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Research article

Comparison of morphological and molecular identifications in ichthyoplankton of the Marmara Sea

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Abstract: One of the crucial points of ichthyoplankton research is the accurate identification of species. Generally, ichthyoplankton species identifications are made according to the external morphological characteristics of eggs and larvae, however identifying ichthyoplankton using morphology can be quite difficult in many groups. In this study, the accuracy of morphological identification of fish eggs and larvae was investigated using DNA barcoding. The correct identification rates (accuracy) for fish eggs, determined through morphological examination method, when referenced to mtDNA barcoding, were calculated as 80%. Similarly, for fish larvae, the correct identification rate was determined to be 82%. Therefore, identifying the species of fish eggs and larvae causes considerable error using only morphological reference points. Morphological identification has an acceptable success rate but has moderate reliability in detecting some groups, especially in some rare species. Species identification cannot be made with only DNA barcoding method in ichthyoplankton studies. The researcher must have a high level of knowledge of morphology. However, morphological identifications should be supported with DNA barcoding using an integrative approach to minimize the error rate and obtain more accurate results.

Keywords: Fish Egg, Fish Larvae, Taxonomy, DNA Barcoding.

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Introduction

In their early life stages, fishes are morphologically very similar, and species identification is difficult during these periods (Victor et al., 2009; Ko et al., 2013). For example, fish eggs are identified according to their characteristics, such as whether the vitellus is homogeneous or granular, whether the egg case is simple or structured, whether the perivitelline space is narrow or wide, egg shape, egg diameter, amount, presence size and location of oil droplets, along with the location of the anus, number of myomeres, and bo if any embryo present dy pigmentation. In addition to some of these embriyonic characteristics, larvae are identified according to fin and spine formation, body proportions, form and timing of development of various organs.

Although many distinctive features are involved in identifying fish eggs and larvae, these features are not always easily noticeable under a microscope. Approximately 80% of eggs sampled in ichthyoplankton surveys are not developed, and their structures are not suitable for identification (Nakatani et al., 2001; Baumgartner et al., 2004; Reynalte-Tataje et al., 2012; Almeida et al., 2018). Hence, in these stages, only order or family-level identification is possible (Shao, 2002). Furthermore, the lack of information in the literature and knowledge about a particular species also complicates

identification (Akbar John et al., 2020). Resources available in the laboratory and the researcher's experience, level of focus, possible exhaustion, and even immediate mental state can cause differences and errors in species identification (Teixeira et al., 2021). Errors in the morphological identification of early developmental stages of fish can have cascading effects on other researchers who rely on the study results as a reference. These errors can lead to incorrect identifications being perpetuated in subsequent studies, creating a chain reaction of mistakes. Species identification of dead eggs is another difficulty. Researchers sometimes identify dead eggs of similar diameter to alive eggs collected from the same station as the same species, and this naturally increases the margin of error in species identification of dead eggs. Most of the time, dead eggs are recorded as unidentified.

The DNA barcoding method was introduced to eliminate the problems caused by morphological identification (Duke et al., 2020). Recently, fish eggs and larvae have been frequently identified with DNA barcoding using cytochrome c oxidase subunit I (COI) (Valdez-Moreno et al., 2010; Frantine-Silva et al., 2015; Hubert et al., 2015; Leyva-Cruz et al., 2016; Burrows et al., 2018; Kerr et al., 2020; Chen et al., 2021; Hou et al., 2021). Morphological identification and DNA barcoding showed 76.9% compatibility at the species level, 96.6% compatibility at the genus level, and 96.6% compatibility at the family level (Taylor, 2016). It was reported in the same study that from 37 samples that were unidentified with the morphological method due to deformation, 35 were identified with DNA barcoding.

In this study, the power of morphological species identification in fish eggs and larvae in distinguishing species was tested using mtDNA barcoding in order to determine whether it is necessary to integrate molecular and morphological methods in ichthyoplankton studies.

Materials and methods

Study area and samplings

Erdek Bay, where the field studies of the present study were carried out, is located between the Kapıdağ Peninsula and the Biga Peninsula of Marmara Sea. A total of 24 stations were selected according to the physical characteristics, freshwater inputs, highly populated areas, and depth conditions of the bay (Figure 1). The sampling was done monthly between April 2019 and March 2020.

The samples were taken using a conical WP-2 type plankton net with a length of 3m, an opening diameter of 57 cm, and if any present mesh size of 500 μm. The collected samples were stored in 330- mL jars in a mixture of 30% seawater and 70% ethanol. The samples were then transferred to containers containing 95% ethanol after 24 hours.

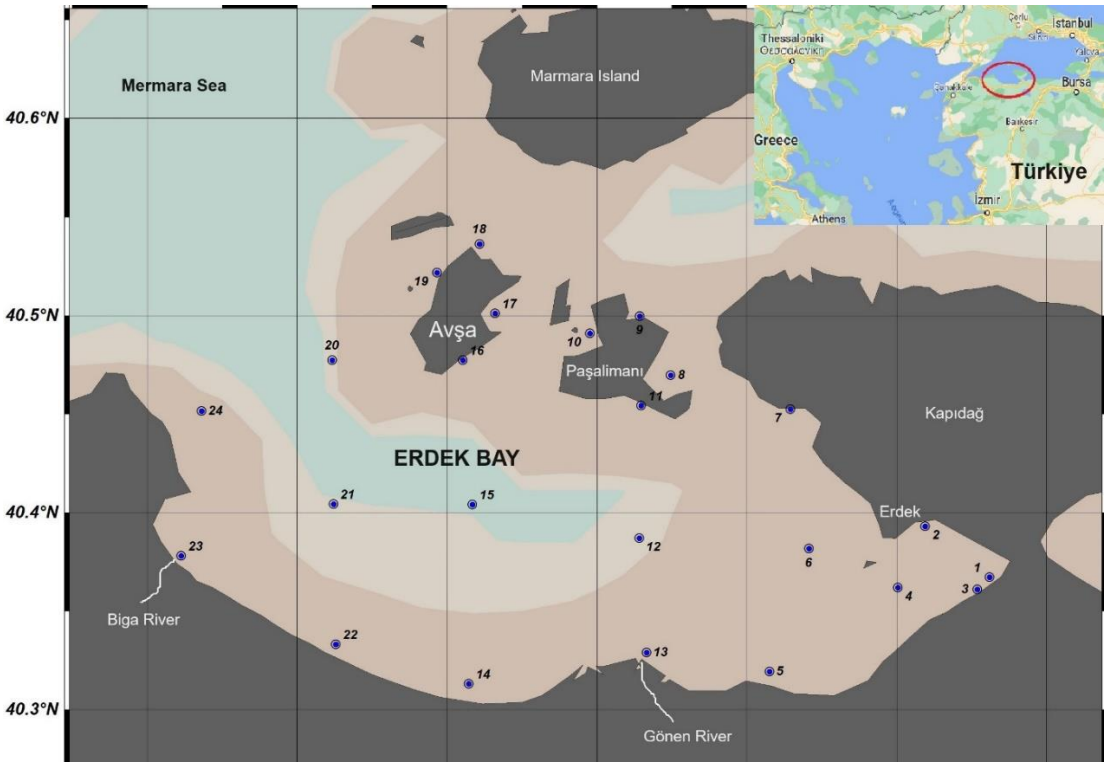

Figure 1. Sampling stations.

Morphological identification

A total of 36,516 eggs and 566 larvae were sampled in the plankton surveys. Preliminary morphological examinations of eggs and larvae were first carried out. In these examinations, eggs and larvae considered as different species or considered the same species but showing different morphological characteristics were selected from each sampling jar, and morphometric measurements were then made. Each ambiguous egg or larva was taken for detailed morphological identification and DNA barcoding to detect the different developmental stages of different species. Aside from live eggs, dead eggs, and deformed larvae were also taken for DNA barcoding following preliminary morphological examinations.

Following characters were used to identify eggs by morphological features; egg shape (spherical or oval), appearance of vitellus (homogeneous or granular), structure of capsule (smooth, structured, thin, thick, transparent, opaque), width of perivitelline space, diameter of egg, presence and the number, size and location of the oil droplet, the location of the anus, the number of myomeres of the embryo and the pigmentation patterns. The characters that we used to identify prelarval stages morphologically as wrere follows; the shape and size of the body, the presence and position of oil droplet/s, the location of the anus, the number of myomeres, the shape and developmental status of the premordial fins, and the pigmentation of the body. Finally, postlarval samples were identified according to their shape, size, body proportions, the form and formation of the fins, spines, and other organs and the number of myomeres. Morphological species identification was performed using a Leica M125 stereo microscope.

Barcoding methodology

The eggs and larvae were cleansed of alcohol, and the samples were rehydrated to eliminate the dehydration caused by the alcohol. Extraction was performed using commercial extraction kits with spin columns. Samples transferred to 1.5-mL capped tubes were incubated on a heater block at 60 °C for lysis after adding 20-µL proteinase-K and 100 µL lysis buffer included in the kits. The incubation period of 10-90 minutes recommended by the kit producer was extended overnight to ensure adequate lysis of fish egg capsules of thicknesses varying from species to species. Again, an additional 20-µL of proteinase-K was added to ensure adequate lysis while the incubation continued, and the tubes were vortexed at regular intervals. The remaining steps were carried out according to the commercial kit's (Qiagen - Qiamp Fast DNA Tissue Kit) procedures.

Following DNA extraction, the obtained DNA was inspected in the laboratory using a spectrometer (NanoQ) to determine the amount of DNA obtained and the contamination rate. It was determined that the amount of DNA obtained was outside the measuring range of the device (significantly less than necessary) and that the contamination rate was above the value of 1.8-2.0, which is the ideal range for PCR at 260/280 nm wavelengths. The measurement results did not change with repeated extraction attempts with an incubation period increased to 24 hours. While the DNA obtained was at low amounts and had no desired purity, experimental PCRs performed on the first isolated samples showed satisfactory PCR results, and extraction processes were continued.

M13-tailed primers designed by Ivanova et al. (2007) were used in the PCR procedures to amplify the 5' region of the mitochondrial COI gene. For amplifying the mitochondrial 16S rRNA and 12S rRNA gene regions, the primers reported by Yang et al. (2014), which were also M13-tailed, were used. PCR procedures were performed on a final volume of 25 µL using standard Taq polymerase, dNTP, Taq buffer, MgCl2, and target-specific primers under various optimization conditions. Sequence analysis operations were performed on an AB-3500 sequence analysis device installed in the molecular genetics laboratory of the Directorate of Sheep Breeding Research Institute.

Morphological identifications were compared with the species identification made using the mitochondrial DNA (mtDNA) barcoding method. In the analyses performed, the mtDNA barcoding method was taken as a reference, and the sensitivity, specificity, positive predictive power, and negative predictive power of morphological species identification compared to genetic barcoding were calculated.

In the DNA barcoding part of the study, a total of 2,594 samples identified by morphological examination were barcoded, including 2,217 fish eggs and 377 larvae (Table 1). PCR was performed on 331 egg samples that could not be identified with morphological examination due to death or deformity. Amongst these individuals, a total of 149 (45%) of the samples produced PCR products of suitable

quality for sequencing and were DNA barcoded. The species identified from dead and deformed eggs and their quantities are given in Table 2.

Table 1. Species barcoded via mtDNA Barcoding method.

No	Species	Egg	Larvae
1	*Arnaglossus thori*	1	2
2	*Atherina hepsetus*	0	2
3	*Belone belone*	0	1
4	*Blennius ocellaris*	0	1
5	*Bothus podas*	2	0
6	*Buglossidium luteum*	4	1
7	*Callionymus lyra*	35	0
8	*Chelon aurata*	15	0
9	*Chelon saliens*	10	0
10	*Chromis chromis*	0	1
11	*Chromogobius quadrivittatus*	0	2
12	*Chromogobius zebratus*	0	1
13	*Diplodus annularis*	83	7
14	*Diplodus puntazzo*	56	0
15	*Diplodus sargus*	38	0
16	*Engraulis encrasicolus*	124	101
17	*Epinephelus mariginatus*	1	0
18	*Eutrigla gurnardus*	53	0
19	*Gaidropsarus mediterraneus*	71	2
20	*Gobius niger*	0	71
21	*Gobius paganellus*	0	2
22	*Lithognathus mormyrus*	124	3
23	*Maurolicus muelleri*	5	0
24	*Microlipophrys dalmatinus*	0	1
25	*Millerigobius macrocephalus*	0	1
26	*Monochirus hispidus*	80	0
27	*Mullus barbatus*	155	1
28	*Nerophis lumbriciformis*	0	2
29	*Pagellus erythrinus*	13	0
30	*Parablennius sanguinolentus*	0	20
31	*Parablennius tentacularis*	0	28
32	*Pegusa impar*	11	1
33	*Pegusa lascaris*	5	0
34	*Pomatomus saltatrix*	15	0
35	*Salaria pavo*	0	2
36	*Sardina pilchardus*	81	9
37	*Sardinella aurita*	53	0
38	*Sciaena umbra*	63	0
39	*Scomber colias*	6	0
40	*Scophthalmus maximus*	48	38
41	*Scopthalmus rhombus*	61	0
42	*Scoroaena porcus*	59	0
43	*Scorpaena notata*	1	0
44	*Serranus cabrilla*	1	0
45	*Serranus hepatus*	155	4
46	*Serranus scriba*	167	0
47	*Sphyraena sphyraena*	2	1
48	*Spicara flexuosum*	0	2
49	*Spicara maena*	2	0
50	*Sprattus sprattus*	64	7
51	*Symphodus ocellatus*	0	11
52	*Synapturichthys kleinii*	17	0
53	*Trachinus draco*	53	0
54	*Trachurus mediterraneus*	179	34
55	*Trachurus trachurus*	119	12
56	*Tripterygion tripteronotum*	0	1
57	*Umbrina cirrosa*	57	0
58	*Uranoscopus scaber*	128	0
59	*Zebrus zebrus*	0	5
Total		2217	377

Table 2. Mitochondrial COI barcode results of fish eggs marked as dead or deformed during morphological examination

Species	No
Engraulis encrasicolus	8
Lithognathus mormyrus	11
Pomatomus saltatrix	1
Scomber colias	1
Serranus hepatus	13
Serranus scriba	21
Trachurus mediterraneus	90
Trachurus trachurus	3
Deformed/dead eggs that cannot be sequenced	182
Total	331

Data analysis

Following set of rules were used to compare the morphological and molecular identifications quantitatively; if a sample identified as species “A” according to mtDNA barcoding was also identified as species “A” via morphological examination, the result was accepted as a True Positive. If a sample identified as a different species according to mtDNA barcoding was identified as species “A” via morphological examination, the result was accepted as a False Positive. If a sample identified as species “A” according to mtDNA barcoding was identified as a different species via morphological examination, the result was accepted as a False Negative. Lastly, if a sample identified as a different species according to mtDNA barcoding was also identified as a different species via morphological examination, the result was accepted as a True Negative. Statistical analyses were performed according to the following formulas, devised by Trevethan (2017).

Sensitivity = $[a/(a + c)] \times 100$

Specificity = $[d/(b + d)] \times 100$

Positive predictive power = $[a/(a + b)] \times 100$

Negative predictive power = $[d/(c + d)] \times 100$Sensitivity, specificity, positive predictive power, and negative predictive power of species identification in fish eggs and larvae via morphological examination were calculated separately for each species and then, finally, for all species.

Table 3. Statistical analysis formulas.

		mtDNA barkod	
		Species "A"	Species "A" NOT
Morphology	Species "A"	True Positive (a)	True Positive (b)
	Species "A" Not	False Negative (c)	False Negative (d)

Results

In the DNA barcoding part of the study, a total of 2,594 samples identified by morphological examination were barcoded, including 2,217 fish eggs and 377 larvae. PCR was performed on 331 egg samples that could not be identified with morphological examination due to death or deformity. A total of 149 (45%) of the samples produced PCR products of suitable quality for sequencing and were DNA barcoded. In Tables 4 and 5, the positive predictive power is the probability of species “A” identified by morphological examination being identified as species “A” by the reference method (mtDNA barcoding). Sensitivity is the percentage of individuals belonging to species “A” in the whole sample group being identified as species “A” by morphological examination. Negative predictive power is the probability of the sample not identified as species “A” by morphological examination not belonging to species “A”. Specificity is the proportion of samples not identified as species “A” to samples not belonging to species “A” in the entire sample group. This method, which serves to measure the reliability of medical diagnostic tests, has been adapted to evaluate the power of species identification of morphological examination compared to the mtDNA barcoding method; the main informative aspects of the method regarding the study subject are the values of sensitivity and specificity.

A total of 59 species belonging to 10 orders and 29 families were identified in this study. The accuracy of species identification by morphological examination taking mtDNA barcoding as a reference across all samples was calculated as 79.6% for fish eggs and 82.2% for fish larvae (Table 4 and 5). The morphological identification specificity was calculated as 94.2% and 95.2% for fish eggs and larvae, respectively. When calculated separately for each species, morphological identification specificity was found to be quite high (96.8%-100%), while striking differences were observed in sensitivity values, indicating the probability of correct identification of species. For example, morphological species identification sensitivity values were 0 for the first eight species in Tables 4 and 5. This indicates that the morphological species identification of all the egg and larva samples belonging to these species was false. Similarly, morphological species identification showed between 20% and 60% accuracy in the egg samples of seven species and between 50% and 60% accuracy in the larvae samples of six species. In summary, the success rate of morphological species identification in egg and larva samples belonging to 29 species in the sample group of 59 species was between 0% and 60%.

Since the samples were taken from seawater, it was observed that there was some unavoidable environmental contamination, and the effects of environmental contamination caused relatively more polluted sequences to appear than expected. However, due to the high power of both gene barcode regions (COI and 16S rRNA) in distinguishing species, a generally high nucleotide matching rate (>98%) using both NCBI and BOLD system databases was detected, even with dirty barcodes. Thus, there were no problems distinguishing species, except in some cases specific to some species.

One larva identified as *Blennius ocellaris* during morphological examination could not be barcoded because PCR could not be successfully performed by COI or 16S rRNA. Exactly 58 of the total 59 species identified during the morphological examination were barcoded by mitochondrial COI and/or 16S rRNA. One egg identified as belonging to the species *Epinephelus mariginatus*, two eggs belonging to *Sphyraena sphyraena*, two eggs belonging to *Spicara maena*, and one larva belonging to *Microlipophrys dalmatinus* could not be identified by PCR through COI but were successfully barcoded by PCR through 16S rRNA. In summary, very few samples (a total of five eggs and one larva) belonging to four species from the 59 species detected in morphological examinations were barcoded through 16S rRNA since PCR could not be performed through COI. One sample belonging to one species did not produce sufficient PCR products related to either gene region and was not barcoded. The barcoded samples entered the NCBI and BOLD systems (Table 4). In addition, a new project was created in the BOLD system and an “online” identification atlas containing information such as egg or larva photos, the geographical area where samples were collected, and COI and 16S rRNA barcode sequences for each identified species were created and submitted for use by the researchers. 347 high-resolution

photos of eggs and larvae belonging to the 59 species identified by morphological examinations and mtDNA coding were uploaded to the BOLD system.

As a result of the BLAST (Basic Local Alignment Search Tool) search comparing the COI sequences barcoded by our team and the sequences in the NCBI, and in BOLD it was determined that the COI sequences of five larvae specimens identified with questionable morphological identification did not match in both databases. When these samples were sequenced using 16S rRNA and after the BLAST search was initiated again, it was observed that the sequence matched with the *Zebrus zebrus* sequence recorded in the NCBI. The COI sequences of the *Zebrus zebrus* species produced through 16S rRNA barcoding were entered into the NCBI (MZ723117) and BOLD (ERDK055-21) systems for the first time as part of this study and submitted for use by other researchers. Similarly, the COI sequences of two larvae samples identified as *Chromogobius quadrivittatus* were not found in the BOLD system but were found in the NCBI database. The COI sequences belonging to this species were submitted to the BOLD system for the first time in this study.

Table 4. Success of identification by morphological examination in fish eggs compared to mtDNA barcoding.

Species	mtDNA Barcoded Egg	Morfological Identification True	Morfolojical Identification False	Sensitivite (%)	Spesifite (%)	Positive predictive power	Negative predictive power	False Identified Species
Bothus podas	2	0	2	0.0	100.0	0.0	99.9	Undefined
Epinephelus mariginatus	1	0	1	0.0	100.0	0.0	100.0	Undefined
Pagellus erythrinus	13	0	13	0.0	100.0	0.0	99.4	Family, Undefined
Pegusa impar	11	0	11	0.0	100.0	0.0	99.5	Family
Scorpaena notata	1	0	1	0.0	100.0	0.0	100.0	*Scorpaena porcus*
Serranus cabrilla	1	0	1	0.0	100.0	0.0	100.0	*Serranus hepatus*
Sphyraena sphyraena	2	0	2	0.0	100.0	0.0	99.9	Undefined
Spicara maena	2	0	2	0.0	100.0	0.0	99.9	Family
Pegusa lascaris	5	1	4	20.0	99.6	11.1	99.8	Undefined
Scopthalmus rhombus	61	23	38	37.7	100.0	100.0	98.3	Family, Undefined
Scomber colias	6	3	3	50.0	100.0	100.0	99.9	*Scomber japonicus*
Synapturichthys kleinii	17	9	8	52.9	100.0	100.0	99.6	*Pegusa lascaris*
Eutrigla gurnardus	53	29	24	54.7	100.0	100.0	98.9	*Trigla lucerna* Family
Chelon aurata	15	9	6	60.0	99.9	81.8	99.7	*Chelon saliens*
Pomatomus saltatrix	15	9	6	60.0	100.0	100.0	99.7	Undefined
Monochirus hispidus	80	54	26	67.5	100.0	100.0	98.8	Family
Serranus hepatus	155	105	50	67.7	99.7	93.8	97.6	Family, *Serranus scriba*
Diplodus puntazzo	56	38	18	67.9	99.6	82.6	99.2	Family, *Diplodus sargus*
Trachurus trachurus	119	82	37	68.9	100.0	100.0	98.3	Family, Undefined
Callionymus lyra	35	25	10	71.4	100.0	100.0	99.5	Family, *Callionymus pusillus*
Scorpaena porcus	59	43	16	72.9	100.0	97.7	99.3	Family, *Scorpaena scrofa*
Gaidropsarus mediterraneus	71	54	17	76.1	100.0	100.0	99.2	Family, Undefined
Lithognathus mormyrus	124	96	28	77.4	99.4	88.9	98.7	Family, *Diplodus annularis*
Mullus barbatus	155	121	34	78.1	100.0	100.0	98.4	Family, *Mullus surmuletus*
Chelon saliens	10	8	2	80.0	99.7	57.1	99.9	Family, *Chelon aurata*
Trachinus draco	53	45	8	84.9	100.0	100.0	99.6	Family
Diplodus annularis	83	71	12	85.5	98.7	71.7	99.4	Family, *Lithognathus mormyrus*
Trachurus mediterraneus	179	155	24	86.6	100.0	100.0	98.8	Family
Uranoscopus scaber	128	111	17	86.7	100.0	100.0	99.2	Undefined
Diplodus sargus	38	33	5	86.8	99.6	80.5	99.8	Family, Undefined
Umbrina cirrosa	57	51	6	89.5	100.0	100.0	99.7	*Sciaena umbra*
Sciaena umbra	63	57	6	90.5	99.7	90.5	99.7	Family
Sardinella aurita	53	48	5	90.6	100.0	100.0	99.8	Undefined
Serranus scriba	167	161	6	96.4	97.6	76.3	99.7	Family, *Serranus hepatus*
Sardina pilchardus	81	79	2	97.5	100.0	100.0	99.9	Undefined
Sprattus sprattus	64	63	1	98.4	100.0	100.0	100.0	Family
Arnaglossus thori	1	1	0	100.0	100.0	100.0	100.0	
Buglossidium luteum	4	4	0	100.0	100.0	100.0	100.0	
Engraulis encrasicolus	124	124	0	100.0	100.0	100.0	100.0	
Maurolicus muelleri	5	5	0	100.0	100.0	100.0	100.0	
Scophthalmus maximus	48	48	0	100.0	100.0	100.0	100.0	
Total	**2217**	**1765**	**452**	**79.6**	**94.2**	**92.8**	**83.1**	

Table 5. Success of identification by morphological examination in fish larvae compared to mtDNA barcoding.

Species	mtDNA Barcoded Egg	Morfological Identification True	Morfolojical Identification False	Sensitivite (%)	Spesifite (%)	Positive predictive power	Negative predictive power	False Identified Species
Blennius ocellaris	1	0	1	0.0	96.8	0.0	99.7	Family
Chromogobius quadrivittatus	2	0	2	0.0	100.0	0.0	99.5	Family
Chromogobius zebratus	1	0	1	0.0	100.0	0.0	99.7	Family
Microlipophrys dalmatinus	1	0	1	0.0	100.0	0.0	99.7	Family
Millerigobius macrocephalus	1	0	1	0.0	100.0	0.0	99.7	Family
Nerophis lumbriciformis	2	0	2	0.0	100.0	0.0	99.5	Family
Tripterygion tripteronotum	1	0	1	0.0	100.0	0.0	99.7	Family
Zebrus zebrus	5	0	5	0.0	100.0	0.0	98.7	Family
Gaidropsarus mediterraneus	2	1	1	50.0	100.0	100.0	99.7	Undefined
Salaria pavo	2	1	1	50.0	100.0	100.0	99.7	*Parablennius tentacularis*
Spicara flexosa	2	1	1	50.0	100.0	100.0	99.7	*Spicara maena*
Trachurus trachurus	12	6	6	50.0	100.0	100.0	98.4	*Trachurus mediterraneus*
Diplodus annularis	7	4	3	57.1	100.0	100.0	99.2	*Atherina hepsetus*
Parablennius tentacularis	28	16	12	57.1	99.7	94.1	96.7	*Blennius ocellaris*
Lithognathus mormyrus	3	2	1	66.7	100.0	100.0	99.7	Family
Serranus hepatus	4	3	1	75.0	100.0	100.0	99.7	Family
Gobius niger	71	55	16	77.5	100.0	100.0	95.0	*Gobius paganellus*
Sardina pilchardus	9	7	2	77.8	100.0	100.0	99.5	Family
Parablennius sanguinolentus	20	18	2	90.0	100.0	100.0	99.4	Family
Symphodus ocellatus	11	10	1	90.9	100.0	100.0	99.7	Family
Trachurus mediterraneus	34	32	2	94.1	98.3	84.2	99.4	Family
Scophthalmus maximus	38	36	2	94.7	100.0	100.0	99.4	Family
Engraulis encrasicolus	101	99	2	98.0	100.0	100.0	99.3	Undefined
Arnaglossus thori	2	2	0	100.0	100.0	100.0	100.0	
Atherina hepsetus	2	2	0	100.0	100.0	100.0	100.0	*Atherina boyeri*
Belone belone	1	1	0	100.0	100.0	100.0	100.0	
Buglossidium luteum	1	1	0	100.0	100.0	100.0	100.0	DNA failed
Chromis chromis	1	1	0	100.0	100.0	100.0	100.0	
Gobius paganellus	2	2	0	100.0	100.0	100.0	100.0	
Mullus barbatus	1	1	0	100.0	100.0	100.0	100.0	
Pegusa impar	1	1	0	100.0	100.0	100.0	100.0	
Sphyraena sphyraena	1	1	0	100.0	100.0	100.0	100.0	
Sprattus sprattus	7	7	0	100.0	100.0	100.0	100.0	
Total	**377**	**310**	**67**	**82.2**	**95.2**	**94.2**	**84.9**	

Discussion

We detected 59 species in this study while a total of 56 species were detected in a study conducted at 19 stations in Erdek Bay for 12 months between 2011-2012 (Kara, 2015). Exactly 28 species detected in the study mentioned above were also detected in our study. Obviously, the local biodiversity has not been changed in the last 10 years. Given that the error rate in this study, conducted with DNA barcoding, is low, it is likely that errors were made in the identification of some species in the study of Kara (2015).

The power of morphological examination performed under a microscope on fish eggs and larvae to distinguish species was tested by taking mtDNA barcoding as a reference. The “true positive”, “true negative”, “false positive”, and “false negative” values in species identification were calculated, and the sensitivity, specificity, positive predictive power, and negative predictive power of the morphological species identification were calculated through these values. The values calculated for each species were then calculated for the entire sample group. Therefore, the species identification power of morphological species identification was evaluated for both separate species and all species. The analyses were performed separately for fish eggs (Table 4) and larvae (Table 5).

The method that we applied to compare molecular and morphological identifications normally serves to measure the reliability of medical diagnostic tests, has been adapted to evaluate the power of species identification of morphological examination compared to the mtDNA barcoding; the main informative aspects of the method regarding the study subject are the values of sensitivity and specificity.

In species identifications made by mtDNA barcoding method, the minimum nucleotide difference threshold value required to distinguish two species is accepted to be roughly 3% (Hebert et al., 2003a; Chakraborty et al., 2014). In this study, we found that a nucleotide difference of 3% is sufficient for distinguishing species.

Currently, the COI gene is considered the most accurate tool and key in DNA barcoding for species identification of fish eggs and larvae (Hebert et al., 2003a, b; DeSalle and Amato, 2004; Triant and Whitehead, 2009; Ko et al., 2013, Hubert et al., 2015; Frantine-Silva et al., 2015; Ayala et al., 2016; Bhattacharya et al., 2016; Briski et al., 2016). However, in our research, it was determined that the COI gene was insufficient for distinguishing some species in species identification using mtDNA, and the nucleotide difference between some detected species and the alternative species with the closest nucleotide similarity to the detected species was well below the 3% threshold value. In this study, PCR processes were performed on all samples' 5' barcode region of the COI gene. Samples with conflicting COI barcode results and samples for which PCR could not amplify the COI gene, underwent another PCR targeting of the mitochondrial 12S rRNA and 16S rRNA gene barcode regions. In the trials, the 12S rRNA gene barcode sequences produced results inconsistent with the NCBI database, and this gene region was ignored. The 16S rRNA barcode region was used as an alternative for barcoding samples with inconsistent results for COI gene barcodes or did not produce PCR results for the COI 5' gene region.

In the genetic analysis, it was observed that the COI barcode sequences of *Pegusa impar* and *Synapturichthys kleinii* were almost identical, and the COI gene could not effectively distinguish these two species. Similarly, some *Gobious* species could only be identified at the "sp" level by COI barcoding. Thus, these samples were sequenced by performing PCR on the mitochondrial 12S rRNA and 16S rRNA genes. It was observed that barcoding performed through 12S rRNA was less successful in species identification than with the COI gene. On the other hand, each sample belonging to the *Pegusa impar* and *Synapturichthys kleinii* species, which could not be separated by COI barcoding, was consistently identified through 16S rRNA. The second possible species with the closest nucleotide similarity to the mentioned species was found to be *Solea solea*, and the difference between the mentioned species and the possible closest species in each sample was well above the 3% threshold (between 5.2% and 5.7%). Similarly, samples of the Gobius species that could only be defined at the "sp" level with COI barcoding were identified as *Gobius paganellus* using 16S rRNA barcoding, and the nucleotide difference between each sample and the closest possible species was found to be over 4% for each sample (Table 6).

Table 6. Species identification power of COI and 16S rRNA barcode regions in some species.

Mitochondrial COI Barkoding					
No	Species Identifed	Nucleotide similarity (%)	Most similar species	Nucleotide similarity (%)	Nucleotide difference, % (threshold, %3)
534	*Pegusa impar*	100	*Synapturichthys kleinii*	100	0.0
623	*Pegusa impar*	100	*Synapturichthys kleinii*	100	0.0
676	*Solea kleini*	100	*Pegusa impar*	100	0.0
764	*Pegusa impar*	99,8	*Synapturichthys kleinii*	99.8	0.0
774	*Pegusa impar*	99,4	*Synapturichthys kleinii*	99.4	0.0
781	*Pegusa impar*	99,6	*Synapturichthys kleinii*	99.6	0.0
813	*Pegusa impar*	100	*Synapturichthys kleinii*	100	0.0
864	*Gobius sp.*	99,4	*Gobius paganellus*	90.4	9.0
865	*Gobius sp.*	99	*Gobius paganellus*	90.6	8.4
Mitochondrial 16S rRNA Barcoding					
No	Species Identifed	Nucleotide similarity (%)	Most similar species	Nucleotide similarity (%)	Nucleotide difference, % (threshold, %3)
534	*Solea kleini*	99,2	*Solea solea*	94.1	5.2
623	*Solea kleini*	99	*Solea solea*	93.9	5.2
676	*Solea kleini*	97,2	*Solea solea*	91.5	5.7
764	*Solea kleini*	97,2	*Solea solea*	91.8	5.4
774	*Solea kleini*	96,7	*Solea solea*	91.5	5.3
781	*Solea kleini*	96,8	*Solea solea*	91.5	5.3
813	*Solea kleini*	97	*Solea solea*	91.7	5.3
864	*Gobius paganellus*	94,8	*Gobius cobitis*	90.6	4.2
865	*Gobius paganellus*	94,2	*Gobius cobitis*	89.5	4.7

It is generally accepted that using only morphological identification in ichthyoplankton surveys can result in major errors (Ahlstrom and Moser, 1976; Nakatani et al., 2001; Shao et al., 2002; Pegg et al., 2006; Ko et al., 2013; Becker et al., 2015; Frantine-Silva et al., 2015; Duke et al., 2020). Fish eggs without precise morphological characteristics or with shared morphologies have proven more difficult to classify (Shao et al., 2002; Gleason and Burton, 2012; Harada et al., 2015; Lin et al., 2016). Classical ichthyoplankton studies are considered as time-consuming, expensive, and even tedious (Kelso et al., 2012; Deters et al., 2013; Hintz et al., 2017). However, DNA barcoding of every egg and larva sampled is also quite time-consuming and significantly costly, but integrating classical morphology based methods with the molecular methods can provide an easy to use and cost effective methodology (Mavruk et al. 2023).

Conclusions

In this study, we found ichthyoplankton species identification success rate with DNA barcoding method is over 80 percent. DNA barcoding of every egg and larva sampled in ichthyoplankton studies is not possible due to both time restrictions and extremely high costs. For this reason, it is not possible to abandon morphological identification information at the moment, but it would be advantageous to integrate molecular methods into such studies to attain the most accurate results. DNA barcoding is also useful in recognizing species diversity, especially in special ecosystems.

The early developmental stages of species belonging to some families are especially similar to each other. Great care should be taken when identifying species using morphological methods in ichthyoplankton studies; a realistic approach should be taken, and, if possible, molecular methods should be used together with taxonomic knowledge.

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Ethical Approval

No need to ethical approval for this study.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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