

Some Marine Tunisian *Atherina boyeri* Populations (Teleostei) have Morphological and Molecular Characteristics of Lagoon Fishes

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Abstract: In this study, analyses of 87 biometric parameters and of genetic variation of the *cytochrome b* gene show that *A. boyeri* populations from shallow waters of Tunisian Islands (Kerkennah) belong, in spite their marine habitat, to the lagoonal group. Moreover, in all the phylogenetic analyses, the sequences of these marine atherines constitute with the lagoon ones a clade strongly statistically supported. Moreover, in accordance with ecological rules, vertebral numbers of wild individuals increased with latitude for all the *Atherina* species, but for *A. boyeri* a low decrease of vertebral number in its more septentrional distribution area is suggested. In addition, the lower values are found for species living in particular habitats, rocky sea beds or lagoons for respectively punctated and lagoonal fish, and a rapid decrease of mean vertebral number by latitude has been found for lagoon fish.

Keywords: *Atherina*, Mediterranean Sea, Tunisian islands, Vertebrae variation, Jordan's rule.

INTRODUCTION

In Europe, the family Atherinidae is represented by two genera and six species, i.e., *Atherinomorus lacunosus* Forster and Schneider 1801, a Lessepsian fish migrant, the sand smelt *Atherina hepsetus* Linnaeus 1758, *Atherina presbyter* Cuvier 1829, and three possible species belonging to the *Atherina boyeri* complex Risso 1810 [1-3]. Their ecological and geographic distributions according to Quignard and Pras [2] and Quignard and Tomasini [4] are briefly presented here. The hardyhead silverside *A. lacunosus* is distributed along sandy shorelines and reef margins of Indo-Pacific Oceans and the Red Sea. It is now found on the Mediterranean coasts from Lebanon to Tunisia [3]. *Atherina hepsetus* is a pelagic brackish and marine fish; it is found in Eastern Atlantic (coasts of Spain, Portugal and Morocco including Madeira and the Canary Islands), in the Western Mediterranean and the Adriatic, and more rarely in the Eastern Mediterranean and the Black Sea. *Atherina presbyter* is mainly a pelagic marine inshore fish that only occasionally enters estuaries and coastal lagoons. Its geographical distribution ranges from the British Isles and Southern North Sea to the Canary Islands, Mauritania and Cape Verde, and in the Western Mediterranean Sea. Members of the *A. boyeri* complex are demersal, amphidromous and extremely euryhaline

fish, which inhabit coastal and estuarine waters as well as lagoon shallow brackish and inland waters. A recent study coupling biometric and mitochondrial DNA (mtDNA) data within this species complex, recognized at least three species: *A. boyeri*, *A. punctata* and *A. lagunae* as respectively non-punctuated marine, punctuated marine and lagoon atherines [5,6]. Moreover, molecular investigations by mtDNA [7-11] and allozyme analysis [12] confirmed our previous hypothesis by which *A. boyeri* can be considered as a complex of two different species (one marine and one living in lagoons and river mouths) [5,6], punctuated fish were not present in all these last studies. The distribution of *A. boyeri* species sensu stricto ranges in the Northeast Atlantic from the Azores to the Northwest coasts of Scotland; it is common in the Southern North Sea and the English Channel, less so further north, and being found throughout the Mediterranean and Black Sea. Two subspecies are recognized: *Atherina boyeri pontica* Eichwald 1838 from the Black Sea and *Atherina boyeri caspia* Eichwald 1838 from the Caspian Sea. To date, *A. punctata* has been found only on some rocky sea beds in the Western Mediterranean Sea [13], whereas *A. lagunae* has been found in lagoons along the Mediterranean coasts [10,13]. Within each species of the *A. boyeri* complex, morphological, DNA and protein studies have detected a high degree of differentiation (e.g. [5,6,11-14]). However, the greatest morphological, morphometric and meristic variations have been found in *A. lagunae* populations suggesting allopatric differentiation [15-20]. Recently, we have proposed an evolutionary pattern of the three species of the *A. boyeri* complex [20], including firstly sympatric speciations

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followed by a post-Pleistocene colonization of the lagoons. Similarly, it has been suggested that during the cold periods, fish of the *A. boyeri* complex probably went extinct from the Portuguese coasts and present populations are due to recent recolonizations from western Mediterranean refugia [21]. The presence of *Atherina* populations around the Tunisian Islands gave us the opportunity to obtain new data concerning the evolution of the *A. boyeri* species complex, constituting thus the aim of this study.

MATERIALS AND METHODOLOGY

Biometric Analyses

A total 220 individuals of *Atherina sp.* were collected from three sites off the Kerkennah Islands coasts: El Attaya (34°44'N, 11°18'E), Sidi Fraj (34°41'N, 11°07'E) and Sidi Youssef (34°38'N, 10°58'E) (Fig. 1). All were preserved in 10% formalin. Several morpho-anatomical parameters were examined, i.e., 10 cephalic, 28 body and 40 body-cephalic ratios and 9 meristic parameters (detailed in [20]). For multivariate analyses Canonical Discrimination Analysis (CAN-DISC) was applied [22]. Ordination of individual samples was defined by the variable canonical components taken two by two [23].

Molecular Analyses

Forty individuals of the *A. boyeri* complex have been collected for molecular investigations (Fig. 1 and Table 1). Moreover, we have added to the data set 6 and 2 specimens of respectively *A. presbyter* and *A. hepsetus*, in order to obtain the closer outgroups of the *A. boyeri* complex. As already described in [6], the procedure is only briefly summarized here. Total DNA was extracted from approximately 0.25 cm² of caudal fin and a section of 376 bp of mtDNA genome from the *cytochrome b* (*cyt b*) gene was amplified by polymerase chain reaction (PCR) using published specific primers New-For 5'-AGCCTACGAAAACCCACCC-3' and 34-Rev 5'-AAACTGCAGCCCCTCAGAATGATATTTGT CCTCA-3'. Using the single-stranded DNA as a template, the nucleotide sequence was determined with an automated DNA sequencer (Macrogen, South Korea). Moreover, three *A. presbyter* sequences have been extracted from GenBank and 120 previously obtained sequences of Atherids were used including *A. lagunae*, *A. boyeri*, and *A. punctuata* issuing from French and Tunisian coasts [5,6] (future accession numbers from AM884397 to AM884563). For phylogenetic reconstructions, three methods have been used: Neighbor-Joining algorithm (NJ) applied to the Kimura 2 parameters implemented in the Phylowin software [24], Maximum parsimony (MP) with Phylowin, and Maximum likelihood (ML) using the algorithm of Guindon and Gascuel [25] implemented in the Phylml software. The model of molecular evolution used for reconstruction was chosen with the Modeltest software using the FindModel website at Los Alamos National Laboratory (<http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html>). This allowed us to choose among 28 nucleotide models with the Akaike Information Criteria. The chosen model was HKY [26] with Gamma distribution of rate variation among sites. The parameter of the Gamma distribution as well as the base frequencies were estimated by the software. The robustness of the tree has been tested by bootstrap analyses with 1000 resamplings.

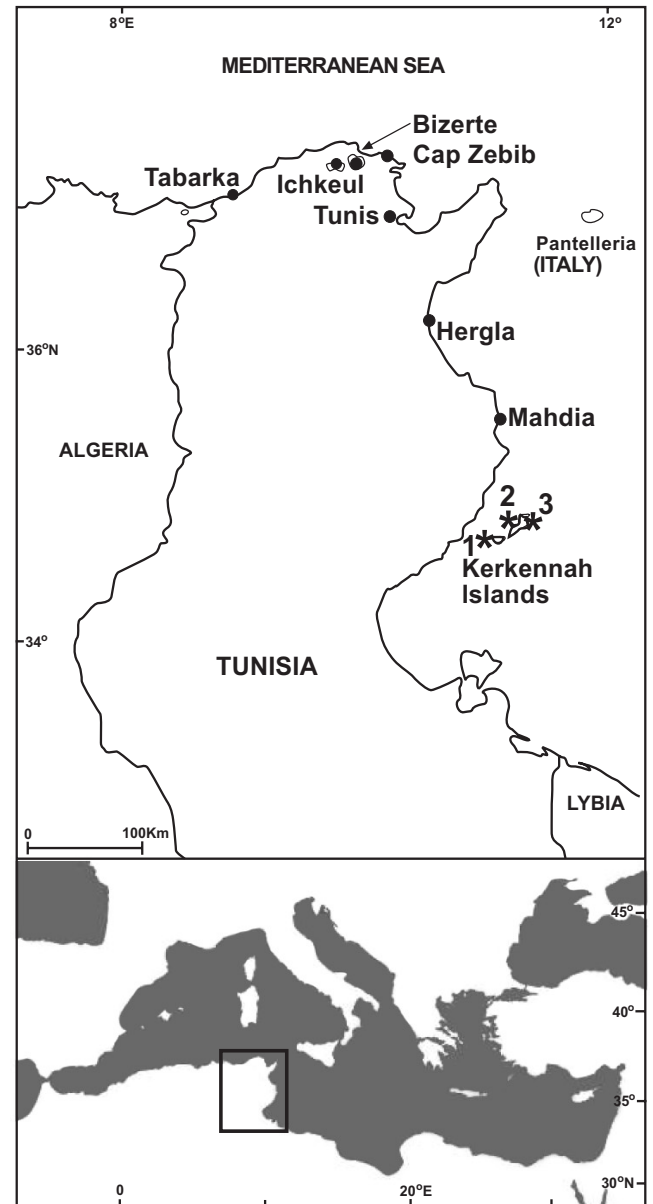


Fig. (1). Geographical distribution of *Atherina sp.* sampling sites in Tunisia. The sampling sites of Kerkennah Islands (1, Sidi Fraj; 2, Sidi Youssef; 3, El Attaya) are indicated by an asterix. The other sampling sites correspond to previous studies [5,6,13,20].

RESULTS

Biometric Analyses

Surprisingly, six out of the 9 meristic parameters analyzed (number of upper, lower and total gillrakers, number of lateral line scales, vertebrae, pectoral fin rays) of the Kerkennah fish are closer to those of lagoon sand smelts (i.e. from France: Biguglia, Camargue, Manguio and Thau, and Tunisia: Bizerte, Ichkeul and Tunis, see Fig. 1) than those of other marine atherines (*A. boyeri* and *A. punctuata*). Moreover, the closest lagoon population is those of Tunis; however, it is possible to distinguish Tunisian island sand smelts from those from islands by the higher number of lower and

Table 1. Data Concerning the Various Sequences Use for Phylogenetic Analyses

Ocean/Sea		Atlantic Ocean			Western Mediterranean Sea	
Country		Spain		France		France
Habitat		Marine			Marine	
Species	Location	Canary Islands	Madeira Archipelago	Roscoff	Arcachon Basin	Gulf of Lion, Sete
<i>A. presbyter</i>		APrAC 1seq DQ197925	APrAM 1seq (2seq) EF439188, EF439189	AprR4 1seq (4seq)	AprAR 1seq (2seq)	
<i>A. hepsetus</i>						Ahe21 1seq (2seq)

Sea		Western Mediterranean Sea								
Country		France			Tunisia					
Habitat		Marine		Lagoon	Marine		Lagoon	Islands coasts		
Species		Gulf of Lion: Thau Corsica: Scandola		Gulf of Lion: Agde Corsica: Lavezzi Scandola	Gulf of Lion: Ayrolle, Maughio Corsica: Biguglia	Zebib Cape, Hergla, Monastir	Zebib Cape, Hergla	Tunis Lake	Kerkennah	
								Sidi Youssef	Sidi Fraj	El Attaya
<i>A. boyeri</i>		11seq (28seq)				9seq (28seq)				
<i>A. punctuata</i>		12seq (15seq)				7seq (9seq)				
<i>A. lagunae</i>				15seq (39seq)			1seq (12seq)	TKSY 5seq (11seq)	TKSF 7seq (12seq)	TKEA 6seq (10seq)

Accession numbers are given for sequences which have not been sequenced in our laboratory (*A. presbyter*). Other accession numbers are in GenBank. Except for island fish, data concerning sequences of the *A. boyeri* complex are in our previous studies [5,20]. For each groups, the number of sequences (seq) uses for phylogenetic analyses (when identical sequences have been found, only one sequence has been used in the dataset) are indicated; whereas, the total number of sequences is in brackets.

total gillrakers, lower number of lateral line scales, vertebrae, pectorals and first dorsal fin rays. Moreover, 71 out of 78 biometric ratios grouped island fish with lagoon sand smelts, whereas only 7 biometric parameters separated them (eye-inside/postorbital length, eye/head, eye/postorbital length, eye-inside/eye, preorbital length/eye, preorbital length/head and postorbital length/head). The island fish are characterized by high values for the first three indices and low values for the last four. Only, three biometric parameters clearly separate island fish from all the other atherines (eye/head, eye/postorbital length and preorbital length/eye).

The statistical multivariate analysis performed in this study is Canonical Discriminant Analysis. The results of the definition of the first three axes of the discriminative canonical analysis by meristic and metric parameters are shown in Fig. (2). The first three axes of the canonical discriminant analysis explained 69.7% of the total variation. The first component (axis 1) with 32.5% total inertia was defined by 25 parameters, among which 14 were positively and 11 negatively positioned. Axis 2 (18.0%) was correlated with 60 parameters among which 40 contributed positively and 20 negatively along with this component (Fig. 2A). Axis 3 (11.6%) was defined by 2 positive parameters (Fig. 2B).

The projection of canonical variables on the plane defined by axes 1-2 (Fig. 2A) draws clear distinction between island atherids, *A. punctuata*, *A. boyeri* and *A. lagunae*. Ordination of axes 1-3 (Fig. 2B) helps to distinguish clearly between *A. boyeri*, *A. punctuata* from Hergla (see Fig. 1) and a group constituted with island and lagoon fish plus two other populations of *A. punctuata*. With respect to remaining

atherinid groups, island fish have the peculiarity of relatively higher individual values of parameters that define the negative parts of axis 1 and 3. Moreover, in the two projections, island fish constitute a relatively well individualized group, although relatively close to Tunisian lagoon atherines.

Variation of Average Vertebrae Number

Relationship between the mean vertebrae number bibliographical data and latitude (Table 2) for the five *Atherina* European species are shown in Fig. (3). The data for *A. hepsetus* are inconclusive since there are only 4 populations in a restricted latitudinal area. The four other species exhibit a general increase in the number of vertebrae from the south to the north. However, differences exist. The average for *A. presbyter* and *A. punctuata* increases slightly with the latitude. For *A. boyeri*, in spite of irregular latitudinal sampling areas [1,13,27,28], the curve suggests that the maximum of average vertebrae count is around 43°-45°N with a slightly decrease at northerly and southerly latitudes. Only two values suggest the Northern decrease, but as they concern fish caught in the Irish Sea and off the Netherland they can be retained. As already suggested by Trabelsi [13], our vertebrae analyses would confirm that populations of the *A. boyeri* complex from Morocco and Menton (France) could be punctuated fish. Our analysis also suggests that fish of the Marmara Sea are *A. punctuata*, this agrees with a photography of one of these fish [29], where a line of black spot is seen below the silvery.

The variation of the vertebral average indicates a relatively complex admixture for *A. lagunae*, with moreover a

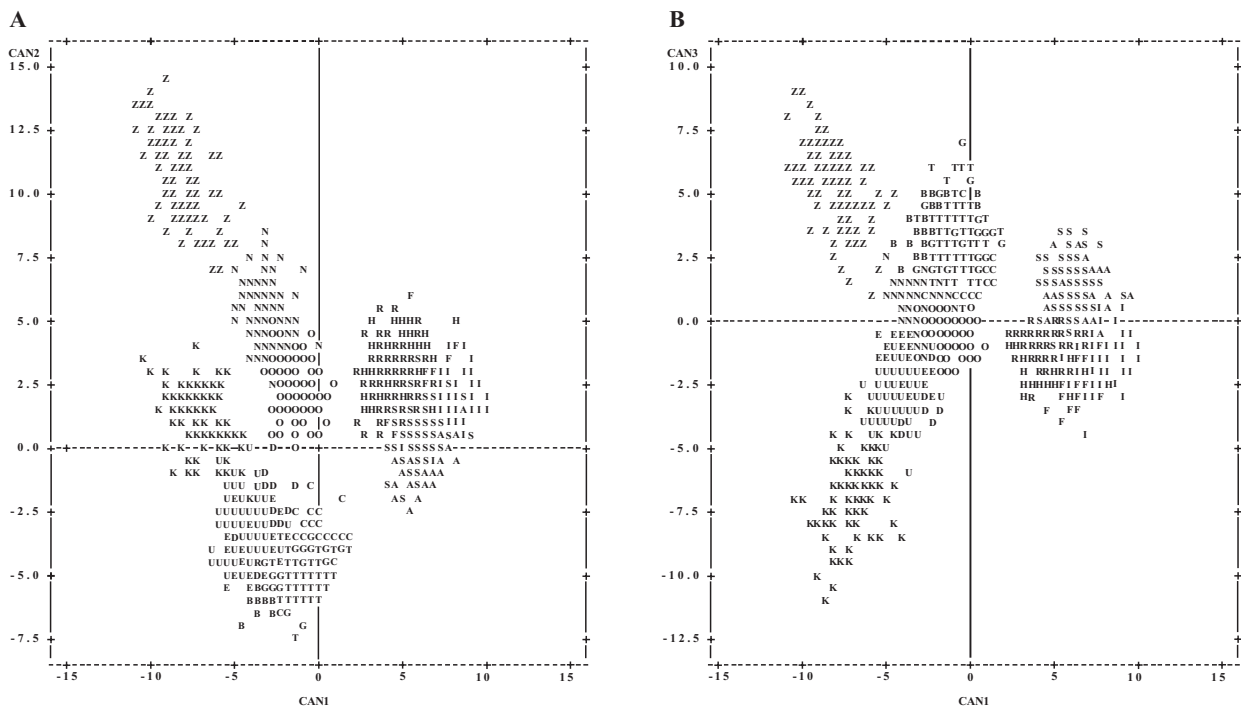


Fig. (2). Graphical representation of point individuals in discriminative canonical analysis of meristic and metric parameters, **(A)** as component 1 (axis 1) and 2 (axis 2), **(B)** as component 1 (axis 1) and 3 (axis 3). Island atherids from Kerkennah Islands (K); *Atherina lagunae* from France: Biguglia (B), Camargue (C), Mauguio (G) and Thau (T), and Tunisia: Bizerte (B), Ichkeul (E) and Tunis (U); *A. punctuata* from France: Pinarello (N) and Lavezzi (O) and Tunisia: Zebib Cape (Z); *A. boyeri* from France: Palavas (A), Pinarello (I), Sete (S) and Tunisia: Hergla (H), Mahdia (F) and Tabarka (R). Complementary data are in [13].

rapid latitudinal variation trend. The low average value of Kerkennah fish is due to the low latitude of these islands and, at least according to this meristic character, island fish could belong to *A. lagunae*. Similarly, as already suggested by Trabelsi [13], Israeli fish from the Alexander river mouth [1] also belong to this species. The *A. boyeri* population from the freshwater Iznik Lake (Turkey) has been introduced at an unknown time [30]. According to Kosswig [31], this population has a Mediterranean origin, which is in agreement with its position in Fig. (3) (a Greek or Turkish origin could be hypothesized owing to the vertebral number), this suggests they belong to *A. lagunae*. This low value excludes a Black Sea origin; indeed, *A. boyeri pontica* or even its related form *A. boyeri caspia* from the Caspian Sea have high vertebral average value, generally around 46 (see [1] and Iranian fish value in Table 2). Moreover, owing to its vertebral value, *Atherina lacustris* Bonaparte 1831 could belong to the *lagunae* group.

In addition, one vertebral value concerning Iranian *A. boyeri* from the Caspian Sea [32] has been removed from the dataset for the construction of Fig. (3). These fish present a average value of 46.49 which is in agreement with those found for the subspecies of this area *A. boyeri caspia* (45-48) [33], and also for fish with a Black Sea origin, *A. boyeri pontica*. This also corresponds to those of *Atherina mochon* Cuvier 1829 (now synonymous of *A. boyeri*) of the Black and Caspian Sea ([1] and references therein). Moreover, according to Kiener and Spillmann [1], some large *Atherina* from the Caspian and Aral Seas are *A. boyeri* with characteristics “approaching *A. presbyter*”, indeed a Moroccan *A.*

presbyter population living at about the same latitude as those of the south of the Caspian Sea has a vertebral value of 47.5 (Table 2).

Molecular Analyses

The partial sequence (376 pb) of the mitochondrial *cyt b* gene for 131 French and Tunisian *Atherina boyeri* complex individuals including *A. lagunae* has been previously determined [6,20]. For this study, 23 sequences from the three stations of the Kerkennah Islands have been obtained. Preliminary analyses demonstrated that 121 out of the 361 positions were variable, whereas 97 were informative for unweighted parsimony. Molecular analyses using three reconstruction methods (MP, ML and NJ) gave the same general topology with the same main groups (Fig. 4); only the topology of the ML analysis has been shown with bootstrap results of the three methods. Bootstrap values (BP) were generally good for inner nodes, thereby supporting the main clades.

In our molecular analyses, when *A. hepsetus* and *A. presbyter* sequences are used as outgroups, species of the *A. boyeri* complex form a clade supported by high bootstrap (BP) values (100% in ML, NJ and MP). Surprisingly, in these analyses, *A. presbyter* could not be monophyletic. Sequences of Atlantic fish from Canary Islands and Madeira cluster with one sequence of Mediterranean *A. hepsetus*. These last sequences are the sister group of those of *A. presbyter* from Arcachon and Roscoff (Atlantic French coasts).

The topologies of the sequences of the *A. boyeri* complex are similar to those already published [5,6,14]. The species

Table 2. Average Vertebrae Number and Approximative Degree of Latitude North for Several Populations of Atherina Family

<i>A. boyeri</i>				<i>A. hepsetus</i>			
Sample site	n	Lat. N	Mean	Sample site	n	Lat. N	Mean
Maarn Veerse meer, Rhine (N) [1]	10	52°04'	45.1	Menton (F) [1]	10	43°50'	55.1
Oldbury, Uskmouth and Somerset (UK) [26]	39	51.63'	45.2	Marseilles Islands (F) [1]	20	43°15'	55.2
Chioggia, Venice Lagoon (I) [25]	842	45°13'	45.9	Banuyls (F) [1]	10	42°46'	55.2
Lagoons of Liguria (I) [1]	10	44°18'	45.4	Bastia (F) [1]	10	42°42'	53.8
Palavas-les-Flots (F) [12]	100	43°31'	45.78	<i>A. punctuata</i>			
Sète (F) [12]	100	43°24'	45.81	Sample site	n	Lat. N	Mean
Marseille (F) [1]	10	43°16'	45.9	Menton (F) [1]	10	43°50'	43.5
Bastia (F) [1]	20	42°42'	44.9	Pinarello (F) [12]	100	41°40'	43.13
Pinarello (F) [12]	100	41°40'	45.39	Lavezzi Islands (F) [12]	278	41°20'	43.21
Caspian Sea (Ir) [30]	34	37°	46.59	Marmara Sea (Tk) [27]	69	41°	43.38
Tabarka (T) [12]	100	36°57'	45.41	Zebib Cape (T) [12]	100	37°15'	43.12
Hergla (T) [12]	100	36°10'	45.23	Monastir (T) [12]	100	35°40'	42.90
Monastir (T) [12]	650	35°40'	45.14	Maroc (Atlantic coasts) [1]	5	34°	42.2
Madhia (T) [12]	309	35°30'	45.24	<i>A. lagunae</i>			
<i>A. presbyter</i>				<i>A. lagunae</i>			
Sample site	n	Lat. N	Mean	Sample site	n	Lat. N	Mean
Oban and Blyth (UK) [26]	5	55°11'	51.9	Mauguio Lagoon (F) [12]	200	43°35'	44.54
Isle of Man (UK) [26]	10	54°14'	50.2	Camargue lagoon (F) [12]	100	43°34'	44.53
Maarn Veerse meer, Rhine (N) [1]	20	52°04'	50.5	Olivier Lagoon (F) [1]	20	43°32'	44.2
Burnham and Kingsnorth (UK) [26]	8	51°32'	49.8	Vic Lagoon (F) [1]	10	43°30'	44.0
Lough Hyne (E) [26]	7	51°29'	49.1	Berre Lagoon (F) [1]	10	43°27'	44.8
Gravelines (F) [26]	15	50°59'	49.5	Thau Lagoon (F) [12]	110	43°25'	44.62
Dungeness in Kent (UK) [26]	10	50°54'	50.3	Bolmon Lagoon (F) [1]	30	43°24'	43.8
Fawley, Southampton Water (UK) [26]	16	50°49'	50.6	Ceinturon Lagoon (F) [1]	10	43°12'	44.8
Fleet, in south Dorset (UK) [26]	15	50°35'	50.3	Biguglia lagoon (F) [12]	110	42°37'	44.47
Salcombe (UK) [26]	10	50°14'	50.0	Canet Lagoon (F) [1]	10	42°36'	43.9
Bretagne and Vendée (F) [1]	16	47°	49.8	Diana Lagoon (F) [1]	10	42°07'	42.4
Arcachon Basin (F) [1]	20	44°39'	49.2	A. lacustris Albano-Nemi (I) [33]	?	41°45'	44
Biarritz (F) [1]	20	43°29'	49.9	Porto Vecchio Salines (F) [1]	10	41°35'	41.3
Maroc (Atlantic coasts) [1]	14	34°	47.5	Iznik Lake (Tk) [27]	103	40°26'	44.42
				Bizerte lagoon (F) [12]	100	37°11'	42.15
				Ichkeul lagoon (F) [12]	326	37°10'	43.05
				Tunis Lake (T) [12]	510	36°50'	41.26
				Kerkennah Islands (T)*	220	34°41'	40.43
				Haïfa, Alexander River (Is) [1]	10	32°49'	41.1

To our knowledge, all the average number of vertebrae for populations for which the number of specimens is greater than or equal than 5 have been summerized. When the number of individuals is slow, when both the values of the vertebrate number and latitude location are closer, some populations have been grouped. Abbreviations and symbols: n, number of specimens; Mean, Mean vertebrae count; *, this study; the initials of the country are in brackets, (F), France; (I), Italy; (Ir), Iran; (Is), Israel; (N) Netherlands; (T), Tunisia; (Tk), Turkey; (UK), United Kingdom.

complex is divided into three clades corresponding to three species [5,6], strongly supported statistically (BP always \geq 99%). Briefly, the *A. boyeri* clade contains sequences from France and Tunisia without distinction of their geographical range. The *A. punctata* clade is divided into two groups, one containing the sequences of Tunisian punctuated fish with strong bootstrap values (ML: 97%, NJ: 98% and MP: 86%)

and the other those of French fish, but only statistically supported using the NJ method (94%). The last group contains all the sequences of lagoon fish added to those of the *A. boyeri* complex of Kerkennah Islands. French lagoon fish constitute the sister group of Tunis Lake and island fish, these two groups are strongly supported (BP from 84 to 100%).

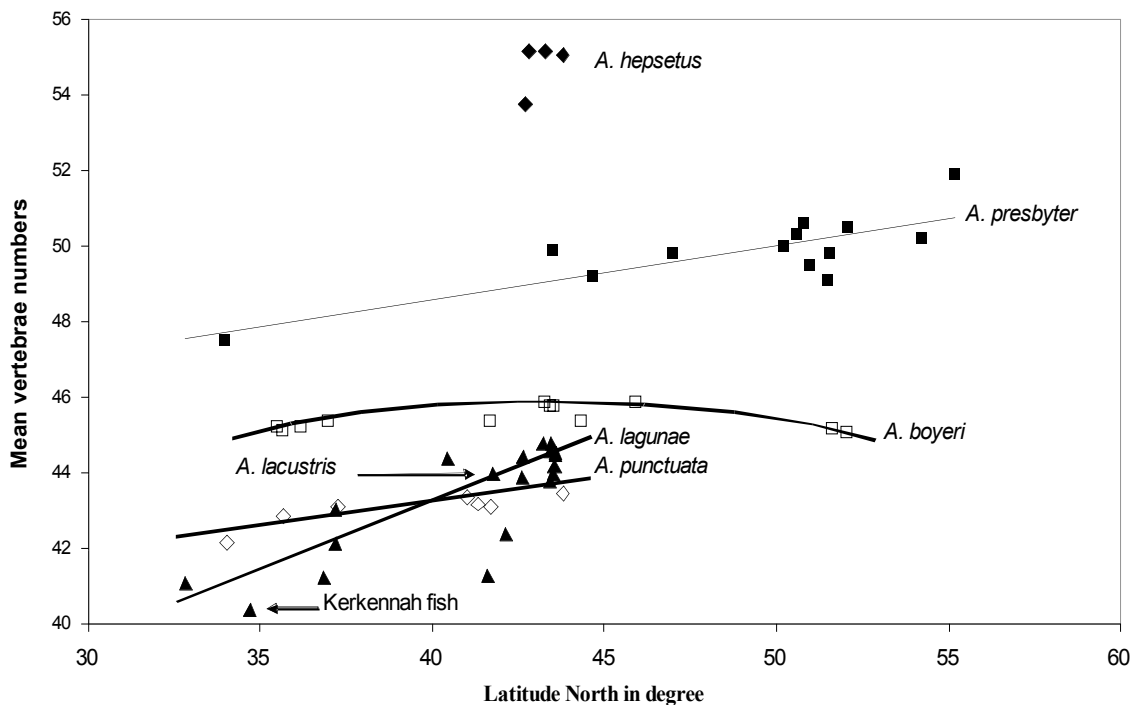


Fig. (3). Variation in vertebral number in *Atherina* genus in relation to latitude of populations. Values are in Table 2.

The most surprising result of this phylogenetic analysis is the grouping of sequences of island fish with those of *A. lagunae* from the Tunis Lake. Twelve individuals from this Lake have been sequenced and all of them have strictly the same sequence. Moreover, the level of genetical divergence of fish from Kerkennah is similar to those observed between respectively, *A. boyeri*, *A. punctata*, or French lagoon fish.

Nomenclature Remarks

Responding to suggestions of Dr Scott Federhen (NCBI), the name *Atherina punctata* Trabelsi 2002 is invalid since *Atherina punctata* Bennet 1833 (in synonymy with *Atherinomorus lacunosus*). Also, *Atherina punctuata* has been proposed as a replacement name. Moreover, on the past, studies of 19th Century naturalists were possibly in agreement with the subdivision of the *A. boyeri* complex into three different species. Indeed, in 1835 Valenciennes [34] had described atherines with black spots, but he did not distinguish this fish from *A. boyeri*. This character has been also described by Bonaparte [35], Moreau [36] and Tortonese [37]. Moreover, *Atherina lacustris* described by Bonaparte [35] from Italian volcanic crater lakes Albano and Nemi now considered as a junior synonym of *A. boyeri* Risso 1810, could correspond to the lagoon species. Table 3 shows that owing to the four meristic characters given by Bonaparte [35], the *A. lacustris* fish would be closer to the French *A. lagunae* fish than to other populations or species. Lastly, the fish identified as *A. lacustris* by Günther [38] is less close to French *A. lagunae* than those described by Bonaparte, and its number of lateral line scales is very high for a fish of the *A. boyeri* complex.

DISCUSSION AND CONCLUSION

Both morphological and molecular analyses suggest that island sand smelts from Kerkennah are *A. lagunae*. However, although the former suggest a close relationships be-

tween island and lagoon individuals especially those of the Tunis Lake, the consideration of 87 meristic and metric parameters of island fish highlights the discriminating value of certain meristic parameters (low numbers of vertebrae and of lateral line scales) and metric (7 ratios previously cited). Moreover, morphological relationships between island fishes and those of inshore continental waters has been already found for *Sardina pilchardus* Walbaum 1792 off the Mallorcan, Corsican, Sardinian and Sicilian coasts [39], *Sardinella aurita* Valenciennes 1847 off the Balearic and Rhodian Islands coasts [40] and *Alosa fallax nilotica* Geoffroy 1827 off the Sardinian Islands coasts [41].

In molecular phylogeny, the island-Tunis Lake clade is supported by high bootstrap values (100% using the three methods). However, within this clade, island fish do not constitute an individualized group versus those of Tunis Lake; in addition, the *cyt b* sequences of fish from Tunis Lake exhibit only three nucleotide differences with one from Kerkennah (TKEA28B). Moreover, these analyses do not allow to differentiate fish from the various sites of the Kerkennah Islands which are distant to each other from 9 to 19 km, indicating gene flow between populations.

In morphological analyses, the level of divergence of island fish is similar to those found in lagoon populations taken individually, contrarily, in molecular analyses, island sand smelts sequences exhibit a remarkable level of divergence between them, which is similar to that observed between French and Tunisian of both *A. boyeri* and *A. punctuata* [6,20].

However, an alternative hypothesis could not be excluded, indeed, Tunis Lake population could be introgressed fish with mtDNA from Kerkennah fish; moreover, it has been passed through a bottle-neck with a severe decrease of the genetic diversity. For *A. lagunae*, island fish which have the

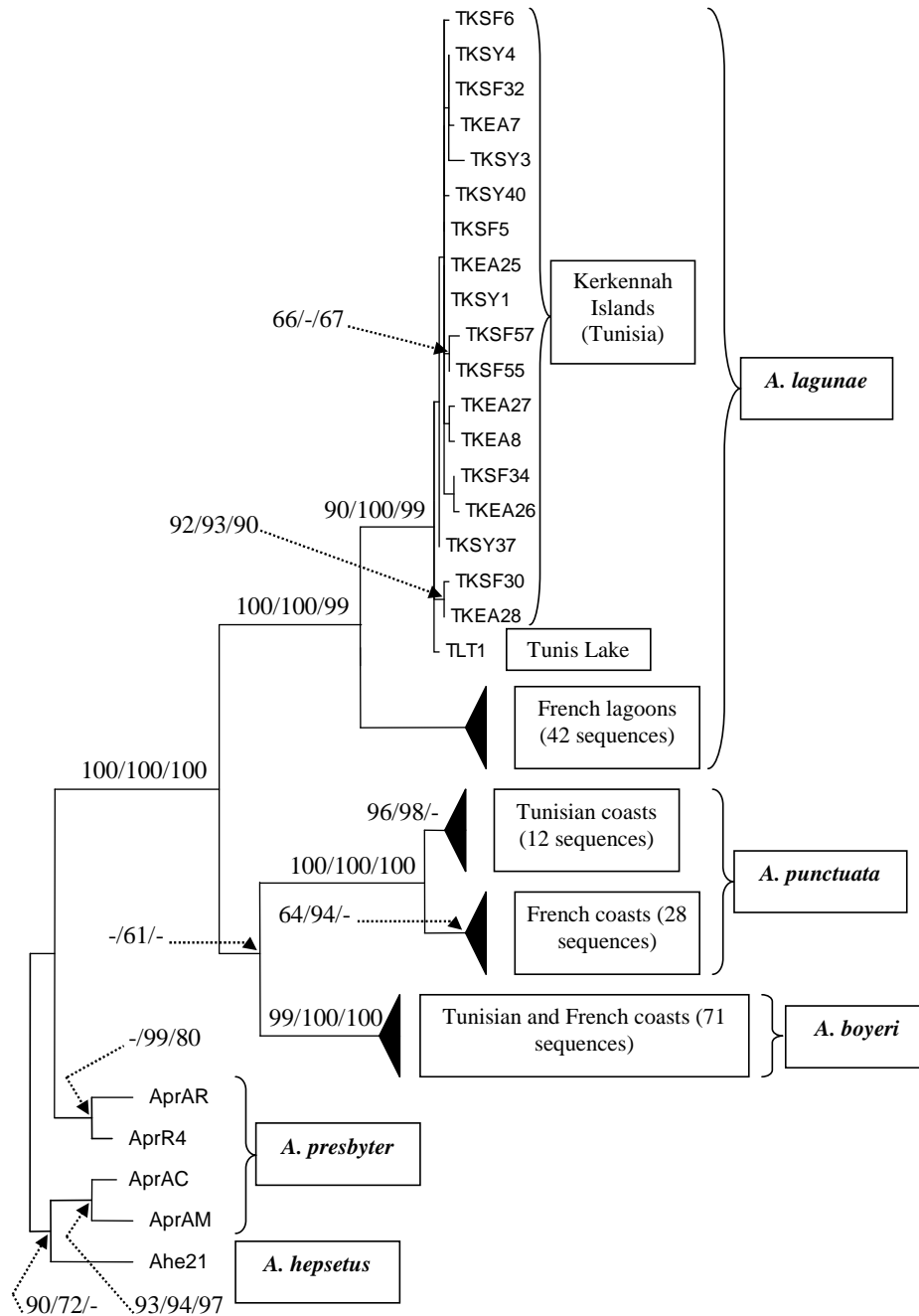


Fig. (4). Phylogenetic tree using the Maximum Likelihood method of *Atherina* species. Sequences of both *A. presbyter* and *A. hepsetus* have been used as outgroups. Bootstrap values (BP in %), carried out with 1000 iterations, are given for each node only if they exceed 60, Maximum Likelihood (left BP), Neighbor Joining (middle BP) and Maximum Parsimony (right BP).

greatest genetic diversity, suggesting thus numerous gene flows, could be the core populations, whereas lagoon fish would constitute the satellite populations. Indeed, satellite populations show a low level of genetic diversity and all the partial *cyt b* regions from the Tunis Lake fish sequenced are strictly identical between them. This last data could suggest that the gene flow from islands to lagoon would be broken; moreover, distances between the Tunis Lake to Kerkennah Islands which are respectively around 400 km could explain the limitation of the gene flow and the possibility of a bottleneck event. However, if gene flow is broken, this induces an increase of the consanguinity and a potential loss of adaptive

abilities facing environmental change. Deleterious effect of consanguinity has also been studied by several authors, who have observed that this favors the build-up of anomalies during development [42,43]. Interestingly, a very high level of spinal deformities has been found in Tunis Lake atherines since $\approx 10\%$ of the fish exhibit severe kyphosis, this could be due, at least in part, to the presence of some deleterious alleles added to recent anthropical physico-chemicals changes [44]. Contrarily, no vertebral deformity has been found in *A. lagunae* from three French lagoons where the level of nucleotide variations can reach 4.3% [20].

Speciation in shallow-water has been shown or strongly suggested for numerous marine organisms including fish and invertebrates (e.g., [45-47]). Differentiation in numerous inshore *A. lagunae* populations could lead to give incipient species, but this induced possible speciation is counterbalanced by the partial homogenization of the populations during the course of time; this homogenisation could be due to the selection of the "better" alleles for a particular lagoon. This scenario could explain the phylogenetic structuration of the island fish which constitute an undifferentiated group with lagoon Tunisian fish. An ecological feature is in favor of this hypothesis for the Kerkennah Islands where the very particular aspect of phanerogames *Posidonia oceanica* fields named "herbier tigré" constitutes a disrupted habitat which facilitates isolation of several micropopulations: strips of several tens meters long separated by one to two meters wide weave beds of *Cymodocea nodosa* and *Caulerpa prolifera*, between 0.5 and 3 m deep [48,49]. Interestingly, a recent study using mtDNA analyses shown evidences that two *Atherina* populations of the *A. boyeri* complex sampled from two islands of the Aegean Sea grouped with those of lagoon/lake environments, whereas the other island populations grouped together [11]. According to the authors, this fact could be due to the physical-chemical conditions existing in the sampling sites; indeed, one of them is a small shallow and narrow bay where freshwater is gushing out, whereas in the other there are hot springs and moreover mineral water. The authors concluded that these local environmental conditions are possibly similar to those existing in the lagoon sites.

As for the evolutionary history life of lagoon fish, there are two possible scenarios, either the ancestors of the *A. lagunae* were marine fish adapted in a first step to shallow waters of the island coasts and after, they having colonized lagoons, or some lagoon fish could have migrated to adapt in island shallow coasts with possible secondary contacts and introgression. According to the core-satellite hypothesis, the first scenario would be the most plausible owing to the reduced gene diversity in lagoons. However, statistical study of metric and meristic characters show that islands fish exhibit differences versus lagoon fish, even for the closest population of Tunis Lake, this could suggest a secondary introgression of mtDNA from island fish to lagoon populations.

Our molecular analyses suggest that *A. lagunae* and *A. punctuata* constitute a monophyletic clade, even if it is not supported by high bootstrap values; similarly, the projection of canonical variables on the plane defined by axes 1-3 (Fig. 2B) clearly distinguish a group constituted by island and lagoon fish plus French populations of *A. punctuata*. Interestingly, an insertion of c. 200 bp in length has been found in the same localisation in the mtDNAs of both lagoon and marine punctuated fish but not in the marine non-punctuated specimens, as well as in other two congeneric species, *A. hepsetus* and *A. presbyter*, and in the atheriniform *Menidia menidia* [50]. This intergenic spacer is located between the tRNA(Glu) and *cyt b* genes, and shares approximately 50% sequence similarity with *cyt b*, suggesting that it might have originated from an event of gene duplication involved at least a part of the *cyt b* gene and which took place in the common ancestor of the lagoon and the marine punctuated specimens, confirming our morphological and

phylogenetic results. In addition, our molecular analyses suggest that *A. presbyter* could not be monophyletic. Similarly, in another recent molecular study [14], involving the same species of *Atherina* have been analyzed, the lower bootstrap values have been found for *A. presbyter* and the monophyly of this species lacks the support by the Bayesian analysis. As for *A. hepsetus*, our results are unconvincing since only two identical sequences have been used; however, according to a recent study, *A. hepsetus* could be a Mediterranean derivative of a mainly Atlantic stock and no geographical patterns have been detected in mtDNA analyses of samples from the Mediterranean Sea [14], whereas, another study show that *A. hepsetus* from the Grecian coasts share the same two main composite haplotypes, leading to the assumption of the existence of a single panmictic population [51].

It was interesting to know if the relations between molecular data of the different species or populations and environmental conditions fit with meristic ones. According to Jordan's rule [52], the number of vertebrae increase with the latitudes as does the size for animals (Bergman's rule) [53]. The higher number of vertebrae in cold waters has been found for several fish families, including Atherinopsidae [54,55]. However, the number of different factors that influence the number of vertebrae in fishes makes for highly complex patterns of variation, and means that unraveling causes is difficult (reviewed in [56]). Jordan's rule has been amended by Casanova [57] as follow: "the number of vertebrae increases with latitude for boreal fish and decreases for tropical ones". This new formulation suggests that the greatest number is found in the "better adapted habitat" and due to global latitudinal temperature gradient, the relationship between average vertebrae number and latitude would give a Gaussian curve (also called "bell-shaped curve") with a maximum in the better adapted habitat. It is in keeping with a more general definition of Bergmann's rule: "a species reaches its maximal size in high, intermediate or low latitudes of its area if it has a boreal, temperate or tropical distribution" [57]. *Atherina* populations from northern waters, British and Netherlands coasts, for both *A. presbyter* and *A. boyeri*, and off Northern Mediterranean coasts for both *A. punctuata* and *A. lagunae* have a higher average vertebrae number than the Southern Mediterranean populations, in agreement with Jordan's rule. In return, according to Casanova's rules, the presence of a north-south negative cline for the average vertebral numbers could suggest a temperate origin of the ancestor of both *A. presbyter* and species of the *A. boyeri* complex although, they have now a temperate-subtropical distribution (indeed, no decrease of the vertebrae number has been found in the latitudes studied). In our analyses, the curve obtained using *A. boyeri* data suggests that the optimal latitudinal range could be around 42-45°N. For this species, populations near the northern area distribution have been analyzed (52°N versus ≈56°N), contrarily, for *A. presbyter*, the latitude of the caught sites are globally in the intermediate latitudinal range (34°N-55°N versus 26°-60°N). Concerning *A. punctuata* and *A. lagunae*, other Northern populations are needed, e.g. North Adriatic. Moreover, in our analyses, the ranges of vertebrae numbers clearly differentiate *A. hepsetus*, *A. presbyter* or *A. boyeri*, and this is useful character to distinguish each species. The regression line of *A. lagunae* crosses that of *A. punctuata*;

Table 3. Comparisons of Some Meristic Characters of Populations of Western Mediterranean Species of the *A. boyeri* Complex with those of *Atherina lacustris* Bonaparte 1836

Species	Sample Site	n	Latitude	D1	D2	Anal	Verteb.	References
<i>A. boyeri</i>	Palavas (F)	100	43°31'N	7.97	11.67	13.87	45.78	[13]
	Sète (F)	100	43°24'N	8.00	11.76	13.64	45.81	[13]
	Pinarello (F)	100	41°40'N	7.47	11.28	13.26	45.39	[13]
	Tabarka (T)	100	36°57'N	7.55	11.42	13.48	45.41	[13]
	Hergla (T)	100	36°10'N	7.54	11.51	13.47	45.23	[13]
	Monastir (T)	650	35°40'N	7.58	11.46	13.44	45.14	[13]
	Madhia (T)	309	35°30'N	7.59	11.31	13.42	45.24	[13]
<i>A. punctuata</i>	Pinarello (F)	100	41°40'N	6.58	11.64	13.17	43.13	[13]
	Lavezzi Islands (F)	278	41°20'N	6.85	10.68	12.98	43.21	[13]
	Zebib Cape (T)	100	37°15'N	6.89	10.75	13.25	43.12	[13]
	Monastir (T)	100	35°40'N	6.88	10.84	13.16	42.90	[13]
<i>A. lagunae</i>	Mauguio Lagoon (F)	100	43°35'N	7.79	11.23	13.14	44.51	[13]
	Camargue Lagoon (F)	100	43°34'N	7.78	11.30	13.38	44.51	[13]
	Thau Lagoon (F)	100	43°25'N	7.49	11.35	13.49	44.53	[13]
	Biguglia Lagoon (F)	100	42°37'N	7.52	11.35	13.23	44.49	[13]
<i>A. lacustris</i>	Albano and Nemi Lakes (I)	?	41°45'N	7-8	11	12	44	[35]
	Italy	?	36°42'N-45°13'N	(5)6-8	10-11	12-13(14)	43-44	[38]
<i>A. lagunae</i>	Bizerte Lagoon (F)	100	37°11'N	7.48	11.38	13.41	42.15	[13]
	Ichkeul Lagoon (F)	326	37°10'N	7.59	11.67	13.45	43.05	[13]
	Tunis Lake (T)	480	36°50'N	7.02	11.27	13.41	41.23	[13]
	Kerkennah Islands (T)	220	34°41'N	6.95	11.28	13.47	40.43	this study

The closer characters between *A. lacustris* and populations of *A. boyeri* complex are in bold letters. Abbreviations: Anal, average of anal fin rays; D1, average of first dorsal fin rays; D2, average of second dorsal fin rays; n, number of specimens; verteb., average of vertebrae counts. For the abbreviations of the countries names, see legend of the Table 2. The asterisk corresponds to value do not use in graphical representation.

these two species with low vertebrae values are linked to particular habitat: lagoons and some islands coasts for the former and rocky sea beds for the later. Moreover, numerous morphological analyses suggest relatively close relationships between these two species.

Moreover, for a same latitude range, contrarily to data obtained for *A. boyeri*, *A. presbyter* and *A. punctuata*, average vertebrae values for *A. lagunae* show great differences between populations. Similar results have been already shown between diadromous stocks of *Galaxias brevipinnis* Günther 1866 versus lacustrine populations of this species [58,59]. Brackish water ecosystems are often exposed to wide variations in environmental parameters such as temperature and salinity which may cause strong selective pressures on organisms. These pressures, in association with geographical discontinuity, can play an important role in separating species inhabiting these environments into different populations. Moreover, great differences in the physico-chemical parameters could be found between lagoons even geographically close. Inversely, constancy of the major abiotic parameters and the physical continuum of oceanic biota retrain the build up of ecological barriers and of isolated biotopes.

In conclusion, this study shows evidence that island sand smelts are molecular and morphological characteristics of Tunisian lagoonal fish. In the future, in order to investigate genetic structure and population history of this species using microsatellite markers, populations of all other Tunisian lagoons and islands will be studied and compared to those of France. Moreover, vertebral numbers of *Atherina* spp. vary with latitude in accordance with ecological rules and this parameter is a good diagnostic specific character in *Atherina*. The great difference in vertebrae counts found for *A. lagunae* suggest strong physico-chemical constraints; the average vertebrae numbers of each population is a consequence of these constraints. Moreover, other populations covering the whole range of each species are needed, for both for morphological and molecular analyses. This will shed more light on the evolution of the genus *Atherina*. Moreover, this study strongly strengthen that the Southern Mediterranean marine coastal lagoons and islands are valuable biological reservoirs deserving more investigation and preservation.

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