*International Journal of Latest Trends in Engineering and Technology*
*Vol.(13)Issue(3), pp.010-015*
*DOI: http://dx.doi.org/10.21172/1.133.02*
*e-ISSN:2278-621X*

# DESCRIPTION OF COCHLIOPODIUM MASSILIENSIS SP. NOV., ISOLATED IN BRONCHOALVEOLAR LAVAGE OF A TUNISIAN PATIENT WITH PNEUMONIA.

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**Abstract- In this work, we describe the isolation of Cochliopodium massiliensis sp nov in a bronchoalveolar lavage from a patient with pneumonia. It is the first international isolate of this genus of amoeba in a human sample but also the first case described during this pathology. This new species is the smallest of the Cochliopodium species already described. Cochliopodium massiliensis sp nov is described on the basis of morphological observations in light microscopy, ultrastructure by TEM microscopy and molecular genetic evidence of SSU rDNA. Cochliopodium massiliensis n. sp (length ~ 16µm) is characterized by the presence on the surface of complex structures called Scale. Containing a apical funnel shaped collar (~560 nm), dorsal and ventral bases plate of the Scale (with radius ~ 420 nm). Based on morphological and molecular genetic data of SSU-DNA gene, we describe our isolate as a new species, the first isolate of this genus from a human biological sample, and the first case described during pneumonia.**
**Key words: Cochliopodiidae, Cochliopoduim, ultrastructure, comparative morphology, phylogeny, SSU-rDNA, taxonomy.**

## 1. INTRODUCTION

Amoebae are micro-organisms that have a complex spatial and temporal distribution in all ecological niches, from freshwater (Acanthamoeba sp) to the most extreme environments (Halophilic Heterolobosean). Some families of amoebae are recognized as human pathogens such as Entamoeba histolytica, Entamoeba gengivalis, Balamuthia mandrillaris and Naegleria fowleri. Others have a dual role as human pathogens responsible for keratitis as Acanthamoeba keratitis, but also a Trojan horse for many amoeba-resistant pathogenic bacteria (Szenasi Z et al 1998, Linder E and Winiecka KJ, 2001). Recently, for the first time, Cochliopodium minus is recognized as host of a symbiotic bacterium Candidatus Cochliophilus cryoturris (Tsao HF et al 2017). The pathogenic role of this bacterium remains unclear. This group of amoebae is particularly interesting by its mode of reproduction. Evidence from both light microscopy and immunocytochemistry demonstrates that Cochliopodium undergoes cell fusion (plasmogamy) and nuclear fusion (caryogamy) (Tekle YI et al 2014). This process leads to a non-meiotic and parasexual exchange of chromosomes in Cochliopodium (Tekle YI et al 2014). This strongly suggests that Cochliopodium is involved in parasexual activity and that it should no longer be considered strictly asexual like the majority of eukaryotic microbes (Wood FC et al 2017). This study based on the analysis of large amounts of transcriptomic data for 2 species of Cochliopodium, known for their high frequency of cell and nuclear fusion (Wood FC et al 2017). This work has demonstrated the existence of 60 sex-related genes, including 11 genes specific to meiosis and 31 genes involved in fusion and karyogamy (Wood FC et al 2017). The expressed genes form an almost complete recombination mechanism indicating that Cochliopodium is an actively recombinant sex line (Wood FC et al 2017). But also by the presence of a complex and spectacular protein structure called Scale for the genus Cochliopoduim. The latter belongs to the family Cochliopodiidae described by De Saedeleer H (1934), then revised by Page FC (1987, 1988), which comprises four genera, Cochliopodium (Hertwig R and Lesser E 1847), Gocevia (Valkanov 1932), Paragocevia (Page FC 1987) and Ovalopodium. (Sawyer TK 1980). Although it has few species, the genus Cochliopodium is better known for Cochliopodiides. Amoebae of this type are discoidal or globular enclosed in a flexible system, cuticle or tectum composed of complex micro-Scale. The dorsal surface is covered with a tectum and only the ventral surface applied to the substrate (Tekle YI et al 2013). Or a fibrous cuticle for Gocevia and Paragocevia ( Pussard M et al 1977). However, the structure of the genus Ovalopodium remains uncertain. Locomotive forms of this genus Cochliopoduim (ranging in size from less than 20 microns to more than 50 microns) are flattened with a hyaline margin of sub-pseudopods running that may lengthen or partially surround a granular hump (Page FC 1988). Besides the size, the fine structure of tower-shaped micro-Scales (Bark AW 1973) is a distinctive element. In the post-molecular era, Page (1987, 1988) placed Cochliopodiums in the phylum Rhizopoda, subclass Testacealobosia (De Saedeleer H 1934) and order Himatismenida (Page 1987). Currently, they are classified in the Amoebozoa and Amorphea super-group (Adl et al., 2012), with typical characteristics of amoeboids, notably non-eruptive and extended pseudopods, stable locomotion characteristic of Acanthamoeba, Amoeba Proteus, Vannella and Vexillifera. Molecular phylogenetic analysis shows that the genus Cochliopodium is robust holophyletic within Amoebozoa and is fully compatible with observational holophyletic morphological data (Kudryavtsev A et al., 2005). There are five known species in

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the literature review with a size of less than 20 microns of the genus Cochliopodium: Cochliopodium gallicum, Cochliopodium minutum, Cochliopodium Minutoidum, Cochliopodium kieliense and Cochliopodium maeoticum (Kudryavtsev A 2006, Page FC 1988). Two new species recently described have a size greater than 20 µm, Cochliopodium pentatrifurcatum, with a size of 25 µm (Tekle YI et al 2013) and Cochliopodium arabianum with an average length of 35 µm and a width of 51 µm (Tekle YI et al 2015).Others are much larger, such as Cochliopodium gulosum (~ 56-90 microns) (Schaeffer 1926), Cochliopodium spiniferum (~ 70 microns) (Kudryavtsev A 2004) and Cochliopodium bilimbosum (~ 90 microns) (Sadakane et al., 1996). The genus Cochliopoduim is present in many environment such as freshwater, Bottom sediments from the freshwater pond near, Bottom sediments from the Gulf of Taganrog, the Azov Sea, Southern Russia (Tekle YI et al 2013), Saline water (Kudryavtsev A and Smirnov A 2006), and from animals such as gills and other organs of the perch, Perca fluviatilis (Dykova I et al, 1998). We describe the isolation of Cochlipodium massiliensis sp nov, isolated from bronchoalveolar lavage of a patient with pneumonia. It is the first human isolate of this genus of amoeba and the first isolate of Cochliopodium during pneumonia. With structural features studied by electron and optical microscopy (~ 16 microns long) with beautiful Scales unique to ensure its taxonomy but also the phylogenetic evidence based on the molecular genetics of the 18S rRNA gene for a precise idea of this new species.

## 2. RESULTS

*2.1 Structure by light microscopy with phase contrast*

The strain Cochliopodium massiliensis sp nov has a length of 14-18 microns with an average of 16 microns and a width of 7-11 microns with an average of 9 microns. The ratio of width to length was 0.5 to 0.61 micrometer with an average of 0.55 micron. The average rate of locomotion was 12 mm per minute. During locomotion, the amoeba exposed various oval and angular forms (Fig. 1a). Sometimes, during locomotion, the posterior portion of the amoeba is attached to the substrate, allowing the cell to lie down, expand, and advance forward (Figure 1b). During the mobilization of the amoeba, we witness the emission of sub-pseudopods (Fig. 1a). The nucleus was vesicular and the cytoplasm was granular and contained numerous vacuoles with a wide range of sizes ranging from 0.8 to 1.9 µm, sometimes broadly aggregated as a halo at the periphery of the granuloplasm (Fig. 1b). During locomotion, it was observed that amoebae were in contact, sometimes momentarily glued, to form aggregates of 2 amoeba. Amoeba cysts were observed with sizes ranging from 7 to 11 microns (Fig. 2), depending on their stage of maturation after 3-4 weeks in liquid culture.

*2.2 Ultrastructure by TEM*

The fine ultra structure and morphology of the Scales are identified which shows that our isolate belongs to the Cochliopodidae. A cross section of an amoeba (Fig. 3a) shows a nucleus (~ 4.5µm). Different characteristics of this isolate, Cochliopodium massiliensis, including of Amoebozoa. Same, the presence of subpseudopodia in the peripheral (Fig.3a). The forms of Mitochondria are tubulo- cristate with a typical diameter of 1,7-1,9 micron (Fig.3a). The Scales (560nm in height) have a particular morphology for this new species (Fig.3a), comprising a base plate and dorsal base plate radius (~420nm) (Fig.3b) and apical funnel shaped collar parts (~560nm) (Fig.3b) in a tangential section.

*2.3 Giemnez, Hemacolor and Gram staining of Cochliopodium massiliensis*

Our isolate themselves in Giemnez, Hemacolor and gram staining with a nucleus individualized by this techniques (Fig.4a,b, and c).

*2.4 Phylogeny by 18S ribosomal RNA gene*

Cochliopodium massiliensis sp nov, is an out group with the genus Cochliopodium with full support (Fig5). The GenBank access numbers for our nucleotide sequence is MK734144.

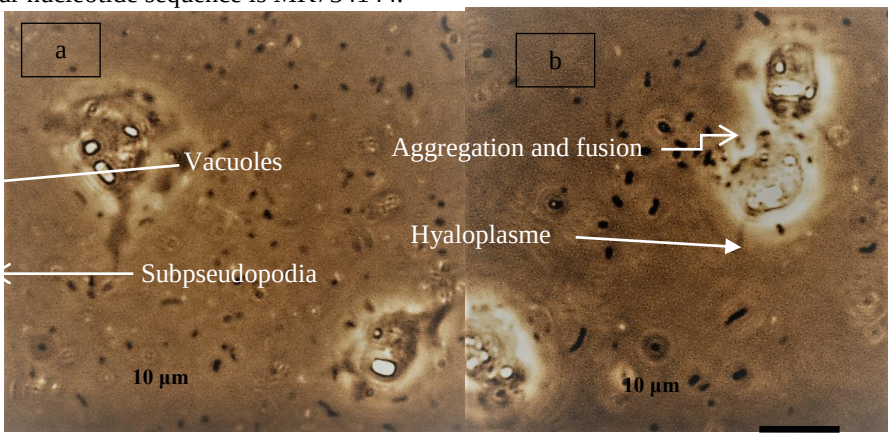


Figure1: Images of Cochliopodiumtun massiliensis sp. nov., by light microscopic with phase contrast a–b. Morphology of living C massiliensis n. sp. during locomotion. a – Several forms of amoeba are observable during locomotion (oval,

triangular).a- locomoting amoebae with anterior hyaloplasm fringe; a – morphology of the relatively short subpseudopodia that become adhesive laterally and posteriorly, and attach to the substratum; b – aggregation and fusion of amoebae, scale bars: 10 µm.

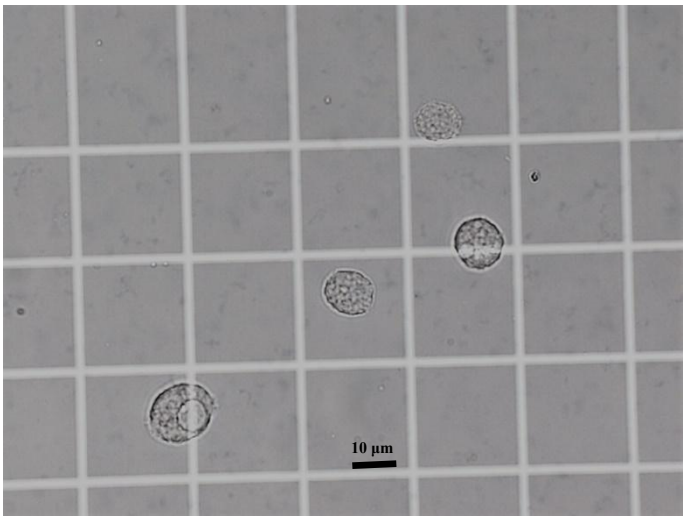


Figure2: Mature cysts of Cochliopodium massiliensis sp. nov., Scale bars = 10 µm

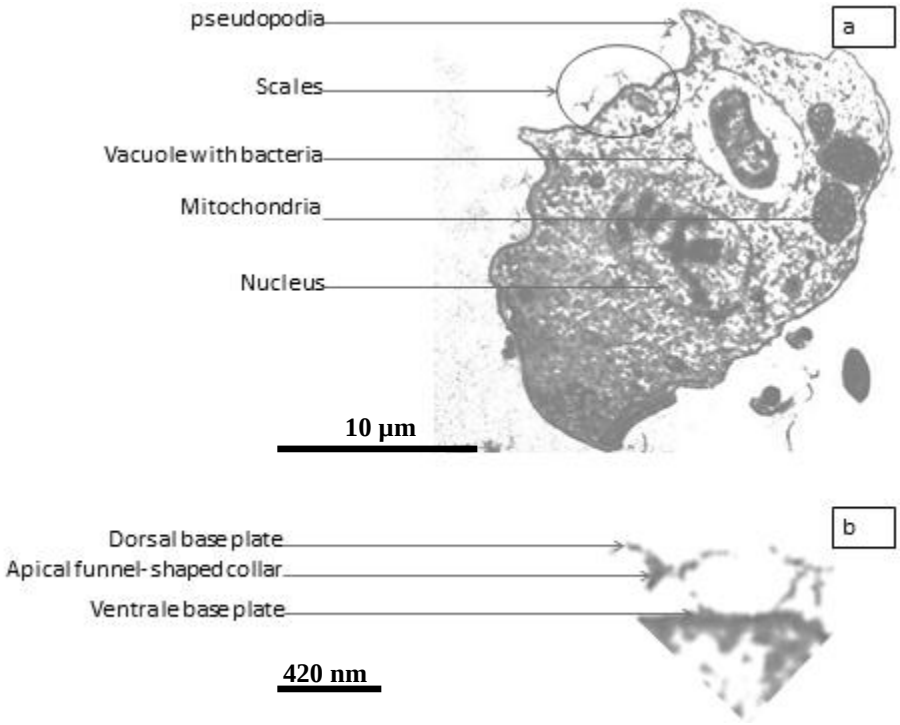


Figure3: a- Cell of Cochliopodium massiliensis sp. nov., in cross-section showing fine structure of the nucleus, vacuole, emergent pseudopodia from the ventral surface, tubulo-cristate mitochondria in the cell periphery and Scales. b- The fine structure and details of Scale in the periphery of amoeba. Scale bars = 10 µm

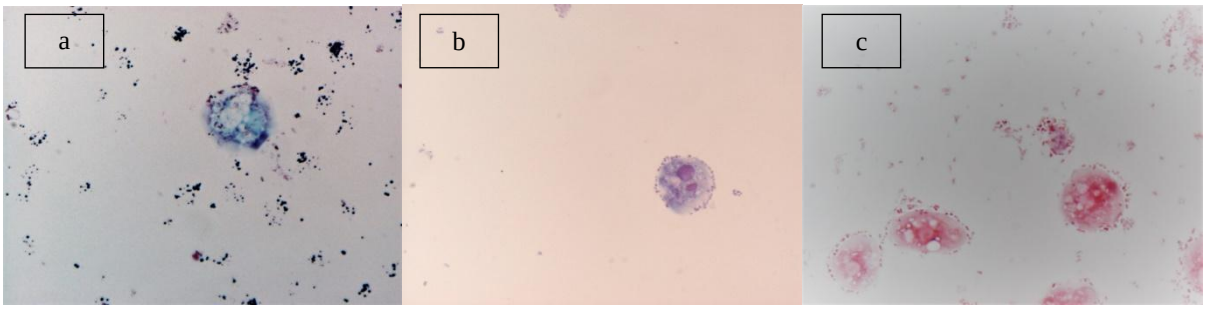


Figure4: Cochliopodium massiliensis sp. nov., by a-Giemnez, b-Hemacolor and c-Gram staining.

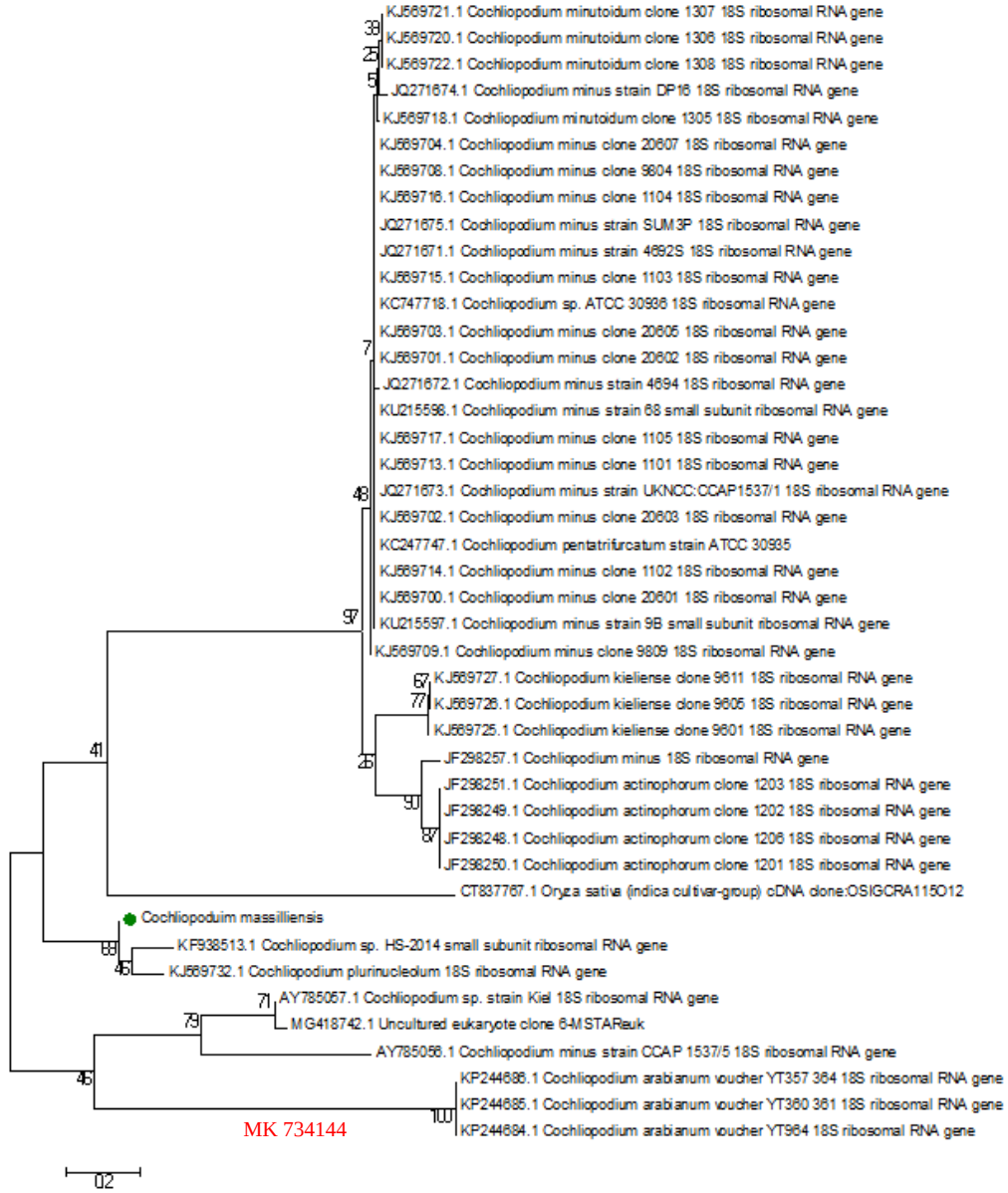


Figure5: Phylogram tree made from the alignment of the gene ortholog sequence of gene SSU-rRNA sequence. Using nucleotide BLAST program, gene homologous sequences related to SSU rRNA sequence (MK734144 is the number assigned by genbank) from Cochliopodium massiliensis sp nov, Phylogram tree using the Mega5.04 program (NJ method, bootstrapped 500 times) constructed from the ClustalW alignment of the SSU-rDNA gene sequence of different species of Cochliopodium (indicated on the tree). The gene sequence of interest has a node in green. The bar at the bottom indicates 0.2% amino acid divergence.

## 3. DISCUSSION

In the literature, we can count up to 21 described species published for the genus Cochliopodium, for which there are 13 species officially having a species name and the other simply called sp which three described by Bark AW (1973). In addition, three other species mentioned, Cochliopodium echinatum (Korotneff A, 1879), Cochliopodium pilosum (Hertwig and Lesser 1874) and Cochliopodium longispinum (West GS 1901) are described by Kudryavtsev A (2005) by complete morphological analysis using transmission electron microscopy as synonym of Cochliopodium longitum (West 1901), originally described by Archer W (1871, 1877). In the literature many types of Scales are known. Indeed, two new species recently reported in the literature show remarkable differences in the structure of Scales. Cochliopodium pentatrifurcatum has a scale showing the surface of a base with a narrow rim and areolate internal flat five styles Supporting a central column, and an apical funnel -shaped collar with radial rays and trifurcate a relatively short vertical spine (Tekle YI et al, 2013). And Cochliopodium megatetrastylus has a scale showing the grid -like base plate and the apical conical collar supported on oven styles (Anderson OR and Tekle YI, 2013). But it must be emphasized that these forms of Scales are commonly found in other species of the genus Cochliopodium. Cochliopodium massiliensis sp. nov., has a Scales shaped tower without terminal spine completely different in size of other species published. But the overall shape of the Scales of this new strain reminder the form of C. minus. The discovery of Cochliopodium massilliensis sp nov in a patient with pneumonia highlights the important role of amoebae in this pathology. Indeed this amoeba can be infected by Legionella and therefore other microorganisms that can play an important role in this disease. This discovery shows the need to broaden the spectrum of research on emerging microbes in pneumonia, 40% of which remain unknown origin by relying on the search for amoebae other than Acanthamoeba. Based on the morphology observation by optical microscopy, the results of fine structure by TEM microscopy, the structure and the size of Scales, and the phylogeny based on the molecular genetics of SSU rDNA gene, we describe our isolated strain as a new specie.

## 4. MATERIALS AND METHODS

*4.1 Isolation Cochliopodium massiliensis sp. nov.,*

The strain Cochliopodium massiliensis sp nov. Has been isolated from a Tunisian collection of LBA used to search emerging viruses. The presence of viruses is observed under an optical microscope (× 40 magnifications) by a cytopathic effect on Acanthamoeba spp. Observation of the culture wells led us to identify an amoeba of unusual morphology that Acanthamoeba spp used as a cellular support. Initially, we tried to inoculate in a petri dish containing fresh Klebsiella aerogenes used in the usual laboratory diagnosis for the isolation of amoebae in a corneal fluid. After two days of agar monitoring, a small fragment is cut with a scalpel after identification of the amoeba on the agar plate and placed in liquid culture containing PAS buffer (Page amoeba saline) with fresh Klebsiella aerogenes. It is thanks to this technique that we have been able to isolate this new species. After their transfer in a liquid medium. The amoeba was cultured in the presence of heat-neutralizing Klebsiella aerogenes to promote passage and adaptation to an axenic medium such as PYG (Peptone Yeast Extract). All observations and measurements reported here were performed on live amoebae with a target x 40; further observations have been made with the use of Giemnez, Gram and Hemacolor staining with a 100x objective using oil emersion.

*4.2 Light microscopy*

To study the behavior and morphology of Cochliopodium massiliensis sp nov., (> 200 cells), amoebae grown in plastic Petri dish. Cultures were recorded over a period of time 8 months. The rate of locomotion of amoebae and other morphological characteristics were observed using an inverted optical microscope (Zuzi 1615225- Camera) with software (Image j). The calculation of locomotion amoebae in culture was carried out in plastic Petri dish for solid medium in non-nutritive agar coated with 1 ml of fresh Klebsiella aerogenes and in plastic plates 6 well for liquid medium with PAS buffer and 500 µl of fresh Klebsiella aerogenes. The propagation speed of the amoeba and the distance of migration are calculated in average speed (V T2-T1=d T2-T1/t T2-T1) and not instantaneous speed (VI=dt/tt).

*4.3 TEM Electron Microscopy*

The washes of Cochliopodium massiliensis cells were made in PBS buffer (Phosphate-buffered saline). After resuspended in 2% paraformaldehyde, 0.5 % glutaraldehyde in PBS buffer for 1 h at room temperature. It performs by three successive washes i n PBS buffer. The cell pellet was fixed in 2% osmium tetroxide, washed once in PBS buffer, dehydrated in 70, 95 and 100 % ethanol and embedded in Epon 812. Ultrathin sections were post stained with 4% uranyl acetate and lead citrate and were observed using FEI Tecnai TEM operating at 120 kV.

*4.4 DNA extraction, PCR amplification, alignment of sequence, and phylogenetic analysis*

DNA was extracted from 200µl of the material obtained of Cochliopoduim massiliensis culture in PAS buffer. The extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) according to manufacturer‟s instructions. For the molecular characterization, we use one primer pairs to amplify the Cochliopoduim massiliensis by targeting the SSU- rDNA (F= ccagctccaagagtgtatatt / R=gttgagtcgaattaagccgc). The primers were used for sequencing in an ABI PRISM 3130xl genetic sequencer (Applied Biosystems). The sequences were assembled, analysed using Chromaspro

software (Technelysium) and compared with sequences in the GenBank database using BLAST software (http://blast.ncbi.nlm.nih.gov/). To build the phylogenetic tree, the sequences were aligned using ClustalW alignement software and the tree were created using Mega5.04 (Tamura K et al 2011).

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