their taxonomy (subfamily, genus), and nomenclature based on the information from the NCBI database. The mtDNA sequences were aligned by using Kalign software (*Lassmann*, 2020) which is able to perform multiple sequence alignments (MSA) with thousands of nucleotide sequences, which was considered optimal for our study. MEGA-X 10.2.2 (*Kumar et al.*, 2018) was used to remove every gap and misread, including those whole sequences too that finally did not comply with our conditions. For the determination of cyt *b* gene polymorphism and the formation of the haplotype network of sequences, the DNA Sequence Polymorphism v.06 (DnaSP) (*Rozas et al.*, 2017) and NETWORK 10 (*Bandelt et al.*, 1999) software were applied. Population genetic structure was calculated among and within subfamilies and different species in haplotypes by an analysis of molecular variance (AMOVA), and neutrality tests (Tajima's D and Fu's FS test) using ARLEQUIN 3.5.2.2 (*Excoffier and Lischer*, 2010) software. The phylogenetic association of the founded haplotypes was calculated by the MEGA-X 10.2.2 software (*Kumar et al.*, 2018). The phylogenetic tree was built using the maximum likelihood (ML) of the Tamura-Nei model (*Tamura and Nei*, 1993) with 500 bootstrap replicates (highest log likelihood -73606.21) *(Fig. 1)*. For this, an outgroup sequence from the *Acheilognathus argenteus* species (access. no.: AB366479.1) was employed for this analysis, which belongs to the Acheilognathidae family.

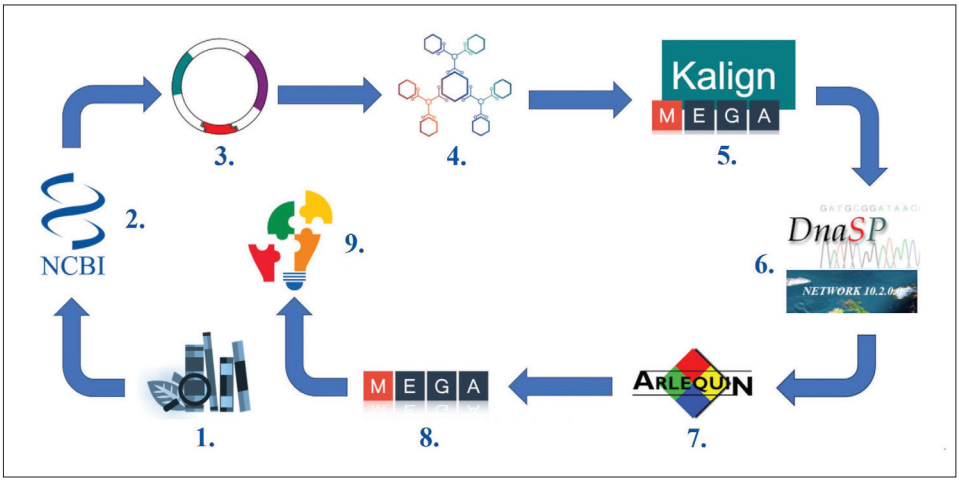
3. Results

In this study, taxonomical groups based on the NCBI database were used, which contained 9 subfamilies (Acrossocheilinae, Barbinae, Cyprininae, Labeoninae, Probarbinae, Schizopygopsinae, Schizothoracinae, Smiliogastrinae, Spinibarbinae), 1 clade (Poropuntiinae), and 2 other groups (Cyprinidae incertae sedis, unclassified Cyprinidae) that belong directly under the Cyprinidae family (*Yang et al.*, 2015; *Sayers et al.*, 2022). Applying the MEGA-X 10.2.2 (*Kumar et al.*, 2018) program after the last filtering, 1025 cyt *b* sequences were selected, representing 219 species, with a final sequence length of 1109 bp. The DnaSP software found 684 haplotypes among the 1025 sequences (Supplementary file 1), which are distributed according to the number of sequences within each haplotype in Supplementary file 2.

This shows that the majority of the sequences (n=564) are classified into separate haplotypes, implying that most of those are unique. Out of the 684 haplotypes, only 2.3% (n=16) belongs to more than one species, which are mostly belongs to the same genus. However, the haplotype Hap_6 swas linked to sequences from different subfamilies (based on NCBI data), namely from the Torinae and Barbinae (Table 1).

Numerous species had the same genetic variations in our research. For example, the *Poropuntius bolovenensis, the Poropuntius lobocheiloides* and the

Figure 1. *Process of the phylogenetic meta-analysis study*



1. Literature review; 2. International database screening; 3. Cyt *b* sequence selection; 4. Preparation of databases; 5. Multiple sequence alignment, removement of every gap and misread; 6. Determination of gene polymorphism and the formation of the haplotype network; 7. Calculation of population genetic structure; 8. Structure of the phylogenetic tree; 9. Comparison of results with the literature, drawing conclusions

1. ábra. A filogenetikai metaanalízis vizsgálatának folyamata
1. szakirodalom áttekintése; 2. nemzetközi adatbázisok szűrése; 3. Cyt *B* szekvencia kiválasztása; 4. adatbázis készítése; 5. több szekvencia illesztése, minden hiányos régió és félreolvasás eltávolítása; 6. génpolimorfizmus meghatározása, és a haplotípusok kapcsolatainak ábrázolása; 7. populáció genetikai struktúrák számítása; 8. filogenetikai fa készítése; 9. kapott eredmények összehasonlítása a szakirodalommal, következtetések levonása

Poropuntius solitus species shared 6 haplotypes, whereas the *C. auratus* and the *Carassius gibelio* (Gibel carp) had a total of 5 with each other. Noteworthy, that some species were listed in the NCBI database with their synonym names (Supplementary file 3), which could lead to confusion in research. A good instance is that in our analysis it looked like two different species had the same haplotype (Hap_342), while it turned out, those sequences belonged to the *Crossocheilus burmanicus* fishes. Table 2 presents the statistical data for our full set of sequences, meanwhile emphasizing the groups of species that share haplotypes. In this table, and throughout the paper, these groups will be referred to as follows: HG1: containing all species; HG2: *C. auratus, C. gibelio*; HG3*: C. auratus, C. gibelio, C. cuvieri, C. carpio*; HG4: *P. bolovenensis, P. lobocheiloides, P. solitus*; HG5: *B. barbus, B. balcanicus*; HG6: *B. bynni, B. petitjeani*; HG7: *P. jerdoni, H. lithopidos*; HG8: *H. dubius, H. micropogon*.

Table 2 also shows the results of the polymorphism analysis. Visibly the polymorph sites (S=704) haplotype diversity (Hd=0.996), and the nucleotide diversity (ϖ=0.137) values were the highest in the case of the largest analysed group of species (HG1). This was expected, as it contains sequences (n=1025) from 13 subfamily-ranked taxonomy groups, and all their haplotypes (n=684). To better understand the second most common genetic variations shared by

Table 1a

Haplotypes with more than one species

Haplotype (1)	Sample size (2)	Taxonomy group (3)	1. Species (4)	2. Species (4)	3. Species (4)
Hap_493	20	Species	*P. bolov.*	*P. loboch.*	*P. solitus*
		Genus	*Poropuntius*	*Poropuntius*	*Poropuntius*
		Subfamily	Poropuntiinae	Poropuntiinae	Poropuntiinae
Hap_491	15	Species	*P. bolov.*	*P. loboch.*	
		Genus	*Poropuntius*	*Poropuntius*	
		Subfamily	Poropuntiinae	Poropuntiinae	
Hap_116	10	Species	*C. auratus*	*C. gibelio*	
		Genus	*Carassius*	*Carassius*	
		Subfamily	Cyprininae	Cyprininae	
Hap_115	7	Species	*C. auratus*	*C. gibelio*	
		Genus	*Carassius*	Carassius	
		Subfamily	Cyprininae	Cyprininae	
Hap_119	3	Species	*C. auratus*	*C. gibelio*	
		Genus	*Carassius*	*Carassius*	
		Subfamily	Cyprininae	Cyprininae	
Hap_485	3	Species	*P. bolov.*	*P. loboch.*	*P. solitus*
		Genus	*Poropuntius*	*Poropuntius*	*Poropuntius*
		Subfamily	Poropuntiinae	Poropuntiinae	Poropuntiinae
Hap_6	2	Species	*B. bynni*	*B. petitjeani*	
		Genus	*Labeobarbus*	*Barbus*	
		Subfamily	Torinae	Barbinae	
Hap_117	2	Species	*C. auratus*	*C. gibelio*	
		Genus	*Carassius*	*Carassius*	
		Subfamily	Cyprininae	Cyprininae	

P. bolov. = P. bolovenensis; P. loboch. = P. lobocheiloides

1a. táblázat: Az egynél több fajt tartalmazó haplotípusok
P. bolov. = P. bolovenensis; P. loboch. = P. lobocheiloides
haplotípus (1); minta mérete (2); taxonómiai csoport (3); faj (4)

different species (HG2), we included sequences from *Carassius cuvieri*, which inherited identical genetic variations with goldfish, and from the species often used in similar research, the *C. carpio* (HG3). This helped us recognize how their genetic variations are connected (Table 2; Fig. 2). Interestingly, despite the HG4 group containing 75 sequences from 3 species, it had the fewest polymorph sites (S=19) and the lowest nucleotide diversity (ϖ=0.003) among all the studied groups.

Returning to the HG1 group, the AMOVA analysis (Table 3) showed that only 62.2% of the variation came from among the populations, which is remarkable

Table 1b

Haplotypes with more than one species

Haplotype (1)	Sample size (2)	Taxonomy group (3)	1. Species (4)	2. Species (4)	3. Species (4)
Hap_118	2	Species	*C. auratus*	*C. gibelio*	
		Genus	*Carassius*	*Carassius*	
		Subfamily	Cyprininae	Cyprininae	
Hap_157	2	Species	*C. auratus*	*C. cuvieri*	
		Genus	*Carassius*	*Carassius*	
		Subfamily	Cyprininae	Cyprininae	
Hap_166	2	Species	*H. dubius*	*H. micropogon*	
		Genus	*Hypselobarbus*	*Hypselobarbus*	
		Subfamily	Torinae	Torinae	
Hap_167	2	Species	*P. jerdoni*	*H. lithopidos*	
		Genus	*Hypselobarbus*	*Hypselobarbus*	
		Subfamily	Torinae	Torinae	
Hap_487	2	Species	*P. bolov.*	*P. solitus*	
		Genus	*Poropuntius*	*Poropuntius*	
		Subfamily	Poropuntiinae	Poropuntiinae	
Hap_488	2	Species	*P. bolov.*	*P. solitus*	
		Genus	*Poropuntius*	*Poropuntius*	
		Subfamily	Poropuntiinae	Poropuntiinae	
Hap_489	2	Species	*P. bolov.*	*P. solitus*	
		Genus	*Poropuntius*	*Poropuntius*	
		Subfamily	Poropuntiinae	Poropuntiinae	
Hap_515	2	Species	*B. barbus*	*B. balcanicus*	
		Genus	*Barbus*	*Barbus*	
		Subfamily	Barbinae	Barbinae	

P. bolov. = P. bolovenensis; P. loboch. = P. lobocheiloides

1b. táblázat: Az egynél több fajt tartalmazó haplotípusok 2
P. bolov. = P. bolovenensis; P. loboch. = P. lobocheiloides
haplotípus (1); minta mérete (2); taxonómiai csoport (3); faj (4)

considering the comparison of data gained from different subfamilies. The HG2 and HG4 groups of species indicated that their variation is more likely to occur within the populations (Vb=60.6% and 74.5%). This means, that in the case of the 2 *Carassius* and 3 *Poropuntius species*, the polymorphism is greater inside the species than between them, which indicates close phylogenetic relatedness. However, for the HG6, HG7, and HG8, the results of the AMOVA analysis could not be considered due to the low number of sequences (low sample numbers). The results of the neutral tests were consistently below zero in all species groups but were never at a significance level. Thus, we did not have strong enough

Table 2

Statistical data of different groups of species and their haplotypes

Groups (1)	Sample size (2)	S (3)	h (4)	Hd ± SD (5)	ϖ (6)
HG1	1025	704	684	0.996 ± 0.001	0.137 ± n.d.
HG2	262	228	94	0.965 ± 0.006	0.017 ± 0,001
HG3	268	269	95	0.967 ± 0.006	0.021± 0.002
HG4	75	19	18	0.873 ± 0.024	0.003± 0.0002
HG5	50	332	46	0.996 ± 0.005	0.064± 0.008
HG6	3	25	2	0.667 ± 0.314	0.015± 0.007
HG7	3	71	2	0.667 ± 0.314	0.043± 0.020
HG8	2	0	0	0	0

S = Number of polymorph sites; h = Number of Haplotypes; Hd = Haplotype (gene) diversity; SD = Standard deviation; ϖ = Nucleotide diversity (per site); n.d. = no data

2. táblázat: Statisztikai adatok a különböző haplotípus-csoportokról, amelyek mindegyike eltérő fajösszetételű
S = polimorf helyek száma; h = haplotípusok száma; Hd = haplotípus (gén) diverzitás; SD = szórás; ϖ = nukleotid diverzitás (pozíciónként); n.d. = nincs adat
csoportok (1); minta mérete (2); S = polimorf helyek száma (3); h = haplotípusok száma (4); Hd = haplotípus (gén) diverzitás (5); SD = szórás (6); ω = nukleotid diverzitás (pozícionként) (7); n.d. = nincs adat (8)

Table 3

Results of the AMOVA and neutrality analysis

Groups (1)	Va (2)	Vb (3)	Fixation Index FST (4)	Tajima's D (5)	Fu's FS (6)
HG1	62.2	37.8	0.622	-0.542	-8.985
HG2	39.4	60.6	0.394	-0.645	-11.929
HG3	64.5	35.5	0.645	-0.323	-5.964
HG4	25.5	74.5	0.255	-0.955	-1.329
HG5	64.8	35.2	0.648	-1.210	-9.436

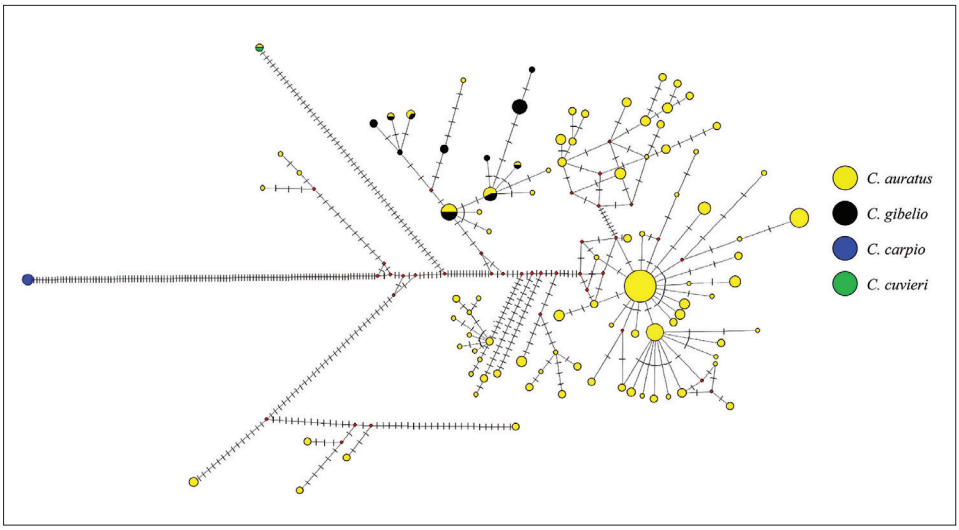
Va = Percentage of variation among populations (2); Vb = Percentage of variation within populations (3)

3. táblázat: Az AMOVA és a neutrális tesztek eredményei
csoportok (1); a populációk közötti eltérés százalékos aránya (2); a populációkon belüli eltérés százalékos aránya (3); fixációs index (4); Tajima D érték (5); Fu FS értéke (6)

evidence to draw any firm conclusions about genetic variations or population changes in these species (e.g. the possibility of recent population expansion or genetic drift).

In the median-joining network tree of the HG3 group of species (Fig. 2), one can observe that the haplotype of *C. carpio* displays a high number of mutations when comparing it to species of the *Carassius* genus. Particularly, *C. gibelio* does not appear to be completely distinct from the groups of *C. auratus*, while the shared haplotype of the *C. cuvieri* species shows an increased number of polymorphic sites in comparison to them.

Figure 2. **Median-joining network of HG3**



The different colors represent the species. Branch length is not representative of evolutionary distance. The frequency of the crossing lines indicates the number of mutations, while the size of the circle is proportional to the number of sequences in the haplotypes.

2. ábra. A HG3 median-joining hálózata
A különböző színek a fajokat jelölik. Az ágak hossza nem reprezentálja az evolúciós távolságot. Az egymást keresztező vonalak gyakorisága a mutációk számát jelzi, míg a kör mérete a haplotípusok szekvenciáinak számával arányos.

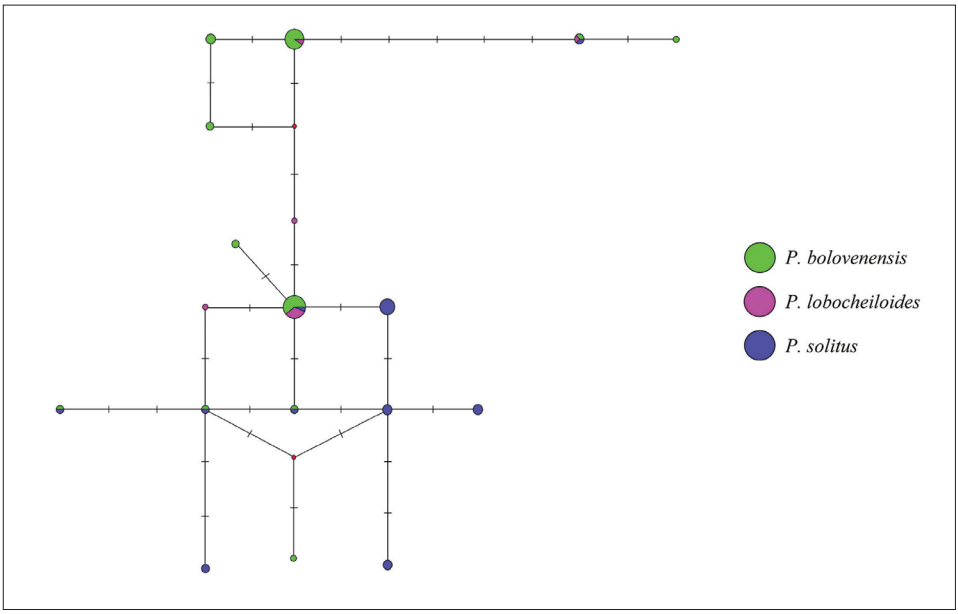
The HG4 group is presented in the following figure, demonstrating that the 3 *Poropuntius* species often have identical genetic variation on the haplotype sites, without high number of mutations between them (Fig. 3).

To grasp the genetic relations between the haplotypes, a ML tree was structured and used to measure polymorphism within and between the observed distinct haplotypes. In most cases, the different genetic variations showed the expected relationships on the phylogenetic tree according to the species they represented. This arrangement was most apparent, as the subfamilies appeared on the tree in a well-differentiated order. One exception was the haplotype belonging to 2 different subfamilies (Hap_6), which was classified under the Torinae subfamily. In our study, there is a less classified subfamily-ranked group, the Cyprinidae incertae sedis, from which we had 2 sequences, but these were found in separate haplotypes (Hap_329 and Hap_330). In the end, it was possible to determine their potential position in the classification tree, as they were also associated with haplotypes belonging to the Barbinae and Poropuntiinae subfamilies (Fig. 4).

4. Discussion

The literature mainly focuses on smaller taxonomic units within the families, thus broad phylogenetic research covering a whole family is rare. So, to our best knowledge, this study is the first within the family Cyprinidae to include

Figure 3. **Median-joining network of HG4**



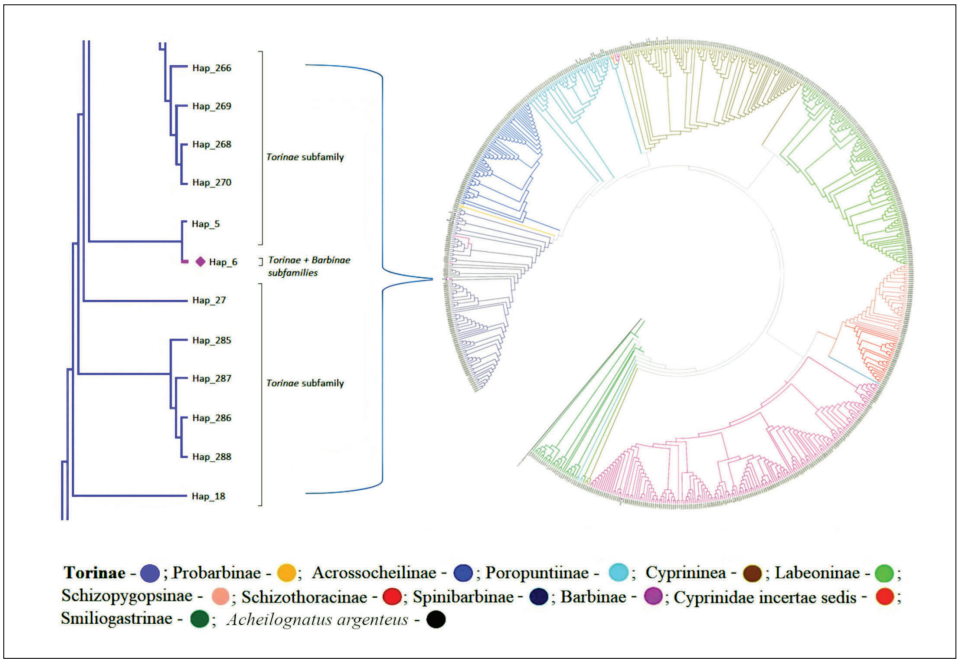
The different colors represent the species. Branch length is not representative of evolutionary distance. The frequency of the crossing lines indicates the number of mutations, while the size of the circle is proportional to the number of sequences in the haplotypes.

3. ábra. A HG4 median-joining hálózata
A különböző színek a fajokat jelölik. Az ágak hossza nem reprezentálja az evolúciós távolságot. Az egymást keresztező vonalak gyakorisága a mutációk számát jelzi, míg a kör mérete a haplotípusok szekvenciáinak számával arányos.

sequences from all subfamilies, involving 219 species. In this analysis, most of the 1025 cyt *b* sequences examined within the 12 subfamily-ranked groups, contained only one sequence, which is consistent with the expected result, as international databases (e.g.: NCBI) mostly contain sequences from separate haplotypes. In this meta-analysis, a total of 684 unique haplotypes were found. Among them, only 16 contained sequences from different species (Table 1), which verified that they were not just synonyms of each other (e.g.: *Crossocheilus burmanicus* and *Tariqilabeo burmanicus* in Hap_342) (Supplementary file 1; Supplementary file 3).

Species belonging to these shared haplotypes were further investigated separately. In the case of the species of *C. auratus* and *C. gibelio*, they had 94 haplotypes with 5 (Hap_115, Hap_116, Hap_117, Hap_118, Hap_119) of them being shared with each other. The species had relatively high haplotype diversity (Hd=0.965) with 60.6% of variation within, and 39.4% among populations with the second lowest Fixation index value (Fst=0.394). These numbers were not surprising, as the literature shows that it is still questionable whether we can consider *C. gibelio (*synonym: *C. auratus gibelio)* as a separate species from *C. auratus (*synonym: *C. auratus auratus)*, or they are simply subspecies (*Apalikova et al.*, 2011; *Cheng*

Figure 4. **Phylogenetic tree of the Cyprinidae haplotypes**



The colors represent different subfamilies in clockwise order.

4. ábra. Filogenetikai fa a Cyprinidae haplotípusokról
A színek a különböző alcsaládokat jelölik az óramutató járásával megegyező sorrendben.

et al., 2020). The controversy arises from the fact that the goldfish was domesticated in China and believed its ancestor is the Gibel carp, but there are studies that indicate they were developed from a sister lineage (*Rylková et al.*, 2010; *Chen et al.*, 2020). Other studies reported (*Cheng et al.*, 2012) that they found shared haplotypes in other mitochondrial regions (COI) too, and attention was drawn to the ploidy as a disruption factor for the classification of *Carassius* species.

This analysis includes the comparison (HG3) of the already mentioned *C. auratus, C. gibelio* species with the *C. cuvieri* (1 shared haplotype with goldfish), and *C. carpio* which is often studied together with them (*Apalikova et al.*, 2011; *Cheng et al.*, 2020; *Wang et al.*, 2022). Among them, 95 haplotypes were identified, with 64.5% variation among populations. This compared to the results of the previous analysis (Va=39.4%) of HG2 mostly shows the effect of the genetic difference of the common carp when studied alongside *Carassius* species (Table 2, Table 3). On the network tree (Fig. 2), the haplotype of the *C. carpio* is well separated with high number of mutations, which was expected as this species belongs to another genus. However, a strong link can be found between the haplotypes of Gibel carp (black) and goldfish (yellow), with no more than 5 mutations between them. On the contrary, the haplotype occurring in *C. cuvieri* and goldfish contains 54 mutations from the main body of the tree. These results in these haplotypes correlate with the already existing literature.

The most frequent species with identical haplotypes (n=6 out of 18) were *P. bolovenensis*, *P. lobocheiloides*, and *P. solitus*. Further into our meta-analysis, they were referred to together as HG4 group. The analyses found that these species had 74.5% within-population variance, while their nucleotide diversity value (ϖ =0.003), the number of variable sites (S=19), and the Fst value (Fst=0.255) was the lowest among the studied groups (Table 2, Table 3). In their network tree (Fig. 3) there were at most 5 mutations between the haplotypes, which indicates that they are in close relations. Questions were raised in their first reports (*Roberts*, 1998; *Kottelat*, 2000) about the studied *Poropuntius* species, as it was not clear, whether they are separate or subspecies because some of their morphological features (e.g. lateral line scales, predorsal scales) are not suitable for distinguish. This issue cannot be precisely defined from our results, but it goes back to the assumption proposed by Kang et al. (2016). Namely, which suggests that there is a possibility that *P. lobocheiloides* and *P. solitus* are the same species as *P. bolovenensis* and that their phenotypic divergence is simply the result of intraspecific trophic polymorphism.

Group HG5 included 2 species: *Barbus balcanicus* and *Barbus barbus*, with a total of 46 haplotypes, one of which was found in both species. This group had the second highest haplotype (Hd=0.996) and nucleotide (ϖ =0.064) diversity with 64.8% of among-population variance (Table 1, Table 2). From these data, it can be observed that these species are not as closely related as the previously studied ones and are well separated within the Cyprinidae family, which is supported by the available literature too (*Levin et al.*, 2019).

The HG6 analysed group encloses one haplotype called Hap_6, which was found in 2 species: *Labeobarbus bynni bynni* (access. no.: AF287420.1) from the Torinae, and the *Barbus petitjeani (*synonym: *Labeobarbus petitjeani)* (access. no.: AF287443.1) from the Barbinae subfamily. The possible reason why this could occur, is that these species are hexaploids (*Guegan et al.*, 1995), and as *Yang et al.* (2015) proposed that these *'Barbus'* hexaploid species should be moved into the *Labeobarbus* genus under the Torinae subfamily. Nowadays some of the literature already classify this species under this genus (*Diallo*, 2020). It can be noticed that following this, our Hap_6 haplotype can be found under the Torinae branch of the ML tree (Fig. 4). This means, that the taxonomy classification of these species not yet been perfectly clarified. Still, our results support the proposal of *Yang et al.* (2015).

The species in HG7 and HG8 groups belong to the same *Hypselobarbus* genus, and they formed haplotypes according to the clade where they belong based on the literature: the Hap_166: *Hypselobarbus curmuca - Hypselobarbus dubius - Hypselobarbus micropogon*, and the Hap_167: *Hypselobarbus lithopidos - Puntius jerdoni* (synonym: *Hypselobarbus jerdoni*) clade (*Arunachalam et al.*, 2012). Unfortunately, in this case, the low number of sequences in the groups (Table 2) prevented valid results from the statistical tests. Although it should be mentioned that the difference in the number of sequences in each species may affect our results.

5. Conclusion

A large-scale meta-analysis was carried out based on the available sequences of the cytochrome *b* (cyt *b*) region of the Cyprinidae family from the National Center for Biotechnology Information (NCBI) database. This family is the largest among the vertebrates, comprising several subfamilies, genera, and eventually more than 3000 species. For the study, 1025 sequences were selected from the database according to their quality, which eventually covered all subfamilies and a total of 219 species. The analyses were carried out using different software, resulting in 684 haplotypes within the family, from which 16 haplotypes occurred in at least 2 species. A close relationship among the haplotypes of *C. auratus* and *C. gibelio* species had been shown, as well as between the haplotypes of *P. bolovenensis*, *P. lobocheiloides* and *P. solitus*, where it is still unclear whether they should be considered as species or subspecies. In addition, the suggestion of a previous study that the *Barbus petitjeani* may need to be reclassified in the subfamily Torinae because of its hexaploidy was supported by our results. Furthermore, the validity of this current study was supported by the fact that species belonging to the genus *Hypselobarbus* shared a common haplotype with species belonging to the same clade. An unclassified group at the subfamily level, Cyprinidae incertae sedis was included in the study and we were able to predict its possible position in the phylogenetic tree because of its association with haplotypes of the subfamilies Barbinae and Poropuntiinae. Finally, further analysis showed that the taxonomic classifications used today are mostly correct, as the relationships of the haplotypes correspond to known classifications and associations between the species under study, but the different kinds of synonyms of species can make the work harder with them. To the best of our knowledge, the present study is the first attempt to investigate the cytochrome *b* region of the whole Cyprinidae family, by phylogenetic analysis of the sequences available from the NCBI database

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8. Supplementary files

Supplementary files are available from the Authors and on the journal's website.

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