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First description of two moderately halophilic and psychrotolerant *Mycoplasma* species isolated from cephalopods and proposal of *Mycoplasma marinum* sp. nov. and *Mycoplasma todarodis* sp. nov

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ABSTRACT

Two moderately halophilic and psychrotolerant new *Mycoplasma* species were isolated from common cephalopods. Three strains were isolated in pure culture from two individual European flying squid (*Todarodes sagittatus*), and two individual octopuses (*Octopus vulgaris*). The strains showed optimal growth at 25 °C and a salinity of 3% (w/v) NaCl. Molecular analyses revealed that the isolates belonged to two new, but phylogenetically related species, divergent from all previously described *Mollicutes*, representing the first marine isolates of the class, and also the first *Mycoplasma* strains for which NaCl requirement has been demonstrated. A genome search against all available marine metagenomes and 16S rRNA gene databases indicated that these two species represent a novel non-free-living marine lineage of *Mollicutes*, specifically associated with marine animals. Morphology and physiology were compatible with other members of this group, and genomic and phenotypic analyses demonstrated that these organisms represent two novel species of the genus *Mycoplasma*, for which the names *Mycoplasma marinum* sp. nov. and *Mycoplasma todarodis* sp. nov. are proposed; the type strains are PE^T (DSM 105487^T, CIP 111404^T) and 5H^T (DSM 105,488^T, CIP 111405^T), respectively.

Introduction

The Class *Mollicutes* is the monotype of the phylum *Tenericutes*. The common morphological characteristics of these prokaryotes are their very small size and the lack of a cell wall, being bounded only by a plasma membrane. *Mollicutes* currently consist of nine genera (*Mycoplasma, Ureaplasma, Entomoplasma, Mesoplasma, Spiroplasma, Acholeplasma*, "Candidatus *Phytoplasma*", *Anaeroplasma* and *Asteroleplasma*), and have been isolated from various vertebrate, invertebrate, insect and plant hosts. The genus *Mycoplasma* (family *Mycoplasmataceae)* is sterol-requiring, unable to hydrolyse urea, and the cells usually present a coccoidal cellular morphology. Mycoplasmas encompass more than one hundred species and are reported to be commensals or pathogens (including causative agents of notifiable diseases) in vertebrate hosts, some being significant pathogens of human and animals [6,8].

Abbreviations: DGGE, denaturing gradient gel electrophoresis; RAPD, randomly amplified polymorphic DNA; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; ANI, average nucleotide identity; AAI, average amino acid identity; MAG, metagenome assembled genomes.

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Although molecular techniques have successfully documented the existence of *Mycoplasma* in various non-mammal marine aquatic animals such as salmon [20], long-jawed mudsucker [2], cod [37], Antarctic notothenioid fish [68], abalone [21,61], coral [44], lobster [38] and octopus [22], no cultured representative exists currently. Cephalopods are of worldwide commercial interest for human consumption and records of *Mollicutes* in such animals are scarce [22] and squid digestive tract [4]. Therefore, our goal was to isolate mycoplasmas from marine hosts.

In the current work we describe the isolation of two novel mycoplasmas, one from European flying squid (*Todarodes sagittatus*) [66] and the other from octopuses (*Octopus vulgaris*) [64]. The two groups of isolates were phylogenetically related, and differed enough from the known mycoplasmas to be classified as two new species. To our knowledge this is the first isolation and characterization of mycoplasmas from cephalopods. Our study fulfilled the guidelines of the revised minimal standards for the description of new species of the class *Mollicutes* [9] that recommended suitable molecular methods to be acceptable characteristics in place of serology [14]. The names *Mycoplasma marinum* sp. nov. and *Mycoplasma todarodis* sp. nov. are proposed for the two new species with the designated type strains PE^T (DSM 105487^T = CIP 111404^T) and 5H^T (DSM 105,488 ^{T} = CIP 111405^T), respectively. The Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession numbers PSZO00000000 (*M. marinum* sp. PE) and PSZP00000000 (*M. todarodis* sp. 5H).

Materials and methods

Isolation and culture conditions

Nine dead cephalopods were sampled at the anatomopathology service (Veterinary Faculty, Universidad de Las Palmas de Gran Canaria, Spain) where they were being studied for other purposes. Two dead European flying squid, *Todarodes sagittatus* (*Cephalopoda*: *Ommastrephidae*), collected from the south coast of El Hierro island (Canary Islands, Spain), had been frozen before examination, while seven common octopuses, *Octopus vulgaris* (*Cephalopoda*: *Octopodidae)* were from aquaculture from Gran Canaria island (Canary Islands, Spain) and had been kept refrigerated before examination. Thirty-three swabs were collected from octopus mouth/oesophagus (7), gills (7), stomach (4), digestive gland (7), testicles (1), funnel (3), eyes (3) and intestine (1), and three swabs were taken from squid gills (2) and intestine (1). Swabs were inoculated in SP4-II broth medium [51] supplemented with 1.5% (w/v) NaCl, and incubated at 18 °C for 24 h. After incubation, cultures were filtered through 0.45 μm pore size sterile membranes into fresh medium. The inoculated tubes were sealed with paraffin and incubated as above for a minimum of one and a half months and a maximum of three months.

DNA extraction, real time PCR and culture cloning

DNA was extracted from cultures using a Realpure Genomic DNA extraction kit (Durviz, Spain) to obtain purified genomic DNA, following the manufacturer's instructions. The *Mollicutes* PCR method used was described by Vega-Orellana et al. [65], and consisted of a real time application of the conventional PCR described by Botes et al. [5]. From each positive culture, one typical fried egg-shaped colony was isolated in pure culture by filter-cloning three times [62]. Once the cloned cultures were obtained, a new DNA

PCR, sequencing and phylogenetic analysis

Denaturing gradient gel electrophoresis (DGGE) [36] and randomly amplified genomic DNA (RAPD) using the primer RAPD1 (5′-TGCGAACTGTTGGGAAGGG-3′) [58] were performed following the respective protocols. Sequencing 16 S–23 S rRNA Intergenic Spacer Region (ISR) and 16S rDNA were performed as published previously [52,74]. Amplifications were done using the illustra Ready-To-Go^{TM} RT-PCR Beads kit (GE Healthcare Lifescience, Barcelona, Spain), and sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands). New sequences were added to the LTP 132 [72] and SILVA REF NR 132 [50] databases, aligned using the SINA aligner [49], and further manually improved using the universal alignment implemented in the ARB program package [29]. Different trees were reconstructed using the neighbor-joining [57] and RAxML [59] algorithms with the Jukes Cantor and the GTR-GAMMA substitution models respectively. For the neighbor-joining trees a supporting set of 757 high-quality sequences [41] was used, whereas for the RAxML reconstructions only sequences from the representative *Mollicutes* branch were used. In both cases we used different conservation filters implemented in the LTP [72] to remove hypervariable regions to reduce the phylogenetic noise. Partial sequences were inserted using the parsimony tool implemented in the ARB program package [29].

Maldi-Tof ms

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analyses of the proposed new species together with the type strains of the phylogenetically closely related mycoplasma species were performed as follows: protein extracts from 2 ml of late-exponential phase broth cultures were obtained as previously described [45]. Mass spectra were generated using a microflex LT Biotyper under control of FlexControl 3.4 software (Bruker Daltonics GmbH, Leipzig, Germany). Main spectra peak lists (MSPs) of each strain were created using the MSP creation functionality of MBT Compass Explorer 4.1. For each strain, 30 individual mass spectrum measurements from ten different spots of protein extracts were performed. Mass spectra were processed using FlexAnalysis 3.4 and a minimum of 20 spectra with high quality were selected for MSP creation. A dendrogram based on the distance matrix of generated MSP was created with MBT Compass Explorer 4.1 using the correlation distance measure with the average linkage algorithm and a threshold value for a single organism of 300.

Genome sequencing, assembly and annotation

PE^T and 5H^T genomes were sequenced using Illumina Hiseq (PE 100x2) technology. Raw reads were trimmed using SolexaQA v3.1.4 software using the Phred score threshold of quality 20 as previously suggested [11]. The assembly was performed using IDBA v1.1.1 [43] tool. Assembled contigs longer than >500 bps were selected for further analysis. Gene prediction and automatic annotation were conducted using RAST annotation server and compared with KAAS-KEGG [40] predictions. The almost complete 5S, 16S and 23S rRNA gene sequences were extracted from the genome sequences using the RNAmmer 1.2 Server [27]. Completeness and contamination levels of the genomes were determined using the "HMM.essential.rb" script downloaded from Enveomics collection [55].

Core genome (genes shared between all genomes) and pan

minimum identity ≥45% and ≥50% of the query sequence length. The orthologous gene groups (OGs) shared among all genomes were extracted using the script "ogs.mcl.rb" also from the Enveomics collection. Pseudogenes in the *Mycoplasma* genomes were identified as the reciprocal best matches in BLASTP pairwise comparisons by examining the proteins of the orthologous genes. Proteins that contained a stop codon in one of the query genomes were tagged as pseudogene. The presence or absence of variable genes was used to cluster the genomes with the Euclidian distance using Ggplot2 package from R [71]. The genes forming the core genome were individually aligned using the aligner MUSCLE v3.8.31 [13]. The aligned genes were concatenated in order to reconstruct trees using the neighbor joining and RAxML algorithms implemented in ARB program package [29]. ANI (average nucleotide identity) value between all-versus-all genomes was calculated using the JSpeciesWS tool [54], and the AAI (average amino acid identity) using the webserver available through http://enve-omics.gatech.edu/ [55].

Screening all marine TARA oceans metagenomic datasets

TARA Expeditions (https://oceans.taraexpeditions.org/en/) are global scientific voyages to explore the impact of climate change on ocean life, collecting samples from the photic and (secondarily) subphotic layers. Molecular data are obtained and made available to other researchers (https://figshare.com/articles/TARA-NON-REDUNDANT-MAGs/4902923/1). We screened all marine TARA oceans metagenomic datasets available under the accession ID PRJEB1787 as well as the 957 non-redundant metagenome assembled genomes (MAGs) derived from these metagenomes [12] for the presence of close relatives of the *Mycoplasma* genomes recovered by our study. Screening was done by searching all the predicted genes of the isolate genome against the curated metagenome database or the MAGs through a megablast search. Only high quality matches (>95% ID and >70% alignment) were retained for further analysis. This analysis included 60 selected, representative marine surface and deep-sea sediment samples to capture different regions of the world and depths that were made available by the Tara Oceans expedition [46], and water column metagenomic data previously recovered from 0 to 1500 m below sea level across the Gulf of Mexico [32,33], and our own published [56], and unpublished metagenomes retrieved from MG-RAST (https://www.mg-rast.org/) with accession numbers 4510162.3–4510175.3, 4537092.3–4537094.3.

Growth conditions and phenotypic tests

Isolates adapted to the SP4-II broth supplemented with 1.5% (w/v) NaCl were analysed for different variables to calculate the optimal growth conditions at different temperatures (4 °C, 10 °C, 18 °C, 25 °C, 30 °C and 37 °C), NaCl concentrations (0.0–6.0% (w/v) at 0.5% intervals at 18 °C and 25 °C), and aerobic and anaerobic conditions (using an overlay of sterile paraffin). To analyse their filterability, cultures were filtered using 0.45 μm and 0.22 μm pore size sterile membranes. Growth was evaluated by acidification of the broth containing phenol red as a pH indicator [35] after 14 days of incubation. Colony morphology of the isolates was assessed on SP4-II plates [51] for seven days at 25 °C. Cultures were also adapted to Mycoplasma Liquid Medium (ML; Mycoplasma Experience Ltd, Surrey, UK) supplemented with 3.0% (w/v) NaCl and growth was tested on Mycoplasma Agar & Supplement (MS; Mycoplasma Experience Ltd) under aerobic, microaerophilic (5% CO_2) and anaerobic, nitrogen-rich environment (95% N_2, 5% CO_2) conditions at 25 °C.

Microscopy

Gliding motility was assessed with a microcinematography motility assay as described elsewhere [23]. An Olympus AX70 microscope equipped with a Color View CCD digital camera controlled using CellF software package (Olympus Soft Imaging Solutions GmbH, Muenster, Germany) was used to capture 1-s interval phase-contrast images for 200 s. Cultures were centrifuged at 1000 × *g* for 30 min and pellets were resuspended and fixed in an equal volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and submitted for transmission electronic microscopy at the Diagnostic Pathology Services (Institute of Veterinary Science, University of Liverpool, UK). The pellet was prepared for transmission electronic microscope analyses according to their standard operating procedure [69] and examined under a Philips EM208S transmission electron microscope (Philips UK Limited, Guildford, UK), observed at 80 kV.

Histology

Squid and octopus samples were fixed in 4% formaldehyde overnight, washed in phosphate buffered saline (PBS) and then stored in 70% ethanol until further processing. The fixed tissues were further dehydrated through graded alcohols and xylene, and finally embedded in paraffin. Tissue sections of 5 μm were then stained with haematoxylin and eosin (H&E) [31] and evaluated under an Olympus CX41 light microscope (Olympus Iberia SAU, Barcelona, Spain).

Results and discussion

Molecular analyses

In a previous culture-independent survey using specific *Mollicutes* PCR [65], *Mollicutes* DNA was successfully amplified in samples from octopus and squid. In an attempt to isolate such *Mollicutes*, SP4-II medium [51] supplemented with a 1.5% NaCl was used in combination with anaerobic incubation at 18 °C. During the enrichment incubation, three samples from two squid (*Todarodes sagittatus*, gills (2) and intestine (1)) and seven octopuses (*Octopus vulgaris*, oesophagus (1), stomach (2), digestive gland (1), intestine (1) and eyes (2)) tested positive using real-time PCR [65]. Two months of incubation were required for the primary isolations, and three isolates were recovered from the gills (2) and intestine (1) of the two squid, and also two from oesophagus, stomach of one octopus and a further isolate from the digestive gland of a second octopus. Unfortunately, the sample from stomach could never be properly filter-cloned and was excluded from further studies as it was always in co-culture with *Vibrio* sp., known contaminants able to pass 0.45 μm [10]. Colonies of cephalopod mycoplasmas are shown in Fig. 1.

The five cultures were checked for purity by analysing the 16S rRNA genes [74], the ISR [52] and by MALDI-TOF MS. The 16S rRNA genes and ISR of the three squid mycoplasma strains were identical (100%); the sequences of the two octopus strains were also identical but differed from the squid isolates by lowering the percentage of identity to 98.7% (data not shown). These results were confirmed by the RAPD and DGGE profiles (Supplementary Fig. S1 and S2) which showed distinct patterns between the octopus and squid mycoplasmas. However, the profiles were identical among isolates for the same host, demonstrating that the isolates were clonal variants, but originated from different animals. Similarly, the ISR amplifica-

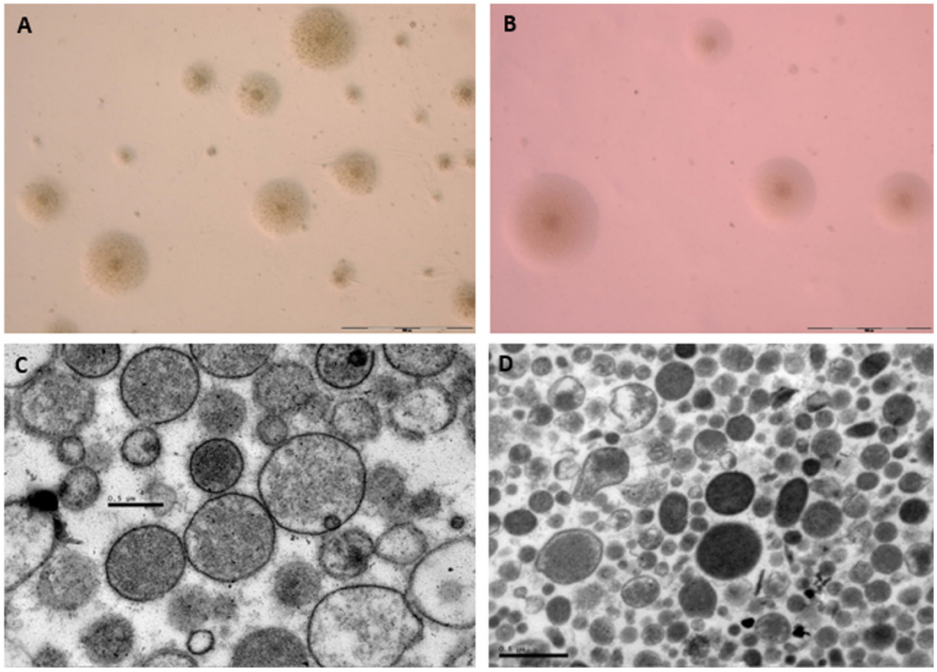

Fig. 1. Morphology of cephalopod mycoplasmas. SP4-II agar supplemented with 3% NaCl was used to grow colonies of *Mycoplasma* sp. PE^T (A) and *Mycoplasma* sp. 5H^T (B) magnification: ×40. Transmission electron micrographs show pleomorphic cells for *Mycoplasma* sp. PE^T cells (C) and *Mycoplasma* sp. 5H^T (D); length bar represents 500 μm in A and B and 0,5 μm in C and D.

Table 1
Most relevant genomic features of *Mycoplasma* sp. PE^T and *Mycoplasma* sp. 5H^T with respect to *M. mobile* 163K^T.

	Mycoplasma sp. PE^T	*Mycoplasma* sp. 5H^T	*M. mobile* (163K^T)
16S rRNA gene sequence (bp)	LT716014 (1525 bp)	LT716015 (1523 bp)	NR074620 (1520 bp)
rRNA gene intergenic spacer sequence (bp)	LT716016 (521 bp)	LT716017 (496 bp)	AY737011 (304 bp)
Genome accession number	PSZO00000000	PSZP00000000	NC_006908.1
Completeness	97.1%	85%	92.2%
Genome size (Mb)	1,171,149	1,007,879	780,000
Number of contigs	128	84	1
%GC	28.41	30.95	25.0
Total no. CDS	1003	914	689 (652)
Hypothetical genes	328	296	90
16S rRNA genes	1	1	1
23S rRNA genes	1	1	1
5S rRNA genes	2	2	1
Number of RNAs	34	30	31
tRNA genes	42	39	28
Number of pseudogenes	6	32	3
AAI *M. marinum* (% shared proteins)	100 (100%)	76.34 (49.21%)	60.53 (30.16%)
AAI *M. todarodis* (% shared proteins)	76.34 (49.21%)	100 (100%)	60.59 (29.23%)

both strains was 72.1% (data not shown). The almost complete 16S rRNA gene sequences (LT716014 (octopus PE^T strain) and LT716015 (squid 5H^T strain)) showed less than 90% identity to the closest type strain mycoplasma sequences (Supplementary Table S1).

The 16S rRNA gene sequences differed significantly from all known *Mycoplasmas* species, and the phylogenetic reconstruction always showed both type strains clustered together as a deep branching lineage, close to the *M. hominis* and *M. pulmonis* groups, and with the unstable branch of *M. mobile* (Fig. 2 and Supplementary Fig. S3). The highest sequence identities were with *M. mobile* (M24480), *M. moatsii* (AF412984) and *M. sualvi* (AF412988), and ranged between 86.61% and 87.71% (Supplementary Table S1). In this regard, the relatively low (<90%) sequence identities with the closest relatives could indicate that this new lineage might represent belonging to two different new species of the monotypic genus *Mycoplasma*.

It was noticeable that all relative sequences from non-mammal marine surveys retrieved from the SILVA REF NR 132 database, as well as some of the short partial sequences retrieved from *Octopus mimus* in a culture-independent survey [22] inserted by parsimony (Supplementary Fig. S4), form a monophyletic lineage, with identities ranging from 90.2% to 85.4% (Fig. 2, Supplementary Fig. S3 and Table S1). In addition, both 16S rRNA genes were specifically identified in squid samples used to feed marine mammals in a gut microbiome study of dolphins and sea lions [4]. The authors did not report any abnormalities in the squid used as food source in the US Navy Marine Mammal Program (MMP) in San Diego Bay, San Diego, CA facility, thus the squids appeared to be healthy.

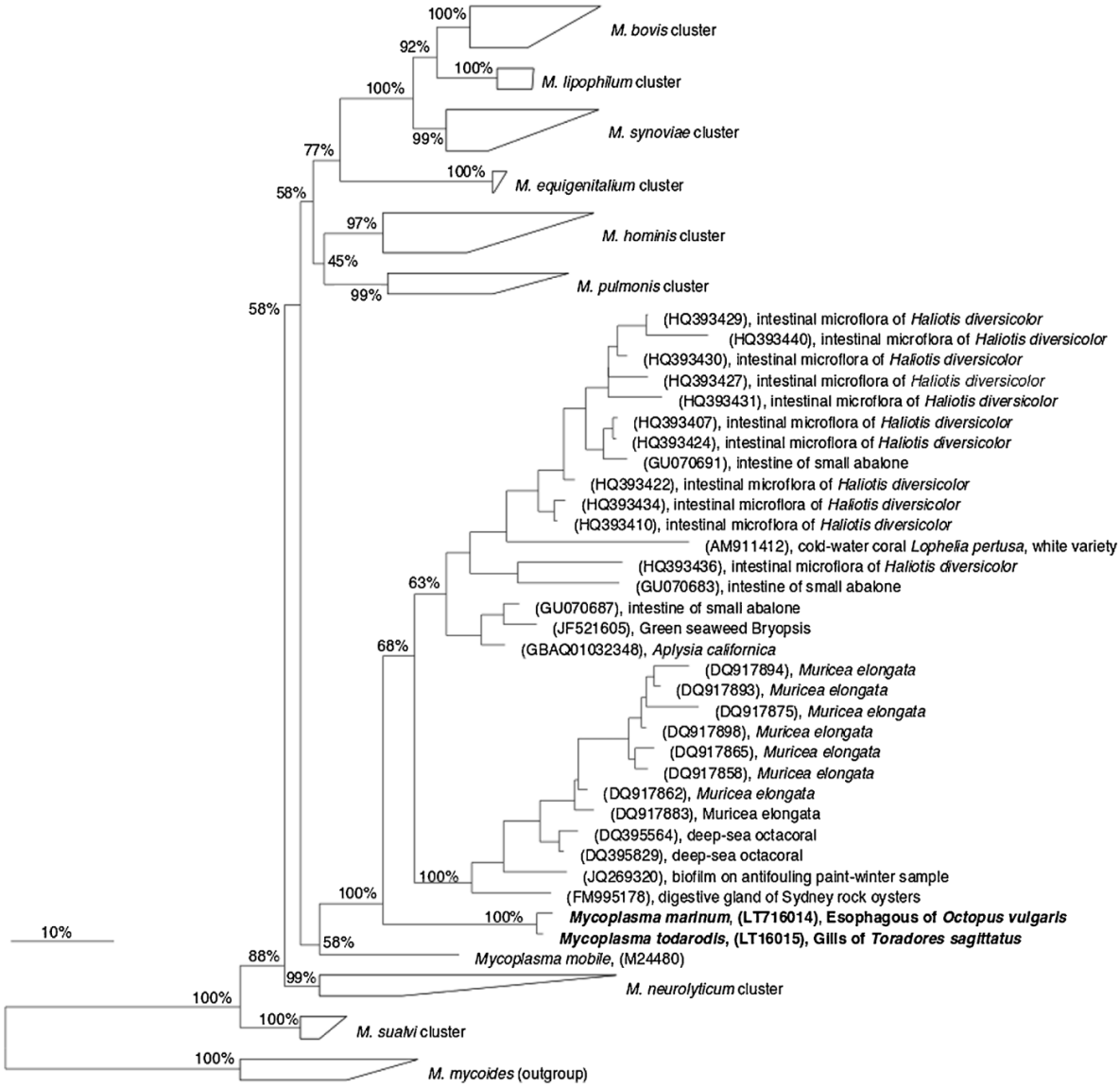

Fig. 2. Phylogenetic reconstruction of the new cephalopod mycoplasma strains. 16S rRNA gene tree reconstruction based on all almost complete sequences of *Mycoplasma* type strains with the addition of all those of the closest relatives retrieved from culture-independent surveys close to the two new *Mycoplasma* lineages studied in this work. The tree is based on a maximum likelihood reconstruction using the RAxML algorithm [59], with the bacterial conservational filter as implemented in the LTP_132 [72] that removes all positions not conserved for the bacterial domain. Bootstrap values in the nodes indicate branch stability. The monophyletic origin of *M. mobile* (acc. M24480) was consistent in all treeing approaches using maximum likelihood algorithm with different conservational filters. The distinct clades of mycoplasmas are indicated by each reference species, and the full tree can be seen in the Supplementary Fig. S5. The sequences retrieved by culture independent methods affiliating with the sister branch to the new isolates, are indicated by their accession number (in brackets) and the sample origin according to their publication or the gene entry description.

MS profiles comparing the mass spectra of the type strains with an extended mycoplasma database, showed that the new mycoplasma strains formed a cohesive and homogeneous cluster clearly separated from type strains of closely related species, confirming that each could represent a different species (Supplementary Fig. S5).

Since these results suggested that the five strains represent two different species, and that each species was formed by the same clonal variant despite originating from different individual animals we selected only one representative from each species for whole-genome sequencing (for details on quality and gene content see below in Section "Genomic characteristics of the new isolates"). The ANI value between genomes was not calculated because the has been proposed as a putative boun dary for species circumscription [25,26,54]. In addition, the AAI value with the closest relative *M. mobile* genome was about 60.53% and 60.59% for the octopus and squid isolates, respectively; lower values were obtained for other known *Mollicutes* taxa (Tables 1 and 2). Again, and in accordance with the 16S rRNA gene identity observations, AAI values below 70% would be compatible with these two isolates representing new genus within the *Mollicutes* [26]. However, as already mentioned, we prefer to consider them just as new species and to retain their genus status as *Mycoplasma*. The similarity dendrogram based on the amino acid identity of the core genome genes (Fig. 3) confirmed our observations showing the cephalopod isolates to be distinct

cleotide identity (ANI; upper right) and average amino acid identity (AAI; lower left in bold) between selected *Mycoplasma hominis*, *Spiroplasma* and *Mesoplasma* genomes. Values in brackets represent the number of teins and the % of shared proteins. The ANI value was not calculated (ND - Not determined) because the number of genes shared was lower than 20% of the genomes.

	Mycoplasma sp. 5H^T (PSZP00000000)	*Mycoplasma* sp. PE^T (PSZO00000000)	*Mesoplasma florum* L1 (AE017263.1)	*Mycoplasma anatis* 1340 (AFVJ00000000.1)	*Mycoplasma canis* PG14 (CP014281.1)	*Mycoplasma lipofaciens* R171 (JMKY00000000.1)	*Spiroplasma citri* GII3-3X (CP013197.1)	*Mycoplasma mobile* 163K (AE017308.1)
ma sp.	–	**ND (194)**	ND (8)	ND (11)	ND (8)	ND (5)	ND (4)	ND (6)
00000)								
ma sp. PE^T)0000)	**57.37 (282–18.81%)**	–	ND (15)	ND (10)	ND (18)	ND (16)	ND (9)	ND (11)
na florum 17263.1)	**57.5 (254–16.94%)**	**57.37 (282–18.81%)**	–	ND (7)	ND (13)	ND (11)	ND (16)	ND (13)
ma anatis	**59.9 (371–24.9%)**	**60.15 (398–26.76%)**	**55.56 (219–14.72%)**	–	ND (46)	ND (28)	ND (4)	ND (13)
00000.1)								
ma canis	**59.7 (342–24.03%)**	**59.52 (369–25.93%)**	**57.13 (204–14.33%)**	**66.37 (517–36.33%)**	–	ND (33)	ND (4)	ND (15)
:1.1)								
ma s R171^T)00000.1)	**59.95 (375–28.19%)**	**60.1 (403–30.3%)**	**57.08 (236–17.74%)**	**64.8 (230–39.8%)**	**64.13 (474–35.64%)**	–	ND (6)	ND (22)
na citri	**58.36 (269–14.01%)**	**57.63 (282–12.97%)**	**60.82 (425–28.35%)**	**56.7 (230–15.47%)**	**57.16 (201–14.12%)**	**57.47 (234–17.59%)**	–	ND (6)
17.1)								
ma mobile	**60.59 (378–29.23)**	**60.53 (390–30.16%)**	**57.04 (207–16%)**	**61.13 (356–27.53%)**	**60.91 (331–25.59%)**	**60.24 (356–27.53%)**	**56.84 (233–18.02%)**	–
18.1)								

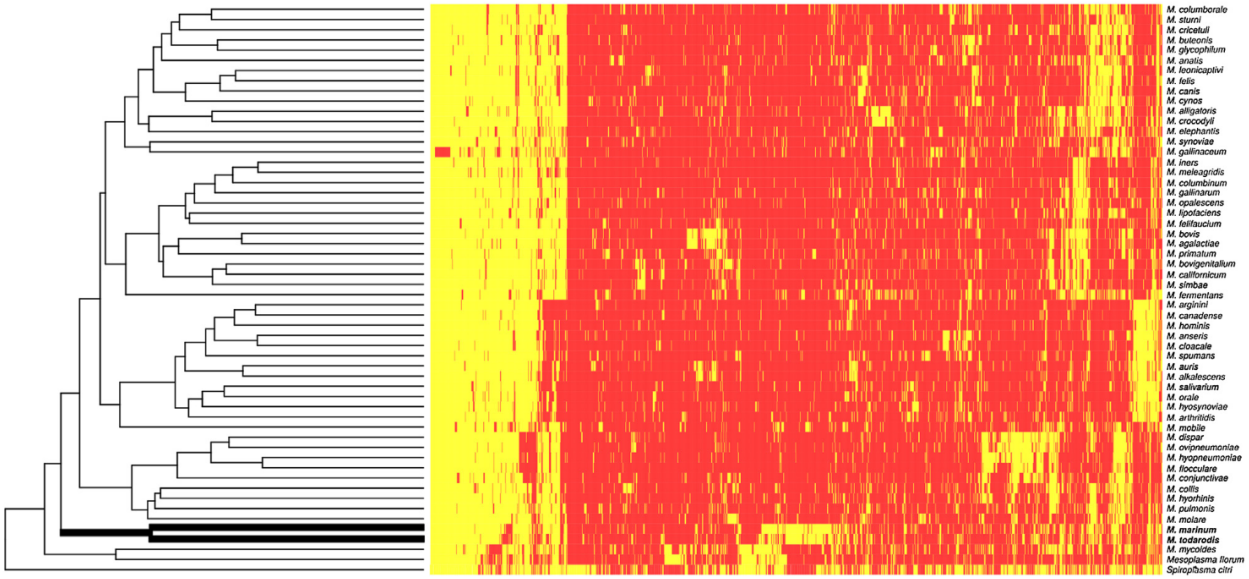

Fig. 3. Hierarchical clustering of cephalopod mycoplasmas genomes based on shared gene content. Clustering was based on the presence (yellow) or absence (red) of the orthologous variable genetic groups (orthologous groups are formed by genes shared between 2 or more genomes) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

teins (Supplementary Fig. S6) mirrored our previous observations using the 16S rRNA gene (Supplementary Fig. S3).

Phenotypic distinction of the new isolates from their closest relatives

The original isolates were fastidious organisms but they grew more rapidly once the strains adapted to the ML — Mycoplasma Liquid Medium (Mycoplasma Experience Ltd, Surrey, UK) medium and the growth conditions were adjusted to meet the optimal conditions (3% NaCl, 25 °C, aerobic incubation). On solid media, the colonies appeared mainly with central spots (<50 μm), especially after their recovery from freezing and lyophilization, but once adapted to the medium they produced small colonies with the typical circular and umbonate (fried egg-shape) morphology after 7 days of incubation at 25 °C (Fig. 1). The diameters varied from 50 to 300 μm (Octopus strain PE^T) or 400 μm (squid strain 5H^T). Colonies could be seen on plates incubated aerobically and microaerobically, however, slightly more robust growth was seen when plates were incubated anaerobically. Well-defined colony margins and absence of satellite colony formation indicated that the organisms were non-motile [9]. Furthermore, gliding motility was not observed for any cephalopod mycoplasma despite several attempts at various temperatures and at various viscosities of the medium. Transmission electron micrographs of ultrathin sections showed pleomorphic cells of approximately 200–1100 nm (PE^T) and 80–480 nm (5H^T) in length with a cell membrane, absence of cell wall and granular, moderately electron-dense nucleosome (Fig. 1C and D). Although the majority of motile mycoplasmas are members of the *M. pneumoniae* group, a few species in the *M. hominis* group have also been shown to glide over solid surfaces [7,39]. However, as suspected from the rounded cell morphology, the lack of an elongated flask shape, the absence of a noticeable attachment tip structure and the lack of motility genes, the cephalopodian mycoplasma seem to be non-motile.

In liquid media, strains grew both aerobically and anaerobically (Table 3), with aerobic growth being preferred. The isolates passed tolerated was 4.5% (at 18 °C) and 5% (at 25 °C) and the minimum 0.5–1% (at 18 °C) and 1.0% (at 25 °C), the optimum 3% (w/v). No growth at all was visible without the addition of NaCl. *M. mobile* [24], *M. moatsii* [16,30] and *M. sualvi* [18] were compared with our isolates as these were the closest relatives (Table 3). Octopus strain (PE^T) and squid strain (5H^T) differed from the other *Mycoplasmas* and *Mollicutes* in their need for salt, and represent the first description of a moderate halophilic *Mollicutes*. The optimum incubation temperature of 25 °C was the same as for *M. mobile* [24], and close to the 30 °C for reptile mycoplasmas (*M. aggassizii, M. alligatoris, M. crocodyli, M. testudineum, M. testudinis* and *M. insons*), while it differed from the 37 °C optimum temperature of other mycoplasmas [8]. Another phenotypic characteristic that differentiated the cephalopod mycoplasmas from nearly all other *Mollicutes* (except *M. mobile*) was their ability to grow below 20 °C. Growth at 4 °C was observed, although sparse. They were typically psychrophylic, being able to grow at 0–5 °C, but with an optimum growth temperature above 15 °C [17]. The isolates required sterol for growth, and fermented glucose and mannose, but did not possess urease activity or reduce tetrazolium. Arginine hydrolysis was negative for both the octopus strain (PE^T) and squid strain (5H^T). Film and spots were produced on agar by the octopus strain (PE^T), but not by the squid strain. Octopus and squid strains differed only in this test.

Genomic characteristics of the new isolates

The most relevant genomic features of the PE^T and 5H^T strains with respect to *M. mobile* (163K^T) can be seen in Table 1. After assembly, the genome of the octopus strain (PE^T) presented 128 contigs (>500 bp length). The total length of the genome was 1.17 Mbp and encoded for 1003 genes, of which 328 were hypothetical proteins and 42 were transfer RNAs. The genome contained a single copy of the 16S and 23S rRNA genes and two copies of the 5S rRNA gene. On the other hand, the genome of the squid strain (5H^T) presented 84 contigs with a size of 1.008 Mbp. This genome presented 914 genes that included 296 genes for hypothetical proteins, 39 transfer RNAs, one copy of the 16S and 23S rRNA genes

phenotypic characters of *Mycoplasma* sp. PE^T and *Mycoplasma* sp. 5H^T with respect to *M. mobile* 163K^T, *M. moatsii* MK 405^T and *M. sualvi* Mayfield B^T.

	Mycoplasma sp. PE^T	*Mycoplasma sp.* 5H^T	*M. mobile* 163K^T	*M. moatsii* MK 405^T	*M. sualvi* Mayfield B^T
	Octopus (*Octopus vulgaris*)	Squid (*Toradores saggitattus*)	Tench (*Tinca tinca*)	Grivit monkey (*Cercopithecus aethiops*) and rats (*Rattus norvegicus*)	Pig (*Sus scrofa*)
o host	Commensal	Commensal	Pathogen	Commensal	Commensal
	Oesophagus, digestive tract	Gills, intestine	Gills	Urogenitary and respiratory tracts	Intestine and vagina
norphology	Fried-egg	Fried-egg	Fried-egg	Fried-egg	Fried-egg
urface	Highly granular	Fine granular	Microsatelites	Fine granular	NM
ize	20–260 µm	30–390 µm	10–500 µm	50–200 µm	NM
norphology	Coccoidal	Coccoidal	Flask shaped	Spheroidal	Flask shaped
ize	200–1100 nm length	80–480 nm length	300–1600 nm length	300–1200 nm	670–870 nm length
	200–900 nm width	80–440 nm width	100–500 nm width		250–350 nm width
on	Anaerobic facultative	Anaerobic facultative	Anaerobic facultative	Anaerobic facultative	Anaerobic facultative
(range) growth ure	25 °C (4–25 °C)	25 °C (4–25 °C)	25 °C (4–30 °C)	37 °C	37 °C (30–37 °C)
irement	+	+	–	–	–
(range) NaCl % (w/v) ent	3% (1–4.5%)	3% (1–4.5%)	ND	ND	ND
ermentation	–	–	+	–	–
fermentation	+	+	+	+	+
hydrolysis	+	+	+	ND	ND
is of urea	–	–	–	+	+
n of	–	–	–	–	–
henyltetrazolium					
on of film and spots	+	–	+	ND	–
es	This paper	This paper	[24]	[16,30]	[18]

ne; NM-not mentioned in the publications.

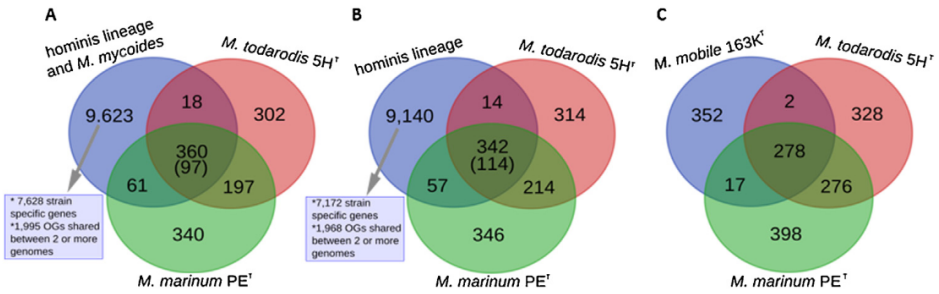

Fig. 4. Shared orthologous gene groups (COGc) of cephalopod mycoplasmas. Venn diagrams showing the number of shared orthologous genetic groups (OGs) between the genome of *Mycoplasma* sp. 5H^T, *Mycoplasma* sp. PE^T with: (A) members of *Mycoplasmas* affiliated to the hominis lineage adding the *M. mycoides* subsp. *mycoides*; (B) just with hominis lineage; and (C) with *M. mobile*. The number of shared genes between all genomes (core-genes) is shown in brackets for each Venn diagram.

Table 4
Description of *Mycoplasma marinum* sp. nov. and *Mycoplasma todarodis* sp. nov.

Taxonumber	TA00412	TA00413
Species name	*Mycoplasma marinum*	*Mycoplasma todarodis*
Genus name	*Mycoplasma*	*Mycoplasma*
Specific epithet	*marinum*	*todarodis*
Species status	sp. nov.	sp. nov.
Species etymology	ma.ri'num. L. neut. adj. marinum of or belonging to the sea, marine	to.da.ro'dis. N.L. gen. n. todarodis of the squid *Todarodes sagittatus*, as the type strain was isolated from the flying squid, *Todarodes sagittatus*
Strain collection numbers	DSM 105487^T = CIP 111404^T	DSM 105,488^T = CIP 111405^T
16S rRNA gene accession number	LT716014	LT716015
Genome accession number [RefSeq]	PSZO00000000	PSZP00000000
Genome status	Draft	Draft
Genome size bp	1,171,149	1,007,879
GC mol %	28.41	30.95
Country of origin	Spain	Spain
Region of origin	Canary Islands, Gran Canaria	Canary Islands, El Hierro
Date of isolation	2012-04-01	2012-01-18
Source of isolation	*Octopus vulgaris*, oesophagus	*Todarodes sagittatus*, gills
Sampling date	2012-02-15	2011-12-05
Geographic location	Gran Canaria	El Hierro
Number of strains in study	2	3
Source of isolation of non-type strains	*Octopus vulgaris*, digestive gland	*Todarodes sagittatus*, gills, intestine
Growth medium, incubation conditions	medium SP4-II + 3% (w/v) NaCl, 25 °C, aerobic.	medium SP4-II + 3% (w/v) NaCl, 25°, aerobic.
[Temperature, ph, and further information] used for standard cultivation	Primary isolation needed 2 months	Primary isolation needed 1.5-2 months
Is a defined medium available	ML - Mycoplasma Liquid Medium (Mycoplasma Experience) supplemented with 3.0% (w/v) NaCl	ML - Mycoplasma Liquid Medium (Mycoplasma Experience) supplemented with 3.0% (w/v) NaCl
Motility	Nonmotile	Nonmotile
Sporulation (resting cells)	None	None
Colony morphology	Fried-egg shape	Fried-egg shape
Temperature range	4-25 °C	4-25 °C
Lowest temperature for growth	4 °C	4 °C
Highest temperature for growth	25 °C	25 °C
Temperature optimum	25 °C	25 °C
pH optimum	7	7
Lowest NaCl concentration for growth	1%	1%
Highest NaCl concentration for growth	5%	5%
Salinity optimum	3%	3%
Salinity category	Mild halophile (optimum 1-6 % NaCl)	Mild halophile (optimum 1-6 % NaCl)
Relationship to O2	Facultative aerobe	Facultative aerobe
O2 conditions for strain testing	Aerobiosis	Aerobiosis
Carbon source used [class of compounds]	Sugars	Sugars
Acid formation from carbohydrates (all positive)	Glucose, mannose	Glucose, mannose
Energy metabolism	Chemoorganotroph	Chemoorganotroph
Biosafety level	1	1
Habitat	Oesophagus (UBERON:0,001,043)	Gills (UBERON:0,000,171)
Biotic relationship	Commensal	Commensal

with or without *M. mycoides*. The organisms shared 278 genes with *M. mobile*, 342 genes with the hominis lineage and 360 with the hominis lineage plus *M. mycoides mycoides*.

The central metabolism of the *Mycoplasma* is the core reactions of the Embden–Meyerhof–Parnas pathway (synthesis of pyruvate (EC 1.1.1.28 and EC 1.1.1.27 respectively) [47,53] and for this reason they are facultative anaerobe.

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related 16S rRNA gene sequences (*e.g.*, >97% identity) were detected associated in datasets originating from squid [4] and octopus [22]. Coupled to the histopathological studies revealing no lesions in the squid or octopus studied here, and the apparent healthy state of the cephalopods in the previous studies, these findings suggest that the two proposed new *Mycoplasma* species are host-associated and are possibly natural members of their microbiome.

The authors wish to emphasize that *Mycoplasma* taxonomy is a sensitive and complex issue [7]. Since the late eighties, the polyphyly of this genus has been known [70] and it has been a recurring topic of debate [15,28,63,67]. A recent proposal for changes attempts to reconcile phylogeny and taxonomy [19] but within the genus *Mycoplasma* there are many important human and animal pathogens whose names are related to a large body of publications and government regulations. For this reason, and according to the International Code of Nomenclature of Prokaryotes, changes in the taxonomy of this group would have to be considered with caution [28,42]. Thus we have carefully evaluated the alternative of proposing a new genus within the *Mollicutes*. However, according to the majority opinion of the members of the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Mollicutes* [34], and to avoid taxonomic confusion and even produce a rejection by the scientific community, as previously occurred with the genus *Chlamydia* [60], we have preferred to retain a traditional monotypic genus and classify new species instead.

Based on the biochemical, chemotaxonomic, and phylogenetic characteristics, strain PE^T and 5H^T, representing two and three clonal isolates from distinct individuals respectively, can be distinguished from each other and other members of the genus *Mycoplasma* as different species. They represent the first mycoplasmas isolated in pure culture from a non-mammal marine origin; both have been shown to be psychrotolerant and to have a unique characteristic within the genus of being moderately halophilic. For them we propose two novel species within the genus *Mycoplasma*, family *Mycoplasmataceae*, and order *Mycoplasmatales.* We propose *M. marinum* sp. nov. and *M. todarodis* sp. nov. for the new marine species. The species description according to the digital protologue are shown in Table 4.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.04.003.

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