



RESEARCH ARTICLE

Investigation of taxonomic relationship and effect of seasonal temperature changes based on protein profiles of fishes from Beyşehir, Suğla lakes and Dam Apa

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ABSTRACT

Besides traditional methods based on morphological characters, electrophoretic methods such as SDS-PAGE are preferred by taxonomists to make the right decision in the species identification process. In addition, the effect of environmental factors, such as pH, salinity, heat, and temperature on protein profiles are essential in various studies. In this study, we aimed to determine the degree of relationship in some fish species, such as *Squalius lepidus*, *Cyprinus carpio*, *Carassius gibelio*, *Pseudophoxinus anatolicus*, *Tinca tinca*, *Alburnus orontis*, *Scardinius erythrophthalmus*, *Capoeta capoeta*, *Vimba vimba*, *Sander lucioperca* living in Beyşehir, Suğla lakes and Apa Dam by SDS-PAGE method, and to examine seasonal differences by evaluating the effect of hot/cold water on protein profiles in fish. Although there were common major protein bands in all fish species studied, the presence of species-specific minor protein bands led to the separation of the species. The same fish species distributed in different lakes and dams were different both in minor bands, and changes in protein profiles were observed consequently on the same fish species synthesizing different proteins in different seasons. The data obtained from this study can contribute to systematic classification studies of fish.

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Introduction

The family Cyprinidae, represented by 200 genera and 2010 species, constitutes the largest family among freshwater fish (Nelson, 1994; He et al., 2008). Although Cyprinidae members are not found in South America, Australia, and Antarctica, they include many fish with cultural and economic importance (He et al., 2008). A total of 236 species and subspecies belonging to 26 families are found and it constitutes approximately 8% of all fish species in the inland waters of Türkiye (Kuru, 2004).

Mostly in taxonomic studies, the species identification process is based on morphological and anatomical characteristics (Theophilus & Rao, 1998; Yılmaz et al., 2005). Some morphological features may subsequently change as a result of environmental conditions (Fowler, 1970; Ganai et al., 2014). During the definitive identification of a species, classical morphological characters can be misleading due to the existence of these changes over time (Menon, 1989; Ganai et al., 2014). For this reason, comparisons based on morphological characters are not sufficient for taxonomists to make the right decision for determining the species (Hua et al., 2019; Şalcıoğlu et al., 2020). Proteins are used as genetic markers that play an important role in determining taxonomic relationships (Crick, 1963; Nirenberg et al., 1963; Ochoa, 1963; Ganai et al., 2014). In previous systematic studies on fish species, successful identifications were made by electrophoresis of serum proteins, and these studies brought a new perspective to taxonomic evolution (Theophilus & Rao, 1998, Yılmaz et al., 2007).

The aim of this study to evaluate the degree of relationship of some fish species distributed in Beyşehir, Suğla lakes and Apa Dam according to their total protein profiles by using the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method and to determine the effects of seasonal changes on the protein profiles of fish.

Material and Methods

Table 1 indicates the different fish species obtained from Beyşehir, Suğla lakes and Apa Dam, their localities and the seasonal period in which they were obtained.

Protein Isolation

The protocol proposed by Hoffman & Penny (1973) was used, partially modified, for protein isolation from muscle tissue of fish. Five grams of muscle was taken from each fish and

thoroughly crushed in a mortar. The shredded muscles were transferred to the falcon tube and 10 ml of distilled water was added. After vortexing, the samples were kept at +4°C overnight. The falcons were thoroughly vortexed the next day and centrifuged for 25 min at 5000 rpm at +4°C in a refrigerated centrifuge (Hettich Universal, Zentrifugen). The supernatant was transferred to a new falcon tube and stored at -20°C until use.

SDS-PAGE Electrophoresis

The SDS-PAGE method was carried out by modifying the method of Laemmli (1970). Before electrophoresis, protein lysate and sample buffer (0.125 M Tris-HCl, 4% SDS, 20% Glycerol, 10%, Mercaptoethanol 2%) were mixed in a 1:1 ratio and boiled in a water bath for five min. The separating gel was a 10% polyacrylamide gel in 1.2 M Tris-HCl (pH 8.8) and 0.3% SDS. The stacking gel contained 3% acrylamide in 0.25 mol/l Tris-HCl (pH 6.8) and 0.2% SDS. The electrode buffer contained 0.025 mol/l Tris-HCl, 0.192 M glycine and 0.15% SDS at pH 8.16. The protein standard (Fermantas SM 0431) was used as the molecular weight standard. Running of the proteins was performed using the Biometra vertical electrophoresis system (Biometra, Göttingen) with the size of 120×110×1 mm. The samples were run in electrophoresis at 36 mA until bromophenol blue, which was used as an indicator, reached the end of the running gel. When the electrophoresis process was completed, the gels were removed from the glass plates and placed in staining cuvettes containing the staining solution (50% methanol, 10% Acetic acid, 0.1% Coomassie Brilliant Blue G-250 M, water). After staining, the gels were washed with a washing solution (5% methanol, 7% acetic acid, water). Finally, images of all gels were taken using a gel imaging system (Vilber Lourmat, France).

Data Analysis

Scoring was done according to the absence (0) and presence (1) of protein bands. Similarities were calculated with the Bio1D++ computer program according to Nei's genetic similarity (Nei, 1978). To construct a dendrogram with the UPGMA (unweighted pair-group method and arithmetic averages) method, the degree of relationship and protein differences in summer/winter months were evaluated by cluster analysis.

Table 1. Names and localities of the fish species used for protein analysis

Order	Family	Genus	Species	Sample Number	Localities	Seasonal Change		
Cypriniformes	Cyprinidae	<i>Cyprinus</i>	<i>Cyprinus carpio</i>	1,2	Beyşehir lake	Winter		
				3,4	Apa Dam	Winter		
				5,6	Apa Dam	Summer		
		<i>Carassius</i>	<i>Carassius gibelio</i>	7	Beyşehir lake	Winter		
				8,9	Suğla lake	Winter		
				10,11	Apa Dam	Winter		
				12	Apa Dam	Summer		
				<i>Alburnus</i>	<i>Alburnus akili</i>	23, 24	Beyşehir lake	Winter
						<i>Vimba</i>	<i>Vimba vimba</i>	34
				<i>Scardinius</i>	<i>Scardinius erythrophthalmus</i>	30	Beyşehir lake	Winter
						31, 32	Beyşehir lake	Summer
				<i>Pseudophoxinus</i>	<i>Pseudophoxinus anatolicus</i>	17,18	Beyşehir lake	Winter
						19,20	Beyşehir lake	Summer
		21, 22	Suğla lake			Winter		
		<i>Squalius</i>	<i>Squalius lepidus</i>			25	Beyşehir lake	Winter
				26, 27	Apa Dam	Winter		
				28,29	Apa Dam	Summer		
		<i>Gobio</i>	<i>Gobio microlepidotus</i>	43	Beyşehir lake	Winter		
				<i>Tinca</i>	<i>Tinca tinca</i>	13, 14, 15	Beyşehir lake	Summer
		16	Suğla lake			Winter		
		<i>Capoeta</i>	<i>Capoeta capoeta</i>	33	Suğla lake	Winter		
	Cobitidae	<i>Cobitis</i>	<i>Cobitis bilseli</i>	42	Beyşehir lake	Winter		
Mugiliformes	Atherinidae	<i>Atherina</i>	<i>Atherina boyeri</i>	44	Beyşehir lake	Winter		
Perciformes	Percidae	<i>Sander</i>	<i>Sander lucioperca</i>	35, 36	Beyşehir lake	Winter		
				37, 38	Suğla lake	Winter		
				39, 40	Apa Dam	Winter		
				41	Apa Dam	Summer		

Results

Total proteins belonging to fish species used in this study were isolated and SDS-PAGE electropherograms were taken. (Figure 1). A total of 96 polypeptide bands, ranging in size from 18.4 kDa to 116 kDa, were observed in thirteen different fish species.

Cluster analysis was performed using the UPGMA method with the Bio1D++ computer program. According to the dendrogram obtained (Figure 2), fish species were divided into two main groups with 38% similarity. While *Cyprinus carpio*, *Pseudophoxinus anatolicus*, *Squalius lepidus*, *Alburnus orontis*,

Scardinius erythrophthalmus, *Carassius gibelio*, *Tinca tinca*, *Capoeta capoeta*, *Vimba vimba*, *Gobio microlepidotus*, *Sander lucioperca*, *Atherina boyeri* were in the first main branch, *Cobitis bilseli* species were in the second main branch. Fish species belonging to Cyprinidae and Cobitidae families in Cypriniformes order were clustered and divided into two branches. But members of the Mugiliformes and Perciformes clustered and separated within the first main branch. In other words, while protein profiles distinguish two families belonging to Cypriniformes order with 38% similarity, *A. boyeri* (Mugiliformes) and *S. lucioperca* (Perciformes) species belonging to different orders and are placed in Cyprinidae family of Cypriniformes.

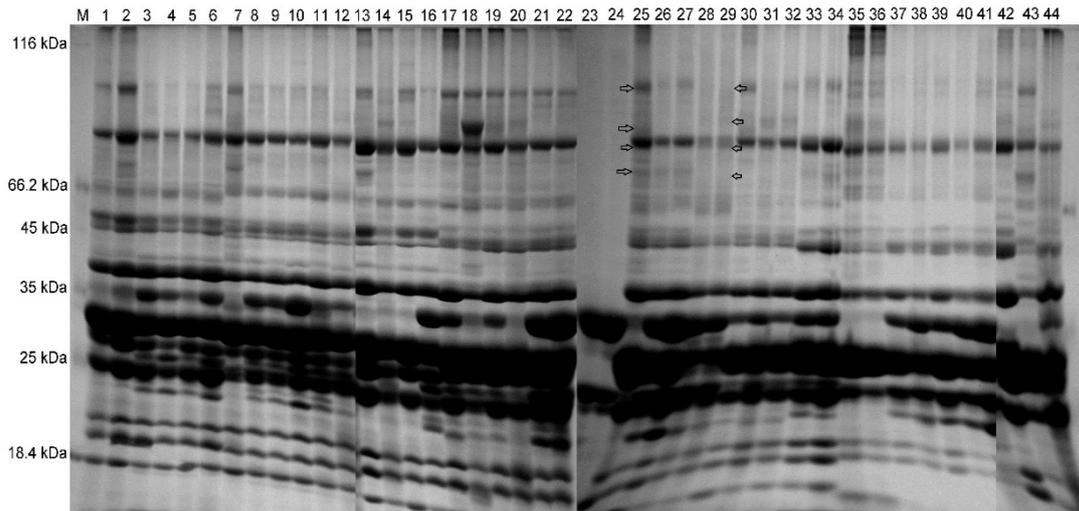


Figure 1. SDS-PAGE protein profiles of the fish species

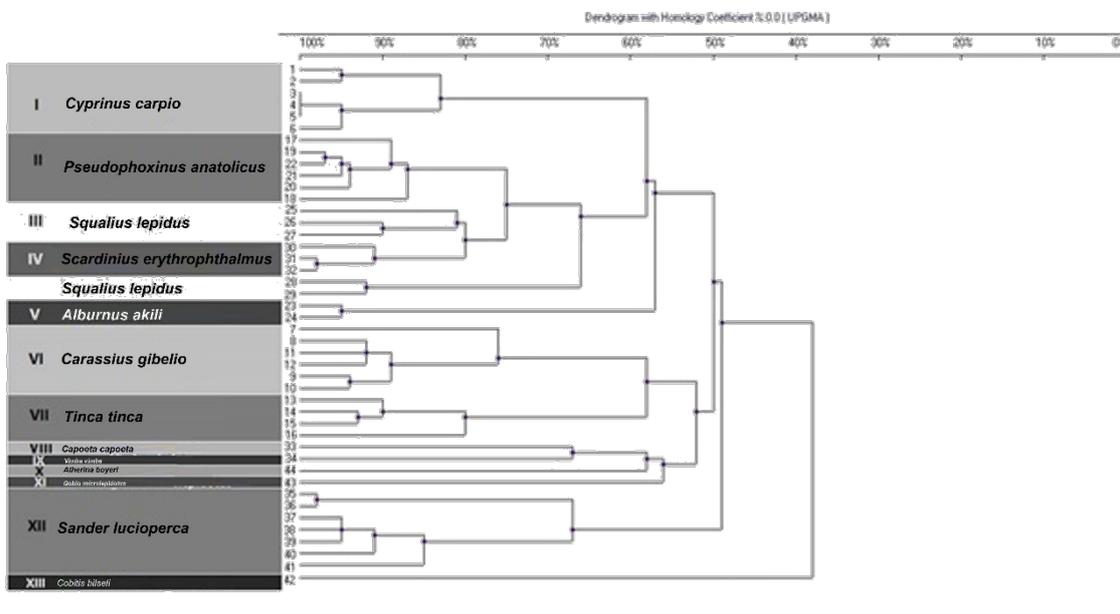


Figure 2. UPGMA dendrogram showing relationship between fish species based on protein profiles

In the first group, different populations (obtained from Beyşehir Lake in winter season, Suğla Lake in Winter, from Apa Dam in Winter and Summer) of *S. lucioperca* (pikeperch) were clustered with a similarity rate of 48%. *C. carpio*, *P. anatolicus*, *S. lepidus*, *S. erythrophthalmus*, and *A. akili* were included in one clade, with 50% similarity, while the other clade consisted of *C. gibelio*, *T. tinca*, *C. capoeta*, *V. vimba*, *A. boyeri* and *G. microlepidotus*. In the first clade, *P. anatolicus*, *S. lepidus* and *S. erythrophthalmus* species had closely related each other than carp. Contrary to expectations, *C. gibelio*, *C. capoeta*, *V. vimba*, *A. boyeri*, and *G. microlepidotus* were in the second clade, 50% away from the grass carp and located closer to the *T. tinca*.

When different fish species and populations of the same species were evaluated in total, 13 small groups were formed in

the dendrogram (Figure 2). The same fish species collected from different regions were included in the same group. The fact that the species in these different populations in each group are 10-20% distant from each other can be attributed to the seasonal changes and their collection from different geographical regions.

Based on the UPGMA dendrogram similarity levels results, while the *S. lepidus* species (25, 26, 27) were quite similar to each other (81-89%), the *S. lepidus* 28 and 29 were found to be less similar than the others at the rate of 67.5%. This is due to differences in protein profiles as a result of seasonal changes (as indicated in Figure 1 different bands are indicated by arrows).

When we evaluate the seasonal changes within the species, the synthesis of some proteins increased while others decreased.

The most obvious change was observed in *S. lepidus* species. Other types have minor changes.

Discussion

Many researchers have successfully differentiated fish species using serum protein profiles via SDS-PAGE, isoelectric focusing and two-dimensional electrophoresis methods. In this study, we evaluated protein identification SDS-page method to understand taxonomy of Cyprinid and other Order fishes. The most important finding for this study is that species-specific minor protein bands provide good differentiation between species. In addition, another important finding is that the minor band profile changes even in the same fish species depending on seasonal changes. In the electropherogram obtained from some studies revealed that protein bands are characteristic to the species (Yilmaz et al., 2005; Berrini et al., 2006; Ganai et al., 2014). Yilmaz et al. (2000) examined the serum proteins of 66 *Capoeta trutta* and 92 *Capoeta umbla* by SDS-PAGE method. It has been stated that there are taxonomically significant differences between the serum proteins of these two fish species. They reported that these differences were due to differentiation of the genes which synthesizes different proteins.

On the other hand, Yilmaz et al. (2007) evaluated the differentiation of *S. lepidus*, *Acanthobrama marmid*, and *Chondrostoma regium* fish species from Karakaya (Malatya) Dam Lake according to serum protein profiles obtained as a result of Native-PAGE and SDS-PAGE. In this study, they observed 13 protein bands from *S. lepidus* and 11 protein bands from the *A. marmid* and *C. regium*. In addition to the 80.3 kD protein band observed in the electrophoretograms obtained from the Native-PAGE method, the protein bands of the three fish species were observed to be 21.7 kD in the results obtained from the SDS-PAGE method, where the molecular weights of the protein bands were different. In addition, although the protein bands obtained mostly have similar molecular weights, those with 63.4, 52.3 and 49.5 kD weights were observed only in *S. lepidus* and *C. regium* species.

In DNA-based phylogenetic studies, there are some studies do not agree with the degree of relationship obtained as a result of the protein profiles in this study. For example, in a phylogenetic tree based on mitochondrial genome analysis, *T. tinca*, and *C. carpio* were separately located in distant clades, consistent with our results. However, contrary to our results, *Alburnus alburnus* was found to be closer to *T. tinca* and much farther from *C. carpio* (Imoto et al., 2013). Consistent with our

results, in a phylogenetic tree based on *cyt b* gene, *Alburnus escherichii* and *Squalius lepidus* were closely located in a clade, while *C. carpio* and *C. capoeta* were located in the same clade, although not very closely (Durand et al., 2002).

While *C. carpio* and *Carassius auratus* species are quite close to each other, *A. alburnus*, *T. tinca* and *Gobio gobio* have taken place separately in different clades farther away from them (Tang et al., 2010, 2011). Similarly, in another phylogenetic analysis based on mitochondrial 16S rRNA, contrary to the results of our study, it was shown that *C. carpio* and *Carassius carassius* species, and *Leuciscus* genus and *Gobio* genus are closely related each other (Li et al., 2008). Similar to current result, Imoto et al. (2013) found the *Cobitis striata* to be the most distant species from other Cyprinidae family members.

It is known that environmental conditions lead to changes in the amount and number of proteins, which are the expression products of the gene. It has been stated that Native-PAGE and SDS-PAGE methods are useful in separating the proteins of fish samples whose structure changes as a result of exposure to high pressure (Etienne et al., 2001). Muhammad et al. (2018) analyzed the liver proteins of three different fish species using the SDS-PAGE technique and the similarities and differences between the species were determined. In addition, according to the results of their studies, they suggested that the SDS-PAGE method could be used to examine the toxicological aspects of the species (Muhammad et al., 2018). Another group of researchers were able to distinguish processed fish samples using different staining methods of SDS-PAGE (Martinez et al., 2001). Tokur & Kandemir (2008) analyzed the effect of different thawing methods of frozen *Oncorhynchus mykiss* and *Sardina pilchardus* on protein quality by SDS-PAGE and the differences were determined between two species.

Thuğ et al. (2020) evaluated the seasonal changes in blood biochemical parameters of *Hucho hucho*, and it was observed that some blood parameters increased significantly in the spring and decreased in the summer. On the other hand, Abolfathi et al. (2022) found the presence of proteins with molecular weights ranging from 7-224 kDa in a study in which the seasonal changes in skin-epidermal structure and mucosal immunity parameters of the skin of *O. mykiss* were examined by SDS-PAGE method. They also reported that there were noticeable differences between the number and size of protein bands in seasonal changes. In the study, they reported that small proteins with a molecular weight of less than 35 kDa were found in high proportion in the late summer and spring, while

proteins with a larger molecular weight (> 35 kDa) were clearly observed in the winter season. Similarly, in our study, the synthesis of some proteins increased in the winter season, while others decreased in the summer season (for example: Figure 1 indicated by the arrow). This change was most prominently observed in *S. lepidus* species.

Conclusion

Although the electrophoretic results of the proteins are incompatible with DNA-based methods, in taxonomic studies, it allows successful differentiation at the level of species and higher characters, as well as the determination of seasonal changes in protein number and amount.

The presence of species-specific protein bands in this study led to the determination of the degree of relatedness. In addition, it was determined that protein profiles changed according to seasonal changes in the same fish species. It was observed that this change was quite evident in the protein profile of *S. lepidus*. In further studies, specific protein determination and quantification by methods such as Westernblot or typing by methods such as peptide mapping can be performed.

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Compliance With Ethical Standards

Authors' Contributions

EA: Study design, Writing

EGM: Writing, Data analysis and management

Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statements

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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