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## A diversity of amoebae colonise the gills of farmed Atlantic salmon (*Salmo salar*) with amoebic gill disease (AGD)

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### Abstract

*Neoparamoeba perurans* is the aetiological agent of amoebic gill disease (AGD) in salmonids, however multiple other amoeba species colonise the gills and their role in AGD is unknown. Taxonomic assessments of these accompanying amoebae on AGD-affected salmon have previously been based on gross morphology alone. The aim of the present study was to document the diversity of amoebae colonising the gills of AGD-affected farmed Atlantic salmon using a combination of morphological and sequence-based taxonomic methods. Amoebae were characterised morphologically via light microscopy and transmission electron microscopy, and by phylogenetic analyses based on the 18S rRNA gene and cytochrome oxidase subunit I (COI) gene. In addition to *N. perurans*, 11 other amoebozoans were isolated from the gills, and were classified within the genera *Neoparamoeba*, *Paramoeba*, *Vexillifera*, *Pseudoparamoeba*, *Vannella* and *Nolandella*. In some cases, such as *Paramoeba eilhardi*, this is the first time this species has been isolated from the gills of teleost fish. Furthermore, sequencing of both the 18S rRNA and COI gene revealed significant genetic variation within genera. We highlight that there is a far greater diversity of amoebae colonising AGD-affected gills than previously established.

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**Keywords:** Amoebozoa; AGD; Aquaculture; Atlantic salmon; Discosea; Tubulinea

### Introduction

The gills of teleost fish play a vital role in a number of essential physiological processes including respiration,

osmoregulation, ammonia secretion and acid–base regulation (Evans 2005). However, by virtue of their position and physical structure the gill represent an important yet vulnerable barrier that is in constant and intimate contact with the external environment. As such the gills can be subjected to a variety of environmental and pathogenic insults which, under the appropriate conditions, may result in gill pathology and or injury. While in some cases a single pathological agent can

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be responsible, a number of more complex gill diseases with apparent mixed aetiologies have emerged in farmed teleosts (Gjessing et al. 2017; Herrero et al. 2018). The increasing list of proven and putative gill pathogens, and the complexity of disease expression gives reason to consider gill disease in the context of dysbiosis of microbial community structure, rather than focusing on a single agent (Downes et al. 2018; Egan and Gardiner 2016; Gjessing et al. 2017; Herrero et al. 2018).

Amoebic gill disease remains one of the most important diseases affecting Atlantic salmon aquaculture in Tasmania, Australia. Caused by the free-living protozoan parasite, *Neoparamoeba perurans*, AGD affects Atlantic salmon during the marine grow-out phase (Crosbie et al. 2012; Young et al. 2007). Attachment of *N. perurans* to gill epithelium causes epithelial hyperplasia, oedema and lamellar fusion and, ultimately if not treated, mortality (Adams and Nowak 2001). *N. perurans* however is not the only amoeba species capable of colonising the gills of marine cultured Atlantic salmon. Early reports, based on gross morphology, identified five different genera accompanying *Neoparamoeba* spp. on the gills of farmed Atlantic salmon in Tasmania, Australia, including *Acanthamoeba*, *Flabellula*, *Heteroamoeba*, *Vannella* and *Vexillifera* (Howard 2001). Similarly, five genera were isolated from the gills of farmed Atlantic salmon in Ireland, including *Flabellula*, *Mayorella*, *Nolandella*, *Vannella* and *Vexillifera* (Bermingham and Mulcahy 2007). While it is evident that the gills of Atlantic salmon may be colonised by a variety of Amoebozoa, the role that non-*N. perurans* amoebae play in AGD, as either a secondary invader, primary pathogen or commensal bystander, remains unclear (Morrison et al. 2005; Nowak and Archibald 2018).

Dysbiosis describes a microbial community shift that has a negative impact on the host (Petersen and Round 2014). In the context of microbial communities in teleost gills, recent studies have shown pronounced shifts in bacterial communities associated with disease status (Legrand et al. 2018). While it is clear that *N. perurans* alone is capable of causing AGD, it remains uncertain if AGD promotes a more global Amoebozoan dysbiosis and ultimately how such a community shift contributes to disease onset or progression. In some cases of gill disease in salmonids, for example nodular gill disease (NGD), a single pathogen has not been attributed as the aetiological agent, rather a multi-amoeba aetiology is proposed (Dyková et al. 2010; Dyková and Tým 2015).

Traditionally the identification of Amoebozoan communities through gross morphological features alone has been difficult, largely due to the inherent plastic morphology of Amoebozoa (Dyková and Lom 2004). More recently however, the application of genetic approaches, improved culture practices and advanced microscopy has facilitated more extensive profiling of the diversity of amoebozoan communities. Using these methodological improvements, the goal of the present study was to document the diversity of Amoebozoa capable of colonising the gills of AGD-affected Atlantic salmon using both morphological and molecu-

lar taxonomic approaches. Specifically, we isolated and established mixed-cultures and monocultures from AGD-affected gills, then recorded the amoebae morphology via light microscopy and, where possible, transmission electron microscopy (TEM). The diversity of amoebae was further assessed by sequencing the 18S ribosomal RNA (18S rRNA) gene and cytochrome oxidase subunit I (COI) gene, and each newly obtained amoeba sequence was identified by sequence homology and supported by phylogenetic analysis.

## Material and Methods

### Sampling AGD-affected gills to establish amoeba cultures

The gill basket of five farmed Tasmanian Atlantic salmon that displayed clinical signs of AGD were collected during each of the four sampling events: June and October 2015, May 2016, and August 2017. For this purpose, gills with a gill score greater than three (Taylor et al. 2009) were dissected and transported in chilled, filtered seawater to the laboratory. All fish used in this study were approved for sampling by CSIRO Queensland Animal Ethics Committee (AEC number A13/2015 and A9/2016).

### Primary isolation and maintenance of cultures

Amoebae were isolated from the gill baskets by inoculating culture flasks with either mucus scrapes or small tissue samples. These primary isolates were cultured in 0.2 µm filtered, sterile 33 ppt seawater at 14 °C, and regularly observed for two weeks for the presence of amoebae using an inverted microscope (Olympus CK2) at 200× magnification. Once the amoebae attached to the bottom of the culture flask, the overlaid seawater was carefully removed by pipetting, followed by gentle rinsing with filtered, sterile seawater to remove excess bacteria and tissue debris, then replaced with an aliquot of 1% malt yeast broth (MYB; 0.01% (w/v) malt extract and 0.01% (w/v) yeast extract in filtered, sterile seawater). Successfully established mixed-amoebae-cultures were maintained weekly, which involved media exchange, contaminant checks and splitting cultures as necessary. The amoeba cultures were sampled for DNA extraction when they were approximately 70% confluent.

### Establishing monocultures

Single amoeba cells were isolated using an adapted pipette and dilution technique from Smirnov (1999). The presence of one cell in 0.1 µL of seawater was confirmed using a light microscope at 200× magnification. The single amoeba cell was then transferred to a 96-well culture plate and grown in 1% MYB at 14 °C. Once 70% confluent, each monoculture was sampled for DNA extraction. To prevent the potential

loss of virulence, antibiotics were not used to control bacterial overgrowth (Bowman and Nowak 2004; Embar-Gopinath et al. 2005). As a result, the monocultures were not axenic and contained associated bacterial communities.

### Morphological characterisation of amoebae grown in monoculture

Gross morphology of 20 amoebae from each monoculture was documented using an inverted microscope (Olympus CK2) under 200× magnification. Images were obtained with a Luminoptic camera and processed using ISCapter (Tucson) software. The dimensions of the attached and floating forms of each amoeba strain were measured and expressed as mean values, with range and standard deviation indicating variation of intra-strain size. A key to marine gymnamoebae by Page (1983) was employed to assist with morphology-based identification.

For transmission electron microscopy (TEM), amoebae were cultured on carbon-coated 3 mm sapphire discs (Wohlwend, Switzerland) and frozen in a cryoprotectant (20% BSA in artificial seawater) in an HPM 010 high pressure freezer (Baltec, Liechtenstein) before undergoing freeze-substitution in 1% osmium tetroxide and 0.5% uranyl acetate in acetone for 48 h at −90 °C in a Leica AFS2 automated freeze substitution machine (Leica, Austria). Samples were then embedded in Epon, polymerised at 60 °C for 48 h and sectioned at 80 nm using a Leica UC6 ultramicrotome (Leica Microsystems, Austria) before viewing at 80 kV on a Jeol JSM 1011 transmission electron microscope (Jeol, Japan).

### Identification of amoebae by sequencing

DNA extractions from both mixed and monocultures was performed using a DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions. Extracted DNA was quantified with a Nanodrop ND-1000 spectrophotometer (Life Technologies) and stored at −20 °C. A 669–950 bp fragment of the 18S rRNA gene or COI gene was amplified by PCR using one of five universal eukaryotic primers (Table 1). PCR reactions followed the Kapa Taq PCR Protocol (KapaBiosystems) for a 25 µL reaction, with a thermal profile of denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 2 min with an annealing temperature specific to each primer set (Table 1), 72 °C for 2 min, with a final extension at 72 °C for 2 min. Successful amplification was confirmed by visualising the amplified products in a 1.2% agarose gel.

Amplified DNA was purified with the QIAGEN PCR purification kit following the manufacturer's instructions. The purified DNA was ligated into a pGEM-T Easy Vector (Promega) then transformed using Alpha-Select Silver Efficiency Competent Cells (Bioline) according to the man-

ufacturer's protocol. The transformed cells were plated onto LB Agar plates containing ampicillin (100 mg/L) and incubated overnight at 37 °C. Successful ligation and transformation was confirmed by PCR of multiple colonies using T7 and SP6 vector specific primers. Colonies with inserts were grown in LB broth overnight at 37 °C. Corresponding glycerol (20%) stocks were prepared for archiving and plasmid was purified using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmids were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, country) and purified with the Agencourt CleanSEQ (Beckman Coulter) as per the manufacturer's instructions. Sequences were generated by the ABI 3130xl genetic analyser (Applied Biosystems). The sequences were edited and aligned using ChromasPro (Version 1.5, Technelysium Pty Ltd.) and CLC Main Workbench 7 (Version 7.6.4, QIAGEN Aarhus A/S).

### Sequence analysis

Each newly sequenced 18S rRNA and COI gene fragment were subjected to a BLASTn (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) search to find the top hit. The 18S rRNA sequences were only accepted as Amoebozoa sequences if they had an e-value of zero. In contrast, to determine if the COI sequences were amoebae or contaminants such as fungi or bacteria, preliminary taxonomic positions of all COI sequences was determined by phylogenetic analysis. This is because there is a paucity of Amoebozoa COI reference sequences on public databases to infer their identity based on BLAST analysis alone. The sequences found to be Amoebozoa were then cloned and sequenced twice to account for potential mixed base calls in some positions in the original sequence. In total, 19 18S rRNA gene fragments and 17 COI gene fragments obtained from mixed-cultures and monocultures were used in the following phylogenetic analysis.

### Phylogenetic analysis of 18S rRNA amoeba sequences

Initially we aimed to construct a phylogenetic analysis based on 18S rRNA that comprised all Amoebozoa groups so that the most likely identity and phylogenetic position of each of the newly sequenced strains were shown within one analysis. Despite trialling a number of different sequence datasets, alignments, and trimming methods this approach proved unsuitable because it did not resolve the currently accepted Amoebozoa phylogeny which has been established in more sophisticated, multi-gene analyses (Kang et al. 2017; Tekle et al. 2008). As our aim was to determine the most closely related species to our newly sequenced strains, not reconstruct the full Amoebozoan phylogeny, we focused our analysis on lower taxonomic groups of interest.

**Table 1.** Universal eukaryotic 18S rRNA and COI primer sets used to amplify amoebae.

| Gene     | Primer                | Sequence (5'-3')                                        | Annealing temperature (°C) | Expected product size | Reference               |
|----------|-----------------------|---------------------------------------------------------|----------------------------|-----------------------|-------------------------|
| 18S rRNA | RibB<br>S12.2         | TGATCCATCTGCAGGTTACCTAC<br>GATYAGATACCGTCG TAGTC        | 50                         | 800–900               | Smirnov et al. (2007)   |
| 18S rRNA | Ami6F1<br>Ami9R       | CCAGCTCCAATAGCGTATATT<br>GTTGAGTCGAATTAAGCCGC           | 60                         | 700–900               | Thomas et al. (2006)    |
| 18S rRNA | 570C<br>1137R         | GTAATTCCAGCTCCAATAGC<br>GTGCCCTTCCGTCAAT                | 58                         | 700–900               | Schroeder et al. (2001) |
| COI      | Eucox1F<br>Euglycox1R | GAYATGGCKTTNCCAAGATTAAA<br>AGCACCCATTGAHAAAACRTAATG     | 50                         | 800–1000              | Heger et al. (2011)     |
| COI      | LCO1490<br>HCO2198    | GGTCAACAAATCATAAAGATATTGG<br>TAAACTTCAGGGTGACCAAAAAATCA | 50                         | 600–700               | Folmer et al. (1994)    |

All newly obtained 18S rRNA sequences were either Tubulinea or Discosea based on the closest BLASTn hit. Thus, a separate phylogenetic analysis was performed for each group. The most recent phylogenetic models were used as the backbone to the analysis, which included those of Tým and Dyková (2017) for Tubulinea, Sibbald et al. (2017), Kudryavtsev and Gladkikh (2017) and Udalov et al. (2016) for Discosea, in addition to an overall Amoebozoa guide from Kang et al. (2017). Several different sequence datasets were assembled using publicly available databases (NCBI; [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/), EMBL; [www.ebi.ac.uk/ena/](http://www.ebi.ac.uk/ena/), DDBJ; [www.ddbj.nig.ac.jp/index-e.html](http://www.ddbj.nig.ac.jp/index-e.html)) to varying taxonomic extents. Most of the reference sequences chosen from the databases were as long as possible, around 2000 bp, and were derived from well-characterised strains from recognised culture collections such as the American Type Culture Collection (ATCC; [www.atcc.org/](http://www.atcc.org/)), the Culture Collection of Algae and Protozoa (CCAP; [www.ccap.ac.uk/](http://www.ccap.ac.uk/)) or the Institute of Parasitology – Biology Centre, Czech Republic (Dyková and Kotska 2013).

The sequence datasets were aligned in MAFFT v. 7 (Katoh et al. 2017) using the G-INS-I algorithm for the Tubulinea dataset, and the E-INS-I algorithm for the Discosea dataset. The alignments were trimmed using trimAl v.12 (Capella-Gutiérrez et al. 2009) with -gt 0.3 -st 0.001 restrictions for both the Tubulinea and Discosea analysis. All alignments were inspected in AliView v. 1.18.1 (Larsson 2014). The tree was constructed in IQ Tree online version (<http://iqtree.cibiv.univie.ac.at>) (Trifinopoulos et al. 2016) with a model selected in the built-in Model Finder (Kalyaanamoorthy et al. 2017), standard bootstrapping (100 bootstrap alignment (Felsenstein 1985)) and an approximate Bayes test (Anisimova et al. 2011). The labelling of higher taxa on the final two analyses was based on Kang et al. (2017). The focus group of the Tubulinea tree was Elardia, and the outgroup was Echinamoebidia and Leptomyxida. While the focus group of the Discosea tree was Vannellida and Dactylopodida, and the outgroup included Acanthamoebidae, Dermamoebidia and Thecamoebidia.

## Phylogenetic analysis of amoeba COI sequences

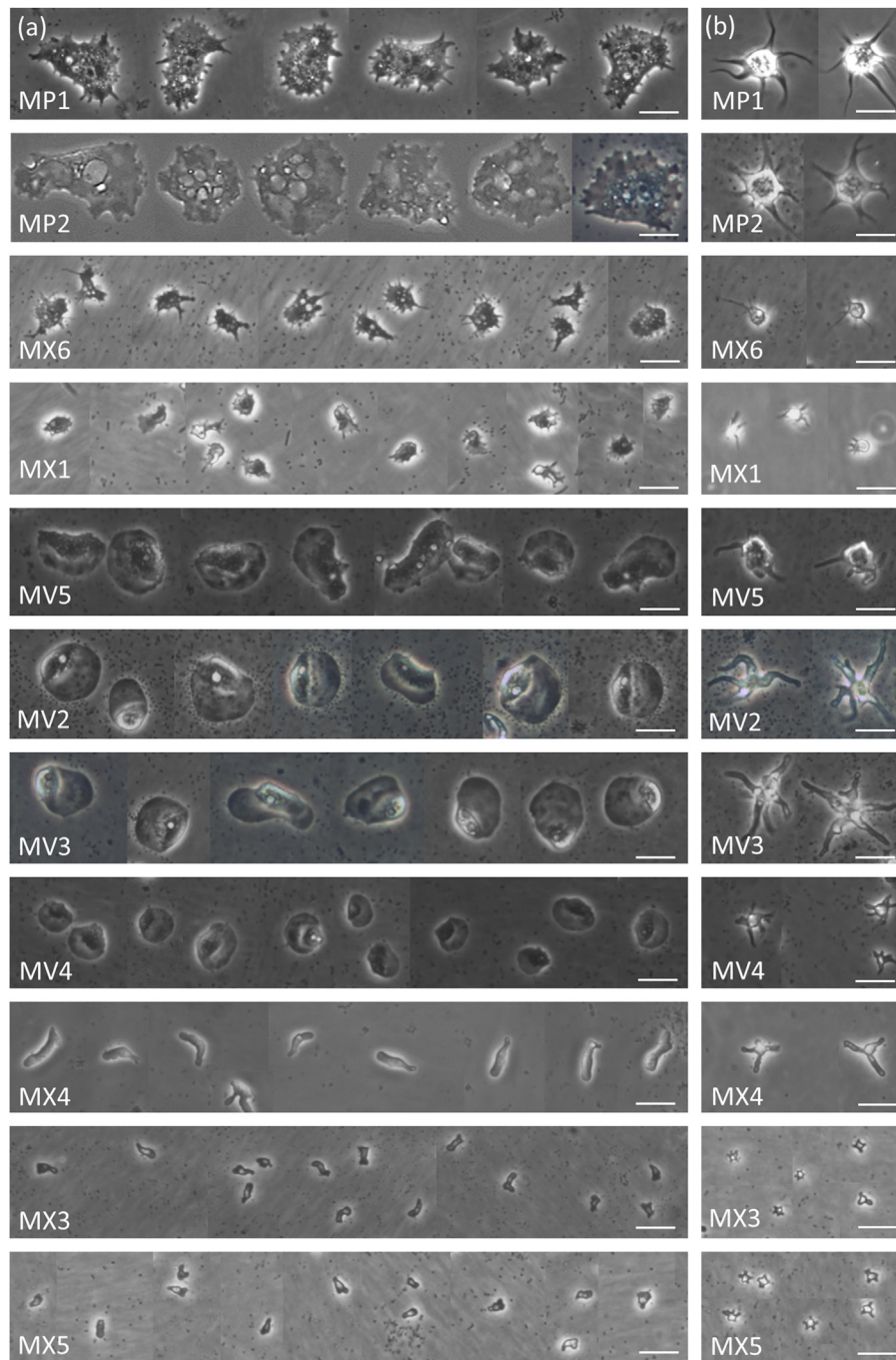
To determine the most likely identity of each newly obtained amoeba COI sequence the analysis was constructed using all major Amoebozoa groups currently characterised by COI. The analysis was carried out as for the Tubulinea dataset with one variation in that trimming was with the -automated1 restriction. The labelling of higher taxa of interest on the final tree was based on Kang et al. (2017), with the main focus group being Vannellida and Dactylopodida, and the outgroup comprised of fungi.

## Results

### Morphological characterisation of amoeba monocultures

The attached and floating form of 11 monocultures established from the primary mixed-cultures were documented under light microscopy (Fig. 1) to support assigning each strain to its lowest practical taxonomic level. The amoeba strains displayed morphological characteristics consistent with either Dactylopodida, Vannellida or Tubulinea (Smirnov et al. 2011). The Dactylopodida strains, MP1, MP2, MX6 and MX1, were all laterally flattened with finger-like subpseudopodia. While strain MV2, MV3, MV4 and MV5 had the distinct fan-shaped morphotype of Vannellida. Strain MX4, MX3 and MX5 were classed as Tubulinea based on their tubular, elongated morphotype and hyaline cap at the anterior during locomotion. Cyst-like forms were observed in all 11 strains, most commonly one week post sub-culture. Vast inter- and intra-strain size variation was evidenced by trophozoite dimensions available in Table A of Supplementary material.

The attached forms of six of the 11 monocultures were further characterised by imaging ultrastructure via TEM. Not all monocultures were documented, partially to avoid repeat processing of the same species, but also due to loss of cultures.



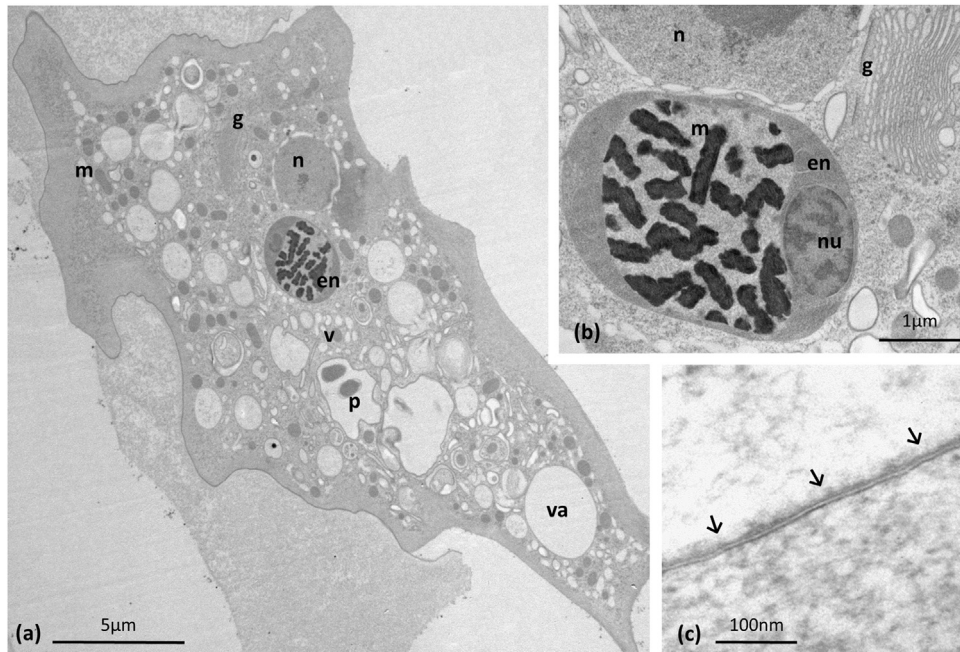
**Fig. 1.** a–b Light microscopy of cultured amoeba strains isolated from gills of farmed Atlantic salmon *Salmo salar* with signs of AGD, including attached trophozoite (a) and floating form (b). Strain MP1 and MP2 is *Neoparamoeba perurans*, MX6 is *Vexillifera* sp., MX1 is *Pseudoparamoeba* sp., MV5, MV2, MV3 and MV4 is *Vannella* sp., MX4 is Tubulinea-like Amoebozoa, MX3 and MX5 is *Nolandella* sp. All scale bars = 20  $\mu$ m.

Despite not all monocultures being documented, each genus is represented.

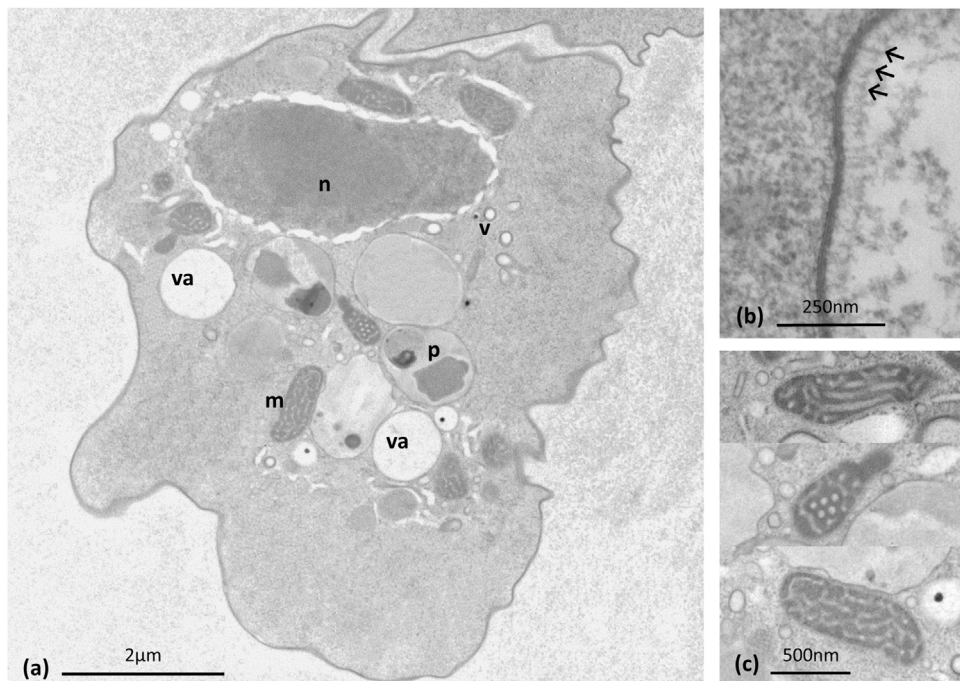
The general ultrastructure of MP1 corresponded to previous description of *N. perurans* (Wiik-Nielsen et al. 2016) and other *Neoparamoeba* species (Dyková et al. 2005b). The most conspicuous characteristic of MP1 was the *Perkinsella amoebae*-like endosymbiont lying adjacent to the nucleus

(Fig. 2a, b). Other defining characteristics were the lack of scales, a rather thin 10 nm amorphous glycocalyx (Fig. 2c), the relatively small spherical mitochondria (Fig. 2a), and the golgi apparatus located in the perinuclear zone (Fig. 2b).

MX6 did not respond well to freeze fixation, which was evident by the cytoplasmic degradation and dilation of nuclear membranes (Fig. 3a). We included these images



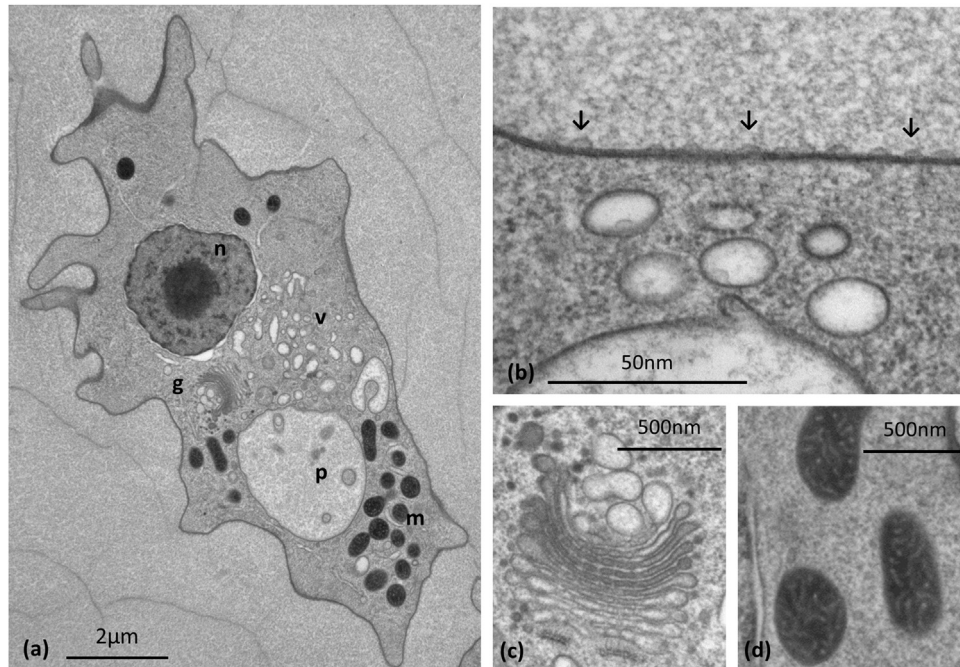
**Fig. 2.** a–c Ultrastructure of *Neoparamoeba perurans* (strain MP1) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructure: nucleus (n) adjacent to endosymbiont (en), mitochondria (m), golgi apparatus (g), vesicles (v), vacuole (va), phagosome (p). **b.** Ultrastructure of *Perkinsela amoebae*-like endosymbiont (en) adjacent to trophozoite nucleus (n) and golgi apparatus (g): nucleus of endosymbiont (nu) and mitochondrion (m) with darkly stained kinetoplast DNA. **c.** Cell surface with a very thin amorphous glycocalyx (arrows).



**Fig. 3.** a–c Ultrastructure of *Vexillifera* sp. (strain MX6) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructure: nucleus (n), mitochondria (m), phagosome (p), vacuoles (va) vesicle (v). **b.** Cell surface with approximately 60 nm thick glycocalyx made up of cylinder-like glycostyles (arrows). **c.** Mitochondria with tubular cristae.

alongside the higher quality figures because they show the defining ultrastructure features, such as structure of mitochondria cristae and cell surface glycocalyx. These

ultrastructures were congruent with previous descriptions of *Vexillifera* sp. (Dyková et al. 2011a; Page 1983). For instance the mitochondria had tubular branching cristae (Fig. 3c) and



**Fig. 4.** a–d Ultrastructure of *Pseudoparamoeba* sp. (strain MX1) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructures: nucleus (n), mitochondria (m), golgi apparatus (g) vesicles (v), phagosome (p). **b.** Trophozoite cell surface lined with domed scales (arrows) **c.** Golgi apparatus with parallel arrangement of cisternae. **d.** Mitochondria with branching tubular cristae.

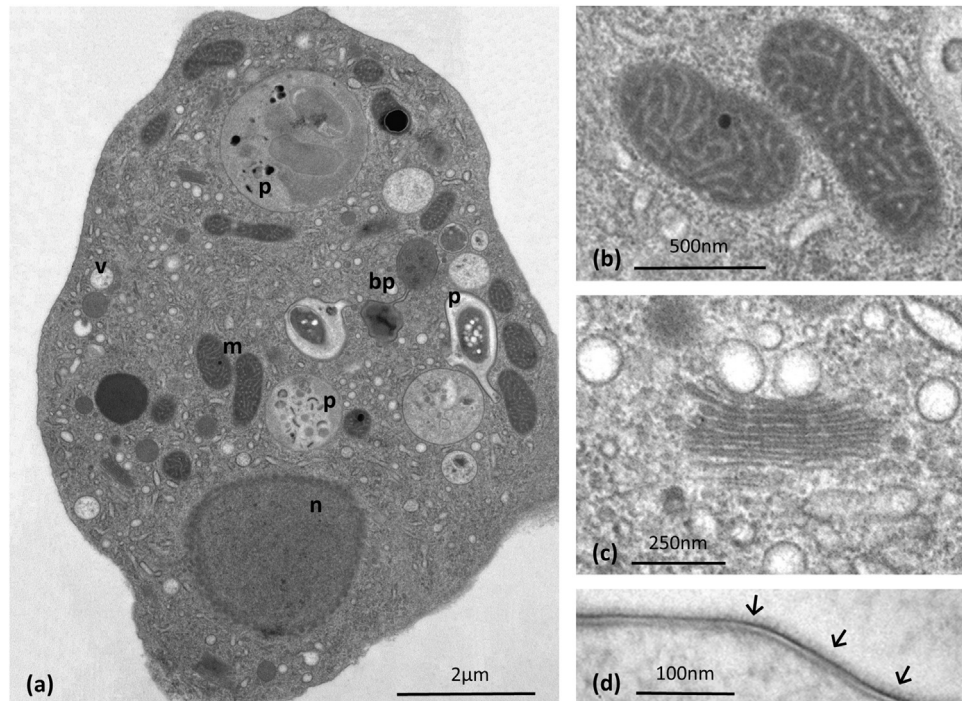
the nuclei had a slightly darker stained nucleolus (Fig. 3a) (Dyková et al. 2011a). The glycocalyx (Fig. 3b) was approximately 60 nm thick and comprised of glycostyles (arrows) that looked like cylinders in longitudinal sections. This glycocalyx thickness and form aligned with the descriptions of *Vexillifera* sp. by Page (1983) (60–70 nm), however in this instance the freeze fixation was not adequate to determine whether each glycostyle had a hexagonal form in cross section, which is also a key feature of the *Vexillifera* genus (Page 1983).

MX1 had several pseudopodia extending from the hyaloplasm (Fig. 4a), indicating the amoeba was likely fixed during locomotion, and belongs to the Dactylopodida taxa. The MX1 trophozoite cell surface was lined with domed scales (arrows Fig. 4b) similar to those described in *Pseudoparamoeba microlepis* by Udalov (2016) and for the genus *Pseudoparamoeba* by Page (1983). However the scales of MX1 were not as elevated from the cell membrane compared with those imaged for *Pseudoparamoeba microlepis* (Udalov 2016). The cytoplasm contained a prominent vesicular nucleus and many darkly stained mitochondria (Fig. 4a) that appeared to have branching tubular cristae (Fig. 4d). The golgi apparatus featured a parallel arrangement of cisternae (Fig. 4c).

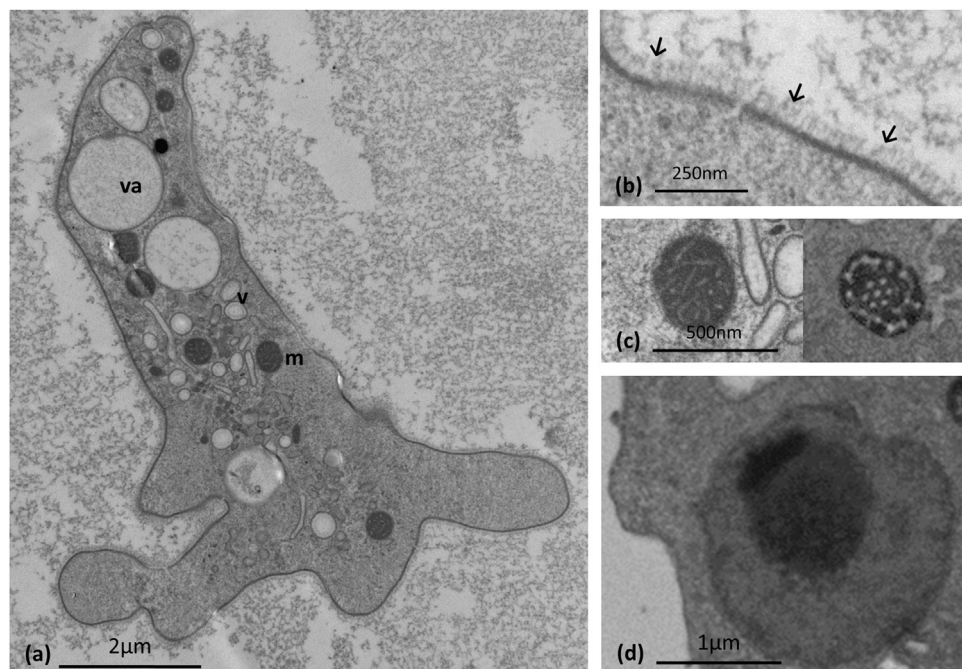
The ultrastructure of MV3 (Fig. 5) and MV4 (Fig. 6) had features consistent with the genus *Vannella*, including an extensive hyaloplasm and distinct glycocalyx (Bovee 1965; Page 1983). The main differentiating features of these

*Vannella* strains is the trophozoite size, with MV3 approximately two times larger than MV4, and the cell surface structure. MV3 cell surfaces had a thin amorphous glycocalyx, approximately 10 nm thick (Fig. 5d), while MV4 had a thick glycocalyx differentiated into distinct glycostyles projecting approximately 60 nm from the cell membrane (Fig. 6b). Both *Vannella* strains had mitochondria with tubular branching cristae (Figs. 5b, 6c). We are unsure whether the darkly stained inclusions in the mitochondria of MV3 (Fig. 5a, b) are an unusual and yet to be described ultrastructure, or a processing artefact. However, the dark round structures appeared to be site-specific, as they were seen in a number of different mitochondria and not in any other organelle.

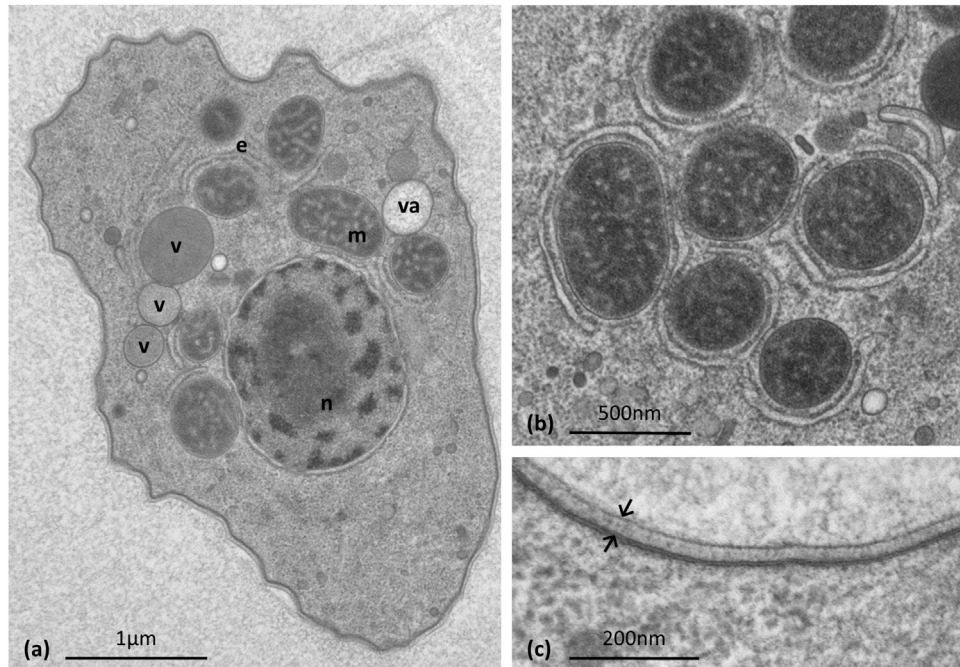
MX5 looked similar to images of *Nolandella* sp. published in Dyková and Kostka (2013) and Bermingham and Mulcahy (2007). Similarities included the relatively large mitochondria in relation to the size of the nucleus (Fig. 7a), and the presence of granular endoplasmic reticulum curled around each mitochondrion with tubular branching cristae (Fig. 7b). According to Page (1983) the cell surface of *Nolandella* comprises tightly packed hexagonal elements rising 30 nm above the membrane. The thickness of this strain's glycocalyx was consistent with the Page (1983) description (25 nm), however we did not manage to section a plane that confirmed or ruled-out the presence of hexagonal glycostyles (Fig. 7c). Other notable features include the vesicular nuclei with a prominent bi-layered nuclear membrane (Fig. 7a).



**Fig. 5.** **a–d** Ultrastructure of *Vannella* sp. (strain MV3) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructure: nucleus (n), mitochondria (m), vesicles (v), early-stage phagosome (p), late-stage budding phagosome (bp). **b.** Mitochondria with tubular branching cristae. **c.** Golgi apparatus with parallel arrangement of cisternae. **d.** Cell surface with amorphous glycocalyx (arrows).



**Fig. 6.** **a–d** Ultrastructure of *Vannella* sp. (strain MV4) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructure: mitochondria (m), vacuoles (va) vesicle (v). **b.** Cell surface with amorphous glycocalyx (arrows). **c.** Mitochondria with tubular branching cristae. **d.** Vesicular nucleus.



**Fig. 7.** **a–c** Ultrastructure of *Nolandella* sp. (strain MX5) isolated from gills of Atlantic salmon, *Salmo salar* farmed in Tasmania, Australia. **a.** Overview of trophozoite ultrastructures: vesicular nucleus (n), mitochondria (m), vacuoles (va), vesicles with unknown content (v), endoplasmic reticulum (e). **b.** Granular endoplasmic reticulum encircles mitochondria with tubular cristae. **c.** Trophozoite cell surface with a 25 nm thick glycocalyx (arrows) covering the cell membrane.

### Genetic characterisation of mixed and monocultures

18S rRNA and COI amplicons of the expected size were obtained from all five primer sets used. The average amplicon length was 841 bp for 18S rRNA and 874 bp for COI sequences. The molecular clones were very similar to the original sequences, with 1–3 different bases detected at the tail ends of 18 out of the 36 sequences cloned. Based on the high similarity between the molecular clones, only one of each was used in the final phylogenetic analysis.

### Phylogenetic analysis of all amoebae detected

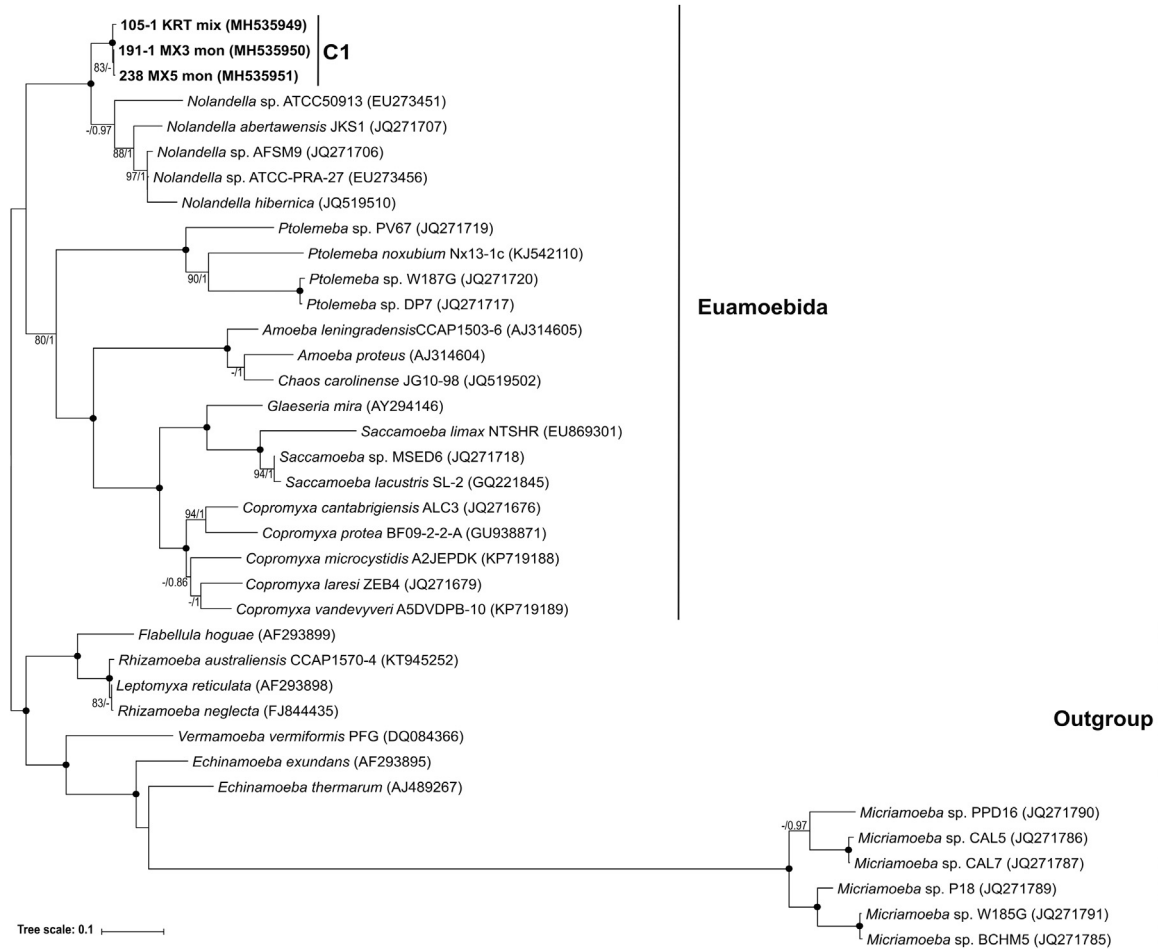
Both the Tubulinea and Discosea 18S rRNA analysis were congruent with former Amoebozoa analysis (Kang et al. 2017; Kudryavtsev and Gladkikh 2017; Tylm and Dyková 2017), as many typical higher taxa were found grouped within the trees presented. All the newly sequenced strains within Tubulinea had high homology with sequences representative of genus *Nolandella*, (Clade 1 (C1) in Fig. 8). They were not identical to any described species, but were nested as a separate, well-supported clade within *Nolandella* (ML bootstrap; ML 100, Bayesian probability; BP 1).

The newly sequenced strains from Discosea were nested in either Dactylopodida or Vannellida (Fig. 9). The sequences identified as Dactylopodida represented four genera, *Neoparamoeba*, *Vexillifera*, *Paramoeba* and *Pseudoparamoeba*. As expected, *N. perurans* was recovered in mixed-cultures

and monocultures, shown by the cluster including strain 183MP1, 249-1MP2 and 279SVA (ML 100, BP 1). Despite this grouping, the three *N. perurans* sequences were not identical, indicating possible intra-species variation within the same geographic region. Of the Dactylopodida monocultures, strain 322MX6 was closely related to *Vexillifera tasmaniana* (ML 74, BP 0.99) and strain 333-1MX1 was nested with the genus *Pseudoparamoeba* (ML 100, BP 1) but was not identical to any currently described species. Finally, strain 106KRT and 107-1HRT obtained from two different mixed-cultures corresponded to the marine amoeba *Paramoeba eilhardi* in a well-supported clade (ML 100, BP 1).

Within Vannellida, strain 285MV5 was closely related to *Vannella australis* (ML 94, BP 0.98), and strain 282-2MV4 as homologous with *Vannella* sp. strain PMCH-II (ML100, BP 1). Additionally, strain 205MV3 and 243MV2 were grouped with *Vannella septentrionalis*, however this relationship was weakly supported (ML 33, BP 0.38). Interestingly, two groups with unclear positions formed part of the basal Vannellida genera, including *Paravannella*, *Ripella*, *Lingulamoeba* and *Clydonella*. The first clade (C2), comprising strain 149-1SVA, 147SVA and 151-1SVA, was quite separate to all other genera within Vannellida. The second clade (C3), including 136SVA and 153-2SVA, was nested within the basal Vannellida genera however this had a poorly supported position (ML 42, BP 0.59).

The analysis derived from COI sequences (Fig. 10) was similar to, but not completely congruent with, former 18S rRNA and multi-gene-based Amoebozoa phylogenies (Kang et al. 2017; Kudryavtsev and Gladkikh 2017; Tylm and

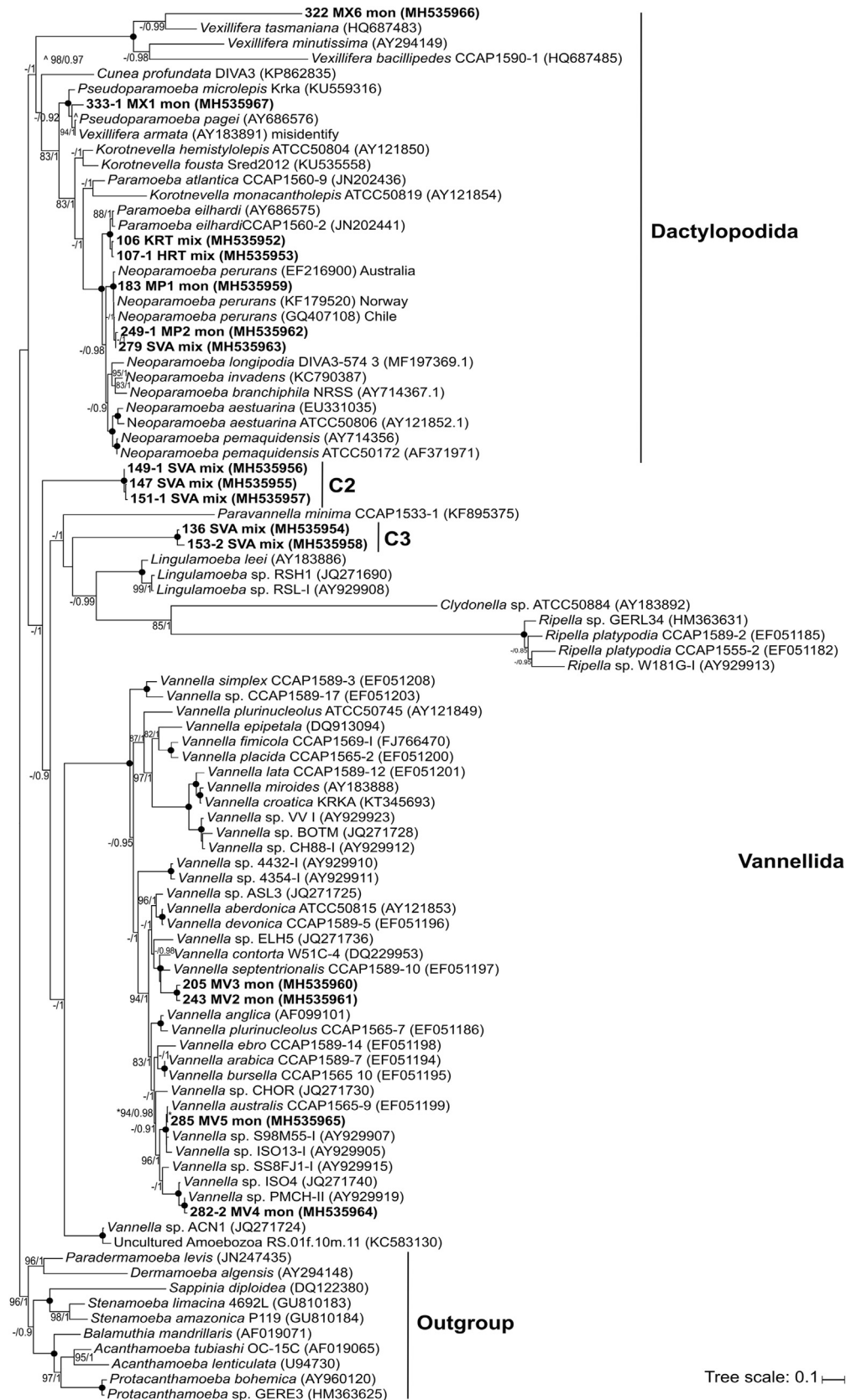


**Fig. 8.** Maximum likelihood analysis of taxa from Tubulinea 18S rRNA gene sequences. Numbers at the nodes represent ML bootstraps (ML) and Bayesian posterior probability (BP). Only values higher than 80 and 0.8 are presented. Black dots indicate 100/1 support values. Echinamoebidia and Leptomyxida serves as the outgroup. Taxon and strain names are listed before GenBank accession numbers. Strains in bold are the newly obtained sequences, and ‘mon’ refers to sequences obtained from monocultures and ‘mix’ refers to sequences from mixed-cultures.

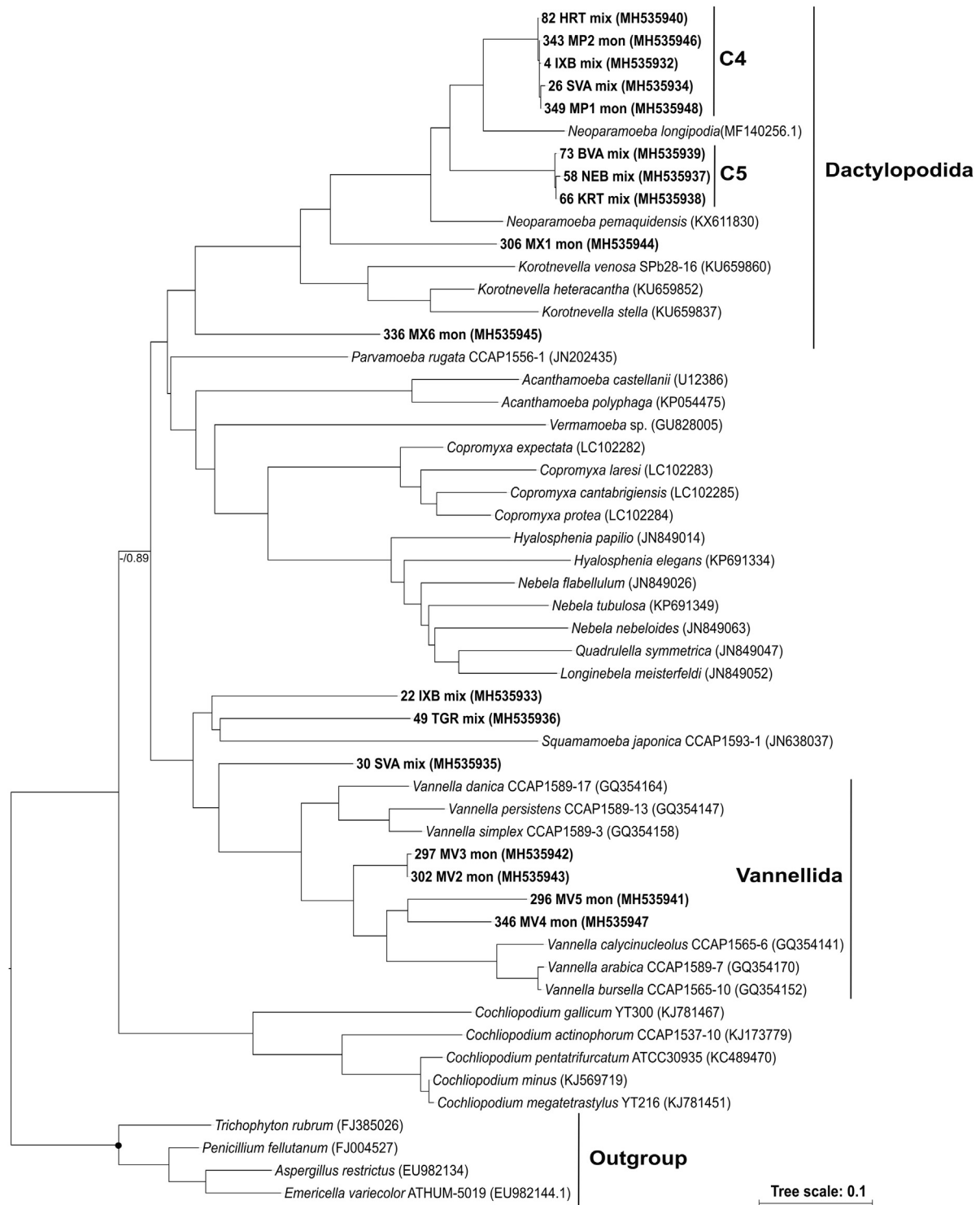
Dyková 2017). For instance, the main focus groups of this study, Dactylopodida and Vannellida, clustered separately, but were not as closely related to each other in terms of phylogenetic distance compared with findings derived from 18S rRNA-based analysis by Udalov et al. (2016) and multi-gene-based analysis by Kang et al. (2017), which cluster these groups together in Discosea. An additional difference is evident by the position of the reference sequences of *Parvamoeba rugata* and *Cochliopodium minus* which were grouped within Himatismenida in Kang et al. (2017) but were quite distant in this analysis. Considering this is one of the few published Amoebozoa phylogenies based on COI rather than 18S rRNA, some disparities should be expected and should not render the analysis redundant, rather an alternative perspective on Amoebozoa evolutionary relationships.

Similar to the 18S rRNA analysis, this COI phylogeny resolved that most newly sequenced amoeba strains on AGD-affected gills were closely related to either Dactylopodida or Vannellida. Due to the lower resolution of the COI phylogeny,

the identities of the monoculture COI sequences were based on the 18S rRNA tree in Fig. 9. Four Dactylopodida species were recovered by sequencing COI, including *N. perurans* which formed a well-supported clade labelled as C4 (ML 100, BP 1), another unidentifiable *Neoparamoeba* species clustered in C5 (ML 100, BP 1), *Pseudoparamoeba* sp. (306MX1) and *Vexillifera* sp. (336MX6). Of the Vannellida species, the four *Vannella* monocultures (297MV3, 302MV2, 296MV5, 346MV4) fell into a well-supported clade (ML 100, BP 1). Finally, there were three additional strains sequenced from mixed-cultures (22IXB, 49TGR, and 30SVA) that appeared to be neither Dactylopodida nor Vannellida, but could not be confidently identified to genus level. Of these three unidentified strains, 22IXB and 49TGR clustered with *Squamamoeba japonica*, however this position was not well supported by ML bootstraps (ML 33, BP 0.98), and the other remaining strain (30SVA) did not group with any currently recognised Amoebozoa COI reference sequence. These three strains may represent additional novel species.



**Fig. 9.** Maximum likelihood analysis of taxa from Discosea 18S rRNA gene sequences. Numbers at the nodes represent ML bootstraps (ML) and Bayesian posterior probability (BP). Only values higher than 80 and 0.8 are presented. Black dots indicate 100/1 support values. Centramoeba serves as the outgroup. Taxon and strain names are listed before GenBank accession numbers. Strains in bold are the newly obtained sequences, and 'mon' refers to sequences obtained from monocultures and 'mix' refers to sequences from mixed-cultures.



**Fig. 10.** Maximum likelihood analysis of taxa from Amoebozoa COI gene sequences. Numbers at the nodes represent ML bootstraps (ML) and Bayesian posterior probability (BP). Only values higher than 80 and 0.8 are presented. Black dots indicate 100/1 support values. Fungi serves as the outgroup. Taxon and strain names are listed before GenBank accession numbers. Strains in bold are the newly obtained sequences, and 'mon' refers to sequences obtained from monocultures and 'mix' refers to sequences from mixed-cultures.

## Diversity of amoebae detected on AGD-affected gills

In total, 12 different species were detected on the gills of AGD-affected Atlantic salmon farmed in Tasmania. There were three additional amoeba strains sequenced with COI primers which could not be identified (22IXB, 49TGR, 30SVA), but they could represent additional novel species. Each of the Amoebozoa listed in Table 2 were characterised to varying taxonomic extents. The most well characterised species were *N. perurans*, *Pseudoparamoeba* strain MX1, *Vexillifera* strain MX6, *Vannella* strain MV3 and strain MV4, with gross morphology and ultrastructure documented, and fragments of both the 18S rRNA and COI genes sequenced. Table 2 also shows the frequency each species was recovered from the gill samples. This information is unlikely to accurately reflect true species abundance on the gills, rather it indicates that some species were recovered more than once at different sample times, in particular *N. perurans* and *Nolandella* sp., and therefore are more likely to frequently colonise AGD-affected gills.

## Discussion

A diversity of amoebae was found accompanying *N. perurans* on the gills of farmed Tasmanian Atlantic salmon with characteristic AGD pathology. Six species were differentiated by both the 18S rRNA and COI gene, four by 18S rRNA gene alone, one by the COI gene only, and one species based on its distinct morphotype compared to all other genera sequenced in this study. The sequence-based taxonomic assessment of the strains grown in monoculture was well supported by trophozoite gross morphology and ultrastructure. Using a combined genetic and morphological approach effectively characterised a variety of amoeba species, in addition to *N. perurans*, colonising the gills of farmed Atlantic salmon with AGD. Significantly, we provide the first report of *P. eilhardi* being isolated from the gills of fish, and of *Pseudoparamoeba* sp. being isolated from the gills of Atlantic salmon, to our knowledge. Furthermore, considerable genetic variability within genera was observed across both the COI and 18S rRNA loci, revealing new phylogenetic clades and lineages not previously described.

Six genera of amoeba were detected on the gills and, of these, *Vannella* offered the greatest species diversity in this study. *Vannella* spp. are frequently isolated from both freshwater and marine environments, and have been cited as the most common amoeba isolated from various teleost organs, in particular the gills (Dyková et al. 2005a; Dyková and Lom 2004; Smirnov et al. 2007). *Vannella* spp. are generally considered non-pathogenic due to their frequent detection on healthy gills (Dyková et al. 2005a), and a challenge trial which found one *Vannella* strain (previously referred to as *Platyamoeba* sp.) isolated from Atlantic salmon in Ireland

was not associated with gill lesions (Nowak et al. 2004). Indeed, while a number of studies have isolated *Vannella* spp. from the gills of both asymptomatic and diseased fish, including Atlantic salmon with AGD and rainbow trout with NGD (Bermingham and Mulcahy 2007; Dyková et al. 2010; Dyková et al. 2005a; Dyková and Tým 2015), the true role *Vannella* spp. may play in these diseases remains unknown.

Within the Vannellida phylogeny presented here, five newly obtained 18S rRNA sequences formed two well separated clades (referred to as C2 and C3) positioned within the basal Vannellida genera (i.e. *Paravannella*, *Ripella*, *Lingulamoeba* and *Clydonella*). Although their position is unstable, C2 and C3 represent two new lineages, possibly genera, because they are quite different from any other currently recognised Vannellida genera. Whether C2 and C3 are in fact new genera cannot be established with certainty from the data currently provided. To confirm this finding the complete 18S rRNA gene sequence is needed, as well as gross and fine-scale morphological features. Unfortunately, the strains that make up C2 and C3 were all sequenced from mixed-cultures, hence type material is not available to carry out the necessary work required to fully characterise these lineages.

Of the Dactylopodida strains isolated, as expected *N. perurans* was detected during multiple sampling events, and from both mixed- and monocultures. This finding likely indicates that *N. perurans* is the most abundant species on salmon gills. *Vexillifera tasmaniana* has been previously detected on Atlantic salmon gills, as it was first isolated from Atlantic salmon held within an AGD experimental facility in Tasmania (Dyková et al. 2011a). *Vexillifera* sp. has also been found on the gills of farmed Atlantic salmon with AGD in Ireland, though this taxonomic assessment was based only on morphology (Bermingham and Mulcahy 2007). While there are a few instances of *Vexillifera* spp. isolated from AGD-affected fish, there have also been multiple detections from various other asymptomatic fish gills, including bitterling *Rhodeus sericeus*, banded leporinus *Leporinus fasciatus*, vimba bream *Vimba vimba*, turbot *Scophthalmus maximus*, and Nile tilapia *Oreochromis niloticus* (Dyková and Kostka 2013).

Interestingly, the *N. perurans* strain (MP1) examined under TEM in this study repeatedly showed ultrastructural features of the endosymbiont which looked different to all other currently published images (Dyková et al. 2003; Nowak and Archibald 2018; Wiik-Nielsen et al. 2016). In particular, the darkly stained arrangement of kinetoplast DNA appear to be in distinct elongated structures which were not as tightly associated with each other compared to previous detailed characterisation by Dyková et al. (2003). This difference was predicted to be caused by methodological approach. High pressure freeze fixation and freeze substitution was used as opposed to the common approach of using glutaraldehyde-based chemical fixation (Bermingham and Mulcahy 2007; Dyková et al. 2005a; Udalov 2016). We also trialled chemical fixation, but found freeze fixation generated images with far greater detail and fewer artefacts. Other studies which compared these methods in detail also found that freeze fixation

**Table 2.** Summary of all amoeba species/strains detected on the gills of Atlantic salmon *Salmo salar* with signs of AGD, and their method of detection and characterisation.

| Taxonomic assignment         | Strain                               | Sample used for amoeba detection (Mix, Mon) <sup>a</sup> | Amoeba characterisation (LM, TEM, SS) <sup>b</sup> | Frequency of recovery from gills |              |          |             | Gene fragment sequenced | Primers                                                       | GenBank accession number                                                                     |
|------------------------------|--------------------------------------|----------------------------------------------------------|----------------------------------------------------|----------------------------------|--------------|----------|-------------|-------------------------|---------------------------------------------------------------|----------------------------------------------------------------------------------------------|
|                              |                                      |                                                          |                                                    | June 2015                        | October 2015 | May 2016 | August 2017 |                         |                                                               |                                                                                              |
| <i>Neoparamoeba perurans</i> | MP1, MP2, 279SVA, 82HRT, 4IXB, 26SVA | Mix, Mon                                                 | LM, TEM, SS                                        | 1                                | 4            | 1        |             | 18S rRNA, COI           | S12.2Y-RibB                                                   | MH535932<br>MH535934                                                                         |
|                              |                                      |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
|                              |                                      |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
|                              |                                      |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
|                              |                                      |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
|                              |                                      |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
| <i>Vexillifera</i> sp.       | MX6                                  | Mon                                                      | LM, TEM, SS                                        |                                  |              |          | 1           | 18S rRNA, COI           | Ami6F1-<br>Ami9R<br>Eucox1F-<br>Euglycox1R                    | MH535940<br>MH535946<br>MH535948<br>MH535959<br>MH535962<br>MH535963<br>MH535945<br>MH535966 |
| <i>Pseudoparamoeba</i> sp.   | MX1                                  | Mon                                                      | LM, TEM, SS                                        |                                  | 1            |          |             | 18S rRNA, COI           | S12.2Y-<br>RibB<br>Eucox1F-<br>Euglycox1R                     | MH535944<br>MH535967                                                                         |
| <i>Vannella australis</i>    | MV5                                  | Mon                                                      | LM, SS                                             |                                  |              | 1        |             | 18S rRNA, COI           | S12.2Y-<br>RibB<br>Eucox1F-<br>Euglycox1R                     | MH535941<br>MH535965                                                                         |
| <i>Vannella</i> sp.          | MV2                                  | Mon                                                      | LM, TEM, SS                                        |                                  |              | 2        |             | 18S rRNA, COI           | S12.2Y-<br>RibB<br>Ami6F1-<br>Ami9R<br>Eucox1F-<br>Euglycox1R | MH535942<br>MH535943<br>MH535960<br>MH535961                                                 |
|                              | MV3                                  |                                                          |                                                    |                                  |              |          |             |                         |                                                               |                                                                                              |
| <i>Vannella</i> sp.          | MV4                                  | Mon                                                      | LM, TEM, SS                                        |                                  |              | 1        |             | 18S rRNA, COI           | 570C-1137R<br>LCO1490-<br>HCO2198                             | MH535947<br>MH535964                                                                         |
| Tubulinea                    | MX4                                  | Mon                                                      | LM                                                 |                                  |              | 1        |             | N/A                     | N/A                                                           | N/A                                                                                          |

|                                     |                                  |          |             |   |   |   |          |                        |                                  |
|-------------------------------------|----------------------------------|----------|-------------|---|---|---|----------|------------------------|----------------------------------|
| <i>Nolandella</i> sp.<br>(C1)       | MX3, MX5,<br>105KRT              | Mix, Mon | LM, TEM, SS | 1 | 1 | 1 | 18S rRNA | S12.2Y-<br>RibB        | MH535949<br>MH535950<br>MH535951 |
| <i>Paramoeba</i><br><i>eilhardi</i> | 106KRT,<br>107-1HRT              | Mix      | SS          | 2 |   |   | 18S rRNA | S12.2Y-<br>RibB        | MH535952<br>MH535953             |
| Vannellida (C2)                     | 149-1SVA,<br>147SVA,<br>151-1SVA | Mix      | SS          |   | 3 |   | 18S rRNA | S12.2Y-<br>RibB        | MH535955<br>MH535956             |
| Vannellida (C3)                     | 136SVA,<br>153-2SVA              | Mix      | SS          |   | 2 |   | 18S rRNA | S12.2Y-<br>RibB        | MH535957<br>MH535954<br>MH535958 |
| <i>Neoparamoeba</i><br>sp. (C5)     | 73BVA,<br>58NEB, 66KRT           | Mix      | SS          | 2 | 1 |   | COI      | Eucox1F-<br>Euglycox1R | MH535937<br>MH535938<br>MH535939 |
| COI strain 22                       | 22IXB                            | Mix      | SS          |   | 1 |   | COI      | Eucox1F-<br>Euglycox1R | MH535933                         |
| COI strain 49                       | 49TGR                            | Mix      | SS          |   | 1 |   | COI      | Eucox1F-<br>Euglycox1R | MH535936                         |
| COI strain 30                       | 30SVA                            | Mix      | SS          | 1 |   |   | COI      | Eucox1F-<br>Euglycox1R | MH535935                         |

<sup>a</sup>Mix = mixed-culture, Mon = monoculture.

<sup>b</sup>LM = light microscopy, TEM = transmission electron microscopy, SS = sangersequencing.

and freeze substitution improves the preservation of biological specimens, with less evidence of shrinkage or extraction artefacts (Harahush et al. 2012; Kurth et al. 2012). To our knowledge, this is the first study to document these particular amoebae in their attached form and preserved by high pressure freeze fixation. For these reasons the TEM images published in this study present an alternative perspective on some Amoebozoa ultrastructures, and possibly represent a more accurate depiction.

*Nolandella* sp. was the only well-characterised Tubulinea species isolated, and was the second most frequently isolated species in this study. *Nolandella* sp. has also previously been found on the gills of Atlantic salmon farmed in Ireland (Bermingham and Mulcahy 2007), but again this taxonomic assessment was based on morphology alone. Additionally, two well characterised *Nolandella* strains have colonised the gills of turbot, *Scophthalmus maximus* and rainbow trout, *Oncorhynchus mykiss* (Dyková and Kostka 2013; Dyková and Novoa 2001). *Nolandella* spp. are not considered to cause pathology because several strains closely related to *N. abertawensis* (species formerly attributed to *Hartmannella* genus, transferred by Smirnov et al. (2011)) has been isolated from the liver, spleen, kidneys and gills of various healthy teleost species (Dyková and Kostka 2013).

Whilst the results of our study represent the most comprehensive list of amoeba species colonising AGD-affected salmon gills to date it is likely that the diversity of amoebae is higher. For instance, two species that have been isolated from salmon gills in the past, *N. pemaquidensis* and *N. branchiphila*, were not detected in this study (Dyková et al. 2011b; Young et al. 2007). Additionally, there were three COI gene fragments that could not be identified to genus level, hence it is unclear whether they represent novel species thereby elevating species diversity. The reason for missed species is probably due to the low abundance of each species on the gills, and the methodological bias as highlighted below.

Two methods were used to address the aim of this study, including sequencing mixed-cultures, and sequencing alongside morphological characterisation of monocultures. Each approach generated a different community of amoebae species as summarised in Table 2. Establishing monocultures was ideal for comprehensively characterising species, as they provided a robust way of linking the gross and fine-scale morphological features with two variable genetic regions. However, only eight of the total 12 species were established as monocultures, which supports the premise that not all amoebae are easily grown in pure culture. Sequencing mixed-primary isolates from the gills lead to the detection of four additional strains. This approach likely yielded different species because it increased the chance of sequencing species that cannot grow in pure culture, and less abundant cryptic species such as *N. pemaquidensis* and *N. branchiphila* (Young et al. 2007). However, the two methods used to document species diversity in this study do not account for species that cannot grow in vitro, and are biased towards selecting amoe-

bae that are more capable of growing in the chosen culture conditions.

In this study two Amoebozoa barcoding regions, 18S rRNA and COI, were employed to examine the sequence diversity of amoebae on the gills of Atlantic salmon. 18S rRNA is the most commonly used gene to decipher Amoebozoa phylogenetic relationships and identify species (Tymł and Dyková 2017; Udalov et al. 2016; Volkova and Kudryavtsev 2017). Accordingly, 18S rRNA was the most taxonomically informative gene in this study due to the many reference sequences available on public databases. However 18S rRNA can be problematic for barcoding amoebae and developing molecular detection assays because it is too conserved between species and can contain intra-strain polymorphism (Nassonova et al. 2010; Smirnov et al. 2007; Young et al. 2014). Indeed, large conserved regions were evident throughout our newly sequenced amplicon of 18S rRNA representing multiple distinct species. For instance, 58% of base pairs were conserved between the 15 strains sequenced with the RibB-S12.2 primers. Intra-species polymorphisms were also detected within the 18S rRNA fragments representing three *N. perurans* strains and three *Nolandella* strains sequenced in this study.

COI is currently considered a promising gene for amoeba species barcoding because it can provide higher phylogenetic resolution between closely related species compared to 18S rRNA (Nassonova et al. 2010). However this finding has only been evidenced in a few amoeba genera, *Vannella* (Nassonova et al. 2010), *Cochliopodium* (Tekle 2014) and nebelid testate amoebae (*Hyalosphenia*, *Nebela*, *Quadrullella*, *Padaungiella*) (Kosakyan et al. 2012). This indicates that more amoeba COI sequences are needed to further validate the utility of this gene for amoeba barcoding and taxonomy. We have contributed 17 new Amoebozoa COI fragments to GenBank, all of which represent amoeba that have not been previously characterised with the COI gene sequence. These sequences will contribute to future Amoebozoa sequenced-based taxonomy.

## Conclusion

*N. perurans* and up to 11 other amoeba species colonise the gills of farmed Atlantic salmon with AGD. While *N. perurans* is the primary pathogen of AGD, the role of these accompanying amoebae in AGD remains unclear. Future research should investigate whether amoeba species other than *N. perurans* are parasitic or commensal, and whether AGD is associated with Amoebozoa dysbiosis which potentially influences disease onset or progression.

## Author contributions

M.C., P.L. and A.B. conceptualised the project. C.E. refined the project design and carried out the research inves-

tigation, with training and resource administration provided by N.B., P.L. and J.W. C.E. wrote the manuscript under the supervision of J.W., N. B., A. B., P. L. and M.C. T.T. and C.E. carried out the phylogenetic analysis and T.T. advised on how to write the methods and results in relation to this work. All authors contributed to reviewing and editing the final manuscript.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ejop.2018.10.003>.

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