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Short Communication

Evolutionary history of the genus *Trisopterus* ☆

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ABSTRACT

The group of small poor cods and pouts from the genus *Trisopterus*, belonging to the Gadidae family, comprises four described benthopelagic species that occur across the North-eastern Atlantic, from the Baltic Sea to the coast of Morocco, and the Mediterranean. Here, we combined molecular data from mitochondrial (cytochrome *b*) and nuclear (rhodopsin) genes to confirm the taxonomic status of the described species and to disentangle the evolutionary history of the genus. Our analyses supported the monophyly of the genus *Trisopterus* and confirmed the recently described species *Trisopterus capelanus*. A relaxed molecular clock analysis estimated an Oligocene origin for the group (~30 million years ago; mya) indicating this genus as one of the most ancestral within the Gadidae family. The closure and re-opening of the Strait of Gibraltar after the Messinian Salinity Crisis (MSC) probably triggered the speciation process that resulted in the recently described *T. capelanus*.

1. Introduction

The marine environment offers a perfect baseline scenario for studying how different factors such as historical processes or life-history features have shaped the current genetic structure of marine fish populations. The history of evolutionary diversification in the Mediterranean Sea and Atlantic Ocean transition has been greatly linked to two main speciation events of vicariance and dispersion, respectively: the Messinian/Zanclean episode of desiccation and replenishment of the Mediterranean Sea (~6.0 and 5.3 mya), and the eustatic sea level fluctuation throughout the Pleistocene (~10,000 years ago). In this context, the genus *Trisopterus*, Rafinesque-Schmaltz, 1814, presents an interesting opportunity to test different biogeographic and evolutionary hypotheses of speciation. This genus includes only four species with a wide and overlapping distribution area in two different water bodies, the North-eastern Atlantic and the Mediterranean (Choen et al., 1990), i.e. an area exhibiting important topological and hydrological complexities and transition zones (Patarnello et al., 2007). All DNA-based phylogenies recovered *Micromesistius* as the sister group of *Trisopterus* (Bakke and Johansen, 2005; Roa-Varon and Orti, 2009; Teletchea et al., 2006) and identified *Gadiculus* as the stem group of all other Gadidae species (Bakke and Johansen, 2005). An ancient origin of the *Trisopterus* group within the gadids is not only suggested by its basal position in the Gadidae phylogeny (Bakke and Johansen, 2005), but also by fossil evidence, which indicates that the genus already inhabited the North Sea waters in the Oligocene (Woydack and Morales-Nin, 2001). However, despite several recent taxonomical works (e.g. see Mattiangeli et al., 2000), the taxonomic status of the genus needs further confirmation.

Originally, based on morphological characteristics, three *Trisopterus* species were described: *Trisopterus luscus*, Linnaeus, 1758, *Trisopterus minutus*, Linnaeus, 1758, and *Trisopterus esmarkii*, Nilsson, 1855. These species exhibit a large overlapping area across their distribution ranges. Thus, the Norway pout, *T. esmarkii*, is restricted to the Northern Atlantic Seas from Skagerrak and Kattegat (in the Baltic Sea) to around Iceland as well as south to the English Channel and Bay of Biscay (Choen et al., 1990). The pouting, *T. luscus*, is distributed from Skagerrak, North Sea, and the British Isles to about 25°N along the West African coast and, reportedly, also in the western Mediterranean (Choen et al., 1990). Finally, the poor cod, *T. minutus*, is known to have the widest distribution, from the Trondheim Fjord and the Faeroe Islands to the southern coast of Morocco, and into the Mediterranean Sea (Choen et al., 1990).

☆ This paper is dedicated to the memory of Prof. Ignacio Lozano, from Universidad de La Laguna (Tenerife), an enthusiast of fish biology and ecology and always an extraordinary support for the FishTrace project.

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Studies based on differences in parasites and morphology, i.e. vertebrae and gill raker counts, dorsal-fin and anal-fin formulae, together with information from fossil otoliths, established the basis for the morphological distinction of two morphs within the poor cod: *T. minutus minutus* in the North-eastern Atlantic Ocean and *T. m. capelanus* (La Cepède, 1800) in the Mediterranean Sea (Gaemers, 1976; Svetodivov, 1986; Tirard et al., 1992). However, recent molecular studies have suggested that the subspecies *T. minutus capelanus* from the Mediterranean may be considered as the separate species *T. capelanus* and consequently that *T. m. minutus* may be also considered as *T. minutus* (Mattiangeli et al., 2000). The main goal of this study is to elucidate the evolutionary history of the genus making an attempt to analyze the factors underlying the speciation processes. To achieve this goal, we used partial sequences of mitochondrial (mtDNA) (cytochrome *b*) and nuclear (nDNA) genes using specimens from all four described species of *Trisopterus*. To address the historical time frame of *Trisopterous* diversification we applied a relaxed molecular clock approach to disentangle if any of the two evolutionary scenarios described above (Miocene vicariance vs. Pleistocene dispersal) have left a significant imprint on the extant genetic variability of the genus or if other events caused the current diversity.

2. Materials and methods

2.1. Sampling collection and laboratory procedures

At least two (but as many as 12) samples were collected from nine locations of the Mediterranean and Baltic Seas, and from the Atlantic Ocean (Fig. 1A and Supplementary Table S1). Given the

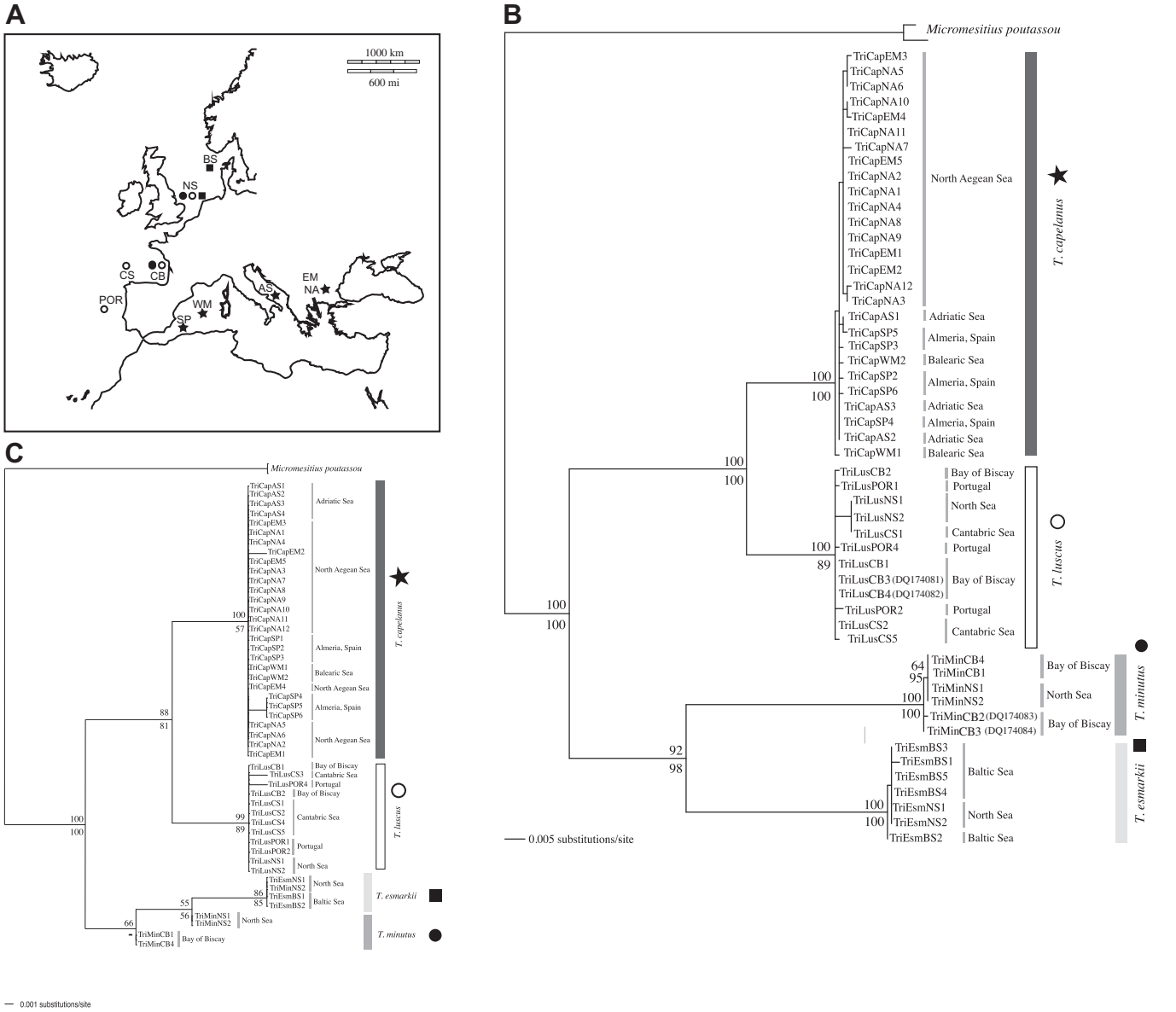

Fig. 1. (A) Sample locations of *Trisopterus* species included in this study. The acronyms used in A, B and C refers to the geographical origin of the samples (NS: North Sea; BS: Baltic Sea; CB: Bay of Biscay; CS: Cantabrian Sea; POR: Portugal coast; NA/EM: Two close sampling localities at the North Aegean Sea; AS: Adriatic Sea; SP: Santa Pola, Alicante, Spain; WM: Balearic Sea). Species in A, B and C are indicated as follows: square: *T. esmarkii*; open circles: *T. luscus*; solid circles: *T. minutus*; stars: *T. capelanus*. Details for sample sizes are listed in Table S1. (B) Phylogenetic relationships within *Trisopterus* based on a maximum likelihood (ML) analysis of the *cytb* data set using the TIM + Γ evolutionary model. Numbers above and below nodes correspond to maximum likelihood bootstrap proportions – BP, and Bayesian posterior probabilities – BPP, respectively. Only BP and BPP values above 50 and 75%, respectively, are represented. GenBank accession numbers are also included in brackets. (C) Phylogenetic relationships within *Trisopterus* based on a ML analysis of the Rho fragment using the HKY + I evolutionary model. Numbers above and below nodes correspond to maximum likelihood BP, and BPP, respectively. Only BP and BPP values above 50% and 75%, respectively, are represented.

uncertain taxonomic status of *T. minutus capelanus*, samples were obtained from four localities across the Mediterranean Sea (the Aegean Sea, the Adriatic Sea, the Alicante coast and the Balearic Sea) (Fig. 1A). Voucher numbers and other morphological characters obtained for most of the specimens can be found at http://www.fishtrace.org/. The complete mtDNA cytochrome *b* (*cytb*) and the nDNA rhodopsin (Rho) genes were amplified for all species using the primers and PCR conditions specified in (Sevilla et al., 2007).

2.2. Phylogenetic analyses

Alignments of nucleotide sequences were constructed with Clustal X version 1.83 using default parameters (Thompson et al., 1997), and verified by eye in order to maximize positional homology. Two different data sets were analyzed (see Table S1 for GenBank accession numbers): (1) partial nucleotide sequences of the mtDNA *cytb* gene from 52 specimens of *Trisopterus* produced an alignment of 1103 base pairs (bp). Of these, 821 were constant and 271 were parsimony informative, and (2) partial nucleotide sequences of the nuclear DNA Rho gene from 49 specimens of *Trisopterus* produced an alignment of 450 bp. Of these, 414 were constant and 33 were parsimony-informative. The putative sister species *Micromesistius poutassou* was selected as outgroup (Bakke and Johansen, 2005) for both data sets.

Bayesian inferences (BI) were conducted with MrBayes v3.1.2 (Huelsenbeck et al., 2001) using both data sets. The Akaike Information Criterion (AIC) implemented in Modeltest (Posada and Crandall, 1998) selected TIM + Γ and HKY + I as the best-fit models for the mtDNA and nDNA analysis, respectively. PhyML v2.4.4 (Guindon and Gascuel, 2003) was used to estimate the maximum likelihood (ML) tree. Because TIM + Γ model is not available in PhyML, the TrN + Γ (the second best-fit model, according to Modeltest) was used in the ML analysis of the mtDNA data set.

In order to further evaluate the taxonomic status of *T. minutus*, the approximate unbiased (AU), the Kishino–Hasegawa (KH), and the Shimodaira–Hasegawa (SH) tests were performed based on the nuclear data set. These tests are implemented in Consel v0.1i and use log-likelihoods of site-patterns of the trees estimated with Paml v.4.

2.3. Divergence time estimation

Divergence times within the genus *Trisopterus* were estimated using a Bayesian relaxed molecular-clock approach, as implemented in Beast version 1.4.8 (Drummond and Rambaut, 2007) using both data sets. Dating analyses were performed using the evolutionary models described above for each data set. Two calibrations based on the available fossil record were used in both data sets. One, was based on the first appearance in the fossil record of the genus *Trisopterus* dated from the Lower Oligocene–Rupelian [33.9–28.4 mya] (Woydack and Morales-Nin, 2001). The other, was set at the Miocene [23–5.3 mya] corresponding to the minimum age of *T. luscus* (Girone et al., 2006). Calibrations were modeled with a lognormal distribution, where 95% of the prior weight fell within the geological interval in which each fossil was discovered. Accordingly, and to establish the parameters of the lognormal calibration of the first appearance of the genus *Trisopterus* we set the hard minimum bound at 28.4, log mean 1.012, and standard deviation at 0.423. The minimum age of *T. luscus* was also modeled with a lognormal calibration in which the hard minimum bound was set at 5.3 log mean 2.18, and standard deviation 0.4225. MCMC were performed in Beast with 2×10^7, following a discarded burn-in of 2.000,000 steps for both data sets. The convergence to the stationary distribution was confirmed by inspection of the MCMC samples using the program Tracer v1.5 (Rambaut and Drummond, 2007).

All ML, BI and dating analyses were carried out on the freely available Bioportal (http://www.bioportal.uio.no).

2.4. Population genetic structure and historical demography

Mean haplotype genetic distances (*D*) were calculated with Mega5.0 software (Tamura et al., 2007), for all the mtDNA dataset within and between species. Because the TIM + Γ model was not implemented in Mega5.0, to calculate *D* we applied the Kimura 2-parameters model. The standard errors (*SE*) of the genetic distances were calculated using bootstrapping with 1.000 replicates. The sequences for *M. poutassou* were used to estimate *D* between species.

An analysis of molecular variance (AMOVA) and mtDNA pairwise haplotype divergences (using the fixation index Φ_{ST}) between *T. capelanus* sampling sites (which accounts for the larger number of samples, $n = 27$) were estimated with Arlequin 3.11 (Excoffier et al., 2005). Significance of pairwise comparison was tested by 10,000 permutations. Moreover, D^* (Tajima, 1989), R^2 (Ramos-Onsins and Rozas, 2006) and *Fu's Fs* (Fu, 1997) tests of mutation/drift equilibrium were performed with DnaSP 5.10 (Librado and Rozas, 2009) for this species. Finally, the *T. capelanus* sequences were analyzed for possible historical events of population growth or decline, i.e. the mismatch distribution (Rogers and Harpending, 1992), as implemented in DnaSP 5.10. Deviations from the sudden population expansion model were further tested using the *H*ri index (Harpending, 1994). Time and magnitude of the inferred population expansion was determined by calculating τ (the age of the population expansion), Θ_0 and Θ_1, which correspond to the effective female population size before and after the expansion, respectively.

3. Results

3.1. Genetic diversity and gene divergence

The higher divergence value was observed among *T. capelanus*/*T. minutus* (mtDNA *p* values raging from 3.8% to 15%). Congruently, the overall *p* distance from *Trisopterus* mtDNA haplotypes to the outgroup (*M. poutassou*) was almost a magnitude higher (average ± *SE* = 17.4% ± 1.9%) than the intraspecific distances. The values for the nuclear sequence divergence between sister species were shown to be lower than the mtDNA values (average ± *SE* = 2.4% ± 0.7%), but yet five-fold lower than the intraspecific distance values with the outgroup (average ± *SE* = 11.3% ± 1.7%). The low variability of the nuclear gene is reflected in the interspecific genetic distances obtained, which are close to zero (Table 1).

3.2. Phylogenetic relationships within Trisopterus

The ML ($-\ln L = 3337.37$) and BI ($-\ln L = 3353.08$) analyses based on the mtDNA data set arrived at an identical topology and clearly differentiated four clades corresponding to *T. luscus*, *T. capelanus*, *T. esmarkii*, and *T. minutus*. ML ($-\ln L = 998.11$) and BI ($-\ln L = 979.40$) analyses based on the nDNA data set recovered three well-supported clades corresponding to the three first abovementioned species but *T. minutus* was not recovered as monophyletic. Nevertheless, the AU, SH, and KH tests of alternative tree topologies using the nuclear data set did not reject the monophyly of *T. minutus* (AU: $P = 0.069$; KH: $P = 0.094$; SH: $P = 0.094$). All topologies described a sister species relationship between *T. luscus* and *T. capelanus* and between *T. minutus* and *T. esmarkii*.

3.3. Splitting events within Trisopterus

Date estimates for the origin of the genus *Trisopterus* were quite similar either using mitochondrial or nuclear data (Fig. 2A and B).

Table 1
Mean genetic divergences (±*SE*) values within and between species, based on mitochondrial and nuclear genes.

Species	*cytb*	Rho
T. luscus/*T. capelanus*	0.038 ± 0.007	0.019 ± 0.007
T. esmarkii/*T. minutus*	0.098 ± 0.013	0.009 ± 0.004
T. capelanus/*T. minutus*	0.149 ± 0.018	0.025 ± 0.008
T. luscus/*T. minutus*	0.136 ± 0.016	0.025 ± 0.008
T. luscus/*T. esmarkii*	0.124 ± 0.016	0.032 ± 0.009
T. capelanus/*T. esmarkii*	0.136 ± 0.017	0.035 ± 0.009
T. capelanus (East Med.)/*T. capelanus* (West Med.)	0.001 ± 0.000	0.001 ± 0.001
T. minutus (North Sea)/*T. minutus* (Bay of Biscay)	0.000 ± 0.013	0.008 ± 0.004
T. minutus (Bay of Biscay)/*T. esmarkii*	0.098 ± 0.014	0.013 ± 0.006
T. minutus (North Sea)/*T. esmarkii*	0.097 ± 0.014	0.005 ± 0.004
T. esmarkii/*M. poutassou*	0.175 ± 0.020	0.046 ± 0.011
T. luscus/*M. poutassou*	0.178 ± 0.020	0.049 ± 0.011
T. minutus/*M. poutassou*	0.169 ± 0.019	0.040 ± 0.010
T. capelanus/*M. poutassou*	0.175 ± 0.019	0.054 ± 0.012
T. luscus	0.004 ± 0.001	0.000 ± 0.000
T. esmarkii	0.000 ± 0.000	0.000 ± 0.000
T. capelanus	0.001 ± 0.000	0.000 ± 0.000
T. minutus	0.001 ± 0.001	0.005 ± 0.003

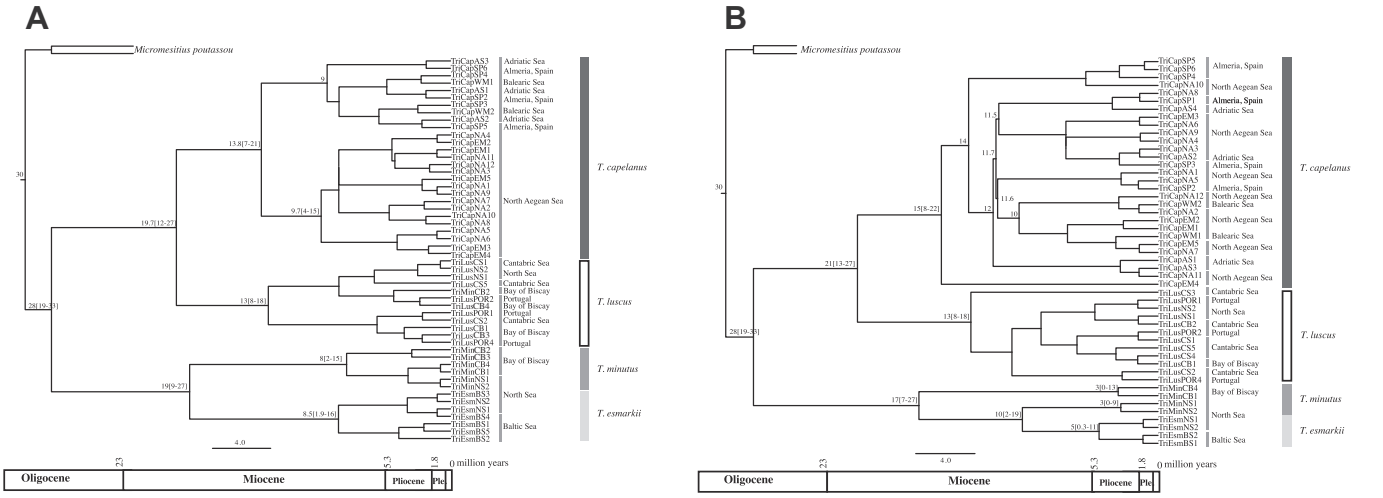

Fig. 2. BEAST maximum clade credibility chronogram based on the *cytb* (A) and the Rhod (B) dataset, respectively. Age estimates in million years and corresponding 95% highest posterior density intervals (values in square brackets) are depicted.

According to our estimates, the diversification of the genus began in the Oligocene at 28 mya (Fig. 2). The divergence between *T. luscus* and *T. capelanus* occurred at about 21 [13–28] or 19.7 [12–27] mya depending on whether estimates were based on the nDNA or the mtDNA data sets. The divergence of *T. esmarkii* and *T. minutus* is estimated to have occurred at approximately 17 [7–28] or 19 [9–27] mya and of *T. luscus* was 13 mya, using nDNA or mtDNA data, respectively. The age of the MRCA of *T. minutus* was estimated at 8 [1.8–15] mya using the mtDNA data set. This is the only available estimate for the origin of *T. minutus* because the species was not recovered as monophyletic using the nuclear data set. The origin of *T. esmarkii* was estimated at 4.8 [0.3–11] mya using the nuclear data set (or at 8.5 [1.9–16] mya with mitochondrial data).

3.4. Demographic analysis of T. capelaus

Results of AMOVA indicated the higher intrapopulation variation (% of variation within populations = 99.41 %; $\Phi_{ST} = 0.25$; $P < 0.027$) when *T. capelanus* samples were separated by the North Aegean Sea (NA and EM, thereafter named as East Mediterranean) and Alicante, Balearic and Adriatic Seas (AS, SP and WM, thereafter named as West Mediterranean). The neutrality test gave significant negative results for *T. capelanus* and the two putative *T. capelanus* populations (East and West Mediterranean Sea) previously identified (Table 2), which along with the low and significant R^2 index obtained for this species and populations, are consistent with a model of recent expansion (Table 2). Conversely, the unimodal distribution of the *T. capelanus* data set was not significantly different from that predicted by the growth expansion model (as measured by the PD value; $p = 0.402$, Table 2). The observed unimodal shape mainly corresponds with the number of haplotype differences within the eastern individuals (that are defined by two unique haplotypes not shared by any other sample from the West Mediterranean, Fig. S1). The separate analysis of East and West *T. capelanus* yielded in both cases a unimodal distribution (Fig. S1B and C) with a significant mismatch distribution (Table 2). However the interpretation of this signal for population expansion differs when the rest of the demographic variables are compared. Estimated Θ_0 was higher than Θ_1 indicating a process of expansion. However, this difference was almost 20 times higher for the East Mediterranean population in comparison with the West Mediterranean population. Moreover, estimated τ-values were very different (and lower for the East samples), indicating that population expansion in both groups may date from different historical periods. Similarly, the *H*ri was almost five fold lower in the case of the East Mediterranean population, indicating a significant fit of the observed

Table 2
Demographic parameter estimates for *T. capelanus* (and samples from East and West Mediterranean *T. capelanus* localities) cytochrome *b* (*cytb*) data set.

Statistics	All species	*T. capelanus*	East Mediterranean samples	West Mediterranean samples
Fu's Fs	13.56	**−7.920*****	**−3.892****	**−4.523****
FS p-value	0.999	0.000	0.00107	0.009
Ramos-Onsins & Rozas' R^2	0.156	**0.056*****	**0.1017***	**0.0915*****
R^2 *p*-value	0.958	0.000	0.0245	0.000
Tajimas'D	1.179	**−1.865****	**−1.436***	**−1.839****
T'D *p*-value	0.928	0.001	0.046	0.003
PD		No sign	***	*
Hri		0.16	0.08	0.39
Θ_0		0.799	0	2.95
Θ_1		51,643	99,782	17,878
τ		4.16	0.02	19.67

PD: mean number of pairwise differences; no sign: non-significant.
* *p*-Value < 0.05.
** *p*-Value < 0.01.
*** *p*-Value < 0.001.

and expected distributions, and therefore further evidence of population expansion (Table 2). Finally, a Θ_0 greater than zero before expansion was observed in the West Mediterranean samples, possibly indicating an older age for this population.

4. Discussion

The existence of four monophyletic groups corresponding with the three nominal species *T. esmarkii*, *T. luscus* and *T. minutus* and the recently described *T. capelanus* were recovered in all our mtDNA analyses, in agreement with previous species classification based on morphological and genetic data (Mattiangeli et al., 2000). The two morphotypes of *T. minutus*, traditionally considered as two subspecies (*T. m. capelanus* and *T. m. minutus*), are genetically identified as two different species (Fig. 1), confirming the existence of the recently described species, *T. capelanus*. Evidence obtained from the genetic divergence among sister species also supports these results (Table 1). The new distribution of *T. capelanus* would be restricted to the Mediterranean Sea whereas *T. minutus* occurs in the Atlantic Ocean. However, a more thorough sampling of these two species in the contact zone (probably around the Strait of Gibraltar) is necessary to better define their distributional ranges. *T. minutus* was not recovered as monophyletic in our nuclear-based ML and BI trees (Fig. 1). Specimens from the North Sea clustered with *T. esmarkii* although with low statistical support, instead of grouping with specimens from the Bay of Biscay. In contrast, the individuals of *T. minutus* from the North Sea population have an almost identical mtDNA sequence to that of the *T. minutus* from the Bay of Biscay (Fig. 1). Nevertheless, the non-parametric likelihood-based AU, SH, and KH tests using the nuclear data set did not reject the monophyly of *T. minutus*. Considering these results, *T. minutus* taxonomic status remains unequivocal.

Regarding the divergence time estimations, ancestral *Trisopterus* species are thought to have originated and diversified in the North Sea Basin throughout the Tertiary Period (Woydack and Morales-Nin, 2001). By using a sequence divergence rate of 3.5% for the *cytb* gene and applying a molecular clock to calibrate the phylogenetic tree, Bakke and Johansen (2005) determined the time of divergence of *Trisopterus* and *Micromesistius* sister groups at about 11 mya. Considering that the fossil record of Gadidae (in which the genus *Trisopterus* is included) dates back to the Oligocene, Bakke and Johansen (2005) refer that this divergence date might be underestimated as a result from saturation of nucleotide positions. We obtained a significantly earlier divergence estimate for this splitting in the Lower Oligocene, at about 32 mya (Fig. 2). Our age estimates were based on both nDNA and mtDNA using a Bayesian relaxed molecular-clock approach, which by accommodating some uncertainty in evolutionary rates and calibration times, is expected to yield more reliable estimates than rate constancy. Moreover, nuclear and mitochondrial genes yielded very similar age estimates, although their different coalescent times (Barrowclough and Zink, 2009), and estimates fell within the fossil record available for this genus. Bakke and Johansen (2005) based their estimates on a single gene (*cytb*) and assumed rate constancy, which recognizably might produce inaccurate estimates, as rates could vary among lineages (Ayala, 1997; Britten, 1986). Nevertheless, as in any estimation, our dating analysis should be considered cautiously, particularly the nodes that exhibit higher confidence intervals.

All reconstructed phylogenies recovered *T. capelanus* and *T. luscus* as sister taxa (Fig. 1). This pattern is somehow intriguing considering their geographic distribution. A sister relationship between (*T. minutus* + *T. esmarkii*) and *T. luscus* would be more expectable considering that all three lineages inhabit the Atlantic Ocean, and *T. capelanus* occurs in the Mediterranean. According to our estimates, the MRCA of *T. capelanus* and *T. luscus* occurred at about 21 mya (or 19.7 mya depending on whether estimates were based on nuclear or mitochondrial data – Fig. 2). These divergence dates coincide fairly with the final closure of the Tethys Ocean that ceased the connection between the Mediterranean and the Indian Ocean (Ricou, 1987). This genus is not represented in the Indian Ocean thus, if this vicariant event triggered the speciation process that resulted in the formation of *T. luscus* and *T. capelanus*, the expected phylogeographic pattern would be a sister relationship between Atlantic and Mediterranean species. Considering that the geographic distribution of the MRCA of *T. capelanus* and *T. luscus* included the Mediterranean Sea, the present-day occurrence of *T. capelanus* in this area needs further explanation, given the episode of desiccation and replenishment of the Mediterranean Sea during the MSC (~6.0 and 5.3 mya). A possible evolutionary scenario would involve a re-colonization of the Mediterranean after its refilling by the ancestral lineage that later evolved into that species, while the Atlantic populations diverged into *T. luscus*. The alternative scenario of a postglacial invasion of the Atlantic Ocean from the warm-adapted *T. capelanus*, as has been hypothesized for other marine fishes (Bargelloni et al., 2005; Charrier et al., 2006), seems more unlikely considering the extreme conditions in the Mediterranean during MSC, which had probably conducted to the extinction of most of the pelagic fauna in the area.

Finally, demographic analysis and Φ_{ST} pairwise comparisons supported genetic differentiation of *T. capelanus* populations, one occurring in the East Mediterranean Sea (North Aegean Sea) and the other in the West/(Central) Mediterranean Sea (Alicante, Baleric and Adriatic Seas). Indeed, the mismatch pairwise distribu-

tions rendered a significant signal for population expansion (Fig. S1), while the demographic parameters obtained indicated an earlier timing in the expansion process for the West Mediterranean population in comparison with the East Mediterranean one (Table 2). Although the genetic divergence between the two populations is lower than that observed in other gadoid's subspecies divergence (0.09–0.25%, Van Houdt et al., 2003) they still might be constitute distinct evolutionary significant units and not a single panmictic population in the Mediterranean Sea. However, since the recovered trees in any of the ML or BL analyses, had no phylogeographic structure this assumption should be taken cautiously. Additional samples and studies on the population dynamics of *T. capelanus* samples across the Mediterranean Sea are needed to further understand the factors that might be promoting the geographical split between the two (East and West) genetic population groups.

In conclusion, the deeper lineage splitting events within the genus *Trisopterus* took place during the Upper Oligocene (e.g. the MRCA of the four currently described lineages occurred at about 28 mya, Fig. 2) and seem to be unrelated to the vicariance vs. dispersal hypotheses associated to the Messinian/Zanclean episode of desiccation and replenishment of the Mediterranean Sea (~6.0 and 5.3 mya) or the eustatic sea level fluctuations throughout the Pleistocene (~10,000 years ago). Nevertheless, the closure and reopening of the Strait of Gibraltar after the MSC probably triggered the speciation process that resulted in the recently described *T. capelanus*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.11.032.

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