Vibrio tapetis-like strain isolated from introduced Manila clams *Ruditapes philippinarum* showing symptoms of brown ring disease in Norway

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ABSTRACT: The Manila clam *Ruditapes philippinarum* was introduced to Norway in 1987 and was produced in 2 hatcheries until 1991. Clam seed was planted at 6 sites. Two sites were on the Island of Tysnes, south of Bergen. Surviving adult Manila clams were recovered in 1995 and 1996. In the present study, Manila clams from the original seeding that displayed morphological signs of brown ring disease (BRD) were recovered in June 2003 (n = 7) and in June 2004 (n = 17). Samples from extrapallial fluid, tissues and haemolymph were inoculated on marine agar. Replicate subcultures on selective media were used to select potential *Vibrio tapetis* strains, and in total, 190 bacterial strains were isolated. One of these strains clustered within the *V. tapetis* clade and was named NRP 45. DNA:DNA hybridisation with the type strain CECT4600 showed 52.7 and 57.3% DNA:DNA similarity. Hybridisation of NRP 45 and the *V. tapetis* LP2 strain, isolated from corkwing wrasse *Symphodus melops*, produced 46.6 and 44.4% re-association. Partial gene segments encoding 16S rRNA, gyrase B protein (GyrB) and chaperonin 60 protein (Cpn60) were characterised and compared to CECT 4600. NRP 45 showed 5 differences in the 1416 nucleotides (nt) of the 16S rRNA encoding gene (99.6% similarity), while the GyrB encoding gene had 62 substitutions of 1181 nt compared (94.8% similarity) and the Cpn60 encoding gene had 22 substitutions out of 548 nt compared (96% similarity). This is the first finding of BRD and the first isolation of a *V. tapetis*-like bacterial strain from a bivalve in Norway.

KEY WORDS: Brown ring disease · *Vibrio tapetis* · Bivalve · Manila clams · Bacteria · Norway

INTRODUCTION

The Manila clam *Ruditapes philippinarum* (Adams & Reeve 1850), which is originally from Asia, has been introduced to many areas in North America and Europe. In many cultivation areas, the Manila clam has been severely affected by brown ring disease (BRD; reviewed by Paillard 2004). BRD is a bacterial shell disease,

caused by *Vibrio tapetis* (Paillard & Maes 1990, Borrego et al. 1996). The symptom characterising this disease is an abnormal conchiolin deposit, which generally adheres to the inner shell between the pallial line and the growth edge of the shell, forming the characteristic brown ring (Paillard et al. 1989). The Manila clam was introduced to France in 1972, and BRD appeared for the first time in clam beds in Brittany (France) in 1987. The

disease has subsequently spread along the East Atlantic coast from England to North Africa (Paillard 2004). BRD was first observed in Spain in 1994, in England in 1997 and in Ireland in 1998 (Paillard & Maes 1994, Castro et al. 1996, Novoa et al. 1998). The highest prevalence is encountered in the northernmost countries, and BRD is generally classified as a cold-water disease (Paillard et al. 1994). In Norway, the Manila clam was introduced in 1987 (Mortensen & Strand 2000). Approximately 500 broodstock specimens were imported from Scottish Seafarms Ltd to 2 Norwegian hatcheries, located in Espevik on the Island of Tysnes, south of Bergen, and in Vallersund in South Trøndelag County (Fig. 1A). From 1987 to 1991, more than 200 million seed were produced, with ongrowth done in nurseries at Espevik, Rong, Vallersund and Vågstanda. Most of the Manila clam seed were exported to Spain and Ireland. In Espevik, the native clam *R. decussatus* was also produced by the same methods and during the same period as the Manila clams. Clam production was terminated in 1991. Between 1988 and 1991, Manila clam seed were released on beaches at 6 sites. At Seløy (Fig.1B), approximately 2000 Manila clam spat were released in 1988, and a total of 28 specimens were recaptured in 1995 (Mortensen & Strand 2000). The objective of the present study was to investigate if survivors of Manila clams planted along Seløy in 1988 exhibit clinical signs of BRD and harbour *V. tapetis* strains.

MATERIALS AND METHODS

Sampling site. Seløy is a small island outside the island of Tysnes, in Hordaland County, south of Bergen, Norway (59° 55′ N, 5° 43′ E). The sampling was performed in the Seløy poll (lagoon) at the south side of the island (Fig. 1A). This site consists of a sheltered, relatively small poll with a maximum depth of approximately 5 m, a narrow channel with a slow tidal current, and a sheltered bay as an entrance to the outside fjord (Fig. 1B). The poll has been used as a traditional, heliothermic oyster poll in conjunction with intensive flat oyster culture carried out in the nearby Espevik poll and nursery. There are at present few bivalves in the Seløy poll, probably due to relatively dense populations of predator species such as wrasse, eel and shore crabs. The channel has a sand and gravel substrate, suitable for cockles and clams with a moderate density of these species.

Clam sampling and identification. In May 2003, Manila clams were collected in a restricted area by digging from the upper 15 cm of the substrate in the sandy part of the tidal current, and were kept on ice until dissection in the laboratory. In June 2004, sampling for Manila clams was conducted in the whole narrow channel and also along the shoreline around the poll by diving. All recovered Manila clams and native clam species were identified by morphological features of shells and soft parts, as described by Cesari
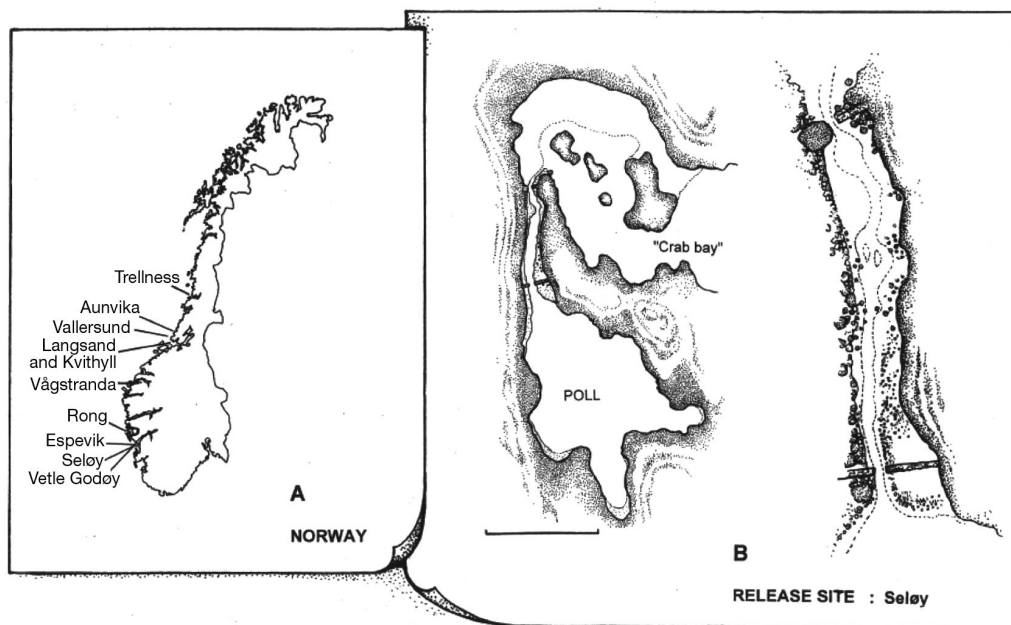

Fig. 1. (A) Manila clams *Ruditapes philippinarum* were originally imported to hatcheries at Vallersund and Espevik, produced in nurseries at Vallersund, Vågstranda, Rong and Espevik, and seeded at Trellnes, Aunvika, Langsand and Kvithyll, Vetle Godøy and Seløy. (B) Sampling site at Seløy consisting of a poll (lagoon), a shallow tidal stream and an outside bay facing the fjord. The tidal current (enlarged) runs into a partially man-made channel. Clams were sampled in the widest part of the channel. Scale bar = 100 m

& Pellizato (1990) and Holme (1961). Shell length of Manila clams was measured with callipers to the nearest mm, and age was determined from growth rings according to Johannessen (1973).

BRD diagnostics. Development and/or recovery of BRD were judged by scaling macroscopic symptoms according to the classification system established by Paillard & Maes (1994), with modifications by Paillard (2004).

Bacterial isolation protocol. Bacteria were isolated from extrapallial fluid (EPF; Paillard et al. 2006), digestive gland and mantle (Allam et al. 1996). Briefly, serial dilutions of EPF, digestive gland and mantle suspensions were prepared in sterile seawater (SSW). Serial dilutions (0.1 ml) were plated on Difco marine agar and incubated for 72 h at 20°C. Total heterotrophic bacteria (THB) concentrations were counted and are presented as colony-forming units (CFU) ml^{-1} or g^{-1} fresh weight of whole clams. The selection of isolates carried forward for further analyses was performed according to Maes & Paillard (1992). Briefly, replicate subcultures on selective media were used to select potential *Vibrio tapetis* strains, based on 4 important characteristics: non-utilisation of sucrose and mannitol, growth on thiosulfate citrate bile sucrose (TCBS) agar, and failure to grow above 30°C. Colonies meeting these 4 requirements were isolated and purified, and then subjected to phenotypic, serological and molecular characterisation as described below.

Phenotypic characterisation. A series of biochemical tests were carried out using commercial API 20E and API 50CH kits (Bio-Merieux). The tests were conducted according to the manufacturer's protocol, with the following exceptions: with the API 20E Kit, bacteria were suspended in autoclaved, 30 ppt seawater. Bacteria were suspended in the medium provided by the supplier for the API 50CH kit that had been adjusted to a final salinity of 20 ppt using NaCl. Kits were then incubated at 20°C and read for 48 h prior to interpreting each test strip. Bacterial surface antigen properties were addressed by using an agglutination assay using a polyclonal antiserum (Pasteur Institute), raised in a rabbit against cells of *Vibrio tapetis* strain IS1. Prior to agglutination, bacteria were cultured for 72 h, and colonies that developed on marine agar were harvested in 0.5 ml SSW. Finally, 5 µl of an anti-*V. tapetis* (CECT 4600) polyclonal antibody solution (Pasteur Institute) were added for visual observation of agglutination. An antibody against *V. cholerae* (Pasteur Institute) was used as a negative control.

PCR amplification and sequencing. Using the protocol described by Paillard et al. (2006), all bacterial strains were screened using specific primers for *Vibrio tapetis*. This screening was used to select strains for further characterisation. DNA from the isolated strain was extracted with a DNAeasy Tissue Kit (Qiagen) according to the manufacturer's instructions, and stored at –20°C. Selected type strains of *Vibrio* spp. were subject to PCR amplification of 16S rRNA (Weisburg et al. 1991), GyrB (Yamamoto & Harayama 1995) and Cpn60 (Rusanganwa et al. 1992) partial gene fragments. The bacteria included in this study are summarised in Table 1. All PCR reactions used approximately 100 ng DNA in a 20 µl reaction and were performed with an ABI 9700 thermal cycler (Applied Biosystems). PCR amplification of the 16S rRNA encoding gene was performed with final reaction concentrations of 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 1 unit *Taq* polymerase (Promega). The temperature conditions consisted of an

Table 1. Bacterial strains and accession numbers for all sequences included in the phylogenetic analysis

Strain	16S rRNA	Gyrase B (GyrB)	Chaperonin 60 (Cpn60)
NRP 45	AM402968	AM399070	AM399071
Vibrio tapetis CECT4600	Y08430	AM162571	AM162561
Vibrio tapetis LP2	AY129278	AM162570	AM162560
Vibrio lentus CIP 107166	AM162659	AM162564	AM162554
Vibrio chagasii LMG 21353	AJ490157	AM162568	AM162558
Vibrio splendidus LP1	AY129277	AM235734	AM235733
Vibrio fischeri NCMB 1281	AY292923	AY455874	AM235730
Vibrio parahaemolyticus NCMB 1902	AF388387	AM235735	AM235731
Vibrio kanaloae LMG 20539	AM162657	AM162563	AM162553
Vibrio cyclitrophicus LMG 21359	AM162656	AM162562	AM162552
Listonella anguillarum strain 610	AM162655	AM162569	AM162559
Listonella anguillarum NCMB 6	AM235737	AM235736	AM235732
Aeromonas salmonicida subsp. *salmonicida* NCBM 1102	AB027541	AY294485	DQ074967
Photobacterium damsela	AY147857	AJ249850	AF230942
Pseudomonas fluorescens	DQ178230	NC004129	NC007492
Escherichia coli K12	U00096	U00096	U00096

initial denaturation step of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR amplification of Cpn60 was performed with final reaction concentrations of 1.88 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 µM of each primer and 1 unit *Taq* polymerase (Promega). Thermocycling consisted of an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension hold at 72°C for 10 min. PCR amplification of GyrB was performed with final reaction concentrations of 1.88 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 µM of each primer and 2.5 units *Taq* polymerase (Promega). Thermocycling consisted of an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 7 min.

Sequencing was performed using the ABI Prism™ Big-Dye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems), and reactions were analysed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). The gene fragments were assembled into complete sequences in ContigExpress in the Vector NTI Advance 9.0 computer package (Invitrogen). The 16S rRNA encoding gene sequence was BLAST-searched for nucleotide-nucleotide matches in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/BLAST/) to establish initial strain identity (Altschul et al. 1990). The 16S rRNA, GyrB and Cpn60 sequences obtained were submitted to GenBank (accession nos. AM402968, AM399070 and AM399071, respectively).

DNA:DNA hybridisation. DNA:DNA hybridisation of NRP 45 and *Vibrio tapetis* type strain CECT 4600 and *V. tapetis* strain LP2 was performed by the DSMZ laboratory (Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA:DNA hybridisation was carried out as described by De Ley et al. (1970), with modifications described by Huss et al. (1983), using a model Cary 100 Bio ultraviolet-visible (UV/VIS)-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). Re-association was carried out in 2× saline sodium citrate (SSC) at 67°C, and the percentage of re-hybridisation was calculated.

Phylogenetic inference. Phylogenetic analyses of a selection of bacteria were performed, using the 16S rRNA, GyrB and Cpn60 partial gene fragments obtained from GenBank. The alignments were manually adjusted using the sequence alignment editors GeneDoc (Nicholas et al. 1997) and BIOEDIT (Hall 1999). Genetic distance calculations and phylogenetic

tree estimations under maximum parsimony (MP) and maximum likelihood (ML) were made with PAUP* (Swofford 1998). Hierarchical log-likelihood ratio testing was performed with Modeltest version 3.7 (Posada & Crandall 1998) to infer the best evolutionary model for ML estimates of phylogenetic trees (Posada & Crandall 2001). Similar procedures were followed with MrModeltest version 2.2 (Nylander 2004) in order to infer appropriate model settings and prior probability definitions for Bayesian analysis with MrBayes3 (Ronquist & Huelsenbeck 2003). The Bayesian computation was done by 2 parallel runs, each with 4 Markov chains sampling parameter states every 200 of 2 million generations in total. The 'burn-in' was performed on 19 700 trees. Phylogenetic inference was made from each gene fragment separately and additionally from a concatenated matrix of the alignments from each gene. Parsimony and ML heuristic searches were executed with 100 replicate random additions of sequences and with the TBR algorithm and MulTrees in effect (Swofford 1998). Branch support was calculated under MP with 250 bootstrap replicates. Tree graphics were made with utilities in Mesquite (Maddison & Maddison 2004) and Adobe Photoshop®.

RESULTS

Recovery of clams at Seløy. In total, 24 Manila clams were recovered at the Seløy poll (Fig. 1B), including 7 individuals in May 2003 and 17 in June 2004. The Manila clams measured 46 to 62 mm, weighed 33 to 66 g and were estimated to be 15 yr old. No juvenile Manila clams were found. A total of 12 individuals were female, 6 were males and 6 had undifferentiated gonads.

BRD diagnostics. In May 2003, the BRD prevalence in Manila clams was 71% (5 were diseased and 2 had completely recovered). In the diseased clams, the conchiolin stages varied between the individuals examined, depending on the shell repair stages (SRS); 3 had the lowest conchiolin deposit stage (CDS 1 or 2), and 2 showed extensive development of BRD signs (CDS 4 and 7). The majority of diseased clams exhibited signs of a shell repair process, and observations suggested the clams to be in various stages of this process (Fig. 2). In contrast, the clams collected in June 2004 had a lower prevalence of BRD: only 1 clam displayed morphological BRD signs (BRD prevalence = 6%), and 7 clams had recovered completely (recovery prevalence = 41%).

Isolation and characteristics of bacteria. Total heterotrophic bacterial microflora (THBM) varied between individuals: digestive gland bacterial loads ranged from 10^4 to 4.3×10^5 CFU g^{-1} while those in extrapallial fluids ranged from 1.4×10^3 to $1.4 \times$

Fig. 2. *Ruditapes philippinarum*. Manila clam from Seløy, Norway, with characteristic brown ring symptoms showing recovery (shell repair stage = 2.5) on the inner shell surface. Scale bar is in cm

Table 2. Important biochemical characteristics distinguishing the type strain CECT 4600 and strain NRP 45 by using API 20E and API 50CH. AMY: AMYgdaline; DXYL: D-XYLose

Biochemical property	Type strain CECT 4600	Strain NRP 45
Mannitol	–	+
AMY	–	+
Indole production	–	+
Cellobiose	–	+
DXYL	–	+

with mannitol, this strain was the dominant bacterium from the primary isolation on MA and was harvested after 48 h. The colony morphology on these media was similar to that of *V. tapetis* type strain CECT 4600 as both produced green (sucrose negative) colonies on TCBS media and did not grow at 30°C. The isolated strain was given the strain identity NRP 45.

Phenotypic characterisation. The NRP 45 strain showed differences in 5 biochemical properties compared to the type strain CECT 4600 (Table 2). The agglutination assay revealed agglutination in strain NRP 45 when specific antibodies (against *Vibrio tapetis* CECT 4600) were used, and no agglutination in the control antiserum.

PCR product and sequencing. Using primers targeting the genes 16S rRNA, GyrB and Cpn60, the sizes of the PCR products were approximately 1500, 1200 and 600 nucleotides (nt), respectively. Sequence comparison of the 16S rRNA-encoding gene from strain NRP 45 (AM402968) and the type strain CECT 4600 (Y08430) revealed 99.6% similarity in the 1416 nt compared. Comparison of GyrB partial gene sequences from NRP 45 (AM399070) and CECT 4600 (AM162571) revealed 94.8% similarity, with 62 substitutions in the sequence

10^6 CFU ml^{-1}. In one clam, a similar abundance of bacteria to the extrapallial fluid was found in the mantle (3×10^4 CFU g^{-1}). After using selective methods, i.e. growth on thiosulfate citrate bile sucrose (TCBS) agar, mannitol, temperature of 30°C, agglutination tests and species-specific primed (SSP)-PCR identification, only 1 strain fulfilled the criteria defined for *Vibrio tapetis*. This strain was isolated from a plating of extrapallial fluid from 1 clam collected in May 2003 showing signs of shell repair (SRS = 2.5). According to colony morphology on marine agar (MA) and MA supplemented

Table 3. Summary of phylogenetic analyses based on nucleotide sequences from 3 genes in *Vibrio* and other bacteria. The maximum parsimony (MP) score includes steps in uninformative sites. The TrN model (Tamura & Nei 1993) assumes unequal base frequencies and 3 rate categories, K80 (Kimura 1980) assumes equal base frequencies and 2 rates; SYM (symmetrical) assumes equal base frequencies and 6 rates, and GTR is the general time reversible model with unequal base frequencies and 6 rates. G means that rate heterogeneity over the sequences is modelled with the gamma function, and I additionally models invariable sites. p-dist: proportion of nucleotide sites at which 2 sequences being compared are different; ML: maximum likelihood

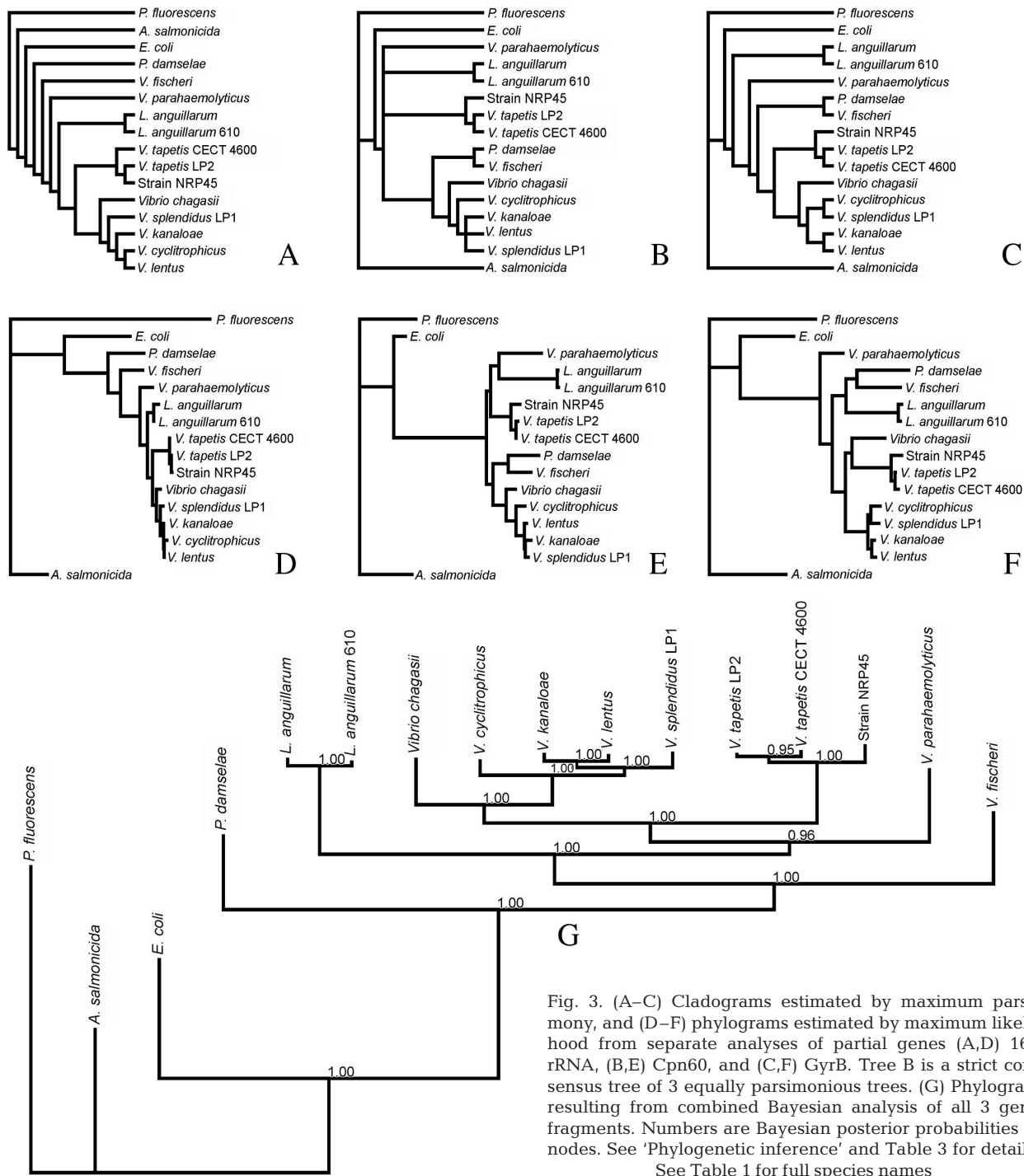|	16S rRNA	Cpn60	GyrB	All
Alignment positions	1358	483	1137	2978
Mean p-dist (variance)	0.062 (0.002)	0.180 (0.005)	0.213 (0.005)	–
Parsimony informative sites	164	170	453	787
Number of MP trees	1	3	1	3
Number of minimum steps	556	550	1641	2787
Model used in ML search	TrN+I+G	K80+G	SYM+I+G	TrN+I+G
Log likelihood score	–4533.25	–3037.52	–8248.51	–16209.42
Model used in Bayesian search	(GTR+G+I)	(SYM+G+I)	(SYM+G+I)	Mixed

Fig. 3. (A–C) Cladograms estimated by maximum parsimony, and (D–F) phylograms estimated by maximum likelihood from separate analyses of partial genes (A,D) 16S rRNA, (B,E) Cpn60, and (C,F) GyrB. Tree B is a strict consensus tree of 3 equally parsimonious trees. (G) Phylogram resulting from combined Bayesian analysis of all 3 gene fragments. Numbers are Bayesian posterior probabilities of nodes. See 'Phylogenetic inference' and Table 3 for details. See Table 1 for full species names

of 1181 nt compared. Comparison of 548 nt of the Cpn60 partial gene fragment revealed 96% similarity, where strain NRP 45 (AM399071) showed substitutions in 22 positions compared to CECT 4600 (AM162561).

Phylogenetic analysis. Separate analyses of each gene (Table 3) with MP produced slightly different tree topologies (Fig. 3A–C), but the closest phyloge-

netic relationship between NRP 45 and the *Vibrio tapetis* strains was indicated by all gene fragments with 100% bootstrap support. ML searches based on model settings and parameter settings inferred with Modeltest 3 (Table 3) returned results that were congruent with the MP tree with respect to the placement of strain NPR 45 (Fig. 3D–F).

The concatenated matrix of 3 gene fragments using ML criterion returned a tree with the same topology as the Bayesian reconstruction (Fig. 3G) with mixed models (Table 3). The Bayesian computation returned a posterior probability of 100% for NPR 45 being at the base of the *Vibrio tapetis* clade. Thus, the evidence from combined analysis of 3 genes suggests that strain NPR 45 diverged from the most recent common ancestor of the LP2 and CECT 4600 strains of *V. tapetis*.

DNA:DNA hybridisation. DNA:DNA hybridisation of strain NRP 45 and the *Vibrio tapetis* type strain CECT 4600 revealed re-hybridisation percentages of 52.7 and 57.3% in 2 replicate hybridisations. The hybridisation of NRP 45 and *V. tapetis* LP2 strain returned 46.6 and 44.4%.

DISCUSSION

We report the first identification of a *Vibrio tapetis*-like strain in Manila clams exhibiting clinical signs of BRD in Norway. In the present work, the Manila clams recovered coincided with both the original seeding in 1988 (Mortensen & Strand 2000) and the recovery in 1995. No spat or young specimens were found during our study in 2003 and 2004. Therefore, there is still no indication of an establishment of this species in the Norwegian fauna.

Macroscopical features on the inner bivalve shell surface are used as indicators of abnormal or disturbed shell growth. A brownish conchiolin deposit is a characteristic feature associated with BRD (Paillard et al. 1989). These organic deposits, which may be found both in *Ruditapes philippinarum* and *R. decussatus*, are a result of a defence reaction and are not exclusively due to the presence of the BRD causative agent *Vibrio tapetis* (Paillard 2004). Other parasites, like fungi, annelids and trematodes, also disturb the pallial edge by shell boring, irritating the epithelium or living within the mantle tissue (Paillard et al. 1994, 1996). Because of the non-specificity of the symptoms, BRD must always be verified by *V. tapetis* detection. The strain NRP 45 showed 99.6% 16S rRNA similarity to the *V. tapetis* CECT 4600 reference strain, 94.8% GyrB similarity and 96% Cpn60 similarity. Nucleotide differences in the Cpn60 fragments were all in synonymous codons when NPR 45 was compared to the *V. tapetis* strains. However, the GyrB differences affected a minimum of 5 nonsynonymous sites, of which 2 involved amino acids with similar physiochemical characteristics (D vs. E, and I vs. V). In contrast to sequence similarities, the mean result from DNA:DNA hybridisation was 55%, which is below the species threshold level of 70% suggested by Wayne et al. (1987). Thus, the strain NRP 45, although clearly related genomically and serologically, as well as with respect to important biochemical characteristics, should not be assigned to the species *V. tapetis* based on the threshold criterion. DNA:DNA hybridisation with the *V. tapetis* strain LP2, isolated from wrasse in Norway, showed an average result of approximately 45%. This further supports that NRP 45 should not be regarded as a *V. tapetis* strain. The high 16S rDNA similarity found demonstrates that this gene alone does not allow discrimination between closely related strains or species. As illustrated by the phylogenetic analysis (Fig. 3), the strain clustered within the *V. tapetis* clade and should be termed *V. tapetis*-like.

It is not known whether strains closely related to NRP 45 or *Vibrio tapetis* were already present in the environment when the clams were seeded at Seløy. BRD symptoms might have been overlooked during previous samplings, during the production in Espevik from 1989 to 1992 (Mortensen 1993). During sampling at Seløy and the nearby Vetle Godøy in 1995 (Mortensen & Strand 2000), no diagnostic tests for *V. tapetis* were carried out. Except for 1 isolation of *V. tapetis* LP2 from 1 fish species, the corkwing wrasse *Symphodus melops* (Jensen et al. 2003), this bacterium has not previously been found in Norway. Thus, the origin of *V. tapetis*–like bacteria in Seløy is not known. There are apparently at least 2 possibilities explaining its presence. One possibility is that *V. tapetis*-like strains were introduced with Manila clams in 1987. If this is the case, Espevik may represent an important site, as the centre of previous Manila clam production. Between 1988 and 1992, a large biomass of *Ruditapes philippinarum* and *R. decussatus* was present in Espevik during the hatchery and nursery operations (Mortensen 1993, Strand 1996). The nursery and a semi-recirculation system, using poll water as a food source (Strand 1996), are particularly interesting from a zoosanitary point of view. Any pathogenic agent present in clams, or other bivalve species in the nursery or in the poll, would have had time and opportunity to become established in susceptible or vector species present, or within the environment. The Manila clams from Seløy were originally hatched and nursed at this site and could therefore have been exposed to a number of bacteria, either indigenous or introduced from other areas. Following the seeding, few primary hosts for *V. tapetis*-like bacteria were available at Seløy. After seeded clams died, and since no more clams have been introduced in these waters, we presume that the availability of hosts has been low. However, *V. tapetis* might have colonised new hosts, and other clam species such as *Tapes pullastra* could potentially harbour this pathogen (Paillard et al. 1989, Maes & Paillard 1992). The cockle *Cardium edule* has been recognised as a reservoir species (Maes & Paillard 1992, Paillard

2004) and could also potentially contribute to the persistence of *V. tapetis* at the site. The other possibility is that NRP 45 represents an indigenous bacterial species, which colonises indigenous host species, such as several clam species and cockles. A coevolution process between strains like NRP 45 or *V. tapetis* and native bivalves might have occurred in many areas in Europe. The introductions of Manila clams might then have represented the exposure of a new, highly susceptible host, resulting in the described BRD outbreaks.

During the present sampling, only a few bacterial samples were taken from native *Ruditapes decussatus*, from which *Vibrio tapetis* or *V. tapetis*-like bacteria were not detected (authors′ unpubl. data). A more thorough sampling should therefore be carried out in Seløy, Espevik and at clam sites where Manila clams have not been present, in order to clarify the possible presence of *V. tapetis* and *V. tapetis*-like bacteria in the indigenous Norwegian bivalve fauna.

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