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Atherina punctata and *Atherina lagunae* (Pisces, Atherinidae), new species found in the Mediterranean Sea. 2. Molecular investigations of three Atherinid species

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Abstract – On the basis of morphoanatomical parameters, the sand smelt species (*Atherina boyeri* Risso, 1810) is viewed as a highly polymorphic complex. In this study, intraspecific sequence variation in a portion of the cytochrome *b* gene was examined in 88 individuals from Tunisia and France. The correlation between the results of statistical analysis of the sequence data using a variety of tree-building algorithms and morphoanatomical analyses demonstrated the subdivision into three putative species: *A. boyeri*, which only includes non-punctuated fishes, *A. punctata*, which corresponds to punctuated fishes and *A. lagunae*, which corresponds to atherines living in lagoons. ***To cite this article: M. Trabelsi et al., C. R. Biologies 325 (2002) 1119–1128.*** © 2002 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

***Atherina boyeri* / *Atherina lagunae* / *Atherina punctata* / cytochrome *b* / sand smelts / Mediterranean Sea / speciation**

Résumé – ***Atherina punctata* et *Atherina lagunae* (Pisces, Atherinidae), nouvelles espèces trouvées en Méditerranée. Investigations moléculaires de trois espèces d'Athérines.** En se basant sur l'étude des caractères morphologiques, l'espèce *Atherina boyeri* Risso, 1810 peut être considérée comme un complexe très polymorphe. Dans cet article, les variations intraspécifiques de séquence d'une portion du gène cytochrome *b* ont été examinées chez 88 individus français et tunisiens. La corrélation entre les résultats des analyses statistiques de ces séquences, effectuées en utilisant plusieurs algorithmes de construction phylogénétique, et les données morphoanatomiques met en évidence une subdivision en trois espèces putatives : *A. boyeri*, ne comprenant que les populations non ponctuées marines, *A. punctata*, correspondant aux individus ponctués, et *A. lagunae*, les athérines lacustres. ***Pour citer cet article : M. Trabelsi et al., C. R. Biologies 325 (2002) 1119–1128.*** © 2002 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

***Atherina boyeri* / *Atherina lagunae* / *Atherina punctata* / Athérines / cytochrome *b* / mer Méditerranée / spéciation**

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Version abrégée

Atherina boyeri (Risso, 1810) est un petit poisson téléostéen vivant dans les zones côtières, les estuaires et les lagunes. Il peut supporter de grandes variations de salinité, allant de l'eau douce à des lacs hyper-salés. Ce poisson est présent en Méditerranée, dans les mers adjacentes et dans la façade est de l'océan Atlantique. *Atherina boyeri* forme un complexe taxonomique qui a été divisé par certains auteurs en diverses espèces et sous-espèces, ces confusions taxonomiques venant du fait que de grandes différences au niveau des caractères métriques et méristiques sont observées. Divers travaux sur *A. boyeri* de Méditerranée ont montré que cette espèce forme des populations distinctes, qui peuvent être séparées en deux grands groupes homogènes, l'un étant constitué des individus marins et l'autre des individus lagunaires. L'identification de ces deux groupes est essentiellement basée sur des caractères méristiques. De plus, des travaux récents ont montré que sur les côtes de France (Corse comprise), de Sardaigne, de Sicile et de Tunisie, le groupe marin se différencie en deux populations, partiellement sympatriques. Cette distinction est basée sur la coloration, la morphologie et les caractéristiques biochimiques. Les individus de la nouvelle population se différencient des autres Athérines marines par la présence d'une ligne de points noirs située le long des flancs, au-dessous de la bande argentée, d'yeux remarquablement développés, d'un espace interorbitaire large, d'un museau court, d'un corps robuste et trapu, de nageoires dorsales rejetées à l'arrière, d'un nombre plus faible de vertèbres et de branchiospines, ainsi que par l'absence de parvalbumine V. Un travail d'investigation moléculaire associé à l'analyse complète des données morphoanatomiques, préalablement publiée, était donc indispensable avant d'affirmer le statut exact de ces trois groupes.

Notre équipe a récolté 88 spécimens dans des lagunes et sur les côtes méditerranéennes françaises (languedocienne et corse) et tunisiennes. Approximativement, 350 bp du gène mitochondrial codant le cytochrome *b* ont été amplifiés. Les analyses phylogénétiques ont été effectuées en utilisant trois approches: le *Neighbour-Joining*, avec une matrice basée sur la distance à deux paramètres de Kimura, une approche cladiste, utilisant le maximum de parcimonie non pondéré, et, enfin, le *Maximum Likelihood*. Mille *bootstraps* ont été effectués lors des analyses en *Neighbour-Joining* et en *Maximum Likelihood*. Les séquences provenant d'*A. boyeri* ont été alignées avec les séquences correspondantes de *L. wallacei* et de *L. presbyteroides*, qui ont été trouvées être les plus proches de celles des Athérines. Sur 362 positions, 102 sites ont été trouvés informatifs. La composition en base de la troisième position de chaque codon montre une forte hétérogénéité. D'une manière générale, la composition en base du gène de cytochrome *b* pour les trois positions des codons est proche des valeurs de pourcentage connues pour les Téléostéens. Comme aucune saturation en base n'a été détectée, toutes les positions ont été utilisées lors des reconstructions phylogénétiques. Une recherche heuristique des arbres les plus parcimonieux a fait apparaître 3815 arbres également parcimonieux (214 pas). L'index de cohérence (CI) est égal à 0,696, suggérant un faible taux d'homoplasie ; l'index de rétention (RI) est égal à 0,924.

Les trois méthodes de construction phylogénétique produisent des topologies similaires. Seulement 36 haplotypes différents ont été observés sur 88 individus. Après enracinement, sur les groupes extérieurs, les trois topologies mettent en évidence deux groupes distincts. Le premier groupe est uniquement constitué des haplotypes appartenant aux populations lagunaires et forme un groupe monophylétique qui présente des pourcentages de *bootstrap* (PB) de 99% et un *decay index* de 8. Le second, qui est constitué de l'ensemble des populations marines, n'est pas fortement supporté ; il se sépare toutefois en deux groupes, qui sont, quant à eux, bien différenciés : les individus ponctués, d'une part (PB de 76 à 100%), et les non ponctués, d'autre part (PB de 95 à 100%). Aucun haplotype n'est partagé entre les différents groupes, ce qui suggère qu'il n'y a pas eu de flux génique récent entre eux. De plus, chacun de ces groupes semble avoir des caractéristiques d'évolution différentes.

La structure géographique des trois sous-groupes a aussi été analysée. La différentiation génétique au sein des athérines ponctuées est faible, mais, excepté pour un individu, la séparation France–Tunisie est nette : une explication écologique pourrait expliquer ceci, car ces poissons sont inféodés à des milieux rocheux. Au sein des athérines marines non ponctuées, les relations intra-groupe ne sont pas significatives, suggérant que ces populations étaient panmictiques dans un passé récent. Les athérines lagunaires se séparent en individus tunisiens et français selon deux sous-groupes monophylétiques. Les longueurs des branches sont similaires dans les deux cas, suggérant des vitesses d'évolution équivalentes. Il est intéressant de noter que les données morphoanatomiques ont aussi mis en évidence le fort degré de polymorphisme au sein des poissons lagunaires.

L'étude moléculaire d'athérines provenant de la mer et des lagunes des côtes nord et est de la Tunisie, de Corse et du Languedoc confirme les résultats obtenus par les études morphoanatomiques. Ces travaux avaient

montré que trois grands groupes s'individualisent nettement, tant par les caractères métriques que méristiques, quelle que soit la localisation géographique. Au niveau marin, des divergences entre des spécimens sympatriques ou sub-sympatriques sont décelables par la coloration (présence de tâches noires sous la ligne argentée). La prise en considération des caractères métriques, méristiques et biochimiques de ces spécimens a confirmé la valeur discriminante du caractère chromatique, en mettant en évidence des différences morphologiques concernant l'œil, l'espace interorbitaire, le préorbitaire, la position des nageoires dorsales, la taille de la première nageoire dorsale, la hauteur du corps et du pédoncule caudal. Aucune structuration n'est observée au sein des Athérines marines non ponctuées, et cela en dépit du fait que les régions de collecte sont séparées de plus de 1000 km (France–Tunisie). L'explication la plus plausible serait que les diverses populations ne seraient issues que d'une seule, qui aurait envahi toutes les autres régions. Cependant, aucun des divers sous-groupes ne donne d'informations concernant l'origine de la population ancestrale. Ces résultats suggèrent que des flux géniques récents se sont produits, voire même que des migrations d'individus entre la France et Tunisie restent encore possibles.

La variabilité génétique au sein des populations d'*A. boyeri* qui est observée dans cette étude est donc congruente avec tous les autres travaux, qui ont démontré le fort taux de plasticité phénotypique au sein de ce complexe taxonomique. Les différences phénotypiques sont telles qu'il a été suggéré que ce complexe pourrait être constitué d'au moins 20 espèces. Un important critère dans la reconnaissance d'espèce est l'isolement reproductif, ce qui semble confirmé par l'ensemble des données moléculaires et morphoanatomiques ; il est, de plus, intéressant de noter que cette barrière reproductive existe aussi au sein des populations marines ponctuées/non ponctuées, en dépit du fait qu'elles vivent en sympatrie.

Dans le futur, l'analyse des nombreux types de populations du genre *Atherina* offre une opportunité unique d'étudier les diverses étapes de radiations adaptatives et aussi d'essayer de déceler les divers mécanismes conduisant aux phénomènes de spéciation. D'une manière générale, de nombreux *Atherinidae* présentent des niveaux peu communs de diversification écologique et de spéciation rapide. À ce jour, nous n'avons pas d'indication concernant les forces impliquées dans ces phénomènes de spéciation. L'évolution des phénotypes divergents et des conditions d'utilisation des ressources alimentaires pourrait aussi précéder ou initier des processus de spéciation ou pourrait apparaître après un isolement reproductif (modification du choix du partenaire sexuel ?), et ainsi faciliter la coexistence entre les espèces. D'autres hypothèses de spéciation favorisent l'importance de facteurs extrinsèques dans la spéciation, tels que le degré de salinité dans les lagunes. Dans nos recherches futures, les facteurs impliqués dans ces phénomènes de spéciation seront recherchés à l'intérieur de ce complexe.

En conclusion, l'analyse moléculaire a mis en évidence le fait que les nœuds séparant les trois groupes sont très profonds et sont comparables à ceux observés lors de la séparation de deux espèces de Téléostéens appartenant au même genre. Les constructions phylogénétiques suggèrent donc à elles seules la présence de trois espèces. Ces analyses sont complètement congruentes avec celles obtenues sur la base des caractères métriques, méristiques et biochimiques. Certaines populations du complexe *A. boyeri* sont donc engagées dans des phénomènes de spéciation et les trois sous-groupes étudiés ont dépassé le stade d'espèce in statu nascendi ; les divergences sont suffisamment significatives pour admettre que chacun d'eux a atteint le niveau spécifique. Donc, en Méditerranée, en plus d'*Atherina presbyter* (rare et certainement localisée à l'extrême Ouest du bassin occidental) et d'*A. hepsetus*, le complexe *A. boyeri* peut être divisé en trois espèces. L'appellation *A. boyeri* étant conservée uniquement pour les individus lagunaires non ponctués, nous proposons de nommer les populations ponctuées *Atherina punctata* et *Atherina lagunae* pour les individus lagunaires. Les diagnoses de ces espèces ont été publiées dans un article précédent, consacré à l'étude des caractères morphoanatomiques.

1. Introduction

The sand smelt, *Atherina boyeri* (Risso, 1810) is an atherinid fish that principally inhabits coastal and estuarine waters, including coastal lagoons, with a wide range of salinities, from freshwater to seawater, or hypersaline lakes. For example, the population of the Bardawil lagoon, whose unique supersaline environment has salinity ranges between 45 and 70‰ [1] in most of its area, with the maximum recorded salinity being 110‰ [2]. *A. boyeri* is found throughout the Mediterranean and adjacent seas and the east Atlantic Ocean.

Historically, a number of species and subspecies have been recognised, which are now included within *A. boyeri* [3–5]. This taxonomic confusion is attributable to

the great morphometric and meristic characteristics shown by the fish. Thus, *A. boyeri* as a species is found over a wide range of temperature and salinity conditions in the coastal, estuarine and lagoon habitats and its subdivided into semi-isolated populations, each one with a characteristic morphology and life history. The works of Kiener and Spillmann [3] and Marfin [6] on *Atherina boyeri* of the western Mediterranean Sea showed that this species is formed of distinct populations, which can be arranged into two broad groups. The first is homogeneous and is composed of sand smelts living in marine waters, whilst the other is heterogeneous and includes those living in lagoon waters. The identification of these two groups was based essentially on the study of meristic characteristics. Using the same criteria, various more recent studies came to a similar conclusion in the eastern Mediterranean Sea [7–9]. In addition to these groups, more recent studies have demonstrated that another group of populations exist along the French, Corsican, Sardinian, Sicilian and Tunisian coasts and are different from the sympatric 'marine group' [10, 11]. The difference between these two marine groups is based on colouring, morphological and biochemical characteristics. The specimens of this 'new marine group' mainly differ from those of the other group by the presence of dark spots along the lateral line (spotted marine sand smelt), a large eye, a lower number of vertebrae and gill-rackers, and the absence of parvalbumin V. However, in spite of all these morphological and biochemical differences [11, 12], a genetic investigation must be conducted in order to find the exact taxonomic status of the 'spotted sand smelt'. In addition, studies of the *A. boyeri* complex provide an excellent and probably unique opportunity within marine fishes to study speciation and adaptative radiation. This study has concentrated on Atherines from Tunisian and French Sea and lagoon water. In this study, we have determined and compared partial sequences of the mitochondrial cytochrome *b* gene (cyt *b*) to analyse the phylogenetic relationships between the sand smelt complex. This marker displays enough sequence variation to assess the phylogenetic relationships in fishes and other vertebrates at intraspecific levels and to study recent speciation [13, 14].

2. Materials and methods

2.1. Biological samples

Eighty-eight DNA sequences were obtained from specimens collected by our team in the Mediterranean Sea (Tunisian and French coasts and lagoons). The fishes were identified by us and were then stored in

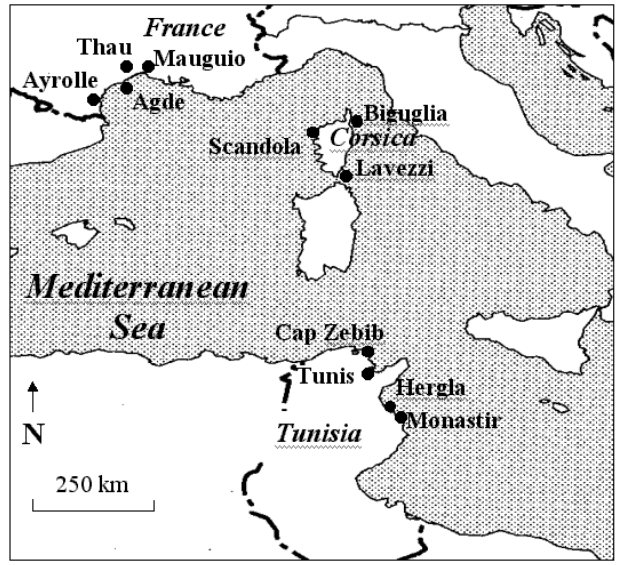

Fig. 1. Map of the western Mediterranean Sea, showing the locations of samples collected.

70% ethyl alcohol at ambient temperature or at –20 °C as soon as possible. Data for the specimens examined are given in the map of collecting locations shown in Fig. 1. For each type of population, we have used a three-letter code; the first one indicates the country: Corsica (C), France (F) and Tunisia (T); the second one indicates the type of sand smelts: punctuated (P), marine non punctuated (N) and lagoon (L); the last one shows the locations of each population, Agde (A), Biguglia (B), Cap Zebib (Z), Hergla (H), Lavezzi (L), Mauguio (G), Monastir (N), Thau (T), Tunis (U), and Scandolla (S). The name of each population is summarised in Table 1.

2.2. DNA extraction and PCR reaction

Total DNA was extracted from approximately 0.25 cm^2 of caudal fin by using modifications of Taberlet and Bouvet's method [15]. A section of approximately 350 bp of mitochondrial (mt) DNA genome from the cyt *b* gene was amplified using published specific primers New-For 5'-AGCCTACGAAAACCCACCC-3' [16] and 34-Rev 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' [17]. Polymerase chain reaction (PCR) components per 50 µl reaction were as follows: 50 ng template DNA, 0.2 µM of each primer, 2.0 U. HiTaq *Taq* polymerase, dNTPs 0.2 mM, 5 µl of the reaction buffer provided by the *Taq* manufacturer (Bioprobe, France). The cycling parameters were as follows: 92 °C for 2 min, 5 times (92 °C for 15 s, 48 °C for 45 s, and 72 °C for 1.5 min), 30 times (92 °C for 15 s, 52 °C for 45 s, and 72 °C for 1.5 min), and 72 °C for 8 min. Using the single-stranded DNA as

Table 1. Nomenclature of each sampling location.

	Country	Corsica (C)			France (except Corsica) (F)					Tunisia (T)		
	Habitat	lagoon	marine		lagoon		marine		lagoon	marine		
Type of populations	Locations	Biguglia (B)	Lavezzi (L)	Scandola (S)	Ayrolle (A)	Mauguio (M)	Agde (A)	Thau (T)	Tunis (U)	Cap Zebib (Z)	Hergla (H)	Monastir (N)
Punctuated (P)			**CPL**	**CPS**			*FPG*			*TPZ*	**TPH**	
Marine non-punctuated (N)				*CNS*				**FNT**		**TNZ**	**TNH**	**TNN**
Lagoonal (L)		*CLB*			**FLA**	**FLM**			**TLU**			

a template, the nucleotide sequence was determined with an automated DNA sequencer (Genome Express, Grenoble, France).

2.3. Sequence analysis

Phylogenetic analyses were performed using three different approaches: (1) the Neighbour-Joining (NJ) method [18], based on a matrix of the Kimura two parameters distance [19] in Mega [20]; (2) a cladistic approach using the maximum parsimony (MP) criterion (heuristic search of PAUP* [21]); (3) the Maximum Likelihood (MLH) reconstruction was performed using estimations of all model parameters from the dataset. The choice of model is based on the use of Modeltest 3.0 [22], which enables the selection of the simplest model, which does not significantly differ from a model with a higher number of parameters. The Rogers–Swofford [23] procedure – a method that allows calculation of initial branch lengths which approximate maximum-likelihood estimates – was used to reduce the preliminary computing time. Robustness of nodes was estimated by running a bootstrap test with 1000 replicates for NJ trees, 1000 replicates for MP trees (heuristic search of PAUP* [21] with 10 random additions of taxa and TBR branch-swapping and 1000 replicates for MLH trees). To test the robustness of branches in the tree, Bremer's decay index [24] was computed. We used the Templeton's test (Wilcoxon sign-rank tests) [25] to test topological differences using maximum parsimony (unweighted parsimony). Two cyt *b* sequences of Atherinidae were extracted from GenBank as outgroups (*Leptatherina wallacei*, accession number: AY026174 and *Leptatherina presbyteroides*, accession number: AY026120).

3. Results

3.1. Nucleotide polymorphism

We determined the partial sequence (362 pb) of the mitochondrial cyt *b* gene for 88 Atherina individuals. In various preliminary tests, all atherinimorph sequences available from Genbank were selected in turn as outgroups. In all trees obtained, the topology and approximate value of the nodes are similar (data not shown). Also, for the phylogeographic analysis of western Mediterranean Sea sand smelts, the 88 partial cyt *b* sequences of *A. boyeri* were aligned with those from *L. wallacei* and *L. presbyteroides*, which were some of the closest to the cyt *b* sequences of sand smelt. These sequences were chosen as outgroups. Of the 362 positions, 102 were informative for unweighted parsimony. In addition, the display of saturation was examined for each codon position. No saturation effects were observed for the different substitution patterns (Fig. 2). The base composition of the third position of codons shows a strong heterogeneity between bases. For the second positions, a slight fluctuation is apparent and there is a strong homogeneity for the first (Fig. 3). Mean base composition in partial cyt *b* sequences was similar to those previously reported for teleostean species [17]. This is why we used an unweighted parsimony and the Kimura two parameters distance for the NJ method.

As no saturation was observed, all positions were used for the construction of phylogenetic trees (alignment is available from the authors upon request). The cyt *b* gene sequences were analysed using three phylogenetic methods: unweighted Maximum Parsimony (MP), Neighbour Joining (NJ) and Maximum Likehood (MLH). A heuristic search of the most parsimonious tree [19] showed 3815 equally parsimonious trees (214 steps). The CI (consistency index) was equal to 0.696, suggesting a low level of homoplasy, and RI (retention index) was equal to 0.924.

3.2. Phylogenetic relationships

The three tree-making methods (NJ, MP and MLH) produced similar topologies, and the Templeton test showed that the three trees were statistically indistinguishable according to the maximum parsimony model. Only 36 different haplotypes were observed in the 88 individuals sequenced. Rooting on the two outgroup

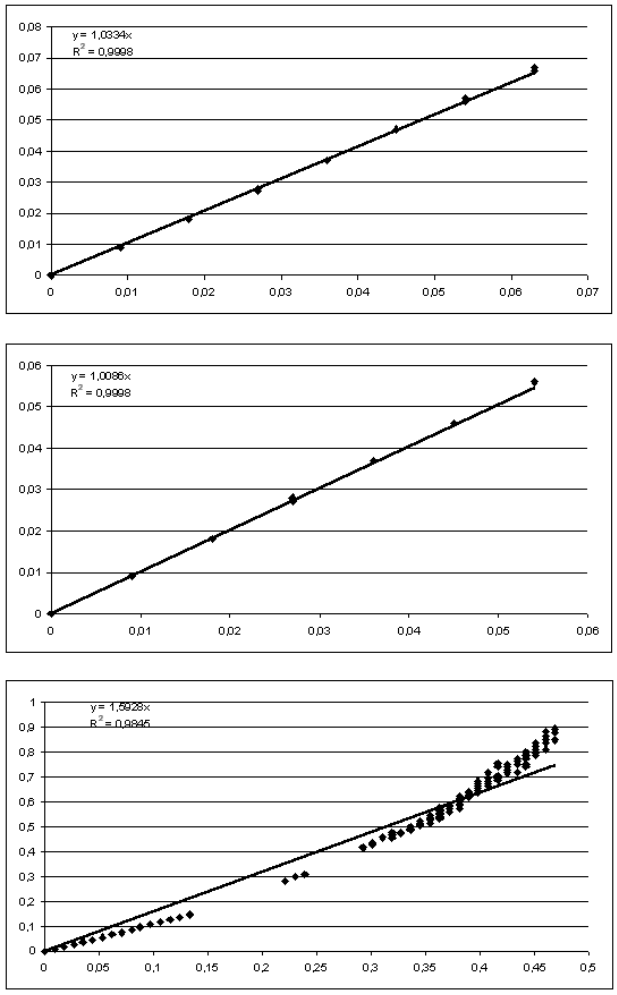

Fig. 2. Scatter plots obtained from 362 positions for the first, second, and third position of the codons of the cytochrome *b*, using 23 different haplotypes. *X*-axis: pairwise number of substitutions in using the p-distance; *Y*-axis: pairwise number of substitutions in using the Kimura two-parameter model.

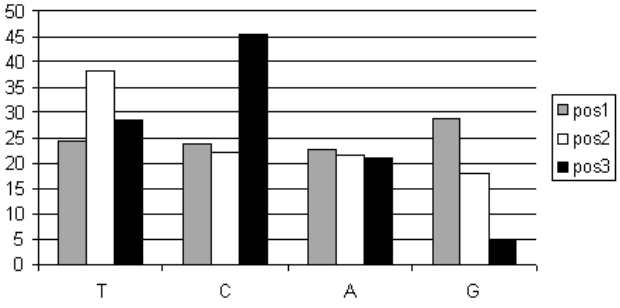

Fig. 3. Base composition of the cytochrome *b* sequence for *A. boyeri* complex for each codon position taken separately.

species, the three topologies displayed two groups (Fig. 4). The first group only consisted of the haplotypes belonging to the lagoon populations and constituted a monophyletic group (BP = 99/99/99, decay index, DI = 8). The second group consisted of the haplotypes belonging to the punctuated marine specimens (BP = 100/99/76, DI = 14) and the non-punctuated marine specimens (BP = 100/100/95, DI = 6), but this group was not strongly supported (BP = 76/51/–, DI = 0). No haplotype was shared by the different groups, suggesting that there had not been any recent hybridisation among the different species within the *A. boyeri* complex.

We estimated the mean genetic distance within and between the different populations using a Kimura two parameters distance. The mean genetic distance within populations ranged from 0.000 ± 0.000 (same haplotype for Tunisian lagoon population) to $0.017\,75 \pm 0.004\,02$ for the Tunisian punctuated marine population (Table 2). The mean genetic distance between populations ranged from 0.0023 ± 0.0008 to 0.2013 ± 0.0295 suggesting an intra- and interspecific polymophism (Table 3).

3.3. Geographical structure of mtDNA variation within the three subgroups

3.3.1. Punctuated sand smelts

As far as the punctuated fishes were concerned, all Tunisian punctuated sand smelts, except one, are grouped together, although this is not supported statistically. The structure of the other subgroups, including all French fishes and one Tunisian punctuated fish suggests recent gene flow.

3.3.2. Non-punctuated marine sand smelts

Within the non-punctuated marine fishes, the intra group relationships were shown to be non-significant, having bootstrap values always below 67%. There is no evidence of significant population differentiation in haplotype frequencies, suggesting that these populations have been entirely panmictic in the recent past.

3.3.3. Lagoon sand smelts

Within the lagoon sand smelts, the bifurcation in the NJ, MP and MLH analyses clearly differentiate the Tunisian fishes (seven sequences with the same haplotype) from the French fishes into two monophyletic subgroups. Bootstrapped distance analysis clearly supports this monophyly (100/100/76%). The branches in the two different subgroups have similar lengths, indicating a similar evolution rate. In addition, the Corsican (seven individuals with the same haplotype) and the Metropolitan France lagoon sand smelts are sister groups.

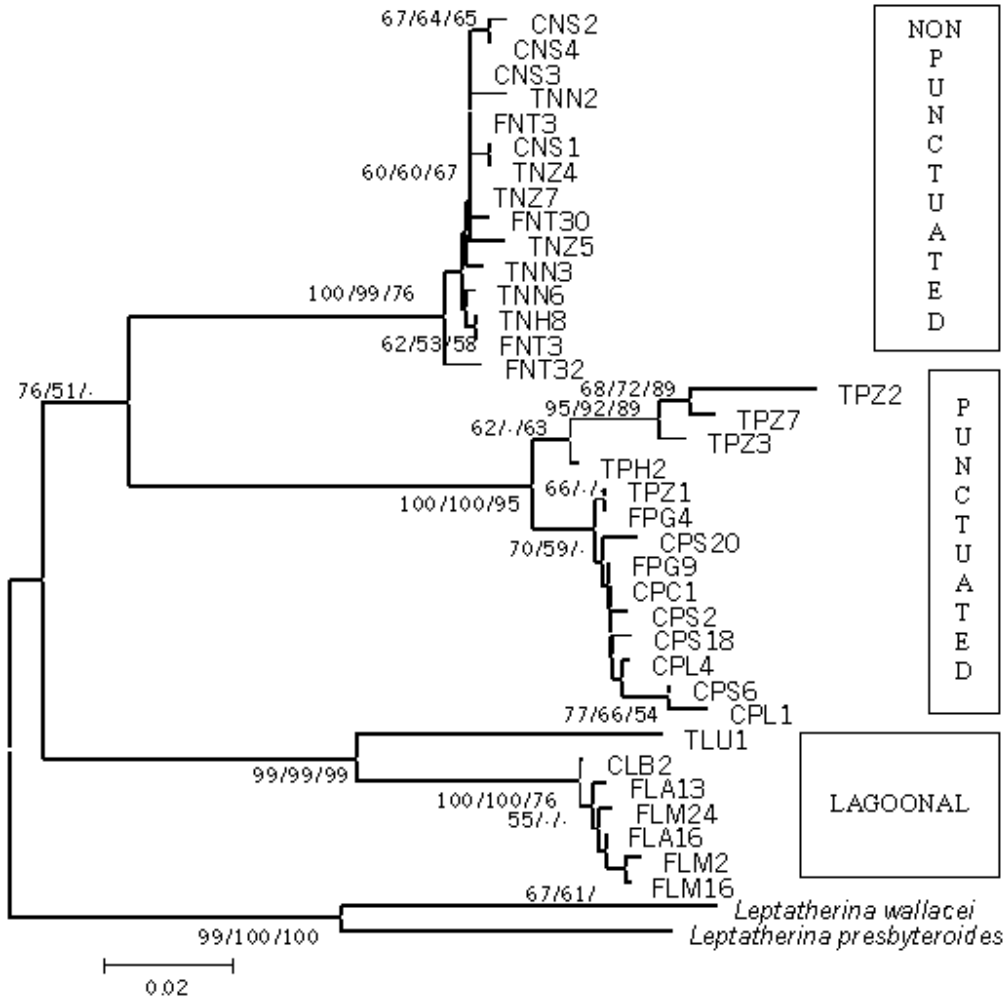

Fig. 4. Templeton's test indicates that the three trees are not significantly different in topology, so only the Neighbour-Joining tree is shown. Bootstrap analyses carried out with 1000 iterations using the 362 pb of the cytochrome *b* among 23 'haplotypes' of the *Atherina* complex: using the Neighbour-Joining method on a matrix of the Kimura two-parameter model (bootstrap value on the top left), Maximum Parsimony (middle bootstrap value) and Maximum Likelihood (right bootstrap value). The two *Atherinidae* (*Leptatherina wallacei* and *Leptatherina presbyteroides*) were used as outgroups and corresponded (in this case) to a midpoint rooting.

Table 2. Mean distance within group using the Kimura two-parameter model. Standard error estimated by the bootstrap method (500 replications).

Type of populations	*d*	S.E.
Punctuated (Tunisia)	0.017 75	0.004 02
Punctuated (France excepted Corsica)	0.001 98	0.001 92
Punctuated (Corsica)	0.007 71	0.002 45
Lagoonal (Tunisia)	0.000 00	0.000 00
Lagoonal (France excepted Corsica)	0.002 71	0.001 35
Lagoonal (Corsica)	0.000 00	0.000 00
Non-punctuated (Tunisia)	0.001 98	0.000 76
Non-punctuated (France excepted Corsica)	0.002 68	0.001 25
Non-punctuated (Corsica)	0.004 98	0.002 84

4. Discussion

4.1. Molecular analyses suggest the presence of three putative species within the *A. boyeri* complex

This study has revealed a remarkable level of divergence in the mitochondrial cyt *b* gene of sand smelts. The phylogenetic divergence between the three groups of sand smelts is very deep, comparable to, or greater than those seen between two species of Teleostei belonging to the same *genus* [13] (and references therein). Mitochondrial DNA only represents a small part of the genome and this should be kept in mind when making evolutionary inferences about populations and species [26]. However, these molecular results are strongly correlated to various other studies that, on the basis of morphological, morphometric and biochemical parameters, all clearly demonstrated three groups [12] (and references therein). As suggested by Trabelsi et al. [12], these three groups could be elevated to the rank of species. The first species consisted of non-punctuated marine specimens (*A. boyeri*), the second species of punctuated marine specimen (*A. punctata*) and the third species consisted of the lagoon specimens (*A. lagunae*). An important criterion for recognising species is the achievement of reproductive isolation in nature [14, 26, 27]. As the three putative

Table 3. Between-group average using the Kimura two-parameter model for the eight population samples. Standard error estimated by the bootstrap method (500 replications).

	1	2	3	4	5	6	7	8	9	10
Punctuated (Tunisia)		0.006 76	0.007 24	0.027 46	0.026 22	0.025 79	0.021 74	0.021 74	0.022 00	0.024 90
Punctuated (France excepted Corsica)	0.024 33		0.001 66	0.029 46	0.026 60	0.026 18	0.022 00	0.022 00	0.022 27	0.026 01
Punctuated (Corsica)	0.028 37	0.005 18		0.029 55	0.026 92	0.026 52	0.022 23	0.022 23	0.022 49	0.026 26
Lagoonal (Tunisia)	0.194 36	0.198 20	0.201 31		0.017 34	0.017 81	0.024 12	0.024 13	0.024 21	0.025 44
Lagoonal (France excepted Corsica)	0.183 30	0.179 52	0.184 54	0.084 47		0.003 20	0.025 06	0.025 05	0.025 38	0.026 04
Lagoonal (Corsica)	0.177 88	0.173 82	0.178 86	0.086 54	0.004 47		0.024 62	0.024 61	0.024 95	0.026 54
Non-punctuated (Tunisia)	0.133 12	0.128 85	0.133 57	0.162 65	0.159 85	0.154 99		0.000 82	0.001 82	0.023 56
Non-punctuated (France, excepted Corsica)	0.133 25	0.129 06	0.133 73	0.162 36	0.159 82	0.154 95	0.002 31		0.001 84	0.023 49
Non punctuated (Corsica)	0.136 40	0.132 34	0.137 09	0.165 06	0.163 55	0.158 65	0.003 92	0.004 36		0.023 64
Outgroup	0.194 92	0.200 22	0.204 26	0.200 06	0.207 57	0.210 06	0.181 80	0.180 76	0.182 58	

sand smelt species present morphological and molecular isolates, we could rule out the possibility of gene introgression between these three groups.

In spite of the absence of high bootstrap values, our molecular analyses demonstrated phylogenetic differentiation between lagoon and marine fishes. Numerous authors [6–9, 28–30], basing their argument on morphometric, meristic and biochemical characters, concluded in favour of two conspecific but divergent groups of populations within the *A. boyeri* complex: a 'brackish group' and a 'marine group'. In addition, recent morphoanatomical studies highlighted another group of population in the western Mediterranean Sea. This group differs from the sympatric marine group [10, 11, 31, 32]. The distinction between these two marine groups is based on colouring, morphological and biochemical characteristics. Interestingly, the different groups shared no haplotype; this result suggests that there is no gene flux between marine and lagoon fishes and that, in addition, in spite of the fact that the two marine subgroups are sympatric, the latter are clearly genetically separated.

4.2. Intra-subgroups analyses

If the wide phylogenetic differentiation between the three groups of western Mediterranean Sea sand smelts is the major finding, the analyses of intra-subgroups demonstrated a large difference between the structures of each new species. There was no structure within the non-punctuated sand smelts, little difference in the punctuated species and high degree of divergence between Tunisian versus French lagoon fishes.

4.2.1. Lack of polymorphism within non-punctuated marine sand smelts

The lack of significant variation throughout Tunisian and French non-punctuated fishes is somehow unexpected for populations that were collected in two regions more than 500 km apart from each other (from Tunisia to Corsica). A plausible explanation for the absence of differentiation between these populations seems to be a recent expansion from one of these regions. As this result strongly suggests recent gene flow, we could conclude that *A. boyeri* from Tunisian and French coasts belongs to the same population. In addition, migrations of individuals from France to Tunisia and in the opposite direction could not be excluded.

4.2.2. Weak genetic differentiation within punctuated sand smelts

Except for one Tunisian sequence of an atherine of Cap Zebib, which belongs to the French subgroup, phylogenetic results suggest that contrary to the non-punctuated fishes, weak genetic divergence was demonstrated in this group.

4.2.3. Phylogenetic analyses of lagoon sand smelts revealed a remarkable level of polymorphism

Surprisingly, the phylogenetic divergence between the two monophyletic groups of lagoon sand smelts (Tunisian versus French) is very deep and this is supported by high bootstrap values. Interestingly, the branch lengths in the two different subgroups have similar length ranges that could indicate a similar rate of evolution. The deep phylogenetic break between

Tunisian lineage and French lineage suggests that there has been a long-term barrier to the gene flow between these two populations. We could not exclude a genetic drift, which is frequently observed in closed environments. Interestingly, the high degree of polymorphism within the lagoon sand smelts has been confirmed by the analyses of meristic and morphometric characters. The marine populations are relatively homogeneous and are distinguished by more or less clear gradient values according to the considered features, but the lagoon populations are very heterogeneous and always have mean values smaller than those of the marine media for meristic characters [7, 9, 11]. The evolution of divergent phenotypes and resource use may precede or initiate the speciation process, or it may evolve after reproductive isolation via disruptive sexual selection has occurred and would thus facilitate the coexistence of species. Other hypotheses for speciation emphasise the importance of extrinsic factors for speciation, such as saline levels in lagoons. In the future, within this complex, it will be possible to identify the factors involved in this speciation process. In addition, numerous studies have demonstrated the explosive speciation within the Cichlidae family and various speciation models have been proposed (reviewed in [33]). Once the *A. boyeri* complex is completely analysed, the cichlid studies could help us to understand the evolution of *Atherina*. In addition, in contrast to what is found in cichlid fishes ([34] and references therein), no events of introgressions have been found in our study: this suggests that all three putative species have been reproductively isolated since the beginning of their separation.

5. Conclusion

The molecular genetic variability revealed in the present study between populations of sand smelts is consistent with earlier reports of large amounts of phenotypic variation between populations of the *A. boyeri* complex [6–9, 11, 31, 28, 29, 30, 32]. These phenotypic differences between populations of *A. boyeri* resulted in the proposition of more than 20 species [35]. Our phylogenetic analyses clearly demonstrate that *A. boyeri* is made of at least three monophyletic groups supported by high bootstrap values. In addition, the fishes of these three groups could be widely recognised as distinct species, with clear morphological, ecological and behavioural differences. Trabelsi et al. [12] confirm this proposition, using purely morpho-anatomical analyses. Interestingly, biochemical analyses (of the parvalbumin-distribution type) confirm these results ([12] and references therein).

In the future, the analysis of numerous populations of the *Atherina* genus could offer a unique opportunity to study different stages of adaptative radiations and to detect general and unique characteristics of their species flocks. Several Atherinidae lineages show unique levels of ecological diversification and species packing; fast speciation rates have been suggested by various authors [2, 36–40] (and references therein). At present, we do not have indications concerning the driving forces of atherine speciation.

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References

[1] O. Gon, A. Ben-Tuvia, The biology of Boyer's sand smelt, *Atherina boyeri* Risso in the Bardawil lagoon on the Mediterranean coast of Sinai, J. Fish Biol. 22 (1983) 537–547.

[2] P.A. Henderson, R.N. Bamber, On the reproductive strategy of the sand smelt *Atherina boyeri* Risso and its evolutionary potential, Zool. J. Linn. Soc. 32 (1987) 395–415.

[3] A. Kiener, C.J. Spillman, Contributions à l'étude systématique et écologique des athérines des côtes françaises, Mém. Mus. Hist. nat. Paris, nouv. ser., Ser. A: Zool. 40 (1969) 33–74.

[4] R. Bamber, P.A. Henderson, Morphological variation in British atherinids, and the status of *Atherina presbyter*, Cuvier (Pisces, *Atherinidae*), Biol. J. Linn. Soc. 25 (1985) 61–67.

[5] M. Kotelat, European freshwater fishes, Biologia, Bratislava 52 (1997) 1–271.

[6] J.-P. Marfin, Les problèmes liés au polymorphisme de l'espèce *Atherina boyeri* Risso, 1810, Cybium 6 (1982) 19–26.

[7] M. Trabelsi, F. Kartas, Contribution à l'étude des caractères numériques de l'athérine *Atherina boyeri* Risso, 1810 des côtes tunisiennes, Rapp. Comm. int. Mer. Médit. 29 (1985) 187–189.

[8] M. Trabelsi, Recherches sur le polymorphisme et la biologie de l'athérine *Atherina boyeri*, Risso, 1810 (Poisson, Téléostéen) des côtes de Tunisie, thèse de 3e cycle, Fac. Sci. Tunis, 1989, 239 p.

[9] F. Kartas, M. Trabelsi, Sur le polymorphisme de l'athérine, *Atherina boyeri* Risso, 1810 (Atherinidae) des eaux littorales tunisiennes, Cybium 14 (1990) 295–311.

[10] M. Trabelsi, B. Focant, J.-A. Tomasini, J.-P. Quignard, Originalité du complexe *Atherina boyeri* Risso, 1810 (Poissons, Téléostéens, Atherinides) de la réserve naturelle des îles Lavezzi (Corse), Trav. sci. Parc. nat. reg. Res. nat. Corse, Fr. 56 (1995) 83–105.

[11] M. Trabelsi, J.-P. Quignard, J.-A. Tomasini, M. Boussaid, B. Focant, F. Maamouri, Discriminative value of the meristic characteristics of the punctuated sand smelts marine populations, Obelia 26 (2000) 75–88.

[12] M. Trabelsi, É. Faure, J.-P. Quignard, M. Boussaid, B. Focant, F. Maamouri, *Atherina punctata* and *Atherina lagunae* (Pisces, Atherinidae) new species found in the Mediterranean Sea, 1. Biometric investigations of three Atherinid species, C. R. Biologies 325 (2002) 967–975.

[13] A. Gilles, A. Miquelis, J.-P. Quignard, É. Faure, Intraspecific variation of the cytochrome *b* gene in the dusky grouper (*Epinephelus marginatus*, Pisces, Serranidae, Lowe 1834), C. R. Acad. Sci. Paris, Ser. III 323 (2000) 327–337.

[14] J.C. Avise, Phylogeography: the history and formation of species, Harvard University Press, Cambridge, MA, USA, 2000.

[15] P. Taberlet, J.A. Bouvet, Single plucked feather as a source of DNA for bird genetic studies, The Auk 108 (1991) 58.

[16] A. Meyer, T.D. Kocher, P. Basasibawaki, A.C. Wilson, Monophyletic origin of Lake Victoria's cichlid fishes suggested by mitochondrial DNA sequences, Nature (London) 347 (1990) 550–553.

[17] P. Cantatore, M. Roberti, G. Pesole, A. Ludovico, F. Milella, M.N. Gadaleta, C. Saccone, Evolutionary analysis of cytochrome *b* sequences in some Perciformes evidence for a slower rate of evolution than in mammals, J. Mol. Evol. 39 (1994) 589–597.

[18] N. Saitou, M. Nei, The Neighbor-Joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.

[19] M. Kimura, A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120.

[20] S. Kumar, K. Tamura, M. Nei, MEGA: Molecular Evolutionary Genetics Analysis, Pennsylvania State University, University Park, PA, USA, 1993.

[21] D.L. Swofford, Phylogenetic analysis using parsimony (PAUP), Version 4.0, Sinauer Sunderland, MA, USA, 1998.

[22] D. Posada, K.A. Crandall, Modeltest: testing the model of DNA substitution, Bioinformatics 14 (1998) 817–818.

[23] J.S. Rogers, D.L. Swofford, A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences, Syst. Biol. 47 (1998) 77–89.

[24] K. Bremer, Branch support and tree stability, Cladistics 10 (1994) 294–304.

[25] A.R. Templeton, Convergent evolution and nonparametric inferences from restriction fragment and DNA sequence data, in: B. Weir (Ed.), Statistical analysis of DNA sequence data, Marcel Dekker, New York, 1983, pp. 151–179.

[26] J.C. Avise, K. Wollenberg, Phylogenetics and the origin of species, Proc. Natl Acad. Sci. USA 94 (1997) 7748–7755.

[27] D. Otte, J.A. Andler, Speciation and its consequences, Massachussetts Sinauer, Sunderland, 1989.

[28] B. Focant, E. Rosecchi, A.J. Crivelli, Attemps at biochemical characterization of sand smelt *Atheina boyeri* Risso, 1810 (Pisces, Atherinidae) population from the Camargue (Rhone Delta, France), Comput. Biochem. Phys. B: Biochem. 122 (1991) 261–267.

[29] B. Focant, M. Trabelsi, F. Kartas, J.-P. Quignard, Caractérisation biochimique de trois populations d'*Atherina boyeri* des milieux lagunaires méditerranéens, Rapp. Comm. int. Mer Médit. 33 (1992) 94.

[30] B. Focant, M. Trabelsi, P. Vandewalle, F. Kartas, J.-P. Quignard, Apport de l'analyse biochimique à la caractérisation de populations d'*Atherina boyeri* des milieux lagunaires méditerranéens, Actes du colloque Okeanos, « Le système littoral méditerranéen », Montpellier, 1993, pp. 145–148.

[31] M. Trabelsi, J.P. Quignard, F. Kartas, Atherina boyeri : première mention en Méditerranée de deux populations marines sympatriques, Cybium 18 (1994) 457–459.

[32] M. Trabelsi, J.-P. Quignard, J.-A. Tomasini, M. Boussaid, B. Focant, F. Maamouri, Discriminative value of the meristic characteristics of *Atherina boyeri* Risso, 1810 Lagoon populations, Vie et Milieu 52 (2) (sous-presse).

[33] P.D. Danley, T.D. Kocher, Speciation in rapidly deverging systems: lessons from Lake Malawi, Mol. Ecol. 10 (2001) 1075–1086.

[34] L. Ruber, A. Meyer, C. Sturmbauer, E. Verheyen, Population structure in two sympatric species of the Lake Tanganyika cichlid tribe Eretmodini: evidence for introgression, Mol. Ecol. 10 (2001) 1207–1225.

[35] A. Kiener, C.-J. Spillman, Contribution à l'étude systématique et écologique des athérines des côtes françaises, Mém. Mus. Hist. nat. Paris, Ser. A: Zoologie 60 (1969) 33–74.

[36] M.S. Johnson, R.J. Watts, R. Black, High levels of genetic subdivision in peripherally isolated populations of the atherinid *Craterocephalus capreoli* in the Houtman Abrolhos Islands, western Australia, Mar. Biol. 119 (1994) 179–184.

[37] R. Bamber, P.A. Henderson, Pre-adaptative plasticity in atherinids, and the estuarine seat of teleost evolution, J. Fish Biol. 33 (1988) 17–23.

[38] P. Yakovlevich, Fingerprinting fishes: detection of generic, interspecific and intraspecific variation in the mitochondrial cytochrome *b* gene of silverside fishes (Atherinomorpha: Atherinidae) using PCR/SSCP and DNA sequencing, Fish. Sahul. 13 (1999) 614–620.

[39] D.J. McGlashan, J.M. Hugues, Reconciling patterns of genetic variation with stream structure, earth history and biology in the Australian freshwater fish *Craterocephalus stercusmuscarum* (Atherinidae), Mol. Ecol. 9 (2000) 1737–1751.

[40] L.B. Beheregaray, P. Sunnucks, Fine scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*,, Mol. Ecol. 10 (2001) 2849–2866.