The following supplement accompanies the article

First isolation of *Miamiensis avidus* (Ciliophora: Scuticociliatida) associated with skin ulcers from reared pharaoh cuttlefish *Sepia pharaonis*

Zhen Tao, Lu Liu, Xiangrui Chen, Suming Zhou, Guoliang Wang*

*Corresponding author: wangguoliang@nbu.edu.cn

Diseases of Aquatic Organisms 122: 67–71 (2016)

Procedure for PCR amplification and sequencing. The DNA fragments were amplified with PCR and then sequenced. Genomic DNA was extracted from approximately 10^3 of collected ciliates using the EasyPure Genomic DNA kit (TransGen Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. DNA fragments were amplified using the PCR method according to Gao et al. (Gao et al. 2012). The primers for the SSU rRNA gene and ITS1-5.8S rDNA-ITS2 shown in Table 1 were adapted from previous studies (Medlin et al. 1988, Shang 2004, Moreira et al. 2007). The PCR reaction was performed in a 50-µl volume consisting of 1× PCR buffer plus $MgCl_2$ (Promega), 50 µM each of the deoxynucleoside triphosphates (dNTPs; TransGen), 400 nM of each primer, 1.25 U of Taq DNA polymerase (TransGen), and deionized water up to 50 µl. Amplification followed a thermal cycling profile of an initial denaturation of 94°C for 5 min; 10 cycles of denaturation (94°C for 40 s), annealing (60°C for 60 s), and elongation (72°C for 2 min), followed by 25 cycles of denaturing (94°C for 30 s), annealing (56-58°C for 40 s), and elongation (72°C for 1 min for ITS regions and 2 min SSU rRNA); and a final extension of 72°C for 7 min. PCR products were purified by agarose gel electrophoresis and retrieved from gel using E.Z.N.A.® gel extraction kit (Omega Bio-tek, Inc., Norcross, GA, USA). Purified PCR products were cloned into the pMD™18-T vector (Takara Biotechnology, Dalian Co., Ltd. Dalian, China). Three of PCR-verified clones were subjected to bi-directional sequencing with primers M13F and M13R on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems). In addition, internal sequencing primers (Table 1) were also used to cover the full sequences of SSU rRNA gene fragments. Sequence contigs were assembled from shorter fragments using SeqMan in Lasergene Software suite (DNASTAR Inc., Madison, WI, USA). Sequences of the two DNA fragments were then search against NCBI database using online BLAST (Altschul et al. 1990; http://www.ncbi.nlm.nih.gov/BLAST/) to classify the ciliate based on gene similarities (>97%).

Phylogenetic analyses. The SSU rRNA gene sequences of the isolated ciliate were compared with that of selected Scuticociliate species. The DNA sequences which were compared were all downloaded from GenBank at http://genbank.gov/. Phylogenetic analyses were performed on the phylogeny.fr server (Dereeper et al. 2008) at http://www.phylogeny.fr as follows: sequences were aligned with MUSCLE (Edgar 2004), the phylogenetic tree was inferred with PhyML (Guindon & Gascuel 2003) using the maximum likelihood (ML) method using the Generalized Time Reversible model (Tavaré 1986) plus gamma distribution and invariant sites, and bootstrap support was computed after 100 reiterations.

The systematic position of the ciliate was determined among 39 Scuticociliatia SSU rRNA sequences downloaded from GenBank and integrated in the phylogenetic analysis (Figure S1). Maximum-likelihood analysis-based SSU rDNA sequences showed that cuttlefish ciliate clustered tightly with other *M. avidus* strains (with a maximal bootstrap value of 100), forming a sister clade to that formed by *Anophyroides haemophila* and *Glauconema trihymene*. The monophyletic clades of Philasterida, Loxocephalida and Pleuronematida in the inferred phylogenetic tree (SSU rRNA) are strongly supported by the maximal bootstrap values, but the relationships within the order level, especially in Philasterida, are not well resolved, as suggested by low node-supporting bootstrap values.

Comparative bacteriological analysis. A total of six cuttlefish were sampled for bacterial analysis including three affected and three non-affected cuttlefish. Culturable bacteria were isolated from lesions and normal cuttlefish skin. To isolate culturable bacteria, the lesion or skin site was rinsed with sterile PBS to remove

seawater leftover. After this wash, a sterile cotton-tipped swab was used to rub the lesion and the substance from the swab was streaked onto Marine 2216E medium agar plates. The plates were incubated at 25°C for 48 hours before picking colonies for bacterial identification. Two colonies from each plate were randomly selected and identified using 16S rDNA sequencing combined with the BLAST tool in NCBI. The 16S rDNA fragments were amplified by PCR using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') according to Goodfellow & Stackebrandt (1991). Species identification was confirmed by comparing the DNA sequence to the GenBank database as described above.

In total, 12 isolates each were obtained from the lesion sites of affected cuttlefish and normal skin sites of unaffected animals. The result revealed no significant difference in bacterial species between the lesion and healthy skin (Figure S2). The majority of isolates from both types of samples were *Pseudoalteromonas* sp.

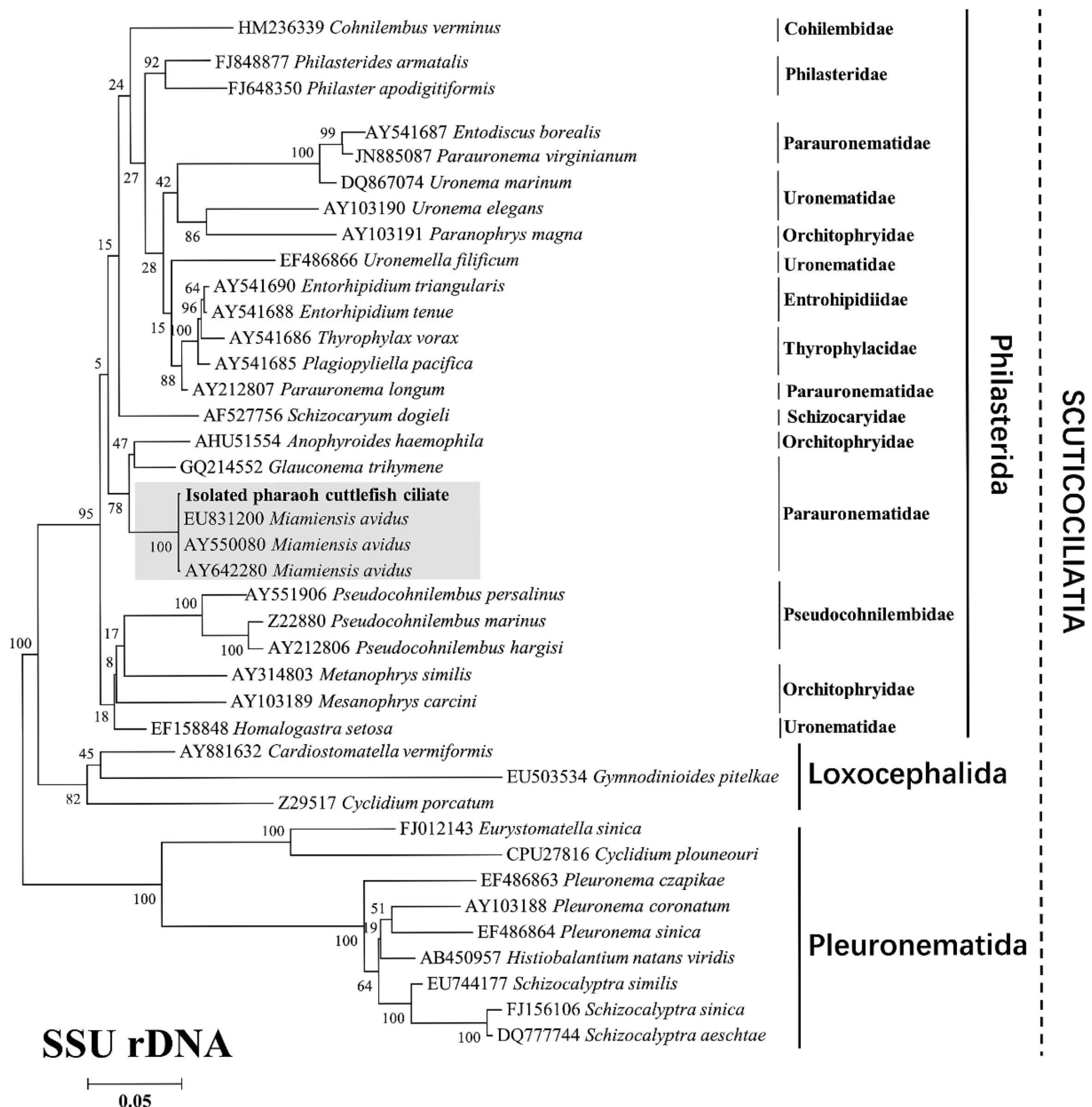

Fig. S1. Maximum likelihood tree showing the phylogenetic position of the ciliates found in the ulcers of reared pharaoh cuttlefish *Sepia pharaonis* (in bold) compared against 39 selected Scuticociliate SSU rRNA sequences available in GenBank. Numbers at nodes represent the bootstrap values of maximum likelihood out of 100 replicates. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Terminology and systematic classification follows Lynn's description (Lynn 2008).

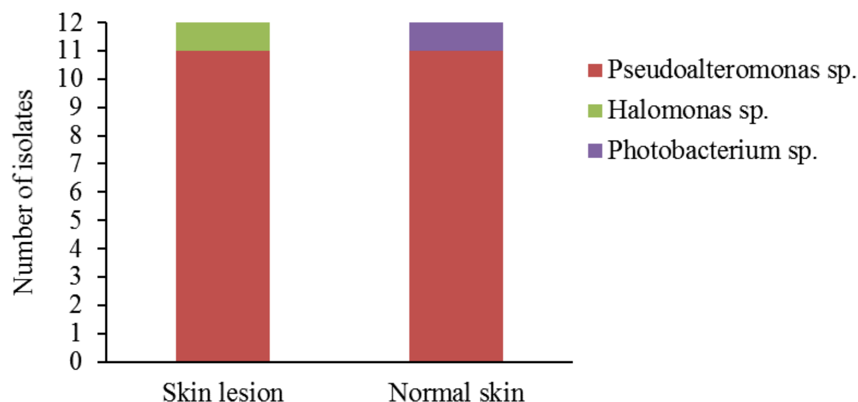

Fig. S2. Bacterial isolates yield from skin lesion and normal skin.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of molecular biology 215:403-410

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard J-F, Guindon S, Lefort V, Lescot M (2008) Phylogeny. fr: robust phylogenetic analysis for the non-specialist. Nucleic acids research 36:W465-W469

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics 5:1

Gao F, Katz LA, Song W (2012) Insights into the phylogenetic and taxonomy of philasterid ciliates (Protozoa, Ciliophora, Scuticociliatia) based on analyses of multiple molecular markers. Molecular phylogenetics and evolution 64:308-317

Goodfellow M, Stackebrandt E (1991) Nucleic acid techniques in bacterial systematics, Vol 5. J. Wiley

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic biology 52:696-704

Lynn DH (2008) The ciliated protozoa: characterization, classification, and guide to the literature, Vol. Springer Science & Business Media

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71:491-499

Moreira D, von der Heyden S, Bass D, López-García P, Chao E, Cavalier-Smith T (2007) Global eukaryote phylogeny: combined small-and large-subunit ribosomal DNA trees support monophyly of Rhizaria, Retaria and Excavata. Molecular phylogenetics and evolution 44:255-266

Shang H (2004) Molecular Phylogeny of Scuticociliatia (Ciliophora, Oligohymenophorea). PhD Dissertation Ocean University of China

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on mathematics in the life sciences 17:57-86