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Original Article

Genetic, haplotype and phylogenetic analysis of *Ligula intestinalis* by using mt-CO1 gene marker: ecological implications, climate change and eco-genetic diversity

Análise genética, haplótipo e filogenética de *Ligula intestinalis* usando marcador do gene mt-CO1: implicações ecológicas, mudanças climáticas e diversidade ecogenética

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Abstract

Ligula intestinalis is a cestode parasite that affects freshwater fish in different countries of the world. The current study aims to reveal the phylogenetic, genetic and haplotype diversity of mt-CO1 gene sequences sent to the NCBI database from different countries by using in-silico analysis. The 105 mt-CO1 (371 bp) gene sequences of *L. intestinalis* obtained from NCBI were used for bioinformatics analyses. Sequences were subjected to phylogenetic and haplotype analysis. As a result of the haplotype analysis of *L. intestinalis*, 38 haplotypes were obtained from 13 different countries. Hap24 constituted 44.76% of the obtained haplotype network. Changes in nucleotides between haplotypes occurred at 1-84 different points. China and Turkey have highest fixation index (Fst) values of 0.59761, while the lowest (-0.10526) was found between Russia and Turkey. This study provides a baseline for future studies on extensive scale on the epidemiology, ecological aspects, distribution pattern, transmission dynamics and population dispersion of *L. intestinalis* worldwide.

Keywords: *Ligula intestinalis*, genetic variation, mt-CO1, in-silico analysis.

Resumo

Ligula intestinalis é um parasita cestódeo que acomete peixes de água doce em diversos países do mundo. O presente estudo visa revelar a diversidade filogenética, genética e de haplótipos das sequências do gene mt-CO1 enviadas ao banco de dados do NCBI de diferentes países, por meio de análise in-silico. As sequências gênicas de 105 mt-CO1 (371 pb) de *L. intestinalis* obtidas do NCBI foram utilizadas para análises bioinformáticas. As sequências foram submetidas a análise filogenética e de haplótipos. Como resultado da análise de haplótipos de *L. intestinalis*, 38 haplótipos foram obtidos de 13 países diferentes. Hap24 constituiu 44,76% da rede de haplótipos obtida. Mudanças nos nucleotídeos entre os haplótipos ocorreram em 1-84 pontos diferentes. A China e a Turquia apresentam os maiores valores do índice de fixação (Fst), 0,59761, enquanto o menor (-0,10526) foi encontrado entre a Rússia e a Turquia. Este estudo fornece uma linha de base para futuros estudos em larga escala sobre epidemiologia, aspectos ecológicos, padrão de distribuição, dinâmica de transmissão e dispersão populacional de *L. intestinalis* em todo o mundo.

Palavras-chave: *Ligula intestinalis*, variação genética, mt-CO1, análise in-silico.

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1. Introduction

Ligula intestinalis is a pseudophyllidean cestode, which is located in the Diphyllobothridae family. It is a common parasitic disease that causes the most important health problems in fish farming. The final hosts of *L. intestinalis* are waterfowls while the first intermediate host is Crustacea, and the second intermediate host is freshwater fish (Hoole et al., 2010). The eggs, which are excreted in feces of final hosts, develop and hatch in water as coracidium. Free coracidiums entered in first intermediate host from drinking of contaminated water, the crustacean copepods, and develop into procercoids in the abdominal cavities within 2-3 weeks. The second intermediate host ingests copepods containing procercoids. Then procercoids transform into the plerocercoids in the abdominal cavity of the fish. In the waterfowl that eat fish contaminated with plerocercoid, the larvae mature into the adult parasite (Loot et al., 2002).

The most prominent parasitism phase of *L. intestinalis* during its life cycle is the plerocercoid stage. *L. intestinalis* causes important effects such as physical damage, growth retardation, changes in blood parameters, immunological disorders, hormonal disruption and behavioral changes during the development in the abdominal cavity of the fish (Loot et al., 2001; Barber et al., 2000; Britton et al., 2009). The prevalence of *L. intestinalis* in fish species changes according to the regions. The prevalance was 7.2% in Zimbabwe (Barson and Marshall, 2003), 29.00% in Ethiopia (Emaminew et al., 2014), 14.98% in Malawi (Gabagambi and Skorping, 2018), 9.7% in Iran (Shargh et al., 2008), 23.38% in Sri Lanka (Weliange and Amarasinghe, 2001). The prevalence rate of *L. intestinalis* in fish in Turkey was 23% in Balikesir (Koc et al., 2006), 71.5% in Eskisehir (Ozbek and Ozturk, 2010) and 73.33% in Tokat provinces (Turgut et al., 2011).

Ligulosis is a foodborne zoonotic disease. There are various reports regarding this situation (Urdes and Hangan, 2013). Fish production has a special place in trade and economy for the human population, more importantly, there is a greater need in tropical and subtropical countries with nutritional deficiency problems. Parasites that cause diseases in fish cause great economic losses as mortality increases, while expenses incurred for treatment increase farm costs, and a decrease in growth rate depending on the periods of parasitic diseases causes weight loss accordingly. All these situations hinder the progress of fish farms (Osuigwe and Obiekezie, 2007).

Due to the advancement of today's technology and advances in sequencing and computational technologies in the genetic field, the obtained DNA sequences have become a source of information to advance the understanding between genetic relationships and to clarify the evolutionary process (Tibayrenc, 2005).

It has come ready to contribute to taxonomic research, population genetics and phylogenetic by developing DNA barcodes, which are short DNA sequences of a standard gene region. At the same time, many groups around the world are working day by day to obtain an equipped DNA barcode database that covers most of the world's biodiversity (Ali et al., 2014; Hajibabaei et al., 2007; Sarkar and Trizna, 2011). It enables species identification and classification by obtaining nucleotide sequences of the mitochondrial Cytochrome Oxidase subunit 1 (CO1) region of the genome for DNA barcoding purposes (Hebert et al., 2003). Present study was aimed to investigate the haplotype diversity, genetic variation, and phylogeny of *L. intestinalis* by using mt-CO1 genetic marker deposited to GenBank from different geographical zones.

2. Methodology

2.1. Collection of data

The sequences of the partial mt-CO1 gene fragment of *L. intestinalis* had been submitted to the National Center for Biotechnology Information, USA, (NCBI) (www.ncbi.nlm.nih.gov) until May 05, 2021 were used for the bioinformatics analyses. A total of 105 sequences were identified after searching these gene sequences in the NCBI database. The sequences of *L. intestinalis* were used from different countries of Europe, Asia, Africa and North America while the selected sequences were retrieved by focusing on gene region, host and location.

2.2. Data and phylogenetic analysis

The selected sequences were retrieved by using FASTA format from NCBI database to the CLC Sequence Viewer 8 (QIAGEN CLC Main Workbench, 2007). A reference sequence (Access no. NC039445) of *L. intestinalis* was used to align all obtained sequences of different lengths. After all sequences were trimmed and aligned, 105 sequences with a length of 371 bp were obtained and then used for bioinformatics analyses. Following, the phylogenetic tree was created with the Neighbour Joining (NJ) model. For statistical support to specific branches, 1000 bootstrap replicates were obtained.

2.3. Haplotype analysis

After all sequences were saved in FASTA format then DnaSP 6 program was used for the analyses. With the program, statistics about haplotype and nucleotide change values, haplotype numbers, nucleotide content, and mutation amounts between haplotypes were determined (Rozas et al., 2017). In the DnaSP6 tool, it was also recorded in NEXUS format to create the haplotype network with sequences (Maddison et al., 1997). Then, the haplotype network was created by using with PopART (Population Analysis with Reticulate Trees) program (Leigh and Bryant, 2015).

3. Results

In this study, the geographic origin, fish hosts, and GenBank accession numbers of the mt-CO1 sequences (371 bp) of *L. intestinalis* isolates were obtained from the NCBI database (Table 1). From NCBI database, 105 mt-CO1 gene sequences (371 bp) were obtained from 13 countries including Algeria (n=12), China (n=4), Czechia (n=22),

Table 1. Origin, host species and GenBank accession numbers of *Ligula intestinalis* sequences.

Origin	Host species	GenBank accession numbers
Algeria	Barbus sp.	JQ279069/72
	Barbus sp.	JQ279068/75-76-77-78-79
	Barbus setivimensis	JQ279070-71/73
	Barbus sp.	JQ279074
China	Gymnocypris przewalskii	KY321844
	Hemiculter leucisculus-Carassius carassius	AF153910
	-	MT558599/NC_039445
Czechia	Rutilus rutilus	EU241279-80-81
	Rutilus rutilus	EU241240-41-42-43/ EU241277-78
	Abramis brama	EU241269
	Abramis brama	EU241263-64-65-66-67-68/70/83-84-85-86
	Rutilus rutilus	EU241282
Estonia	Abramis brama	JQ279085-86/ EU241275-76/ EU241294
Ethiopia	-	MT558600
France	Rutilus rutilus	EU241261/ EU241299/ EU241300
	Abramis brama	EU241259
	Rutilus rutilus	JQ279082/ JQ279105
	Alburnus alburnus	JQ279087-88
	Rutilus rutilus	JQ279081/83/89-90/ JQ279104/ EU241258
	Abramis brama	EU241260
Germany	Rutilus rutilus	JQ279080/84/ EU241273-74/ EU241301-02
Russia	Abramis brama	EU241251-52-53-54-55-56-57/ EU241309-10
Tunisia	Rutilus rubilio	JQ279098-99
	Scardinius erythrophthalmus	JQ279094/96
	Pseudophoxinus callensis	JQ279100/01
	Pseudophoxinus callensis	JQ279102
	Rutilus rubilio	JQ279091-92/ EU241271/ EU241312-13-14-15
	Scardinius erythrophthalmus	JQ279093/95/97/ JQ279103/ EU241272
Turkey	Tinca tinca	MK286929-30-31-32-33-34
Ukraine	Alburnus alburnus	EU241316
	Carassius carassius	EU241238
	Rutilus rutilus	EU241317
United Kingdom	Phoxinus phoxinus	EU241304
	Rutilus rutilus	EU241303
USA	Oncorhynchus tshawytscha	KY552875

Estonia (n=5), Ethiopia (n=1), France (n=15), Germany (n=6), Russia (n=9), Tunisia (n=19), Turkey (n=6), Ukraine (n=3), England (n=2) and USA (n=1). The distribution of the sequence-collected countries are shown in Figure 1.

3.1. Diversity, neutrality, fixation and gene flow analysis

Table 2 illustrates the diversity and neutrality indices. The 105 mt-CO1 nucleotide sequences (371 bp) of *L. intestinalis* isolates from the NCBI database were used to investigate the haplotype and genetic analysis. The resulting sequences lacked appropriately conserved DNA regions. The highest Fixation index (Fst) (0.59761) was found between China and Turkey, while the lowest (-0.10526) was found between Russia and Turkey (Table 3). A negative Tajima's D means a lot of low-frequency polymorphism. In two cases, we encountered a negative Fu's Fs value, either as evidence for a large number of alleles, as would be expected from a recent population increase or genetic hitchhiking.

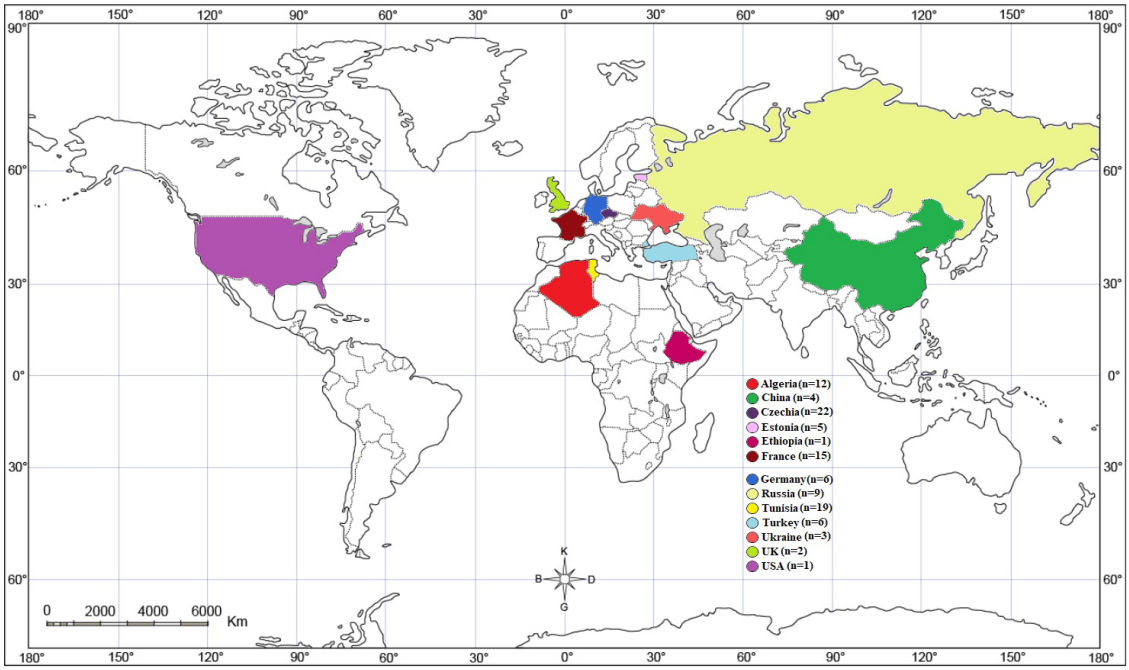

Figure 1. Distribution map of *Ligula intestinalis* sequences according to geographical origins.

Table 2. Diversity and neutrality indices obtained using nucleotide data of the *Ligula intestinalis mt*-CO1 gene (371 bp).

mt-DNA	n	H	hd ± SD	πd ± SD	Tajima's D	p value	Fu's Fs	p value	FLD	p value	FLF	p value
371 bp	105	38	0.791±0.041	0.03849±0.00695	-1.78251	P < 0.05	-2.814	0.022	-0.23814	P > 0.10	-1.09133	P > 0.10

n: number of isolates; H: number of haplotypes; hd: haplotype diversity; πd: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic.

Table 3. Dual fixation index (Fst) for *Ligula intestinalis* isolates compared with those from various geographic regions using nucleotide data of the mt-CO1 gene.

Origin	Algeria	China	Czechia	Estonia	France	Germany	Russia	Tunisia	Turkey	Ukraine
Algeria	-	-	-	-	-	-	-	-	-	-
China	0.34743	-	-	-	-	-	-	-	-	-
Czechia	0.56125	0.57430	-	-	-	-	-	-	-	-
Estonia	0.55800	0.56876	0.01448	-	-	-	-	-	-	-
France	0.56926	0.59140	0.01288	0.00714	-	-	-	-	-	-
Germany	0.56590	0.58533	0.10458	0.07273	0.12245	-	-	-	-	-
Russia	0.56814	0.59495	0.03481	0.00000	0.01714	0.12632	-	-	-	-
Tunisia	0.43159	0.42196	0.14365	0.06951	0.15636	0.16509	0.15426	-	-	-
Turkey	0.56879	0.59761	0.03687	0.00000	0.01905	0.13333	0.10526	0.15594	-	-
Ukraine	0.55821	0.57371	0.02406	0.00000	0.00952	0.08889	0.00000	0.14594	0.00000	-
UK	0.55309	0.55682	0.01524	0.00000	0.00519	0.05714	0.00000	0.13437	0.00000	0.00000

Algeria n=12; China n=4; Czechia n=22; Estonia n=5; France n=15; Germany n=6; Russia n=9; Tunisia n= 19; Turkey n= 6; Ukraine n= 3; UK n= 2.

3.2. Phylogenetic analysis

In this study, 105 sequences retrieved from mt-CO1 gene of *L. intestinalis* isolates were used. *Diphyllobothrium latum* and *Spirometra decipiens* sequences were used as outgroup sequences. Figure 2 depicts the phylogenetic tree. Haplotypes were seperated from themselves by mutation at 1-81 different points among themselves. Hap38 (JQ279072-71-70), both Hap35 (JQ279078) and Hap36 (JQ279077-76-75-74) were the most distant haplotypes, showing mutations at 81 points (Table S1).

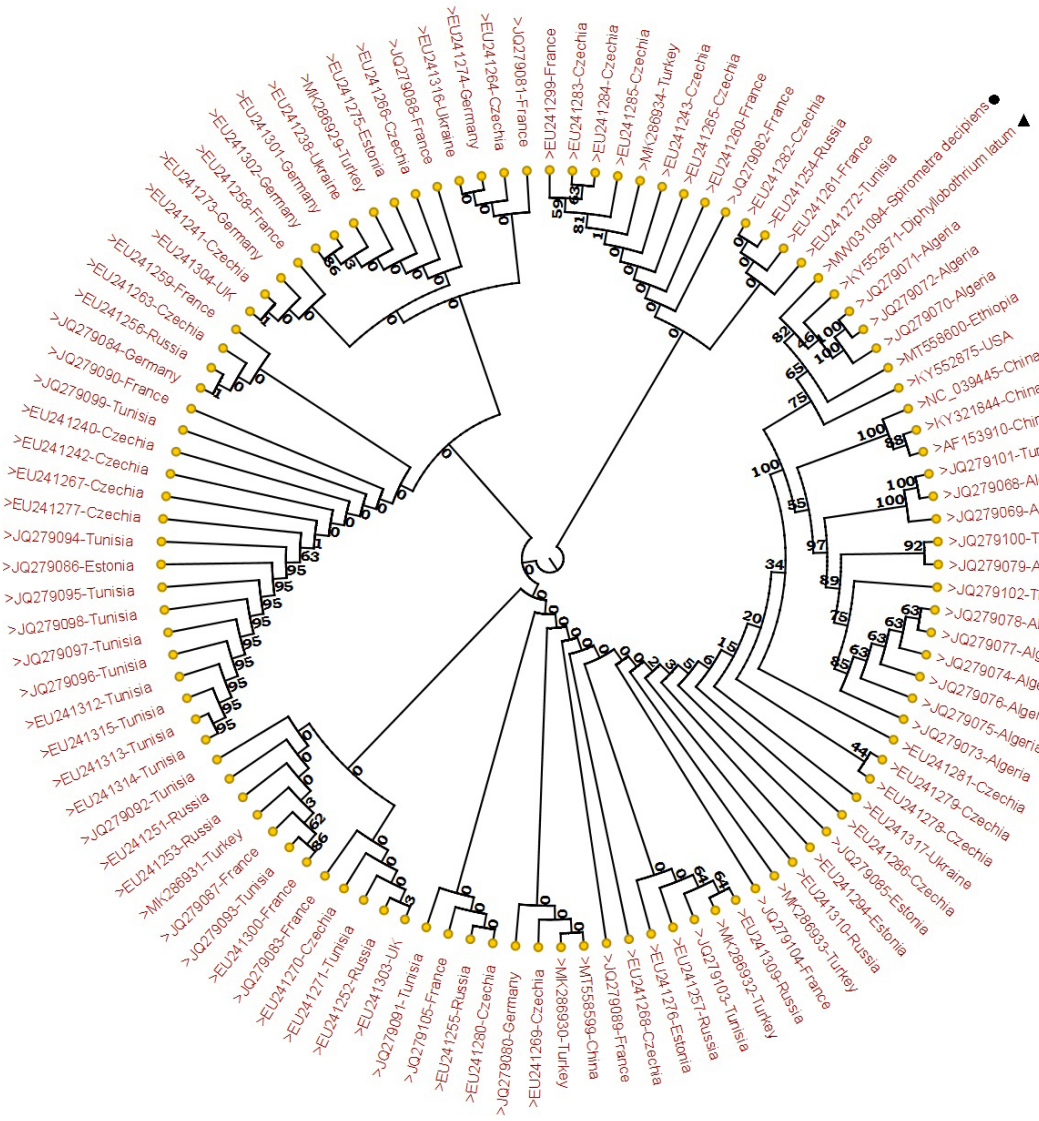

Figure 2. Phylogenetic tree view of *Ligula intestinalis* sequences using mt-CO1 gene (371 bp) sequences and reference sequences. CLC Sequence Viewer 8 was used to generate a Maximum Likelihood tree based on the Neighbor Joining model. The reliability of the tree was evaluated with 1000 bootstrap iterations. ● *Spirometra decipiens*, ▲ *Diphyllobothrium latum*.

3.3. Haplotype networks

Figure 3 illustrates the haplotype network obtained. Analyses of 38 haplotypes revealed high haplotype diversity (0.791±0.041). *L. intestinalis* isolates grouping of haplotypes of the mt-CO1 sequences and the accession numbers of the isolates forming the groups are shown in Table S2. The haplotype network obtained as a result of haplotype analysis constitutes a total of 44.76% of Hap24. This haplotype was consisted of 83.33% of Turkey, 80% of France, 77.77% of Russia, 54.54% of Czechia, 50% of Germany, 40% of Estonia, 33.33% of Ukraine and 26.31% of Tunisia sequences. In the haplotype network, 73.68% were single haplotype, 13.15% were double haplotype, 5.26% were triple haplotype, 2.63% were quadruple haplotype, and 2.63% were decimal haplotype.

4. Discussion

Due to the rapidly increasing population in the world, the need for food has become one of the most important problems. Especially fish are among the most important because they are rich in protein, economically cheap and easily accessible (Oktener et al., 2008). As aquaculture has become important, aquaculture diseases that develop due to various factors such as bacterial, fungal and viral, etc. have become important today. Among these factors, parasitic diseases constitute the most important part (Oktener, 2003). Therefore, in our study, it was determined that *L. intestinalis* isolates, which frequently infect fish, it is aimed to determine gene flows, genetic variations and phylogeny according to geographical regions by obtaining 371-bp long sequences belonging to the mt-CO1 gene region.

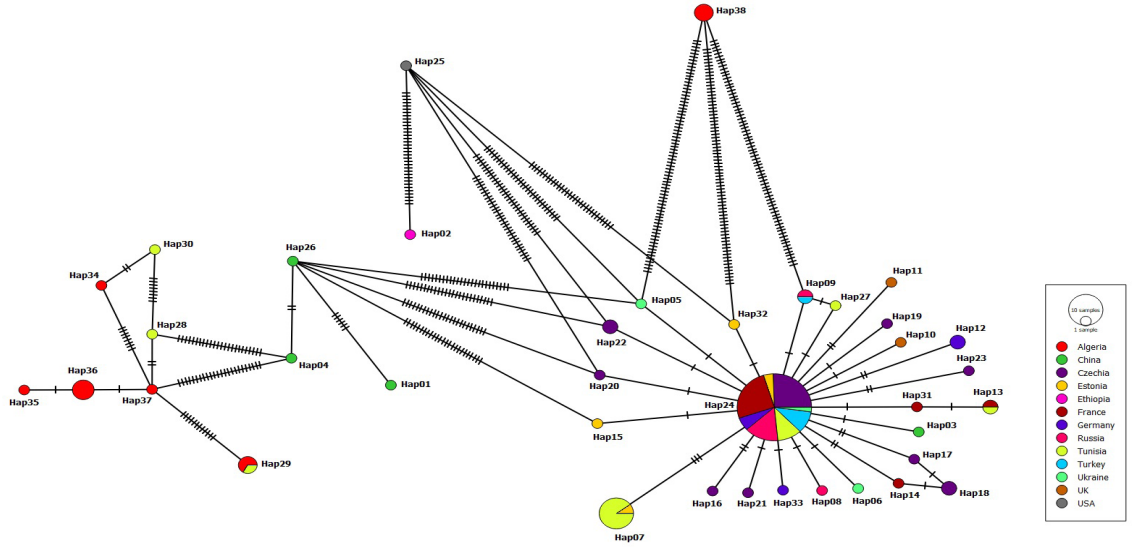

Figure 3. Appearance of mt-CO1 (371 bp) haplotypes of *Ligula intestinalis* sequences. The number of mutations that distinguish haplotypes are indicated by screening marks. The geographical distribution of haplotypes is shown in different colors. The size of the circles is related to the haplotype frequency.

Mitochondrial DNA sequences are one of the most accurate molecular detection marker for the organism differentiation. It is due to higher mutation rates and maternal inheritance, it is feasible to investigate the genetic analysis of population and their systematic studies of closely related species (Jia et al., 2012). Sequence data of mt-CO1 gene can be used to investigate phylogenetic relationships at the genus level and to illuminate systematic biology (Chontananarth et al., 2014).

Ligula intestinalis is one of the most common tapeworms among freshwater fish and is very common in freshwater basins worldwide. Seasonal migration of their final host waterfowl is very effective in this spread. With the in-silico analysis method, the mt-CO1 gene sequences of *L. intestinalis* were obtained according to the geographical regions, and the differences in gene sequences were tried to be found by controlling the close relatedness between these isolates and its subspecies have been checked and at the same time, the presence of a gene flow between different geographies was checked.

Neutrality tests such as Tajima D, Fu's Fs were performed to analyze the neutrality values e.g.h as nucleotide variation and population expansion (Korneliussen et al., 2013). While Tajima D based on previous mutations reflecting population events for a long time. Fu's Fs value is more specific to latest mutation. The sequences of 13 different geographic populations were analyzed reporting low and negative values of Fu's F and Tajima D values. A negative Tajima's D means a lot of low-frequency polymorphism. In two cases, we encounter a negative Fu's Fs value, either as evidence for a large number of alleles, as would be expected from a recent population increase or genetic hitchhiking. It was seen that the main haplotype obtained in haplotype analysis made up 44.76% of the total network and included isolates from eight different countries. Therefore, this main haplotype represents a single ancestor. When the main haplotype and other haplotypes were analyzed, it was seen that *L. intestinalis* isolates exhibited a rapid genetic diversity and could lead to the formation of a subspecies.

China and Turkey showed highest fixation index (Fst) valeu of 0.59761, while the lowest (-0.10526) was found between Russia and Turkey. In this scenario, it can be stated that gen flow between the Chinese and Turkish populations is at a minimum level. It may be an indication that there is little or no fish trade, presumably because of China and Turkey are located by the sea in both countries.

As a result of haplotype analysis, 38 haplotypes were formed among 13 different countries. In these, 28 haplotypes were detected as single haplotype. This situation makes us think that it may be an indication that different subspecies may emerge as a result of the evolutionary period.

Haplotypes were separated from themselves by mutation at 1-81 different points among themselves. Hap38 (JQ279072-71-70), both Hap35 (JQ279078) and Hap36 (JQ279077-76-75-74) were the most distant haplotypes, showing mutations at 81 points. This situation made us think that there may be a gene flow as a result of cross fertilization between the species brought from Europe to North Africa, since the city of Algeria has a coastline to the Mediterranean Sea and is on the migration routes of waterfowl.

5. Conclusion

In conclusion, there have been different molecular studies about *L. intestinalis* carried out to date, but it is the first study done as an in-silico analysis method. With this study, it serves as a stepping-stone for future studies on *L. intestinalis* worldwide on epidemiology, genetic variation,

and evolutionary process. The mt-CO1 sequences sent to the dataset at NCBI included regions of short length, which were the most important limiting factors for us. Therefore, in future studies, it will be useful to target larger gene regions and genetic diversity in more comprehensive and to analyze them in terms of possible new subspecies.

Acknowledgements

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Supplementary Material

Supplementary material accompanies this paper.
Table S1. Nucleotide variation positions of the mt-CO1 (371 bp) gene among 38 haplotypes analyzed.
Table S2. Haplotype of mt-CO1 sequences of Ligula intestinalis and accession numbers of isolates forming groups.

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