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Genetic diversity among geographically distant isolates of *Neoparamoeba perurans*

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ABSTRACT: The present study explored the use of 2 common genetic markers, the mitochondrial cytochrome oxidase I (COI) and the ribosomal internal transcribed spacer (ITS) to infer the relationship between geographically distant isolates of the protozoan gill parasite *Neoparamoeba perurans*, the agent responsible for amoebic gill disease in farmed Atlantic salmon worldwide. Present data confirmed that the ITS marker is suitable for *Neoparamoeba* species discrimination; however, it is not recommended as a population marker due to the presence of multiple copies of ITS within both *N. perurans* clonal and polycultures. On the other hand, in the partial COI gene analysed here, a low variability was observed, with 8 haplotypes recovered from *N. perurans* samples collected from Europe (Ireland, Norway, Scotland) and Tasmania (Australia). In Europe, the COI haplotypes which have more recently been detected in aquaculture are different to the haplotypes associated with the original gill disease emergence in Ireland in 1997 and Norway in 2006. The presence of unique COI haplotypes in different continents suggests the presence of multiple distinct reservoirs of the pathogen in both Europe and Tasmania. Isolates from additional geographical locations are required to fully understand the origins and routes for the spread of *N. perurans* worldwide.

KEY WORDS: Amoebic gill disease · AGD · Gill parasite · Amoeba · COI · Atlantic salmon

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1. INTRODUCTION

Neoparamoeba perurans (syn. *Paramoeba perurans*) Young, Crosbie, Adams, Nowak, Morrison 2007 is the causative agent of amoebic gill disease (AGD), originally described as affecting Atlantic salmon *Salmo salar* L. in Tasmania (Munday et al. 1993) and later reported in most Atlantic salmon-producing countries (Oldham et al. 2016). *N. perurans* is a cosmopolitan organism affecting a wide range of farmed marine fish and has only been sporadically reported in wild fish in both the Northern and Southern Hemispheres (Stagg et al. 2015, Oldham et al. 2016).

Understanding the origin of *N. perurans* in different aquaculture facilities and the epidemiological relationship between individual outbreaks is essential for the development of effective disease management strategies. The mitochondrial cytochrome oxidase I (COI) gene is an informative genetic marker which can reveal differences at the population level and be utilized to investigate the history of introduction for different metazoan fish parasites (see for example

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Hansen et al. 2007, Yazawa et al. 2008). In amoeboid protists, the COI is also used as an informative species barcoding marker, and for some slow-evolving species complexes this marker offers higher resolution compared to other widely used markers such as the ribosomal small subunit (SSU) or internal transcribed spacer (ITS) (Nassonova et al. 2010). Traditionally, variation in the SSU (Dyková et al. 2007) and ITS (Young et al. 2014) has been used to discriminate between closely related *Neoparamoeba* species. However, more recently, COI sequences have been characterized for amphizoic *Neoparamoeba* species (Volkova & Kudryavtsev 2017) as well as for 17 amoeboid species colonizing Atlantic salmon gills (English et al. 2019). The latter study demonstrated the potential of COI for species characterization and highlighted issues related to the incongruence of COI and SSU-based Amoebozoa phylogenies.

In the present study, we explored the use of 2 common genetic markers, the COI mtDNA and ITS region of rRNA, to investigate the origin and relationship of AGD outbreaks worldwide utilizing *N. perurans* samples collected in Tasmania and 3 European Atlantic salmon-producing countries: Ireland, Norway and Scotland.

2. MATERIALS AND METHODS

Total DNA was extracted from 24 *Neoparamoeba perurans*-infected farmed fish gill tissues, 3 polyclonal and 1 clonal culture originally isolated from infected Atlantic salmon, as well as from 2 *N. pemaquidensis* cultures (Page, 1980) Page, 1987 and 1 *N. branchiphila* culture obtained from public depositories (Dyková et al., 2005) (Table 1). Scottish

Table 1. Samples of amoebas available for the characterization of cytochrome oxidase I (COI) diversity. *Neoparamoeba perurans*-infected gills of farmed Atlantic salmon *Salmo salar* and hatchery-reared ballan wrasse *Labrus bergylta* were utilized in the analysis in addition to *N. perurans* poly- and clonal cultures. A and B in the column 'Strain name' signify that samples originated from the same farm. ITS: internal transcribed spacer

Species/Country	Year of isolation	Source	Strain name	COI phylogeny	COI GenBank accession no(s).	ITS GenBank accession no(s).
N. perurans/Ireland	2017	Atl. salmon	IRE_2017_01	Yes	MN025476	
	2017	Atl. salmon	IRE_2017_02	Yes	MN025477	
	1997	Atl. salmon	IRE_1997_17_01A		MN025478	
	1997	Atl. salmon	IRE_1997_17_01B	Yes	MN025479	
N. perurans/Norway	2006	Atl. salmon	NO_2006_09_01A		MN025480	
	2006	Atl. salmon	NO_2006_09_01B	Yes	MN025481	
	2013	Atl. salmon	NO_2013_17_01	Yes	MN025482	
	2013	Atl. salmon	NO_2013_17_02	Yes	MN025483	
	2014	Atl. salmon	NO_2014_17_01	Yes	MN025484	
	2013	Atl. salmon	NO_2013_17_03	Yes	MN025485	
	2014	Atl. salmon	NO_2014_17_02	Yes	MN025486	
	2013	Polyculture	NO_2013_17_06	Yes	MK990580	MN010335–MN010353
	2013	Atl. salmon	NO_2013_17_04	Yes	MN025487	
	2013	Ballan wrasse	NO_2013_17_05	Yes	MN025488	
N. perurans/Scotland	2014	Polyculture	SCO_2014_01	Yes	MK990577	MN010362–MN010376
	2016	Clonal culture	SCO_2016_01		MN025489	MN010377–MN010379
	2012	Atl. salmon	SCO_2012_01A	Yes	MN025490	
	2012	Atl. salmon	SCO_2012_02	Yes	MK990578	
	2012	Atl. salmon	SCO_2012_01B	Yes	MN025491	
	2014	Atl. salmon	SCO_2014_02A	Yes	MK990579	
	2014	Atl. salmon	SCO_2014_02B	Yes	MN025492	
N. perurans/Tasmania	2013	Polyculture	TAS_2013	Yes	MK990581–MK990592	MN010354–MN010361
		Atl. salmon	TAS_2015_4IXB	Yes	MH535932	
		Atl. salmon	TAS_2015_26SVA	Yes	MH535934	
		Atl. salmon	TAS_2015_82HRT	Yes	MH535940	
		Atl. salmon	TAS_2015_MP1	Yes	MH535948	
		Atl. salmon	TAS_2015_MP2	Yes	MH535946	
N. pemaquidensis/Wales	1976	Environmental-cultured	CCAP 1560/4	Yes	MN025475	
N. pemaquidensis/USA		Farmed coho salmon	ATCC50172	Yes	MK990593	
N. branchiphila/USA	2005	Blue crab	RP	Yes	MK990594	EU884491
N. aesturiana						EU884483

polyclonal and clonal cultures were established according to Collins et al. (2017), and the Tasmanian polyclonal culture was established using the protocol of Morrison et al. (2004). All *N. perurans*-infected gill tissue originated from farmed Atlantic salmon or ballan wrasse *Labrus bergylta* collected from a rearing facility (Table 1). Two sets of COI universal primers, LCO1490F and HCO2198R (Folmer et al. 1994) and Eucox 1F and Euglycox 1R (Heger et al. 2011), were used to amplify a partial COI gene. Alternatively, newly designed primers, Pp_CO1_F_HH (5′-CCA CCT TCG TTT TTC TTA CT-3′) and Pp_CO1_R_HH (5′-AGC AAT TGA AAC CAT AGC AT-3′) were used to amplify an overlapping COI segment for a sub-set of isolates which did not amplify for the larger fragment. COI sequence data was obtained by direct sequencing of purified PCR products and submitted to NCBI under accession numbers MK990577–94 and MN025475–92 (Table 1). The ITS region was amplified using the primers PER 1F (5′-CGA TGT TTG GTC CGG TGA AA-3′) and PER 1R (5′-ATA TGC TTA AGT TCA GTG GGT A-3′), which were newly designed based on the sequence DQ660492 (Caraguel et al. 2007). All PCRs used the same cycling conditions: 95°C for 1 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min; and 72°C for 1 min and a final extension of 72°C for 5 min. The ITS product was ligated into bacterial plasmid using the pGEM-T Vector Systems (Promega) as per the manufacturer's instruction prior to sequencing, and sequences were submitted to GenBank under accession numbers MN010335–79.

A final dataset of 39 sequences of *N. perurans*, *N. pemaquidensis* and *N. branchiphila* (Table 1) spanning the partial 475 bp fragment of COI gene was aligned using MAFFT v.7 and the L-INS-I algorithm (Katoh & Standley 2013). The HKY+G substitution model (Hasegawa et al. 1985) was applied as the best-fit model as determined in MEGA v.7 (Kumar et al. 2016). Neighbor joining (NJ) trees were generated in MEGA v.7 with the reliability of the trees tested with 1000 bootstrap replicates. A haplotype network was constructed using the R package pegas (Paradis 2010) based on an infinite site model of DNA sequences.

For ITS phylogenetic analysis, 19 clones from Norwegian, 15 clones from Scottish and 8 clones from Tasmanian polyculture were sequenced, and altogether 44 sequences originating from *N. perurans*, *N. branchiphila* and *N. aesturiana* (Table 1), spanning 410 bp, were aligned as outlined above. FASTA alignments were uploaded into BEAUTI software v2.4.7, and Bayesian phylogenetic analysis was performed in BEAST software v2.4.7 incorporating the HKY + G substitution model under Coalescent Bayesian Skyline priors. Ancestral location reconstruction was implemented using the method outlined in Lemey et al. (2009) to predict the geographic location of isolates at each node of the tree and therefore estimate the origins of *N. perurans* clones/strains. The Markov chain Monte Carlo method (MCMC) was run for 100000000 generations (every 10000 generation was logged), with the first 10% of trees being discarded for burn-in using TreeAnnotator (Drummond et al. 2012). Tracer software v.1.6 was used to assess the convergence of MCMC where all effective sample size statistics (ESS) were >200.

3. RESULTS AND DISCUSSION

3.1. *Neoparamoeba perurans* ITS variation

The partial ITS rDNA (1.1 kb) was amplified from a single Scottish *Neoparamoeba perurans* clonal culture and 1 *N. perurans* polyculture from Scotland, Norway and Tasmania, respectively. Unambiguous sequence data was only obtained following cloning of the ITS PCR products, and a high level of variation was observed among clones originating from each *N. perurans* polyculture (Table 2). Variation in the ITS was also observed among 3 individual clones from a Scottish clonal culture (Table 2), with variation reaching 6.6%, including a 12 bp deletion in the ITS2 spacer.

Numerous deletions observed throughout the ITS region were removed from the alignment, and Bayesian inference analysis showed a lack of monophyletic clustering of clone sequences originating from the individual polycultures (data not shown). In addition, no clustering based on geographic origin of *N. perurans* samples was observed in the present dataset (Fig. A1 in the Appendix).

The high level of heterogeneity within the ITS region was previously described for another *Neoparamoeba* species, *N. pemaquidensis*, and unam-

Table 2. Diversity in the *Neoparamoeba perurans* partial internal transcribed spacer (ITS) rDNA fragment

Source	Country	No. of unique ITS sequences/no. of clones
Polyculture	Scotland	15/15
	Norway	18/19
	Tasmania	10/10
Clonal culture	Scotland	3/3

biguous sequence data was also only obtained from cloned PCR products (Caraguel et al. 2007). The problems of obtaining unambiguous sequence reads by direct sequencing of PCR products and the lack of monophyletic structuring of ITS copies originating from the same *N. perurans* polycultures led us to conclude that the ITS might not be a suitable genetic marker for tracing the origin of AGD outbreaks. Other authors have also concluded that the use of ITS for phylogenetic studies of protists should be treated with caution because, for example, in the case of *Pythium*, a genus of parasitic oomycetes, the concerted evolution did not prevent diversification and the occurrence of multiple copies of ITS within the same isolate (Belbahri et al. 2008).

3.2. *Neoparamoeba pemaquidensis* COI variability

No COI product was obtained with the Folmer et al. (1994) primer set for either of the *N. pemaquidensis* cultures studied while products of approximately 900 bp were amplified using the Eucox 1F and Euglycox 1R (Heger et al. 2011). The COI sequence of *N. pemaquidensis* isolate CCAP 1560/4 (from Wales) was identical to the sequence by Tanifuji et al. (2017) obtained from the same culture strain (accession number KX611830). On the contrary, *N. pemaquidensis* isolate ATCC50172 from the USA showed a difference of 9.89% (79/798 bp) compared to the strain CCAP 1560/4 which originated from Wales. The interspecific variability among the most common European haplotype of *N. perurans* (haplotype 6) (see Section 3.3), *N. branchiphila and Neoparamoeba* C5 (English et al. 2019) used in this study ranged between approximately 7.89 and 12.53% (Table 3). The question of whether the 2 isolates of *N. pemaquidensis* belong to one or to several different species needs to be further investigated. Until further data is available, these isolates can only be reported as separate molecular operational taxonomic units (MOTUs) as was previously suggested for some amoeba morphospecies from the genus *Vanella* (Nassonova et al. 2010).

3.3. *Neoparamoeba perurans* COI phylogeny and haplotype network

No COI product was obtained with the Folmer et al. (1994) primer set, while products of approximately 900 bp were amplified using Eucox 1F and Euglycox 1R (Heger et al. 2011). In addition to the COI sequences generated in this study, a further 5 COI sequences obtained by English et al. (2019) from farmed fish in Tasmania were used to analyse the relationship between geographically distant *N. perurans* populations. In total, 8 haplotypes of *N. perurans* were identified in the present dataset (Table 4). In Tasmania, overall 6 different COI haplotypes were found, with 5 haplotypes (H1–H4 and H7) distinguished from a polyculture isolated from farmed salmon in 2013 and 2 haplotypes (H7 and H8) identified from infected farmed salmon tissue between 2015 and 2017 (Table 4). The polymorphic diversity between haplotypes observed within a single Tasmanian polyculture and among different farm sites (English et al. 2019) was comparable, reaching 0.63%.

In Europe, the main difference was observed between *N. perurans* collected prior to and after the 2009–2010 increase in AGD outbreaks in Europe. Based on both the NJ and haplotype network analysis, the Irish samples collected in 1997 (strains IRE_1997_17_01A and B; Palmer et al. 1997, Downes et al. 2018) and Norwegian samples from 2006 (strain NO_2006_09_01A and B) (Steinum et al. 2008) were shown to belong to a different haplotype (H5) than the more recent *N. perurans* samples collected throughout Europe between 2012 and 2017 (H6) (Figs. 1 & 2, Table 4). Interestingly, *N. perurans* haplotype H6 was found on both farmed Atlantic salmon and hatchery-reared ballan wrasse. The percentage of polymorphic sites between European haplotypes 5 and 6 was 1.26%. The number of mutational steps between the 2 different European haplotypes (H5 and H6) was higher (8 hypothetical steps) than between the European haplotype 6 and any of the Tasmanian haplotypes (2–5 steps) identified in this study, suggesting 2 distinct pathogen

Table 3. Diversity (% variation) in the partial cytochrome oxidase I (COI) gene among *Neoparamoeba* species calculated over the partial COI alignment (798 bp)

Species	Table ID	1	2	3	4	5
N. pemaquidensis 1560	1	–	9.89	11.65	10.90	12.40
N. pemaquidensis ATCC	2		–	11.65	12.66	13.15
N. perurans haplotype 6	3			–	7.89	12.53
N. branchiphila	4				–	12.40
Neoparamoeba C5 (English et al. 2019)	5					–

Table 4. Partial cytochrome oxidase I (COI) gene (475 bp) haplotypes of *Neoparamoeba perurans* identified in the dataset

Haplotype	No. of strains	*N. perurans* strain ID(s)
1	1	TAS_2013_Contig4
2	1	TAS_2013_Contig1
3	1	TAS_2013_Contig15
4	1	TAS_2013_Contig12
5	2	NO_2006_09_01B, IRE_1997_17_01B
6	16	NO_2013_17_01, NO_2013_17_02, NO_2014_17_01, NO_2013_17_03, NO_2014_17_02, NO_2013_17_06, IRE_2017_01, NO_2013_17_04, IRE_2017_02, NO_2013_17_05, SCO_2014_01, SCO_2012_01A, SCO_2012_02, SCO_2012_01B, SCO_2014_02A, SCO_2014_02B
7	13	TAS_2013_Contig3, TAS_2013_Contig5, TAS_2013_Contig2, TAS_2013_Contig6, TAS_2013_Contig8, TAS_2013_Contig10, TAS_2013_Contig11, TAS_2013_Contig13, TA_2013S_Contig16, TAS_2015_4IXB, TAS_2015_82HRT, TAS_2015_MP1, TAS_2015_PM2
8	1	TAS_2015_26SVA

origins present in Europe. The percentage of polymorphic sites in the sequenced COI region between the Tasmanian haplotypes and European haplotypes (H5 and H6) was 1.47 % and 0.84 %, respectively.

Low intraspecific variation of the COI gene even across large geographic distances can be the result of high gene flow and rapid spread, as has also been reported for other amoebae species from the terrestrial genus *Assulina* (Lara et al. 2011). Although higher variability and existence of further COI haplotypes is expected if the complete COI gene region is available, the present study demonstrated the ability of COI to discriminate different *N. perurans* populations within and between different continents and showed no overlap between European and Tasmanian COI haplotypes (Figs. 1 & 2).

A review by Oldham et al. (2016) concluded that AGD is no longer restricted to warm seawater regions, as outbreaks of the disease have now been reported in temperatures below 10°C (Mo et al. 2015). A period of abnormally higher water temperature, relative to each regional average, is one common factor which always precedes the first reports of AGD, corroborating the hypothesis that the increase in water temperature is an important predictor of disease outbreak (see Oldham et al. 2016). Considering the phylogeny and haplotype network analysis inferred from the partial COI region, it appears that distinct reservoirs of the pathogen exist on each continent, with AGD most likely being triggered by unusually high seawater temperatures. In addition, 2 very distinct *N. perurans* forms/haplotypes occur in Europe with the more recent form (H6) rapidly spreading throughout the European aquaculture industry and successfully persisting there since at least 2011. However, further populations from regions impacted by AGD are required to fully understand the origins and routes of the spread of *N. perurans* worldwide.

To our knowledge, this is the first report which describes the intraspecific variability of the *N. perurans* mtDNA, specifically the COI gene, between geographically distant isolates, and demonstrates its power to distinguish between individual outbreaks. It is important to continue to record all relevant abiotic and biotic data associated with AGD outbreaks and to characterise *N. perurans* strains in order to

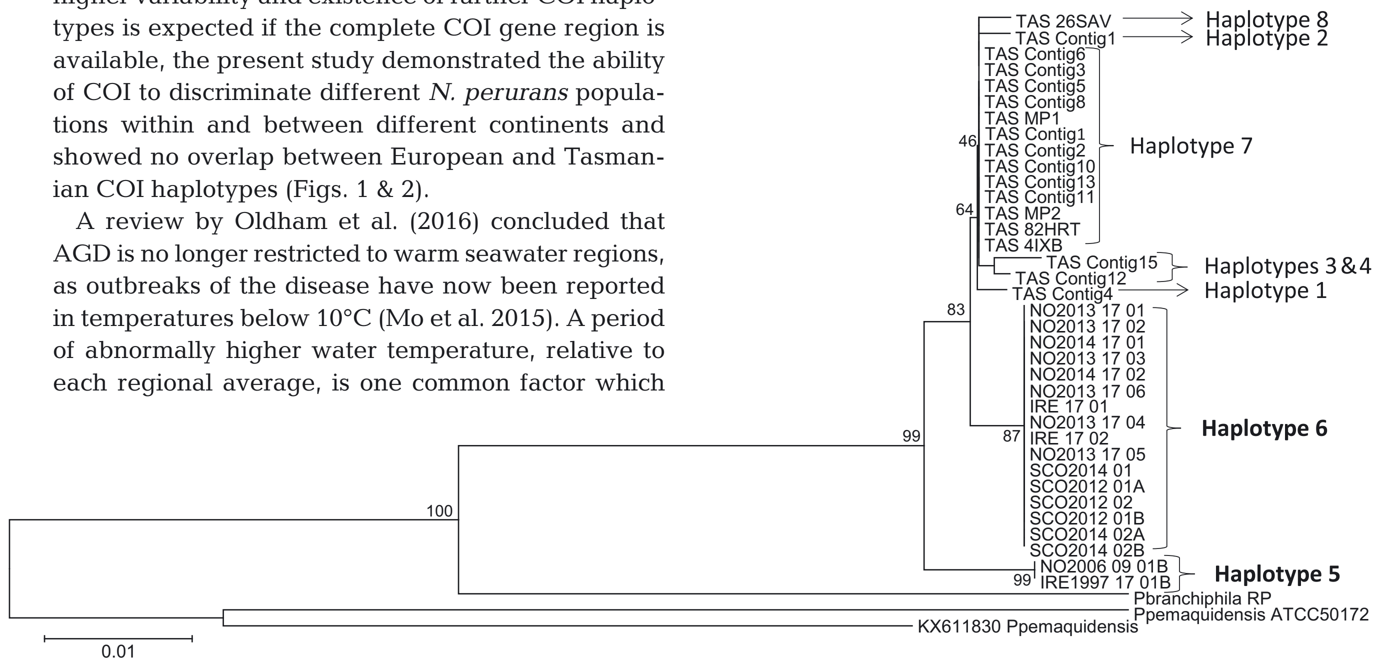

Fig. 1. Neighbor-joining phylogenetic analysis performed with the partial cytochrome oxidase I (COI) gene data set illustrating relationship between European (in **bold**) and Tasmanian *Neoparamoeba perurans* haplotypes

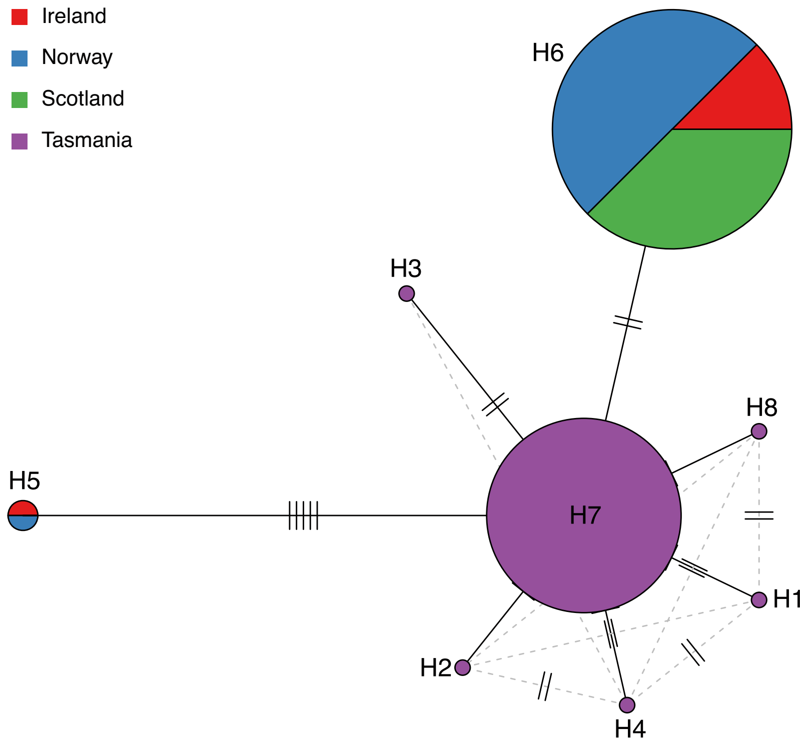

Fig. 2. Randomized minimum spanning haplotype network for the *Neoparamoeba perurans* cytochrome oxidase I (COI). Circle size is proportional to the number of *N. perurans* samples identified as same haplotype. Hatch marks represent the number of mutational steps between nodes (on solid lines) or hypothetical intermediate haplotypes (on dashed lines)

identify the missing hypothetical intermediate haplotypes and fully understand AGD emergence and persistence and the mechanisms by which the disease spreads worldwide.

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Appendix

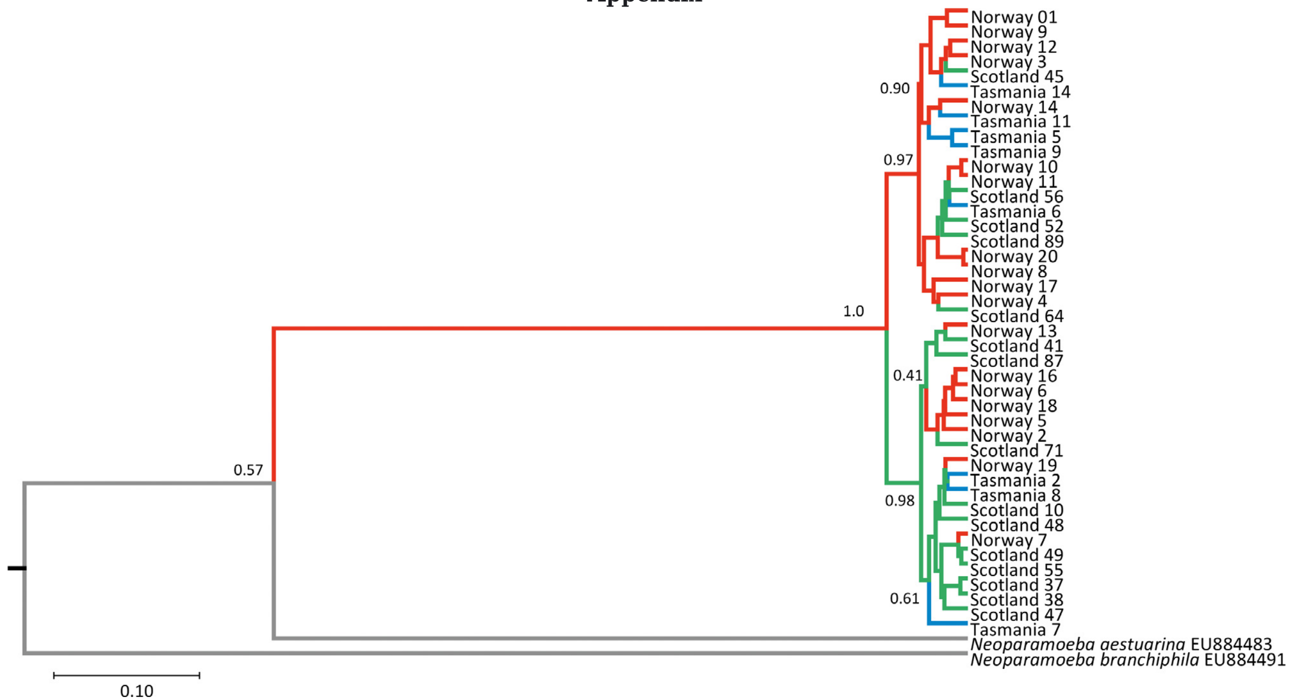

Fig. A1. Bayesian phylogenetic analysis, inferred from the partial internal transcribed spacer (ITS) region sequences obtained from individual clones originating from a single *Neoparamoeba perurans* polyculture each from Norway, Scotland and Tasmania

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