Int. J. Aquat. Biol. (2022) 10(6): 478-488
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com
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Original Article

Morphological and molecular analysis of the freshwater bivalve *Anodonta anatina* in Iran and Finland

Hosein Mohamadzadeh[1], Hadise Kashiri[1*], Ainaz Shirangi[2], Jouni Taskinen[3], Reza Khaleghi[1], Amir Ghadermarzi[1]

[1]Department of Aquatic Ecology, Faculty of Fisheries and Environment, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
[2]Department of Biology, Faculty of Basic Sciences, University of Gonbad, Gonbad Kavus, Iran.
[3]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland.

Abstract: Duck mussel, *Anodonta anatina* is a habitat generalist inhabiting both lentic and lotic aquatic ecosystems. Due to high morphological similarity and phenotypic plasticity, *A. anatina* has sometimes been misidentified as *A. cygnea*. Here, morphological and molecular studies were conducted on *Anodonta* mussels inhabiting North Iran and Finland. The individuals were collected from Anzali Wetland, Tajan River (North Iran) and Jyväsjärvi Lake (Finland). The COI sequence analysis showed the existence of *A. anatina* in the sampling areas. The Iranian and Finland specimens showed three and two haplotypes, respectively. The Iranian haplotypes were placed in a single clade, while the Finland haplotypes were clustered with those of Central Europe. The mean *P*-distance between these two clades was 2.4. The median-joining network showed that the Iranian haplotypes were lumped into a single haplogroup, while the Finland ones were in the same haplogroup as those from Central Europe. The Mediterranean haplotypes were the most divergent haplogroup from both Iranian and Central European haplogroups. In morphological characteristics, the shell pattern of all individuals from both Iranian and Finland specimens was stretched and slightly compact with light/dark brown periostracum. The mean length of the specimens from Anzali Wetland was significantly higher than those of Tajan and Jyväsjärvi. No significant difference was observed in morphometric characteristics between Tajan and Jyväsjärvi populations. The results did not indicate significant variation in shell morphology in the studied groups. In this regard, the conventional linear measurements can be supplemented using more complex geometric morphology in further studies.

Article history:
Received 4 August 2022
Accepted 8 December 2022
Available online 25 December 2022

Keywords:
Anodonta
Haplogroup
Haplotype
Shell morphology

Introduction

Amon different groups of Bivalvia, the family Unionidae is highly distributed in fresh- and brackish-water ecosystems of the world. They are known for their significant ecological role (Vaughn, 2018), specific life cycle, being parasite in the larval stage (Modesto et al., 2018) and unusual doubly (paternal and maternal) mitochondrial inheritance (Guerra et al., 2019). In biogeographical studies, the Unionidae has great potential to study hydrological and geological events (Zieritz et al., 2020). Like many other freshwater fauna, bivalves are currently regarded as threatened groups and globally decreased because of anthropogenic activities (Ferreira-Rodrigues et al., 2019; Riccardi et al., 2019), enhancing their conservation significance. In fact, conserving the Unionidae populations is important due to their major ecological role in freshwater ecosystems (Vaughn, 2018). Among the Unionidae, the duck mussel, *Anodonta anatina* (Linnaeus, 1758), known as the pan-European freshwater mussel, is an important species due to high distribution in the lakes and rivers of Europe and Asia below 65°N latitude down to Sicily and Portugal and expanding as far east as the Siberian region. Therefore, the duck mussel is a suitable model to assess hypotheses in biogeography about freshwater habitats (Graf, 2007; Froufe et al., 2014).

As a habitat generalist, *A. anatina* inhabits both lotic and lentic aquatic ecosystems, from streams and rivers to reservoirs and lakes (Hinzmann et al., 2013). This species has high ecological importance,

*Correspondence: Hadise Kashiri
E-mail: hadiskashiri@gmail.com
DOI: https://doi.org/10.22034/ijab.v10i6.1771
DOR: https://dorl.net/dor/20.1001.1.23830956.2022.10.6.5.8

including decreasing water turbidity and controlling suspended particles (Vaghum, 2010). Moreover, duck mussels can filter some parasitic nematodes and decrease their transition to fish hosts (Gopko et al., 2017). It also has a high ability to filter and digest Cyanobacterial colonies (Bontes et al., 2007). Despite its high ecological importance, there is little information on the conservation status of *A. anatina* worldwide. Some regional studies have indicated a decrease in duck mussel populations in Europe so that it is now regarded as near threatened or threatened species in Austria, Germany, Irland, and Romania. The global decrease in freshwater mussel populations has threatened biodiversity and some habitat services the mussel provides (Hajisafarali et al., 2022). In this regard, some countries, including Germany and Luxemburg, have established conservation programs for *A. anatina* (Byrne et al., 2009; Binot-Hafke et al., 2011).

Characteristics of bivalves' shells, and calcified exoskeletons with protection functions, are considered common morphological markers in population studies (Modestin, 2017). Intraspecific variation in shell morphological characters in some bivalves has been previously studied (Modestin, 2017; Ghozzi et al., 2022; Wu et al., 2022; Mirzoeva and Demchenko, 2022). However, classic morphometric measurements might be insufficient in distinguishing bivalve populations and/or species (Morais et al., 2014). Like other Unionid mussels, *A. anatina* has high morphological plasticity that sometimes has resulted in some mistakes in identifying the species, so more than 400 synonyms are reported for this taxon (Nagel et al., 1996; Graf and Cummings, 2019). The morphological differences between *A. anatina* populations from some aquatic basins in Iberia resulted in the introduction of more than 20 synonyms (Araujo et al., 2009). Applying some morphological characteristics, especially those related to the shells, has caused some errors by taxonomists in characterizing the bivalve species and populations. It seems that morphological attributes traditionally used in identifying taxonomic units have some limitations since some characteristics have high morphological flexibility expressing different ecophenotypes (Zieritz and Aldridge, 2009; Zieritz et al., 2010). However, due to their simplicity, low cost, and no need for complicated facilities, many scientists have widely used morphological markers.

Due to the high morphological similarity between *A. anatina* and *A. cygnea* and high phenotypic plasticity, these two species are sometimes misidentified (Froufe et al., 2014). Until now, the Anodontini individuals inhabiting North Iran have been misidentified as *A. cygnea*. The aim of the present study was to study the morphological traits and examine possible affinities of the Anodontini populations in North Iran and Finland using COI gene data.

Materials and methods

Sampling was done during Jun and July 2020 in Anzali Wetland, Guilan Province and Tajan River, Mazandaran Province, Iran. The samples were also collected from lake Jyväsjärvi, Jyväskylä, Finlan) in November 2019. A small foot tissue was cut from live mussels and immediately preserved in 96% ethanol. Mussel shells of the samples (N= 27, 28 and 12 for Anzali Wetland, Tajan River, and Jyvaskyla Lake, respectively) were also collected for morphological studies.

Morphometry and age estimation: The biometric variables, including shell length (SL), shell height (SH), and shell width (SW), were measured for each specimen to the nearest 0.1 mm using an AACO caliper. The morphological indices, including shell convexity (CI = W/L ratio × 100) and elongation (EI = H/L ratio × 100), were calculated. The age of the samples was determined by counting the growth rings, which were visible on the shell.

Molecular studies: Total genomic DNA was extracted from the foot tissue of each mussel (N=6 and 4 for Iranian and Finland, respectively) using a high-salt procedure (Sambrook et al., 1989) with slight modification. DNA quality and quantity were examined via agarose gel (1%) electrophoresis and a

Table 1. List of COI sequences used in the present study.

Taxon	Accession number	Lineage/Haplotype	Reference
Anodonta anatina	OP905650	Iran/I3	This study
A. anatina	OP905651	Iran/I3	This study
A. anatina	OP905652	Iran/I2	This study
A. anatina	OP905653	Iran/I3	This study
A. anatina	OP905656	Iran/I3	This study
A. anatina	OP905661	Iran/I2	This study
A. anatina	OP905654	Iran/I3	This study
A. anatina	OP905655	Iran/I1	This study
A. anatina	OP905657	Iran/I1	This study
A. anatina	OP905658	Iran/I3	This study
A. anatina	OP905659	Iran/I3	This study
A. anatina	OP905660	Iran/I3	This study
A. anatina	OP906256	Central Europe/E8	This study
A. anatina	OP906257	Central Europe/E9	This study
A. anatina	OP906258	Central Europe/E8	This study
A. anatina	OP906259	Central Europe/E8	This study
A. anatina	KC583482	Central Europe/E6	NCBI's GenBank
A. anatina	KC583483	Central Europe/E3	NCBI's GenBank
A. anatina	KC583484	Central Europe/E7	NCBI's GenBank
A. anatina	KC583485	Central Europe/E2	NCBI's GenBank
A. anatina	KC583487	Central Europe/E10	NCBI's GenBank
A. anatina	KC583501	Central Europe/E4	NCBI's GenBank
A. anatina	MF414222	Central Europe/E1	NCBI's GenBank
A. anatina	EF440346	Central Europe/E11	NCBI's GenBank
A. anatina	MF414221	Central Europe/E5	NCBI's GenBank
A. anatina	EF571394	North Iberia/NI3	NCBI's GenBank
A. anatina	KC583507	North Iberia/NI4	NCBI's GenBank
A. anatina	KC583503	North Iberia/NI2	NCBI's GenBank
A. anatina	KC583462	North Iberia/NI8	NCBI's GenBank
A. anatina	KC583496	North Iberia/NI10	NCBI's GenBank
A. anatina	KC583459	North Iberia/NI11	NCBI's GenBank
A. anatina	KC583458	North Iberia/NI9	NCBI's GenBank
A. anatina	KC583456	North Iberia/NI5	NCBI's GenBank
A. anatina	KC583472	North Iberia/NI1	NCBI's GenBank
A. anatina	KC583470	North Iberia/NI7	NCBI's GenBank
A. anatina	KC583447	North Iberia/NI6	NCBI's GenBank
A. anatina	KC583450	South Iberia/Morocco/IM10	NCBI's GenBank
A. anatina	KC583451	South Iberia/Morocco/IM9	NCBI's GenBank
A. anatina	KC583452	South Iberia/Morocco/IM8	NCBI's GenBank
A. anatina	KC583464	South Iberia/Morocco/IM5	NCBI's GenBank
A. anatina	KC583476	South Iberia/Morocco/IM3	NCBI's GenBank
A. anatina	KJ402054	South Iberia/Morocco/IM4	NCBI's GenBank
A. anatina	KC583479	South Iberia/Morocco/IM1	NCBI's GenBank
A. anatina	KC583481	South Iberia/Morocco/IM2	NCBI's GenBank
A. anatina	MK733420	South Iberia/Morocco/IM11	NCBI's GenBank
A. anatina	EF571396	South Iberia/Morocco/IM6	NCBI's GenBank
A. anatina	EF571397	South Iberia/Morocco/IM7	NCBI's GenBank
A. anatina	MF414241	Mediterranean/M7	NCBI's GenBank
A. anatina	MF414233	Mediterranean/M4	NCBI's GenBank
A. anatina	MF414230	Mediterranean/M1	NCBI's GenBank
A. anatina	KC583518	Mediterranean/M6	NCBI's GenBank
A. anatina	KC583512	Mediterranean/M5	NCBI's GenBank
A. anatina	MF414237	Mediterranean/M3	NCBI's GenBank
A. anatina	KC583475	Mediterranean/M2	NCBI's GenBank

Table 1. Continued.

Taxon	Accession number	Lineage/Haplotype	Reference
A. cygnea	MK034157	-	NCBI's GenBank
A. cygnea	MK034159	-	NCBI's GenBank
A. cygnea	MT027890	-	NCBI's GenBank
Pseudanodonta complanata	MK574186	-	NCBI's GenBank
P. complanata	MK574187	-	NCBI's GenBank
P. complanata	MK034156	-	NCBI's GenBank
P. complanata	MK034155	-	NCBI's GenBank
A. exulcerata	MF414313	-	NCBI's GenBank
A. exulcerata	MF414306	-	NCBI's GenBank
A. exulcerata	MF414301	-	NCBI's GenBank
Anemina arcaeformis	KY561633	-	NCBI's GenBank
Sinanodonta woodiana	KY978735	-	NCBI's GenBank

Biophotometer Spectrophotometer (Eppendorf, Hamburg, Germany), respectively. We used the primers LCO22me2 (5'-GGT CAA CAA AYC ATA ARG ATA TTGG-3') and HCO700dy2 (5'-TCA GGG TGA CCA AAA AAY CA-3') (Walker et al., 2006, 2007) to amplify the partial sequences of cytochrome c oxidase subunit I (COI) gene. PCR was run on a thermal cycler (Bio-RAD MJ Mini Thermal Cycler, Hercules, CA, USA) in 25 µl reaction mix containing 1 µl DNA (20-160 ng/µL), 15 µl Taq 2X master mix red (Amplicon, Denmark), 1 µl of each primer and 7 µl PCR grade water. The PCR condition was set as follows: 4 min at 94°C, 40 cycles at 94°C (30 s), 50°C (40 s) and 72°C (60 S), followed by 10 min at 72°C. The products were assessed using agarose gel (1.5%) electrophoresis in TBE buffer (1X). The high-quality products were sent to the Genetic Codon Company (Tehran, Iran) for Sanger sequencing using the same primers.

Data analysis: The obtained sequences were manually edited in BioEdit 7.0.1 (Hall, 1999). We extracted 334 COI sequences from NCB I's GenBank. Multiple sequence alignment using ClustalW also was implemented in BioEdit. After trimming the sequences, a 592-bp *COI* fragment was left. Similar sequences were removed through the online tool FaBox 1.41 (Villesen, 2007). The phylogenetic tree was constructed based on 53 unique sequences (Table 1); three and two of them were for Iranian and Finland specimens, respectively. We used *Sinanodonta woodiana* (KY978735) and *Anemina arcaeformis* (KY561633) as outgroups.

The phylogenetic tree was reconstructed using Bayesian inference in MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001). The best-fitting models of nucleotide substitution based on the Akaike information criterion (Akaike, 1973) were estimated using MrModelTest v3.7 (Posada and Crandall, 1998) in PAUP v4.0 (Swofford, 2003). Two parallel runs were independently conducted. Each included one cold and three heated Metropolis coupled MCMC chains. The program was run for 10 million generations and sampled once every 10000 generations with 20% burn-in fraction. The obtained tree was visualized through FigTree v1.4.2 (Rambaut, 2008). Genetic divergences based on *P*-distance were assessed in MEGA 6.0 (Tamura et al., 2013). The median-joining network was also constructed using 43 sequences of *A. anatina* (Table 1) through PopArt v1.7 (Leigh and Bryant, 2015) to study the relationships between haplotypes.

Results

The shell pattern of Iranian and Finland samples were stretched and slightly compact with brown/olive-green periostracum. We used COI gene sequencing to identify the species. According to the molecular data, the studied bivalves were *A. anatina* (Fig. 1).

Morphometric data: Morphometric features of the *A. anatina* specimens collected from Anzali

Figure 1. *Anodonta anatina*; a and b represent live duck mussel individuals and exterior/interior view of the mussel, respectively.

Table 2. Morphometric features and age of *Sinanodonta lauta* from Iran and Finland

		Length	Height	Width	Convexity index	Elongation index	Age (year)
Tajan River	Min-Max	31.84-115.38	21.42-52.43	9.63-35.97	29.77-39.74	42.08-67.27	1-7
	Mean±SD	86.30±18.12	41.68±7.38	28.05±5.99	32.57±2.45	49.22±6.34	5±1.3
Anzali Wetland	Min-Max	67.9-134.32	32.45-75.63	21.4-49.35	28.58-38.12	41.63-59.57	2-8
	Mean±SD	97.99±21.77	50.03±15.0	32.49±9.5	32.81±2.46	50.65±5.67	5.11±1.47
Jyväsjärvi Lake	Min-Max	66.43-130.93	33.99-64.19	22.67-39.22	29.95-38	47.96-56.16	
	Mean±SD	84.83±18.75	40.49±8.57	28.04±4.51	33.39±2.2	51.31±2.52	5.83±1.4

Abbreviations: SD (Standard deviation); Min (Minimum); Max (Maximum)

Table 3. Age-length relationships of *Anodonta anatina* from Iran and Finland.

		Age							
		1	2	3	4	5	6	7	8
Length	Tajan	31.84	40.28	62.08	74.76	88.7	98.91	97.09	-
	Anzali	-	68.04	73.4	77.76	92.88	115.58	126.11	133.06
	Jyväsjärvi	-	-	-	73.6	75.7	83.9	-	107.45

Wetland, Tajan River and Jyväsjärvi Lake are shown in Table 2. The age of individuals from Iran and Finland ranged 1-8 and 4-8 years, respectively. The largest mussel was recorded from Anzali with a length of 134.32 cm (8 years old). The mean length of the specimens from Anzali Wetland was significantly higher than those of Tajan and Jyväsjärvi ($P \leq 0.05$). No significant difference was observed in morphometric characteristics of the mussels from Tajan and Jyväsjärvi ($P > 0.05$). In the length-age relationships, Finland bivalves exhibited lower length at a given age than Iranian specimens (Table 3).

The convexity index ranged between 27.49 and 3735.78 for Tajen samples (mean CI: 32.57), 29.48 and 37.57 for Anzali samples (mean CI: 32.81) and 29.96 and 38 for Jyväsjärvi (mean CI: 33.39). The elongation index also ranged from 42.08 to 67.27 for Tajan individuals (mean EI: 49.22), 41.63 to 59.57 for Anzali individuals (mean EI: 50.65) and 47.96 to 56.16 for Jyväsjärvi (mean EI: 51.31) (Table 2).

Molecular data: The COI sequences analysis

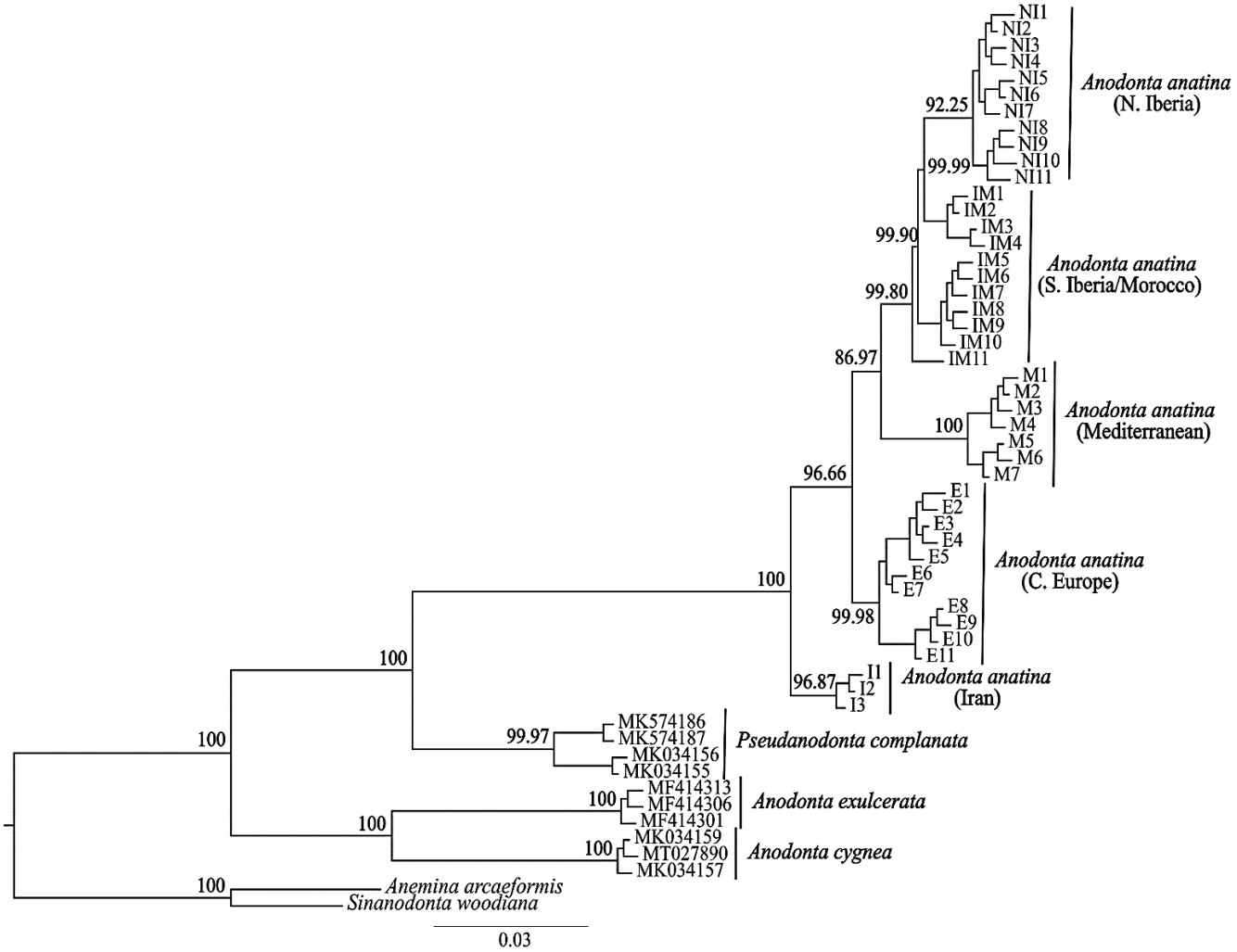

Figure 2. The Bayesian phylogenetic tree on the basis of 53 unique COI sequences of *Anodonta anatina* and related taxa; Two sequences of *Sinanodonta woodiana* and *Anemina arcaeformis* are the outgroups. The numbers above branches represents the bootstrap support values. The scale bar shows the branch lengths.

confirmed the existence of *A. anatina* in the sampling areas. Twelve 705-bp and four 652-bp long fragments of the COI gene were acquired from the Iranian and Finland duck mussel specimens and deposited to the NCBI's GenBank (Table 1). We reconstructed the phylogenetic tree under TIM+I+G model (Fig. 2). The specimens from the studied regions in Iran belonged to three haplotypes, while the samples collected from Finland belonged to two haplotypes. The Finland haplotypes were placed in the same clade as those from Central Europe (Lineage Central Europe). The Iranian haplotypes were also placed in a single clade (Lineage Iran) with strong bootstrap support (100%). Besides these two lineages, there are three more mitochondrial lineages of *A. anatina* (Fig. 2). The mean COI *P*-distances between the *A. anatina* lineages are presented in Table 4. This distance ranged from 1.7 (between the lineages North Iberia and South Iberia/Morocco) to 3.5 (between Iran and the Mediterranean clades). The distance between the lineage comprising the Iranian samples and other lineages ranged from 2.4 to 3.5.

The haplotype network recovered three and two haplotypes for the Iranian and Finland samples (Fig. 3). The haplotypes from Iran were lumped into a single haplogroup separated by 1-2 substitutions. There are four more haplogroups. There was also one mutation site between two haplotypes from Finland. The haplotypes from the Mediterranean group were the most divergent haplogroup from the haplogroups comprising our samples from Iran and Finland.

Table 4. Genetic divergences (mean uncorrected *P*-distance %) among *Anodonta anatina* lineages.

	S. Iberia / Morocco	C. Europe	Mediterranean	N. Iberia
C. Europe	2.1			
Mediterranean	2.8	3.1		
N. Iberia	1.7	2.5	3.4	
Iran	3.0	2.4	3.5	3.1

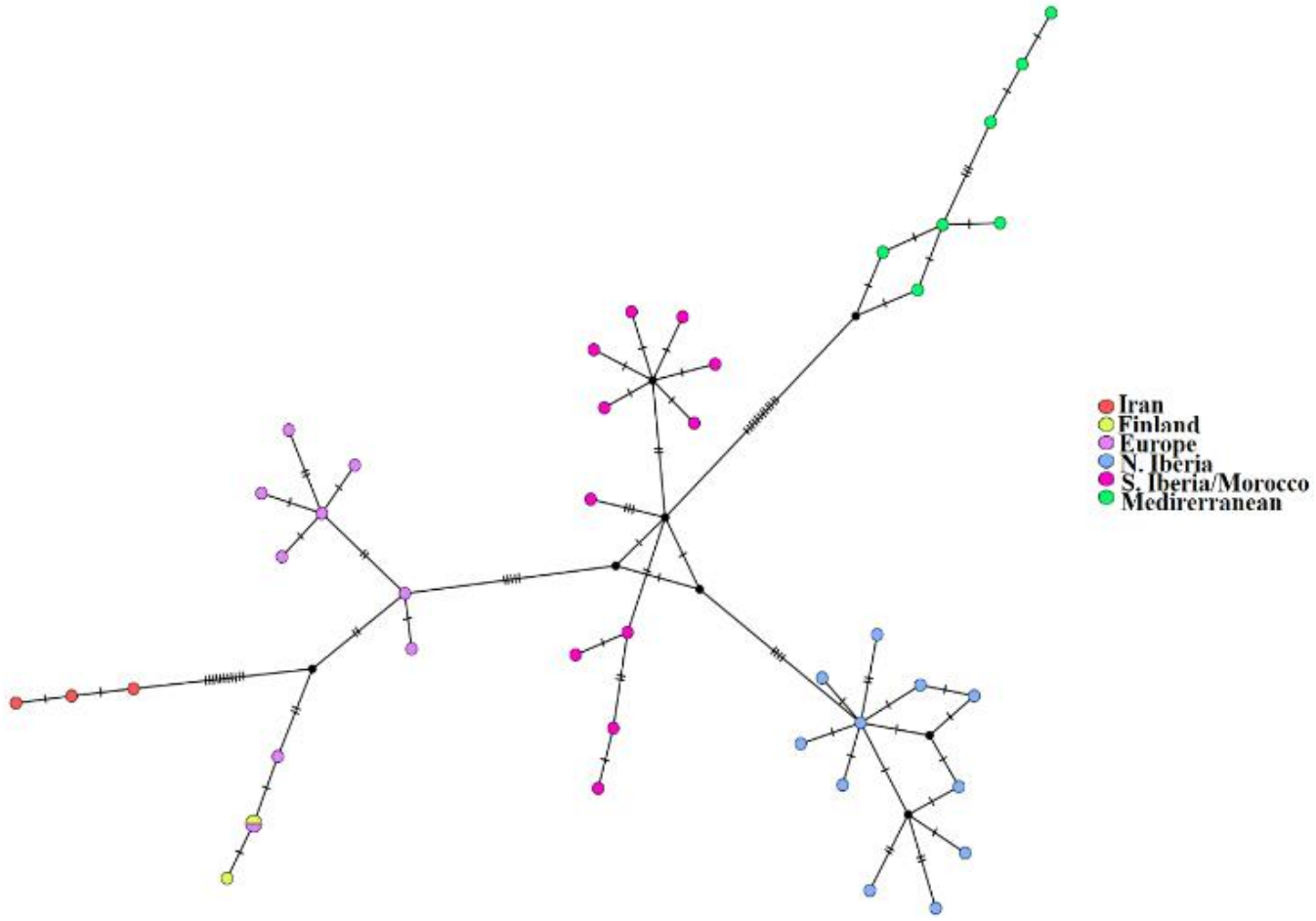

Figure 3. Median joining network for COI sequences of *Anodonta anatina* (N=43). Short lines between the haplotypes indicate the number of mutation sites.

Discussion

Anodontini has consistently been retrieved as a monophyletic group, comprising genera from North America, including *Pseuanodonta* and *Anodonta* spp. (Williams et al., 2017; Riccardi et al., 2019). The genus *Anodonta* comprises *A. californiensis*, *A. kennerlyi*, *A. nuttalliana*, and *A. oregonensis* in North America (Williams et al., 2017), and *A. exulcerata*, *A. cygnea* and *A. anatina* in Europe and some parts in Asia (Graf, 2007; Lopes-Lima et al., 2017). However, the taxonomic status of Unionid bivalves is under discussion due to the high morphological similarity between the cryptic taxa and deficient molecular information (Bolotov et al., 2016; Bespalaya et al., 2018; Riccardi et al., 2019; Kondakov et al., 2020). As reported by Klishko et al. (2018), the difference in shell morphometric variables between *A. anatina* and other *Anodonta* species is weak and cannot support the exact identification. Therefore, due to great plasticity, the differences were even overlooked in most comprehensive classifications, considering

A. anatine and *A. cygnea* as a single species. That is why all nominal taxa in European countries had previously been regarded as *A. cygnea* (Haas, 1969). Furthermore, a recent molecular phylogenetic study has clustered *A. anatina* with *A. cygnea* and *A. nuttalliana* in one monophyletic clade (Araujo et al., 2017). The Anodontini mussel inhabiting the freshwater bodies in the North of Iran has been considered as *A. cygnea* but based on our molecular data, they belong to *A. anatina.*

According to the results, the Iranian and Finland specimens exhibited three and two haplotypes, respectively. The Iranian specimens clustered in separate clade while the Finland haplotypes were placed together with those of Central Europe. Froufe et al. (2014) reported three mitochondrial clades for pan-European freshwater mussels, including Iberia, Europe, Italy, and Ebro, while Riccardi et al. (2019) reported four clades, including N. Iberian, W. Iberian/Moroccan, central European and Mediterranean ones. Tomilova et al. (2020) reported four intraspecific lineages comprising Iberia, Azov, Europe and Italy. In our study, there were five lineages and within each of them, some geographically related haplogroups. However, as low divergences between these lineages (1.7 and 3.5%) and a lack of samples from some areas, we do not decide on their taxonomy. Froufe et al. (2014) reported the highest and lowest divergence between the Iberian with Italian and Europe, respectively. They also demonstrated that the highest and lowest genetic divergence was between Europe with Italy and Azov, respectively. Here, we observed the lowest mean uncorrected COI *P*-distance between the north and south Iberian/Moroccan haplotypes, while the highest difference was between the Iranian and Mediterranean haplotypes. The lineage comprising the Iranian group exhibited the highest and lowest affinity with the lineages Central Europe and Mediterranean, respectively.

Based on the median-joining network, *A. anatina* could be divided into five haplogroups. Consistent with our phylogenetic data, the haplotype network also recovered three and two haplotypes for Iran and Finland individuals, respectively. The specimens from Finland and those from Central Europe are interrelated and comprise a COI haplotype cluster. The Iranian haplotypes are placed in a single cluster. This cluster has a distinct source, likely around the Caspian Sea. However, the Mediterranean was the most distant group from Iran and Finland, while the Central European population was the closest to both.

Different taxa have shown plasticity in phenotype in response to environmental and landscape parameters (e.g., Minton et al., 2008; Inoue et al., 2013; Modestin, 2017). Slow changes in freshwater bivalve's morphology have been reported from upstream to downstream of a river (Graf, 1998; Hornbach et al., 2010). In fact, the shell shape of bivalves can be influenced by their way of life (Alyakrinskaya, 2005). According to Selin (2007), a convexity index more than 0.5 shows the shell is convex, and the lower the elongation index (less than 0.9), the more the mussel is stretched; otherwise, it is truncated. The mean CI of our samples were 32.57, 32.81, and 33.39 for Anzali, Tajan and Jyväsjärvi, respectively and none of the individuals had CI more than 0.5. The mean EI of our samples was low (49.23, 50.65, and 33.39 for Anzali, Tajan and Jyväsjarvi, respectively). No significant EI and CI differences were observed among the Iranian and Finland specimens. The substrate characteristics can significantly influence the CI and EI indices (Modestin, 2017). In both sampling sites in Iran and lake Jyväsjärvi in Finland, the beds were dominated by fine sandy mud and the shells of the samples were stretched and slightly compact. This is in accordance with the results of Modestin (2017), who reported that *Lucina pectinata* was more stretched in fine sandy mud beds compared to the coarse sand and sandy mud hardened by the mangrove tree roots.

Although the *A. anatina* individuals did not display diversity in the shell shape and colour based on their region, the samples from Finland exhibited lower shell length at a given age compared to the Iranian ones. Among the Iranian samples, the Anzali individuals were bigger than the Tajan ones at a given age. Similar to the present study, such a

different length growth at a given age had previously been reported by Girgibo (2013) for *A. anatina* populations in different lakes, Koijarvi and Paijanne, in Finland. This could be related to the suitability of the habitat, especially in terms of food availability (Girgibo, 2013).

Although the morphology is usually applied to identify freshwater bivalves, this could be strongly variable based on habitat properties. The Anodontini species had been considered morphologically as *A. cygnea* in Iran and our data identified it as *A. anatina* based on molecular data. A combination of molecular and morphological data is applied to study the plasticity of freshwater bivalve's phenotype (Zieritz et al., 2010). However, our results did not indicate evidence for significant variation in shell morphology in the studied regions. In this regard, the conventional linear measurements can be supplemented using more complex geometric morphology in further studies.

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