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Research Article

New Species of *Pseudocohnilembus* (*Ciliophora*: Cuticociliate) Identified from a Rock Dove Feces in Algeria

Asma Guilane[1], Tahar Kernif[2], Fadila Tazerouti[1], Rezak Drali[3] and Amina Boutellis[1]*

[1]Laboratory of Biodiversity and Environment: Interactions, Genome, Faculty of Biological Sciences, University of Science and Technology Houari Boumediene, 32 El Alia, Bab Ezouar 16111 Algiers, Algeria

[2]Laboratory of Parasite Eco-Epidemiology and Population Genetics, Pasteur Institute of Algeria, Algeria

[3]Genomics Platform-Bioinformatics, Pasteur Institute of Algeria, Algeria

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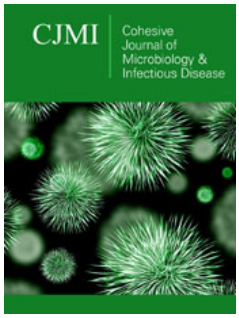

***Corresponding author:** Amina Boutellis, Laboratory of Biodiversity and Environment: Interactions, Genome, Faculty of Biological Sciences, University of Science and Technology Houari Boumediene, 32 El Alia, Bab Ezouar 16111 Algiers, Algeria

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Abstract

Scuticociliates are facultative parasitic ciliates of the subclass *Scuticociliatia* Small, 1967 which cause *scuticociliatosis*, one of the most important parasitological problems in marine aquaculture worldwide. In this study, we report, for the first time, the presence of *Pseudocohnilembus* sp. in Algeria. The unexpected discovery of *Scuticociliate* DNA during our research on protozoan parasites in bird faeces, led to the parasite's molecular and phylogenic characterization. BLAST analysis of the partial small subunit ribosomal DNA sequence (SSU rDNA) showed sequence similarity value of 99.64% with an uncultured *Pseudocohnilembus* clone. The alignment of this sequence with other *Pseudocohnilembus* sequences gives a separated sub-clade among the same uncultured *Pseudocohnilembus* sequences isolated from stool. The distance between our *Pseudocohnilembus* sp. and the uncultured *Pseudocohnilembus* sequences isolated from stool does not exceed 1% when computed using Kimura 2-parameter model, however, it greatly exceeds 1.5% with the other species included in the dataset. These distances, and the phylogenic tree, clearly indicates that *Pseudocohnilembus* sp. isolated from different animal stools corresponds to a new species, and our present sequence of *Pseudocohnilembus* sp. forms an original Algerian haplotype.

Keywords: Animal feces; Birds; Genotype; Protozoa; SSU rDNA; *Scuticociliates*

Introduction

Ciliated protozoa are single-cell eukaryotes with diverse morphologies and extensive distributions [1]. *Scuticociliatia*, one of six subclasses of the class *Oligohymenophorea*, are commonly found in ecosystems worldwide [1,2]. They are known to rapidly invade and establish colonization of the marine host organs like gills, skin, brain, heart, muscles including visceral organs, and intestine [3,4]. *Scuticociliatosis* is highly histophagous and destroys infected tissues [5]. Outbreaks of *scuticociliates* cause mass mortalities in fishes, and has been reported in olive flounder *Paralichthys olivaceus*, rainbow trout *Oncorhynchus mykiss*, southern bluefin tuna *Thunnus maccoyii*, turbot *Scophthalmus maximus*, seabass *Dicentrachus labrax*, and silver pomfret Pampus argenteus [3]. The genus *Pseudocohnilembus* Evans and Thompson (1964) was originally erected for free-living marine ciliate [6,7]. However, since isolation of *P. persalinus* from diseased olive flounder in Korea by Kim et al. [8], it has become recognized as an important facultative parasite causing serious economic losses in marine aquaculture worldwide. Genome analysis showed that *P. persalinus* has acquired many unique prokaryote-derived genes that potentially contribute to the virulence of this organism, including cell adhesion, hemolysis, and heme utilization genes [2]. Moreover, *P. persalinus* is an ideal model organism for a wide variety of biological research [9] and is considered to be a good microorganism to evaluate environmental risks by applying nanomaterial monitoring and bioremediation [10]. The SSU rDNA sequence analysis approach is a universally applicable tool to identify *scuticociliates* because morphological analysis, based upon qualitative staining techniques may obscure subtle variations, and lead to misidentification [3]. This study was first aimed to screen intestinal protozoan parasite present in birds, and

the fortuitous discovery of a *Scuticociliate* DNA led us to a molecular and phylogenic characterization of the parasite.

Materials and Methods

Collection of animal samples

This study was conducted in accordance with the World Animal Health Organization [11] guiding principles on animal welfare included in the OIE Terrestrial Animal Health Code. Fresh fecal samples from different bird species collected from Algiers, North Algeria (36° 45' 9.00" N and 3° 02' 31.09" E), were screened for gastrointestinal parasites using the zinc sulphate flotation method, followed by microscopy [12]. Among the analyzed samples, one belonging to a rock dove Columba livia Gmelin, 1789, presented granular forms similar to *Blastocystis sp* (Figure 1).

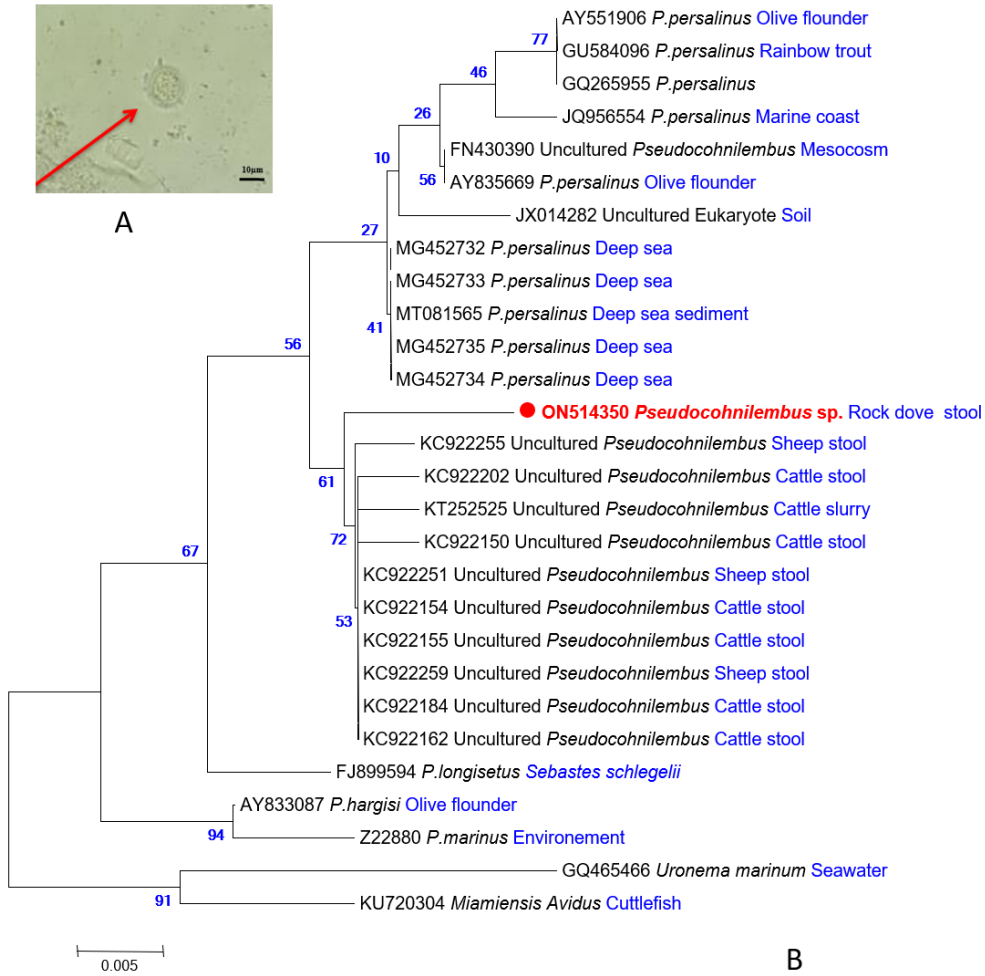

Figure 1:

A. Microscopy of the granular form of *Pseudocohnilembus* species observed on the coprological examination.

B. Molecular Phylogenetic analysis of the SSU rDNA of *Pseudocohnilembus* sequences are designated by the GenBank access number and the name of their hosts or their isolation source. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There are a total of 284 positions in the final dataset. Evolutionary analyses were conducted by ML method in MEGA7.

Molecular studies

The selected sample was conserved in 70% ethanol, and then washed with milliQ water prior to the DNA extraction method from fresh stool. DNA was extracted using the QIAGEN DNA Tissue Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol, with the modifications previously specified [13,14]. The presence of the so-called Blastocystis in DNA samples was determined using PCR targeting a fragment of the SSU rDNA gene using previously described primers [15]. PCR reactions were performed in 25µl reaction mixtures containing 2.5µl of 10× buffer, 2.5mM of MgCl2, 200µM of each Deoxy-Ribonucleoside Triphosphate (dNTPs) mixture, 0.2µM of each primer (Eurogentec, Belgium), 0.025U/µl of Taq DNA polymerase Hot Star (Qiagen, Courtaboeuf, France), and 5µl of genomic DNA using the following thermocycling conditions: 95 °C for 15min, followed by 95 °C for 30s, 54 °C for 30s, and 72 °C for 1min for 35 cycles, and a final extension 72 °C for 5min. PCR amplification result was then verified by electrophoresis migration of PCR product on a 1.5% agarose gel using Ethidium bromide. Nucleo Fast 96PCR Plates

(Macherey-Nagel EURL, France) and BigDye Terminator version 1.1 cycle sequencing-ready reaction mix (Applied Biosystems, Foster City, CA) were then used to purify one positive PCR product to be sequenced directly in both directions with the same primers used in the PCR amplification. The ABI 3100 automated sequencer (Applied Biosystems) resolved the sequenced products. The obtained sequence was edited with Chromas Pro software (Technelysium PTY, Australia), compared to the GenBank database using BLAST, and deposited in GenBank under the accession number ON514350. The phylogeny reconstruction and genetic distances were inferred using the MEGA7 software [16]. Haplotype Diversity (Hd) and nucleotide diversity (π) were calculated using DNA Sequence Polymorphism (DNASP 5.10.01) [17].

Results

The provisional identification of protozoan species using morphological characteristics was challenged by DNA barcoding approach. In the present study, BLAST analysis of the partial SSU rDNA (284bp) sequence of the protozoan showed 99.64% sequence similarity with an uncultured *Pseudocohnilembus* clone (KC922259) isolated from a sheep stool in China. The SSU rDNA partial sequence of *Pseudocohnilembus* sp. was aligned with 25 other *Pseudocohnilembus* sequences (uncultured *Pseudocohnilembus*, *P. persalinus*, *P. longisetus*, *P. hargisi* and *P. marinus*) (Table 1). Sequences of Miamiensis Avidus and Uronema marinum were used as the out-group. The trees inferred using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) led to similar topologies and thus only the ML tree is shown (Figure 1). For the latter, the best model, estimated by MEGA7, was the Tamura 3-parameter with a discrete Gamma Distribution (G) [16]. All *Pseudocohnilembus* species formed a monophyletic clade separated from the out groups (91% bootstrap). The *Pseudocohnilembus* clade exhibits 3 sub-clades: *P. hargisi* and P. *marinus* clustered separately together (94% bootstrap), *P. longisetus* forms a separate cluster (67% bootstrap), and the *Pseudocohnilembus* sequence of the present study is composed with other uncultured *Pseudocohnilembus* sequences, a sister group with *P. persalinus* clade (56% bootstrap). Distances computed using Kimura 2-parameter distance between our *Pseudocohnilembus* sp. sequence and uncultured *Pseudocohnilembus* sequences isolated from stool corresponds to 1%, and distances between our sequence and the other species ranged between 2-3% (*P. persalinus*), 3-4% (remaining *Pseudocohnilembus* species) and 5-6% (out groups) (Table 2). Considering that the interspecific genetic distances of ciliates are more than 1.5% [18], these distances, and the tree, clearly indicate that *Pseudocohnilembus* sp. isolated from different animal stools corresponds to a new species. Multiple sequence alignment of the uncultured *Pseudocohnilembus* species isolated from stool revealed limited heterogeneity among the 11 sequences (nucleotide diversity π = 0.00450), in the form of 7 singleton variable sites leading to the exhibition of four different haplotypes with a rate of gene diversity Hd corresponding to 0.727 (Figure 2). Our present sequence of *Pseudocohnilembus sp*. forms an original algerian haplotype supported by 61% bootstrap value (Figure 1).

Table 1: Species of *Pseudocohnilembus* used in the present molecular study. All species belong to the *Scuticociliatia*.

Species	Genbank	Isolation Source	Location	Reference
Pseudocohnilembus persalinus	MG452735	Deep sea	Pacific Ocean South Korea, to Suva, Fiji	Schoenle et al. [19]
	MG452734			
	MG452733			
	MG452732			
	MT081565	Deep sea sediment	Caribbean Sea	Živaljić et al.
	AY835669	Olive flounder (*Paralichthys olivaceus*)	South Korea	Song et al.[5]
	JQ956554	Marine coast	China	Unpublished
	GU584096	Rainbow trout (*Oncorhynchus mykiss*)	Clear Springs Food USA	Jones et al. [6]
	GQ265955	Unknown	China	Unpublished
	AY551906	Olive flounder (*Paralichthys olivaceus*)	South Korea	Kim et al. [8]
Uncultured Pseudocohnilembus	KC922259	Sheep stool		
	KC922255			
	KC922251			
	KC922154	Cattle stool	China	Unpublished
	KC922162			
	KC922155			
	KC922202			
	KC922184			
	KC922150			
Uncultured Pseudocohnilembus	FN430390	Mesocosm experiment (seawater + fuel oil)	Germany-Helgoland	Gertler et al., 2010

Uncultured Eukaryote	KT252525	Farm anaerobic reactor inoculated with cattle slurry	France	Goux et al. [21]
Uncultured Eukaryote	JX014282	Soil	Yellow River Delta, China	Zhao et al. [20]
Pseudocohnilembus longisetus	FJ899594	*Sebastes schlegelii*	Jeju South Korea	Whang et al. [3]
Pseudocohnilembus hargisi	AY833087	Olive flounder (*Paralichthys olivaceus*)	South Korea	Song et al. [5]
Pseudocohnilembus marinus	Z22880	Environnemental Isolate	United King dom	Unpublished
Uronema marinum	GQ465466	Sea water	China	Pan et al. [1]
Miamiensis avidus	KU720304	Cuttle fish (*Sepia pharaonis*)	China	Tao et al., 2016
Pseudocohnilembus sp.	ON514350	Rock dove stool	Algiers	Present study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 A Y833087 *P.hargisi*																											
2 Z22880 *P.marinus*	1%																										
3 MG452732 *P.persalinus*	3%	4%																									
4 MG452733 *P.persalinus*	3%	4%	0%																								
5 MT081565 *P.persalinus*	3%	4%	0%	0%																							
6 MG452735 *P.persalinus*	3%	4%	0%	0%	0%																						
7 MG452734 *P.persalinus*	3%	4%	0%	0%	0%	0%																					
8 FN430390 Uncultured Mesocosm	3%	3%	0%	0%	0%	0%	0%																				
9 A Y835669 *P.persalinus*	3%	3%	0%	0%	0%	0%	0%	0%																			
10 GQ265955 *P.persalinus*	3%	3%	1%	1%	1%	1%	1%	1%	1%																		
11 A Y551906 *P.persalinus*	3%	3%	1%	1%	1%	1%	1%	1%	1%	0%																	
12 GU584096 *P.persalinus*	3%	3%	1%	1%	1%	1%	1%	1%	1%	0%	0%																
13 JQ956554 *P.persalinus*	3%	3%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%															
14 JX014282 Uncultured soil	4%	4%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	2%														
15 KC922202 Uncultured stool	3%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	2%													
16 KT252525 Uncultured stool	3%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	2%	1%												
17 KC922150 Uncultured stool	3%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	2%	1%	1%											
18 KC922251 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%										
19 KC922154 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%	0%									
20 KC922155 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%	0%	0%								
21 KC922259 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%	0%	0%	0%							
22 KC922184 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%	0%	0%	0%	0%						
23 KC922162 Uncultured stool	2%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	1%	0%	0%	0%	0%	0%	0%	0%	0%					
24 KC922255 Uncultured stool	3%	3%	1%	1%	1%	1%	1%	1%	1%	2%	2%	2%	2%	2%	1%	1%	1%	0%	0%	0%	0%	0%	0%				
25 **ON514350 Rock dove stool**	**3%**	**4%**	**2%**	**2%**	**2%**	**2%**	**2%**	**2%**	**2%**	**3%**	**3%**	**3%**	**2%**	**3%**	**1%**	**1%**	**1%**	**1%**	**1%**	**1%**	**1%**	**1%**	**1%**	**1%**			
26 FJ899594 *P.longisetus*	1%	2%	2%	2%	2%	2%	2%	2%	2%	3%	3%	3%	3%	3%	2%	2%	2%	1%	1%	1%	1%	1%	1%	2%	**3%**		
27 GQ465466 *Uronema marinum*	6%	6%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	6%	6%	6%	6%	6%	6%	6%	6%	6%	6%	6%	**6%**	7%	
28 *KU720304 Miamiensis Avidus*	3%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	4%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	5%	**5%**	4%	3%

Table 2: Estimate of evolutionary divergence between sequences. There were a total of 284 positions in the dataset. Distances are Kimura 2-parameter distances, shown as percentages.

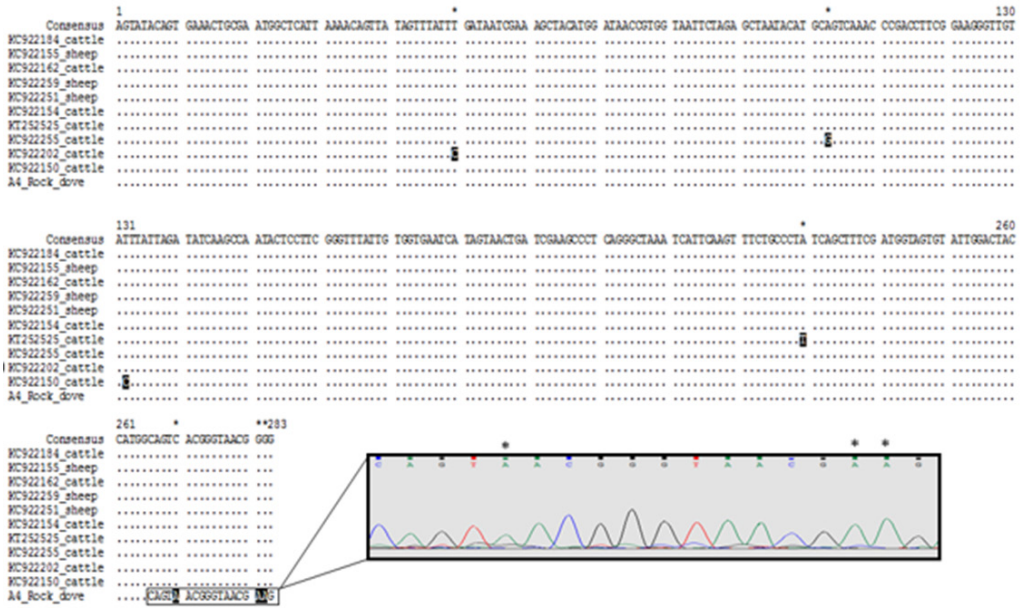

Figure 2: Multiple sequence alignment of the uncultured *Pseudocohnilembus* species isolated from stool.

Discussion

In the present study, a new species of *Pseudocohnilembus* associated to animal stools was obtained by sequencing the partial SSU rDNA. The sequence is clean and much perfectly with Blastocystis primers designed by Poirier et al. [15], as well as the negative control remained negative all along amplification and sequencing. In literature, several *Scuticociliate* species have already been reported in farmed marine fish and deep sea [8,19]. However, only two publications report the presence of eukaryotic *Scuticociliate* out of seawater, especially in soil [20] and in a farm anaerobic reactor inoculated with cattle slurry [21]. Here, the obtained *Pseudocohnilembus* sp. sequence was isolated from a stool sample of a rock dove in Algiers. The phylogenic characterization shows that our sequence clustered with uncultured *Pseudocohnilembus* sequences isolated from cattle and sheep stool from China (https://www.ncbi.nlm.nih.gov/nuccore/KC922255 unpublished data). The uncultured eukaryote identified in the French anaerobic reactor inoculated with stale and cold cattle slurry also clustered on the same clade forming a monophyletic stool *Pseudocohnilembus* clade. The sister clade corresponds to *Pseudocohnilembus* persalinus, which has been recognized as a common facultative pathogen [7]. In recent years, there have been many reports of fatal outbreaks of infection in marine fish by several *Scuticociliate* species including *Pseudocohnilembus persalinus* [2]. The gene sequencing of microeukaryotes isolated from the Yellow River delta in China revealed a higher diversity of ciliate assemblages including P. persalinus in the high salinity soil adjacent to the sea [20], and *Pseudocohnilembus* have been tested as an appropriate indicator of high salinity levels in soil [22].

Rock dove or pigeon belong to order Columbiformes, and family Columbidae have been associated with humans for a long time now. They are raised as ornamental birds, companion animals, meat production or laboratory specimens [23]. Dove's parasitic infections are widespread, especially coccidiosis [24]. The causative agent of this disease is in the genus Eimeria: *E. columbarum*, *E. labbeana*, and *E. columbae* [23]. Another important disease of doves is Trichomoniasis [25]. Doves were considered as the primary reservoir of *Trichomonas* gallinae [26], otherwise, since the report of Colpoda steinii in oral swabs from mourning doves (*Zenaida macroura*) by Toepfer Jr [27] non ciliate was reported in pigeons.

Conclusion

In conclusion, this is the first report of *Pseudocohnilembus* sp. in Algeria. The molecular identification of the parasite in a stool sample of a rock dove leads us to speculate that it may have come from a different water source in the city of Algiers, in particular the Mediterranean Sea. However, the genetic profile of this organism is different from other marine origin species. As a result, we believe that a larger number and variety of birds and other animals should be tested in the future in this area. Moreover, further studies using specific primers of *Scuticociliates* are needed to confirm our study and to determine if this genetic profile of *Pseudocohnilembus* sp is original and specific to birds in Algeria.

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