Origin, diversification, and historical biogeography of the genus *Trachurus* (Perciformes: Carangidae)

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Abstract

We addressed phylogenetic relationships in the genus *Trachurus* using cytochrome *b* gene and D-loop sequences. The trees showed five groups: (1) the Southwest Pacific species (*T. japonicus*, *T. novaezelandiae*, and *T. declivis*); (2) The Mediterranean Sea and Eastern Atlantic species (*T. mediterraneus*); (3) The Atlantic Ocean species (*T. lathami* and *T. trecae*); (4) Eastern Atlantic species (*T. trachurus* and *T. capensis*); and (5) a group of highly mobile pelagic species, two from the Eastern Pacific (*T. symmetricus* and *T. murphyi*) and one from the Eastern Atlantic (*T. picturatus*). The phylogeny based on Cyt b, supports the molecular clock hypothesis and our results agree with the reported fossil indicating that the origin of this genus occur when the Thetys Sea closed (around 18.4 MYA). In addition, a very slow neutral substitution rate is reported identified only two periods of maximum diversification: the first occurring between 18.4 and 15.0 MYA and the second between 8.4 MYA and present day.

Keywords: *Trachurus*; Biodiversity; Molecular clock; Fish evolution; Biogeography; Evolutionary rate

1. Introduction

The species of the genus *Trachurus* are widely distributed along the coasts and throughout oceanic waters of temperate, tropical and subtropical seas (Eschmeyer, 2003). The most recent review of mackerels identified 14 species in the genus *Trachurus* (Nekrasov, 1994): *T. aleevi*, *T. capensis*, *T. declives*, *T. delagoa*, *T. indicus*, *T. japonicus*, *T. lathami*, *T. mediterraneus*, *T. murphyi*, *T. novaezelandiae*, *T. picturatus*, *T. symmetricus*, *T. trachurus*, and *T. trecae*. Later, Eschmeyer (2003) incorporated *T. longimanus*, although this author recognizes that the current status of this species in the genus is questionable given that it is considered a synonym of *T. picturatus* (Smith-Vaniz et al., 1990). Researchers have centered the majority of their efforts on morphological and ecological studies of species in this group, nevertheless, the taxonomic and phylogenetic relationships within this taxon remain controversial (Ben Salem, 1995; Berry and Cohen, 1972; Karaiskou et al., 2003; Kijima et al., 1988; Oyarzún, 1998; Poulin et al., 2004; Shaboneyev, 1981; Stepien and Rosenblatt, 1996; Suda et al., 1995).

The taxonomy of the genus *Trachurus* was first described by Nichols (1920, 1940) who recognized a total of 12 species, and suggested that this taxon should be represented by three geographically isolated races (i.e., trachurus, mediterraneus, and picturatus). Later, Berry and Cohen (1972) used additional morphological

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characteristics to raise the subspecies *T. mediterraneus indicus* to the rank of species (*T. indicus*) and to redescribe *T. delagoa* as a new species (*T. margaretae*), resulting in a total of 13 described species. Shortly after, Nekrasov (1976) revised this taxonomy, considering *T. capensis* to be a subspecies of *T. trachurus*, therefore reducing the count to 12 species. Subsequently, Shaboneyev (1981) proposed a new classification for the genus, in which groups were built by considering that closely related species share communities of origin and have similar morpho-ecological characteristics. Based on this classification, Shaboneyev presented a taxonomy with 11 species and 6 subspecies, which were divided into three groups: (1) the trachurus group (*T. trachurus trachurus*, *T. t. capensis*, *T. novaezelandiae*, *T. delagoa*, *T. japonicus*, and *T. declives*), characterized by having large scutes and remnants of many primitive characters; (2) the picturatus group (*T. picturatus*, *T. symmetricus murphyi*, and *T. s. symmetricus*) characterized by having low body depth, a large number of scutes, and inhabiting areas beyond the continental shelf; and (3) the mediterraneus group (*T. mediterraneus* , *T. m. ponticus*, *T. trecae*, *T. indicus*, and *T. lathami*) characterized by small scutes, high body depth, and inhabiting coastal areas. This author suggested that the genus *Trachurus* originated in the Tethys Sea (present day Mediterranean Sea) during the Miocene. According to the evolutionary pattern proposed by Shaboneyev, the most primitive forms are present in the mediterraneus group, while the most advanced forms occur in the picturatus group. The third group, trachurus, is regarded as a new and independent branch, characterized by the presence of both primitive and advanced forms. To ratify this evolutionary hypothesis, Ben Salem (1988, 1995) and Ben Salem and Ktari (1992) utilized the numerical taxonomy of 13 external morphological features to develop a more theoretical construction of the phylogenetic relationship. As mentioned above, the most recent revision of the genus by Nekrasov (1994) lists 14 species.

Phylogenetic analyses based only on morphology may result in misleading phylogenetic information, since this type of characteristic increases the chance of using homoplasy in the phylogenetic tree reconstruction (e.g., Kocher and Stepien, 1997). A molecular phylogenetic approach decreases the chance of using homoplasy (e.g., Nei and Kumar, 2000). Nevertheless, in the genus *Trachurus* molecular studies have been limited to resolving taxonomic conflicts at local scales (Astorga and Galleguillos, 1998; Kijima et al., 1988; Oyarzún, 1998; Poulin et al., 2004; Smolenski et al., 1994; Stepien and Rosenblatt, 1996). Recently, Karaiskou et al. (2003) studied the phylogeny of three species in this genus (*T. trachurus*, *T. mediterraneus*, and *T. picturatus*) based on partial mitochondrial DNA (mtDNA) sequences of Cyt b and the 16S rDNA segment. These authors found that, among the three species, *T. picturatus* and *T. mediterraneus* were more closely related, and they suggested that the origin of the three species was in the Straits of Gibraltar 2–5 MYA, showing a split during the Pliocene (Messinian). However, in this study the absence of species from other regions and the use of incomplete gene sequences for the phylogenetic analysis, does not allow researchers to draw firm conclusions regarding the evolutionary history of the genus *Trachurus*.

In this study, we assessed the phylogenetic relationships among 11 recognized species of the *Trachurus* genus, using two mitochondrial molecular markers: the Cyt b gene and D-loop. Here we reevaluate the previous hypothesis (Shaboneyev, 1981) regarding the evolutionary history, origin, and diversification patterns of *Trachurus*. We use phylogenetic analyses and molecular clock calibrations to resolve the biogeographic controversy surrounding the divergence time of the genus and between species in the genus.

2. Materials and methods

2.1. Samples

Of the 14 well-recognized species of the genus *Trachurus* we used the 11 species presenting the most representative geographical distribution of the genus and which are of major economic importance (Fig. 1): five from the Pacific Ocean (*T. japonicus*, *T. declives*, *T. novaezelandiae*, *T. symmetricus*, and *T. murphyi*); five species inhabiting the Atlantic Ocean and Mediterranean Sea (*T. mediterraneus*, *T. lathami*, *T. picturatus*, *T. trachurus*, and *T. trecae*) and one species from the coast of South Africa (*T. capensis*). The three species inhabiting the western Indian Ocean (*T. aleevi*, *T. delagoa*, and *T. indicus*) are not included in this study because it was difficult to obtain access to samples.

Cyt b sequences for the molecular phylogenetic analyses of three species were obtained from the GenBank database (*T. japonicus*, Accession No.: AP003092; *T. lathami*, AF363748; and *T. trecae*, AY050740; see Reed et al., 2002). Sequences for the remaining eight species were obtained from tissue samples of specimens (two specimens per species with the exception of *T. murphyi*, in which three specimens were used) collected from their native ranges of distribution.

An additional molecular analysis was performed using the mtDNA Control Region (D-loop). For this analysis we obtained sequences from the same eight species mentioned above, and the sequence for *T. japonicus* was obtained from the GenBank database (Accession No. AP003092).

2.2. DNA extraction, amplification, and sequencing

Total DNA was extracted from muscle tissue using the standard phenol:chloroform:isoamyl alcohol procedure

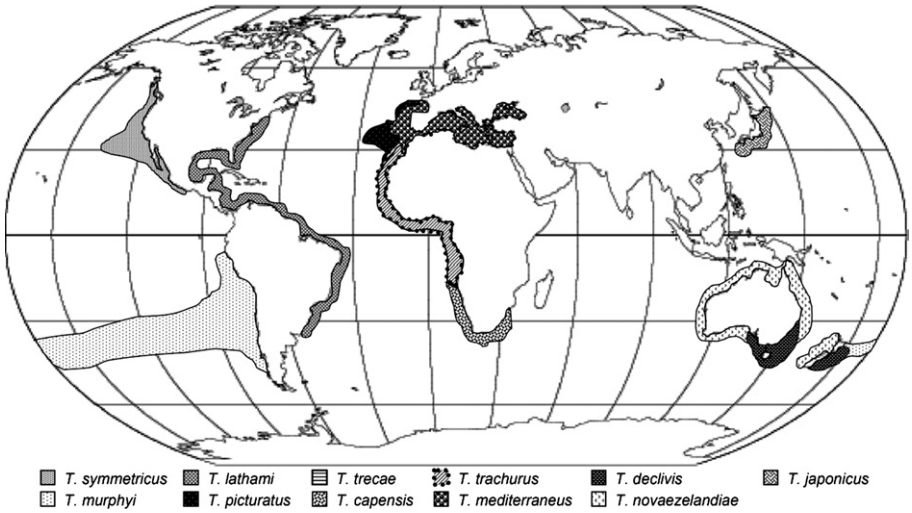

Fig. 1. Map of distribution of *Trachurus* species used in this study.

(Sambrook et al., 1989). Amplifications of the Cyt b gene (1140 bp) were carried out using primers designed from tRNA-Glu and tRNA-Thr sequences of members of the Carangidae family (CTB-F: 5′-ATG GCA AAT CTC CGT AAA ACC C-3′ and CTB-R: 5′-AGG CTC ATC CGA GCA TTT TA-3′). One internal primer was necessary for the total amplification of this gene (F416: 5′-TTT CCG CTG TCC CCT ACG TAG-3′). Subsequently, we amplified and sequenced the D-loop segment (860 bp) of the *Trachurus* specimens using primers described by Poulin et al. (2004).

Double-stranded DNA amplification of Cyt b was performed in 15 µl of reaction volume containing 0.2 U *Tth* polymerase (BIOTOOLS, Spain), 1.5 µl of 10× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, and approximately 5–10 ng of template DNA. The thermalcycling amplification conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of strand denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, primer extension at 72 °C for 45 s, and a final 10 min elongation at 72 °C. The D-loop amplification conditions were: 2.5 µl of 10× buffer reaction, 3.2 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, and approximately 5 ng of template DNA. The thermalcycling amplification conditions for D-loop were the same as for Cyt b, except that the annealing temperature was performed at 55 °C for 90 s. The size of the PCR products for both mitochondrial markers was checked by comparing with a 100 bp DNA ladder (GENCRAFT, Germany) in 1.5% agarose gel, run in 1× TBE buffer, and stained with 0.5 µg/µl ethidium bromide. Amplified DNA was purified with the QIAquick PCR Kit according to the supplier's protocol (Qiagen, USA). Finally, all samples were sequenced in the forward and reverse direction with an automated DNA-sequencer (Model ABI3100, Applied Biosystems) under the following conditions: polymer POP6, Byg Dye Terminator V.3, voltage at 12 V, temperature at 50 °C and run time of 7500 s. The new sequences have been deposited in the GenBank database under the accession numbers shown in Table 1.

2.3. Sequence analyses

Initial alignments of the Cyt b gene and D-loop sequences were performed with the CLUSTAL X program (Thompson et al., 1997), and final alignments were adjusted by eye. Statistics on nucleotide composition were compiled using DnaSP version 3.15 (Rozas and Rozas, 1999) and DAMBE (Xia and Xie, 2001) programs. We performed a test introduced by Xia et al. (2003) to measure substitution saturation in a set of aligned nucleotide sequences, to evaluate whether these sequences are useful for phylogenetic analyses.

2.4. Phylogenetic analysis

For phylogenetic analyses of the genus *Trachurus* based on Cyt b we used sequences of *Decapterus punctatus* (GenBank: AY050732) as an outgroup, since this species has a close phylogenetic relationship with *Trachurus* (Reed et al., 2002). Phylogenetic analyses based on D-loop were conducted without an external group, due to a lack of suitable sequences from the closest related species; therefore we used an unrooted tree for phylogenetic inference.

The reconstruction of phylogenetic trees was performed using PAUP* software (Swofford, 2002). To evaluate phylogenetic relationships we used three distinct phylogenetic approaches: a distance-based method using neighbor joining (NJ) (Saitou and Nei, 1987), maximum parsimony (MP), and maximum likelihood (ML). The simplest ML model that best explained the data were estimated using the Akaike Information Criterion (AIC) in the program MODELTEST 3.0 (Posada and Crandall, 1998). Results of MODELTEST indicated that the Tam-

Table 1
Geographical origin and GenBank Accession No. for mtDNA gene sequences of *Trachurus* species sequenced in this study

Species	Origin		Locality	GenBank Accession No.	
	Latitude	Longitude		Cyt b	D-loop
T. capensis 10	34.29S	18.43E	South Africa	AY526535	AY533480
T. capensis 13	34.22S	17.43E	South Africa	AY526536	AY533481
T. declives I1	37.27S	177.51E	Tauranga, NZ	AY526542	AY533476
T. declives I2	37.27S	177.51E	Tauranga, NZ	AY526543	—
T. mediterraneus 4	38.10N	21.05E	Ionian Sea, Greece	AY526548	AY533479
T. mediterraneus 5	38.10N	21.05E	Ionian Sea, Greece	AY526549	—
T. murphyi IQ	20.07S	70.11W	Iquique, Chile	AY526537	AY532708
T. murphyi TL	39.37S	75.17W	Valdivia, Chile	AY526538	AY532709
T. murphyi NZ	37.25S	178.12E	Tauranga, NZ	AY526539	—
T. novaezelandiae J1	37.25S	178.12E	Tauranga, NZ	AY526544	AY533475
T. novaezelandiae J2	37.25S	178.12E	Tauranga, NZ	AY526545	—
T. picturatus 21	38.50N	20.20E	Ionian Sea, Greece	AY526546	AY533477
T. picturatus 35	38.50N	20.20E	Ionian Sea, Greece	AY526547	AY533478
T. symmetricus 25	33.0N	125.2W	San Diego, USA	AY526540	AY532699
T. symmetricus 07	33.0N	125.2 W	San Diego, USA	AY526541	AY532700
T. trachurus 01	38.50N	20.20E	Ionian Sea, Greece	AY526533	AY533482
T. trachurus 05	38.50N	20.20E	Ionian Sea, Greece	AY526534	AY533483

A hyphen indicates that the D-loop was not sequenced.

ura–Nei model (TrN + I, variable base frequencies, equal transversion frequencies, and variable transition frequencies; see Tamura and Nei, 1993) was the most appropriate model of evolution for this data set. Consequently, we incorporated this model of nucleotide evolution in PAUP* software for ML and NJ analyses. To find the phylogenetic tree in MP we implemented a branch and bound search of the tree space, and in the ML method we implemented an heuristic search using random sequence addition ($n = 20$) and TRB branch swapping (Nei and Kumar, 2000). Additionally, bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 1000 bootstrap replicates.

2.5. Molecular clock

We used the Likelihood Ratio Test (LRT) to evaluate whether our ML phylogenetic tree based on Cyt b reconstruction fit to a molecular clock (Felsenstein, 1981). The LRT statistic is defined as: $\mathrm{LRT} = -2\ln(H_0/H_1)$, with H_0 representing the likelihood score associated with the null hypothesis (clock-like model) that the rate of evolution is homogeneous among all branches in the phylogeny, and H_1 representing the likelihood score associated with the alternative model (non-clock model). The LRT statistic is asymptotically distributed as χ^2. To test for a molecular clock the degrees of freedom are $s - 2$, where s is the number of taxa in the phylogeny (Felsenstein, 1981). We used the χ^2 statistic with a significance level of $p < 0.05$. Our results supported the molecular clock model, allowing us to calibrate the molecular clock to estimate the divergence time of the *Trachurus* species. Two approaches were followed for the calibration of molecular clock. First, in accordance with the suggestion by Karaiskou et al. (2003) we used a conservative value for the rate of divergence (1% per site per million years) of teleost fish obtained from Johns and Avise (1998) and Bermingham et al. (1997). Second, we used two available paleontological records for a fossil calibration. The first point of fossil calibration (C_1) corresponded to the ancestral node in the root of phylogenetic tree of the genus *Trachurus*. In accordance with Shaboneyev (1981), this point was calibrated using the oldest representative of the genus *Trachurus* in the fossil record of Lebanon and Caucasus, which has an estimated age of 16.4–23.8 MYA linked with closure of Tethys Sea (Miocene; Danil'Chenko, 1964). The second point of calibration (C_2) was the node of most recent divergence between *T. symmetricus* and *T. murphyi*, currently the only species inhabiting the Eastern Pacific coast (Poulin et al., 2004). This point was calibrated using the most primitive fossil member of the genus *Trachurus* in the Eastern Pacific, corresponding to specimens from Ecuador (Landini et al., 2002) from the late Pliocene (between 3.7 and 3 MYA, Ibaraki, 1997). The date of divergence between species was calculated for each node with bootstrap support above 50%, in the ML clock tree reconstruction. The divergence time for the nodes (C_1 and C_2) was estimated with the Tree-Explorer program (Tamura, 1999), using the midpoint between the upper and lower bounds of the fossil record date (Marko, 2002).

3. Results

3.1. Sequence variation: Cyt b

No variation in length was detected in Cyt b sequences (1140 bp) among different species of the genus *Trachurus*. Of the 1140 nucleotide positions sampled, 1014 were

Table 2
Pairwise sequence divergences show as percentage for Cyt b

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *T. japonicus*																			
2. *T. lathami*	4.33																		
3. *T. trecae*	4.72	1.19																	
4. *T. trachurus* 01	5.12	4.07	4.72																
5. *T. trachurus* 05	4.84	3.81	4.45	0.63															
6. *T. capensis* 10	5.38	4.07	4.72	0.72	0.63														
7. *T. capensis* 13	5.38	4.07	4.72	0.72	0.63	0.00													
8. *T. murphyi* IQ	5.50	4.79	5.86	4.88	4.60	4.76	4.76												
9. *T. murphyi* TL	5.50	4.79	5.86	4.88	4.60	4.76	4.76	0.00											
10. *T. murphyi* NZ	5.50	4.79	5.86	4.88	4.60	4.76	4.76	0.00	0.00										
11. *T. symmetricus* 25	5.25	4.66	5.73	4.63	4.35	4.76	4.76	0.90	0.90	0.90									
12. *T. symmetricus* 07	5.25	4.66	5.73	4.63	4.35	4.76	4.76	0.90	0.90	0.90	0.00								
13. *T. declivis* I1	1.47	3.64	4.01	4.61	4.35	4.86	4.86	5.36	5.36	5.36	5.11	5.11							
14. *T. declivis* I2	1.37	3.77	4.14	4.75	4.49	5.01	5.01	4.99	4.99	4.99	5.00	5.00	0.44						
15. *T. novaezelandiae* J1	1.09	3.88	4.49	4.87	4.60	5.13	5.13	5.63	5.63	5.63	5.14	5.14	1.66	1.57					
16. *T. novaezelandiae* J2	1.56	4.20	4.82	5.09	4.82	5.35	5.35	5.86	5.86	5.86	5.37	5.37	1.75	1.66	0.81				
17. *T. picturatus* 21	4.84	3.94	4.67	4.37	4.10	4.48	4.48	2.31	2.31	2.31	1.80	1.80	4.82	4.72	4.61	4.95			
18. *T. picturatus* 35	4.86	3.96	4.69	4.38	4.12	4.50	4.50	2.31	2.31	2.31	1.81	1.81	4.84	4.74	4.63	4.97	0.18		
19. *T. mediterraneus* 4	4.36	4.06	4.67	5.23	4.95	4.99	4.99	4.69	4.69	4.69	4.68	4.68	4.12	4.25	4.36	4.69	4.34	4.36	
20. *T. mediterraneus* 5	4.23	3.92	4.53	5.20	4.92	4.96	4.96	4.78	4.78	4.78	4.77	4.77	3.99	4.12	4.23	4.56	4.43	4.45	0.27

ML distance using the model described in the text (TrN + I).

invariant and 126 were variable sites. The total number of mutations was 137, with 116 synonymous mutations, and 111 phylogenetic informative sites. Mean nucleotide composition revealed an unequal structure of bases: 23.5% A, 34.1% C, 27.1% T, and 15.3% G. The substitution saturation test demonstrated that our sequences have little saturation ($Iss = 0.2229 < Iss.c = 0.7682$; $df = 280$ and $p < 0.0001$), thus validating their use for phylogenetic inference. The average number of pairwise differences between sequences was 38.5 ± 14.1, and the mean divergence detected among members of *Trachurus* genus was $4.03 \pm 1.5\%$. The least interspecific divergence was observed between *T. capensis* and *T. trachurus*, and the greatest divergence was found between *T. murphyi* and *T. novaezelandiae* (Table 2).

3.2. Sequence variation: D-loop

No variation in length was detected among *Trachurus* D-loop sequences. Of the 825 nucleotide positions sampled, 745 were invariant and 80 were variable sites with 92 total mutations and 61 phylogenetic informative sites. Base composition was strongly biased: A and T were represented roughly equally, occurring at mean percentages of 31.1 and 30.2%, respectively, while C occurred at 22.2% of the sites, and G at only 16.5%. The substitution saturation test showed little saturation ($Iss = 0.3185 < Iss.c = 0.7467$; $df = 108$, $p < 0.0001$). The mean number of pairwise differences between D-loop sequences was 29.45 ± 10.2, and the mean divergence among members of *Trachurus* was $5.2 \pm 2.2\%$. The greatest interspecific divergence was observed between *T. mediterraneus* and *T. murphyi*, and the least between *T. capensis* and *T. Trachurus* (Table 3).

3.3. Phylogenetic analyses

In the MP approach for Cyt b only intraspecific topological differences (with 0 branch length) were detected between the three most parsimonious trees, resulting in a consensus tree with 210 steps (consistency index $CI = 0.6429$, homoplasy index $HI = 0.3571$, retention index $RI = 0.8231$; Fig. 2). The MP consensus tree showed the same topology as the NJ phylogenetic reconstruction. All nodes on the tree had bootstrap support greater than 52%. The node root showed high bootstrap support (100%) suggesting a monophyletic status of the genus *Trachurus*.

Results of the ModelTest showed that the most descriptive model of evolution for both genes was TrN + I with a proportion of invariable sites. For Cyt b, the TrN + I model contained the following nucleotide substitution rate parameters: A–C = 1.0, A–G = 18.62, A–T = 1.0, C–G = 1.0, C–T = 8.95, and G–T = 1.0. The heuristic search found a single ML phylogenetic tree with a log likelihood ($-\ln L$) score of 3093.97 (Fig. 3A). In this case, the topology showed the same distribution of groups as the MP and NJ trees. However, the difference was that the ML analysis of the bootstrap consensus tree showed a basal politomy (Fig. 3B).

We obtained similar gene tree-topologies using the three different methods. The branching pattern indicated five distinct groups: the first group included all species from the Southwest Pacific (*T. japonicus*, *T. novaezelandiae*, and *T. declivis*: bootstrap support of 66% in MP, 55% in NJ, and 73% in ML); the second group consisted in only one species which inhabits the Mediterranean Sea and Eastern Atlantic Ocean (*T. mediterraneus*: bootstrap support of 100% in MP,

Table 3
Pairwise sequence divergences show as percentage for D-loop

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *T. symetricus* 25													
2. *T. symetricus* 07	0.12												
3. *T. murphyi* IQ	2.53	2.37											
4. *T. murphyi* TL	2.37	2.22	0.12										
5. *T. japonicus*	7.38	7.14	7.63	7.38									
6. *T. novaezelandiae* J1	7.64	7.89	7.64	7.39	1.43								
7. *T. declivis* I1	7.70	7.46	7.21	6.97	2.61	1.87							
8. *T. picturatus* 21	3.85	3.67	3.85	3.67	5.50	6.36	5.82						
9. *T. picturatus* 35	3.80	3.63	3.80	3.63	5.41	5.83	5.32	0.76					
10. *T. mediterraneus* 4	9.06	9.37	10.30	10.00	4.45	4.65	5.73	7.28	7.12				
11. *T. capensis* 13	6.82	6.60	6.13	5.91	5.93	5.92	5.40	6.14	5.16	8.05			
12. *T. capensis* 10	5.90	5.68	5.27	5.06	5.32	5.32	4.46	5.07	4.59	7.20	0.77		
13. *T. trachurus* 01	6.60	6.37	5.91	5.70	5.73	5.73	4.85	5.49	4.59	7.19	1.04	1.03	
14. *T. trachurus* 05	6.60	6.37	5.91	5.70	5.73	5.32	4.46	5.49	4.59	7.19	1.31	1.31	0.25

ML distance using the model described in the text (TrN + I).

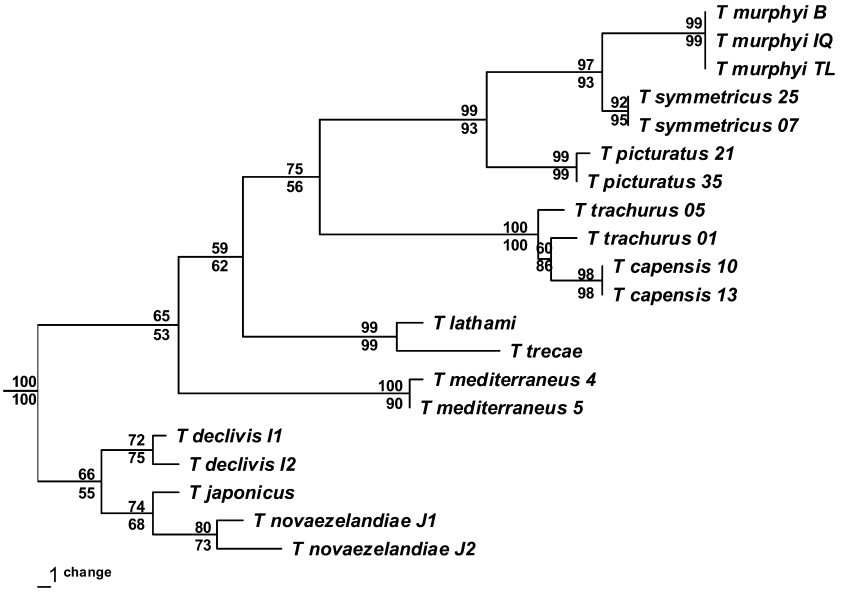

Fig. 2. MP tree of phylogenetic relationships of the genus *Trachurus* based on Cyt b sequences. On the MP tree the value above each branch indicates the bootstrap percentages for the MP analysis, and the value below each branch indicates bootstrap percentages for the NJ analysis. (The MP and NJ trees have the same topology.)

90% in NJ, and 85% in ML); the third cluster exclusively included Atlantic Ocean species (*T. lathami* and *T. trecae*: bootstrap support of 99% in MP, NJ, and ML); the fourth group included two species with broad distributions, covering a wider range of the Mediterranean Sea and Eastern Atlantic Ocean than the second group (*T. trachurus* and *T. capensis*: bootstrap support of 100% in MP and NJ, and 99% in ML); and finally, the fifth group encompassed three highly mobile pelagic species, two from the Eastern Pacific Ocean (*T. symmetricus* and *T. murphyi*) and one with distribution in the Eastern Atlantic Ocean and the Mediterranean Sea (*T. picturatus*) (bootstrap support of 99% in MP, 93% in NJ, and 86% in ML).

The unrooted ML tree reconstruction based on D-loop sequences and an heuristic search resulted in only one phylogenetic tree with $-\ln L = 1869.97$, and bootstrap support between 50 and 100% (Fig. 4). In this reconstruction, two species were excluded due to the lack of available tissue samples and sequences (*T. lathami* and *T. trecae*). Nevertheless, the branching patterns were congruent with four of the five groups resolved by Cyt b tree reconstruction: the Southwest Pacific group with 95% bootstrap support (*T. japonicus*, *T. novaezelandiae*, and *T. declivis*); the clade containing *T. mediterraneus* (61% bootstrap support) which appeared in the Southwest Pacific group, but with large genetic differentiation (4%) relative to all other species separations; the

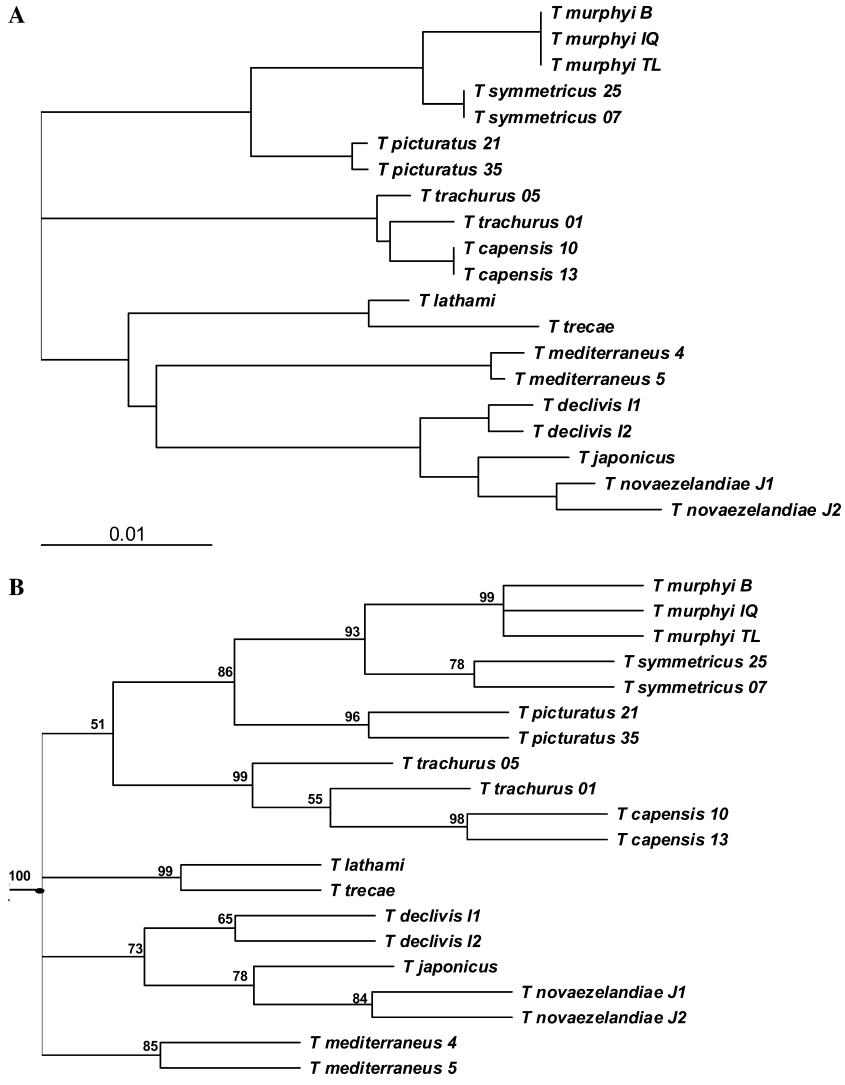

Fig. 3. (A) ML tree of phylogenetic relationships of the genus *Trachurus* based on Cyt b sequences. (B) ML bootstrap consensus tree. The value above each branch indicates the bootstrap percentages. The model for the ML tree (TrN + I) had an $-\ln L$ score of 3095.7905, and AIC of 6201.5811.

group composed by *T. trachurus–T. capensis* (98% bootstrap support); and the clade consisting in *T. symmetricus*, *T. murphyi*, and *T. picturatus* with 95% bootstrap support (Fig. 4).

3.4. Molecular clock

The LRT indicated that the rate of evolution was homogeneous between all the branches in the ML phylogenetic tree based on Cyt b ($-\ln L$ clock = 3101.36, df = 19, $\chi^2 = 14.78$, $p = 0.7365$). In other words, the substitution rate did not vary significantly between branches, therefore indicating that a generalized molecular clock model was appropriate.

Calibration of the molecular clock using the rate of divergence value proposed for teleost fish (1% per site per million years) indicated that the group originated around 2.6 MYA (Late Pliocene, Piacenzian Age). According to this calibration, the most recent divergence times were between *T. trachurus* and *T. capensis*, and *T. murphyi* and *T. symmetricus*, with both divergence events occurring around 400,000 years ago (Pleistocene). In contrast, both fossil calibrations produced divergence times much older than the conservative value of divergence rate for teleost fish (Fig. 5). Both fossil calibrations showed similar rates of molecular divergence between the first (C_1: 0.13%) and second (C_2: 0.15%) calibration points. These calibrations of the molecular clock indicate an origin of the genus *Trachurus* 16.7–20.1 MYA (Early Miocene, Burdigalian Age), the most recent species split between *T. trachurus* and *T. capensis* with a divergence time 2.3–2.8 MYA (Late Pliocene, Piacenzian Age), and a node of divergence for *T. murphyi* and *T. symmetricus* 2.7–3.3 MYA (Late Pliocene, Piacenzian Age).

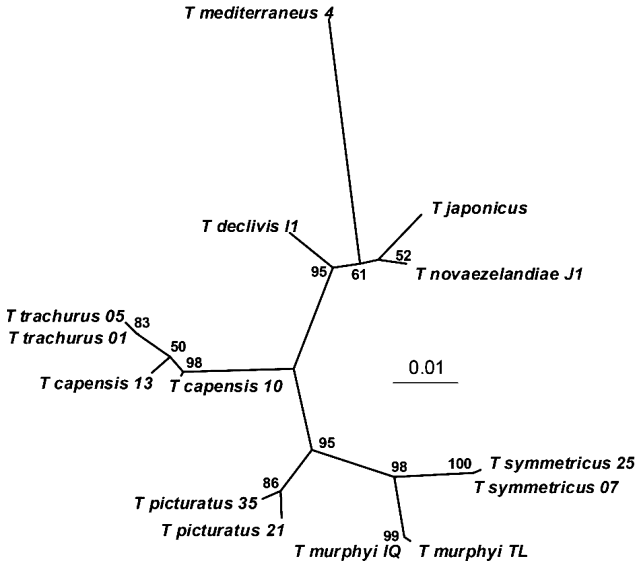

Fig. 4. ML unrooted tree of phylogenetic relationships of the genus *Trachurus* based on D-loop sequences. On the ML tree the value near each node indicates the bootstrap percentages. The model for the ML tree (TrN + I) had an $-\ln L$ score of 1871.4075, and AIC of 3752.8149.

4. Discussion

4.1. Phylogenetic relationships

The only previous hypothesis of evolutionary history of the genus *Trachurus* was presented by Shaboneyev (1981) using morphological characteristics. This author defined three historical groups: trachurus, picturatus, and mediterraneus. In our study, we did not find evidence to support the trachurus and mediterraneus groups; however, the picturatus group was a consistent clade throughout all phylogenetic reconstruction approaches. The picturatus group is composed of *T. picturatus*, *T. murphyi*, and *T. symmetricus*, of which *T. murphyi* and *T. symmetricus* were phylogenetically the closest (Figs. 2–4). The species *T. murphyi* is a highly migratory fish, which is widely distributed in the South Pacific; it is found off the Chilean and Peruvian coasts reaching across to New Zealand and Tasmania (Suda et al., 1995). *T. symmetricus* is distributed in the Northeastern Pacific, resulting in an antitropical distribution of the clade *T. murphyi–T. symmetricus* (Poulin et al., 2004). The latter component of this group, *T. picturatus*, has a more restricted distribution reaching from the Bay of Biscay (France) southward to Morocco and eastward into the Mediterranean Sea (Eschmeyer, 2003; Karaiskou et al., 2003). Shaboneyev (1981) used morphological traits (i.e., low body depth, a large number of scutes) and ecological aspects (i.e., inhabiting far beyond the continental shelf) to characterize the picturatus group as more advanced. Our results confirm this hypothesis indicating a greater number of synapomorphisms with respect to other members of the genus *Trachurus* (Fig. 2). Shaboneyev (1981) considered that the picturatus group was closely related to members of the mediterraneus group, however, our study revealed that the genetic distance between *T. mediterraneus* and the picturatus group was greater than for all other species pairs compared in this study (e.g., D-loop divergence *T. mediterraneus–T. symmetricus* was 9.1%; *T. mediterraneus–T. murphyi* was greater than 9.4%; and *T. mediterraneus–T. picturatus* was greater than 5.7%, see Table 3). Moreover, phylogenetic analyses showed that *T. mediterraneus* has a topological position in the phylogenetic trees that is distant from the picturatus group (Figs. 2–4). Additionally, the

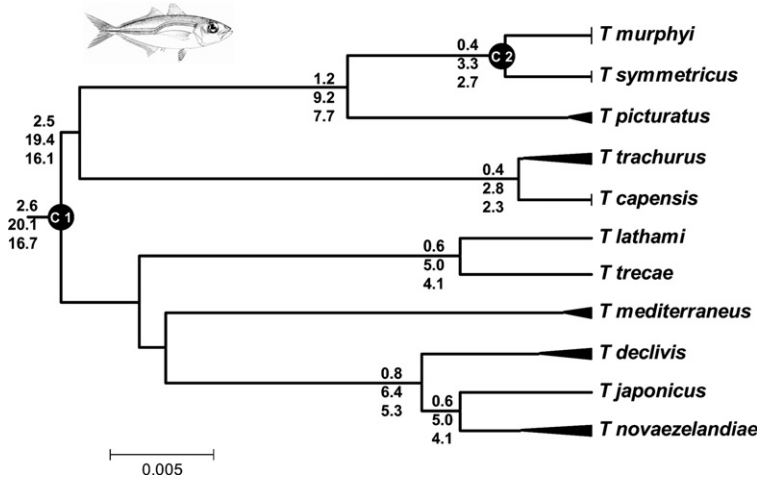

Fig. 5. ML tree with molecular clock calibrations of phylogenetic relationships of the genus *Trachurus* based on Cyt b sequences. The values above each branch indicate the divergence time in millions of years with a calibration of 1% per site per million years. The first value below each branch indicates the divergence time using the first point of fossil calibration (C_1: Miocene of the Lebanon and Caucasus), and the second value below each branch indicates the divergence time using the second point of fossil calibration (C_2: the Late Pliocene of Ecuador). We only indicate divergence time for nodes with bootstrap support over 50%.

other members of Shaboneyev's mediterraneus group, the Atlantic Ocean species (*T. lathami* and *T. trecae*) were located in an independent phylogenetic clade.

The other highly consistent group in our molecular analyses was conformed by the Southwest Pacific species (*T. japonicus*, *T. novaezelandiae*, and *T. declives*; Figs. 2–4). These results reject the hypothesis proposed by Shaboneyev (1981), that *T. japonicus* and *T. novaezelandiae* are members of the trachurus group, and that *T. declives* is an evolutionarily aberrant form relative to other members of the genus. Furthermore, our study demonstrates that all *Trachurus* species from the Southwest Pacific are well-defined species (bootstrap support above 60%, Figs. 2 and 3) and represent a monophyletic lineage (bootstrap support of 66% in MP, 55% in NJ, and 73% in ML). These results contrast with the findings of Kijima et al. (1988), who used isoenzyme loci to suggest that *T. declives* may have been derived from an ancestor of *T. murphyi* from the Southeastern Pacific, and concluded that *T. novaezelandiae* and *T. japonicus* are subspecies that radiated from the Indian Ocean to the entire coasts of the west Pacific Ocean. Karaiskou et al. (2003) showed that *T. mediterraneus* and *T. picturatus* are more closely related than *T. trachurus*, however, our results do not support this hypothesis and showed that the three species belong to different evolutionary clades (Figs. 2–4).

4.2. Divergence time

The percentage of sequence divergence based on Cyt b among *Trachurus* species (mean $3.43 \pm 1\%$, Table 2) constitutes the lowest values reported for marine fish species (mean 3.7–13%, Billington and Hebert, 1991; Johns and Avise, 1998). The D-loop sequences showed the same low level of divergence (mean $3.5 \pm 1\%$, Table 3) suggesting that this may be a common characteristic for the mtDNA of the genus *Trachurus*. In a previous study by Stepien and Rosenblatt (1996), using electrophoresis protein analysis, the researchers reported little genetic divergence between *T. murphyi* and *T. symmetricus*, suggesting that both taxa belong to the same species. At the DNA level, Cantatore et al. (1994), based on Cyt b sequences of seven Perciform fish species (including *T. trachurus*), suggested that the nucleotide substitution rate is three to five times lower than in mammals. Additionally, Smolenski et al. (1994) performed enzyme restriction analyses of mtDNA, observing a low level of genetic diversity in *T. declives*. Finally, Karaiskou et al. (2003), based on partial Cyt b sequences (239 bp), reported a low value of sequence divergence among three Mediterranean *Trachurus* species (2.13–5.25%). These evidently lower levels of genetic divergence between *Trachurus* species may indicate that: (1) these species are very recent and therefore have not had sufficient time to accumulate many mutations, or (2) that the nucleotide substitution rate is intrinsically lower in the mitochondrial genes of the genus *Trachurus*. In our results, the two fossil calibrations of the molecular clock produce much older divergence times than the conservative rate of divergence value estimated for teleost fish (Fig. 5), indicating that the alternative of a lower nucleotide substitution rate is better supported.

Rates of genetic change can vary between taxa or genes, therefore the molecular clock should be calibrated independently for each data set, using a known date of divergence, which is commonly based on evidence from the fossil record (e.g., Marko, 2002; Near and Sanderson, 2004). The oldest paleontological record of the genus *Trachurus* found in the Early Miocene of the Lebanon and Caucasus (Burdigalian Age: Danil'Chenko, 1964), suggests a rate of divergence around 0.13% per million years for Cyt b, the lowest value currently reported for teleost fish species. Furthermore, the second paleontological record, associated with the Eastern Pacific East clade (*T. murphyi–T. symmetricus*) in the late Pliocene of the Ecuador (Canoa formation: Landini et al., 2002), suggests a rate of divergence around 0.15% per million years for Cyt b. Both calibrations, using different fossils, showed similar rates of divergence and times of origin for the genus *Trachurus*. The high congruence between this two independent points of fossil calibration suggests low error in the estimation of a node divergence (see Near and Sanderson, 2004).

Our molecular clock calibration for the evolutionary event linked to the origin and diversification of the genus *Trachurus* was in agreement with Shaboneyev's proposition (1981). This author proposed that the biogeographic history of the genus *Trachurus* began when the ancestral groups present in the Tethys Sea were separated by the division of this single body of water into two parts: the present day Mediterranean Sea (western part of the Tethys) and the present day Indian Ocean (eastern part of the Tethys) during the Early Miocene (18 MYA) (Berggren and Hollister, 1975).

The original fauna of the Tethys Sea was tropical, but the temperatures decreased during the Oligocene and Early Miocene (Berggren and Hollister, 1975). These variations in water temperature potentially increased the diversity of environmental conditions and caused an upsurge in the speciation process, forcing the most primitive forms of the western part, to split into two branches, one moving across the Mediterranean Sea, then southward along the coast of west Africa to warm waters and westward to the Atlantic Ocean. The other group remained in the Northern Hemisphere where new species developed as a consequence of adaptation to harsher environmental conditions. Since the cooling did not affect most of the Indian Ocean, Shaboneyev suggested that the eastern part of the ancestral stock remained in the Indian Ocean.

Part of the ancestral stock of the western Tethys acquired several adaptive traits for an active pelagic mode of life. This is the case for the picturatus group (*T. picturatus*, *T. murphyi*, and *T. symmetricus*) which began to diversify 7.7–9.2 MYA (Late Miocene, Tortonian Age; Fig. 4). One part of the ancestral stock of *T. picturatus*, the ancestor of the Eastern Pacific species (*T. murphyi* and *T. symmetricus*), extended its range westward across the Atlantic. This group then penetrated into the Pacific Ocean passing over the submerged Isthmus of Panama, spreading along the western coasts of North and South America. The northern part of this group originated into *T. symmetricus* and the southern part gave rise to *T. murphyi* about 2.7–3.3 MYA (Late Pliocene, Piacenzian Age; Fig. 5). This event occurred together with the origin of the actual Pacific current system and the Isthmus of Panama, and is confirmed by the paleontological record (Fitch, 1969; Landini et al., 2002). According to Coates and Obando (1996) the emergence of the Central American Isthmus had major consequences on ocean circulation and global climatic patterns. We consider the formation of the Isthmus of Panama to be an important event of diversification in the genus *Trachurus*.

Using a divergence rate for teleosts of about 1% (Bermingham et al., 1997), Karaiskou et al. (2003) suggested that the diversification of the ancient *Trachurus* stock occurred between 2 and 5 MYA. However, our results, based on fossil record calibrations of the molecular clock suggest that the diversification of the ancestral stock of *Trachurus* occurred between 16 and 20 MYA (Fig. 4), probably associated with the division of the Tethys sea. Later, two important events occurred in the Mediterranean Sea that may be associated with the diversification of the *Trachurus* species in this area: (1) the isolation of Mediterranean Sea from the Atlantic Ocean and (2) the division of the Mediterranean and the Paratethys Sea (presently the Black, Caspian, and Aral Seas). The most ancestral species of the genus (*T. mediterraneus*) found refuge in the western part of the Mediterranean Sea, while the ancestral population of *Trachurus* in the Atlantic Ocean differentiated into *T. trachurus*, *T. capensis*, and *T. trecae* in the east Atlantic (i.e., coast of South Africa) and *T. lathami* in the Southwestern Atlantic Ocean.

An analysis of the number of lineages versus mean divergence times for each node shows two periods of maximum diversification (Fig. 6), corresponding to the major geological events described above. The first period occurred between 18.4 and 15.0 MYA (duration 3.4 million years) and the second period occurred between 8.4 MYA and present day, both with very similar diversification rates of 0.77 and 0.78, respectively (for an explanation of diversification rates see Barraclough and Nee, 2001; Nee, 2001). This analysis shows a period of transition of 6.5 million years (15.0–8.4 MYA) with either a very low diversification rate, or a high extinction rate. The first and second period of diversification shows a simple model of constant speciation rate, where the probability of a speciation event occurring in a given time is constant both over time and between species (Barraclough and Nee, 2001; Nee, 2001). The first period includes the origin of the genus *Trachurus*, and of the five principal lineages observed in the phylogenetic reconstruction (Figs. 5 and 6), including the current species, *T. mediterraneus*. The second period includes the diversification processes within each of the five lineages originated in the first period, as well as the origin of the current species of *Trachurus* around the world.

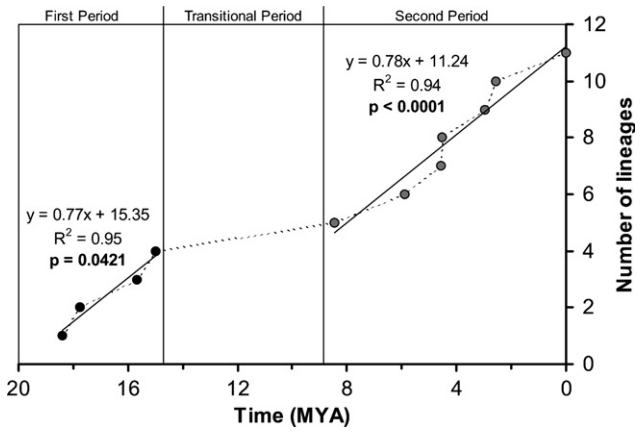

Fig. 6. Number of lineages plotted against mean divergence time (considering both fossil calibration times: $(C_1 + C_2)/2$) of each node. This figure shows the two principal periods of diversification in the genus *Trachurus* (First and Second Period), where under the simplest model (i.e., constant speciation rate model) a straight line with slope equal to the per lineage speciation rate, b, is expected. The regression analysis to assess the diversification rate b was performed in EcoSim 7 software (Gotelli and Entsminger, 2003) using a randomization approach (10,000 random matrices, random seed = 12345). A significance level of $p < 0.05$ was assumed.

In summary, our study resolves the controversy regarding the phylogenetic relationships of the genus *Trachurus*, and our molecular clock calibration supports the paleobiogeographic history originally proposed by Shaboneyev (1981). The contemporary species have a common ancestor which comes from the present day Mediterranean Sea, and the current geographical distribution is related to past events. Here, we propose that the current species originated from five ancient and independent lineages, characterized by an evolutionary history with two large diversification processes. New phylogeographic studies are required to elucidate the microevolutionary processes that potentially explain the macroevolutionary pattern of diversification in the genus *Trachurus*.

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