HYDROBIOLOGICAL RESEARCH

Hydrobiological Research 2(2), 35-41 (2024) https://www.doi.org/10.5281/zenodo.12800103

Research Article

Comparative molecular characterization of Diplozoon species from fishes

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Abstract: In this article, it was aimed to isolate Diplozoon species from naturally infected carp fish from Elâzığ region and to determine their morphological and microscopic characteristics and molecular identification. As a result of the study, it was observed that parasite isolation was realized in all samples of carp fish sampled. It was observed that the isolated parasites were 33-55/27.3-51.3-51.2/24-45 mm in size. DNA isolation and PCR analysis were performed by selecting the most morphologically and different sized samples. In the preliminary identification analysis by Real-Time PCR, both primer sets and DNA interaction were checked. Then, blast results obtained from the sequence data obtained as a result of PCR analysis performed in the thermal cycler device, it was observed that they showed 98% similarity with *Diplozoon paradoxum* and the closest similarity with *Paradiplozoon homoion* and *Paradiplozoon skrjabini* with a maximum similarity of 96%. Phylogenetic analysis of Diplozoon species using sequence data and Neighbor Joining (NJ) methods showed that they occur in 2 different branches. In line with these results, it was observed that 3 different groups were formed in the parasites with the lowest morphological and microscopic similarity. It is thought that genetic differences are the main reasons why Diplozoon parasite species cause different levels of infestation, virulence and mortality in fish.

Diplozoon species in fish are a common cause of infestation worldwide and diplozoon species are also common in the study area. Intraspecific differences in diplozoon species and changes in the host-parasite relationship lead to differences in virulence and pathogenicity, and as a result, cause diseases and economic losses. Knowing the species differences of parasites regionally has an important place in fish diseases for taking prophylactic measures and treatment processes.

Keywords: Diplozoon, Fish parasites, PCR, Phylogenetics, Carp

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(Received: 28.06.2024, Accepted: 12.07.2024, Online Publication: 24.07.2024)

1. INTRODUCTION

Diplozoon species belong to a group of monogenean helminths usually found parasitically in the gills of freshwater fish (Mizelle, 1936). These organisms usually feed by attachment to fish gills and can often adversely affect fish health (Bychowsky, 1957). Diplozoon species usually attach to fish gills and feed on gill tissues, which can affect respiratory ability and cause breathing difficulties in fish (Ergens, 1969). Some species of these parasites can contribute to the spread of infections among fish that can cause disease and mortality (Khotenovsky, 1985). Diplozoon species can cause severe damage to the respiratory system of fish, as they are usually attached to the gills, which can negatively affect the growth and health of fish (Justine et al., 2011).

Molecular characterization of Diplozoon species is an important research topic in parasitology and molecular biology (Dos Santos & Avenant-Oldewage, 2020). Diplozoans are monogenean worms that parasitize the gills of freshwater fish and are characterized by the permanent union of two individuals during mating. These parasites are widespread in freshwater ecosystems, especially in Europe and Asia, and threaten the health of fish species of high economic value. Therefore, molecular characterization of diplozoa is of great importance to determine their genetic diversity, evolutionary relationships and intra- and interspecific differences.

Molecular characterization processes are usually performed using advanced genetic and biotechnological techniques such as polymerase chain reaction (PCR), DNA sequencing, ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) analysis. Thanks to these techniques, the taxonomic position of Diplozoon species can be more clearly established, providing critical data for biodiversity conservation and management of aquatic ecosystems. Furthermore, a better understanding of the life cycles and host-parasite relationships of these species allows the creation of innovative approaches to fish health management and the development of biological control strategies.

At the global level, molecular characterization of Diplozoon species is also of great importance for ecosystem health and biodiversity conservation. These studies contribute not only to the conservation of fish species of high economic value, but also to maintaining the balance of natural aquatic ecosystems. The spread of parasites such as diplozoa can disrupt ecosystem balances, leading to poor water quality and declining fish populations. Therefore, molecular characterization studies provide in-depth knowledge on the biology and ecology of these parasites, helping to develop sustainable water management strategies.

Molecular characterization of Diplozoon species is an indispensable tool for both academic research and applied sciences and is of critical importance for the sustainability of the aquaculture sector in Turkey and globally and for the conservation of biodiversity. Strategic steps need to be taken to control the spread of these parasites, reduce economic losses and protect the health of aquatic ecosystems. Therefore, molecular characterization of Diplozoon species is an important research area in disciplines such as parasitology, evolutionary biology and ecology.

In this study, molecular identification of diplozoon parasite species isolated from carp fish species in Elazığ region was carried out and their similarities or differences with the species available in the database were realized by phylogenetic analysis.

2. MATERIAL AND METHOD

2.1. Total DNA Isolation

350 µl of lysis buffer and steel ball were added to the diplozoon parasite tissue samples obtained from 3 different regions and homogenized with TissueLyser Lt. Then, according to the optimized protocol, 20 µl proteinase K was added to the samples and incubated at 65ºC for 20 min on a shaker. At the end of the time, 4 µl RNease A was added and incubated at 21ºC for 15 min on a shaker. The suspension mixture was centrifuged at 12000 rpm for 3 min and the supernatant was carefully removed and loaded onto a spin column. DNA isolation from the samples was performed with a total DNA isolation kit (Sugenomic Biotechnology) (Figure 1).

Figure 1. Schematized view of DNA isolation steps

After loading into the spin column, centrifugation was performed at 12000 rpm for 1 min. Then 700 µl of the 1st wash buffer was added and centrifuged at 12000 rpm for 1 min. The second wash buffer was added and centrifuged at 12000 rpm for 1 min. 50 µl of elution buffer was added and after waiting for 1 min at room temperature, isolation was terminated by centrifugation at 16000 rpm for 2 min (Karataş, 2024).

2.2. Primer Design and Synthesis

Primer synthesis was performed using 28S rRNA and 5.8S rRNA gene region with accession number AF369759.1 for Diplozoon sp. The designed primer sequence region is presented in the appendix (Table 1).

Table-1. The designed primary sequence region

Primer Name	Sequence 5'-3'	Tm
Diplozoon sp 5.8-23S-F	TGCAAACTGCCTTGAGCATC	60.01
Diplozoon sp 5.8-23S-R	ACCAGTACTTTGCTGTATTGT	59.90

2.3. Real-Time PCR Analysis

The components to be used for Real-Time PCR analysis were prepared as follows (Önalan, 2019).

qPCR Master Mix	12.0 µl
Primer F	1.5 µl
Primer R	1.5 µl
RNase-free water	7.0 µl
Template DNA	3.0 µl
Total volume	25.0 µl

The optimized protocol in Real-Time PCR was performed as follows.

Step- 1. Pre Denaturation	95°C. 15 min. (1 cycle)
Step- 2. Cycling	Number of cycles: 40
Denaturation	94°C 1 min.
Annealing	60.5°C 45 sec.
Extension	72°C 1 min.
Step- 3. Final extension	72°C 10 min. (1 cycle)

3. RESULTS

3.1. Microscopic Findings

Microscopic analyzes were obtained with a Leica binocular microscope. The images obtained revealed that the morphological characteristics of Diplozoon sp. The typical body structure and characteristic images were similarly observed in all isolated parasites. Microscopic images of the isolated parasites are given below (Figure 2).

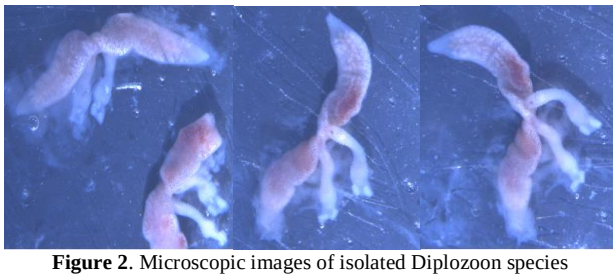

Figure 2. Microscopic images of isolated Diplozoon species

3.2. Morphometric Results

According to the results of morphometric analysis, parasites were isolated from the gill tissues of male and female *Cyprinus carpio* fish from different populations on different dates between 2021 and 2022. The isolated parasites were morphologically identified as Diplozoon paradoxum. Their lengths were found to vary between 33-55 / 27.3-51.2/ 24-45 mm. The morphological data table of the parasites is given below.

3.3. Real-Time PCR Analysis Results

As a result of the study, 1 NTC (negative control) sample was used in Real-Time PCR analysis. Real-Time PCR results are given below for each parasite species separately (Figure 3).

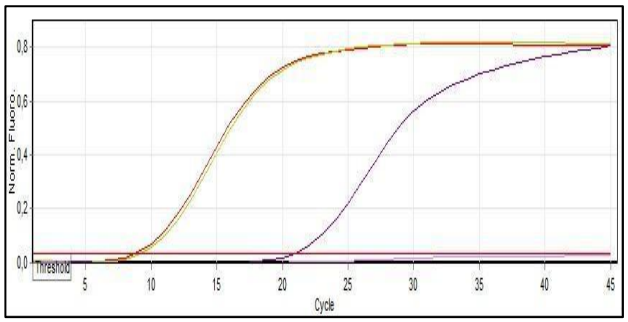

Figure 3. Real-Time PCR pre-identification image

End of Real-Time PCR analysis, the negative control sample (DNA-free components-Non-template control) was negative below the threshold value. The other 3 parasite samples were also positive with sigmoidal curves.

3.4. Sequence Analysis

PCR analysis was performed in a thermal cycler using the DNAs isolated in the study. PCR conditions are given below.

Step- 1 Pre Denaturation	95°C 15 min. (1 cycle)
Step- 2 Cycling	Number of cycles: 40
Denaturation	94°C 1 min.
Annealing	60.5°C 45 sec.
Extension	72°C 1 min.
Step- 3 Final extension	72°C 10 min. 1 cycle

At last of the Sanger sequence analysis of the PCR amplicons obtained as a result of PCR analysis, it was determined that sample-1 and sample-2 were similar to Diplozoon paradoxum and Paradiplozoon homoion species. Sample 3 was identified as Diplozoon sp. As a result of the sequence analysis of PCR amplicons, it was determined that all 3 samples were Diplozoon but samples 1 and 2 were the same. Sample 3 was determined to be diplozoon at the genus level, but the sequence result at the species level was determined at the sp level due to the high similarity with other species. As a result of the sequence, the similarity rates of 3 samples are given below. Dendrogram analysis; Construction Method: UPGMA and Nucleotide Distance Measure: Kimura 80 was obtained with CLC Main Workbench software as a result of statistical evaluation (Table 2).

Table 2: Characteristics of the sampling site and parasites obtained in the study

ID	EVKEM Number	Date	Where It Came From	Fish No	Sex	Length	Weight	Age	Parasite Presence	Number of parasites	Current Organ	Parasite Species
9	13_14	24.05.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Male	33/29.1/26.8	523.13	2+	Positive	1	Gill	*Diplozoon paradoxum*
32	63_	2.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	45.4/39.8/37.4	1313.49	4+	Positive	1	Gill	*Diplozoon paradoxum*
33	64_68	2.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	49/44.5/41	1883.63	3+	Positive	5	Gill	*Diplozoon paradoxum*
34	70_	2.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	46/42/39	1466.	4+	Positive	1	Gill	*Diplozoon paradoxum*
36	75_77	3.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	45/40/38	167.	4+	Positive	3	Gill	*Diplozoon paradoxum*
39	85_	4.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	55/51.2/45	380.7	6+	Positive	1	Gill	*Diplozoon paradoxum*
41		4.06.2021	Yurtbaşı 13th District Uzunova location Hunting area	*Cyprinus carpio*	Female	52/48/44	2648.90	3+	Positive		Gill	*Diplozoon paradoxum*
51	93_80	16.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Male	37.4/33.6/31.6	789.51	3+	Positive		Gill	*Diplozoon paradoxum*
52	81_82	16.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Female	47/43.2/39.3	701.56	3+	Positive	2	Gill	*Diplozoon paradoxum*
53	83_85	16.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Male	43.1/38.8/35.5	1237.88	3+	Positive	3	Gill	*Diplozoon paradoxum*
55	86_	16.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Male	34.6/31.2/28.7	614.82	2+	Positive	1	Gill	*Diplozoon paradoxum*
58	87_91	17.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Female	43/39.2/36.4	416.9	4+	Positive	5	Gill	*Diplozoon paradoxum*
59	92_96	17.06.2021	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (koçkale)	*Cyprinus carpio*	Male	46.3/43/38.7	528.	4+	Positive	5	Gill	*Diplozoon paradoxum*
123	1114_1116	27.10.2021	Keban reservoir 6th region Aydıncık hunting area	*Capoeta trutta*	Female	33.3/29.8/27.6	663,7	6+	Positive	2	Gill	*Diplozoon paradoxum*
124	1117_1123	27.10.2021	Keban reservoir 6th region Aydıncık hunting area	*Capoeta trutta*	Female	30.9/27.3/24.7	523	6+	Positive	6	Gill	*Diplozoon paradoxum*
126	1129_1133	27.10.2021	Keban reservoir 6th region Aydıncık hunting area	*Capoeta trutta*	Male	30.2/27.3/24.6	431,05	5+	Positive	5	Gill	*Diplozoon paradoxum*
127	1136_1141	27.10.2021	Keban reservoir 6th region Aydıncık hunting area	*Capoeta trutta*	Male	31.2/28.2/25.6	425,92	4+	Positive	6	Gill	*Diplozoon paradoxum*
158	1573_	13.05.2022	Keban dam lake Yolüstü Kooparatif 14. region Koçkale	*Cyprinus carpio*	Female	39.5/34.3/31.8	806,32	6+	Positive	1	Mounth	*Diplozoon paradoxum*
166	1756_1758	25.05.2022	Keban Kooparatif 3rd region hunting ground.	*Cyprinus carpio*	Male	39.1/35.2/31.4	894,6	6+	Positive	3	Gill	*Diplozoon paradoxum*
213	2177_2178	10.06.2022	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (Koçkale)	*Cyprinus carpio*	Male	34.4/30.2/28.2	580,8	6+	Positive	2	Gill	*Diplozoon paradoxum*
243	2586_2588	1.07.2022	Keban reservoir 6th region Aydıncık hunting area	*Cyprinus carpio*	Female	44.5/40.5/37.2	1572,42	6+	Positive	3	Gill	*Diplozoon paradoxum*
244	2589_	1.07.2022	Yurtbaşı 15th Region Örencik Kooparatif location hunting area (Koçkale)	*Cyprinus carpio*	Male	39.2/35.1/31.9	974,75	6+	Positive	1	Gill	*Diplozoon paradoxum*

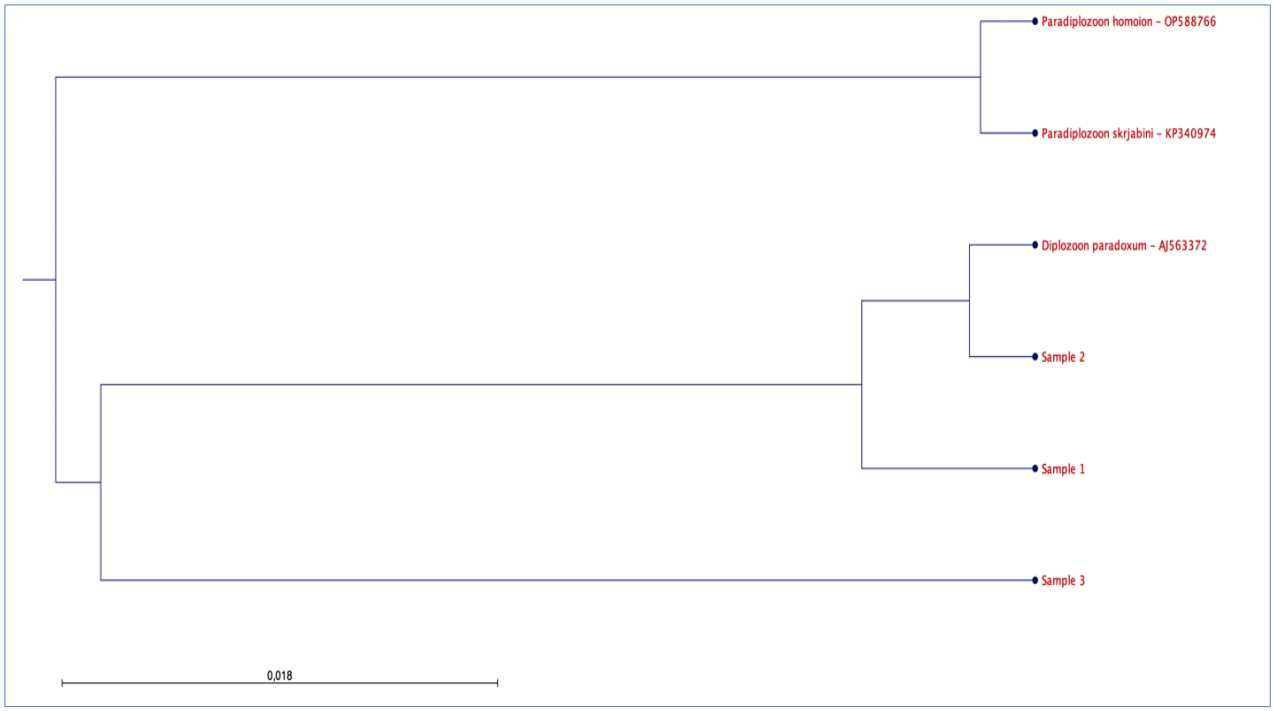

Figure 4. Phylogenetic analysis of the parasites isolated in the study

According to the results of the dendrogram, samples 1 and 2 were 98% similar to *Diplozoon paradoxum* and over 96% similar to *Paradiplozoon homoion* and *Paradiplozoon skrjabini*. Sample 3 was similar to *Diplozoon paradoxum* and *Paradiplozoon homoion*, but more different from Samples 1 and 2 (Figure 4).

4. DISCUSSION

It was observed that parasite isolation was realized in all sampled carp fish samples. It was observed that the isolated parasites varied in size between 33-55/27.3-51.2/24-45 mm. DNA isolation and PCR analysis were performed by selecting the most morphologically different and different sized samples. In the preliminary identification analysis by Real-Time PCR, both primer sets and DNA interaction were checked. Then, as a result of the blast results obtained from the sequence data obtained as a result of PCR analysis performed in the thermal cycler device, it was observed that they showed 98% similarity with Diplozoon paradoxum and the closest similarity with Paradiplozoon homoion and Paradiplozoon skrjabini with a maximum similarity of 96%. Phylogenetic analysis of Diplozoon species using sequence data and Neighbor Joining (NJ) methods showed that they occur in 2 different branches. Ziętara et al. (2010) introduced a protocol for DNA isolation from gyrodactylid species, a monogenean (platyhelminth) parasite. The researchers developed a protocol to extract DNA from samples preserved with 96% ethanol. This protocol supports DNA barcoding for molecular species identification of parasites. Erk'akan et al. (2006) reported the presence of *Diplozoon paradoxum* in Turkey. The researchers carried out DNA isolation and analysis to confirm the presence and molecular characterization of this parasite. This study provided information on the distribution of Diplozoon parasites in Turkey by analyzing their molecular structure.

Levsen et al. (2012) examined the genetic similarities and phylogenetic relationships of different species of Diplozoon. The researchers performed molecular phylogenetic analyses of different Diplozoon species using DNA sequences. The findings showed that Diplozoon species are genetically closely related and that certain genetic traits are important in determining similarities and differences between species. Mendoza-Palmero et al. (2014) examined the chromosome structures and cytogenetic characteristics of Diplozoon species. The researchers compared the karyotypes and chromosome structures of different Diplozoon species. The findings showed that Diplozoon species have genetic similarities as well as certain differences in their karyotype and cytogenetic characteristics. Niewiadomska and Młocicki (2007) conducted molecular studies using 28S rRNA and 5.8S rRNA gene regions of *Diplozoon paradoxum* (Platyhelminthes: Monogenea). By analyzing the rDNA sequences, the researchers examined the genetic structure and evolutionary relationships of *Diplozoon paradoxum.*

Svobodová et al. (2017) examined the genetic diversity and population structure of *Diplozoon paradoxum* using microsatellite markers. The researchers analyzed Diplozoon samples isolated from different Cyprinidae fish species to determine the genetic diversity and population structure of the parasite. Bettim et al., (2019) conducted morphological and molecular characterization of Neoechinorhynchus parasites (Acanthocephala: Neoechinorhynchidae) isolated from *Hoplias malabaricus* fish (Characiformes: Erythrinidae). The researchers identified the parasite species by genetic analysis and studied their distribution in the Paraguay River. Šimková et al., 2003 examined the phylogenetic relationships of the genus Diplozoon using 28S rDNA

sequences. The researchers performed molecular phylogenetic analyses of different Diplozoon species and constructed phylogenetic trees to understand the taxonomic relationships of the genus.

Diplozoon parasites are trematode species commonly found in freshwater fish. These parasites have various effects on their hosts by attaching to the skin and gills of fish. The importance of Diplozoon parasites in freshwater ecosystems in Turkey is determined by the interaction of a number of factors. Diplozoon infections directly affect fish health. They cause damage to the gills and impair respiratory and feeding functions. This can reduce fish growth rates and decrease reproductive success. These effects negatively impact productivity and profitability in the fishing industry. Diplozoon parasites can affect ecological balance. Excessive population increases can cause imbalances in the populations of host fish species and lead to unwanted changes in the ecosystem. This can jeopardize ecosystem services that naturally maintain balance. Diplozoon parasites can indirectly affect human health. Consumption of infected fish can raise food safety concerns. Especially if consumption of freshwater fish is widespread, the risk of transmission of these parasites to humans increases. Finally, Diplozoon parasites have economic impacts. Widespread infections in commercially farmed fish species can increase production costs and reduce profit margins. This can cause economic losses to the fishing industry.

5. CONCLUSION

The importance of Diplozoon parasites in freshwater ecosystems in Turkey is multifaceted. They can have an impact on both fish health and ecosystem balance. Therefore, it is important to control these parasites and prevent their spread. Control strategies include measures such as regular infection monitoring, proper hygiene practices, management of host fish populations and isolation of infected fish. These can help reduce the impacts of Diplozoon parasites and protect the health of freshwater ecosystems. Further research is also needed to better understand the economic and ecological impacts of these parasites. These studies may contribute to the development of effective management strategies and the sustainability of freshwater fisheries. In line with these results, it was observed that 3 different groups were formed in the parasites with the lowest morphological and microscopic similarity. It is thought that genetic differences are the main reasons why diplozoon parasite species cause different levels of infestation, virulence and mortality in fish. In fish, diplozoon species are a common cause of infestation worldwide and diplozoon species are also common in the study area. Intraspecific differences in diplozoon species and changes in host-parasite relationship lead to differences in virulence and pathogenicity, and as a result, cause diseases and economic losses. Knowing the species differences of parasites regionally has an important place in fish diseases for taking prophylactic measures and treatment processes.

Authors' Contributions

AİE: contributed to parasite sampling, determination of morphological characteristics and article writing.
ŞÖ: contributed to molecular characterization and article writing.

Conflict of Interest

The authors declare that there is no conflict of interest.

Statement on the Welfare of Animals

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data Availability Statements

The authors confirm that the data supporting the findings of this study are available within the article.

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