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1 **Otolith shape analysis and mitochondrial DNA markers distinguish three sand smelt**
2 **species in the *Atherina boyeri* species complex in western Mediterranean.**

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1 Abstract

2 *Atherina boyeri* is a common euryhaline teleost fish in the Mediterranean and adjacent areas,
3 which inhabits coastal and estuarine waters, including coastal lagoons and more rarely inland
4 waters. Several recent studies have pointed the possible existence of three distinct groups or
5 species, one lagoon/freshwater group and two ‘punctuated and unpunctuated on the flanks’
6 marine groups, within an *A. boyeri* species complex. This study is a combined approach using
7 otolith shape and molecular markers to better define the structure of the species in the western
8 Mediterranean.

9 Genetic differentiation and species delimitation among nine *Atherina boyeri* populations from
10 several marine and lagoon/brackish habitat sites in Algeria, Tunisia and France were
11 investigated using three mitochondrial (control region, *Cyt b* and 16S) and one nuclear
12 markers (2nd intron of *S7*). For further phylogenetic and phylogeographic study, we added
13 sequences from Genbank covering more areas (Ionian Sea, Adriatic Sea, Tyrrhenian Sea,
14 Black Sea, Atlantic). Five groups were found. Two of them perfectly corresponded to two
15 species already recognized *Atherina presbyter* and *Atherina hepsetus*, both living in marine
16 waters; and three additional, including *Atherina boyeri* (brackish and freshwater
17 environments) and two independent groups of marine punctuated and unpunctuated individuals.
18 Those findings are corroborated by the study of the otolith contour shape of 362 individuals
19 of seven populations from different habitats using Fourier analysis. Individuals could be
20 discriminated into five groups based on the first two functions (Wilk’s lambda = 0.07, $p <$
21 0.001). Samples from Ziamia inlet, marine punctuated individuals and unpunctuated marine
22 specimens from Annaba’s Gulf formed three well separated groups. Specimens from Mellah
23 and Manguio lagoons formed another group. The last one includes individuals from Bizerte
24 and Thau lagoons. The divergences between them strongly support the potential species
25 within the *A. boyeri* species complex.

26
27 **Keywords:** *Atherina boyeri* species complex; mt-DNA; Mediterranean Sea; Fourier analysis;
28 genetic divergence

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1 **1. Introduction**

2 The Mediterranean Sea had a complex history, marked by the Messinian salinity crisis and
3 climatic fluctuations of the recent Quaternary, characterized with the succession of glacial and
4 interglacial periods. These events induced habitat fragmentation and alteration of connections
5 between populations, and resulted in the establishment of its current biodiversity and of
6 different biogeographical zones (Krijgsman et al., 1999; Patarnello et al., 2007). The
7 Mediterranean Sea is now characterized by a high biodiversity with a quarter of endemic
8 species (Bianchi et al., 2012). Despite decades of dedicated studies, knowledges on
9 biodiversity still evolving, even for well known groups such as fishes (Bianchi et al., 2012).
10 The development of genetic approaches allows a better analysis of species limits, for example
11 by identifying cryptic species (Borsa et al., 2004; Pante et al., 2015). The comparison of
12 genetic and morphological data can bring useful information on the evolution of characters
13 used for species delimitation, on morphological plasticity, and on species interactions
14 (differentiation *vs* gene flow).

15 The *Atherina* Linnaeus, 1758 genus has been a subject of several major systematic revisions
16 (Kiener and Spillmann, 1969), where two genera and 20 species were condensed into a single
17 genus. In Europe the Atherinidae family is represented by a single genus and three species:
18 *Atherina boyeri* Risso, 1810, *Atherina hepsetus* Linnaeus, 1758 and *Atherina presbyter*
19 Cuvier, 1829. *Atherina hepsetus* is restricted to the Mediterranean, although it has also been
20 reported in the eastern Atlantic coasts of Spain, Morocco, Madeira and Canary Islands
21 (Quignard and Pras, 1986). The distribution of *A. presbyter* ranges from the British Isles and
22 southern North Sea to the Canary Islands, Mauritania and Cape Verde (Quignard and Pras,
23 1986). It has also been reported from the Azores archipelago (Santos et al., 1997) and rarely
24 from the western Mediterranean (Kiener and Spillmann, 1969; Quignard and Pras, 1986).
25 *Atherina boyeri* has been described as an extremely euryhaline teleost fish which can take a
26 wide range of salinities, from freshwater to hypersaline conditions (110 ‰ maximum
27 recorded) (Gon and Ben-Tuvia, 1983). It mainly inhabits coastal waters of the East Atlantic
28 Ocean as well as in the Mediterranean, Black, Caspian and Aral seas, including estuarine
29 waters, coastal lagoons and more rarely inland waters (Gon and Ben-Tuvia, 1983; Henderson
30 and Bamber, 1987). This small fish forms local semi-isolated populations each with a
31 characteristic morphology and life history traits (Henderson and Bamber, 1987). This species
32 could be a good model to study differentiation and speciation in the Mediterranean Sea with a
33 range spanning important phylogeographical limits and with important morphological
34 diversity according to geography and habitats. Kottelat (1997) pointed out the possible

1 existence of two different groups in *A. boyeri s.l.*, one belonging to the lagoon / freshwater
2 environment and the other to the marine one. This author limited the name of *A. boyeri* to the
3 lagoonal form and proposed *A. mochon* Cuvier, 1829 for the marine one.

4 Several investigations based on morphometry (Kartas and Trabelsi 1990; Trabelsi et al.,
5 2002a, 2004, Boudinar et al., 2015), and allozymic analyses (Focant et al., 1992, 1993, 1999;
6 Cammarata et al., 1996) supported the hypothesis that *A. boyeri* could be subdivided into
7 separate sub-populations or species. Data based on allozymic analysis alone (Mauro et al.,
8 2007), or combined with morphometry (Trabelsi et al., 2002a, 2002b, 2004) pointed out the
9 existence of two (Klossa-Kilia et al., 2002, 2007; Kraitsek et al., 2008) or even three
10 Mediterranean species (Trabelsi et al., 2002a, 2002b, 2004; Astolfi et al., 2005; Francisco et
11 al., 2008, 2011; Milana et al., 2008), including one lagoon form (*A. lagunae*) and one or two,
12 (punctuated on the flanks "*A. punctata*" and non-punctuated "*A. boyeri*") marine forms. Since
13 the genetic divergence values estimated between marine and lagoon *A. boyeri* populations
14 were found to be similar to those existing between well-defined species of the same genus, the
15 authors elevated them to the species rank. All of these studies pointed out the existence of an
16 *A. boyeri* species complex, but for now there is no formal nor extensive description of the
17 biology and distribution range of these putative species. The under-representation of southern
18 populations from the western Mediterranean might have hindered the detection of additional
19 genetic structure (e.g. Chaoui et al., 2009) or a good estimation of the potential species range,
20 as the only data on this species in the Algerian coast are the study of *A. boyeri* species
21 complex using otolith shape and somatic morphology at a local scale (Boudinar et al. 2015),
22 and the age, growth and reproduction of Mellah lagoon population (Boudinar et al., 2016).
23 Additionally the use of a single genetic marker, mostly mitochondrial, is not sufficient for
24 precise species delimitation nor the identification of putative hybridization events. Finally an
25 evaluation of the morphological differentiation of these taxa has not been performed in all
26 cases. Indeed an integrative study including large geographical scope, multilocus and non
27 molecular approaches appears as the best approach for species delimitation (Puillandre et al.,
28 2012; Pante et al., 2015).

29 The purpose of the present work was to use otolith shape analysis and mitochondrial (Control
30 Region: CR, Cytochrome b: Cyt *b*, ribosomal DNA 16S) and nuclear DNA (*S7* intron 2)
31 markers to study the morphological and genetic diversity of *A. boyeri* species complex from
32 different habitat (lagoon, inlet, sea) in the western Mediterranean. This combination of

1 investigative tools was attempted for the first time in this species complex and allowed
2 precisising the species repartition and species limits in western Mediterranean.

3 **2. Materials and methods**

4 *2.1. Sampling sites*

5 Nine populations of *Atherina boyeri* including freshwater/brackish, marine punctuated (Fig.
6 1a) and marine unpunctuated fishes (Fig. 1b) were sampled from eight locations in the
7 northern and southern banks of the western Mediterranean Sea (Fig. 2 and Table 1)
8 representing different geographical areas (Algerian, Tunisian and French coasts and lagoons)
9 and different environments: inshore brackish lagoons and the nearshore marine environment
10 (hereafter called lagoon and sea habitats respectively) and inlets. All specimens were
11 morphologically identified, according to Marfin (1981) and stored in 96% ethanol.

12

13 *2.2. Otoliths morphometry*

14 Otolith shape analysis was performed on six of the nine populations, the same specimens
15 being used for molecular analyses. Samples from Lion's Gulf and Mafragh inlet were
16 discarded: as sampling size was too small for statistical analyses ($N = 7$) and also because the
17 specimens were fingerlings, which did not permit comparisons with adult fish. In order to
18 eliminate fish size effects on otolith shape, all individuals used in this analysis belonged to the
19 same size interval (5 to 8.5 cm Total Length, TL). A total of 362 fish were used for otolith
20 shape analysis (Table 1). The sagittal otoliths were taken as pairs, washed in clean water, air-
21 dried and stored in plastic tubes. Both right and left otoliths were examined under a
22 stereomicroscope fitted with a numerical camera (Lumenera's INFINITY lite) linked to a
23 computer monitor. Otoliths were orientated in a consistent manner, with the sulcus side up.
24 Numerical pictures were then acquired with TNPC software (Mahé et al., 2011). For each
25 numerical image, the software Shape 1.2 (Iwata and Ukai, 2002) calculated the Fourier
26 coefficients in order to make them invariants to the otolith size, orientation and position,
27 regarding the beginning of the outline, which is arbitrarily defined (Crampton, 1995). Also,
28 the Fourier Power (FP) spectrum was calculated to estimate the sufficient number of
29 harmonics accounting for the best reconstruction of the otolith outline (Pothin et al., 2006). A
30 subsample of 90 otoliths (10 samples \times 7 populations) was randomly created for this purpose.
31 As the first 13 harmonics reached 99.99% of the mean cumulated power, the Fourier analysis
32 indicated that the otolith shape could be summarized by these thirteen harmonics. A Factorial
33 Discriminante Analysis (FDA) was performed with Fourier descriptors to compare otolith

1 shape variations among the sampling areas using the SPSS software version 13 (SPSS, 2004).
2 Using Ward's method, a hierarchical clustering was performed with the FactoMineR package
3 with R[®] software (R Core Team 2014). The classification success of the discriminant analysis
4 was checked using jackknifed cross-validation.

5

6 2.3. Molecular analyses

7 For molecular analysis, nine populations of *A. boyeri* species complex were collected from
8 eight locations in the northern and southern banks of the western Mediterranean Sea
9 representing variety in environment kinds of habitats (Fig. 1). Total DNA was extracted from
10 caudal fin using 10% Chelex[®] with a 1 h proteinase K digestion followed by 15 min at 95°C.
11 Mitochondrial fragments of the control region (CR), 16rRNA, cytochrome *b* (Cyt *b*) genes
12 and nuclear fragment of the *S7* intron 2 were amplified by polymerase chain reaction (PCR);
13 the PCR conditions and primers sequences and corresponding references are given in Table 2.
14 For each marker, other available sequences from *Atherina* were retrieved from GenBank. The
15 PCR products were sent for Sanger sequencing at Eurofins MWG. The numbers of sequences
16 used for each marker in each locality are given in Table 1.

17

18 2.4. Species delimitation and population genetics analyses

19 The sequences were aligned with ClustalW (Thompson et al., 1994). In addition to the
20 alignments for each gene, a CR and Cyt *b* concatenated alignment was performed as these
21 markers presented the highest number of common sequences. Sequences of two
22 Atheriniformes were used as outgroups: *Leptatherina wallacei* Prince, Ivantsoff & Potter,
23 1982 belonging to the family Atherinidae (GenBank accession numbers AY326785.1 for CR,
24 AY313118.1 for Cyt *b*) and *Iso natalensis* Regan, 1919 belonging to the family Isonidae
25 (GenBank accession numbers HM855075.1 for 16S). We also added sequences of the only
26 two other congeneric species in the area, *A. presbyter* (GenBank accession numbers
27 JF309618.1) and *A. hepsetus* (GenBank accession numbers JF309620.1), in order to obtain
28 the closest outgroups of the *A. boyeri* species complex. For further phylogenetic and
29 phylogeographic analyses we added sequences from Genbank covering more localities (Table
30 3, Fig. 3, Table S1-S3; supplementary material). We used Jmodeltest 3.7 (Guindon and
31 Gascuel, 2003; Posada, 2008) with the Akaike Information Criterion to identify the best-fit
32 model of evolution for each locus to be used in Maximum-Likelihood (ML) analysis:
33 TVM+G for CR (gamma distribution shape parameter $G = 0.584$), TVM+I+G for Cyt *b* ($I =$
34 0.252 , $G = 0.649$), TPM3uf+G for 16S ($G = 0.171$), and TIM3+G for *S7* ($G = 1.221$). ML

1 analyses were performed with PhyML 3.0 (ref) and we tested the robustness of the inferred
2 trees with 1000 bootstrap replicates (Felsenstein, 1985). Bayesian analyses (BA) were
3 implemented in MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck,
4 2003). The best-fit model of evolution for each locus was set using MrModeltest 2.3
5 (Nylander, 2004): HKY+G for CR (gamma distribution shape parameter $G = 0.522$),
6 GTR+I+G for Cyt *b* ($I = 0.332$, $G = 0.786$), SYM+G for 16S ($G = 0.171$), and SYM+G for S7
7 ($G = 1.016$). We used one independent run of two million generations of Markov chain Monte
8 Carlo (MCMC) simulations for each marker to approximate posterior probabilities of
9 phylogenetic trees. Topologies were sampled every 1000 generations, and a majority-rule
10 consensus tree was estimated after discarding the first 2500 sampled generations.
11 The obtained sequences were used for a phylogeographic analysis. For each marker data set,
12 we analyzed the total amount of genetic variance into three variance components: among
13 groups, among populations within groups and within populations, using an analysis of
14 molecular variance (AMOVA) in Arlequin v3.5 (Excoffier and Lisher, 2010). Samples were
15 grouped by geographic proximity, habitat type or a combination of both, resulting in five
16 different groupings (Table 4). According to the analyzed marker, some test combination may
17 be absent due to the lack of sequence in those localities. The analysis was based on Φ_{st}
18 (Excoffier et al., 1992), and its significance was tested through 1000 permutations. The
19 significance of tests involving multiple comparisons was adjusted with a Bonferroni
20 correction. Average number of nucleotide substitutions per site, Dxy (Nei, 1987) were used to
21 estimate differentiation between populations using DNAsp v5 (Librado and Rozas, 2009).

22 **3. Results**

23 *3.1. Otoliths morphometry*

24 The first two discriminant functions of the FDA performed with Fourier descriptors accounted
25 for 89.40% of the variance. Individuals could be discriminated into five groups based on the
26 first two functions (Wilk's lambda = 0.07, $p < 0.001$). Samples from Ziama inlet, marine
27 punctuated individuals and unpunctuated marine specimens from Annaba's Gulf formed well
28 separated groups. Specimens from Mellah and Manguio lagoons formed another group. The
29 last one includes individuals from Bizerte and Thau lagoons (Fig. 5). Overall area-
30 classification success was 84.30%. The best reclassification rate is 84.03% for Annaba's Gulf
31 punctuated marine specimens, then specimens from Ziama Inlet 75%, Bizerte Lagoon
32 71.92%, Manguio Lagoon 66.07%, Annaba's Gulf unpunctuated marine specimens 64.75%,
33 Mellah Lagoon specimens 55%, and the Thau Lagoon individuals 53.48% (Table 4). As for

1 the molecular analysis, some of the Thau Lagoon specimens are scattered between lagoon and
2 marine environment.

3

4 3.2. *Species delimitation*

5 In total, 373 bp (379 sequences) were sequenced from the CR, 464 bp (81 sequences) from
6 the 16S, 315 bp (217 sequences) from the Cyt *b* and 187 from the S7 (149 sequences)
7 (including indels), yielding 170, 35, 72 and 27 haplotypes in *A. boyeri* species complex,
8 respectively. The number of sequences used for the analysis includes the Genbank sequences.
9 BA and ML species delimitation trees gave similar results in all cases.

10 For the Cyt *b*, CR, 16S, and the concatenated fragments CR and Cyt *b*, converged on a similar
11 tree and showed the same topology. Seven clades were observed, *A. presbyter* and *A.*
12 *hepsetus*, appeared as a sister group of the *A. boyeri* species complex. The five clades of the
13 *A. boyeri* species complex: marine punctuated form, marine unpunctuated form, and two or
14 three clades of the lagoonal form, are well supported by high bootstrap values.

15 The different clades of the lagoonal form correspond to different geographical areas: for the
16 Cyt *b* the first one grouped specimens from Greece (Nea Peramos-Kavala), the second
17 grouped lagoon and insular Tunisian specimens, and the third clade grouped other of lagoonal
18 populations. Four specimens from Thau lagoon, were grouped with lagoonal samples while
19 the other specimens were grouped with the marine unpunctuated individuals (Figure S1;
20 supplementary material).

21 For the 16S, three clades inside the lagoonal form, the first group exclusively Tunisian
22 specimens from Bizerte lagoon, the second specimens from Bulgaria and Turkey, and the third
23 one grouped other of lagoonal populations (Figure S1; supplementary material).

24 For the CR, three clades were observed, the first one grouped specimens from Tunisia, and
25 Italy (Marsala and one specimen from Cagliari), the second grouped specimens from Turkey
26 (Danube, Lake Sapanca) and one specimen from Croatia (Spalato). The third clade grouped
27 other lagoonal samples.

28 The tree for CR includes the most localities and sample size is shown in Fig. 5, along with
29 bootstrap and posterior probabilities values.

30 For the concatenated fragments CR and Cyt *b*, the lagoonal form is separated into two clades,
31 one with the tunisian specimens and the other with those sampled from Algerian and French
32 lagoons (Figure S1; supplementary material).

1 The nuclear S7 marker did not lead to a well resolved phylogeny, but it allowed us to confirm
2 the grouping of some specimens from Thau with the lagoonal samples (presented in a
3 rectangle on the tree) (Figure S2; supplementary material).

4 5 3.3. Population genetics

6 AMOVA results (Table 5) confirmed the existence of a significant genetic structure based on
7 the three mt-DNA markers and showed a highly significant global structure when comparing
8 lagoonal, marine unpunctuated and marine punctuated samples (1), CR ($F_{SC} = 0.66$; $F_{ST} =$
9 0.92 ; $F_{CT} = 0.78$; $P < 0.001$); Cyt *b* ($F_{SC} = 0.77$; $F_{ST} = 0.94$; $F_{CT} = 0.76$; $P < 0.001$); 16S ($F_{SC} =$
10 0.77 ; $F_{ST} = 0.96$; $F_{CT} = 0.82$; $P < 0.001$).

11 The analysis based on Cyt *b* between marine punctuated samples (2) shows a significant
12 divergence among populations within groups ($F_{SC} = 0.06$; $P < 0.001$). No differences are
13 observed within populations ($F_{ST} = 0.61$; $P = 0.06$) or among groups ($F_{CT} = 0.58$; $P = 0.1$).

14 Comparison based on Cyt *b*, between marine unpunctuated samples (3) show a significant
15 differences among populations within groups ($F_{SC} = 0.14$; $P < 0.001$) and within populations
16 ($F_{ST} = 0.38$; $P = 0.06$), no significant differences are observed among groups ($F_{CT} = 0.28$; $P =$
17 0.16).

18 The comparison between North, South Ouest and South East Mediterranean lagoonal samples
19 (4) also revealed significant differences, CR ($F_{SC} = 0.43$; $F_{ST} = 0.82$; $P < 0.001$; $F_{CT} = 0.68$; $P =$
20 0.01); Cyt *b* ($F_{SC} = 0.16$; $F_{ST} = 0.83$; $F_{CT} = 0.80$; $P < 0.001$); 16S ($F_{SC} = 0.17$; $P < 0.001$; $F_{ST} =$
21 0.83 ; $P = 0.02$), based on 16S no significant differences were observed among groups ($F_{CT} =$
22 0.80 ; $P = 0.38$).

23 Significant differences were also found while comparing lagoonal samples as observed in the
24 tree topology, based on CR ($F_{SC} = 0.70$; $F_{ST} = 0.82$; $P < 0.001$; $F_{CT} = 0.41$; $P = 0.01$).

25 The average number of nucleotide substitutions per site between populations for the Cyt *b*
26 was respectively $D_{xy} = 0.14$, $D_{xy} = 0.15$ when comparing lagoonal sequences with marine
27 unpunctuated and punctuated ones, and $D_{xy} = 0.11$ when comparing marine unpunctuated
28 and punctuated sequences. When we compare respectively *A. hepsetus* with lagoonal, marine
29 unpunctuated and punctuated samples, the average number of nucleotide substitutions is D_{xy}
30 $= 0.17$, $D_{xy} = 0.13$, $D_{xy} = 0.14$. Those values are lower when comparing *A. presbyter* with
31 lagoonal ($D_{xy} = 0.14$), marine unpunctuated ($D_{xy} = 0.10$) and punctuated ($D_{xy} = 0.13$)
32 samples. The average number of nucleotide substitutions is the lowest when comparing *A.*
33 *hepsetus* and *A. presbyter* ($D_{xy} = 0.06$).

1 For the CR, when comparing lagoonal sequences with marine unpunctuated and punctuated
2 ones Dxy is respectively equal to 0.15 and 0.18, and Dxy = 0.17 when comparing marine
3 unpunctuated and punctuated sequences. When we compare respectively *A. hepsetus* with
4 lagoonal, marine unpunctuated and punctuated samples, the average number of nucleotide
5 substitutions is Dxy = 0.21, Dxy = 0.16, Dxy = 0.18. In the other hand when we compare *A.*
6 *presbyter* with lagoonal, marine unpunctuated and punctuated samples the average number of
7 nucleotide substitutions is respectively, Dxy = 0.20, Dxy = 0.19, Dxy = 0.16, its lowest value is
8 set when comparing *A. hepsetus* and *A. presbyter* (Dxy = 0.11).

9 For the 16S, when comparing lagoonal sequences with marine unpunctuated and punctuated
10 ones Dxy is respectively equal to 0.08 and 0.07, and Dxy = 0.07 when comparing marine
11 unpunctuated and punctuated sequences. When we compare respectively *A. hepsetus* or *A.*
12 *presbyter* with lagoonal, marine unpunctuated and punctuated samples, the average number of
13 nucleotide substitutions is Dxy = 0.08, Dxy = 0.07, Dxy = 0.07, and Dxy = 0.02 when
14 comparing *A. hepsetus* and *A. presbyter*.

15

16 **4. Discussion**

17 Otoliths are widely used in different studies, such as species identification (Aguirre and
18 Lombarte, 1999; Parmentier et al., 2001) and may vary within species according to fish size,
19 site, depth and/or other environmental factors (Begg et al., 2001; Stransky and MacLellan,
20 2005; Hüseyin, 2008). Several studies have shown that shape analysis allowed discrimination of
21 local fish stocks according to ecological factors (Smith, 1992; Friedland and Reddin, 1994;
22 Bolles and Begg, 2000; Cardinale et al., 2004). But this raises the question of the genetic
23 versus environmental determinism (i.e. plasticity) of otolith shape.

24 In the present study, otolith shape analysis showed five groups: The first one included Mellah
25 and Manguio lagoons samples, when the second gathered individuals from Bizerte and Thau
26 lagoons, the remaining samples (Ziama inlet, marine punctuated specimens and marine
27 unpunctuated specimens from Annaba's gulf) constitute each a well separated group. Those
28 results correspond to what have been found by Boudinar et al. (2015) in a local study on the
29 same Algerian populations, where differences were observed between Mellah lagoon, Ziama
30 inlet, marine punctuated specimens and marine unpunctuated specimens from Annaba's gulf,
31 using otolith shape and somatic morphology. Three different mtDNA markers (CR, Cyt *b*,
32 16S) corroborated otolith shape findings. Both gave us information about how much the
33 punctuated and unpunctuated marine forms are different. Besides, an intra-specific variation into
34 the lagoonal form of *A. boyeri* species complex using both methods was apparent. Molecular

1 results for the three mtDNA markers using different tree construction methods are consistent
2 and allow us to distinguish seven separated groups. Two of them perfectly corresponded to
3 two species already recognized as valid (*A. hepsetus* [Ⓐ] and *A. presbyter* [Ⓑ]) and three
4 additional corresponded to the marine punctuated *A. punctata* [Ⓒ], marine unpunctuated *A.*
5 *boyeri* [Ⓓ] and lagoonal *A. lagunae* [Ⓔ] traditionally included in the *A. boyeri* species complex
6 (Trabelsi et al., 2002b). The main clade grouped lagoon samples (Bizerte, Mauguio, Mellah)
7 and fresh water samples (Ziama and Mafragh inlets). The remaining two clades correspond to
8 punctuated and unpunctuated marine groups, in which we found respectively marine
9 punctuated samples from Annaba Gulf and marine unpunctuated samples from Annaba and
10 Lion's Gulf. Those clades are supported with high bootstraps and posterior probabilities. The
11 divergence between them was comparable to that separating *A. presbyter* from *A. hepsetus*
12 thereby supporting the potential species status of the different clades inside the *A. boyeri*
13 species complex. The observation of misplaced individuals in the species tree was interpreted
14 as the possibility of rare migration events and supported the hypothesis of gene flow among
15 populations. The inter-lagoon difference has already been reported by Trabelsi et al., (2002b)
16 who found an important genetic divergence between Tunisian and French lagoon sand smelts
17 which is supported by high bootstrap values, suggesting that there has been a long-term
18 barrier to the gene flow between these two populations. This was also reported by Astolfi et
19 al., (2005) in a larger scale considering populations from Atlantic, Mediterranean and Black
20 Sea lagoon, where an important and significant genetic structure was observed, with some
21 groupings displaying low bootstraps values. Milana et al., (2012) along the Italian and
22 Albanian coasts of the Mediterranean Sea where a pronounced spatial genetic structure has
23 been detected by microsatellites among lagoon populations of *A. boyeri* species complex with
24 different statistical approaches used.

25 The distinctiveness of the three species of the *A. boyeri* species complex using both molecular
26 and otoliths shape analysis is observed across the eastern and western basins of the
27 Mediterranean Sea. These results support the findings based on morphometric data (Kartas
28 and Trabelsi 1990; Trabelsi et al., 2002a, 2004), *Cyt b* gene (Trabelsi et al., 2002b), and
29 mtDNA control region (Astolfi et al., 2005). Francisco et al., (2008) confirmed these results
30 with further mtDNA fragments (control region and 12S), while Milana et al., (2008) in their
31 analysis of portion of the mitochondrial cytochrome *b*, detected the presence of an insertion
32 common to lagoon and punctuated forms of *A. boyeri* species complex. (88% sequence
33 identity of the insertion was found for marine punctuated form. 93% sequence identity of the

1 insertion for the lagoon form. And 47% similarity when comparing the insertions for the two
2 forms). Using three mtDNA markers (CR, 12S, 16S) and two nuclear markers (Rhodopsin and
3 S7 intron 2) Francisco et al., (2011) sustained the previous the previous results.

4 These differences may have a genetic determinism linked to a local selective process
5 potentially and / or habitat choice or (Lemaire et al., 2000; Chaoui et al., 2012). This could
6 also correspond to morphological plasticity (e.g. Capoccioni et al., 2011) or to a combination
7 of environmental and genetic effects. Most likely, the reason for *A. boyeri* to appear as a
8 complex clade is related to its distinct life cycle which is, among the other species, the one
9 most associated with more fragmented environments (lagoons, estuaries and freshwater
10 bodies). The combination between this fragmentation in its habitats linked to the low dispersal
11 ability of the species is probably responsible for the highly phylogeographic pattern observed.

12 Our results reinforce the species status of the three Mediterranean forms traditionally included
13 in the *A. boyeri* species complex using both of otolith shape and mitochondrial DNA markers.
14 A formal description and range distribution definition should be considered as a priority for
15 this species, with further phylogeographic investigations including wider sampling and
16 additional molecular genetic markers.

17

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3 Fig. 1. Marine punctuated (A) and marine unpunctuated (B) forms belonging to *Atherina boyeri* species
4 complex.

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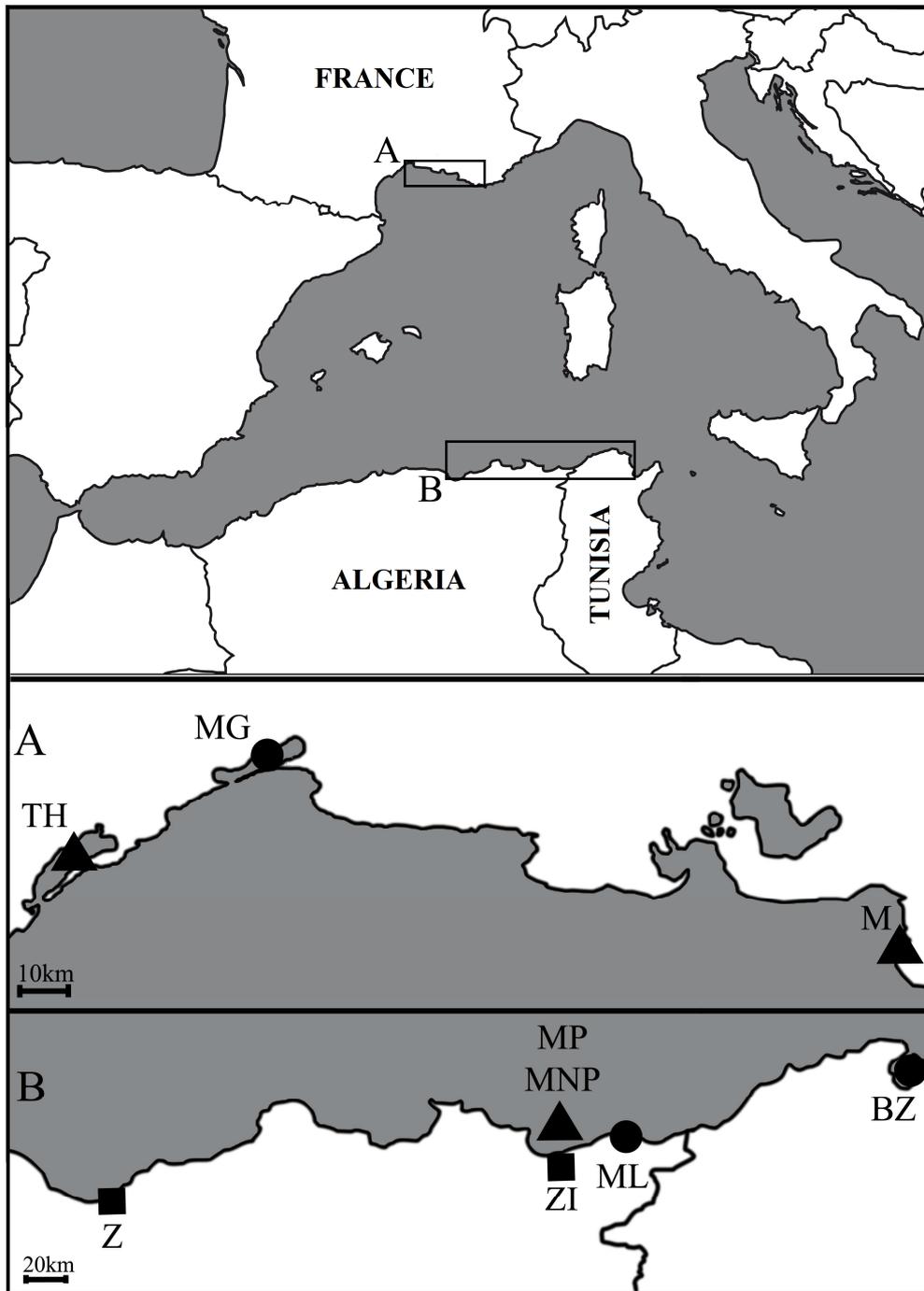
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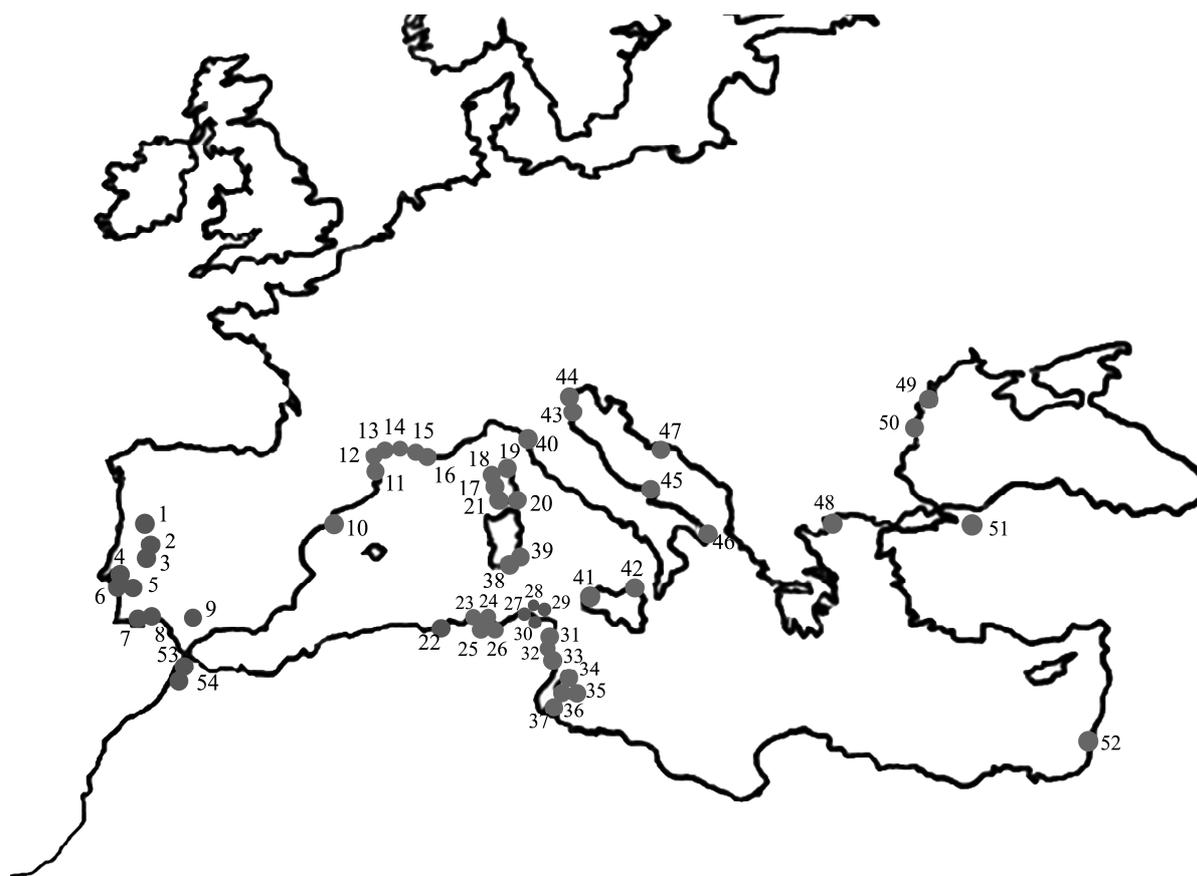
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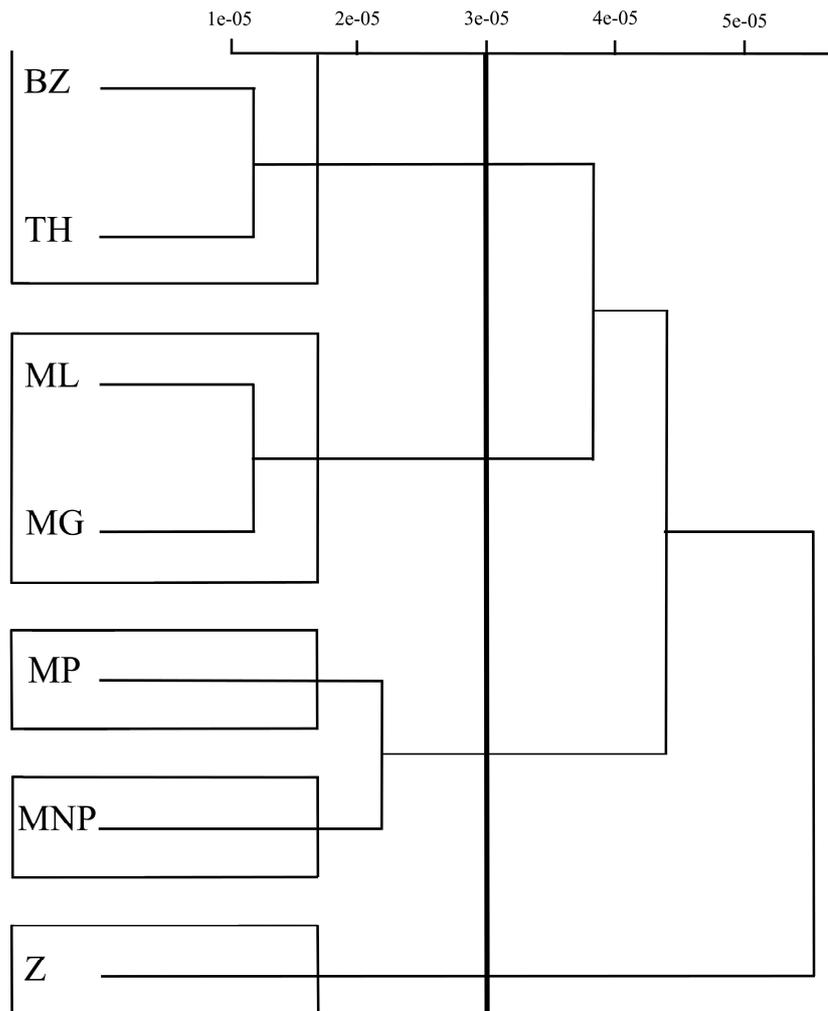
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3 Fig. 2. Map of the study area. The eight sampling locations are indicated by their codes as
 4 defined in Table 2. Circles indicate inshore lagoonal habitat, triangles marine habitat and
 5 square inlet/brakish habitat. The upper map shows the north-western Mediterranean sampling
 6 area, and the lower map shows the south-western Mediterranean sampling area.

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2 Fig. 3. Map of sampling locations for the extended analysis. The location numbers details are
3 presented in table 3.
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2 Fig. 4. Hierarchical clustering using Fourier's descriptors on otolith shape of *A. boyeri* species
3 complex. Sampling locations are indicated by their codes as defined in Table 2.

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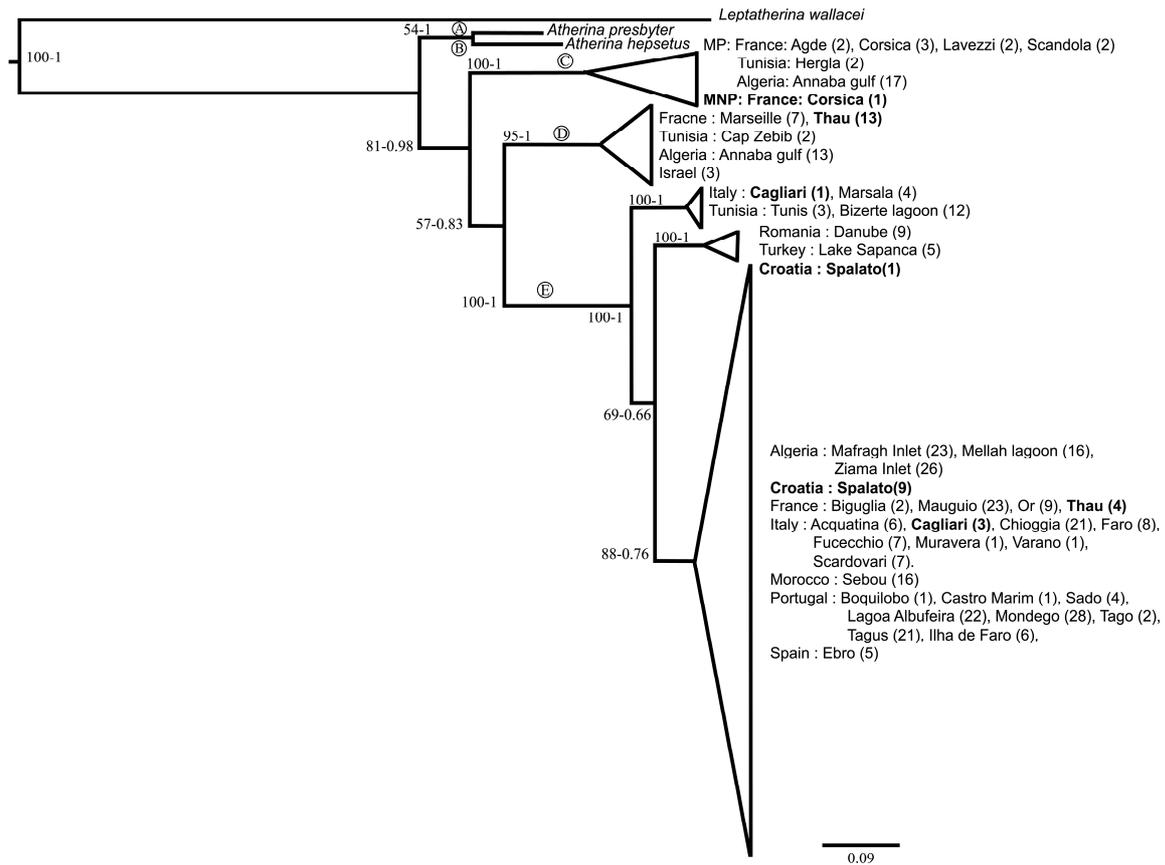
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2 Fig. 5. Phylogenetic relationships within *Atherina* for the Control region (CR). Each main
 3 node is represented by circled capital letters. (A) : *A. hepsetus*, (B) : *A. presbyter*, (C) : *A.*
 4 *punctata*, (D) : *A. boyeri* and (E) : *A. lagunae*). Percentages of bootstrap for maximum
 5 likelihood are in bold followed by the posterior probability for Bayesian analysis.

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1 Table 1. Description of *A. boyeri* species complex samples including sampling locations,
 2 samples codes, number of used sequences for each marker and number of otoliths for the
 3 shape analysis in the western Mediterranean Sea region.

Sampling locations	Country	Sample code	CR	Cyt <i>b</i>	16S	S7	Otoliths
Annaba Gulf	Algeria	MP	16	12	7	7	61
Annaba Gulf		MNP	18	16	4	8	60
Mellah lagoon		ML	15	10	4	9	50
Mafragh Inlet		MI	22	24	9	10	
Ziama Inlet		Z	24	28	6	8	56
Bizerte lagoon	Tunisia	BZ	17	8	4	7	57
Thau lagoon	France	TH	15	7	6	11	22
Mauguio lagoon		MG	21	20	8	9	56
Lion's Gulf		M	7	5	7	6	

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1 Table 2. Primers and polymerase chain reaction conditions for each fragment.

Fragment	Primer name	Sequence	Sequence Source	PCR conditions
Control region	L-proI	5'-ACTCTCACCCCTAGCTCCCAAAG-3'	(Ostellari et al., 1996)	35 x (94 °C 30", 55 °C 30", 72 °C 1') 72 °C 5'
	H-DL1	5'-CCTGAAGTAGGAACCAGATGCCAG-3'		
Cytochrome b	New-For	5'-AGCCTACGAAAACCCACCC-3'	(Meyer et al., 1990)	30 x (92 °C 15", 52 °C 45", 72 °C 1.5") 72 °C 8"
	34-Rev	5'- AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA- 3'	(Cantatore et al., 1994)	
16S	16SFor	5'-AAGCCTCGCCTGTTTACCAA-3'	(Almada et al., 2005)	35 x (94 °C 45", 50 °C 1', 72 °C 1') 72 °C 10'
	16SRev	5'-CTGAACTCAGATCACGTAGG-3'		
S7 intron 2	S7RPEX2F	5'-AGCGCCAAAATAGTGAAGCC-3'	(Chow and Hazama, 1998)	40 x (95 °C 45", 52 °C 1', 72 °C 45') 72 °C 10'
	S7RPEX3R	5'-GCCTTCAGGTCAGAGTTCAT-3'		

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1 Table 3. Sampling location and number of *Atherina boyeri* species complex individuals used
 2 in the extended phylogenetic analyses.

N° in Fig.	Collection site	N	lagoonal fish			unpunctuated fish			punctuated fish		
			Cyt <i>b</i>	16S	CR	Cyt <i>b</i>	16S	CR	Cyt <i>b</i>	16S	CR
1	Portugal	Mondego	28		28						
2		Tagus	21		3	21					
3		Boquilobo	1			1					
4		Tago	2			2					
5		Sado	4			4					
6		Lagoa Albufeira	22			22					
7		Ilha de Faro	6			6					
8		Castro Marim	1			1					
9	Spain	Zonar	2		2						
10		Ebro	5		5						
		Barcelona	4		4						
11	France	Ayrolle	6	6							
12		Agde	3						3	3	2
13		Thau	20				20	3	17		
14		Mauguio	23	18	8	23					
15		Or	9			9					
16		Marseille	7						7		
17		Scandola	7				7				
18		Scandola	2							2	2
19		Biguglia	9	9		2					
20		Lavezzi	3							3	2
21		Corsica									2
22	Algeria	Ziama Inlet	27	25	5	27					
23		Annaba Gulf	17							10	4
24		Annaba Gulf	13				2	1	13		
25		Mafragh Inlet	23	22	9	23					
26		Mellah lagoon	16	6	5	16					
27	Tunisia	Bizerte lagoon	12	5	3	12					
28		Cap Zebib	4							4	
29		Cap Zebib	8				8	3	2		
30		Tunis	3			3					
31		Hergla	10				10				
32		Hergla	3							3	2
33		Monastir	10				10				
34		Karkennah Sidi Fraj	9	9							
35		Karkennah El Attaya	7	7							
36		Karkennah Sidi Youssef	6	6							
37		Djerba	8	8							
38	Italy	Cagliari	4			4					
39		Muravera	1	1		1					
40		Fucecchio	7			7					
41		Marsala	4			4					
42		Faro	8			8					
43		Scardovari	7			7					
44		Chioggia	21		4	21					
45		Varano	1	1		1					
46		Acquatina	6			6					
47	Croatia	Spalato	10		4	10					
48	Greece	Nea Peramos-Kavala	2					1		1	
49	Romania	Danube	9			9					
50	Bulgaria	Bulgaria	5		5						
51	Turkey	Turkey	3		3						
		Lake Sapanca	5			5					
52	Israel	Israel	3				2	3			
53	Morocco	Loukkos	4		4						
54		Sebou	16			16					

1 Table 4. Classification success rate using based on otolith shape of *A. boyeri* species complex
2 using jackknifed cross-validation. Sampling locations are indicated by their codes as defined
3 in Table 2.

Sites	ML	MNP	MP	BZ	Z	MG	TH
ML	55	8	1	11	6	14	5
MNP	5	79	9	0	11	10	8
MP	1	8	100	3	3	1	3
BZ	8	1	6	82	4	5	8
Z	5	5	2	1	84	12	3
MG	10	7	3	8	5	74	5
TH	3	3	2	7	0	5	23

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- 1 Table 5. AMOVA results for five different groupings, as a function of geographic proximity,
 2 habitat type or a combination of both.

	CR				Cyt <i>b</i>				16S			
	ddl	Variation	F-statistics	P-value	ddl	Variation	F-statistics	P-value	ddl	Variation	F-statistics	P-value
(1) MP/MNP/Lagoon												
Among populations within groups	2	78.67	FSC=0.66	0	2	76.67	FSC=0.77	0	2	82.81	FSC=0.77	0
Within populations	38	14.16	FST=0.92	0	24	18.1	FST=0.94	0	16	13.31	FST=0.96	0
Among groups	333	7.18	FCT=0.78	0	181	5.23	FCT=0.76	0	54	3.88	FCT=0.82	0
(2) Within MP												
Among populations within groups					2	58.94	FSC=0.06					0
Within populations					3	2.52	FST=0.61					0.0645
Among groups					19	38.54	FCT=0.58					0.109
(3) Within MNP												
Among populations within groups					2	28.77	FSC=0.14					0
Within populations					3	10.07	FST=0.38					0.0009
Among groups					51	61.16	FCT=0.28					0.1632
(4) Lagoons: Med North/Med South Ouest/Med South East												
Among populations within groups	2	68.17	FSC=0.43	0	2	80.02	FSC=0.16	0	2	80.12	FSC=0.17	0
Within populations	7	13.95	FST=0.82	0	8	3.25	FST=0.83	0	2	3.49	FST=0.83	0.028
Among groups	114	17.88	FCT=0.68	0.014	110	16.73	FCT=0.80	0	24	16.39	FCT=0.80	0.385
(5) Lagoon populations as in tree topology												
Among populations within groups	2	41.63	FSC=0.70	0								
Within populations	27	41.16	FST=0.82	0								
Among groups	275	17.21	FCT=0.41	0.017								

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