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Phylogenetic relationships of *Atherina hepsetus* and *Atherina boyeri* (Pisces: Atherinidae) populations from Greece, based on mtDNA sequences

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The genetic divergence and the phylogenetic relationships of six *Atherina boyeri* (freshwater and marine origin) and five *Atherina hepsetus* populations from Greece were investigated using partial sequence analysis of 12s rRNA, 16s rRNA and control region mtDNA segments. Three different well divergent groups were revealed; the first one includes *A. boyeri* populations living in the sea, the second includes *A. boyeri* populations living in the lakes and lagoons whereas the third one includes all *A. hepsetus* populations. Fifty-seven different haplotypes were detected among the populations studied. In all three mtDNA segments examined, sequence analysis revealed the existence of fixed haplotypic differences discriminating *A. boyeri* populations inhabiting the lagoon and the lakes from both the coastal *A. boyeri* and the *A. hepsetus* populations. The genetic divergence values estimated between coastal (marine) *A. boyeri* populations and those living in the lagoon and the lakes are of the same order of magnitude as those observed among coastal *A. boyeri* and *A. hepsetus* populations. The results obtained by different phylogenetic methods were identical. The deep sequence divergence with the fixed different haplotypes observed suggests the occurrence of a cryptic or sibling species within *A. boyeri* complex.

ADDITIONAL KEYWORDS: 12srRNA – 16srRNA – D-loop – genetic divergence – lakes – lagoons – marine – sibling species.

INTRODUCTION

The Atherinidae family is represented by a single genus in Europe consisting of three species, *Atherina boyeri* Risso 1810, *Atherina hepsetus* L. 1758 and *Atherina presbyter* Cuvier 1829 (Kiener & Spillman, 1969). *Atherina boyeri* is found over a wide range of temperature and salinity conditions, in coastal, estuarine, lagoon, and inland waters and it is subdivided to local semi-isolated populations that may be quite different in respect to their population biology and morphology (Henderson & Bamber, 1987).

Several studies based on morphometric data revealed differences among *A. boyeri* populations living in the sea compared to those living in the lagoons (Kartas & Trabelsi, 1990; Focant *et al*., 1992; Trabelsi *et al*., 2002a), whereas such differences were also existed between neighbouring marine populations (Trabelsi, Quignard & Kartas, 1994). A high degree of genetic differentiation has also been revealed through protein electrophoresis, among different *A. boyeri* marine populations (Berrebi & Britton-Davidian, 1980; Creech, 1991). Moreover, Focant, Rosecchi & Crivelli (1999) found different electrophoretic patterns sufficient to discriminate the populations living in lagoons from the marine ones. Consequently, *A. boyeri* is viewed by many as a polymorphic complex species. (Focant *et al*., 1999).

In our previous work (Klossa-Kilia *et al*., 2002), high nucleotide divergence values have been detected, through restriction fragment length polymorphism (RFLP) analysis, among *A. boyeri* populations living in

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the sea and those living in lagoons and lakes, leading to the conclusion that there are two different sibling species or at least subspecies or semispecies colonizing 'lagoon' and 'marine' habitats, respectively. The same conclusion was also reached by Trabelsi *et al*. (2002b) using a partial sequence of the mitochondrial cytochrome segment (cyt *b*) and they proposed to include the atherinids living in the lagoons (in France) in a separate new species (*Atherina laguna*), whereas they also distinguished the marine *A. boyeri* populations into two different species *Atherina punctata* and *A. boyeri* (depending on the presence or absent of dark spots along the lateral line, respectively). However, because the proposed taxonomic revision is based (at the molecular level) only on the sequence of a small mtDNA segment, and until this revision be widely accepted, we will still continue to use the current term for *A. boyeri* populations living in the lagoons or the sea. Recently Trabelsi *et al*. (2004) assumed the existence of different taxonomic units among *A. boyeri* populations living in different (and distant) lagoons. High genetic structure among *A. boyeri* populations originated from different lagoons of a wide geographical range has also been shown by Astolfi *et al*. (2005) and Congiu, Rossi & Colombo (2002), although the later authors found no significant differences among populations originated from different lagoons along the coasts of Adriatic Sea (possibly due to migration movements). The high degree of genetic differentiation among different *A. boyeri* populations revealed by all these studies has led to a confusion regarding the taxonomic status of this species, making it obvious that more data are needed to elucidate this problem. Moreover, the literature concerning phylogenetic relationships of *A. boyeri* with the other sympatric species, *A. hepsetus*, which prefer to live in the sea, is limited.

In the present study, we used the partial sequence of three mtDNA segments, a total of 1329 bp from 12s rRNA, 16s rRNA and control region, to investigate the phylogenetic relationships of *A. hepsetus* and *A. boyeri* populations from Greece. The majority of populations analysed were collected from different marine sites, while two populations of *A. boyeri* originated from different freshwater lakes and one from a lagoon. With this experimental approach, we aimed to: (1) explore the phylogenetic relationship among *A. hepsetus* and *A. boyeri* (lagoon, lakes and marine) Greek populations; (2) estimate the magnitude of divergence existing between *A. hepsetus* and *A. boyeri* populations in an attempt to determine the taxonomic status of the 'lagoon' *A. boyeri* populations; (3) ensure the high degree of nucleotide divergence exhibited among *A. boyeri* populations (marine versus lakes and lagoon), using sequencing analysis, in the light of its higher resolution power compared with RFLP analysis; and (4) determine whether there is any important genetic difference between *A. boyeri* populations living in distant isolated lakes.

MATERIAL AND METHODS

SAMPLING

Five *A. hepsetus* populations and six *A. boyeri* ones, were collected from regions located mainly in western Greece (Fig. 1) using traps. Three *A. hepsetus* individuals were examined from each population originating from the coastal areas of Tinos (TIN), Panagopoula (PAN), Monastiraki (MON), and Kiparisi (KIS),

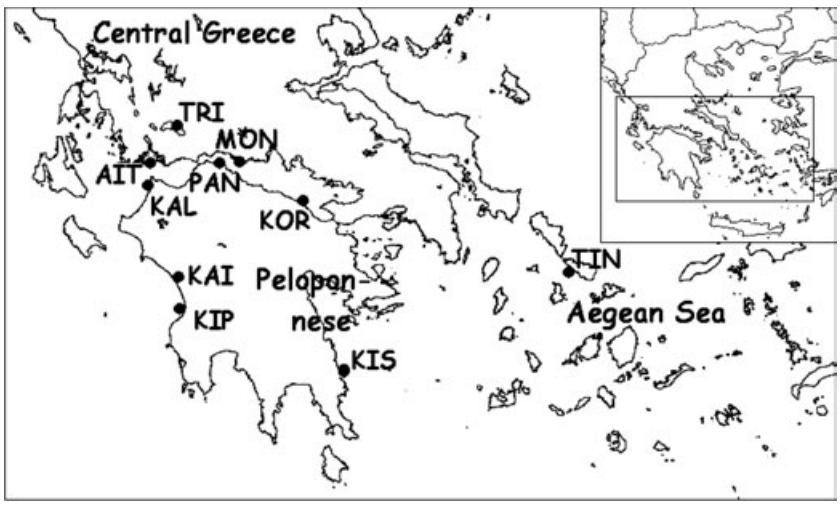

Figure 1. The sampling localities. AIT, Aitoliko lagoon; KAI, Lake Kaiafas; KAL, Kalamaki; KIP, Kiparissia; KIS, Kiparisi; KOR, Korinthiakos; MON, Monastiraki; PAN, Panagopoula; TIN, Tinos; TRI, Lake Trichonida.

Table 1. DNA sequence variability in the 12s rRNA region examined among the eight haplotypes

	11111	1122222222	222333
	22302667	8822235555	578111
	2934120579	4902810123	430269
Ah12s1	ACATGCTTAG	AGTAGAGACC	TAAGTA
Ah12s2	....	C.........	......
Ah12s3	....	....C.....	......
Ab12s1	.A......C.	.ACG..AG.T	AGCACC
Ab12s2	.A......C.	.ACG..AGTT	AGCACC
Ab12s3	.T.C.TCCGA	.ACG.G.GTT	AC.AC.
Ab12s4	GT.CATCCGA	.ACG.G.GTT	AC.AC.
Ab12s5	.TTC.TCCGA	.ACG.G.GTT	AC.AC.

Italics indicate lagoon type haplotypes.

whereas four were examined from Korinthiakos (KOR). *Atherina boyeri* specimens were sampled from the coasts of Kalamaki (KAL), Kiparissia (KIP) and Korinthiakos (KOR), the lakes Trichonida (TRI) and Kaiafas (KAI), and the Aitoliko lagoon (AIT) (Table 1). From each *A. boyeri* population, three individuals were used, except from the populations of Korinthiakos and Aitoliko, where five and four specimens, respectively, were used. All individuals were transferred in dry ice to the laboratory and stored at –75 °C until use.

DNA extraction, polymerase chain reaction (PCR) amplification, and DNA sequencing

Total DNA was isolated from the muscle tissue according to the protocol included in the DNAeasy Tissue kit (Qiagen) and the resulting DNA was examined via agarose electrophoresis.

For the amplification of the three mtDNA regions studied, three sets of primers were used. The universal primers sets 12SAL-12SBH and 16SARL-16SBRH described by Palumbi *et al*. (1991) were used for the 12s rRNA and 16s rRNA segments, respectively, whereas we used the primer set CRA-CRB described by Lee *et al*. (1995) for the control region. PCR amplification included one preliminary denaturation step at 94 °C for 4 min followed by 35 cycles comprising denaturation at 94 °C for 1 min, annealing at 57 °C (12s rRNA)/50 °C (16s rRNA)/51 °C (control region) for 1 min, and primer extention at 72 °C for 1.5 min, ending with a final step at 72 °C for 5 min. The PCR products from the three regions amplified were confirmed via agarose electrophoresis and purified using the QIAquick PCR Purification kit (Qiagen). The light strand sequencing for the three mtDNA regions was performed by MWG Biotech-Germany.

Sequence data analysis

We performed the analysis using each dataset (mtDNA segment) seperately as well as the combination of them. The sequence alignment was performed by Clustal W included in BioEdit, version 5.0.9 (Hall, 1999). Unread nucleotides and gaps were treated as missing data and as the fifth character, respectively. For each dataset, the evolutionary distances (d) were calculated among the 11 populations examined considering the Kimura 2 parameter (Kimura, 1980) model using MEGA, version 3.1 (Kumar, Tamura & Nei, 2004).

The proper substitution models for each dataset and for the combination of them were selected by Modeltest (Posada & Crandall, 1998). Neighbour-joining (NJ) trees (Saitou & Nei, 1987) were generated using MEGA and PAUP* v4.b10 (Swofford, 2002). Maximum likelihood (ML) trees were generated using PAUP* and considering the substitution models selected by Modeltest. Maximum parsimony (MP) analysis was also performed using PAUP*. For the heuristic search, the stepwise addition and the branch swapping options were selected. For each option, a 50% majority rule tree was generated. For all trees plotted, bootstrap values (Felsenstein, 1985) were based on 10 000 pseudoreplicates. The merging of individuals haplotypes to populations in the combined data set was made using McClade, version 4.03 (Maddison & Maddison, 2001). To check the phylogenetic signal of the data the g_1 method (Hillis & Huelsenbeck, 1992) was applied. From the random trees option of PAUP*, 10 million trees were evaluated.

RESULTS

After PCR amplification and sequencing, 351 bp for 12s rRNA, 497 bp for 16s rRNA, and 481 bp for the control region were obtained. For the 12s rRNA segment, 26 variable (21 informative) sites were found. The 16s rRNA segment exhibited 69 variable (57 informative) sites whereas the control region 121 (115 informative) sites, respectively (Tables 1, 2, 3). The *A. hepsetus* individuals were sequenced and resulted in three, ten, and ten different haplotypes for the 12s rRNA, 16s rRNA, and control region, respectively, whereas *A. boyeri* individuals resulted in five, ten, and 19 haplotypes for the same segments, respectively (Table 4). No haplotype was shared between *A. hepsetus* and *A. boyeri* individuals; furthermore, no common haplotype was found between the coastal individuals of *A. boyeri* and those from the lakes and the lagoon. GenBank accession numbers for the 57 haplotypes we obtained are AY749048-AY749104.

The evolutionary distances calculated for the 12s rRNA region revealed that the marine *A. boyeri* pop-

Table 2. DNA sequence variability in the 16s rRNA region examined among the 20 haplotypes; dashes indicate deletions

	1111	1111111111	1111111111	1112222222	2222222222	2222222233	333333334
	345881114	5566677777	8888888999	9990000011	1124455677	7778999901	111234594
	9390386789	4602912368	2346789023	7890356901	3907906302	5898014880	258811448
Ah16s1	TACAACGAGT	AGAATCAACC	-TCCCGGGGT	GGAAAACCCC	TACTACCCCA	GTCACTGTAT	GGTAAAAGT
Ah16s2	....	....	C.........	....	....	....	....
Ah16s3	....	....	TC..G...TA	....	....	....	....
Ah16s4	......A...	....	TC..G...TA	....	....	....	....
Ah16s5	....G.A...	....	CCT..C...G	....	....	....	....
Ah16s6	......A...	....	C.........	....	....	....	....
Ah16s7	....	....	CCT..C...G	....	....	......A...	.......A.
Ah16s8	......A...	....	-.........	....	....	....T.....	....G....
Ah16s9	....	....	-........C	....	....	....	....
Ah16s10	....	....	-.........	....	....	....	....
Ab16s1	C.T..AAC.C	GA.GGT.CT.	---T.A...G	CA..GGTA..	C.TGG.TTTG	ACA.G..CG.	C.C..TG.C
Ab16s2	C.T..AAC.C	GA.GGT.CT.	---T.A...G	CA.GGGTA..	C.TGG.TTTG	ACA.G..CG.	C.C..TG.C
Ab16s3	C.T..AAC.C	GAGGGT.CT.	---T.A...G	CA....TTTT	C.TGG.TTTG	A.A.G..CGG	C.C..TG.C
Ab16s4	C..GGA.GA.	.A.GCTG...	--------.G	..GC-GG...	C..A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s5	C..GGA.GA.	.A.GCTG...	C-------.G	..GC-GG...	C..A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s6	C..GGA.GA.	.A.GCTG...	C------C.G	..GC-GG...	C..A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s7	CG.GGA.GA.	.A.GCTG...	CC-------.G	..GC-GG...	C..A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s8	C..GGA..A.	.A.GCTG..T	CC-......G	..GC-GG...	C..A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s9	C..GGA.GA.	.A.GCTG...	C-------.G	..GC-GG...	CC.A.TT...	.C.T.C.CG.	C.CG.CG..
Ab16s10	C..GGA.GA.	.A.GCTG...	C-----C..G	..GC-GG...	C..A.TT...	.C.T.C.CG.	CACG.CGA.

Italics indicate lagoon type haplotypes.

ulations differed almost equally from both the lagoon *A. boyeri* and the *A. hepsetus* populations, whereas the lagoon *A. boyeri* populations exhibited the greatest distance from the *A. hepsetus* ones. More specifically, *A. hepsetus* populations differed by 4.11–4.30% from the coastal *A. boyeri* ones and 5.38–5.79% from the lake and lagoon populations. *Atherina boyeri* coastal populations differed by 3.70–4.11% from the lake and lagoon ones (Table 5). Analogous differences were also observed for the 16s rRNA as well as for the control region and the combined data set (Tables 6, 7, 8), with the control reagion revealing the most extreme values as expected. In every case, the distance values estimated among *A. boyeri* populations (coastal versus lake and lagoon) are of the same order of magnitude compared with those estimated among the other two well defined species (*A. boyeri* and *A. hepsetus*).

The Akaike Information Theoretical Criterion (AIC) performed by Modeltest for the 12s rRNA haplotypes selected the Kimura 2 parameter model, with equal base frequencies, $Ti/Tv = 2.4513$ and a proportion of invariable sites (I) equaling to 0.8126. The same model was selected for the 16s rRNA haplotypes, with equal base frequencies, $Ti/Tv = 3.4317$ and $I = 0.7552$. The test for the control region haplotypes selected the HKY model with unequal base frequencies, $Ti/Tv = 1.8293$, $\alpha = 0.8486$ and $I = 0.5752$. For the combined dataset, the AIC criterion selected the TrN model with unequal base frequencies, $\alpha = 0.8681$ and $I = 0.6812$.

The sequence data of the four dataset studied revealed a significant phylogenetic signal. The values calculated by the g_1 method was $g_1 = -0.4095$ ($P < 0.001$) for 12s rRNA, $g_1 = -0.5075$ ($P < 0.001$) for 16s rRNA, $g_1 = -0.4233$ ($P < 0.001$) for the control region and $g_1 = -0.4121$ ($P < 0.001$) for the combined data.

For each mtDNA region studied as well as the combined data set, the NJ trees plotted resulted in the same topology. According to this, *A. boyeri* haplotypes are clustered in two groups, one containing the haplotypes from the coastal areas of KAL, KOR, and KIP, and the second containing the haplotypes from the lakes of TRI, KAI, and the AIT lagoon. All the *A. hepsetus* haplotypes were clustered together (Fig. 2). The MP trees plotted for each mtDNA region as well as for the combined data set (Fig. 3) resulted in the same topology, very similar to the topologies of the NJ trees. The same topology was also obtained with the ML analysis. In conclusion, in all distance, parsimony, and likelihood trees plotted, the lake and lagoon haplotypes of *A. boyeri* were clustered together, apart from the coastal *A. boyeri* haplotypes whereas *A. hepsetus* haplotypes were clustered together in a separate clade

Table 3. DNA sequence variability in the control region examined among the 29 haplotypes; dashes indicate deletions

				1111111	1111111111	1111111111	1111111111	1111111112	2222222222	2222222222	2333333333	3333333344	4444444444	4
	1112222223	3333344555	6780000001	1111222222	3333444445	5577888888	8999999990	0022233344	5556677777	8122233333	5566777801	1123444666	6
	2363567890	1235905019	6820345690	1238234589	1467345781	8945123478	9012456797	8903515907	4896702349	0678901348	1545067273	4506345567	8
AbCR1	TAATTTTATC	TACCCCTTCT	CGTTTACCTT	GAAGTACACC	GTGAATTATG	GTAAGTATAG	TAATGAATTC	CAATACTTAA	ACTCTTAACA	ACACTCTATT	CTCGCACCTT	ATATACGGAT	A
AbCR2	....	....	....	A...G.....	.........A	....	....	........G.	........T.	....	.C........	.C.....A..	.
AbCR3	....	....	....	A...G.....	.........A	....	....	....	........T.	....	.C........	.C.....A..	.
AbCR8	....	....	....	....G.....	..A.......	....	....	....	....	....	..A.......	..G....A..	.
AbCR9	....	....	....	....G.....	..A.......	....	....	....	....	....	....	..G.......	.
AbCR10	....	.....T....	....	....	..A.......	....	C.G.......	.....T....	..C.....T.	....	.C........	.C.....A..	.
AbCR13	....	.....T....	....	....	..A.......	....	C.........	.....T....	..C.......	....	.C........	.C.....A..	.
AbCR14	....	....	....	.G........	..A.......	....	....	....	....	....	....	..G....A..	.
AbCR15	....C.....	....	....	....G.....	..A......A	....	....	.....T....	........T.	....	.C........	.C.....A..	.
AbCR16	....	....	.......T..	....G.....	..A.......	....	........A.	....	........T.	....	.C........	.C.....A..	.
AbCR17	....	....	....	....A.....	..A......A	....	....	....	T.......T.	....	.C........	.CG....A..	.
AbCR4	ATC.C.CCC.	CTA.AA....	.TA...AAC.	A.C.A.T.TA	...GTA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR5	ATC.C.CC..	CTA.AA....	.TA...AAC.	A.C.A.T.TA	..A.TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR6	ATC.C.CC..	CTA.AA...C	.TA...AAC.	A.C.A.T.TA	....TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR7	ATC.C.CC..	CTA.AA....	.TA...AAC.	A.C.A.T.TA	....TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR11	ATC.C.CC..	CTA.AAC...	.TA...AAC.	A.C.A.T.TA	..A.TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR12	ATC.C.CC..	CTA.AA....	.TA...AA..	A.C.A.T.TA	....TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	....	.
AbCR18	ATC.C.CC..	CTA.AA....	.TA...AAC.	A.C.A.T.TA	..T.TA...A	....TATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	........T.	.
AbCR19	ATC.C.CC..	CTA.AA....	TTA...AAC.	A.C.A.T.TA	....TA...A	...GTATA.A	CTC....AA.	...GTTCATT	TT.TCCGTAG	..TG.ACCCA	ACA.GT.T..	........T.	.
AhCR1	CTCC.C..AT	...TATCCTC	...CGT.A.C	T..AACACA.	T.T..ACGG.	ACC..-.GCT	AT.C..C..T	TCC..TCA..	..C.....TC	TT..GT....	..TTT...GA	CC.CCGCT.A	T
AhCR2	CTCC.C..AT	...GATCCTC	...C.C.A.C	T.G.ACACA.	T.T..A.GG.	ACT..-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	T...GT....	..TTT.T.GA	CC.CCGCT.A	T
AhCR3	CTCC.C..AT	...AATCCTC	...C.C.A.C	T.G.ACACA.	T.T..A.GG.	ACC..-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	T...GT....	..TTT...GA	CC.CCGCT.A	T
AhCR4	CTCC.C..AT	...AATCCTC	...C.C.A.C	T..AACACA.	T.A..A.GG.	ACC.A-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	T...GT....	..TTT...GA	CC.CCGCT.A	T
AhCR5	CTCC.C..AT	...AATCCTC	...C.C.A.C	C...ACACA.	T.A..A.GG.	ACC..-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	T...GT....	..TTT...GA	CC..CGCT.A	T
AhCR6	CTCC.C..AT	...AATCCTC	...CGT.A.C	T..AACACA.	T.T..ACGG.	ACC..-.GCT	AT.C..C..T	TCC..TCA..	..C.....TC	TT..GT....	..TTT...GA	CC.CCGCT.A	T
AhCR7	CTCC.C..AT	...AATCCTC	T..C...A.C	C...ACACA.	T.A..A.GG.	ACC..-.GCT	AT.CAGC..T	TCC..TCA..	..C.....TC	T...GT....	..TTT...GA	CC.CCGCT.A	T
AhCR8	CTCC.C..AT	...AATCCTC	...CCC.A..	T...ACACA.	TCA..A.GG.	ACT.A-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	T...GT....	..TTT...GA	CC.CCGCT.A	T
AhCR9	CTCC.C..AT	...AATCCTC	...C.C.A.C	T.GAACACA.	T.A..A.GG.	ACC..-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	CT..GT....	..TTT...GA	CC.CCGCT.A	T
AhCR10	CTCC.C..AT	...AATCCTC	T..C.C.A.C	T..AACACA.	T.A..A.GG.	ACC..-.GCT	AT.CA.C..T	TCC..TCA..	..C.....TC	C...GT....	..TTT...GA	CC.CCGCT.A	T

Italics indicate lagoon type haplotypes.

Table 4. Sampling size and haplotype distribution per locality for the *Atherina boyeri* and *Atherina hepsetus* populations examined

	12s					16s										Control																	
Sampling locality*	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Atherina boyeri haplotypes																																		
Kalamaki (3)	2	1										2	1			1	1	1																
Korinthiakos (5)	5													3	2													1	1	1	1	1		
Kiparissia (3)	2	1							1	1	1												1	1	1									
Aitoliko (4)			4			4													1	1	1	1												
Trichonida (3)			3			2	1															1				1	1							
Kaiafas (3)			1	1	1	2		1														1										1	1	
Atherina hepsetus haplotypes																																		
Tinos (3)	3					2	1														3												
Panagopoula (3)	3							2	1							1	1	1																
Monastiraki (3)	2	1					2			1									2	1														
Kiparisi (3)	3										1	1	1								1	1	1											
Korinthiakos (4)	3		1								2			1	1						2			1	1								

*Numbers in parentheses indicate the number of specimens examined from each population.

Table 5. Genetic distances (%) (below the diagonal) and standard errors (%) (above the diagonal) calculated for 12s rRNA between the populations examined, considering the Kimura 2 parameter substitution model

	AhTin	AhPan	AhMon	AhKis	AhKor	*AbTri*	*AbKai*	*AbAit*	AbKor	AbKal	AbKip
AhTin		0.00	0.09	0.00	0.09	1.26	1.28	1.26	1.04	1.05	1.05
AhPan	0.00		0.09	0.00	0.09	1.26	1.28	1.26	1.04	1.05	1.05
AhMon	0.10	0.10		0.09	0.13	1.27	1.29	1.27	1.04	1.05	1.05
AhKis	0.00	0.00	0.10		0.09	1.26	1.28	1.26	1.04	1.05	1.05
AhKor	0.10	0.10	0.19	0.10		1.27	1.29	1.27	1.04	1.05	1.05
AbTri	5.38	5.38	5.48	5.38	5.48		0.16	0.00	1.07	1.05	1.05
AbKai	5.69	5.69	5.79	5.69	5.79	0.29		0.16	1.09	1.07	1.07
AbAit	5.38	5.38	5.48	5.38	5.48	0.00	0.29		1.07	1.05	1.05
AbKor	4.11	4.11	4.21	4.11	4.21	3.81	4.11	3.81		0.09	0.09
AbKal	4.21	4.21	4.30	4.21	4.30	3.70	4.01	3.71	0.10		0.12
AbKip	4.21	4.21	4.30	4.21	4.30	3.70	4.01	3.71	0.10	0.13	

Table 6. Genetic distances (%) (below the diagonal) and standard errors (%) (above the diagonal) calculated for 16s rRNA between the populations examined, considering the Kimura 2 parameter substitution model

	AhTin	AhPan	AhMon	AhKis	AhKor	*AbTri*	*AbKai*	*AbAit*	AbKor	AbKal	AbKip
AhTin		0.30	0.18	0.20	0.12	1.43	1.42	1.42	1.23	1.22	1.22
AhPan	0.52		0.32	0.33	0.30	1.43	1.42	1.42	1.26	1.25	1.25
AhMon	0.31	0.63		0.21	0.19	1.41	1.39	1.40	1.19	1.19	1.18
AhKis	0.49	0.81	0.60		0.19	1.41	1.39	1.40	1.24	1.24	1.23
AhKor	0.16	0.52	0.34	0.49		1.41	1.40	1.41	1.24	1.23	1.23
AbTri	8.58	8.70	8.46	8.58	8.46		0.21	0.10	1.26	1.28	1.26
AbKai	8.62	8.74	8.50	8.62	8.50	0.60		0.17	1.29	1.31	1.29
AbAit	8.46	8.58	8.34	8.46	8.34	0.10	0.49		1.26	1.28	1.26
AbKor	6.94	7.30	6.83	7.26	7.06	7.63	8.12	7.63		0.25	0.17
AbKal	6.71	7.07	6.60	7.11	6.83	7.52	8.00	7.52	0.63		0.18
AbKip	6.60	6.95	6.48	6.99	6.71	7.28	7.76	7.28	0.31	0.31	

Table 7. Genetic distances (%) (below the diagonal) and standard errors (%) (above the diagonal) calculated for the control region between the populations examined, considering the Kimura 2 parameter substitution model

	AhTin	AhPan	AhMon	AhKis	AhKor	*AbTri*	*AbKai*	*AbAit*	AbKor	AbKal	AbKip
AhTin		0.42	0.57	0.43	0.35	2.58	2.61	2.59	1.85	1.86	1.82
AhPan	1.28		0.42	0.44	0.39	2.52	2.55	2.54	1.81	1.82	1.79
AhMon	1.63	1.39		0.37	0.33	2.50	2.55	2.51	1.80	1.83	1.78
AhKis	1.49	1.68	1.32		0.41	2.51	2.55	2.53	1.78	1.81	1.77
AhKor	1.06	1.39	1.13	1.56		2.54	2.57	2.55	1.81	1.83	1.79
AbTri	24.34	23.86	23.48	23.89	24.08		0.23	0.15	1.80	1.80	1.82
AbKai	24.83	24.34	24.15	24.44	24.63	0.54		0.22	1.83	1.82	1.85
AbAit	24.53	24.05	23.69	24.10	24.29	0.39	0.54		1.82	1.82	1.83
AbKor	16.31	16.05	15.54	15.88	16.06	14.67	15.10	14.88		0.40	0.37
AbKal	16.40	16.14	15.89	16.15	16.32	14.41	14.75	14.58	1.51		0.46
AbKip	16.23	15.97	15.45	15.80	15.97	14.84	15.27	15.05	1.47	1.68	

Table 8. Genetic distances (%) (below the diagonal) and standard errors (%) (above the diagonal) calculated for the combined data between the populations examined, considering the Kimura 2 parameter substitution model

	AhTin	AhPan	AhMon	AhKis	AhKor	*AbTri*	*AbKai*	*AbAit*	AbKor	AbKal	AbKip
AhTin		0.19	0.21	0.18	0.16	0.94	0.94	0.95	0.88	0.88	0.88
AhPan	0.64		0.19	0.21	0.19	0.93	0.93	0.93	0.88	0.89	0.88
AhMon	0.69	0.74		0.16	0.13	0.91	0.91	0.91	0.85	0.87	0.85
AhKis	0.72	0.91	0.71		0.17	0.91	0.91	0.91	0.86	0.88	0.86
AhKor	0.52	0.73	0.52	0.76		0.92	0.93	0.93	0.87	0.89	0.87
AbTri	13.00	12.91	12.73	12.86	12.89		0.11	0.06	0.78	0.77	0.77
AbKai	13.28	13.19	13.06	13.16	13.18	0.48		0.11	0.80	0.79	0.79
AbAit	13.03	12.94	12.77	12.90	12.92	0.17	0.45		0.79	0.78	0.77
AbKor	9.41	9.45	9.16	9.40	9.37	9.03	9.44	9.10		0.16	0.14
AbKal	9.41	9.44	9.24	9.48	9.44	8.88	9.26	8.94	0.79		0.17
AbKip	9.29	9.32	9.03	9.30	9.25	8.94	9.35	9.01	0.67	0.74	

DISCUSSION

In the present study, we explored the genetic divergence and the phylogenetic relationships among different *A. boyeri* and *A. hepsetus* populations using mtDNA sequence analysis. The existence of three independent evolutionary units has been demonstrated by our data. The first one consists from *A. hepsetus* populations, whereas the *A. boyeri* populations are split into two separate genetic lineages, the first one containing the populations living in the sea and the second those living in the lakes and the lagoon. Genetic differentiation was found to be significantly high among those groups with the values observed between marine *A. boyeri* and *A. boyeri* living in the lagoon and the lakes being in the same order of magnitude as those estimated among the other two well defined species *A. hepsetus* and coastal *A. boyeri*; in any case, these values were found to be analogous to those existing among different species of the same genus (Karaiskou *et al.*, 2003; Infante, Catanese & Manchando, 2004).

The clear genetic differentiation detected among the three groups mentioned above, is also highlighted by all phylogenetic trees constructed in this study. All trees drawn, either by NJ, MP or ML analysis for each of the three mtDNA segments examined, as well as for the combined data set, exhibited almost identical topology. All these methods resolved the same three distinct and deep divergent groups, a situation well supported from the high bootstrap values, reaching up to 100% in the majority of the cases. It must be noted that, our previous study (Klossa-Kilia *et al.*, 2002), where the diversity of *A. boyeri* populations in Greece was tested using RFLP analysis, resulted in the conclusion that there are two different sibling species or at least subspecies or semispecies colonizing 'lagoon' and 'marine' habitats, respectively. Now it is clearer that the deep sequence divergence revealed in the present study, among the three groups tested, in combination with the fixed haplotypic differences found, suggests the occurrence of a different taxon in the *A. boyeri* species complex.

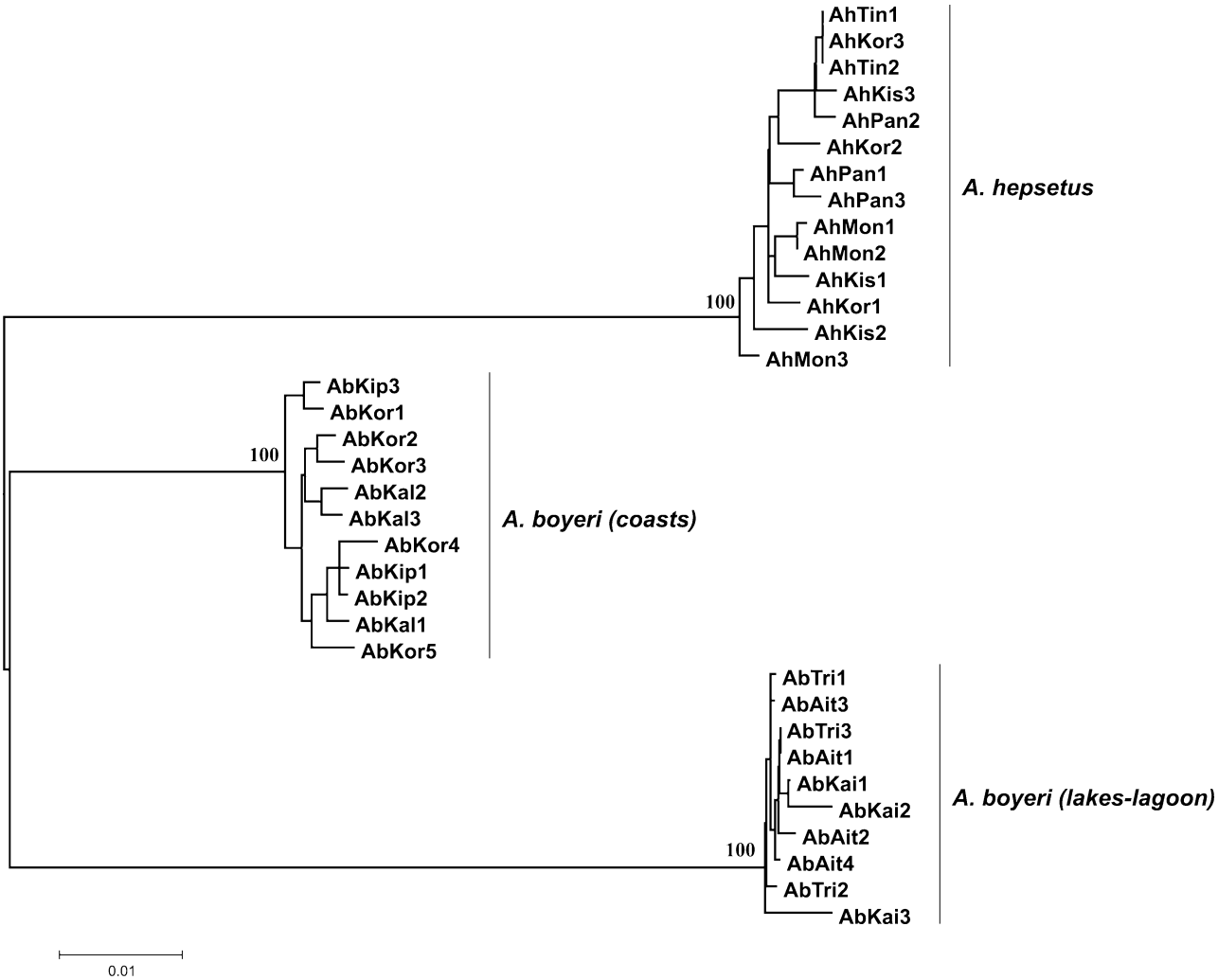

Figure 2. The Neighbour-joining tree of the relationships between the haplotypes for the combined data. The numbers at the tree nodes indicate the bootstrap values from 10 000 pseudoreplicates.

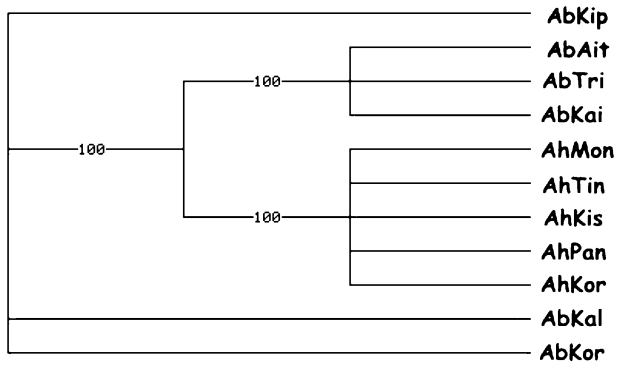

Figure 3. The maximum parsimony tree of the relationships between the populations for the combined data. The numbers at the tree nodes indicate the bootstrap values from 10 000 pseudoreplicates.

Several studies have recently shown that different fish ecotypes have experienced rapid genetic divergence and are, in many cases, on the brink of speciation (Taylor *et al*., 1997; Market *et al*., 1999; Lee, 2000; Staton, Foltz & Felder, 2000; Perdices *et al*., 2001). Significant genetic divergence has also been observed between estuarine and nearby marine populations in the silverside fish *Odontesthes argentinensis*. In this case, the authors assume that the colonization of estuarine habitats has promoted rapid adaptive divergence and reproductive isolation in estuarine populations, which are being considered as incipient ecological species (Beheregaray & Levy, 2000; Beheregaray & Sunnucks, 2001; Beheregaray, Sunnucks & Briscoe, 2002).

A growing body of evidence exists to suggest that estuarine life may promote speciation (Bilton, Paula &

Bishop, 2002). Thus, brackish water ecosystems are often exposed to a wide variation of environmental parameters such as temperature and salinity, which may cause strong selective pressures on organisms (Congiu *et al*., 2002). There is also evidence, pointing in the same direction, that estuarine environments tend to restrict gene flow imposing distinct selective regimes, which contributes to generate physiologically adapted populations, increasing the potential for *in situ* speciation in complete or partial isolation (Bilton *et al*., 2002). According to the same authors, the resulting taxa may be quite divergent from their marine counterparts and represent sibling or cryptic species of truly estuarine origin rather than simply estuarine populations of marine eurytopes. Furthermore, it appears that the phenomenon of specialized estuarine species does exist in a wide range of taxa, which, rather than being populated wholly by mobile marine eurytopes, often harbour endemic species whose recognition may have hampered by the morphological similarity to their fully marine siblings (Bilton *et al*., 2002).

By taking into consideration all of the above, we conclude that *A. boyeri* populations living in the lagoon as well as in the lakes are in essence allopatric compared with those of coastal *A. boyeri* ones. A similar situation has been recognized by many authors (Richardson, Baverstock & Adams, 1986; Allibone *et al*., 1996; McGlashan & Hughes, 2000) and it appears to make the determination of specific status under the biological species concept of Mayr (1942) rather difficult because there is no available evidence regarding the potential to inbreed in sympatry. Although the specific genetic status of the divergent *A. boyeri* 'lagoon' populations is difficult to judge definitively, the situation described, in combination with analogous ones in the literature, can be considered as generating difficulties regarding the biological species concept. However, apart from this theoretical discussion and taking into consideration our clear data, according to which the high genetic distances observed among marine and 'lagoon' *A. boyeri* populations are of the same order of magnitude as those found among the two well defined species *A. hepsetus* and coastal *A. boyeri*, as well as the finding that no haplotypes of marine origin were found among lagoon/lake *A. boyeri* populations, we tend to believe the existence of different species (cryptic or sibling) occupying freshwater environments rather than strongly differentiated populations of the same species. Similar data for *A. boyeri* populations were also provided by Trabelsi *et al*. (2002b).

A great amount of genetic divergence existing among *A. boyeri* populations originating from different (distant) lagoons has been reported by Trabelsi *et al*. (2004) and Astolfi *et al*. (2005). This was not confirmed in our case because the same haplotypes appeared to be present in both lakes that were studied. This situation might be due to recent restocking activities, although there are no data available concerning this. In any case, more studies are needed to elucidate this finding by using more individuals, as well as more populations, from different lakes and lagoons in Greece.

Molecular genetic studies have revealed 'cryptic' taxa existing in several organisms, which exhibit considerable genetic divergence from their closest relatives and show little or no morphological differentiation (Jaarola & Searle, 2004). The existence of cryptic biological diversity has a wide range of implications for both ecological and evolutionary studies as well as for the preservation of biodiversity. Thus, the identification in our study of two discrete evolutionary lineages of *A. boyeri* at the intraspecific level has important implications for the conservation of this species because, from the management point of view, estuarine and marine populations should be treated as individual stocks when formulating fisheries policies (Beheregaray & Sunnuck, 2001).

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