Francisella noatunensis

– Taxonomy and ecology

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Aknowlegdement

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List of Papers

Paper-I. Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossøy B (2006) *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol 5:383-92. Epub 2006 Apr 14.

Paper-II. Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Krossøy B (2007) Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). Arch Microbiol 5:343-50. Epub 2006 Dec 12.

Paper-III. Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Krossøy B, Knappskog D (2007) New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). Arch Microbiol 5:547-50. Epub 2007 Jul 10.

Paper-IV. Ottem KF, Nylund A, Isaksen TE, Karlsbakk E, Bergh Ø (2008) Occurence of *Francisella piscicida* in farmed and wild Atlantic cod *Gadus morhua* L. in Norway. J Fish Dis 7:525-34. Epub 2008 May 13.

Paper-V. Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Kamaishi T (2009) Elevation of *Francisella philomiragia* ssp. *noatunensis* Mikalsen et al. 2007 to *Francisella noatunensis* comb. nov. (syn. *Francisella piscicida* Ottem et al. 2008 syn. nov.) and characterization of *Francisella noatunensis* ssp. *orientalis* ssp. nov., two important fish pathogens. J Appl Microbiol 4: 1231-1243.

CONTENTS

INTRODUCTION

Norwegian fish-farming

The Norwegian fish-farming industry has grown substantially during the last 30-40 years from the onset as a small industry until today where it represents a multi million dollar industry. During this time of development the industry has experienced numerous problems affecting the profitability, in particular those related to fish-health. From an industry mainly producing Atlantic salmon (*S. salar*), Norwegian aquaculture are now producing several marine species, such as Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), turbot (*Psetta maxima*), blue mussel (*Mytilus edulis*), oyster (*Ostrea edulis*) and the great scallop (*Pecten maximus*). In 2008 the sale of farmed Atlantic cod was the largest of these species. When including catch-based aquaculture, it amounted to 18052 metric tons or 2.14 % out of a total of 841776 metric tons of farmed fish sold (Statistics from Norwegian Directorate of Fisheries 2009). It was expected that the production of farmed Atlantic cod should increase further in the coming years, with a prognose for 2010 in the order 150-200 000 tons (Kjesbu et al. 2006). However, in 2009 and 2010 the industry experienced a serious set-back mainly due to poor economy as a combined result of low prices and disease-problems.

Diseases of Atlantic cod

Like the production of farmed Atlantic salmon cod-aquaculture has met several obstacles, in particularly losses due to both disease and disease-management. Most of the knowledge regarding infectious disease and disease-management in the Norwegian aquaculture industry arose during 30-40 years of mainly salmonid farming, but this knowledge is also relevant for the cod-culture (Bricknell et al. 2006). Except for Betanodavirus-infections that cause Viral Nervous Necrosis (VNN) in farmed cod (Patel et al. 2007, Nylund et al. 2008), viral diseases have not been a major concern so far. But, Cod have been shown more or less susceptible to Viral

Haemorrhagic Septicaemia Virus (VHSV) and Infectious Pancreas Necrosis Virus (IPNV) (Snow et al. 2005, Garcia et al. 2006, Samuelsen et al. 2006). However, the main disease-problems in cod-farming are due to bacterial infections. Listonellosis ('classical vibriosis') caused by *Listonella anguillarum* has been an important problem for the industry (Larsen et al. 1994, Pedersen et al. 1999). The disease caused significant losses in the 80-ies in the production of cod-larvae and juveniles, and was the largest disease threat in the infancy of the industry (Samuelsen et al. 2006). All life-stages of cod can develop listonellosis, manifested as an enteritis or septicemia. In hatcheries the bacterium is associated with enteritis after the onset of start-feeding with *Brachionus* spp. and *Artemia* spp. (Korsnes et al. 2006). In vitro challenge experiment on cod larvae has resulted in acute mortality without significant pathological changes (Engelsen et al. 2008). Since cod in early stages has not developed immuno-competence, the effect of vaccination in this phase has been doubtful (Schrøder et al. 1998). Good protection due to vaccination is first obtained in cod larger than 2 grams (Schrøder et al. 2006). Hence, the phase when cod are less than 2 grams is a critical period where infections by both *L. anguillarum* and other opportunistic pathogenic bacteria are difficult to prevent. Also *Aliivibrio* spp. have been associated with enteritis and septicaemia in cod larvae and juveniles (Karlsbakk et al. 2003, Brevik 2008). *Moritella viscosa* and *Aliivibrio* spp. aff. *wodanis* are associated with ulcers (Colquhoun et al. 2004, Brevik 2008). *Aeromonas salmonicida* ssp. *achromogenes* and *Francisella* sp. infections in large cod are often chronic and show systemic granulomatous manifestations (Samuelsen et al. 2006, Paper-I). Furunculosis and listonellosis both caused problems in salmon-culture, but were successfully controlled by the employment of effective vaccines in the 90-ies (Sommerset et al. 2005). Good protection is obtained in cod vaccinated against both *L. anguillarum* serotype O2-β and atypical *A. salmonicida* bacterins (Mikkelsen et al. 2004). However, few vaccines are available for Atlantic cod in Norway and considerable work regarding vaccine-development remains, and bacterial infections are therefore controlled using antibiotics (Samuelsen et al. 2006). Hence, the annual use of antibiotics has increased with the increasing production of cod. Resistance to

some antibiotics has already been detected in some strains of *L. anguillarum* (Colquhoun et al. 2007).

Experiences from 30-40 years of expanding salmon-culture show a continuous emergence of new diseases and pathogens. Hence, our knowledge on the pathogen repertoire of the infant cod-farming industry is scant. Recent previously unrecognized pathogens include novel *Aliivibrio* spp., *Pseudomonas* spp. and Chlamydiaceae (Are Nylund Pers. Com.). There is also a range of lesions and syndromes with unknown causes, occasionally associated with significant disease, such as lateral-line-necrosis, pseudobranchial tumours, and gill cysts of unknown etiology ('CUE's'). The most serious threat to the cod-farming in Norway is a recently described disease characterized by extensive chronic inflammation and granuloma formation associated with intracellular bacteria belonging in the genus *Francisella* (Paper-I, Nylund et al. 2005, Olsen et al. 2006).

Francisella spp. in fish

Several cases of diseased fish showing extensive chronic inflammation and granuloma formation have been described, associated with organisms referred to as *Rickettsia*-Like-Organisms (RLO`s) (Chen et al. 1994, Chern et al. 1994, Fryer & Mauel 1997). This commonly used term was coined by Mohamed (1939) due to the intracellular localization of the associated bacteria and their morphological resemblance to *Rickettsia* spp. (Fryer & Mauel 1997). However, most of these RLO`s can today be identified as either *Piscericketsia salmonis* (Fryer et al. 1990, Cvitanich et al. 1991, Fryer et al. 1992) or bacteria belonging in the genus *Francisella* (Kamaishi et al. 2005, Nylund et al. 2005, Hsieh et al. 2006, Birckbeck et al. 2007, Paper-I, Olsen et al. 2006, Mauel et al. 2007). The genera *Piscirickettsia* and *Francisella* belong within the γ-proteobacteria (Fryer et al. 1992, Forsman et al. 1994, Sjøstedt 2005a), while the true *Rickettsia* spp. belongs within the α-proteobacteria (Mauel et al. 1999). There are some records of RLO`s of which the clinical

significance and aetiology is still not known; in puffers (*Tetraodon lineatus*) (syn. *T. fahaka*) from the Nile River in Egypt (Mohamed 1939), in cultured rainbow trout (*Onchorhyncus mykiss*) from Germany (Ozel and Schwanz-Pfitzner 1975), in dragonets (*Callionymus lyra*) caught of the coast of Wales (Davies 1986) and from blue-eyed panaque (*Panaque suttonarum*) from Colombia (Khoo et al. 1995). However, *P. salmonis* and the *Francisella* spp. are the most significant RLO's in fish-aquaculture. *Piscirickettsia salmonis* cause the serious disease piscirickettsiosis (also called Salmonid Rickettsial Syndrome, SRS), usually with a chronic granulomatous pathology (Fryer et al. 1990, Cvitanich et al. 1991, Garcés et al. 1991, Fryer et al. 1992). The bacterium have been identified in numerous fish species worldwide (Comps et al. 1996, Fryer and Mauel 1997a, Olsen et al. 1997, Athanassopoulou et al. 1999, Chen et al. 2000, Arkush et al. 2005, McCarthy et al. 2005, Corbeil al. 2005). Infections in fish by bacteria belonging to the genus *Francisella* was first recognized in association with extensive granulomatous inflammatory diseases in their hosts (Kamaishi et al. 2005, Nylund et al. 2005, Ostland et al. 2006, Mauel et al. 2007). Infected fish usually displayed few external signs of disease, but skin haemorrhages and ulcers may occur (Alfjorden et al. 2006, Nylund et al. 2006). Despite different host species and environments the internal pathology associated with francisellosis in fish is similar and characteristic; white foci or nodules in the kidney, spleen and sometimes liver (figure 1) (Paper-I, Fukuda et al. 2002, Kamaishi et al. 2005, Hsieh et al. 2006). These nodules consisted of granulomas with macrophages containing bacteria, or in advanced lesions with necrosis and few or no bacteria present. The organs particularly perfused by granulomas were the head-kidney and spleen, which at the extreme could become almost 10 times larger than normal (Ostland et al. 2006). Granulomas in other organs are less frequent (Hsieh et al. 2006). Mortalities associated with francisellosis-outbreaks in farms vary from a few to 40 % (Kamaishi et al. 2005, Olsen et al. 2006, Ostland et al. 2006).

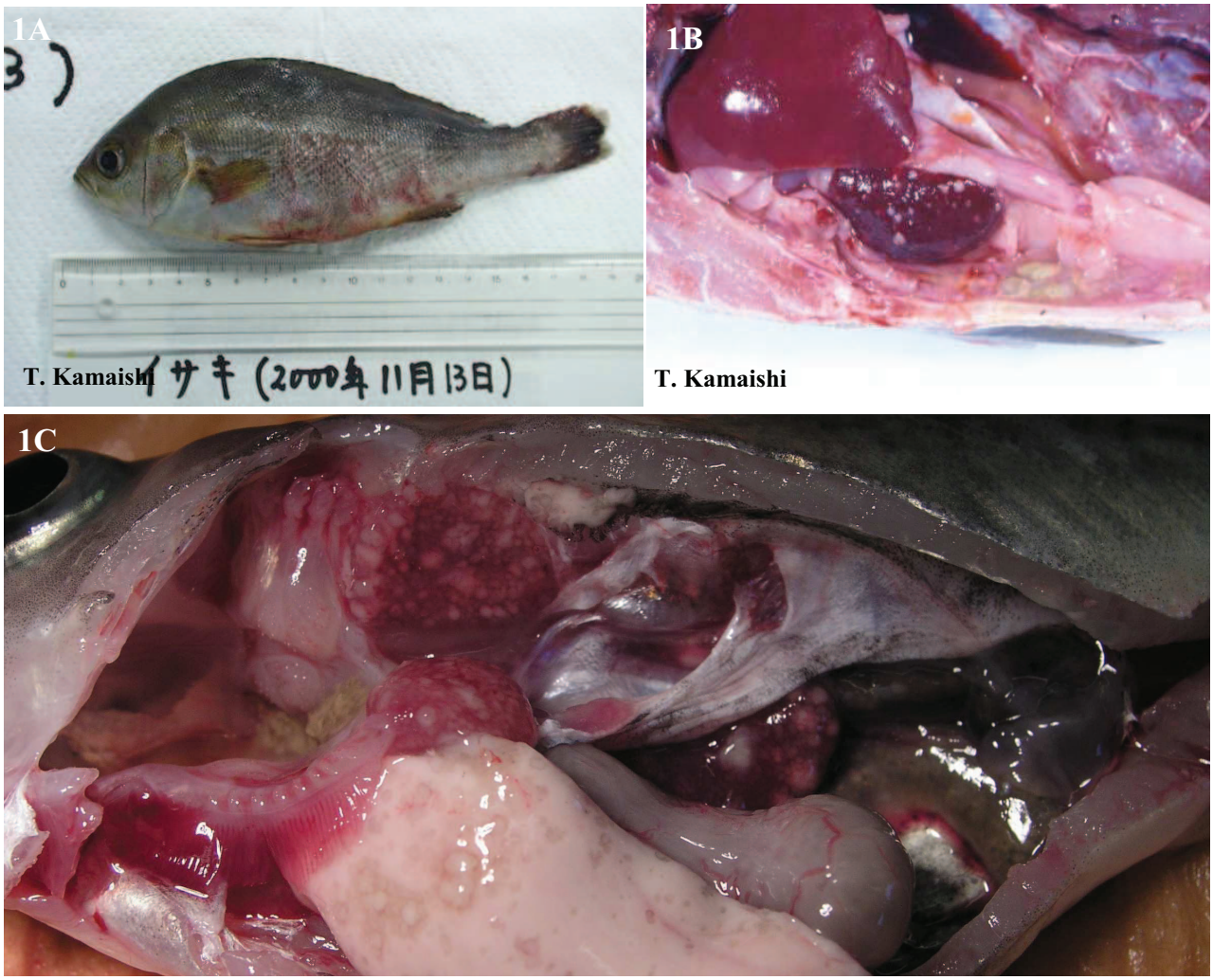

Figure 1. A) and B) Three-line grunt (*Parapristipoma trilineatum*) infected with *Francisella* sp. B, granulomas in spleen (pictures reproduced with the permission of T. Kamaishi) C) Atlantic cod infected with a *Francisella* sp. detected in 2004. Typical internal lesions with granulomas in kidney (svollen), spleen and liver.

It is now clear that francisellosis in fish are caused by three distinguishable lineages of *Francisella* bacteria, currently assigned to two subspecies; *F. noatunensis* ssp. *noatunensis* (= *F. piscicida*) present in Norway, a distinct *F. noatunensis* ssp. *noatunensis* strain in Chile and *F. noatunensis* ssp. *orientalis* originally found only in Asia and America (Paper-II, Paper-III, Paper-V, Kamaishi et al. 2005, Birckbeck et al. 2007, Mikalsen 2008). These bacteria have been detected in numerous species of fish using a variety of detection-methods (summarized in table 1).

Table 1. Table shows in which fish-species *F. noatunensis* sspp. *noatunensis* and *orientalis* have been identified or detected, the tools used and geographical origin of fish-species.

Francisella spp.	Fish-species	Geographic origin of fish-species	Method of detection	References
F. noatunensis ssp. *noatunensis*	Atlantic salmon (*Salmo salar*)	Chile	Histopathology , PCR	Birkbeck et al. 2007
„	„	Norway	Real-time PCR, PCR, Sequencing	Paper-III, Paper-IV
„	Atlantic cod (*Gadus morhua*)	Norway, Denmark, Sweden, North Sea	Histopathology , PCR, Real-time PCR, Immunohistochemistry	Paper-I, Paper-III, Paper-IV, Karlsbakk et al. 2010 (current study figure 3), Alfjorden et al. 2006, Olsen et al. 2006, Zerihoun et al. 2008
F. noatunensis ssp. *orientalis*	firebird (*Aulonocara rubescens*)	Taiwan	Histopathology, In situ hybridization	Hsieh et al. 2007
„	elegans (*Pseudothropheus elegans*)	„	Histopathology, In situ hybridization, PCR	„
„	zebra (*Pseudothropheus zebra*)	„	„	„
„	bream (*Chilotilapia rhoadesii*)	„	Histopathology, In situ hybridization	„
„	Malawi eyebiter (*Dimidiochromis compressiceps*)	„	„	„
„	blue discus (*Symphysodon aequifasciata*)	„	„	„
„	deep-water hap (*Haplochromis electra*)	„	Histopathology, In situ hybridization, PCR	„
„	electric blue hap (*Sciaenochromis fryeri*)	„	„	„
„	blue streak hap (*Labidochromis caeruleus*)	„	„	„
„	super VC-10 hap (*Placidochromis milomo*)	„	„	„
„	humphead cichlid (*Cyphotilapia frontosa*)	„	„	„
”/*F. victoria*/*F. asiatica*	Nile tilapia (*Oreochromis niloticus*)	Taiwan, Costa Rica, Indonesia, Scotland	Histopathology, In situ hybridization, PCR, Sequencing	Hsieh et al. 2006, Kay et al. 2006, Mauel et al. 2007, Paper-V, Soto et al. 2009a, Jeffery et al. 2010
„	Mozambique tilapia (*O. mossambicus*)	Taiwan	”	Hsieh et al. 2006
„	three-line grunt (*Parapristipoma trilineatum*)	Japan	Histopathology , PCR	Kamaishi et al. 2005
„	hybrid striped bass (*Morone chrysops* x *M. saxatilis*)	USA	”	Ostland et al. 2006

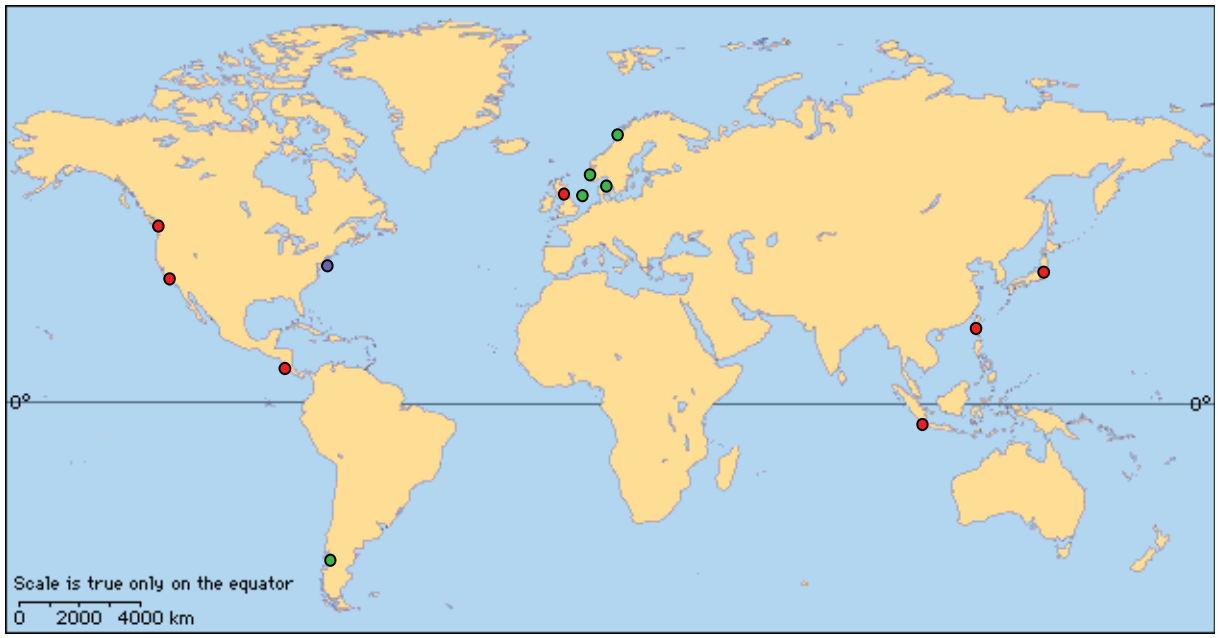

Figure 2. Worldwide distribution of fish pathogenic *Francisella* (red dots = *F. noatunensis* ssp. *orientalis,* green dots = *F. noatunensis* spp. *noatunensis* and blue dot = a partial *Francisella* 16S rRNA sequence-clone aff. *F. noatunensis* ssp. *noatunensis*).

Infections now recognized as *Francisella noatunensis* ssp. *orientalis* was first observed in tilapia (*Oreochromis* spp.) from Taiwan (as RLO's) (Chern et al. 1994, Chen et al. 2000), but has since been detected in several other species of perciform fish (Kamaishi et al. 2005, Hiseh et al. 2006, Ostland et al. 2006, Hsieh et al. 2007, Mauel et al. 2007, Soto et al. 2009a). Geographically these records occur over a large area on both sides of the Pacific Ocean (figure 2) and in both salt-, brackish-, and fresh-water environments. An investigation of old slides also retrospectively identifies the bacterium in several ornamental cichlids in Taiwan (Hsieh et al. 2007).

The known distribution and host-specter of *F. noatunensis* ssp. *noatunensis* is more restricted. Most records of the bacterium are from farmed cod suffering from francisellosis, but infections have also been detected in wild cod populations (Paper-III, Paper-IV, Alfjorden 2006, Zerihoun et al. 2008, Alfjorden et al. 2009, Karlsbakk et al. 2010). The presence of the bacterium has retrospectively been demonstrated in wild cod from the southern North Sea (van Banning 1987 cf. Zerihoun et al. 2008), and in farmed and wild cod from the coast of Norway (figure 3).

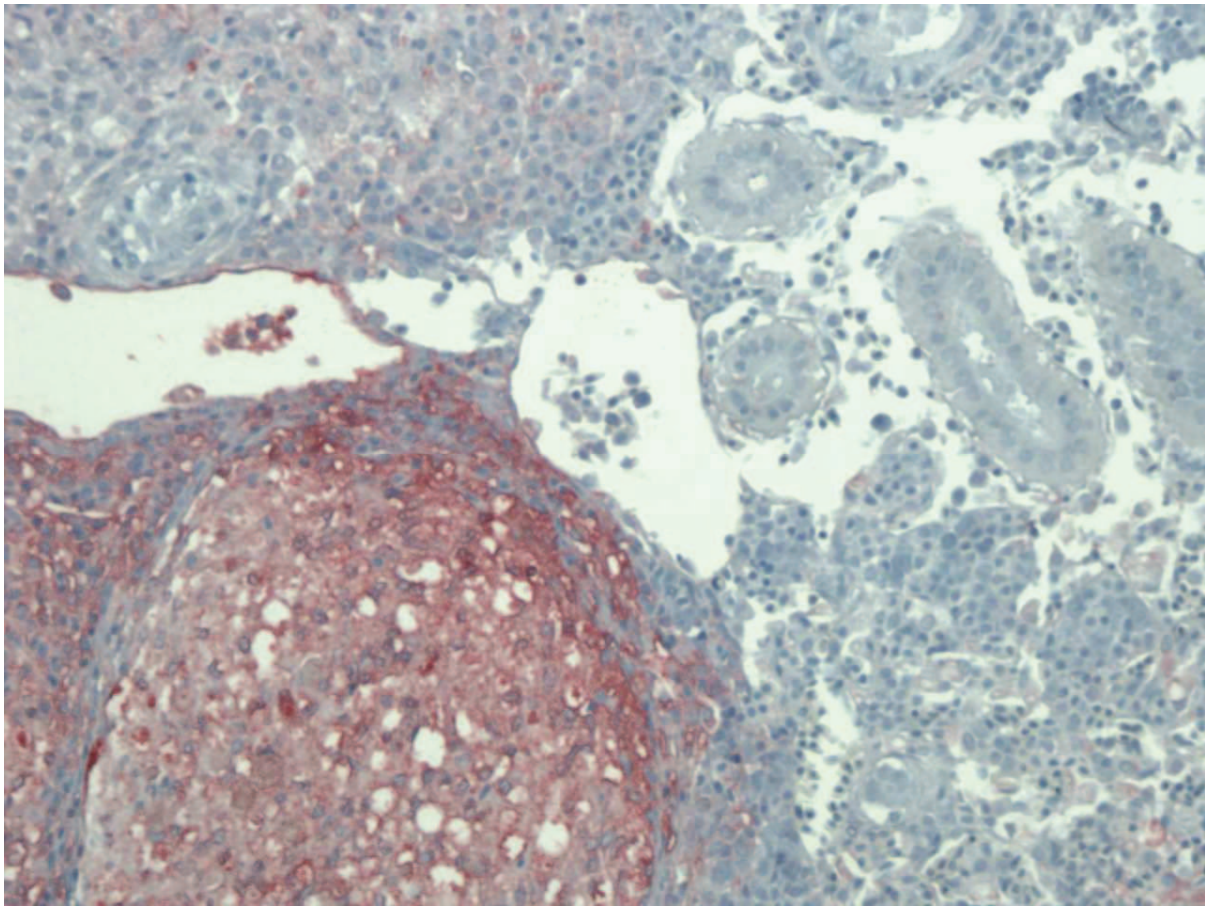

Figure 3. Atlantic cod (*G. morhua*) kidney section with granuloma (Karlsbakk et al. 2010). Immunohistochemical staining of a paraphine section from a farmed cod (Hordaland) with granulomatous disease from 1988. Staining was performed using the Avidine-biotin-alkaline phosphatase method with the rabbit anti *Francisella noatunensis* ssp. *noatunensis* (=*F. piscicida*) GM2212 antisera from Paper-II as the primary antiserum. Positive staining is evident as red coloration and indicates presence of *F. noatunensis* ssp. *noatunensis*.

Cod is also the only species of fish in Norway from which the bacterium has been isolated (Paper-I, Paper-III, Nylund et al. 2005, Olsen et al. 2006). All of these observations suggest that cod is the main host for *F. noatunensis* ssp. *noatunensis* along the Norwegian south coast, and in the Skagerakk and North-Sea areas. Several species of fish and some invertebrates were also weakly positive using the Real-time PCR assay Fc50 which only detects *F. noatunensis* among the known *Francisella* spp. (Paper-IV). A lineage of *F. noatunensis* ssp. *noatunensis* is also present in Chile (table 2, Birckbeck et al. 2007). This lineage has not been identified from fish other than Atlantic salmon parr from Chile (Birckbeck et al. 2007, Bohle et al. 2009).

The ecology of *Francisella* spp.

Prior to the identification of the fish-pathogenic *Francisella* spp., the genus *Francisella* comprised three widely recognized species; *F. tularensis*, *F. philomiragia* and *F. novicida. Francisella tularensis* is usually considered to comprise three subspecies, *F. tularensis* sspp., *tularensis*, *holarctica*, and *mediasiatica.* The former, *F. tularensis* ssp. *tularensis*, is found mainly in North-America and is considered the most virulent for humans and animals (Farlow et al. 2005). A few isolates of *F. tularensis* isolated from ticks and fleas in Europe also show biochemical properties similar to that of *F. tularensis* ssp. *tularensis* (Gurycova 1998). *F. tularensis* ssp. *tularensis* infects mammals including humans with an often fatal outcome. Ticks and biting flies are important vectors for the *F. tularensis* ssp. *tularensis*. There is evidence for two phylogroups of *F. tularensis* ssp. *tularensis*, AI and AII, as suggested by Multiple-Locus Variable-Number Tandem Repeat analysis (Farlow et al. 2001). These phylogroups show distinct geographical distributions matching seemingly well with that of both hosts and vectors (Farlow et al. 2005). The distribution of the *F. tularensis* ssp. *tularensis* AI group closely matches the southern, central and eastern distribution of the American dog tick (*Dermacentor variabilis*), the lone star tick (*Amblyoma americium*) and the eastern cottontail rabbit (*Sylvilagus floridanus*). In contrast, the *F. tularensis* ssp. *tularensis* AII group seems to match the more western, north-central and northern distribution of the Rocky Mountain Wood Tick (*Dermacentor andersoni*), the deer fly (*Chrysops discalis*) and the mountain cottontail rabbit (*Sylvilagus nuttalli*) (Farlow et al. 2005). *Francisella tularensis* ssp. *holarctica* on the other hand, is distributed in a larger geographic area, throughout North America and the whole of Eurasia. Unlike *F. tularensis* ssp. *tularensis*, *F. tularensis* ssp. *holarctica* is only moderately virulent for humans and animals and infections rarely result in fatalities. In comparison to *F. tularensis* ssp. *tularensis,* isolates of ssp. *holarctica* are more homogenic (genetically) despite being distributed in a larger geographic area (Larsson et al. 2004, Farlow et al. 2005). In North-America common hosts are voles (*Microtus* spp.), American beavers (*Castor canadensis*) and muskrats (*Ondatra zibethicus*), while in Europe and Asia hares

(genus *Lepus*), and voles are known hosts (Bell 1980). Blood feeding arthropods such as ticks (genera *Ixodes* and *Dermacentor*), mosquitoes (genera *Aedes*, *Culex* and *Anopheles*) and biting flies (*Chrysops* and *Tabanus* spp., *Haematopota pluvialis*) have been shown to be involved in transmission of *holarctica* (Petersen et al. 2008). Both *F. tularensis* sspp. *tularensis* and *holarctica* appear to be associated with lakes, streams and rivers where infection in humans may occur after contact with or by ingestion of contaminated water (Karphoff et al. 1936, Greco et al. 1987). Most often the sources of such infections are carcasses of rodents or lagomorphs in or near the water (Hoel et al. 1991, Reintjes et al. 2000, Anda et al. 2001). In addition *F. tularensis* sspp. *tularensis* and *holarctica* may show prolonged survival in protists acting as reservoirs (Berdal et al. 1996, Abd et al. 2003, Thelaus et al. 2008). The bacterial cells may also show extended survival in the environment in a viable but non-culturable state (VBNC) (Forsman et al. 2000), the Gram-negative bacterial equivalent to a Gram-postive bacterial spore (Colwell 2009). Considering the apparent link between *F. tularensis* and aquatic environments, fish and amphibians have been considered likely reservoirs aswell (Parker et al. 1951), despite challenge experiments suggests otherwise (Morgan 1946). Black bullhead (*Ameirus melas*), black crappie (*Pomoxis nigro-maculatus*), large mouth bass (*Huro salmoides*), northern pike (*Esox lucius*), yellow perch (*Perca flavescens*) and rainbow trout (*O. mykiss*) did not develop disease after challenge by a highly virulent variant of *F. tularensis* (Morgan 1946). It was not possible to re-isolate *F. tularensis* from any of these fish, or induce disease in guinea pigs injected with tissues from challenged fish (Morgan 1946). These studies show that *F. tularensis* cannot proliferate in these species. However, since *F. tularensis* may persist in protists in aquatic environments (Berdal et al. 1996, Abd et al. 2003, Thelaus et al. 2008) it cannot be excluded that protists on the surface of fish may be involved in infecting animals with *F. tularensis* which come into contact with them (Miller 1939). Fish may thereby act as indirect reservoirs for *F. tularensis* as substrats for infected amoeba or other protists.

In comparison to the relative wide distribution of *F. tularensis* sspp. *tularensis* and *holarctica,* and their clinical significance for humans, *F. tularensis* ssp. *mediasiatica*

and *F. novicida* have a more restricted distribution and are clinically less significant. *F. tularensis* ssp. *mediasiatica* is only recovered sporadically from ticks and animals and seems confined to deltas and valleys of Amu-Darya and Tshu rivers in republics of the former USSR in Central Asia (Olsufjev & Meshcheryakova 1983). Both *F. novicida* found in North-America and a *novicida*-like bacterium from Australia are associated with water environments and rarely result in human infections (Whipp et al. 2003).

In contrast to all the knowledge that is available for *F. tularensis,* relatively little is known on the ecology and distribution of *F. philomiragia*. Only a few isolates of the bacterium exist, chiefly from North-America (Wenger et al. 1989). However, there are some isolates from Europe (Friis-Møller et al. 2004, Mikalsen et al. 2007) and a single *F. philomiragia*-like isolate from Australia (Whipp et al. 2003). *Francisella philomiragia* has been found in voles and water rats and there is an apparent connection to diverse saline-, brackish- and sea-water environments (Jensen et al. 1969, Hollis et al. 1989, Berrada et al. 2009). *F. philomiragia* appears more resistant than *F. tularensis* to grazing by protists (Thelaus et al. 2008) which suggests that the *F. philomiragia* is better adapted to survive in aquatic environments without a host than *F. tularensis*. While infections with *F. tularensis* are not restricted to any particular risk groups in the population, infections with *F. philomiragia* seems to occur only in immuno-supressed individuals (Seger et al. 1982, Wenger et al. 1989, Friis-Møller et al. 2004, Mailman et al. 2005). Infections with *F. philomiragia* are considered to be serious even though rarely leading to a fatal outcome, indicating that the bacterium is an opportunist rather than a primary pathogen.

In addition to these three recognized species a number of uncharacterized *Francisella* spp. has been discovered during the last two decades in environmental samples and as endosymbionts in ticks, but very little is known about their biology (Niebylski et al. 1997, Noda et al. 1997, Scoles 2004, Barns et al. 2005, Venzal et al. 2008). The fastidious nature of many *Francisella* spp. may cause some challenges when trying to isolate them (Petersen et al. 2004). This is the case with many of the *Francisella* spp.

detected in environmental sources and in ticks. Most of these have not been obtained in pure culture and hence, are poorly characterized. Endosymbiotic *Francisella* bacteria have been detected in a range of ticks from most continents; in North-America (Scoles 2004, Goethert & Telford 2005), South-America (Venzal et al. 2008, Machado-Ferreira et al. 2009), Africa (Nylund & Ottem, unpublished results) and Europe (Sréter-Lancz et al. 2008). It is currently not known if these bacteria have any clinical relevance for humans, but their presence in ticks that are known vectors of *F. tularensis* may give false positives in PCR diagnostics of *F. tularensis* (Kügeler et al. 2005). The distribution of *Francisella*-like bacteria in soil and water samples are poorly known and remains to be elucidated since investigations performed so far only have covered limited geographic areas (Barns et al. 2005, Kügeler et al. 2005, Petersen et al. 2009). One such area with *F. tularensis* epizootics is Martha`s Vineyard (Berrada et al. 2009). Berrada et al. (2009) have also detected *F. philomiragia*–like bacteria in their environmental samples from Martha`s Vineyard. Previously unrecognized *Francisella* spp. have also been directly isolated from seawater (Petersen et al. 2009), which indicate that environmental *Francisella* spp. may be more abundant than previously shown (Barns et al. 2005). A new *Francisella* sp. was also recently identified and isolated from diseased edible sea-snails (*Abalone* spp.) from Japan (Kamaishi et al. 2010).

Transmission and reservoirs of *F. noatunensis*

The association of *Franciella* spp. to aquatic environments with regards to source of infection, transmission and reservoirs is well established (Karphoff et al. 1936, Parker et al. 1940, Jellison et al. 1942, Jellison et al. 1950, Forsman et al. 1990, Berdal et al. 1996, Whipp et al. 2003, Wenger et al. 1989, Friis-Møller et al. 2004, Mailman et al. 2005). However, as jugded by field observations but also as shown in numerous challenge experiments *F. noatunensis* seem to be the only member within the genus able to induce disease in fish (Kamaishi et al. 2005, Paper-I, Ostland et al. 2006, Mikalsen et al. 2009, Kamaishi et al. 2010). Several of these challenge experiments

show the bacterium is transmitted directly between fish (Paper-I, Mikalsen et al. 2009). Whether *F. noatunensis* can proliferate in aquatic environments outside of their hosts is poorly known. Like *F. tularensis*, *F. noatunensis* have been demonstrated to enter a viable but none-culturable state (VBNC) after a few weeks in both freshwater and seawater microcosms (Wangen 2009, Duodu & Colquhoun 2010). However, as the VBNC state of *F. noatunensis* could not be resuscitated in cod (Duodu & Colquhoun 2010) the bacterium may have to be activated before becoming infective. Using a *F. noatunensis* specific real-time PCR assay, positive signal was seen in tissue samples from fish, blue mussels and crabs (Paper-IV). Hence *F. noatunensis* may be present in blue-mussels and crabs collected near cod-farms during a francisellosis outbreak (Paper-IV). Blue mussels filter bacteria from the water including *F. noatunensis* subsp. *noatunensis* (Wangen 2009). Bacteria shed to the environment from the diseased cod may therefore be accumulated in mussels nearby. Crabs feed on blue mussels and hence may be contaminated with *F. noatunensis* in their digestive glands. The bacterium is also accumulated in symbiotic ciliates associated with blue-mussels exposed to the bacterium (Wangen 2009). These observations raise the possibility that survival of *F. noatunensis* outside a fish-host may involve residence in phagosomes of bacterivores and transfer in the food-web. This aspect of the *F. noatunensis* life cycle needs further studies. Observations so far strongly suggest that certain fish species are the the primary hosts and main reservoirs for *F. noatunensis* sspp. Asymptomatic carrier wild and farmed populations of fish likely represent reservoirs (Paper-IV). It has been suggested that farmed asymptomatic carrier fish or ornamental fish could play a role in the spread of both subspecies of *F. noatunensis* to areas where they are not endemic. Tilapias (*Oreochromis* spp.), some of the most important hosts of *F. noatunensis* ssp. *orientalis,* have been introduced several times from Africa into Asia and the Pacific; to Indonesia, Taiwan, China, Japan, Thailand and Fiji (De Silva et al. 2004). The presence of *F. noatunensis* ssp. *orientalis* in several of these countries (Kamaishi et al. 2005, Hsieh et al. 2006, Paper-V), may be the result of the spreading of its host. Also, asymptomatic farmed cod carrying *F. noatunensis* ssp. *noatunensis* transported

from South-western to Northern parts of Norway are believed to explain the presence of the bacterium in this area (Paper-IV). Asymptomatic carrier broodfish could also have implications for vertical transmission. Using real-time PCR, broodfish of cod have been found as asymptomatic carriers of *F. noatunensis ,* and the bacterium have been detected in egg-batches from these (Pers Obs, Unpublished results). Although it has not been demonstrated that vertical transmission of *F. noatunensis* occur, the possiblity should not be excluded since this has been demonstrated for other members within the genus *Francisella.* The presence of the dog tick (*Dermacentor variabilis*)-*Francisella* (DVF) in larval progeny of this tick is considered due to transovarial transmission (Goethert & Telford 2005).

Francisella spp. and host-cell interactions

Primary target cells for the known *Francisella* spp. in vertebrates are phagocytes (Proctor et al. 1975, Löfgren et al. 1983, Anthony et al. 1991, Paper-I, Vojtech et al. 2008) and epithelial and dendritic cells (Paper-I, Hall et al. 2007, Kamaishi et al. 2010). Phagocytes are important in the initial control of infections by internalizing the pathogenic agents and in the forming of phagolysosomes which eventually degrade the content. Intracellular bacteria including members of genus *Francisella* have evolved ways to evade this phagolysosomal degradation (Golovliov et al. 2003, Clemens et al. 2004, Santic et al. 2005a). After engulfment by host-cells the phagosomal membrane which initially surrounds the *Francisella*-bacterium are disrupted followed by gradual escape of the bacterium from the phagolysosom into the cytosol, where they may multiply (Golovliov et al. 2003, Clemens et al. 2004, Santic et al. 2005a). The events leading to the escape into the cytosol seem to be identical for *F. tularensis* sspp. *tularensis*, *holarctica* and *F. novicida*, and an important part of this mechanism are the IglC protein and its regulator MglA (Santic et al. 2005b). These genes are located in a 30 kilo base (Kb) Pathogenicity Island which have been detected in *F. tularensis* (Nano et al. 2004, Santic et al. 2005b), in *F. philomiragia* and *F. noatunensis* ssp. *noatunensis* (GenBank records EU489821 and

EU492905) and *F. noatunensis* ssp. *orientalis* (Soto et al. 2009b). The latter contribution clearly showed the importance of the IglC-protein in the pathogenicity of the bacterium *F. noatunensis* ssp. *orientalis* in tilapia (Soto et al. 2009b). A homologue of the IglC-gene has also been detected in a *Francisella*-endosymbiont from a Brazilian tick (Machado-Ferreira et al. 2009). Both the IglC- and MglA-proteins also seems to contribute to the intracellular survival of *F. tularensis* within *Acanthamoebae castellanii* (Lauriano et al. 2004), and are required for replication in mosquito cells (Read et al. 2008).

Taxonomy of genus *Francisella*

Considerable scientific research has focused on *Francisella* ecology and virulence, but there have been surprisingly few studies on the taxonomy of the genus. The *Francisella* taxonomy has been a complex and an ever changing issue since McCoy and Chapin first described *Bacterium tularense* in 1912 (McCoy and Chapin 1912). The bacterium was later placed in the genera *Pasteurella* and *Brucella* (Bergey et al. 1923, Topley & Wilson 1929) until Dorofeev in 1947 finally transferred the bacterium to a new genus, *Francisella* (named after Edward Francis), as *F. tularensis* (Dorofeev 1947). This name was validly published by Skerman et al. (1980). After studying more strains Olsufjev et al. (1959) suggested that several variants or biovars of the bacterium existed. Based on differences in a few characters, notably citrulline ureidase activity, fermentation of glycerol, susceptibility to erythromycin and differences in virulence for mammals (rabbits and humans) Olsufjev et al. 1959 designated these *F. tularensis* vars. *nearctica* and *palaeartica*. These correspond to *F. tularensis* sspp. *tularensis* and *holarctica* (Olsufjev 1970, Olsufjev & Meshcheryakova 1983). Based on slight variations from *F. tularensis* sspp. *tularensis* and *holarctica* in phenotypic and biochemical characteristics, Aikimbaev (1966) designated strains of *F. tularensis* only found in the Central Asian area as *F. tularensis* ssp. *mediasiatica* (see also Olsufjev & Meshcheryakova 1983). Furthermore, on the basis of variation within *F. tularensis* ssp. *holarctica* in

erythromycin resistance and geographical distribution, Olsufjev & Meshcheryakova (1983) suggested that isolates of this subspecies could be designated as the variants; I) Erythromycin susceptible (Ery^s), II) Erythromycin resistant (Ery^r) and III) japonica (strains distributed in the Japanese islands) (Olsufjev & Meshcheryakova 1983). Such a division have not been validly published and is consequently not on the approved list of bacterial names. The bacterium *Pasteurella novicida,* initially isolated from water in Utah (Larson et al. 1955), was established as a species of the genus *Francisella*, *F. novicida,* after comparisons to *F. tularensis* ssp. *tularensis* (Olsufjev 1959, Owen et al. 1964). Hollis et al. (1989) also proposed that *F. novicida* should represent a biogroup of *F. tularensis,* and currently the bacterium is considered to be a subspecies *F. tularensis* ssp. *novicida* rather than a separate species (Sjøstedt 2005a, Rohmer et al. 2007, Hüber et al. 2009). Although the status of *F. novicida* as a subspecies of *F. tularensis* has been in use for a long time, the status was not formerly proposed until more recently (Hüber et al. 2009). In this contribution, the name *F. novicida* will be used. The bacterium named *Yersinia philomiragia*, isolated from water and from a dying muskrat (*Ondatra zibethica*) in Utah (Jensen et al. 1969), was included in the genus *Francisella* by Hollis et al. (1989) due to similarities in biochemical and phenotypic characters, following DNA-DNA hybridizations with *F. tularensis* and *F. novicida* (Hollis et al. 1989). An overview of names and the characteristics of the members of genus *Francisella* as presented in Sjøstedt (2005a) is shown in table 2. The latest characterized members of the the genus are *F. noatunensis* subsp. *noatunensis* (=*F. piscicida*) (Mikalsen et al. 2007, Paper-I, -II, -III, -V), *F. noatunensis* subsp. *orientalis* (Paper-V, Euzeby 2009), *Candidatus* F. noatunensis ssp. endociliophora (Schrallhammer et al. 2010) and *F. hispaniensis* (Hüber et al. 2009). In addition there are some uncharacterized novel *Francisella* spp. isolated from water (Petersen et al. 2009) and a *Francisella* sp. (Shimane-1) pathogenic to edible sea-snails (*Abalone* spp.) (Kamaishi et al. 2010).

Table 2. Physiological and biochemical characteristics of the members of genus *Francisella* as presented in Sjøstedt 2005a; 1 *F. tularensis* ssp. *tularensis*; 2 *F. tularensis* ssp. *holarctica*; 3 *F. tularensis* ssp. *mediasiatica*; 4 *F. novicida* and 5 *F. philomiragia.*

Characteristic	1	2	3	4	5
Size	<0.5µm	<0.5µm	<0.5µm	<1.5µm	<1.5µm
Capsule	Y	Y	Y	N	Nt
Gram Stain	W	W	W	W	W
Growth on MacConkey agar	-	-	-	W	W
Cysteine required for growth	+	+	+	-	-
H_2S production in cysteine-supplemented medium	+	+	+	+	+
B-lactamase	+	+	-	+	+
Acid production from:					
Maltose	+	+	-	W	+
Lactose	-	-	-	-	-
Sucrose	-	-	-	+	+
D-Glucose	+	+	-	+	W
Glycerol	+	-	+	W	-
Citrulline ureidase pathway	+	-	+	+	Nt
Agglutination of *F. tularensis* antiserum	+	+	+	W	-
Presence of *F. tularensis* 17-kDa lipoprotein	+	+	+	+	+
Mol% G + C of DNA	33-36	33-36	33-36	34	33-34
Aerobic, microaerophilic	+	+	+	+	+
CO_2 enhances growth	+	+	+	+	+
Optimum temperature, °C	37	37	37	37	25or37*
Growth in nutrient broth 0% NaCl	-	-	-	-	-
Growth in nutrient broth 6% NaCl	-	-	-	W	W
Catalase activity	W	W	W	W	W
Oxidase ”	-	-	-	-	+
Indole production	-	-	-	-	+
Urease activity	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
H_2S slant, Triple Sugar Iron	-	-	-	-	+
Gelantin hydrolysis	-	-	-	-	+
Motility	-	-	-	-	-
LD50 rabbits	$<10^1$	$<10^6$	$<10^6$	$<10^6$	Nt
Median infectious dose for mice, $<10^3$	+	+	+	-	Nt

* As presented in Sjøsted 2005a + = Positive, - = Negative, N = No, Nt = Not tested, W = Weak positive, Y = Yes

Phylogenetic relationship among *Francisella* spp.

The distinction of species within the *Francisella* genus was mainly based on phenotypic- and biochemical characteristics (table 2), and rarely the geographical origin of strains, e.g. *F. tularensis* ssp. *mediasiatica*. However, in the last decades gene-sequences are being used to discriminate between species and subspecies within the genus (Forsman et al. 1990, Forsman et al. 1994, Farlow et al. 2001, Johansson et al. 2001, Johansson et al. 2004, Nübel et al. 2006). According to analysis of 16S rRNA gene-sequences, *F. tularensis* and members of the genus *Francisella* are placed within the γ-subclass of the proteobacteria representing a separate lineage (Forsman et al. 1994, Sjøstedt 2005a). The closest related genera are the genera *Fangia, Caedibacter* and *Piscirickettsia* see Lau et al. (2007). In a Bayesian phylogenetic analysis using 16S rRNA gene-sequences from a number of *Francisella* spp. and sequence-isolates (figure 4), there are evidences of several clades represented by the roman numericals ranging from I to XX. These clades can be diveded into two main lineages ML-1 (clades I-IX) and ML-2 (clades X-XX). Basal in ML-1 is a well supported branch leading to a group containing; 1) clade IX with sequence-isolates from soil (Barns et al. 2005). 2) clade VIII with two *Francisella* sp. isolates from a human clinical infection (Kügeler et al. 2008), and a *Francisella* sp. isolated from an air condition system (GenBank: FJ591095). Immediately above is another well supported branch leading to a group consisting of; 1) clade VII with two *Francisella* sp. isolates from diseased *Abalone* spp. (Kamaishi et al. 2010) and *Candidatus* F. noatunensis ssp. endociliophora (Schrallhammer et al. 2010). 2) clade VI a sequence-isolate from soil (Barns et al. 2005) and clade V with two novel *Francisella* spp. isolated from sea-water (Petersen et al. 2009). 3) clade IV with numerous sequence-isolates from soil (Barns et al. 2005). Finally there is a well supported branch leading to a group consisting of; 1) clade III with isolates of the species *F. philomiragia* (Jensen et al. 1969, Hollis et al. 1989) and a number of sequence-isolates from water-samples (Berrada et al. 2009). 2) clade II with isolates of the species *F. noatunensis* ssp. *noatunensis* (=*F. piscicida*) (Paper-I, -II, -III, -V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009) and a sequence-isolate from sea-water (Berrada et al. 2009). 3)

clade I with isolates of *F. noatunensis* ssp. *orientalis* (Paper-V). Basal in ML-2 is XX '*Wolbachia persica',* (Suitor & Weiss 1961) which should be transferred to the genus *Francisella* (Niebylski et al. 1997). Immediately following is a highly supported branch leading to a group of *Francisella* sequence-isolates from ticks; 1) XIX a novel *Francisella* sp. in *Dermacentor reticulatus* (Sreter-Lancz et al. 2008). 2) clade XVIII with the *Ornithodoros moubata* symbiont B (Noda et al. 1997), and a sequence-isolate in *Hyalomma truncatum* from Namibia (Unpublished results, current study). In addition there is a branch without support leading to groups consisting of; 1) XVII a sequence-isolate in *Amblyomma maculatum* (Scoles 2004). 2) clade XVI consisting of several sequence-isolates from *Dermacentor occidentalis* and *D. variabilis* (Scoles 2004, Goethert and Telford 2005, Kügeler et al. 2005). 3) clade XV consisting of sequence-isolates from *D. andersoni* and *D. hunteri* (Niebylski et al. 1997, Scoles 2004). 4) XIV a sequence-isolates from *D. albipictus* (Rowland et al. 2008). 5) clades XIII and XII consisting of sequence-isolates from *D. albipictus, D. variabilis* and *D. andersoni*, respectively (Scoles 2004). Intermediate between the *Francisella* endosymbionts in ticks and the final group is XI the species *F. hispaniensis* (Hüber et al. 2010). Finally in ML-2 there is a highly supported branch leading to a group X consisting of all the subspecies of *F. tularensis,* numerous *Francisella* sequence-isolates from water and soil (Barns et al. 2005, Thelaus et al. 2006), and a *Francisella* sp. isolated from sea-water (Petersen et al. 2009).

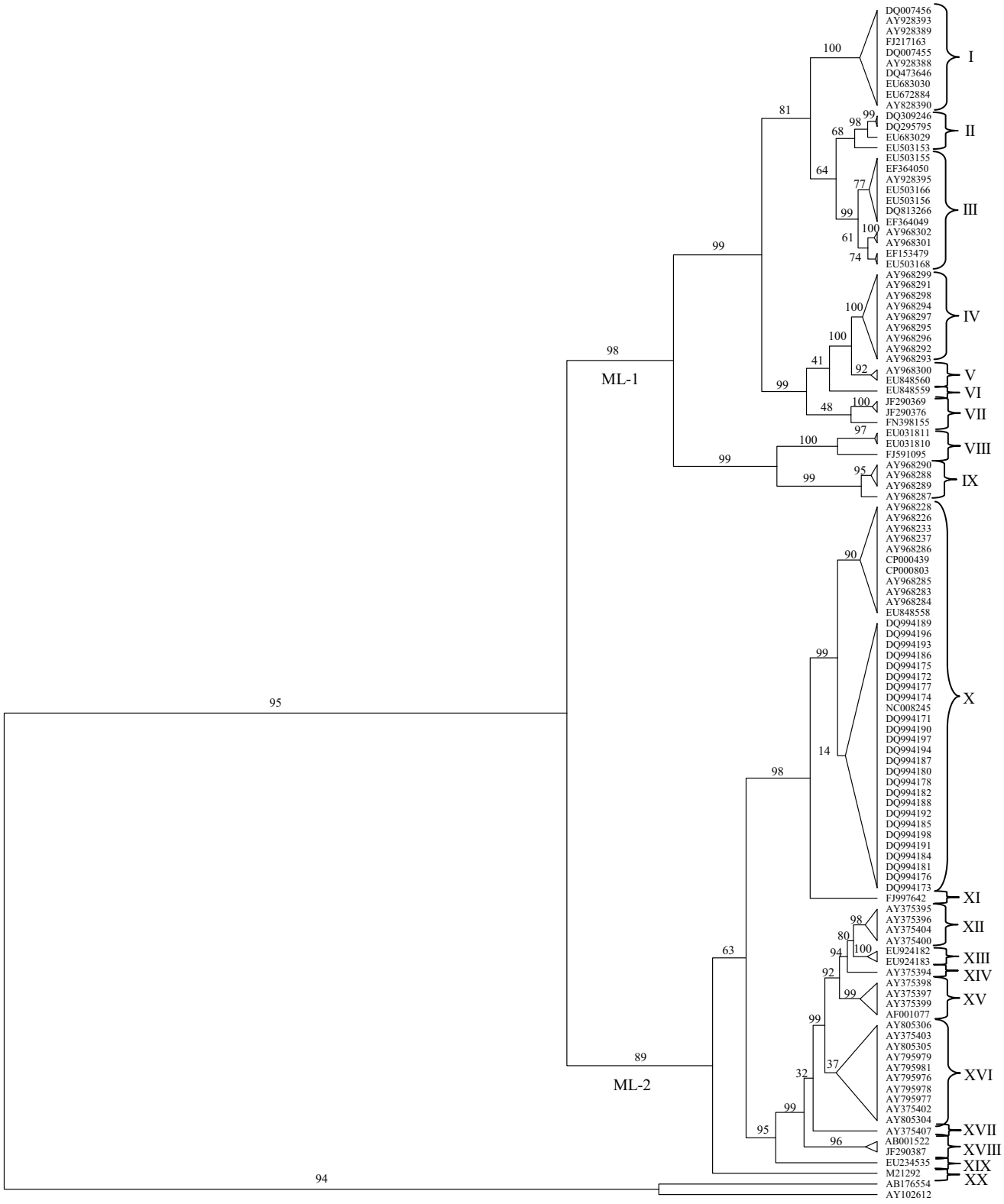

Figure 4. Genus *Francisella* phylogeny inferred from 16S rRNA-gene sequences (1052 nt), with *Caedibacter taerniospirales* and *Fangia hongkongensis* as outgroups. The Bayesian tree was constructed in the BEAST package v.1.6.1 (Drummond AJ, Rambaut A 2007) using the HKY model with gamma distribution and a relaxed uncorrelated lognormal molecular clock (Drummond AJ, Ho SYW, Phillips MJ & Rambaut A 2006) (N=45 000 000 generations, posterior probabilities in % are presented above branches). Phylogenetic trees were drawn using FigTree v.1.3.1 (Rambaut A, Available online: http://tree.bio.ed.ac.uk/software/figtree/).

Bacterial taxonomy and current practices

Although great progress has been made in the field of bacterial taxonomy, there are still no widely accepted concepts of procaryotic species (Rosselló-Mora & Amann 2001). The evolutionary processes involved in shaping the procaryotic genomes have also become better understood, such as recombination (Fraser et al. 2007) and lateral gene transfer (LGT) (Doolittle & Papke 2006). The discovery of recombination and LGT between bacteria challenges the view that procaryotes are asexual organisms only receiving genetic material vertically from its parent cell. What procaryotic species are, how they should be defined, or if the procaryotic species category exists are therefore topics that have received the attention of some authors (Doolittle & Papke 2006, Nesbø et al. 2006). There are currently several opinions on what procaryotic species are (in table 3), but these fall into mainly four categories; recombinational (Dykhuizen & Green 1991, Dykhuizen 2005), ecological (Cohan 2002), phylogenetic (Rosselló-Mora & Amann 2001) and nominalist (Rosselló-Mora & Amann 2001, Stackebrandt et al. 2002, Hanage et al. 2005). In the lack of a widely accepted species concept, however, the goals in the current practices of bacterial taxonomy is to provide stability, reproducibility and coherence. These goals are obtained through standards and rules in terms of; 1) Classification, the arrangement of organisms into taxonomic groups (taxa) comprised of various ranks (Domain, Phylum, Class, Order, Family, Genus, Species and Subspecies). 2) Nomenclature, the assignment of names to taxa (a Linnean style Latin binominal name for species) according to rules that conform to the International Code of Nomenclature of Bacteria (Sneath 1992), and the 1990 revision of The Bacteriological Code. 3) Identification, the process of determining that an isolate belongs to one of the established, named taxa. Identification and classification of bacteria have mainly been based on morphological, phenotypic and biochemical-characeristics and pathogenicity (Krieg 2005). However, DNA-characteristics have become an increasingly important part of bacterial taxonomy such as G+C content, ribosomal gene sequences, protein sequences and whole genome sequences.

Table 3 Species concepts.

Species concept	Definition	References
Biological species	Group of individuals that share the same genetic pool and that are isolated from other similar groups by reproductive barriers	Mayr, 1963, Dobzhansky et al. 1977, White 1978
Ecological species	A species in the bacterial world maybe understood as an evolutionary lineage bound by ecotype-periodic selection	Cohan 2002
Recombination species	A species is a group of individuals where the observed lateral gene transfer within the group is much greater than the transfer between groups	Dykhuizen & Green 1991, Dykhuizen 2005
Phylo-phenetic species	A species could be described as a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property	Roselló-Mora & Amann 2001
Nominal species	Cluster of strains to which it is convenient to give a species name on the basis other than taxonomical	Ravin et al. 1963
Genomo species	Bacteria showing 70 % or greater DNA-DNA hybridization and with 5 °C or less difference in ΔTm	Wayne et al. 1987
Species are none existing	Microbes do no form natural clusters to which the term "species" can be universally and sensibly applied	Nesbø et al. 2005, Doolittle & Papke 2006
Species are metapopulation lineages	A set of connected subpopulations that are maximally inclusive and the limits of which are set by evolutionary cohesive forces.	deQuirez 2005, Achtman & Wagner 2008

With regards to the latter, the method of DNA-DNA hybridization (DDH) is considered to be the corner stones of bacterial taxonomy (Stackebrandt et al. 2002). In the DDH analysis of two bacterial strains, a resulting homology between the strains above 70 % is considered to 'define' a bacterial species (the genomo-species). The DDH method has been implemented in bacterial taxonomy since the 70-ies (Johnson 1973, Wayne et al. 1987). Genetic characters that have become equally important are sequencing and comparison of the 16S rRNA gene, where an identity of two strains below 97 % have been found to correspond to the 70 % DDH-threshold-value to delineate strains as species (Stackebrand & Goebel 1994). Multi Locus Sequence Typing (MLST) is another method which has proven useful in the approaches to bacterial taxonomy. MLST is based on comparison of numerous house-keeping, typically 5 or more, that are evolutionary conserved between bacterial taxa (Maiden et al. 1998, Stackebrandt et al. 2002). The best choice of genes for MLST is considered to be the combination made up of genes from different locations, i.e evenly

distributed in the bacterial genome, that are part of the core genome (Maiden et al. 1998, Stackebrandt et al. 2002). As house-keeping/core-genes are considered less likely to be the result of lateral gene transfer, unlike auxiliary genes, some authors believe they most likely indicate an organism's true phylogeny (Lan & Reeves 2001). A combination of house-keeping/core-genes are therefore also considered to be representative of a genome`s Average Nucleotide Identity (ANI) (Konstantinidis & Tiedje 2004). ANI values between two genomes of less than 95 % have been found to correspond to 70 % DDH-threshold-value to delineate strains as species (Goris et al. 2007). Although these DNA based methods have become important in bacterial taxonomic considerations, scientists are encouraged to use a polyphasic taxonomic approach which includes both phenotypic and phylogenetic comparison to other known strains (Vandamme et al. 1996, Stackebrandt et al. 2002, Gillis et al. 2005, Tindall et al. 2010)

Bacterial taxonomy and critiques

DNA-DNA hybridization

The method of DDH as a basis to delineate procaryotic species was initially based on the study of *Enterobacteriaceae* by Brenner (1973), and he adopted the cut-off value of 70 % for defining species because strains of biochemically well-defined species usually exhibited 70 % or greater nucleotide-sequence relatedness (Brenner et al. 1973). However, some authors are of the opinion that the cut-off value of 70 % does not make any provision for bacterial groups related at different DNA-DNA pairing values (Sneath 1989, Moreno 1997, Dijkshoorn et al. 2000), i.e. that the cut-off value of 70 % in DHH may not be approporiate for all genera. The method of DDH and its use has also some limitations, it is unapplicable to bacteria that have not been cultivated and can only be performed using two strains at the time. Hence the method is time consuming and expensive if several strains are going to be hybridized. Results may also be influenced by a significant number of physico-chemical parameters,

differing genome size, the presence of large plasmids, purity of DNA and which of the hybridized bacteria that are chosen as probe or target (Stackebrandt & Ebers 2006, Achtman & Wagner 2008). Despite of these shortcomings, DDH is still being used as a standard when delineating species. In addition, new DNA-based methods for the use in delineation of species are calibrated to the DDH 70 % cut-off value, e.g. the tresholds of 97 % similarity in the 16S rRNA-gene and ANI of 95 %, respectively (Stackebrand & Goebel 1994, Goris et al. 2007).

Gene-sequence comparison and analysis

The use of gene-sequence comparison and analysis in separation of bacterial species (16S rRNA- and housekeeping genes) are advantegous over the DDH in a number of ways. Gene-sequencing is more cost and time efficiencient, reproducable, hence they are easily standarizable and portable between laboratories. However, the threshold values are calibrated to the DDH threshold value. Although a threshold value in the 16S rRNA gene of 97 % is widely used when defining bacterial species, some authors point out that there are no clear-cut consensus definitions, in terms of exact degree of genetic difference, of bacterial species by 16S rRNA gene-sequence comparison (Fox et al. 1992, Lan & Reeves 2001, Stackebrandt et al. 2002, Stackebrandt & Ebers 2006). Several authors have operated with different values of 16S rRNA gene-sequence similarity or differences to define species. Values such as 0.5 to 1 % have been used to differentiate *Clostridia* (Song et al 2003), or ≥99 % similarity between some Gram-positive rods (Bossard et al. 2003). Others have used 5 to 15 bp difference in the whole 16S rRNA gene-sequence of *Bacillus* spp. to differentiate species (Fox et al. 1992), or differences of 4 bp or fewer between *Mycobacterium* spp. (Tortoli 2003). Similarities such as ≥99.8 % and ≥98.1 % in the 16S rRNA gene-sequence have been used as differential between rickettsial-isolates (Fouriner et al. 2003). On the basis of 16S rRNA gene-sequence data from a number of species descriptions, Stackebrandt & Ebers (2006) have proposed that it could be appropriate to adjust the initial value of 97 % to define species. Another issue which is not clear in terms of 16S rRNA gene-sequence analysis is how much intra-species variability

that should be allowed between strains within a species before it is appropriate to delineate these strains as species. According to some authors (Fox et al. 1992, Song et al. 2003), the intra-species variability in the 16S rRNA-gene should be as close as possible but no more than a 1-1,5 % difference (Fox et al. 1992, Song et al. 2003). All of these aspects indicate that it may not be straight forward to agree upon a single value for the definition of species on the basis of 16S rRNA gene-sequences, or that it perhaps may not be appropriate to use a single value for all genera.

With regards to house-keeping genes, recombinational events or gene exchange may have huge implications for the resulting phylogenies inferred using these genes. Unlike animal eucaryotes where reproductive barriers prevent gene-exchange across species, bacteria do not form closed gene-pools and gene-exhange even among bacteria from distantly related taxa may occur (Xu 2004, Gogarten and Townsend 2005). This means that different parts of an organism's genome could have different evolutionary histories. Analysis based on sequences from different sets of genes may result in markedly different phylogenies even analysis of house-keeping/core-genes from different locations in the same genome (Wetz et al. 2003). As a consequence it may be hard to know which of the innferred phylogenies that reflects the organism´s true phylogeny.

Some authors are of the opinion that the current approaches to define bacterial species is operational and pragmatic, and that the rules, definitions and criteria less likely reflect the true nature of the bacteria and their ecology (Moreno et al. 2002, Achtman & Wagner 2008). As a solution Achtman & Wagner (2008) has proposed that a more naturally based classification system of bacteria should be adopted, 'a method-free species concept', where data from bacterial genomics and ecology are placed in an evolutionary context. According to this view procaryotic species are "metapopulation lineages". A metapopulation lineage can be viewed as a lineage that extends through time and that occupyies an adaptive zone minimally different from that of any other lineage in its range, and at the same time is evolving separately from all lineages outside its range (Achtman & Wagner 2008). These lineages may not exhibit

exclusively the properties of a recombination species, an ecological species or a phylogenetic species. As a result microbiologists are not bound to use particular methods or techniques to study their target organisms, but are free to choose those that are suitable for the organism in question. The reason for this is that such a classification system does not specify a specific method or absolute cut-off value for species delineation, but rather invokes a general evolutionary criterion for speciation (Achtman & Wagner 2008).

Evolution of bacteria

Bacteria reproduce by binary fission, but unlike in animals and plants bacterial reproduction is a function discrete and independent from recombination (LGT or homologous recombination). However, their genomes are still exposed to the same evolutionary mechanisms; point mutations, deletion, duplication, gene degradation, transposition and slippage mutation of DNA repeats (Maynard Smith et al. 1993, Feil et al. 1999, Feil et al. 2000, Lawrence & Hendrickson 2003). The selectional forces acting upon an organism, e.g. their natural habitat or environment, seem to decide to what extent and which of the evolutionary mechanisms are the most influential in shaping the organism's genome. Organisms living under environmental conditions with low levels of nutrients, at extreme temperatures or salinities (high or low) may require genetical material encoding a wide variety of synthesis and regulatory pathways in order to survive. Aquisition of genetic material through LGT or recombination could turn out benifitial for an organism in terms of survival in such environments. In strong contrast, gene-degradation and deletions are less likely to be major influential mechanims in shaping such genomes and could result in loss of gene-functions important for survival. The highly nutritious and stable intracellular environment on the other hand may make genetic material encoding some synthesis or regulatory pathways, otherwise required for survival in a less nutritious environment, redundant (Casadevall 2008). Genome degradation has been shown highly influential in shaping the genomes of intracellular bacteria (Casadevall 2008). However, there

are some intracellular bacteria where recombination has been shown to be a highly influential evolutionary mechanism e.g. the genera *Wolbachia* and *Mycoplasma* (Baldo et al. 2006, Sirand-Pugnet et al. 2007). Still, differences in life-style can generally be reflected by the genome size of the bacteria (Borderstein & Reznikoff 2005). Intracellular bacteria have relative small genomes spanning from obligate intracellular (*Rickettsia sibrica;* with 1.2 Mb) to facultative intracellular (*F. tularensis, F. philomiragia;* with 1.8 and 2.0 Mb). Free living bacteria capable of occupying diverse ecological niches on the other hand have larger genomes (*Pseudomonas aeruginosa* with 6.5 Mb). Most of the previously recognized *Francisella* spp. have been considered facultative intracellular pathogens (Sjøstedt 2005b). To date, according to the *Francisella* spp. genomes available from the GenBank, this lifestyle seem to be reflected by the size of their genomes. Currently, the largest genomes in the *Francisella* genus are found in *F. philomiragia* (ATCC25015 and ATCC25017) with 1.9-2.0 Mega bases (Mb) and *F. novicida* with 1.9 Mb, while the smallest genomes are found in *F. tularensis* sspp. *holarctica*, *tularensis* and *mediasiatica* ranging between 1.8-1.9 Mb. Deletions of region of differences (RD`s) (Svensson et al. 2005), the expansion of insertion elements (e.g. ISFtu1) (Larsson et al. 2007) and gene-degradation (as jugded by the number of pseudo-genes present) have been found to be highly influential in shaping genomes from *Francisella* spp.. These are all considered consequences of an adaptation to intracellular parasitism and niche restriction for the subspecies of *F. tularensis* (Larsson 2007, 2009, Rohmer et al. 2007). The roles of recombination and LGT in shaping *Francisella* genomes are still poorly known although there are indications of such events in the genomes of *F. novicida* and *F. tularensis* ssp. *mediasiatica* (Nübel et al. 2006).

Olsufjev was the first who where dealing with possible evolutionary scenarios of bacteria within the genus *Francisella* (Olsufjev 1970). On the basis of geographical distribution, some biochemical characteristics and differences in hosts he depicted that the *Francisellaceae*, *Brucellaceae* and *Pasteurellaceae* shared a common ancestor of which the two first originally split off and differentiated into *Brucella* and

Francisella Olsufjev (1970). Furthermore, he deduced that *F. tularensis* in the past split into the diverse geographically forms known as sspp. *mediasiatica*, *tularensis* and *holarctica* (including biovar japonica). The ancient form undergoing this split Olsufjev (1970) considered to be closest to *F. tularensis* ssp. *mediasiatica*. However, he could not accurately place *F. novicida* in relation to the subspecies of *F. tularensis.* At the time of his studies no detailed genetic evidence was available which could help explain the events or support this hypothesis. Today the genera *Brucella* and *Francisella* are placed within entirely separate lineages within the alpha- and gamma-proteobacteria, respectively (Moreno 1990, Sjøstedt 2005a). There are also other genera of bacteria that are more closely related to the genus *Francisella* (Lau et al. 2007). However, the evolutionary scenario within *F. tularensis,* as depicted by Olsufjev (1970), match seemingly well with more recent genomic data (Svensson et al. 2005, Larsson 2007). On the basis of these data it has been proposed an evolutionary scenario where *F. novicida* is considered closest to the ancestral form while *F. tularensis* sspp. *tularensis* and *mediasiatica* precedes the appearance of *F. tularensis* ssp. *holarctica* (Svensson et al. 2005).

AIMS OF THE PRESENT STUDY

When this work was commenced (2006), evidence had already been presented that environmental *Francisella* are more widespread than previosuly recognized; both in terrestrial and aquatic environments and in association with organisms like ticks and fish. The first reports on the association of *Francisella* bacteria and disease in fish were from Taiwan and Japan. *Francisella* sp. was also detected in diseased farmed Atlantic cod from Norway in 2004, and subsequently a *Francisella* sp. was detected in Chilean farmed Atlantic salmon. Infections with *Francisella* bacteria in fish have since been identified in several species of fish from around the world. The biology of these novel species remains poorly understood, in particular their ecology and characteristics.

The primary aim of the present study was to;

- Characterize the fish-pathogenic *Francisella* spp. in comparison to related previously described *Francisella* spp. using a polyphasic taxonomic approach.

SUMMARY OF PAPERS

Paper-I. In the autumn of 2004 in the County of Rogaland Atlantic cod (*G. morhua*) showing loss of appetite, reduced swimming performance and dark pigmentation were observed in some farms. There were few external signs of disease, but white granulomas could be found in the skin, gills and in the mouth cavity. Internal investigation revealed pathology ranging from just slightly swollen spleens and kidneys to most organs being perfused by white granulomas. In terminal stages of the disease spleens two to three times the normal size being completely covered with and penetrated by granulomas could be observed. From such cod a bacterium belonging to the genus *Francisella* was detected and isolated. Challenge experiments in Atlantic cod (*G. morhua*) with this *Francisella* sp. resulted in high mortalities. The bacterium used in the challenge was re-isolated from moribund and dead fish. It was not possible to detect any other causes of mortality, and the pathology were the same as those observed in the fish from where the bacterium first was isolated. Hence it was concluded the bacterium was the cause of the mortality and the observed disease on farmed cod. Sequencing of the 16S rRNA-gene revealed that the bacterium was closely related to a *Francisella* sp. previously isolated from fish in Asia and to *F. philomiragia*, among the valid described species most likely representing a novel species within genus *Francisella*.

Paper-II. The *Francisella* sp. GM2212 isolated from cod was characterized. Several genes including the complete 16S rDNA, 16S-23S intergenic spacer and 23S rDNA, malate dehydrogenase (mdh), FopA, tul4 (LpnA) and a hypothetical lipoprotein (LpnB) were sequenced; the biochemical and phenotypical characteristics were determined and compared to *F. philomiragia* and *F. tularensis*. The results show a closer affinity of the *Francisella* sp. GM2212 to *F. philomiragia* than *F. tularensis* with evidence suggesting that GM2212 should represent a new species within the genus.

Paper-III. DNA-DNA hybridization and fatty acid analysis of *Francisella* sp. GM2212 and *F. philomiragia* were performed. In addition 16S rDNA sequences were obtained from *Francisella* sp. infected cod in a larger area in the North-Atlantic and compared to GM2212. Results show only minor differences between the *Francisella* sp. GM2212 from cod and *F. philomiragia* in the fatty acid content, and 70 % similarity in the DNA-DNA hybridization, which is inconclusive evidence for a species status of GM2212. The 16S rDNA sequences from all cod-*Francisella* showed 100 % identity, all containing a unique signature sequence demonstrating the use of 16S rDNA as conserved specific character and together with previous molecular, phenotypic and biochemical characteristics support the establishment of GM2212 as a separate species. *Francisella piscicida* sp. nov. was proposed for GM2212.

Paper-IV. The distribution of *F. piscicida* in farmed and wild Atlantic cod along the Norwegian coast was determined. Real-time PCR assays targeting the 16S rRNA-gene and FopA was developed since sensitive and specific tools are required for detection of asymptomatic carriers of *F. piscicida.* From 2004 to 2007 422 wild Atlantic cod, 955 farmed Atlantic cod and a few other wild species caught along the Norwegian coastline were screened using a Real-time PCR assay (Fc50) against *F. picicida* 16S rRNA. Results show a presence of *F. piscicida* in wild populations of Cod only south of Sognefjorden, while the bacterium is present in farmed populations of cod in most counties with cod-farming. A widespread distribution of the bacterium in wild populations in the south of Norway and an apparant absence in populations in the north is suggested to be due to differences in sea-water temperatures. The presence of the bacterium in farmed populations of cod in the north is also believed to be related to transport of carrier fish from the south.

Paper-V. Further investigations were performed in which *F. piscicida* and *F. philomiragia* ssp. *noatunensis* from cod were compared to the *Francisella* sp. Ehime-1 from three-line grunt from Japan, and several isolates of *F. philomiragia* ssp. *philomiragia.* Biochemical and phenotypic properties were determined, DNA-DNA

hybridization between *Francisella* sp. Ehime-1, *F. philomiragia* ssp. *philomiragia* and *F. piscicida* was performed. The 16S rDNA and 9 house-keeping genes dnaA, mdh, mutS, pgm, prfB, putA, rpoA, rpoB and tpiA were sequenced from each isolate involved in the comparison including a *Francisella* sp. sequence isolate from an Indonesian tilapia which show that *F. piscicida* and *F. philomiragia* ssp. *noatunensis* are identical. The *Francisella* sp. from fish in Asia is genetically distinct from *F. philomiragia* ssp. *philomiragia* and is more similar to *F. piscicida*/*F. philomiragia* ssp. *noatunensis.* Based on these data *F. philomiragia* ssp. *noatunensis* is elevated to species status as *F. noatunensis* comb. nov. In addition the *Francisella* sp. from fish in Asia are described and proposed as a subspecies of *F. noatunensis* for which the name *F. noatunensis* ssp. *orientalis* subsp. nov. is proposed.

SYNTHESIS

The taxonomy of genus *Francisella*

The major aim of this study was to characterize and describe the taxonomy of fish-pathogenic *Francisella* spp.. In order to follow current practices in bacterial taxonomy this work was performed using a polyphasic taxonomic approach and resulted in the proposal of *F. noatunensis* sspp. *noatunensis* (=*F. piscicida*) and *orientalis* (Paper-II, Paper-III, Paper-V). Prior to their discoveries, the taxonomy of the genus has mainly been based on all the knowlegde of the three species *F. tularensis*, *F. novicida* and *F. philomiragia* (Sjøstedt 2005a). Unlike the related genus *Pasteurella* (Christensen et al. 2007), a minimal standard for the description of species or subspecies within genus *Francisella* has not yet been published. This may be explained by the fact that until recently genus *Francisella,* consisted of three validated species only. Furthermore, since *F. tularensis* is a serious human pathogen, the risk of laboratory aquired infections was proposed by Clarrigde et al. (1996) as the main reason for the poorly developed taxonomy of the genus. However, an increasing number of novel *Francisella* spp. have been detected the last decades; ranging from uncultivatable environmental bacteria and endosymbionts in ticks (Noda et al. 1997, Barns et al. 2005) and in a ciliate (Schrallhammer et al. 2010), to pathogens of fish (Kamaishi et al. 2005, Nylund et al. 2005, Paper-I, Olsen et al. 2006), humans (Kügeler et al. 2008, Hüber et al. 2009) and molluscs (Kamaishi et al. 2010). In light of these discoveries a re-examination of the taxonomy of the genus seems to be in order, with particular emphasis on the characters used to differentiate between *Francisella* species and subspecies.

Phenotypic characteristics of *Francisella* spp.

Phenotype and biochemical properties

Bacterial classification has mainly been based on the study of the biochemical and the phenotypic properties of bacteria; their growth characteristics and utilization of

specific substrates (Cohan 2002). With respect to *Francisella* spp. their intracellular habitat, fastidious natures and their ability to cause serious diseases in their host organisms seems to be common although there are some exceptions. The less fastidious *F. novicida* and *F. philomiragia* that can be grown on less nutritious media (Jensen et al. 1969, Hollis et al. 1989), seem to have less specialized life-cycles and resemble environmental bacteria. The more fastidious *F. tularensis* sspp. are not restricted to one host, but there seem to be evidences of preferences for some host groups and vectors (Farlow et al. 2005, Peterson et al. 2010). Some tick species have their distinct *Francisella*-endosymbiont (Scoles 2004, Goethert & Telford 2005, Venzal et al. 2008). *F. noatunensis* ssp. *noatunensis* (=*F. piscicida*) seems to be restricted to cod and Atlantic salmon, like *F. noatunensis* ssp. *orientalis* seems to be restricted to a few species of fish (tilapia, three-line grunt). The association with a limited number of hosts or environments indicate high degrees of niche restriction for several *Francisella* spp.. However, as taxonomic characters these have only had a limited use in taxonomical considerations of the *Francisella;* i.e. in the separation of subspecies of *F. tularensis* (Olsufjev & Meshcheryakova 1983), and in separating *F. noatunensis* and *F. philomiragia* (Paper-III). Mainly biochemical tests have been applied in the characterization of the *Francisella* spp., including fermentation of carbohydrates (Downs & Bond 1935, Francis 1941, Owen et al. 1964, Jensen et al. 1969, Olsufjev & Meshcheryakova 1983, Hollis et al. 1989, Paper-II, Paper-III,) and utilization of different substrates (Downs & Bond 1935, Marchette et al. 1961, Jensen et al. 1969, Hollis et al. 1989, Paper-II, Paper-III). *F. philomiragia* is by far the most diverging according to these characters and can be separated from *F. tularensis* and *F. novicida* on the basis of the production of indole, absence of a citrulline ureidase pathway, presence of oxidase activity, gelatine hydrolysis and growth in Triple Sugar Iron agar (TSI) (table 1) (Hollis et al. 1989). The absence of the three latter characteristics in the closely related *F. noatunensis* also distinguishes this bacterium from *F. philomiragia* (Paper-II, Paper-III). However, there are only minor differences between all of these species in the ability to utilize glucose, maltose, sucrose and the alcohol glycerol as the single carbon source (Jensen et al. 1969, Olsufjev &

Meshcheryakova 1983, Hollis et al. 1989, Paper-II, Paper-III). Furthermore, the bacteria *F. tularensis*, *F. novicida* and novel *Francisella* spp. isolated from human blood are highly similar in these phenotypic characters (Kügeler et al. 2008). Although these phenotypic characters offer poor differentiation between some members of the genus *Francisella*, they have still been important in the taxonomy of the genus. Several of these phenotypic characters have eventually been supplemented or replaced by standardizable biochemcial test-kits notably the APIZYM, API Rapid ID 32A and 32E (bioMeriéux) and the GN2 MicroPlate System (Biolog) (Paper-V, Bohle et al. 2009, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009, Hüber et al. 2009). Enzymatic profiling of *Francisella* spp. using these kits result in a more detailed picture of *F. novicida*, *F. philomiragia* and the recent named *F. hispaniensis* as biochemically more reactive and less fastidious than their counterparts *F. noatunensis* and *F. tularensis* (Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009, Hüber et al. 2009). However, these kits are not optimized for *Francisella* bacteria and misidentification using such kits have occured (O`Hara 2005). In addition, there seem to be minor inconsistencies between studies, as judged by slightly different results, using these kits (Mikalsen et al. 2007, Paper-V, Mikalsen & Colquhoun 2009). Furthermore, despite of the ecological constraints that exist between *F. tularensis* and *F. noatunensis,* i.e. mammal hosts vs. fish, the resolution between these two species offered by these kits is poor (Hüber et al. 2009). Similarly, despite of being separated geographically and having distinct hosts, the subspecies of *F. noatunensis* are virtually indistinguishable when examined using the APIZYM, API Rapid ID 32A and 32E kits (table 4, Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009).

Fatty acids content

Besides biochemical and phenotypic characterization, chemotaxonomic data such as fatty acids -content and -profiling has been used to differentiate between members within genus *Francisella* (Jantzen et al. 1979).

Table 4. Biochemical and other phenotypic characteristics at 22°C of *Francisella philomiragia* and *F. noatunensis* as determined using the API rapid ID 32A & 32E and APIZYM kits (bioMérieux). The table is presented in Paper-V, but the chilean variant of *F. noatunensis* ssp. *noatunensis* UA2660, and two *F. philomiragia* isolates from Marthas Vineyard (Berrada et al. 2009) have been included. Strains; 1 *F. noatunensis* ssp. *orientalis* Ehime-1, 2 *F. noatunensis* ssp. *noatunensis* chilean variant UA2660, 3 *F. noatunensis* ssp. *noatunensis* (syn. *F. piscicida*) and *F. philomiragia* 4-10; 4 strain 1951, 5 CCUG12603, 6 CCUG13404, 7 CCUG19701, 8 ATCC25015^T, 9 strain 080107 (Berrada et al. 2009) direct isolation and 10 strain 080107 after passage through mouse.

	1	2	3	4	5	6	7	8	9	10
B-Galactosidase 6-phoshatase	-	-	-	-	+	-	-	+	-	+
α-Glucosidase	-	-	-	-	-	-	-	+	-	-
Mannose and Raffinose fermentation	-	-	-	+	+	+	+	+	+	+
Arginine arylamidase	+w	+w	+w	+	+	+	+	+	+	+
Proline arylamidase	+	-	+	+	+	+	+	+	+	+
Arylamidases; Leucyl glycine, Leucine, Pyroglutamic acid, Glutamyl glutamic acid, Glycine, Valine, Cystine, Serine	-	-	-	+	+	+	+	+	+	+
Phenylalanine arylamidase	+w	-	-	+	+	+	+	+	+	+
Tyrosine arylamidase	-	+w	+w	+	+	+	+	+	+	+
Alanine arylamidase	+w	+w	+	+	+	+	+	+	+	+
Histidine arylamidase	-	+w	+w	+	+	+	+	+	+	+
D-maltose, D-trehalose, Colistin, Coumarate, O-Nitrophenyl N-Acetyl-BD-Glucosaminide	-	-	-	+	+	+	+	+	+	+
P-Nitrophenyl-BD-galactopyranoside	-	-	-	-	+	-	-	+	-	+
Indoxyl phosphate	+	-	-	+	+	+	+	+	+	+
Napthol-AS-BI-phosphohydrolase	+	+w	-	+	+	+	+	+	+	+
β-galactosidase	-	-	-	-	+	-	-	+	-	+
N-acetyl-β-glucosaminidase, oxidase, H_2S slant, Triple Sugar Iron, gelatine hydrolysis	-	-	-	+	+	+	+	+	+	+
Agglutination with *F. noatunensis* antiserum	+	+	+	-	-	-	-	-	-	-
Cysteine required for growth	+	+	+*	-	-	-	-	-	-	-

+ = positive, - = negative, w = weak, * = negative or slight growth on TSA and BHIA agars without added cysteine

All isolates were positive for Catalase, Tryptophanase, D-glucose, D-saccharose, Alkaline phosphatase, Esterase, Esterase lipase, acid phosphatase and H_2S-production. All isolates were negative for Motility, Urease, Arginine dehydrolase, α-Arabinose, β-N-Acetyl-β-Glucosaminidase, Glutamic acid Decarboxylase, α-fucosidase, Nitrate reduction, Lysine decarboxylase, Ornithine decarboxylase, Esculin, L-arabinose, adonitol, L-rhamnose, D-mannitol, D-sorbitol, D-cellobiose, D-melibiose, Sodium glucuronate, Para-phenylalanine deaminase, 5-Ketogluconate, Palatinose, Galacturonate, Tetra-thionate reductase, Raffinose, Lipase, Trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase and α-mannosidase.

The members of genus *Francisella* has a unique fatty-acid compositons separating the genus from other genera (Jantzen et al. 1979), and has been considered sufficiently unique to serve as a signature of *F. tularensis* in environmental samples (Nichols et al. 1985). The major fatty acid constitituents are long-chain saturated and monosaturated C18 to C26 acids, relatively large amounts of saturated even-chain acids (C10:0, C14:0, and C16:0), and two longchain hydroxy acids (C16:0 3OH and C18:0 3OH) (Jantzen et al. 1979, Nichols et al. 1985, Hollis et al. 1989). The amount of several of these fatty-acid do vary between and within species and subspecies of the *Francisella* (Jantzen et al. 1979, Hollis et al. 1989, Paper-III, Whittaker et al. 2007, Hüber et al. 2009).Within the subspecies of *F. tularensis; tularensis, holarctica* and *mediasiatica* there are particularly variations in the fatty acids C14:0 (6.5-7.7 %, 9-11 % and 4.2-4.5 %) and C24:0, (12-16 %, 7-11 % and 12-16 %) in Olsufjev & Meshcheryakova (1983). The two species *F. philomiragia* and *F. noatunensis* are in comparison more similar in the same fatty acids (Paper-III, Hüber et al. 2009). Maximum divergence between the two latter is in the fatty acids C14:0 (3.4 %), C22:0 (3.5 %) and C24:0 (3.4 %) (Paper-III, Hüber et al. 2009). One of the disadvantages using fatty acid profiling however, is the inconsistency in the fatty acid content within species or subspecies that may be as high as between species and subspecies. Furthermore, the results from a fatty-acid analysis may vary from time to time depending on the methodology used to determine the fatty acid content, and the growth medium used to grow the bacterium prior to analysis (Hollis et al. 1989, Whittaker et al. 2007). Unless fatty-acid profiling of the *Francisella* spp. is properly standarized (strains, growth condition, methodology), as that of the MIDI system (Miller 1982), the taxonomic value will be limited.

Serology

A character which has been very important in the diagnosis of tularemia; serology, has also been applied to distinguish members of the genus *Francisella* (Francis & Evans 1926, Owen et al. 1964, Ohara et al. 1974, Clarrigde et al. 1996, Kügeler et al.

2008, Paper-II, Paper-III, Paper-V). However, the results from the earliest studies are ambiguous, as there have been observed some degrees of cross-agglutination of both human and animal anti-*F. tularensis* sera with the bacteria *Brucella melitensis* and *B. abortus* (Francis & Evans 1926, Ohara et al. 1974). The latter observations explain why *F. tularensis* for some time was considered to belong in the genus *Brucella* (Topley & Wilson 1929). The lack of cross-agglutination of *F. tularensis* antisera with *F. novicida* is also one of the reasons the latter was considered a separate species (Owen et al. 1964). However, despite the apparent lack of agglutination of *F. tularensis* rabbit-antisera with *F. novicida* or *F. philomiragia,* they were determined to share similar antigenic properties (Ohara et al. 1974). A commercially available *F. tularensis* antiserum (Becton, Dickinson and Company) have been shown to agglutinate most *F. tularensis* strains and *F. novicida* (Clarrigde et al. 1996), but not closely related unnamed *Francisella* spp. (Clarrigde et al. 1996, Kügeler et al. 2008) or *F. philomiragia* and *F. noatunensis* (Paper-III). There is a slight agglutination of *F. philomiragia* using the anti *F. noatunensis* rabbit antiserum (GM2212) if the bacterium is fixed in isopropanol or ethanol (Paper-II), but not on *F. philomiragia* from fresh cultures (Paper-III, Paper-V). All of the strains of *F. noatunensis* tested so far from Norway, Asia and Chile are agglutinated by the GM2212 antisera (Paper-V, current study, table 2, Pers Obs). These results indicate a high degree of serological similarity between some of the *Francisella* bacteria but the characters seem to be separative at the species level, at least according to the current taxonomy (Olsufjev & Meshcheryakova 1983, Paper-II, Paper-V, Hüber et al. 2009).

Conclusions phenotypic characteristics

Phenotypic properties used in genus *Francisella* taxonomical consideration seem to offer poor resolution between members, and there may be minor inconsistancy within groups according to some of the characters. But, generally the characters which have been used are valuable as they suggest a high degree of phenotypic consistency within each biochemically and phenotypically defined species and subspecies; e.g. *F.*

philomiragia (Table 2, Paper-V) and the subspecies of *F. tularensis* (Olsufjev & Meshcheryakova 1983). In comparison only a few isolates of the species *F. noatunensis* ssp. *orientalis*, *F. novicida* and *F. hispaniensis* have been studied phenotypically (Paper-V, Mikalsen et al. 2007, 2009, Hüber et al. 2009). Future biochemical and phenotypic studies of more isolates within each of these species is needed.

Several of the detected *Francisella* spp. have not been cultivated and hence, are not phenotypically characterized. According to general applicable criteria and rules governing naming of bacterial species, they cannot currently be assigned to species. This situation is very unsatisfactory. Except for the organism '*Wolbachia' persica* (Weiss et al. 1962), little is known about the biochemical properties of *Francisella* spp. associated with ticks (Scoles 2004), or as of yet unculturable *Francisella* spp..

Genetic characteristics of *Francisella* spp.

16S rRNA gene-sequences

A genetic character widely used in taxonomic considerations of the *Francisella,* has been the 16S rRNA-gene (Forsman et al. 1990, Forsman et al. 1994, Paper-I, Paper-III, Paper-V, Mikalsen et al. 2007, Mikalsen & Colqhoun 2009, Hüber et al. 2009, Schrallhammer et al. 2010). According to 16S rRNA gene-sequence analysis there is a clear difference between the species *F. tularensis* and *F. philomiragia*, although the similarity is high (98.3 %) (Forsman et al. 1994). *F. novicida* and *F. tularensis* show high similarity, as much as 99.8 % (Forsman et al. 1994), and this is one of the reasons that the former has been considered to be a subspecies of *F. tularensis* rather than a separate species (Hollis et al. 1989, Hüber et al. 2010). A sequence similarity between *F. noatunensis* and *F. philomiragia* of 99.3 % is also one of the reasons the former was considered a subspecies of the latter (Mikalsen et al. 2007). There are several recently sequenced members of the genus *Francisella* that exhibit 16S rRNA-gene similarities in the same order either of *F. tularensis* or *F. philomiragia*

(Clarrigde et al. 1996, Noda et al. 1997, Barns et al. 2005, Kamaishi et al. 2005, Nylund et al. 2005, Olsen et al. 2006, Paper-I, Paper-III, Paper-V, Berrada et al. 2009, Kamaishi et al. 2010, Schrallhammer et al. 2010, summarized in Supplementary table 1-1 to 1-6). According to 16S rRNA gene-sequence analysis none of the nominal *Francisella* spp. exhibit similarities below the species criterion of 97 % previously defined for delineation of bacterial species (Stackebrand & Goebel 1994). However, some *Francisella* spp. symbiotic in ticks or detected in environmental samples, show 16S rRNA-gene sequence divergence from the nominal *Francisella* spp. above 3 % (Scoles 2004, Barns et al. 2005). Hence they are likely new species (Scoles 2004, Barns et al. 2005). Due to the low diversity that exists in the 16S rRNA-gene between isolates within known *Francisella* species or subspecies, we consider the 16S rRNA-gene a highly conserved and specific taxonomic character (Paper-III, Paper-V).

DNA-DNA hybridization

The high similarities between some of the *Francisella* spp., as jugded by results from phenotypic and biochemcial profiling and 16S rRNA gene-sequence analysis, is revealed in DNA-DNA hybridization (DDH) studies (Hollis et al. 1989, Paper-III, Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009, Hüber et al. 2010). An overview is given in table 5. From the available DDH results the most diverging species are *F. tularensis* and *F. hispaniensis* vs. *F. philomiragia,* with 39 % similarity (Hollis et al. 1989, Hüber et al. 2009). The species *F. hispaniensis* is also relative distantly related to *F. novicida* (52 % similarity) and *F. noatunensis* ssp. *noatunensis* (47 % similarity) as jugded by DDH-results (Hüber et al. 2009). These four latter DDH-pairings are the only available DDH-values among the *Francisella* that is well below the 70 % criterion proposed as a cut-off for the delineation of bacterial species (Wayne et al. 1987).

Table 5. An overview of DNA-DNA hybridization similarities (%) between species in the genus *Francisella.*

	F. tularensis ssp. *tularensis*	*F. novicida*	*F. philomiragia*	*F. noatunensis* ssp. *noatunensis*
F. novicida	87 Hollis et al. 1989			
F. philomiragia	39 Hollis et al. 1989	51 Hollis et al. 1989		
F. noatunensis ssp. *noatunensis*	NA	NA	70 (68 & 74) Paper-III, 68 (65.2 & 70.8) Mikalsen et al. 2007	
F. noatunensis ssp. *noatunensis* from Chile (UA2660)	NA	NA	70.75 (74.5 & 67) current study	84.1 (87.2 & 81) current study
F. noatunensis ssp. *orientalis*	NA	NA	60.8 (57.3 & 64.3) Paper-V	68.7 (64 & 73.4) Paper-V, 69.9 (69.7 and 70.1) current study
F. asiatica	NA	NA	72.6 (68.7 & 76.5) Mikalsen & Colquhoun 2009	60.3 (58.4 & 62.1) Mikalsen & Colquhoun 2009
F. hispaniensis	NA	52 Hüber et al.2010	39 Hüber et al. 2010	47 Hüber et al. 2010

NA= not available

From the other available DDH-values of the *Francisella* spp. the intraspecies similarities seem to be characterized by values above 80 %; within *F. tularensis,* i.e. between *F. tularensis* and *F. novicida* (Hollis et al. 1989) and between the *Francisella* sp. from Chilean salmon and *F. noatunensis* ssp *noatunensis* (current study). Like data from 16S rRNA-gene sequence analysis and as jugded by the current practice within bacterial taxonomic considerations, results from DDH between *F. novicida* and *F. tularensis* suggests that the former represent a subspecies taxon in *F. tularensis* (Hollis et al. 1989, Hüber et al. 2009). The DDH similarity range within *F. philomiragia* (72-80 %) and between *F. noatunensis* sspp. *noatunensis* and *orientalis* are lower (Hollis et al. 1989, Paper-V). The latter two display DDH similarity of 69 % which is close to the DDH criterion for the delineation of species (Paper-V). It seems that, like 16S rRNA-gene analysis, closely related *Francisella* spp. e.g. *F. philomiragia* and *F. noatunensis,* cannot clearly be discerned as species according to the criterion of 70 % threshold in DDH, generally weighing heavily in bacterial species delineations (Hollis et al. 1989, Paper-III, Paper-V, Mikalsen et al. 2007). There are still several *Francisella* spp. and strains that have not yet been hybridized; *F. tularensis* and *F. novicida* vs. *F. noatunensis* sspp. *noatunensis* and *orientalis,*

several strains of *F. noatunensis* ssp. *noatunensis* vs. each other, strains of *F. noatunensis* ssp. *orientalis* vs. each other, in addition to several novel *Francisella* spp. isolates from various sources cf. (Kugeler et al. 2008, Petersen et al. 2009). DDH-pairings between several of these should be performed.

House-keeping genes

Studies that utilize house-keeping or core-genes analysis of *Francisella* spp. are still very few (Nübel et al. 2005, Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009). These studies demonstrate a resolution far higher than obtained by the 16S rRNA-gene (Nübel et al. 2006, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009, Paper-II, Paper-V and summarized in supplemantary table 2). There is a clear separation of *F. tularensis* and its subspecies in relation to *F. philomragia* and *F. noatunensis* with an Average Nucleotide Identity (ANI) of 86-87 % (Mikalsen & Colquhoun 2009), or 83-84 % (Paper-V, supplementary table 1). *F. philomiragia* and *F. noatunensis* show ANI similarities ranging from 95.5-96.5 % (Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009). The ANI between *F. noatunensis* ssp. *noatunensis* vs. *orientalis* and *F. noatunensis* vs. *F. asiatica* is in the same order (95.5/95.6 %) (Paper-V, Mikalsen & Colquhoun 2009), both of which is borderline according to the ANI criterion for delineation of bacterial species (Konstantinidis & Tiedje 2004, Goris et al. 2007). Except for the differences between isolates of *F. philomiragia* according to these genes (Supplementary table 1), and the differences between the two subspecies of *F. noatunensis,* the similarities between different isolates within subspecies analyzed this far seem to be comparable to that of 16S rRNA gene-sequence analysis, i.e. 99-100 % similarity (Nübel et al. 2006, Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009). The excisting data on *Francisella* house-keeping genes are still scant and is currently based on a few members only. A phylogenetic approach to *Francisella* taxonomy using house-keeping genes in the future would hugely benefit from data from several novel members i.e. endosymbionts in ticks and environmental bacteria. A phylogenetic tree

based on the rpoA-gene from *F. tularensis*, *F. philomiragia*, *F. noatunensis, F. hispaniensis*, a novel *Francisella* sp. isolated from edible sea-snails *Abalone* spp. from Japan (Kamaishi et al. 2010, Brevik in prep) and a *Francisella* sequence-isolate in the tick species *Hyalomma truncatum* from Namibia (Unpublished results) can be viewed in figure 5.

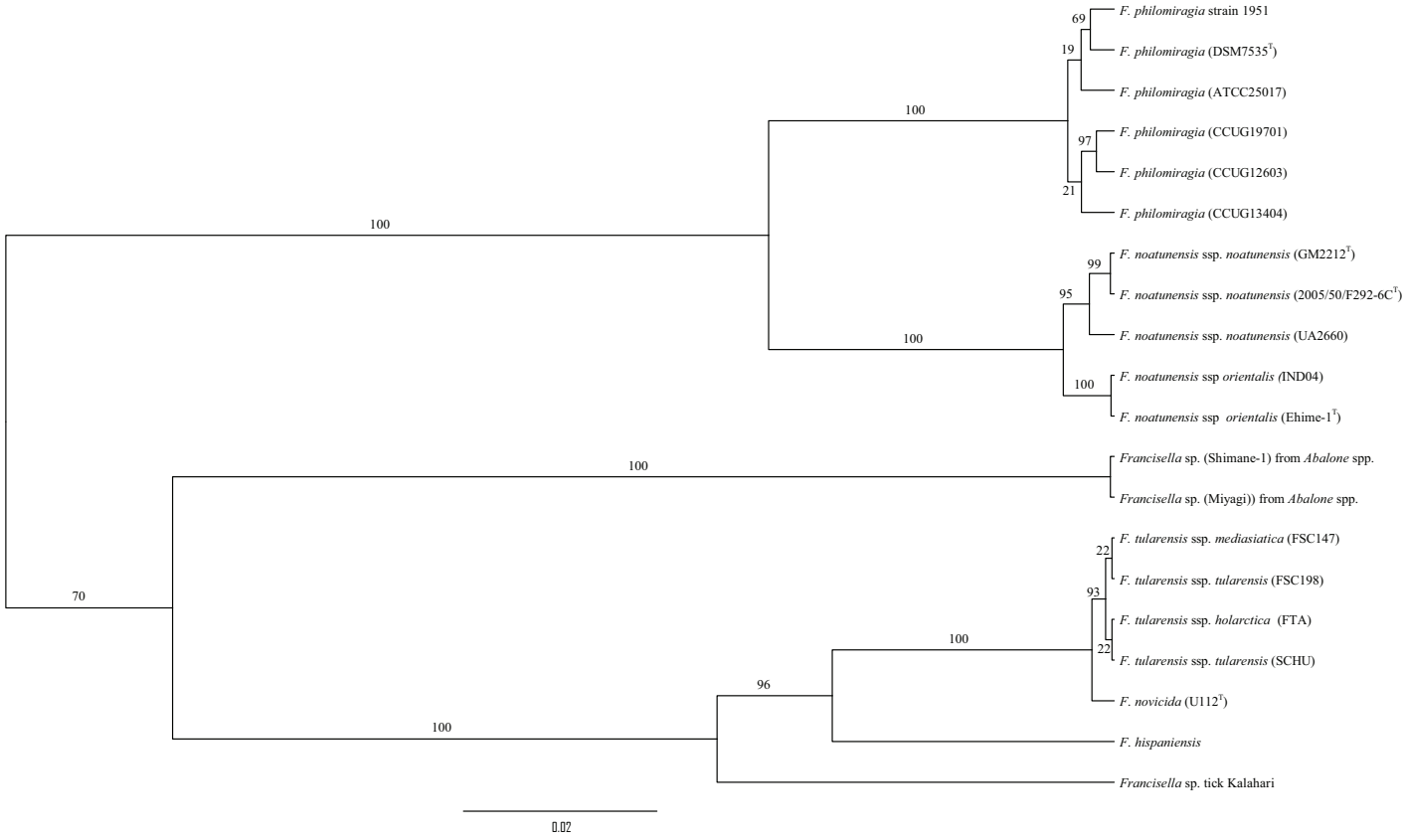

Figure 5. Phylogeny inferred from the rpoA-gene (803 nt) from some *Francisella* members (midpoint rooting). The Bayesian tree was constructed in the BEAST package v.1.6.1 (Drummond AJ, Rambaut A 2007) using the HKY model with gamma distribution and a relaxed uncorrelated lognormal molecular clock (Drummond AJ, Ho SYW, Phillips MJ & Rambaut A 2006) (N=40 000 000 generations, posterior probabilities in % are presented above branches). Phylogenetic trees were drawn using FigTree v.1.3.1 (Rambaut A (2009) Available online: http://tree.bio.ed.ac.uk/software/figtree/).

Conclusions genetic characteristics

It seems that the *Francisella* are genetically relative homogenic (Forsman et al. 1994, Nübel et al. 2006, Paper-III, Paper-V, Mikalsen et al. 2007, Mikalsen & Colquhoun 2009). Still, phylogenetic analyses of members of the *Francisella* results in clades that correspond well with the phenotypically and biochemically defined species or

subspecies. In addition there is evidence of several genotypically definable clades within the genus *Francisella,* which have not been phenotypically and biochemically defined according to conventional methods as they have not been successfully cultivated yet. There are however, strong indications that these clades correlate with an ecologically distinct niche, e.g. members of the *Francisella* associated with certain ticks (Niebylski et al. 1997, Noda et al. 1997, Scoles 2004, Sjøstedt 2005a) or preferences for different hosts and vectors as within *F. tularensis* ssp. *tularensis* (see Farlow et al. 2005). Low genetic diversity between some members within genus *Francisella*, could perhaps be explained by the fact that several of the *Francisella* spp. are obligate intracellular parasites. Obligate intracellular parasites such as *F. tularensis, F. noatunensis,* and *Francisella*-endosymbionts in ticks, seem to have limited opportunities of taking up heterologous DNA since they typically are found intracellularely except in the moment that preceeds dissemination. A possibility is that *Francisella* spp. could aquire DNA from bacteria present in phago-lysosomes within their host-cell. Evidence for recombination in genus *Francisella* is still scant. However, incongruence in a few phylogenetic trees within the *F. tularensis*-group (Nübel et al. 2006) and the *F. philomiragia*-group (Paper-V, Mikalsen et al. 2007, 2009) have been interpreted as possibly due to recombination. Preliminary analysis within the *F. philomiragia*-group by (Mikalsen 2008) using the Sequence type analysis and recombinational tests (START) software (Jolley et al. 2001), showed the presence of significant linkage disequilibrium on the gene-sets under study, indicating a principally clonal population structure. However, linkage disequilibrium may be detected in bacterial populations where recombination is frequent due to; 1) too few isolates in the analysis, 2) the isolates are separated in time, 3) isolates have different ecologies, or 4) isolates have different geographic distributions. These criteria apply to all of the isolates within the *F. philomiragia*-group studied to date, and hence possibly explain the observed linkage disequilibrium. When a slightly different house-keeping gene-set (Paper-V) are analyzed using the Recombination Detection Program 3 (RPD3) package (Martin et al. 2005), there are indications of recombinational events in several *Francisella* spp.; in the pgm- and putA genes from *F. philomiragia,*

in the prfB-gene from *F. noatunensis* ssp. *orientalis, F. tularensis* ssp. *mediasiatica* and *F. novicida* and in the rpoB-gene from *F. noatunensis* ssp. *orientalis* (Unpublished results). Although there seem to be evidences of LGT events in the draft-genomes of *F. noatunensis* (F. Nilsen Pers. Com.), and possibly of recombinational events in some house-keeping genes (Nübel et al. 2006, current study), the main underlying population structure of the few *Francisella* spp. studied to date seem to be clonal as jugded by house-keeping gene analysis (Nübel et al. 2006, Mikalsen 2008), and Variable Number of Tandem Repeat analysis (Farlow et al. 2001, Johansson et al. 2001, Johansson et al. 2004, Brevik et al. 2011). However, further investigations on recombinational or LGT events that will include members of the genus *Francisella* from a wide range of sources seem to be required before any conclusions regarding their roles in *Francisella* spp. genetics can be drawn.

Criteria for species separation in the genus *Francisella*

As jugded by several methods utilized in bacterial taxonmic considerations there are strong indications of relative high biochemical and genetical homogeneity within the genus *Francisella*, (Paper-II, Paper-III, Paper-V, Mikalsen et al. 2007, 2009, Kügeler et al. 2008). Most likely due to the stabilizing selectional forces in the intracellular environments to which they are adapted. As a consequence the criteria that are a measure of biochemical and genetic diversity, considered to be "generally applicable" in bacterial taxonomical considerations (Wayne et al. 1987, Stackebrandt et al. 2002), may not be suitable in taxonomical considerations of the genus *Francisella*. It seems however, that geographical distributions and ecological constraints such as niche or host restriction vary significantly among the members in genus *Francisella*. Hence, the different geographical distributions and ecologies of *Francisella* spp., i.e. specialization to specific hosts, seem to be useful taxonomic characters for members of this genus. Most of the factors that cause specialization of *Francisella* spp. have not yet been explored, and remains to be elucidated. Interupted or lost synthesis or enzymatic pathways, genome-architectures and the expansion of insertion sequences

have been proposed as possible causes of the specialization and niche-restriction seen in *F. tularensis* sspp. (Svensson et al. 2005, Larsson 2007, Rohmer et al. 2007, Svensson 2009). Whole genome data is currently available for only two species of the *Francisella,* namely *F. tularensis* (including *F. novicida*) and *F. philomiragia*. Whole-genome studies- and comparison of more *Francisella* spp. will not only offer unprecedented phylogenetic resolution among *Francisella* bacteria, but will most likely also reveal genomic markers that can be valuable characters in future taxonomic studies of members in the genus *Francisella*. However, until such genomic markers are detected or found in a larger number of *Francisella* spp., two *Francisella*-strains with different geographical distribution and niches, that are biochemically and genetically borderline to recognized species criteria, could be separately evolving species. This way of viewing *Francisella* spp. is very similar to the species in the classification system/method-free species concept outlined be Achtman & Wagner (2008). Implementing the classification system proposed by Achtman & Wagner (2008), could aid in the definition of several of the novel *Francisella* spp. for which most conventional taxonomic criteria seem unapplicable.

Based on differences between *F. novicida* and the subspecies of *F. tularensis* in niches and at the genomic level, in concordance with the view outlined by Achtman & Wagner (2008), Svennson et al. (2009) has proposed that it may be justifiable to keep the species status for *F. novicida* rather than reclassifying it as a subspecies in *F. tularensis*. This has led Johannson et al. (2010) to raise an objection to the proposed classification by Hüber et al. (2010) of the species *F. novicida* to subspecies rank of *F. tularensis* (Johannson et al. 2010). Although the objection by Johannson et al. (2010) will not have any consequences on the classification of *F. novicida* (Busse et al. 2010), some important issues have been raised; that there should be a place for meta-population genomics in future *Francisella* taxonomy.

Some clarifications regarding the taxonomy and nomenclature of fish-parasitic *Francisella* spp..

Fish-parasitic *Francisella* bacteria have been described as both separate species and as subspecies of their closest relatives (Paper-III, Mikalsen et al. 2007, Paper-V, Mikalsen & Colquhoun 2009). This has resulted in some confusion. The *Francisella* sp. identified in cod differed sufficiently from *F. philomiragia*, and was first described as the species *F. piscicida* (Paper-I, -II, -III, Euzeby 2008). The bacterium was simultaneously named a subspecies of that species, *F. philomiragia* ssp. *noatunensis* (Mikalsen et al. 2007), a name which was validated prior to the former. These bacteria are, however, different isolates of the same species for which the name *F. noatunensis* (syn. *F. piscicida*) has priority (Paper-V, Euzeby 2009). Whether the fish-pathogenic *Francisella* spp. isolated mainly from tilapia represents a species separate from *F. noatunensis,* however, has been somewhat uncertain. These bacteria innhabit similar niches i.e. are fish-parasites, they are serologically cross-reactive (Paper-V), almost indistinguishable by phenotype, and genetically they are border-line according to the DDH and ANI criterions for delineation of species (Paper-V, Mikalsen & Colquhoun 2009). As a result, in the current taxonomical standing, the bacterium is placed as a subspecies of *F. noatunensis,* ssp. *orientalis* (Paper-V, Euzeby 2009). Another isolate, from diseased tilapia in Costa Rica, was recently named *F. asiatica*, a name which has been used by some authors, but still awaits validation (Colquhoun et al. 2008, Mikalsen 2008, Mikalsen & Colquhoun 2009, Soto et al. 2010a, Soto et al. 2010b, Soto et al. 2011). The descriptions of *F. noatunensis* ssp. *orientalis* (Paper-V) and *F. asiatica* (Mikalsen & Colquhoun 2009) both obtained very similar results; 1) in biochemical profiles using the API rapid ID 32A & 32E and API ZYM kits (bioMérieux), and 2) in the genetic characteristics independent of the genes investigated except in the DDH results (Mikalsen 2008, Paper-V). Furthermore, the Ehime-1 strain, the type-strain of *F. noatunensis* ssp. *orientalis* (Paper-V), was included in the description by Mikalsen & Colquhoun (2009) of *F. asiatica,* and results showed the Ehime-1 strain was almost identical in phenotype and genotype to

the type-strain of *F. asiatica*, PQ1104. Characters of *F. noatunensis* ssp. *orientalis* and *F. asiatica* are summarized in table 6.

Table 6. Comparison of *F. noatunensis* ssp. *orientalis* (Paper-V) and *F. asiatica* (Mikalsen & Colquhoun 2009).

	F. noatunensis ssp. *orientalis*	*F.asiatica*
Type strain	Ehime-1 (Japan)	PQ1104 (Costa Rica)
Temperature optima for growth	25 ºC (Paper-V)	22 ºC (Mikalsen & Colquhoun 2009)
API rapid 32A & 32E, API ZYM (bioMérieux) profiles	Almost identical (Paper-V, Mikalsen & Colquhoun 2009)	
Gene-sequence similarity;		
16S rRNA, rpoA, rpoB, pgm, sdhA, groEL	100 % (Mikalsen & Colquhoun 2009)	
Average Nucleotide Identity (ANI) to:		
F. philomiragia	94.2-94.4 % (Paper-V)	93.1-93.6 % (Mikalsen & Colquhoun 2009)
F. noatunensis ssp. *noatunensis* (=*F. piscicida*)	95.5 % (Paper-V)	95.6 % (Mikalsen & Colquhoun 2009)
DNA-DNA re-association values to:		
F. philomiragia	60.8 % (57.3 % & 64.3 %) (Paper-V)	72.6 % (68.7 % & 76.5%) (Mikalsen & Colquhoun 2009)
F. noatunensis ssp. noatunensis *(=F. piscicida)*	68.7 % (64 % & 73.4%) (Paper-V)	60.3 % (58.4% & 62.1%) (Mikalsen & Colquhoun 2009)

On the basis of almost identical biochemical profiles and 100 % similarity in several gene-sequences, *F. noatunensis* ssp. *orientalis* (Paper-V) and *F. asiatica* (Mikalsen & Colquhoun 2009) can be considerer to be conspecific. Hence, according to the Bacteriological Code and the rules of priority, the epithet *orientalis* in *F. noatunensis* ssp. *orientalis* has priority over the epithet *asiatica* in *F. asiatica,* whether a

subspecies of *F. noatunensis* or a separate species. The name *F. asiatica* (Mikalsen & Colquhoun 2009) once validated must therefore be considered a later heterotypic synonym of *F. noatunensis* ssp. *orientalis* (Paper-V, Euzeby 2009).

There is however, one contoversy arising when the descriptions of *F. noatunensis* ssp. *orientalis* (Paper-V) and *F. asiatica* (Mikalsen & Colquhoun 2009) are compared. The DDH results between the pairings of *F. noatunensis* ssp. *orientalis* vs. *F. philomiragia* and *F. noatunensis* (=*F. piscicida*) in Paper-V, and the DDH results from the pairing of *F. asiatica* vs. *F. philomiragia* and *F. noatunensis* (=*F. piscicida*) in Mikalsen et al. (2009) do not correlate. The divergence exceeds the 10 % that might occur between DDH parallels (Dr. Spröer pers. com. at Deutsche Sammlung von Mikroorganismen und Zellkulturen). Several factors are known to influence the outcome when a DDH between two bacteria are performed, like a significant number of physico-chemical parameters, differing genome size, the presence of large plasmids, purity of DNA, which of the hybridized bacteria that are chosen as probe or target (Stackebrandt & Ebers 2006, Achtman & Wagner 2008). There is a one degree Celsius difference between the studies in the temperatures used in the DDH; 62 ºC in Mikalsen & Colquhoun (2009) vs. 63 ºC in Paper-V. A similar temperature difference is also present in the DDH studies between *F. noatunensis* (=*F. piscicida*) vs. *F. philomiragia* in Mikalsen et al. (2007) and in Paper-III, without this affecting the outcome significantly as jugded by the results. The small temperature difference seems therefore unlikely to make up for the huge divergence between the DDH pairing presented in Mikalsen & Colquhoun (2009) and Paper-V. The different results can be ascribed to other physico-chemical parameters than temperature or due to differences in purity of DNA from the isolates. Another plausible explanation for the divergence is that different isolates of *F. noatunensis* ssp. *orientalis* is utilized in their respective pairings where PQ1104 in Mikalsen & Colquhoun (2009) originates from Costa Rica, while the Ehime-1 isolate in Paper-V originates from Japan. Although the isolates PQ1104 and Ehime-1 (Mikalsen & Colquhoun 2009) and Ehime-1 and Ind04 (Paper-V) are indistinguishable in house-keeping gene identity, it does not rule out the possibly that these isolates may have differing genome size, or there is a presence of

large plasmids in one of the bacteria that could influence the DDH-results. However, it has previously been demonstrated that DDH-results and the ANI between two bacteria correlate (Goris et al. 2007). Mikalsen & Colquhoun (2009) points out that their obtained ANI between *F. philomiragia* and *F. asiatica* (=*F. noatunensis* ssp. *orientalis*) "are in conflict with their obtained DDH result, but that both values are border-line according to their respective criterions". If the DDH results of *F. philomiragia* vs. *F. asiatica* (=*F. noatunensis* ssp. *orientalis*) in Mikalsen & Colquhoun (2009) in stead are compared to the pairing of *F. noatunensis* sspp. *noatunensis* and *orientalis* in Paper-V and vice versa, the DDH- and the ANI results seems to make sense. Another explanation of the DDH results controversy between Paper-V and Mikalsen & Colquhoun (2009) is erroneous sample labelling or mixing of the results. Therefore, subsequent rounds of DDH at 62 °C between *F. noatunensis* ssp. *orientalis* and *F. noatunensis* ssp. *noatunensis* were performed. These resulted in an overall similarity of 69.9 % (69.7% and 70.1%) between these two bacteria (unpublished results, current study), which is almost identical to the DDH result obtained in Paper-V. However, the *F. asiatica* type strain is not yet available for study.

According to "the formal requirements" used in the current practices in the delineation of procaryotic species the taxonomic standings of *F. noatunensis* sspp. *noatunensis* (=*F. psicicida*) and *orientalis* (=*F. asiatica*) are justifiable. However, although few phenotypic characters distinguish them, they form separate phylogenetic clusters as jugded by analysis of 16S rRNA- and house-keeping gene-sequences (Paper-V, Mikalsen 2008, Mikalsen & Colquhoun 2009). Furthermore they are separated geographically. *Francisella noatunensis* ssp. *orientalis* has been identified in several warm-water fish species in a wide geographical area in Asia and America (Kamaishi et al. 2005, Hsieh et al. 2006, Hsieh et al. 2007, Ostland et al. 2006, Mauel et al. 2007). *Francisella noatunensis* ssp. *noatunensis* (=*F. piscicida*) on the other hand, have only been detected in cold-water fish (Paper-I, Paper-IV, Nylund et al. 2005, Olsen et al. 2006), and with the exception of the isolate found in Chile (Cvitanich et al. 1995, Birckbeck et al. 2007, Mikalsen 2008), seems to be limited to

the North Atlantic Ocean. On the basis of these differences, and in the light of the preceeding considerations, it is possible that the bacterium *F. noatunensis* ssp. *orientalis* (=*F. asiatica*) in the future will be elevated to the rank of species. Furthermore, on the basis of the differences between the Norwegian and the Chilean isolates of *F. noatunensis* (table 4 & 5, Mikalsen 2008, Bohle et al. 2009), like the fact that latter has not been found elsewhere than in the southern hemisphere, the Chilean isolate may represent a subspecies of *F. noatunensis.*

CONCLUSION

Several members of genus *Francisella* are influenced by major ecological constraints such as host- and niche- restrictions and these have impact on the current approaches to the *Francisella* taxonomy;

- Several of the members of genus *Francisella* appear phenotypically and genotypically homogenic according to the current approaches used in bacterial taxonomy.
- Many of the methodologies and characters used in the current approaches to bacterial taxonomy are unapplicable to several of the novel members of the genus *Francisella.*
- The cut-off values used in the delineation of bacterial species in the current approaches to bacterial taxonomy may not be suitable for members of the genus *Francisella*.
- Host restriction and ecological constraints should be recognized as important taxonomical characters for members of the genus *Francisella.*

OUTLOOK

The information available on the host-interactions, reservoirs, virulence and genomes of several of the members in the genus *Francisella* is still scarce and needs to be addressed in future studies. Adressing these issues will provide information on their mode of spread and survival, and as for *F. noatunensis* may perhaps provide crucial knowledge useful in future management strategies. Future genomic studies and or metapopulation genomic studies of the members in the genus *Francisella* will also provide insights into how these intracellular parasitic bacteria has evolved and the mechanisms underlying the virulence in their hosts. This knowledge should be useful in future development of therapeutics and combat strategies against pathogenic *Francisella* spp.. For *Francisella*-sequence isolates detected in ticks or environmental samples, efforts should be directed into the development of cultivation systems. This should facilitate their study, which in turn could contribute to resolve their taxonomy.

SUPPLEMENTARY MATERIAL

Supplementary tables 1-1 to 1-6

Supplementary table 2

Supplementary table 1-1. Table show 16S rRNA gene-sequence similarity in percent (%) between *Francisella* spp. and numerous *Francisella* sequence-isolates available from the Genbank. Strain number/Origin of sequence and accession number in the two columns to the right corresponds to the numbers 1-37 in first row.

Strain number/ Origin	Accesion no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1 novel *Francisella* sp. from Tick	AB001522																																					
2"	AF001077	99,2																																				
3"	AY375394	99,3	99,3																																			
4"	AY375395	99,1	99,1	99,4																																		
5"	AY375396	99,2	99,2	99,5	99,7																																	
6"	AY375397	99,5	99,7	99,6	99,4	99,5																																
7"	AY375398	99,5	99,7	99,6	99,4	99,5	100																															
8"	AY375399	99,1	99,3	99,2	99,0	99,1	99,6	99,6																														
9"	AY375400	99,2	99,2	99,5	99,7	99,8	99,5	99,5	99,1																													
10"	AY375402	99,5	99,3	99,4	99,2	99,3	99,6	99,6	99,2	99,3																												
11"	AY375403	99,5	99,3	99,4	99,2	99,3	99,6	99,6	99,2	99,3	99,6																											
12"	AY375404	99,2	99,2	99,5	99,7	100	99,5	99,5	99,1	99,8	99,3	99,3																										
13"	AY375407	99,2	98,7	98,8	98,6	98,7	99,0	98,9	98,6	98,7	99,0	99,1	98,7																									
14"	AY795976	97,0	96,8	96,9	96,7	96,8	97,1	97,0	96,7	96,8	97,1	97,1	96,8	96,4																								
15"	AY795977	96,9	96,7	96,8	96,6	96,7	97,0	97,0	96,6	96,7	97,0	97,0	96,7	96,3	99,9																							
16"	AY795978	97,0	96,8	96,9	96,7	96,8	97,1	97,0	96,7	96,8	97,1	97,1	96,8	96,4	100	99,9																						
17"	AY795979	99,6	99,4	99,5	99,3	99,4	99,7	99,7	99,3	99,4	99,7	99,9	99,4	99,0	97,1	97,1	97,1																					
18"	AY795981	99,6	99,4	99,5	99,3	99,4	99,7	99,7	99,3	99,4	99,7	99,9	99,4	99,0	97,1	97,1	97,1	100																				
19"	AY805304	99,5	99,3	99,4	99,2	99,3	99,6	99,6	99,2	99,3	100	99,6	99,3	99,0	97,1	97,0	97,1	99,7	99,7																			
20"	AY805305	99,5	99,3	99,4	99,2	99,3	99,6	99,6	99,2	99,3	99,6	100	99,3	99,1	97,1	97,0	97,1	99,9	99,9	99,6																		
21"	AY805306	99,5	99,3	99,4	99,2	99,3	99,6	99,6	99,2	99,3	99,6	100	99,3	99,1	97,1	97,0	97,1	99,9	99,9	99,6	100																	
22"	EU234535	99,2	98,6	98,7	98,5	98,7	98,9	98,9	98,5	98,6	98,9	98,9	98,7	98,6	96,3	96,2	96,3	99,0	99,0	98,9	98,9	98,9															
23"	JF290387	99,7	99,0	99,0	98,9	98,9	99,2	99,2	98,9	99,1	99,2	99,2	98,9	99,0	96,7	96,6	96,7	99,3	99,3	99,2	99,2	99,2	99,0															
24 novel *Francisella* sp. *Abalone* spp.	JF290369	96,8	96,1	96,4	96,2	96,3	96,4	96,4	96,0	96,3	96,4	96,4	96,3	96,4	93,9	93,8	93,9	96,5	96,5	96,4	96,4	96,4	96,4	96,5														
25"	JF290376	96,7	96,0	96,3	96,1	96,2	96,3	96,3	95,9	96,2	96,3	96,3	96,2	96,3	93,8	93,7	93,8	96,4	96,4	96,3	96,3	96,3	96,3	96,4	99,9													
26 novel *Francisella* sp. Air condition	FJ591095	95,0	94,3	94,5	94,2	94,3	94,6	94,6	94,2	94,3	94,6	94,6	94,3	94,5	92,4	92,3	92,4	94,7	94,7	94,6	94,6	94,6	94,7	94,7	95,2	95,1												
27 *F. hispaniensis*	FJ997642	99,3	98,7	98,8	98,6	98,7	99,0	99,0	98,6	98,7	99,0	99,0	98,7	98,7	96,4	96,3	96,4	99,0	99,0	99,0	99,0	99,0	99,1	99,0	96,8	96,7	95,1											
28 *F. noatunensis* ssp. *noatunensis*	DQ295795	97,5	96,9	97,0	96,8	96,8	97,1	97,1	96,8	96,8	97,1	97,1	96,8	96,9	94,6	94,5	94,6	97,2	97,2	97,1	97,1	97,1	97,1	97,2	98,6	98,5	95,5	97,7										
29 "	DQ309246	97,5	96,9	97,0	96,8	96,8	97,1	97,1	96,8	96,8	97,1	97,1	96,8	96,9	94,6	94,5	94,6	97,2	97,2	97,1	97,1	97,1	97,1	97,2	98,6	98,5	95,5	97,7	100									
30 "	EU683029	97,5	96,9	97,0	96,8	96,8	97,1	97,1	96,8	96,8	97,1	97,1	96,8	96,9	94,6	94,5	94,6	97,2	97,2	97,1	97,1	97,1	97,1	97,2	98,6	98,5	95,3	97,7	99,8	99,8								
31 *F. noatunensis* ssp. *orientalis*	DQ007456	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	98,2	98,1	95,2	97,4	99,0	99,0	99,0							
32 "	AY928393	97,1	96,5	96,6	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,5	94,2	94,1	94,2	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,1	98,0	95,1	97,3	99,0	99,0	99,0	99,9						
33 "	AY928389	97,1	96,5	96,6	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,5	94,2	94,1	94,2	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,1	98,0	95,1	97,3	99,0	99,0	99,0	99,9	99,8					
34 "	FJ217163	97,0	96,4	96,5	96,5	96,6	96,7	96,7	96,3	96,6	96,7	96,7	96,6	96,4	94,1	94,0	94,1	96,8	96,8	96,7	96,7	96,7	96,7	96,8	98,0	97,9	95,0	97,2	98,9	98,9	98,9	99,8	99,7	99,7				
35 "	DQ007455	97,1	96,5	96,6	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,5	94,2	94,1	94,2	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,1	98,0	95,1	97,3	99,0	99,0	99,0	99,9	99,8	99,8	99,7			
36 "	AY928388	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	98,2	98,1	95,2	97,4	99,0	99,0	99,0	100	99,9	99,9	99,8	99,9		
37 "	DQ473646	97,1	96,5	96,6	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,5	94,2	94,1	94,2	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,1	98,0	95,1	97,3	99,0	99,0	99,0	99,9	99,8	99,8	99,7	99,8	99,9	
38 "	EU683030	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	98,2	98,1	95,2	97,4	99,0	99,0	99,0	100	99,9	99,9	99,8	99,9	100	99,9
39 "	EU672884	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	98,2	98,1	95,2	97,4	99,0	99,0	99,0	100	99,9	99,9	99,8	99,9	100	99,9
40 "	AY828390	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	98,2	98,1	95,2	97,4	99,0	99,0	99,0	100	99,9	99,9	99,8	99,9	100	99,9

Supplementary table 1-2. Table show 16S rRNA gene-sequence similarity in percent (%) between strains 41-74 (first column) to strains 1-37 (first row).

Strain number/Origin	Accesion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
41 *F. philomiragia*	AY928395	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
42 "	DQ813266	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
43 "	EF153479	97,8	97,1	97,2	97,0	97,1	97,4	97,4	97,0	97,1	97,4	97,4	97,1	97,1	94,9	94,8	94,9	97,5	97,5	97,4	97,4	97,4	97,4	97,5	98,5	98,4	95,7	98,0	99,5	99,5	99,5	99,0	99,0	99,0	98,9	99,0	99,0	99,0
44 "	EF364049	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
45 "	EF364050	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
46 "	EU503153	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,5	97,9	99,6	99,6	99,6	99,0	99,0	99,0	98,9	99,0	99,0	99,0
47 "	EU503155	97,6	97,0	97,0	96,9	96,9	97,2	97,2	96,9	96,9	97,2	97,2	96,9	96,9	94,7	94,6	94,7	97,3	97,3	97,2	97,2	97,2	97,2	97,3	98,5	98,4	95,6	97,8	99,3	99,3	99,3	99,0	99,0	99,0	98,9	99,0	99,0	99,0
48 "	EU503156	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
49 "	EU503166	97,7	97,0	97,1	97,0	97,0	97,3	97,3	97,0	97,0	97,3	97,3	97,0	97,0	94,8	94,7	94,8	97,4	97,4	97,3	97,3	97,3	97,3	97,4	98,6	98,5	95,7	97,9	99,4	99,4	99,4	99,1	99,0	99,0	99,0	99,0	99,1	99,0
50 "	EU503168	97,8	97,1	97,2	97,0	97,1	97,4	97,4	97,0	97,1	97,4	97,4	97,1	97,1	94,9	94,8	94,9	97,5	97,5	97,4	97,4	97,4	97,4	97,5	98,5	98,4	95,7	98,0	99,5	99,5	99,5	99,0	99,0	99,0	98,9	99,0	99,0	99,0
51 novel *Francisella* sp. from seawater	EU848558	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,9	96,8	95,0	99,4	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
52 "	EU848559	97,2	96,6	96,7	96,7	96,7	96,9	96,9	96,5	96,8	96,9	96,9	96,7	96,7	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	99,1	99,0	95,0	97,2	98,9	98,9	98,9	98,5	98,4	98,4	98,3	98,4	98,5	98,4
53 "	EU848560	96,9	96,2	96,5	96,3	96,4	96,5	96,5	96,1	96,4	96,5	96,5	96,4	96,4	94,0	93,9	94,0	96,6	96,6	96,5	96,5	96,5	96,5	96,6	99,1	99,0	95,0	96,9	98,3	98,3	98,3	97,9	97,8	97,8	97,7	97,8	97,9	97,8
54 *F. tularensis* ssp. *tularensis*	AY968226	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
55 "	AY968228	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
56 *F. tularensis* ssp. *mediasiatica*	AY968233	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
57 *F. novicida*	CP000439	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
58 *F. tularensis* ssp. *holarctica*	CP000803	98,9	98,4	98,5	98,3	98,4	98,7	98,7	98,3	98,4	98,5	98,5	98,4	98,2	95,9	95,8	95,9	98,6	98,6	98,5	98,5	98,5	98,7	98,6	96,8	96,7	95,0	99,5	97,5	97,5	97,5	97,2	97,1	97,1	97,0	97,1	97,2	97,1
59 *F. tularensis* ssp. *tularensis*	NC008245	99,0	98,4	98,5	98,3	98,4	98,7	98,7	98,3	98,4	98,7	98,7	98,4	98,4	96,1	96,0	96,1	98,8	98,8	98,7	98,7	98,7	98,9	98,8	97,0	96,9	95,1	99,7	97,7	97,7	97,7	97,4	97,3	97,3	97,2	97,3	97,4	97,3
60 *F. novicida*	AY968237	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
61 novel *Francisella* sp. from human	EU031810	97,2	96,6	96,8	96,5	96,6	96,9	96,9	96,5	96,6	96,9	96,9	96,6	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	96,6	96,5	96,5	97,4	97,4	97,4	97,2	96,9	96,8	96,8	96,7	96,8	96,9	96,8
62 "	EU031811	97,2	96,6	96,8	96,5	96,6	96,9	96,9	96,5	96,6	96,9	96,9	96,6	96,6	94,3	94,2	94,3	97,0	97,0	96,9	96,9	96,9	96,9	97,0	96,6	96,5	96,5	97,4	97,4	97,4	97,2	96,9	96,8	96,8	96,7	96,8	96,9	96,8
63 Uncultivated *Francisella* sp.	AY968284	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,7	96,6	94,9	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
64 "	AY968288	97,9	97,2	97,3	97,1	97,2	97,5	97,5	97,1	97,2	97,5	97,5	97,2	97,2	95,0	94,9	95,0	97,6	97,6	97,5	97,5	97,5	97,5	97,6	97,4	97,3	95,3	98,1	98,0	98,0	97,8	97,4	97,3	97,3	97,2	97,3	97,4	97,3
65 "	AY968292	97,1	96,5	96,8	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,7	94,3	94,2	94,3	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,7	98,6	95,0	97,1	98,2	98,2	98,2	97,8	97,7	97,7	97,6	97,7	97,8	97,7
66 "	AY968295	97,0	96,4	96,7	96,5	96,6	96,7	96,7	96,3	96,6	96,7	96,7	96,6	96,6	94,2	94,1	94,2	96,8	96,8	96,7	96,7	96,7	96,7	96,8	98,6	98,5	94,9	97,0	98,1	98,1	98,1	97,7	97,6	97,6	97,5	97,6	97,7	97,6
67 "	AY968298	96,9	96,2	96,5	96,3	96,4	96,5	96,5	96,1	96,4	96,5	96,5	96,4	96,4	94,0	93,9	94,0	96,6	96,6	96,5	96,5	96,5	96,5	96,6	98,4	98,3	94,7	96,9	97,9	97,9	97,9	97,5	97,4	97,4	97,3	97,4	97,5	97,4
68 "	DQ994171	99,0	98,4	98,5	98,3	98,4	98,7	98,7	98,3	98,4	98,7	98,7	98,4	98,4	96,1	96,0	96,1	98,8	98,8	98,7	98,7	98,7	98,9	98,8	97,0	96,9	95,1	99,7	97,7	97,7	97,7	97,4	97,3	97,3	97,2	97,3	97,4	97,3
69 "	DQ994172	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
70 "	DQ994173	98,9	98,2	98,3	98,1	98,2	98,5	98,5	98,1	98,2	98,5	98,5	98,2	98,2	95,9	95,8	95,9	98,6	98,6	98,5	98,5	98,5	98,7	98,6	96,8	96,7	95,0	99,5	97,5	97,5	97,5	97,2	97,1	97,1	97,0	97,1	97,2	97,1
71 "	DQ994174	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
72 "	DQ994175	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
73 "	DQ994176	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
74 "	DQ994177	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2

Supplementary table 1-3. Table show 16S rRNA gene-sequence similarity in percent (%) between strains 38-74 (first column) to strains 38-72 (first row).

Origin	Accesion	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
38 *Fn ssp. orientalis*	EU683030																																			
39 "	EU672884	100																																		
40 "	AY828390	100	100																																	
41 *F. philomiragia*	AY928395	99,1	99,1	99,1																																
42 "	DQ813266	99,1	99,1	99,1	100																															
43 "	EF153479	99,0	99,0	99,0	99,9	99,9																														
44 "	EF364049	99,1	99,1	99,1	100	100	99,9																													
45 "	EF364050	99,1	99,1	99,1	100	100	99,9	100																												
46 "	EU503153	99,0	99,0	99,0	99,4	99,4	99,5	99,4	99,4																											
47 "	EU503155	99,0	99,0	99,0	99,9	99,9	99,8	99,9	99,9	99,5																										
48 "	EU503156	99,1	99,1	99,1	100	100	99,9	100	100	99,4	99,9																									
49 "	EU503166	99,1	99,1	99,1	100	100	99,9	100	100	99,4	99,9	100																								
50 "	EU503168	99,0	99,0	99,0	99,9	99,9	100	99,9	99,9	99,5	99,8	99,9	99,9																							
51 novel *Francisella* sp.	EU848558	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9																						
52 "	EU848559	98,5	98,5	98,5	98,9	98,9	98,8	98,9	98,9	98,9	98,8	98,9	98,9	98,8	97,1																					
53 "	EU848560	97,9	97,9	97,9	98,3	98,3	98,2	98,3	98,3	98,3	98,2	98,3	98,3	98,2	97,0	99,0																				
54 *Ft* ssp. *tularensis*	AY968226	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,8	97,1	97,0																			
55 "	AY968228	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,8	97,1	97,0	100																		
56 *Ft* ssp. *mediasiatica*	AY968233	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,8	97,1	97,0	100	100																	
57 *F. novicida*	CP000439	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,8	97,1	97,0	100	100	100																
58 *Ft* ssp. *holarctica*	CP000803	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,7	97,6	97,7	97,7	97,8	99,7	97,0	96,9	99,9	99,9	99,9	99,9															
59 *Ft* ssp. *tularensis*	NC008245	97,4	97,4	97,4	97,9	97,9	98,0	97,9	97,9	97,9	97,8	97,9	97,9	98,0	99,7	97,2	97,0	99,9	99,9	99,9	99,9	99,8														
60 *F. novicida*	AY968237	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,8	97,1	97,0	100	100	100	100	99,9	99,9													
61 novel *Francisella* sp.	EU031810	96,9	96,9	96,9	97,2	97,2	97,1	97,2	97,2	97,4	97,1	97,2	97,2	97,1	97,5	96,9	96,9	97,7	97,7	97,7	97,7	97,6	97,6	97,7												
62 "	EU031811	96,9	96,9	96,9	97,2	97,2	97,1	97,2	97,2	97,4	97,1	97,2	97,2	97,1	97,5	96,9	96,9	97,7	97,7	97,7	97,7	97,6	97,6	97,7	100											
63 Uncult. *Francisella* sp.	AY968284	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	96,8	99,8	99,8	99,8	99,8	99,7	99,7	99,8	97,5	97,5										
64 "	AY968288	97,4	97,4	97,4	97,8	97,8	97,7	97,8	97,8	98,0	97,7	97,8	97,8	97,7	98,2	97,7	97,7	98,4	98,4	98,4	98,4	98,3	98,3	98,4	98,7	98,7	98,2									
65 "	AY968292	97,8	97,8	97,8	98,2	98,2	98,1	98,2	98,2	98,2	98,1	98,2	98,2	98,1	96,9	98,8	99,5	96,9	96,9	96,9	96,9	96,8	97,0	96,9	96,8	96,8	96,9	97,6								
66 "	AY968295	97,7	97,7	97,7	98,1	98,1	98,0	98,1	98,1	98,1	98,0	98,1	98,1	98,0	96,8	98,7	99,4	96,8	96,8	96,8	96,8	96,7	96,9	96,8	96,7	96,7	96,8	97,5	99,9							
67 "	AY968298	97,5	97,5	97,5	97,9	97,9	97,8	97,9	97,9	97,9	97,8	97,9	97,9	97,8	96,6	98,5	99,2	96,6	96,6	96,6	96,6	96,5	96,7	96,6	96,5	96,5	96,6	97,3	99,7	99,6						
68 "	DQ994171	97,4	97,4	97,4	97,9	97,9	98,0	97,9	97,9	97,9	97,8	97,9	97,9	98,0	99,7	97,2	97,0	99,9	99,9	99,9	99,9	99,8	100	99,9	97,6	97,6	99,7	98,3	97,0	96,9	96,7					
69 "	DQ994172	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9				
70 "	DQ994173	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,7	97,6	97,7	97,7	97,8	99,5	97,0	96,9	99,7	99,7	99,7	99,7	99,6	99,8	99,7	97,4	97,4	99,5	98,1	96,8	96,7	96,5	99,8	99,9			
71 "	DQ994174	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,7		
72 "	DQ994175	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	100	99,9	99,8	
73 "	DQ994176	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,8	99,9	99,6	99,8
74 "	DQ994177	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,7	100	99,8

Supplementary table 1-4. Table show 16S rRNA gene-sequence similarity in percent (%) between strains 75-113 (first column) to strains 1-37 (first row).

Strain number/Origin	Accesion no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
75 Uncultivated *Francisella* sp.	DQ994178	98,9	98,2	98,3	98,1	98,2	98,5	98,5	98,1	98,2	98,5	98,5	98,2	98,2	95,9	95,8	95,9	98,6	98,6	98,5	98,5	98,5	98,7	98,6	96,8	96,7	95,0	99,5	97,5	97,5	97,5	97,2	97,1	97,1	97,0	97,1	97,2	97,1
76 "	DQ994180	98,6	97,9	98,0	97,8	97,9	98,2	98,2	97,8	97,9	98,2	98,2	97,9	97,9	95,6	95,5	95,6	98,3	98,3	98,2	98,2	98,2	98,4	98,3	96,7	96,6	94,8	99,2	97,2	97,2	97,2	97,1	97,0	97,0	97,0	97,0	97,1	97,0
77 "	DQ994181	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
78 "	DQ994182	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
79 "	DQ994184	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
80 "	DQ994185	98,6	97,9	98,0	97,8	97,9	98,2	98,2	97,8	98,0	98,2	98,2	97,9	97,9	95,8	95,7	95,8	98,3	98,3	98,2	98,2	98,2	98,4	98,3	96,5	96,4	94,7	99,2	97,2	97,2	97,2	97,0	96,9	96,9	96,8	96,9	97,0	96,9
81 "	DQ994186	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
82 "	DQ994187	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
83 "	DQ994188	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
84 "	DQ994189	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
85 "	DQ994190	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	97,0	97,0	95,1	99,6	97,6	97,6	97,6	97,5	97,4	97,4	97,3	97,4	97,5	97,4
86 "	DQ994191	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
87 "	DQ994192	98,5	97,8	97,9	97,7	97,8	98,1	98,1	97,7	97,9	98,1	98,1	97,8	97,8	95,7	95,6	95,7	98,2	98,2	98,1	98,1	98,1	98,3	98,2	96,4	96,3	94,6	99,1	97,1	97,1	97,1	96,9	96,8	96,8	96,7	96,8	96,9	96,8
88 "	DQ994193	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,9	96,8	95,0	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
89 "	DQ994194	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
90 "	DQ994196	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
91 "	DQ994197	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	97,0	97,0	95,1	99,6	97,6	97,6	97,6	97,5	97,4	97,4	97,3	97,4	97,5	97,4
92 "	DQ994198	98,8	98,1	98,2	98,0	98,1	98,4	98,4	98,0	98,1	98,4	98,4	98,1	98,1	95,8	95,7	95,8	98,5	98,5	98,4	98,4	98,4	98,6	98,5	96,7	96,6	94,9	99,4	97,4	97,4	97,4	97,1	97,0	97,0	97,0	97,0	97,1	97,0
93 "	EU924182	98,2	98,2	98,5	98,5	98,6	98,5	98,5	98,1	98,6	98,3	98,3	98,6	97,8	95,7	95,6	95,7	98,4	98,4	98,3	98,3	98,3	97,5	97,9	95,0	95,0	93,2	97,6	95,8	95,8	95,8	95,7	95,6	95,6	95,5	95,6	95,7	95,6
94 "	EU924183	98,2	98,2	98,5	98,5	98,6	98,5	98,5	98,1	98,6	98,3	98,3	98,6	97,8	95,7	95,6	95,7	98,4	98,4	98,3	98,3	98,3	97,5	97,9	95,0	95,0	93,2	97,6	95,8	95,8	95,8	95,7	95,6	95,6	95,5	95,6	95,7	95,6
95 Uncult. Gammaproteobacteria	AY968283	99,0	98,3	98,4	98,2	98,3	98,6	98,6	98,2	98,3	98,6	98,6	98,3	98,3	96,0	95,9	96,0	98,7	98,7	98,6	98,6	98,6	98,8	98,7	96,7	96,6	94,9	99,6	97,6	97,6	97,6	97,3	97,2	97,2	97,1	97,2	97,3	97,2
96 "	AY968285	98,9	98,2	98,3	98,1	98,2	98,5	98,5	98,1	98,2	98,5	98,5	98,2	98,2	96,1	96,0	96,1	98,6	98,6	98,5	98,5	98,5	98,7	98,6	96,6	96,5	94,8	99,5	97,5	97,5	97,5	97,2	97,1	97,1	97,0	97,1	97,2	97,1
97 "	AY968286	99,0	98,4	98,5	98,3	98,4	98,7	98,7	98,3	98,4	98,7	98,7	98,4	98,4	96,1	96,0	96,1	98,8	98,8	98,7	98,7	98,7	98,9	98,8	96,8	96,7	95,1	99,7	97,5	97,5	97,5	97,2	97,1	97,1	97,0	97,1	97,2	97,1
98 "	AY968287	98,0	97,3	97,4	97,2	97,3	97,6	97,6	97,2	97,3	97,6	97,6	97,3	97,3	95,0	95,0	95,0	97,7	97,7	97,6	97,6	97,6	97,6	97,7	97,5	97,4	95,4	98,2	98,1	98,1	97,9	97,5	97,4	97,4	97,3	97,4	97,5	97,4
99 "	AY968289	97,8	97,1	97,2	97,0	97,1	97,4	97,4	97,0	97,1	97,4	97,4	97,1	97,1	94,9	94,8	94,9	97,5	97,5	97,4	97,4	97,4	97,4	97,5	97,3	97,2	95,2	98,0	97,9	97,9	97,7	97,3	97,2	97,2	97,1	97,2	97,3	97,2
100 "	AY968290	97,8	97,1	97,2	97,0	97,1	97,4	97,4	97,0	97,1	97,4	97,4	97,1	97,1	94,9	94,8	94,9	97,5	97,5	97,4	97,4	97,4	97,4	97,5	97,3	97,2	95,2	98,0	97,9	97,9	97,7	97,3	97,2	97,2	97,1	97,2	97,3	97,2
101 "	AY968291	97,1	96,5	96,8	96,6	96,7	96,8	96,8	96,4	96,7	96,8	96,8	96,7	96,7	94,3	94,2	94,3	96,9	96,9	96,8	96,8	96,8	96,8	96,9	98,7	98,6	95,0	97,1	98,2	98,2	98,2	97,8	97,7	97,7	97,6	97,7	97,8	97,7
102 "	AY968293	97,0	96,4	96,7	96,5	96,6	96,7	96,7	96,3	96,6	96,7	96,7	96,6	96,6	94,2	94,1	94,2	96,8	96,8	96,7	96,7	96,7	96,7	96,8	98,6	98,5	94,9	97,0	98,1	98,1	98,1	97,7	97,6	97,6	97,5	97,6	97,7	97,6
103 "	AY968294	97,0	96,4	96,7	96,5	96,6	96,7	96,7	96,3	96,6	96,7	96,7	96,6	96,6	94,2	94,1	94,2	96,8	96,8	96,7	96,7	96,7	96,7	96,8	98,6	98,5	94,9	97,0	98,1	98,1	98,1	97,7	97,6	97,6	97,5	97,6	97,7	97,6
104 "	AY968296	97,0	96,4	96,7	96,5	96,7	96,7	96,7	96,3	96,6	96,7	96,7	96,7	96,6	94,2	94,1	94,2	96,8	96,8	96,7	96,7	96,7	96,7	96,8	98,6	98,5	94,9	97,0	98,1	98,1	98,1	97,7	97,6	97,6	97,5	97,6	97,7	97,6
105 "	AY968297	96,9	96,2	96,5	96,3	96,4	96,5	96,5	96,1	96,4	96,5	96,5	96,4	96,4	94,0	93,9	94,0	96,6	96,6	96,5	96,5	96,5	96,5	96,6	98,4	98,3	94,7	96,9	97,9	97,9	97,9	97,5	97,4	97,4	97,3	97,4	97,5	97,4
106 "	AY968299	96,8	96,1	96,4	96,2	96,3	96,4	96,4	96,0	96,3	96,4	96,4	96,3	96,3	93,9	93,8	93,9	96,5	96,5	96,4	96,4	96,4	96,4	96,5	98,3	98,2	94,6	96,8	97,8	97,8	97,8	97,4	97,3	97,3	97,2	97,3	97,4	97,3
107 "	AY968300	96,8	96,1	96,4	96,2	96,3	96,4	96,4	96,0	96,3	96,4	96,4	96,3	96,3	93,9	93,8	93,9	96,5	96,5	96,4	96,4	96,4	96,4	96,5	99,0	99,0	94,9	96,8	98,2	98,2	98,2	97,8	97,7	97,7	97,6	97,7	97,8	97,7
108 "	AY968301	97,6	97,0	97,0	96,9	96,9	97,2	97,2	96,9	96,9	97,2	97,2	96,9	96,9	94,7	94,6	94,7	97,3	97,3	97,2	97,2	97,2	97,2	97,3	98,3	98,2	95,5	97,8	99,3	99,3	99,3	98,9	98,8	98,8	98,7	98,8	98,9	98,8
109 "	AY968302	97,5	96,9	97,0	96,8	96,8	97,1	97,1	96,8	96,8	97,1	97,1	96,8	96,9	94,6	94,5	94,6	97,2	97,2	97,1	97,1	97,1	97,1	97,2	98,2	98,1	95,4	97,7	99,2	99,2	99,2	98,8	98,7	98,7	98,6	98,7	98,8	98,7
110 *Wolbachia persica*	M21292	98,8	98,2	98,3	98,1	98,2	98,5	98,5	98,1	98,2	98,5	98,5	98,2	98,2	96,1	96,0	96,1	98,6	98,6	98,5	98,5	98,5	98,7	98,6	96,7	96,6	95,2	99,1	97,6	97,6	97,6	97,2	97,1	97,1	97,0	97,1	97,2	97,1
111 *Candidatus* F. noatunensis ssp.	FN398155	97,0	96,4	96,5	96,5	96,6	96,7	96,7	96,3	96,6	96,7	96,7	96,6	96,7	94,2	94,1	94,2	96,8	96,8	96,7	96,7	96,7	96,7	96,8	99,5	99,4	95,0	97,0	98,7	98,7	98,7	98,3	98,2	98,2	98,1	98,2	98,3	98,2
112 *Fangia hongkongensis*	AB176554	89,7	89,2	89,4	89,4	89,5	89,5	89,5	89,1	89,4	89,5	89,7	89,5	89,5	87,6	87,5	87,6	89,6	89,6	89,5	89,7	89,7	89,6	89,4	89,0	89,0	88,8	89,9	89,0	89,0	89,0	88,8	88,7	88,7	88,6	88,7	88,8	88,8
113 *Caedibacter taernospiralis*	AY102612	88,2	87,7	87,9	87,9	88,0	88,0	88,0	87,7	87,9	88,0	88,2	87,9	87,9	86,1	86,0	86,1	88,1	88,1	88,0	88,2	88,2	88,0	87,9	87,4	87,3	88,4	88,4	87,7	87,7	87,7	87,5	87,6	87,4	87,3	87,4	87,5	87,5

Supplementary table 1-5. Table show 16S rRNA gene-sequence similarity in percent (%) between strains 75-113 (first column) to strains 38-72 (first row).

Strain number/Origin	Accesion no.	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
75 Uncultivated *Francisella* sp.	DQ994178	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,7	97,6	97,7	97,7	97,8	99,5	97,0	96,9	99,7	99,7	99,7	99,7	99,6	99,8	99,7	97,4	97,4	99,5	98,1	96,8	96,7	96,5	99,8	99,7	99,8	99,7	99,7
76 "	DQ994180	97,1	97,1	97,1	97,6	97,6	97,5	97,6	97,6	97,4	97,5	97,6	97,6	97,5	99,2	97,0	96,8	99,4	99,4	99,4	99,4	99,3	99,5	99,4	97,3	97,3	99,2	98,0	96,7	96,6	96,4	99,5	99,4	99,5	99,4	99,4
77 "	DQ994181	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,8	99,9	99,6	99,8
78 "	DQ994182	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,9	99,8	99,8
79 "	DQ994184	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,8	99,9	99,6	99,8
80 "	DQ994185	97,0	97,0	97,0	97,4	97,4	97,5	97,4	97,4	97,4	97,3	97,4	97,4	97,5	99,2	96,8	96,6	99,4	99,4	99,4	99,4	99,3	99,5	99,4	97,1	97,1	99,2	97,8	96,5	96,4	96,2	99,5	99,4	99,5	99,4	99,4
81 "	DQ994186	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,7	99,8	99,8
82 "	DQ994187	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,6	99,5	99,6	99,6
83 "	DQ994188	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,9	99,8	99,8
84 "	DQ994189	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,6	99,5	99,6	99,6
85 "	DQ994190	97,5	97,5	97,5	98,0	98,0	97,9	98,0	98,0	97,8	97,9	98,0	98,0	97,9	99,6	97,3	97,1	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,7	97,7	99,6	98,4	97,0	97,0	96,8	99,9	99,8	99,7	99,8	99,8
86 "	DQ994191	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,8	99,9	99,6	99,8
87 "	DQ994192	96,9	96,9	96,9	97,3	97,3	97,4	97,3	97,3	97,3	97,2	97,3	97,3	97,4	99,1	96,7	96,5	99,3	99,3	99,3	99,3	99,2	99,4	99,3	97,0	97,0	99,1	97,7	96,4	96,3	96,1	99,4	99,3	99,4	99,3	99,3
88 "	DQ994193	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	97,0	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,5	97,5	99,6	98,2	96,9	96,8	96,6	99,9	99,8	99,7	99,8	99,8
89 "	DQ994194	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,6	99,5	99,6	99,6
90 "	DQ994196	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,6	99,5	99,6	99,6
91 "	DQ994197	97,5	97,5	97,5	98,0	98,0	97,9	98,0	98,0	97,8	97,9	98,0	98,0	97,9	99,6	97,3	97,1	99,8	99,8	99,8	99,8	99,7	99,9	99,8	97,7	97,7	99,6	98,4	97,0	97,0	96,8	99,9	99,8	99,7	99,8	99,8
92 "	DQ994198	97,1	97,1	97,1	97,6	97,6	97,7	97,6	97,6	97,6	97,5	97,6	97,6	97,7	99,4	97,0	96,8	99,6	99,6	99,6	99,6	99,5	99,7	99,6	97,3	97,3	99,4	98,0	96,7	96,6	96,4	99,7	99,8	99,9	99,6	99,8
93 "	EU924182	95,7	95,7	95,7	96,0	96,0	96,1	96,0	96,0	96,0	95,9	96,0	96,0	96,1	97,0	95,5	95,1	97,2	97,2	97,2	97,2	97,3	97,3	97,2	95,5	95,5	97,2	96,2	95,4	95,3	95,1	97,3	97,2	97,1	97,2	97,2
94 "	EU924183	95,7	95,7	95,7	96,0	96,0	96,1	96,0	96,0	96,0	95,9	96,0	96,0	96,1	97,0	95,5	95,1	97,2	97,2	97,2	97,2	97,3	97,3	97,2	95,5	95,5	97,2	96,2	95,4	95,3	95,1	97,3	97,2	97,1	97,2	97,2
95 Uncult. gammaproteobacteria	AY968283	97,3	97,3	97,3	97,8	97,8	97,9	97,8	97,8	97,8	97,7	97,8	97,8	97,9	99,6	97,1	96,8	99,8	99,8	99,8	99,8	99,7	99,7	99,8	97,5	97,5	99,8	98,2	96,9	96,8	96,6	99,7	99,6	99,5	99,6	99,6
96 "	AY968285	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,7	97,6	97,7	97,7	97,8	99,5	97,0	96,7	99,7	99,7	99,7	99,7	99,6	99,6	99,7	97,4	97,4	99,7	98,1	96,8	96,7	96,5	99,6	99,5	99,4	99,5	99,5
97 "	AY968286	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,7	97,6	97,7	97,7	97,8	99,7	97,0	96,9	99,9	99,9	99,9	99,9	99,8	99,8	99,9	97,6	97,6	99,7	98,3	97,0	96,9	96,7	99,8	99,7	99,6	99,7	99,7
98 "	AY968287	97,5	97,5	97,5	97,9	97,9	97,8	97,9	97,9	98,1	97,8	97,9	97,9	97,8	98,3	97,8	97,8	98,5	98,5	98,5	98,5	98,4	98,4	98,5	98,8	98,8	98,3	99,9	97,7	97,6	97,4	98,4	98,3	98,2	98,3	98,3
99 "	AY968289	97,3	97,3	97,3	97,7	97,7	97,6	97,7	97,7	97,9	97,6	97,7	97,7	97,6	98,1	97,6	97,6	98,3	98,3	98,3	98,3	98,2	98,2	98,3	98,6	98,6	98,1	99,9	97,5	97,4	97,2	98,2	98,1	98,0	98,1	98,1
100 "	AY968290	97,3	97,3	97,3	97,7	97,7	97,6	97,7	97,7	97,9	97,6	97,7	97,7	97,6	98,1	97,6	97,6	98,3	98,3	98,3	98,3	98,2	98,2	98,3	98,6	98,6	98,1	99,9	97,5	97,4	97,2	98,2	98,1	98,0	98,1	98,1
101 "	AY968291	97,8	97,8	97,8	98,2	98,2	98,1	98,2	98,2	98,2	98,1	98,2	98,2	98,1	96,9	98,8	99,5	96,9	96,9	96,9	96,9	96,8	97,0	96,9	96,8	96,8	96,9	97,6	100	99,9	99,7	97,0	96,9	96,8	96,9	96,9
102 "	AY968293	97,7	97,7	97,7	98,1	98,1	98,0	98,1	98,1	98,1	98,0	98,1	98,1	98,0	96,8	98,7	99,4	96,8	96,8	96,8	96,8	96,7	96,9	96,8	96,7	96,7	96,8	97,5	99,9	99,8	99,6	96,9	96,8	96,7	96,8	96,8
103 "	AY968294	97,7	97,7	97,7	98,1	98,1	98,0	98,1	98,1	98,1	98,0	98,1	98,1	98,0	96,8	98,7	99,4	96,8	96,8	96,8	96,8	96,7	96,9	96,8	96,7	96,7	96,8	97,5	99,9	99,8	99,6	96,9	96,8	96,7	96,8	96,8
104 "	AY968296	97,7	97,7	97,7	98,1	98,1	98,0	98,1	98,1	98,1	98,0	98,1	98,1	98,0	96,8	98,7	99,4	96,8	96,8	96,8	96,8	96,7	96,9	96,8	96,7	96,7	96,8	97,5	99,9	99,8	99,6	96,9	96,8	96,7	96,8	96,8
105 "	AY968297	97,5	97,5	97,5	97,9	97,9	97,8	97,9	97,9	97,8	97,8	97,9	97,9	97,8	96,6	98,5	99,2	96,6	96,6	96,6	96,6	96,5	96,7	96,6	96,5	96,5	96,6	97,3	99,7	99,6	99,4	96,7	96,6	96,5	96,6	96,6
106 "	AY968299	97,4	97,4	97,4	97,8	97,8	97,7	97,8	97,8	97,8	97,7	97,8	97,8	97,7	96,5	98,4	99,1	96,5	96,5	96,5	96,5	96,4	96,6	96,5	96,4	96,4	96,5	97,2	99,6	99,5	99,3	96,6	96,5	96,4	96,5	96,5
107 "	AY968300	97,8	97,8	97,8	98,2	98,2	98,1	98,2	98,2	98,2	98,1	98,2	98,2	98,1	96,9	99,0	99,9	96,9	96,9	96,9	96,9	96,8	97,0	96,9	96,8	96,8	96,7	97,6	99,4	99,3	99,1	97,0	96,9	96,8	96,9	96,9
108 "	AY968301	98,9	98,9	98,9	99,7	99,7	99,8	99,7	99,7	99,3	99,6	99,7	99,7	99,8	97,7	98,6	98,0	97,7	97,7	97,7	97,7	97,6	97,8	97,7	97,0	97,0	97,7	97,5	97,9	97,8	97,6	97,8	97,7	97,6	97,7	97,7
109 "	AY968302	98,8	98,8	98,8	99,6	99,6	99,7	99,6	99,6	99,2	99,5	99,6	99,6	99,7	97,6	98,5	97,9	97,6	97,6	97,6	97,6	97,5	97,7	97,6	96,9	96,9	97,6	97,4	97,8	97,7	97,5	97,7	97,6	97,5	97,6	97,6
110 *Wolbachia persica*	M212292	97,2	97,2	97,2	97,7	97,7	97,8	97,7	97,7	97,8	97,6	97,7	97,7	97,8	98,6	97,2	96,7	98,7	98,7	98,7	98,7	98,7	98,8	98,7	97,1	97,1	98,7	97,8	97,0	96,9	96,7	98,8	98,7	98,7	98,7	98,7
111 *Candidatus* F. noatunensis	FN398155	98,3	98,3	98,3	98,7	98,7	98,6	98,7	98,7	98,7	98,6	98,7	98,7	98,6	97,1	99,4	99,0	97,1	97,1	97,1	97,1	97,0	97,2	97,1	96,9	96,9	97,0	97,7	98,6	98,5	98,3	97,2	97,1	97,0	97,1	97,1
112 *Fangia hongkongensis*	AB176554	88,8	88,8	88,8	89,3	89,3	89,4	89,3	89,3	89,0	89,3	89,3	89,3	89,4	89,5	89,0	89,3	89,7	89,7	89,7	89,7	89,6	89,7	89,7	89,1	89,1	89,6	89,0	89,2	89,1	89,0	89,7	89,6	89,5	89,6	89,6
113 *Caedibacter taernospiralis*	AY102612	87,5	87,5	87,5	87,7	87,7	87,8	87,7	87,7	87,7	87,7	87,7	87,7	87,8	88,0	87,7	87,5	88,2	88,2	88,2	88,2	88,1	88,2	88,2	88,4	88,4	88,1	87,8	87,6	87,5	87,3	88,2	88,1	88,0	88,1	88,1

Supplementary table 1-6. Table show 16S rRNA gene-sequence similarity in percent (%) between strains 73-113 (first column) to strains 1-112 (first row).

Stran number/Origin	Accesion no.	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
73 "	DQ994176																																								
74 "	DQ994177	99,6																																								
75 "	DQ994178	99,7	99,7																																							
76 "	DQ994180	99,4	99,4	99,5																																						
77 "	DQ994181	99,8	99,6	99,7	99,4																																					
78 "	DQ994182	99,8	99,8	99,9	99,6	99,8																																			
79 "	DQ994184	99,8	99,6	99,7	99,4	99,8	99,8																																		
80 "	DQ994185	99,4	99,4	99,5	99,2	99,4	99,6	99,4																																	
81 "	DQ994186	99,6	99,8	99,7	99,4	99,6	99,8	99,6	99,4																																
82"	DQ994187	99,4	99,6	99,5	99,2	99,4	99,6	99,4	99,2	99,6																															
83 "	DQ994188	99,8	99,8	99,9	99,6	99,8	100	99,8	99,6	99,8	99,6																														
84 "	DQ994189	99,4	99,6	99,5	99,2	99,4	99,6	99,4	99,2	99,6	99,4	99,6																													
85 "	DQ994190	99,6	99,8	99,7	99,6	99,6	99,8	99,6	99,4	99,8	99,6	99,8	99,6																												
86 "	DQ994191	99,8	99,6	99,7	99,4	99,8	99,8	99,8	99,4	99,6	99,4	99,8	99,4	99,6																											
87 "	DQ994192	99,3	99,3	99,4	99,1	99,3	99,5	99,3	99,9	99,3	99,1	99,5	99,1	99,3	99,3																										
88 "	DQ994193	99,6	99,8	99,7	99,4	99,6	99,8	99,6	99,4	100	99,6	99,8	99,6	99,8	99,6	99,3																									
89 "	DQ994194	99,4	99,6	99,5	99,2	99,4	99,6	99,4	99,2	99,6	100	99,6	99,4	99,6	99,4	99,1	99,6																								
90 "	DQ994196	99,4	99,6	99,5	99,2	99,4	99,6	99,4	99,2	99,6	99,4	99,6	100	99,6	99,4	99,1	99,6	99,4																							
91 "	DQ994197	99,6	99,8	99,7	99,6	99,6	99,8	99,6	99,4	99,8	99,6	99,8	99,6	100	99,6	99,3	99,8	99,6	99,6																						
92 "	DQ994198	99,8	99,6	99,7	99,4	99,8	99,8	99,8	99,4	99,6	99,4	99,8	99,4	99,6	100	99,3	99,6	99,4	99,4	99,6																					
93 "	EU924182	97,0	97,2	97,1	96,9	97,0	97,2	97,0	96,9	97,2	97,0	97,2	97,0	97,2	97,0	96,8	97,2	97,0	97,0	97,2	97,0																				
94 "	EU924183	97,0	97,2	97,1	96,9	97,0	97,2	97,0	96,9	97,2	97,0	97,2	97,0	97,2	97,0	96,8	97,2	97,0	97,0	97,2	97,0	99,2																			
95Uncult. gam.proteobacteria	AY968283	99,4	99,6	99,5	99,2	99,4	99,6	99,6	99,2	99,6	99,4	99,6	99,4	99,6	99,4	99,1	99,6	99,4	99,4	99,6	99,4	97,2	97,2																		
96 "	AY968285	99,3	99,5	99,4	99,1	99,3	99,5	99,3	99,1	99,5	99,3	99,5	99,4	99,5	99,3	99,0	99,5	99,3	99,4	99,5	99,3	97,1	97,1	99,7																	
97 "	AY968286	99,5	99,7	99,6	99,3	99,5	99,7	99,5	99,3	99,7	99,5	99,7	99,5	99,7	99,5	99,2	99,7	99,5	99,5	99,7	99,5	97,3	97,3	99,7	99,6																
98 "	AY968287	98,1	98,3	98,2	98,1	98,1	98,3	98,1	97,9	98,3	98,1	98,3	98,1	98,5	98,1	97,8	98,3	98,1	98,1	98,5	98,1	96,3	96,3	98,3	98,2	98,4															
99 "	AY968289	97,9	98,1	98,0	97,9	97,9	98,1	97,9	97,7	98,1	97,9	98,1	97,9	98,3	97,9	97,6	98,1	97,9	97,9	98,3	97,9	96,1	96,1	98,1	98,0	98,2	99,8														
100 "	AY968290	97,9	98,1	98,0	97,9	97,9	98,1	97,9	97,7	98,1	97,9	98,1	97,9	98,3	97,9	97,6	98,1	97,9	97,9	98,3	97,9	96,1	96,1	98,1	98,0	98,2	99,8	99,8													
101 "	AY968291	96,7	96,9	96,8	96,7	96,7	96,9	96,7	96,5	96,9	96,7	96,9	96,7	97,0	96,7	96,4	96,9	96,7	96,7	97,0	96,7	95,4	95,4	96,9	96,8	97,0	97,7	97,5	97,5												
102 "	AY968293	96,6	96,8	96,7	96,6	96,6	96,8	96,6	96,4	96,8	96,6	96,8	96,6	97,0	96,6	96,3	96,8	96,6	96,6	97,0	96,6	95,3	95,3	96,8	96,7	96,9	97,6	97,4	97,4	99,9											
103 "	AY968294	96,6	96,8	96,7	96,6	96,6	96,8	96,6	96,4	96,8	96,6	96,8	96,6	97,0	96,6	96,3	96,8	96,6	96,6	97,0	96,6	95,3	95,3	96,8	96,7	96,9	97,6	97,4	97,4	99,9	99,8										
104 "	AY968296	96,6	96,8	96,7	96,6	96,6	96,8	96,6	96,4	96,8	96,6	96,8	96,6	97,0	96,6	96,3	96,8	96,6	96,6	97,0	96,6	95,3	95,3	96,8	96,7	96,9	97,6	97,4	97,4	99,9	99,8	99,8									
105 "	AY968297	96,4	96,6	96,5	96,4	96,4	96,6	96,4	96,2	96,6	96,4	96,6	96,4	96,8	96,4	96,1	96,6	96,4	96,4	96,8	96,4	95,1	95,1	96,6	96,5	96,7	97,4	97,2	97,2	99,7	99,6	99,6	99,6								
106 "	AY968299	96,3	96,5	96,4	96,3	96,3	96,5	96,3	96,1	96,5	96,3	96,5	96,3	96,7	96,3	96,0	96,5	96,3	96,3	96,7	96,3	95,2	95,2	96,5	96,4	96,6	97,3	97,1	97,1	99,6	99,5	99,5	99,5	99,3							
107 "	AY968300	96,7	96,9	96,8	96,7	96,7	96,9	96,7	96,5	96,9	96,7	96,9	96,7	97,0	96,7	96,4	96,9	96,7	96,7	97,0	96,7	95,0	95,0	96,7	96,6	96,8	97,7	97,5	97,5	99,4	99,3	99,3	99,3	99,1	99,0						
108 "	AY968301	97,5	97,7	97,6	97,3	97,5	97,7	97,5	97,5	97,7	97,5	97,7	97,5	97,7	97,5	97,4	97,7	97,5	97,5	97,7	97,5	95,9	95,9	97,7	97,6	97,6	97,6	97,4	97,4	97,9	97,8	97,8	97,8	97,6	97,5	97,9					
109 "	AY968302	97,4	97,6	97,5	97,2	97,4	97,6	97,4	97,4	97,6	97,4	97,6	97,4	97,6	97,4	97,3	97,6	97,4	97,4	97,6	97,4	95,8	95,8	97,6	97,5	97,5	97,5	97,3	97,3	97,8	97,7	97,7	97,7	97,5	97,4	97,8	99,9				
110 *Wolbachia persica*	M21292	98,6	98,7	98,7	98,4	98,6	98,7	98,6	98,4	98,7	98,6	98,7	98,6	98,7	98,6	98,3	98,7	98,6	98,6	98,7	98,6	97,1	97,1	98,7	98,7	98,8	97,9	97,7	97,7	97,0	96,9	96,9	96,9	96,7	96,6	96,6	97,6	97,5			
111 *Candidatus* F. noatunensis	FN398155	97,0	97,1	97,0	97,0	97,0	97,1	97,0	96,8	97,1	97,0	97,1	97,0	97,3	97,0	96,7	97,1	97,0	97,0	97,3	97,0	95,3	95,3	97,0	96,9	97,0	97,8	97,6	97,6	98,6	98,5	98,5	98,5	98,3	98,2	99,0	98,4	98,3	97,0		
112 *Fangia hongkongensis*	AB176554	89,4	89,6	89,5	89,2	89,4	89,6	89,4	89,2	89,6	89,6	89,6	89,4	89,6	89,4	89,2	89,6	89,6	89,4	89,6	89,4	88,3	88,5	89,6	89,5	89,8	89,0	89,0	88,9	89,2	89,1	89,1	89,1	89,0	88,9	89,2	89,2	89,1	89,6	89,4	
113 *Caedibacter taernospiralis*	AY102612	87,9	88,1	88,0	87,7	87,9	88,1	87,9	87,7	88,1	88,1	88,1	87,9	88,1	87,9	87,7	88,1	88,1	87,9	88,1	87,9	86,8	87,0	88,1	88,0	88,3	87,9	87,7	87,7	87,6	87,5	87,5	87,5	87,5	87,3	87,4	87,7	87,6	88,2	87,6	93,4

Supplementary table 2. Table show gene-sequence similarity in percent (%) between some *Francisella* spp.; *Francisella philomiragia* (1=strain 1951, 2=ATCC25017, 3=CCUG12603, 4=CCUG13404, 5=CCUG19701, 6=DSM7535^{T}), *F. noatunensis* ssp. *orientalis* (7=Ehime-1, 8=Ind04), *F. noatunensis* ssp. *noatunensis* (9=2005/50/F292-6C^{T}, 10=GM2212^{T} (=*F. piscicida*), 11=UA2660), *F. tularensis* subsp. *tularensis* (12=strain FSC 198, 13=strain SCHU), *F. tularensis* subsp. *holarctica* (14=strain FTA), *F. tularensis* subsp. *mediasiatica* (15=strain FSC 148) and *F. novicida* (16=strain U112). **A**) concatenated gene-sequences (15687 nt) consisting of the partial Open Reading Frame from; **B**) the dnaA-gene (1305 nt), **C**) the mdh-gene (576 nt), **D**) the mutS-gene (2328 nt), **E**) the pgm-gene (1515 nt), **F**) the prfB-gene (882 nt), **G**) the putA-gene (3789 nt), **H**) the rpoA-gene (870 nt), **I**) the rpoB-gene (3918 nt) and **J**) the tpiA (507 nt).

A) Average Nucleotide Identity (ANI) of concatanated gene-sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	98,61														
3	98,67	98,48													
4	98,90	98,43	98,76												
5	98,88	98,60	98,70	98,87											
6	98,53	98,28	98,41	98,51	98,47										
7	94,24	94,45	94,36	94,38	94,34	94,29									
8	94,25	94,46	94,38	94,40	94,35	94,29	99,99								
9	96,86	96,84	96,81	96,82	96,65	96,58	95,56	95,57							
10	96,86	96,84	96,81	96,82	96,65	96,58	95,56	95,57	100						
11	96,75	96,74	96,69	96,71	96,55	96,47	95,47	95,48	99,67	99,67					
12	83,79	83,77	83,76	83,77	83,66	83,73	83,37	83,38	83,61	83,61	83,53				
13	83,80	83,78	83,77	83,78	83,67	83,74	83,36	83,37	83,62	83,62	83,55	99,95			
14	83,77	83,74	83,73	83,75	83,64	83,70	83,30	83,31	83,60	83,60	83,51	99,73	99,78		
15	83,78	83,74	83,75	83,76	83,66	83,71	83,34	83,35	83,62	83,62	83,54	99,78	99,79	99,66	
16	83,91	83,85	83,83	83,85	83,74	83,81	83,41	83,43	83,59	83,59	83,52	98,58	98,60	98,49	98,50

B) dnaA-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	98,62														
3	99,39	98,93													
4	99,16	98,70	99,46												
5	99,92	98,70	99,31	99,23											
6	99,92	98,54	99,31	99,16	99,85										
7	94,25	94,41	94,41	94,41	94,33	94,18									
8	94,25	94,41	94,41	94,41	94,33	94,18	100								
9	94,10	94,10	94,25	94,25	94,18	94,02	99,23	99,23							
10	94,10	94,10	94,25	94,25	94,18	94,02	99,23	99,23	100						
11	94,25	94,25	94,41	94,41	94,33	94,18	99,31	99,31	99,77	99,77					
12	86,51	86,05	86,59	86,97	86,59	86,51	85,67	85,67	85,59	85,59	85,67				
13	86,51	86,05	86,59	86,97	86,59	86,51	85,67	85,67	85,59	85,59	85,67	100			
14	86,36	85,90	86,44	86,82	86,44	86,36	85,67	85,67	85,59	85,59	85,67	99,85	99,85		
15	86,51	86,05	86,59	86,97	86,59	86,51	85,82	85,82	85,75	85,75	85,82	99,69	99,69	99,69	
16	86,59	86,13	86,67	87,05	86,67	86,59	85,98	85,98	85,90	85,90	85,98	99,31	99,31	99,16	99,01

C) mdh-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	98,96														
3	98,78	99,48													
4	98,96	99,65	99,48												
5	98,61	99,31	99,13	99,65											
6	98,78	99,48	99,31	99,48	99,13										
7	96,35	96,01	95,83	96,01	96,01	96,18									
8	96,35	96,01	95,83	96,01	96,01	96,18	100								
9	96,18	96,18	96,01	96,18	96,18	96,35	98,78	98,78							
10	96,18	96,18	96,01	96,18	96,18	96,35	98,78	98,78	100						
11	95,83	95,83	95,66	95,83	95,83	96,01	98,44	98,44	99,65	99,65					
12	84,38	84,55	84,72	84,72	84,90	85,07	83,68	83,68	83,68	83,68	83,33				
13	84,38	84,55	84,72	84,72	84,90	85,07	83,68	83,68	83,68	83,68	83,33	100			
14	84,38	84,55	84,72	84,72	84,90	85,07	83,68	83,68	83,68	83,68	83,33	100	100		
15	84,38	84,55	84,72	84,72	84,90	85,07	83,68	83,68	83,68	83,68	83,33	100	100	100	
16	84,55	84,72	84,90	84,90	85,07	85,24	83,85	83,85	83,85	83,85	83,51	98,96	98,96	98,96	98,96

D) mutS-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	98,58														
3	98,63	98,67													
4	98,84	98,45	98,50												
5	98,67	98,45	98,32	99,05											
6	99,01	98,71	98,84	98,88	98,97										
7	92,53	92,61	92,87	92,53	92,78	92,96									
8	92,53	92,61	92,87	92,53	92,78	92,96	100								
9	97,03	96,91	97,12	96,99	96,95	96,99	93,47	93,47							
10	97,04	96,91	97,12	96,99	96,95	96,99	93,47	93,47	100						
11	96,91	96,78	96,99	96,86	96,82	96,86	93,43	93,43	99,79	99,79					
12	76,72	76,68	76,89	76,85	76,80	76,80	76,42	76,42	76,75	76,76	76,63				
13	76,72	76,68	76,89	76,85	76,80	76,80	76,42	76,42	76,75	76,76	76,63	100			
14	76,63	76,59	76,80	76,76	76,72	76,72	76,50	76,50	76,67	76,68	76,55	99,66	99,66		
15	76,68	76,63	76,85	76,80	76,76	76,76	76,46	76,46	76,71	76,72	76,59	99,83	99,83	99,74	
16	76,42	76,33	76,50	76,46	76,42	76,50	76,29	76,29	76,06	76,07	75,95	97,12	97,12	97,04	97,21

E) pgm-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	96,24														
3	98,35	95,91													
4	99,27	96,30	98,55												
5	98,94	96,24	98,35	99,14											
6	98,68	95,91	98,61	98,88	98,68										
7	95,38	95,91	95,12	95,51	95,38	95,05									
8	95,38	95,91	95,12	95,51	95,38	95,05	100								
9	95,12	95,78	94,92	95,31	95,18	94,85	97,89	97,89							
10	95,12	95,78	94,92	95,31	95,18	94,85	97,89	97,89	100						
11	94,92	95,51	94,65	95,12	94,92	94,65	97,69	97,69	99,54	99,54					
12	87,39	87,85	87,59	87,52	87,46	87,59	87,66	87,66	87,46	87,46	87,13				
13	87,39	87,85	87,59	87,52	87,46	87,59	87,66	87,66	87,46	87,46	87,13	100			
14	87,13	87,59	87,39	87,26	87,19	87,33	87,39	87,39	87,19	87,19	86,86	99,60	99,60		
15	87,39	87,72	87,59	87,52	87,46	87,59	87,66	87,66	87,46	87,46	87,13	99,87	99,87	99,47	
16	87,52	87,92	87,66	87,59	87,52	87,66	87,72	87,72	87,66	87,66	87,33	98,55	98,55	98,42	98,42

F) prfB-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	97,62														
3	95,92	96,94													
4	96,83	98,07	96,71												
5	97,28	99,66	97,05	98,19											
6	98,07	99,09	97,39	97,17	98,75										
7	93,42	94,56	94,44	94,78	94,67	94,22									
8	93,42	94,56	94,44	94,78	94,67	94,22	100								
9	93,54	93,31	93,31	93,42	93,20	93,54	94,10	94,10							
10	93,54	93,31	93,31	93,42	93,20	93,54	94,10	94,10	100						
11	93,42	93,20	93,20	93,31	93,08	93,42	93,99	93,99	99,66	99,66					
12	86,17	85,60	85,94	85,60	85,49	85,83	84,92	84,92	86,28	86,28	86,17				
13	86,17	85,60	85,94	85,60	85,49	85,83	84,92	84,92	86,28	86,28	86,17	100			
14	86,28	85,71	85,83	85,71	85,60	85,94	84,81	84,81	86,17	86,17	86,05	99,55	99,55		
15	85,94	85,37	85,71	85,37	85,26	85,60	84,69	84,69	86,05	86,05	85,94	99,77	99,77	99,32	
16	87,53	86,96	87,19	86,85	86,73	87,19	85,71	85,71	86,05	86,05	85,94	95,35	95,35	95,12	95,12

G) putA-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	98,71														
3	98,68	98,65													
4	99,02	98,47	99,05												
5	98,60	98,10	98,81	98,63											
6	97,78	98,15	98,05	98,18	97,55										
7	96,33	96,46	96,73	96,67	96,31	96,15									
8	96,38	96,52	96,78	96,73	96,36	96,15	99,95								
9	98,47	98,34	98,47	98,36	97,92	97,55	96,44	96,49							
10	98,47	98,34	98,47	98,36	97,92	97,55	96,44	96,49	100						
11	98,34	98,20	98,34	98,23	97,78	97,41	96,30	96,36	99,66	99,66					
12	82,13	82,29	82,13	82,00	81,82	82,08	81,45	81,50	82,29	82,29	82,26				
13	82,13	82,29	82,13	82,00	81,82	82,08	81,45	81,50	82,29	82,29	82,26	100			
14	82,21	82,32	82,21	82,07	81,89	82,11	81,45	81,50	82,37	82,37	82,34	99,68	99,68		
15	82,19	82,29	82,19	82,05	81,87	82,08	81,45	81,50	82,34	82,34	82,31	99,76	99,76	99,71	
16	82,34	82,40	82,29	82,21	81,97	82,24	81,50	81,55	82,45	82,45	82,42	99,26	99,26	99,21	99,29

H) rpoA-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	99,50														
3	99,25	99,25													
4	99,13	99,38	99,13												
5	99,38	99,63	99,63	99,50											
6	99,38	99,38	99,13	99,25	99,50										
7	92,65	92,90	92,65	93,03	93,03	92,78									
8	92,65	92,90	92,65	93,03	93,03	92,78	100								
9	93,03	93,28	93,03	93,15	93,40	93,15	99,13	99,13							
10	93,03	93,28	93,03	93,15	93,40	93,15	99,13	99,13	100						
11	92,90	93,15	92,90	93,03	93,28	93,03	99,00	99,00	99,63	99,63					
12	83,81	84,06	83,81	84,06	84,06	83,69	83,81	83,81	84,06	84,06	83,94				
13	83,81	84,06	83,81	84,06	84,06	83,69	83,81	83,81	84,06	84,06	83,94	100			
14	83,81	84,06	83,81	84,06	84,06	83,69	83,81	83,81	84,06	84,06	83,94	100	100		
15	83,81	84,06	83,81	84,06	84,06	83,69	83,81	83,81	84,06	84,06	83,94	100	100	100	
16	83,94	84,18	83,94	84,18	84,18	83,81	83,94	83,94	84,18	84,18	84,06	99,75	99,75	99,75	99,75

I) rpoB-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	99,31														
3	99,00	98,98													
4	99,03	98,75	98,80												
5	99,13	99,46	98,85	98,72											
6	98,77	98,70	98,49	98,72	98,57										
7	92,98	93,11	92,83	92,83	92,93	92,88									
8	92,98	93,11	92,83	92,83	92,93	92,88	100								
9	98,70	98,60	98,57	98,57	98,42	98,47	92,62	92,62							
10	98,70	98,60	98,57	98,57	98,42	98,47	92,62	92,62	100						
11	98,57	98,52	98,44	98,44	98,34	98,34	92,55	92,55	99,62	99,62					
12	87,60	87,47	87,31	87,49	87,34	87,37	87,42	87,42	87,09	87,09	87,03				
13	87,60	87,47	87,31	87,49	87,34	87,37	87,42	87,42	87,09	87,09	87,03	100			
14	87,49	87,37	87,21	87,39	87,24	87,26	87,29	87,29	87,01	87,01	86,91	99,80	99,80		
15	87,52	87,39	87,24	87,42	87,26	87,29	87,29	87,29	87,01	87,01	86,96	99,72	99,72	99,57	
16	87,54	87,42	87,21	87,39	87,29	87,26	87,34	87,34	87,06	87,06	87,01	99,00	99,00	98,85	98,77

J) tpiA-gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	99,01														
3	99,01	99,21													
4	98,42	98,62	98,22												
5	99,01	98,82	98,42	98,22											
6	95,07	95,27	94,87	94,87	94,87										
7	93,89	94,08	93,69	93,69	93,69	95,66									
8	93,89	94,08	93,69	93,69	93,69	95,66	100								
9	94,87	95,07	94,67	94,67	94,67	95,86	97,63	97,63							
10	94,87	95,07	94,67	94,67	94,67	95,86	97,63	97,63	100						
11	94,87	95,07	94,67	94,67	94,67	95,86	97,63	97,63	100	100					
12	78,50	78,11	78,11	77,71	78,11	77,91	77,51	77,51	78,70	78,70	78,70				
13	78,50	78,11	78,11	77,71	78,11	77,91	77,51	77,51	78,70	78,70	78,70	100			
14	78,70	78,30	78,30	77,91	78,30	78,11	77,71	77,71	78,90	78,90	78,90	99,41	99,41		
15	78,70	78,30	78,30	77,91	78,30	78,11	77,71	77,71	78,90	78,90	78,90	99,80	99,80	99,21	
16	78,70	78,30	78,30	77,91	78,30	77,71	77,32	77,32	78,50	78,50	78,50	98,82	98,82	98,22	98,62

LITERATURE CITED

Abd H, Johansson T, Golovliov I, Sanström G, Forsman M (2003) Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl Envirion Microbiol 69: 600 – 606.

Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6:431-440.

Aikimbaev MA (1966) Taxonomy of Genus *Francisella.* Rep Acad Sci Kaz SSR Ser Biol 5:42-44. In Russian.

Alfjorden A, Jansson E, Johansson KE (2006) A systemic granulomatous inflammatory disease in wild Atlantic cod, *Gadus morhua* associated with a bacterium of the genus *Francisella*. Dipnet Newsletter 44.

Alfjorden A, Mo TA, Madsen L (2009) Annex 6: Provide a review of new diagnostic techniques for *Francisella* sp. and progress in vaccine development, and an update on the susceptibility of species other than cod to this pathogen (ToR b) in: ICES WGPDMO REP pp 55-58.

Almendras FE, Jones SR, Fuentealba C, Wright GM (1997) In vitro infection of a cell line from *Ictalurus nebulosus* with *Piscirickettsia salmonis*. Can J Vet Res. 61:66-8.

Anda P, del Pozo JS, Garcia JMD et al. (2001) Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis 3: 575 – 582.

Anthony LD, Burke RD, Nano FE (1991) Growth of *Francisella* spp. in rodent macrophages. Infect Immun 59:3291-6.

Arkush KD, McBride AM, Mendonca HL, Okihiro MS, Andree KB, Marshall S, Henriquez V, Hedrick RP (2005) Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*. Dis Aquat Organ. 63:139-49.

Athanassopoulou F, Groman D, Prapas TH, Sabatakou O (2004) Pathological and epidemiological observations on rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece. Jour Appl Ichtyol 20: 525-529.

Baldo L, Bordenstein S, Wernegreen JJ, Werren JH (2006) Widespread recombination throughout *Wolbachia* genomes. Mol Biol Evol 2:437-449.

Barns SM, Grow CC, Okinaka RT, Keim P, Kuske CR (2005) Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl Envir Microbiol 71: 5494 – 5500.

Bell JF (1980) Tularemia, Section A: Bacterial, Rickettsial and Mycotic (In: CRC Handbook series in zoonoses, 2nd edn: ed: Steele JM) CRC Press; Boca Raton; pp:161–193.

Bergey DH, Harrison FC, Breed RS, Hammer BW, Huntoon FM (ed.) (1923). Bergey's manual of determinative bacteriology. A key for the identification of organisms of the class Schizomycetes, 1st edn. The Williams & Wilkins Co. Baltimore.

Berdal BP, Mehl R, Meidell NK, Lorentzen-Styr AM, Scheel O (1996) Field investigations of tularemia in Norway. FEMS Immunol Med Microbiol 3:191-5.

Berrada ZL, Telford SR 3rd (2009) Diversity of *Francisella* species in environmental eamples from Martha's Vineyard, Massachusetts. Microb Ecol 2:277-283.

Birkbeck TH, Bordevik M, Frøystad MK, Baklien A (2007) Identification of *Francisella* sp. from Atlantic salmon, Salmo salar L., in Chile. J Fish Dis 8:505-7.

Bohle H, Tapia E, Martínez A, Rozas M, Figueroa A, Bustos P (2009) *Francisella philomiragia,* bacteria asociada con altas mortalidades en salmones del Atlántico (*Salmo salar*) cultivados en balsas-jaulas en el lago Llanquihue. Arch Med Vet 41:237-244. In Spanish.

Bordenstein SR, Reznikoff WS (2005) Mobile DNA in obligate intracellular bacteria. Nat Rev Microbiol 9:688-699.

Bosshard PP, Abels S, Zbinden R, Bottger EC, Altwegg M (2003) Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 41:4134-4140.

Brenner DJ (1973) Deoxyribonuclic acid reassociation in the taxonomy of enteric bacteria. Int J Syst Bacteriol 23: 298-307.

Brenner DJ, Staley JT, Krieg NR (2005) Classification of procaryotic organisms and the concept of bacterial speciation (Vol 2 A in: Bergeys Manual of Systematic Bacteriology, 2nd edn. ed: GM Garrity). Springer, USA. pp. 27-38.

Bricknell IR, Bron JE, Bowden TJ (2006) Diseases of gadoid fish in cultivation: a review. ICES J Mar Sci 63: 253-266.

Brevik OJ (2007) Polygenetic and phenotypic characterization of *Alivibrio logei*-like isolates from Atlantic cod (*Gadus morhua*). Master of Science thesis, Department of Biology, University of Bergen.

Brevik OJ, Ottem KF, Nylund A (2011) Multiple-locus, variable-number of tandem repeat analysis (MLVA) of the fish pathogen *Francisella noatunensis* BMC Vet Res 1:5.

Busse HJ, Hüber B, Anda P, Escudero R, Scholz HC, Seibold E, Splettstoesser WD, Kämpfer P (2010) Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis* – response to Johansson *et al*. Int J Syst Evol Microbiol 60: 1718 - 1720.

Casadevall A (2008) Evolution of intracellular pathogens. Annu Rev Microbiol 62:19-33.

Chen SC, Tung MC, Chen SP, Tsai JF, Wang PC, Chen RS, Lin SC, Adams A (1994) Sytematic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochromis niloticus* (L.), from southern Taiwan. J Fish Dis 17: 591 – 599.

Chen MF, Yun S, Marty GD, McDowell TS, House ML, Appersen JA, Guenther TA, Arkush KD, Hedrick RP (2000). A Piscirickettsia salmonis-like bacterium associated with mortality of white seabass *Astractoscion nobilis*. Dis Aquat Org 43: 117 – 126.

Chern RS, Chao CB (1994) Outbreaks of a disease caused by rickettsia-like organism in cultured tilapias in Taiwan. Fish Pathol 29: 61 – 71.

Christensen H, Kuhnert P, Busse HJ, Wilhelm C, Frederiksen WC, Bisgaard M (2007) Proposed minimal standards for the description of genera, species and subspecies of the Pasteurellaceae. Int J Syst Evol Microbiol 57:166-178.

Clarridge JE , Raich TJ, Sjösted A, Sandström G, Darouiche RO, Shawar RM, Georghiou PR, Osting C, Vo L (1996) Characterization of two unusual clinically significant *Francisella* strains. J Clin Microbiol 34:1995-2000.

Clemens DL, Lee BY, Horwitz MA (2004) Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect Immun. 2004 72: 3204-3217.

Cohan FM (2002) What are bacterial species? Annu Rev Microbiol 56:457-87.

Colquhoun DJ, Hovland H, Hellberg H, Haug T, Nilsen H. (2004) *Moritella viscosa* isolated from farmed Atlantic cod (*Gadus morhua*). Bull Eur Ass Fish Pathol 24: 109-114.

Colquhoun DJ, Aarflot L, Melvold CF (2007) gyrA and parC Mutations and associated quinolone resistance in Vibrio anguillarum serotype O2b strains isolated from farmed Atlantic cod (Gadus morhua) in Norway. Antimicrob Agents Chemother 51:2597-9.

Colquhoun DJ (2008) Infections with *Francisella* spp. in wild and farmed populations of fish. ICES CM 2008/D:07.

Colwell RR (2009) Viable but not cultivatable bacteria (in: Uncultivated Microorganisms, ed: Epstein SS) Springer, Germany pp. 121-129.

Comps M, Raymond MJ, Plassiart GN (1996) Rickettsia-like organism infecting juvenile sea-bass *Dicentrarchus labrax.* Bull Eur Assoc Fish Pathol 16:30–33.

Corbeil S, Hyatt AD, Crane MStJ (2005) Characterisation of an emerging rickettsia-like organism in Tasmanian farmed Atlantic salmon *Salmo salar*. Dis Aquat Org 64: 37-44.

Cvitanich JD, Garate ON., Smith CE (1991) The isolation of a rickettsia-like organism causing disease and mortality in Chilean Salmonids and its confirmation by Koch's postulate. J Fish Dis 14: 121-145.

Davies AJ (1986) A rickettsia-like organism from dragonets, *Callionymus lyra* L. (Teleosti: Callionymidae) in Wales. Bull Eur Assoc Fish Pathol 6:103

De Silva SS, Subashinge RP, Barthley DM, Lowther A (2004) Tilapias as alien aquatics in Asia and the Pacific. FAO Fisheries Technical Paper. No. 453 Rome, FAO 65p.

DeQuirez K (2005) Different species problems and their resolution. Bio Ess 27:1263-1269.

Dijkshoorn L, Ursing BM, Ursing JB (2000) Strain, clone and species: comments on three basic concepts of bacteriology. J Med Microbiol 49:397-401.

Dobzhansky T, Ayala FJ, Stebbins GL, Valentine JW (1977) Evolution. 1st edn. W.H. Freeman, San Francisco.

Doolittle F, Papke T (2006) Genomics and the species problem. Gen Biol 7:116.

Dorofeev KA (1947) Classification of the causative agent of tularemia. Symp Res Wor Inst Epidem and Microbiol Chita 1:170-180.

Downs CM, Bond GC (1935) Studies on the Cultural Characteristics of *Pasteurella tularense.* J Bacteriol 5: 485–490.

Duodu S, Colquhoun D (2010) Monitoring the survival of fish-pathogenic *Francisella* in water microcosms. FEMS Microbiol Ecol 3:534-541.

Dykhuizen D (2005) Species numbers in bacteria. Proc Cali. Acad Sci. 56:62–71.

Dykhuizen D, Green L (1991) Recombination in *Escherichia coli* and the definition of biological species. J Bacteriol 173:7257-7268.

Engelsen AR, Sandlund N, Fiksdal IU, Bergh Ø (2008) Immunohistochemistry of Atlantic cod larvae *Gadus morhua* experimentally challenged with *Vibrio anguillarum*. Dis Aquat Org 80:13-20.

Euzeby 2008 List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 58: 1-2.

Euzeby 2009 List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 58: 55-56.

Farlow J, Smith KL, Wong J, Abrams M, Lytle M, Keim P (2001) *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. J Clin Microbiol 39:3186-92.

Farlow J, Wagner DM, Dukerich M, Stanley M, Chu M, Kubota K, Petersen J, Keim P (2005) *Francisella tularensis* in the United States Emerg Infect Dis 12:1835-41.

Feil EJ, Maiden MCJ, Achtman M, Spratt BG (1999) The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. Mol Biol Evol 16:1496-1502.

Feil EJ, Enright MC, Spratt BG (2000) Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*. Res Microbiol 151:465-469.

Fournier PE, Dumler JS, Greub G, Zhang J, Wu Y, Raoult D (2003) Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. J Clin Microbiol 41:5456-5465.

Forsman M, Sandström G, Jaurin B (1990) Identification of *Francisella* species and discrimination of type A and type B strains of *F. tularensis* by 16S rRNA analysis. Appl Environ Microbiol 4:949-955.

Forsman M, Sandstrom G, Sjostedt A (1994) Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int J Syst Bacteriol 44: 38 - 46.

Fox G E, Wisotzkey JD, Jurtshuk P (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42**:**166-170

Francis E, Evans AC (1926) Agglutination, cross-agglutination, and agglutinin absorption in tularaemia. Public Health Rep 41:1273-1295.

Francis E (1941) Fermentation of sugars by *Bacterium tularense*. J Bacteriol 3:343-346.

Fraser C, Hange B, Spratt B (2007) Recombination and the nature of bacterial speciation. Sci 325:476-480.

Friis-Møller A, Lemming LE, Valerius NH, Bruun B (2004) Problems in identification of *Francisella philomiragia* associated with fatal bacteremia in a patient with chronic granulomatous disease J Clin Microbiol 4:1840-1842.

Fryer JL, Lannan CN, Garcès LH, Larenas JJ, Smith PA (1990) Isolation of a rickettsia-like organism from diseased coho salmon *Oncorhynchus kisutch* in Chile. Fish Pathol 25: 107–114.

Fryer JL, Lannan CN, Giovannoni SJ, Wood ND (1992) *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes Int J Syst Bacteriol Jan 42: 120 - 126.

Fryer JL, Hedrick RP (2003) *Piscirickettsia salmonis*: a Gram-negative intracellular bacterial pathogen of fish J Fish Dis 5:251-262.

Fryer JL, Mauel M (1997) The Rickettsia: an emerging group of pathogens in fish. Emerg Infect Dis 3:137-144

Fukuda Y, Okamura A, Nishiyama M, Kawakami H, Kamaishi T, Yoshinaga T (2002) Granulomatosis of cultured three-line grunt *Parapristipoma trilineatum* caused by an intracellular bacterium. Fish Pathol 37:119-124.

Garcés LH, Larenas JJ, Smith PA, Sandino S, Lannan CN, Fryer JL (1991) Infectivity of a rickettsia isolated from coho salmon *(Oncorhynchus kisutch*). Dis Aquat Org 11:93–97.

Garcia J, Urquhart K, Ellis AE (2006) Infectious pancreatic necrosis virus establishes an symptomatic carrier state in kidney leucocytes of juvenile Atlantic cod, *Gadus morhua* L. J Fish Dis 7:409-413.

Gillis M, Vandamme P, De Vos P, Swings J, KErsters K (2005) Polyphasic taxonomy (Vol 2 A in: Bergeys Manual of Systematic Bacteriology, 2nd edn. Ed: GM Garrity). Springer, USA. pp. 43-48.

Goethert HK, Telford SR (2009) Nonrandom distribution of vector ticks (*Dermacentor variabilis*) infected by *Francisella tularensis.*PLoS Pathog 2: e1000319.

Gogarten J, Townsend J (2005) Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol 3:679-687.

Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjöstedt A. (2003) An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells.Infect Immun 10:5940-5950.

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81-91.

Greco D, Allegrini G, Tizzi T, Ninu E, Lamanna A, Luzi S (1987) A waterborne tularemia outbreak. Eur J Epidemiol 3:35–38.

Gudmundsdóttir BK, Björnsdóttir B, Gudmundsdóttir S, Bambir SH (2006) A comparative study of susceptibility and induced pathology of cod, Gadus morhua (L.), and halibut, Hippoglossus hippoglossus (L.), following experimental infection with Moritella viscosa. J Fish Dis 8:481-7.

Gurycová D (1998) First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. Eur J Epidemiol 8:797-802.

Hanage W, Fraser C, Spratt B (2005) Fuzzy species among recombinogenic bacteria. BMC Biol 3:6.

Hall JD, Craven RR, Fuller JR, Pickles RJ, Kawula TH (2007) *Francisella tularensis* replicates within alveolar type II epithelial cells in vitro and in vivo following inhalation. Infect Immun 2:1034-9.

Hoel T, Scheel O, Nordahl SH, Sandvik T (1991) Water- and airborne *Francisella tularensis* biovar *palaearctica* isolated from human blood. Infection 5:348-50.

Hollis DG, Weaver RE, Steigerwalt AG, Wenger JD, Moss CW, Brenner DJ (1989) *Francisella philomiragia* comb. Nov. (Formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (Formerly *Francisella novicida*) associated with human disease. J Clin Microbiol 27 7: 1601 – 1608.

Hsieh C.Y., Tung, M.C., Tu, C., Chang, C.D. and Tsai, S.S. (2006) Enzootics of visceral granulomas associated with *Francisella*-like organism infection in tilapia (*Oreochromis* spp.). Aquaculture 254:129–138.

Hsieh C.Y., Wu ZB, Tung MC, Tsai SS (2007) PCR and in-situ hybridization for the detection and localization of a new *Francisella*-like bacterium (FLB) in ornamental cichlids. Dis Aquat Org 75:29-35

Jantzen E, Berdal BP, Omland T (1979) Cellular fatty acid composition of *Francisella tularensis*. J Clin Microbiol. 6:928-30.

Jeffery KR, Stone D, Feist SW, Verner-Jeffreys DW (2010) An outbreak of disease caused by *Francisella* sp. in Nile tilapia *Oreochromis niloticus* at a recirculation fish farm in the UK. Dis Aquat Org 91: 161–165

Jellison WL, Kohls GM, Butler WJ , Weaver JA (1942) Epizootic tularemia in the beaver *Castor canadensis*, and the contamination of stream water with *Pasteurella tularensis*. Amer J Hyg 36:168-182.

Jellison WL, Epler DC, Kuhns E, Kohls GM (1950) Tularemia in man from a domestic rural water supply. Pub Health Rep 38:1219-26.

Jensen WI, Owen CR, Jellison WL (1969) *Yersinia philomiragia* sp.n., a new member of the *Pasturella* group of bacteria, naturally pathogenic for the muskrat (*Ondatra zibethica*). J Bacteriol 100: 1237 – 1241.

Johansson A, Ibrahim A, Göransson I, Eriksson U, Gurycova D, Clarrigde JE, Sjöstedt A (2000) Evaluation of PCR-Based Methods for Discrimination of *Francisella* Species and Subspecies and Development of a Specific PCR That Distinguishes the Two Major Subspecies of *Francisella tularensis.* J Clin Microbiol 38: 4180–4185.

Johansson A, Göransson I, Larsson P, Sjöstedt A (2001) Extensive allelic variation among *Francisella tularensis* strains in a short-sequence tandem repeat region. J Clin Microbiol 9: 3140–3146.

Johansson A, Farlow J, Larsson P, Dukerich M, Chambers E, Byström M, Fox J, Chu M, Forsman M, Sjöstedt A, Keim P (2004) Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol 2004:5808–5818.

Johansson A, Celli J, Conlan W, Elkins KL, Forsman M, Keim PS, Larsson P, Manoil C, Nano FE, Petersen JM, Sjöstedt A (2010) Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis.* Int J Syst Evol Microbiol 60: 1717 - 1718.

Johnson JL (1973) Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. Int J Syst Bacteriol 23: 308 - 315.

Jolley KA, Feil EJ, Chan MS, Maiden MC (2001) Sequence type analysis and recombinational tests (START). Bioinformat 12:1230-1231.

Kamaishi T, Fukuda Y, Nishiyama M, Kawakami H, Matsuyama T, Yoshinaga T, Oseko N (2005) Identification and pathogenicity of intracellular *Francisella* bacterium in three-line Grunt *Parapristipoma trilineatum*. Fish Pathol 40: 67 – 71.

Kamaishi T, Miwa S, Goto E, Matsuyama T, Oseko N (2010) Mass mortality of giant abalone *Haliotis gigantea* caused by a *Francisella* sp. bacterium. Dis Aquat Org 2:145-54.

Karlsbakk E, Handeland SO Devold M, Fridell F, Nylund A, Otterlei E, Blom G (2003) Sykdomsproblemer hos torskelarver og yngel. Norsk Fiskeoppdrett 20:40-43. In Norwegian.

Karlsbakk E, Omdal LM, Wangen IH, Fiksdal IU, Mortensen S, Ottem KF, Nylund A (2010) Smittespredning ved francisellose hos torsk. Havforskningsrapporten 2010. Fisken og Havet 1:101-102. In Norwegian.

Karphoff SP, Antonoff NE (1936) The spread of tularemia through water, as a new factor in its epidemiology. J Bacteriol 32: 243-258.

Kay W, Petersen BO, Duus JO, Perry MB and Vinogradov E (2006) Characterization of the lipopolysaccharide and beta-glucan of the fish pathogen *Francisella victoria*. FEBS J 273:3002–3013.

Khoo L, Dennis PM, Lewbart GA (1995) Rickettsia-like organisms in the blue-eyed plecostomus, *Panaque suttoni* (Eigenmann & Eigenmann). J Fish Dis 18: 157 – 164.

Kjesbu O, Taranger G, Trippel E (2006) Gadoid mariculture: development and future challenges. Introduction. ICES Jour Mar Sci 2:187–191.

Konstantinidis KT, Tiedje JM (2004) Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci USA 7: 2567-2572.

Korsnes K, Nicolaisen O, Skår CK, Nerland AH, Bergh Ø (2006) Bacteria in the gut of juvenile cod *Gadus morhua* fed live feed enriched with four different commercialdiets. ICES Jour Mar Sci 63:296–301.

Krieg NR (2005) Identification of Procaryotes (Vol 2 A in: Bergeys Manual of Systematic Bacteriology, 2nd edn. Ed: GM Garrity). Springer, USA. pp. 33-38.

Kügeler KJ, Gurfield N, Creek JG, Mahoney KS, Versage JL, Petersen JM (2005) Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. Appl Environ Microbiol 11:7594-7.

Kügeler K.J., Mead P.S., McGowan K.L., Burnham J.M., Hogarty M.D., Ruchelli E., Pollard K., Husband B., Conley C., Rivera T., Kelisidis T., Lee W.M., Mabey W., Winchell J.M., Stang H.L., Staples J.E., Chalcraft L.J. and Petersen J.M. (2008) Isolation and characterization of a novel *Francisella* sp. from human CSF and Blood J Clin Microbiol 7:2428-31.

Lan R, Reeves P (2001) When does a clone deserve a name? A perspective on bacterial species based on population genetics. Tren Microbiol 9:419-424.

Lannan CN, Fryer JL (1993) *Piscirickettsia salmonis*, a major pathogen of salmonid fish in Chile. Fish Res 17:115-121.

Larsen JL, Pedersen K, Dalsgaard I (1994) *Vibrio anguillarum* serovars associated with vibriosis in fish. J Fish Dis 12:259–267.

Larson CL, Wicht W, Jellison WL (1955) A new organism resembling *P. tularensis* isolated from water. Pub Health Rep 3:253-8.

Larsson P, Oyston PCF, Chain P, Chu MC, et al. (2005) The complete genome sequence of *Francisella tularensis,* the causative agent of tularemia. Nat Genet 2: 153 – 159.

Larsson P (2007) The genetic composition and diversity of *Francisella tularensis. Umeå University Medical Dissertations* Department of of Clinical Microbiology New Series No 1094 ISSN 0346-6612 ISBN 978-91-7264-288-1.

Lau KWK, Ren J, Fung MC, Woo PCY, Yuen KY, Chan KKM, Qian PY, Wong PK, Wu M (2007) *Fangia hongkongensis* gen. nov., sp. nov., a novel gammaproteobacterium of the order *Thiotrichales* isolated from coastal seawater of Hong Kong. Int J Syst Evol Microbiol 57:2665-2669.

Lauriano CM, Barker JR, Yoon SS, Nano FE, Arulanandam BP, Hassett DJ, Klose KE (2004) MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc Natl Acad Sci 12:4246-9.

Lawrence JG, Hendrickson H (2003) Lateral gene transfer: when will adolescence end? Mol Microbiol 50:739–749.

Löfgren S, Tärnvik A, Bloom GD, Sjöberg W (1983) Phagocytosis and killing of *Francisella tularensis* by human polymorphonuclear leukocytes. Infect Immun 2:715-20.

Machado-Ferreira E, Piesman J, Zeidner NS, Soares CA (2009) *Francisella*-like endosymbiont DNA and *Francisella tularensis* virulence-related genes in Brazilian ticks (Acari: Ixodidae). J Med Entomol 2:369-74.

Magnadottir B, Bambir SH, Gudmundsdottir BK, Pilstrom L, Helgason S (2002) Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. J Fish Dis 25:583-597.

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci 6:3140-5.

Mailman TL, Schmidt MH (2005) *Francisella philomiragia* adenitis and pulomanry nodules in a child with chronic granulomatous disease. Can Jour Infect Dis Med Microbiol 16:245-248.

Marchette NJ, Nicholes PS (1961) Virulence and citrulline ureidase activity of Pasteurella tularensis. J Bacteriol. 82:26-32.

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P (2010) RDP3: a flexible and fast computer program for analyzing recombination. Bioinformat 26:2462-2463.

Mauel MJ, Miller DL, Frazier K, Liggett AD, Styer L, Montgomery-Brock D, Brock J (2003) Characterization of piscirickettsiosis-like disease in Hawaiian tilapia. Dis Aquat Org 53: 249 – 255.

Mauel MJ, Soto E, Moralis JA, Hawke J (2007) A piscirickettsiosis-like syndrome in cultured Nile tilapia in Latin America with *Francisella* spp. as the pathogenic agent. J Aquat Anim Health 19:27-34.

Maynard Smith J, Smith NH, O´rourke M, Spratt BG (1993) How clonal are bacteria? Proc Natl Acad Sci 90:4384-4388.

Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge, MA.

McCarthy U, Steiropoulos NA, Thompson KD, Adams A, Ellis AE, Ferguson HW (2005) Confirmation of *Piscirickettsia salmonis* as a pathogen in European sea bass Dicentrarchus labrax and phylogeneticc comparison with salmonid strains. Dis Aquat Org 64:107-119.

McCoy GW, Chapin VW (1912) *Bacterium tularense* the cause of a plague like disease of rodents. Publ Health Bull 53:17-23.

Miller DH (1939) Transmission of tularemia by the fin prick of a catfish. Milit Surg 84:23-29.

Miller LT (1982) Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy fatty acids. J Clin Microbiol 16: 584-596.

Mikalsen J, Olsen AB, Tengs T, Colquhoun DJ (2007) *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). Int J Syst Evol Microbiol 57:1960-1965.

Mikalsen J (2008) Diagnosis and characterisation of intra-callular Gram-negative pathogens of marine and salmonid fish. Thesis for the degree of Philosophiae Doctor (Ph.D). 55 pages.

Mikalsen J. Olsen AB, Rudra H, Moldal T, Lund H, Djønne B, Bergh O, Colquhoun DJ (2009) Virulence and pathogenicity of *Francisella philomiragia* subsp. *noatunensis* for Atlantic cod, *Gadus morhua* L., and laboratory mice. J Fish Dis 4:377-81.

Mikalsen J, Colquhoun DJ (2009) *Francisella asiatica* sp. nov. isolated from farmed tilapia (*Oreochromis* sp.) and elevation of Francisella philomiragia subsp. noatunensis to species rank as *Francisella noatunensis* comb. nov., sp. nov. Int J Syst Evol Microbiol. Sep 25. Epub ahead of print.

Mikkelsen H, Schrøder MB, Lund V (2004) Vibriosis and atypical furunculosis vaccines; efficacy, specificity and side effects in Atlantic cod, *Gadus morhua* L. Aquaculture 242:81-91.

Moreno E, Stackebrandt E, Dorsch M, Wolters J, Busch M Mayer H(1990) *Brucella abortus* 16S rRNA and lipid A reveal phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria. J Bacteriol 172:3569–3576.

Mohamed Z (1939) The discovery of a rickettsia in a fish. Minist Agric Cairo Tech Sci Serv Vet Sect Bull 214:1-6.

Moreno E (1997) In search of a bacterial species definition. Rev Biol Trop 45: 735-771.

Moreno E, Cloeckaert A, Moriyón I (2002) *Brucella* evolution and taxonomy. Vet Microbiol 90:209-227.

Morgan BB (1947) Experimental Studies with *Pasteurella tularensis* in fish. Am J Trop Med 3: 399-401.

Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KK, Roberts MJ, Ludu JS, Letendre GW, Meierovics AI, Stephens G, Elkins KL (2004) A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol 19:6430-6.

Nesbø C, Dultek M, Doolitle F (2006) Recombination in *Thermotaga*: Implications for species concepts and biogeography. Gen 172:759-769.

Nichols PD, Mayberry WR, Antworth CP, White DC (1985) Determination of monounsaturated double-bond position and geometry in the cellular fatty acids of the pathogenic bacterium *Francisella tularensis*. J Clin Microbiol 5:738-40.

Niebylski ML, Peacock MG, Fisher ER, Porcella SF, Schwan TG (1997). Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the Genus *Francisella*. Appl Envir Microbiol 19: 3933 – 3940.

Noda H, Munderloh UG, Kurtti TJ (1997). Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. Appl. Envir Miscrobiol 19: 3926 – 3932.

Nübel U, Reissbrodt R, Weller A, Grunow R, Porsch-Ozcürümez M, Tomaso H, Hofer E, Splettstoesser W, Finke EJ, Tschäpe H, Witte W (2006) Population structure of *Francisella tularensis*.J Bacteriol 14:5319-24.

Nylund A, Ottem KF, Watanabe K (2005) Ny torskesykdom forårsaket av bakterien *Francisella* n. sp.. Norsk Fiskeoppdrett 22:44-47. In Norwegian.

Nylund A, Karlsbakk E, Nylund S, Isaksen TE, Karlsen M, Korsnes K, Handeland S, Martinsen R, Mork Pedersen T, Ottem KF (2008) New clade of betanodaviruses detected in wild and farmed cod (*Gadus morhua*) in Norway. Arch Virol 3:541-7.

Ohara S, Sato T, Homma M (1974) Serological studies on *Francisella tularensis*, *Francisella novicida*, *Yersenia philomiragia* and *Brucella abortus*. Int J Syst Bacteriol 24:191-196

O'Hara CM (2005) Manual and automated instrumentation for identification of enterobacteriaceae and other serobic gram-negative bacilli. Clin Microbiol Rev 18:147–162.

Olsen AB, Melby HP, Speilberg L, Evensen Ø, Hastein T (1997) *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway; epidemiological, pathological and microbiological findings. Dis Aquat Org 31: 35-48.

Olsen AB, Mikalsen J, Rode M, Alfjorden A, Hoel E , Straum-Lie K, Haldorsen R and Colquhoun DJ (2006) A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, Gadus morhua L., associated with a bacterium belonging to the genus *Francisella*.. J Fish Dis 29:307–311.

Olsufjev NG, Emelyanova OS, Dunaeva TN (1959) Comparative study of strains of *B. tularense* in the Old and New World and their taxonomy. J Hyg Epidemiol Microbiol Immunol 3:138-149.

Olsufjev NG (1970) Taxonomy and characteristics of the genus *Francisella* Dorofeev 1947. J Hyg Epidemiol Microbiol Immunol 14:67-74.

Olsufjev NG, Meshcheryakova IS (1983) Subspecific Taxonomy of *Francisella tularensis* McCoy and Chapin 1912. Int J Syst Bacteriol 33: 872 – 874.

Ostland V.E., Stannard J.A., Creek J.J., Hedrick R.P., Ferguson H.W., Carlberg J.M. and Westerman M.E. (2006) Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* x *M. saxatilis*. Dis Aquat Organ 72:135-45.

Owen CR, Buker EO, Jellison WL, Lackman DB, Bell JF (1964) Comparative studies of *Francisella tularensis* and *Francisella novicida*. J Bacteriol 87:676-83.

Ozel M, Schwanz-Pfitzner I (1975) Vergleichende elektronenmikroskopische Untersuchungen an Rhabdoviren pflanzlicher und tierischer Herkunft: III. Egtved-Virus (VHS) der Regenbogenforelle (*Salmo garidneri*) und Rickettsienahnliche Organismen. Zbl Bakt Hyg I Abt Orig 230:1-14.

Parker RR, Jellison WL, Kohls GM, Davis GE (1940) Tularaemia infection found in streams. Pub Health Rep 55:227.

Parker RR, Steinhaus EA, Kohls GM, Jellison WL (1951) Contamination of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and muskrats in the northwestern United States. Bull Natl Inst Health 193:1-161.

Patel S, Korsnes K, Bergh Ø, Vik-Mo F, Pedersen J, Nerland AH (2007) Nodavirus in farmed Atlantic cod *Gadus morhua* in Norway. Dis Aquat Org 2:169-73.

Pedersen K, Grisez L, van Houdt R, Tiainen T, Ollevier F, Larsen JL (1999) Extended serotyping scheme for *Vibrio anguillarum* with the definition and characterization of seven provisional O-serogroups. Curr Microbiol 38:183-189.

Petersen JM, Mead PS, Schriefer ME (2008) *Francisella tularensis*: an arthropod-borne pathogen. Vet Res 40:1-7.

Petersen JM, Carlson J, Yockey B, Pillai S, Kuske C, Garbalena G, Pottumarthy S, Chalcraft L (2009) Direct isolation of *Francisella* spp. from environmental samples. Lett Appl Microbiol 6:663-7.

Proctor RA, White JD, Ayala E, Canonico PG (1975) Phagocytosis of *Francisella tularensis* by Rhesus monkey peripheral leukocytes. Infect Immun 11:146–51.

Rambaut A FigTree v1.1.2. Available online: http://tree.bio.ed.ac.uk/software/figtree/

Rappé MS, Giovannoni SJ (2003) The uncultured microbial majority. Ann Rev Microbiol 57: 369-394

Ravin A.W (1963) Experimental approaches to the study of bacterial phylogeny. Am Nat 97: 307–318.

Read A, Vogl SJ, Hueffer K, Gallagher LA, Happ GM (2008) *Francisella* genes required for replication in mosquito cells. J Med Entomol 6:1108-16.

Reintjes R, Dedushaj I, Gjini A, Jorgensen TR, Cotter B, Lieftucht A, D'Ancona F, Dennis DT, Kosoy MA, Mulliqi-Osmani G, Grunow R, Kalaveshi A, Gashi L, Humolli I (2002) Tularemia outbreak investigation in Kosovo: case control and environmental studies Emerg Infect Dis 1:69-73.

Rohmer L, Fong C, Abmayr S, Wasnick M, Larson Freeman TJ, Radey M, Guina T, Svensson K, Hayden HS, Jacobs M, Gallagher LA, Manoil C, Ernst RK, Drees B, Buckley D, Haugen E, Bovee D, Zhou Y, Chang J, Levy R, Lim R, Gillett W, Guenthener D, Kang A, Shaffer SA, Taylor G, Chen J, Gallis B, D'Argenio DA, Forsman M, Olson MV, Goodlett DR, Kaul R, Miller SI, Brittnacher MJ (2007) Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains.Genome Biol 6:R102.

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39-67.

Samuelsen, O.B., Nerland, A., Svåsand, T., Jørgensen, T., Schrøder M., and Bergh, Ø., 2006. Viral and bacterial diseases of Atlantic cod Gadus morhua, their prophylaxis and treatment: a review. Dis Aquat Org 71:239-254.

Santic M, Molmeret M, Abu Kwaik Y (2005a) Modulation of biogenesis of the Francisella tularensis subsp. novicida-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell Microbiol 7:957-67.

Santic M, Molmeret M, Klose KE, Jones S, Kwaik YA (2005b) The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiol 7:969–979.

Schrøder MB, Villena AJ, Jørgensen TØ (1998) Ontogeny of lymphoid organs and immunoglobulin producing cells in Atlantic cod (*Gadus morhua* L.). Dev Comp Immunol. 22:507–517.

Schrøder MB, Mikkelsen H, Bordal S, Gravningen K, Lund V (2006) Early vaccination and protection of Atlantic cod (*Gadus morhua* L.) juveniles against classical vibriosis. Aquacultur*e* 254**:**46–53.

Scoles GA (2004) Phylogenetic analysis of the *Francisella*-like Endosymbionts of *Dermacentor* ticks. J Med Entomol 41 3: 277 – 286.

Seger RA, Hollis DG, Weaver RE, Hitzig WH (1982) Chronic granulomatous disease: fatal septicemia caused by an unnamed gram-negative bacterium. J Clin Microbiol 16:821-825.

Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barré A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C (2007) Being Pathogenic, Plastic, and Sexual while Living with a Nearly Minimal Bacterial Genome. PLoS Genet. 5: e75.

Sjöstedt AB (2005a). Family III. Francisellaceae fam.nov (In: Bergeys Manual of Systematic Bacteriology, 2nd edn. Ed: GM Garrity). Springer, USA. pp 199 – 210.

Sjöstedt A (2005b) Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. Microb Infect 2:561-567.

Skerman VBD, McGowan V, Sneath PHA (1980): Approved Lists of Bacterial Names. Int J Sys. Bacteriol 30: 225-420.

Sneath PHA (1989) Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. Syst Appl Microbiol 12:15-31.

Sneath PHA (2005) Numerical Taxonomy (Vol 2 A in: Bergeys Manual of Systematic Bacteriology, 2nd edn. Ed: GM Garrity). Springer, USA. pp. 39-42.

Snow M, King JA, Garden A, Raynard RS (2005) Experimental susceptibility of Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) to different genotypes of viral haemorrhagic septicaemia virus (VHSV). J Fish Dis 12:737-742.

Sommerset I, Krossoey B, Biering E, Frost B (2005) Vaccines for fish in aquaculture. Exp Rev Vac 4:89-101.

Song Y, Liu C, Molitoris DR, Tomzynski TJ, Lawson PA, Collins MD, Finegold SM (2003) *Clostridium bolteae* sp. nov., isolated from human sources. Syst Appl Microbiol 26:84-89.

Soto E, Hawke JP, Fernandez D, Morales JA (2009a) *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. J Fish Dis 8:713-22.

Soto E, Fernandez D, Hawke JP (2009b) Attenuation of the fish pathogen *Francisella* sp. by mutation of the iglC* gene. J Aquat Anim Health 3:140-9.

Soto E, Fernandez D, Thune R, Hawke JP (2010a) Interaction of Francisella asiatica with tilapia (Oreochromis niloticus) innate immunity. Infect Immun 5:2070-8.

Soto E, Endris RG, Hawke JP (2010b) In vitro and in vivo efficacy of florfenicol for treatment of Francisella asiatica infection in tilapia. Antimicrob Agents Chemother 11:4664-70.

Soto E, Wiles J, Elzer P, Macaluso K, Hawke JP (2011) Attenuated Francisella asiatica iglC mutant induces protective immunity to francisellosis in tilapia. Vaccine. 3:593-8.

Sréter-Lancz Z, Széll Z, Sréter T, Márialigeti K (2008) Detection of a novel *Francisella* in *Dermacentor reticulatus*: A need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks. Vect Born Zoonot Dis :Epub ahead of print.

Stackebrand E, Goebel BM (1994) A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846-849.

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kampfer P, Maiden MCJ, Nesme X, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52: 1043 – 1047.

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–155.

Steinum T, Sjåstad K, Falk K, Kvellestad A, Colquhoun DJ (2009) An RT PCR-DGGE survey of gill-associated bacteria in Norwegian seawater-reared Atlantic salmon suffering proliferative gill inflammation. Aquaculture 293:172-179.

Suitor E C & Weiss E (1961). Isolation of a rickettsialike microorganism (*Wolbachia persica*, n.sp.) from *Argas persicus* (Oken). J Infect Dis 108: 95 – 106.

Svensson K, Larsson P, Johansson D, Byström M, Forsman M, Johansson A (2005) Evolution of subspecies of *Francisella tularensis*. J Bacteriol 11:3903-8.

Thelaus J, Andersson A, Mathisen P, Forslund AL, Noppa L, Forsman M (2009) In£uence of nutrient status and grazing pressure on the fate of *Francisella tularensis* in lake water. FEMS Microbiol Ecol 67:69-80.

Timur G, Timur M, Akayli T, Korin J, Thompson KD (2005) First observation of rickettsia-like organisms in cultured sea bass (*Dicentrarchus labrax*) in Turkey. Bull Eur Ass Fish Pathol 25: 196 – 202.

Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60: 249 - 266.

Tomaso H, Dahouk SA, Hofer E, Splettstoesser WD, Treu TM, Dierich MP, Neubauer H (2005) Antimicrobial susceptibilities of Austrian *Francisella tularensis holartica* biovar II strains. Int J Antimicrob Agents 26: 279 – 284.

Topley and Wilson (1929): Principles of Bacteriology and Immunity. New York: William Wood & Co 1: 482, 1929.

Tortoli E (2003) Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev 16:319-354.

Van Banning P (1987) Long-term recording of some fish-diseases using general fishery research surveys south-east part of the North Sea. Dis Aquat Org 3:1-11.

Van Belkum A, Struelens M, de Visser A., Verbrugh H, Tibayrenc M (2001) Role of Genomic Typing in Taxonomy, Evolutionary Genetics, and Microbial Epidemiology. Clin Microbiol Rev 14:547-560.

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 2:407-38.

Venzal JM, Estrada-Peña A, Portillo A, Mangold AJ, Castro O, de Souza CG, Félix ML, Pérez-Martínez L, Santibánez S, Oteo J (2008) Detection of Alpha and Gamma-Proteobacteria in *Amblyomma triste* (Acari: Ixodidae) from Uruguay. Exp Appl Acarol 1:49-56.

Vilcins IM, Fournier PE, Old JM, Deane E (2009) Evidence for the presence of *Francisella* and spotted fever group rickettsia DNA in the tick *Amblyomma fimbriatum* (Acari: Ixodidae), Northern Territory, Australia. J Med Entomol 4:926-33.

Vojtech LN, Sanders GE, Conway C, Ostland V, Hansen JD (2009) Host immune response and acute disease in a zebrafish model of *Francisella* pathogenesis. Infect Immun. 2:914-25.

Wangen IH (2009) Observation on the survival of *Francisella noatunensis* in water and in blue mussel (*Mytilus edulis*). Master of Science thesis, Department of Biology, University of Bergen.

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG (1987) Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int J Syst Bacteriol 37: 463 – 464.

Weiss E, Myers WF, Suitor EC, Neptune EM (1962) Respiration of a rickettsialike microorganism, *Wolbachia persica*. J Infect Dis 110:155-64.

Wenger JD, Hollis DG, Weaver RE, Baker CN, Brown GR, Brenner DJ, Broome CV (1989) Infection caused by *Francisella philomiragia* (formerly *Yersinia philomiragia*). A newly recognized human pathogen. Ann Intern Med 110: 888-892

Wertz J, Goldstone C, Gordon D, Riley M (2003) A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. J Evol Biol 16:1236-1248.

Whipp MJ, Davis JM, Lum G, de Boer J, Zhou Y, Bearden SW, Petersen JM, Chu MC, Hogg G (2003) Characterization of a novicida-like subspecies of *Francisella tularensis* isolated in Australia. J Med Microbiol 52: 839-842.

Whittaker P, Day JB, Curtis SK, Fry FS (2007) Evaluating the use of fatty acid profiles to identify *Francisella tularensis*. J AOAC Int 2:465-9.

White MJD (1978) Modes of speciation, Freeman, San Francisco, pp. 1–20.

Xu J (2004) The prevalence and evolution of sex in microorganisms. Gen 47:775-780.

Zerihoun MA, Feist S, Olsen AB, Bucke D, Wiik Jand Colquhoun DJ (2008). Retrospective identification of francisellosis in Atlantic cod (*Gadus morhua*) sampled from the southern North Sea during the 1980's. Poster ved Havbrukskonferansen i Tromsø.

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ORIGINAL PAPER

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Francisella sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming

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Abstract In 2004, a new disease was detected in cod (*Gadus morhua*) in western Norway. Affected cod had white granulomas in the visceral organs and skin. A species of *Francisella* was isolated on blood agar plates from moribund cod. The bacterium could be grown at temperatures ranging from 6 to 22°C, but did not grow at 37°C. Challenge experiments showed that *Francisella* sp. was the cause for the new disease. The 16S rDNA gene sequence from *Francisella* sp. showed 99.17% similarity to *F. philomiragia*, and the 16S–23S ribosomal RNA intergenic spacer (249 nt), shows a similarity with that from *Francisella* isolated from tilapia and *F. tularensis* of 96.8 and 35.9%, respectively. The 23S sequence is more similar to *F. tularensis*, 97.7% (2,862 nt), compared to the tilapia isolate 96.8% (2,131 nt). The partial putative outer membrane protein (FopA) sequence (781 nt) from *Francisella* sp. shows a similarity with that from *F. tularensis* and *F. philomiragia* of 77.3 and 98.2%, respectively. Based on sequence data, culturing temperatures and pathogenicity for cod, it is suggested that this *Francisella* sp. from cod could be a new species of *Francisella*, Family Francisellaceae.

Keywords Cod · *Gadus morhua* · *Francisella* · rDNA · FopA · Phylogeny

Introduction

Fish farming is a large industry on a worldwide basis. In the north Atlantic, the major species in production are Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss*), Halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*). The production of Atlantic salmon in Norway was 508,000 tons in 2003 while the newly started cod production amounted to 605 tons only (Agnalt et al. 2004). A major problem in cod production is the start feeding phase and mortality associated with this stage in production (Brown et al. 2003; Nylund, University of Bergen, unpublished results). Several species of bacteria have been associated with the problems in the start feeding phase, and during the spring and summer of 2004, the first major disease caused by bacteria occurred on larger cod at different production sites in western Norway. During the spring and summer of 2005, this disease apparently spread to a larger area (Nylund, University of Bergen, unpublished results).

In the autumn of 2004, we received material from a cod farm in Rogaland county, western Norway. The cod, 2–3 kg, showed loss of appetite, reduced swimming performance, and dark pigmentation. There were few other external signs of disease, but subdermal white granulomas could be found on gills and in the mouth cavity. The most prominent internal signs were a swollen spleen, kidney and heart where these blood rich organs were covered with and penetrated by white granulomas. These granulomas contained, in most cases, a transparent liquid. Sections of these tissues show necrosis, degeneration, inflammation and proliferation of cells, and a few bacteria could be present in these granulomas.

PCR, using primers targeting the 16S from proteobacteria, gave a positive result, and sequencing of the PCR products showed that this bacterium was closely related to *Francisella philomiragia* (cf. Jensen et al. 1969; Hollis et al. 1989). Blasting the 16S sequence showed that similar *Francisella*-like sequences had been previously found in *tilapia (Oreochromis* spp.) in Taiwan and Isaki (*Parapristipoma trilineatum*) in Japan. Several studies have also showed that *Francisella* spp. and bacteria with 16S sequences similar to that from *Francisella* species are present in ticks, amoebas, fish and environmental samples (Suitor and Weiss 1961; Niebylski et al. 1997; Noda et al. 1997; Anda et al. 2001; Scoles 2004; Kamaishi et al. 2005; Barns et al. 2005; Sjöstedt 2005a; Tomaso et al. 2005).

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The present study describes culturing conditions, morphology, and sequences of the rRNA genes, the 16S–23S ribosomal RNA intergenic spacer and the partial sequence of a putative outer membrane protein (FopA) from this *Francisella* isolate. A challenge experiment on cod has been performed to show that this bacterium is the causative agent of a new disease, francisellosis, described from farmed cod. The histopathology is described from cod with chronic francisellosis.

Materials and methods

Sample collection

Included in this study are 20 cultured Atlantic cod (*Gadus morhua*) collected in 2004 and 2005 from four different farms in western Norway located in the counties; Rogaland, Hordaland and Møre og Romsdal. The cod came from farms experiencing mortalities associated with white granulomas on skin, gills and internal organs. Tissues; gills, skin, heart, spleen, liver, head kidney and kidney were collected from all fish included in this study. Tissues were stored at (80°C or fixed for later processing for histology and electron microscopy.

Histopathology and transmission electron microscopy (TEM)

All tissues, gills, skin, heart, spleen, liver, head kidney and kidney, from cod collected from farms and from challenged cod (see below), were fixed by immersion, at 6°C, in a modified Karnovsky fixative where the distilled water was replaced by a Ringers solution (Nylund et al. 1995). The fixative contained 4% sucrose. Before embedding in EPON 812, the tissues were stained/post fixed in 2% OsO_4. Semi and ultrathin sections were cut on Reichert-Jung Ultracut E. The ultrathin sections (30–40 nm) were stained for 1.5 h in 2% aqueous uranyl acetate solution and then stained with lead citrate. Semithin sections, 1.5 μm, were stained in toluidine blue.

Isolation, media and growth conditions

The first isolation of the organism was made by streaking tissues (spleen and head kidney) from cod (*Gadus morhua*) on blood agar plates added 0.1% cysteine and 1% glucose (BCG-plates). Stock cultures were maintained on these plates.

The cod from the fish farms were examined bacteriologically by inoculation of kidney and spleen tissues on: (a) BCG-plates, which were incubated for 30 days at 10°C, and (b) on blood agar plates containing 1.5% NaCl which were incubated for 30 days at 15°C. After the isolation of *Francisella* sp. from farmed cod, several different culturing temperatures were tested; 6, 10, 15, 19, 22, 29 and 37°C.

In addition to the isolation of *Francisella* sp., two other bacteria were isolated on the blood agar plates, *Virbio logei* and *Chryseobacter* sp. These two bacteria were identified by sequencing of the 16S rRNA gene; *V. logei* (Accession no.: DQ318955) and *Chryseobacter* sp. (Accession no.: DQ318956). A mycoplasma (Acession no.: DQ318957) was also identified, by PCR on spleen tissues from one farmed cod using the general eubacterial primers, EubA(1518R) 5′-AAG GAG GTG ATC CAN CCR CA and EubB(27F) 5′-AGA GTT TGA TCM TGG CTC AG (Giovannoni 1991).

It was also attempted to culture *Francisella* sp. in cell cultures established from salmonids (cf: Devold et al. 2000; Dannevig et al. 1997). *Francisella* sp. from BCG-plates were suspended in PBS, diluted 1:100, and incubated at 10°C in cell culture flasks with a mono-layer of salmon head-kidney (SHK-1) cells and Atlantic salmon kidney (ASK) cells. The cells were incubated for 30 days or until cytopathic effect (CPE) could be observed. Negative control cell cultures were always included.

DNA extraction and amplification

DNA was extracted using the DNeasy DNA Tissue kit (Qiagen) as recommended by the manufacturer. Elution was performed twice in 50 μl 10 mM Tris–HCl, pH = 8.5 to increase the overall DNA yield, and the DNA was stored at (20°C. DNA was extracted from 20 farmed cod and from different tissues (spleen and kidney) collected from the cod included in the challenge trial. Extraction of DNA from bacterial colonies was achieved by heating the bacteria at 95°C for 5 min followed by centrifugation at 14,000 rpm for 1 min. The supernatant was stored ((20°C) for later use in PCR.

The PCR reaction mixture (50 μl) contained 10 × PCR buffer with 1.5 mM $MgCl_2$ (Amersham Pharmacia Biotech Inc.), 25 mM of each dNTP (Promega), 0.2 μM of each primer (Invitrogen), 1 U Taq DNA polymerase (Amersham Pharmacia Biotech Inc.) and 300 ng DNA. Primers used for amplification of the rDNA genes, the complete 16S–23S ribosomal RNA intergenic spacer and the putative outer membrane protein (FopA) are described in Table 1. Amplification was performed in a GeneAmp PCR System 9700 machine (Applied Biosystems) at 95°C for 5 min; 35 cycles of 94°C for 30 s, X C (dependent on the primer set, see Table 1) for 45 s, 72°C for 1 min followed by extension at 72°C for 10 min and a short storage at 4°C.

DNA sequencing and analysis

PCR products were purified with QIAquick PCR purification kit (Qiagen) as described by the manufacturer. Sequencing was then performed with ABI PRISM BigDye terminator chemistry (version 2) according to Applied Biosystems (ABI). All sequences were assembled using the Vector NTI Suite 7.0 program (InforMax

Table 1 Overview of primers used in for sequencing the rDNA genes, the complete 16S–23S ribosomal RNA intergenic spacer, and the putative outer membrane protein (FopA) from *Francisella* sp. The location of the primers is given in relation to Accession no.: DQ309246 (rDNA genes) and DQ333226 (FopA)

Primer	Sequence	Location
rDNA gene forward		
FC-F1	5′-CCT GGC TCA GAT TGA ACG CTG G	Outside
FC-F2	5′-ACA GGT CTT CGG ATG CTG ACG	42–62
FC-F3	5′-AGA GAT AGA TTG GTG CCT TCG G	973–994
FC-F6	5′-TTC AGA GGC GAT GAA GGA C	844–1,862
FC-F4	5′-GCA AAA GTA TGG GAT GAG CTG TGG	53–2,576
FC-F10	5′-TTC AGA GGC GAT GAA GGA C	675–3,694
Reverse		
FC-R20	5′-GGA AAA AGA TGG CGA CTA CC	Outside
FC-R6	5′-TGT GAT GAG CCG ACA TCG	310–4,293
FC-R7	5′-CAC TGC ATC TTC ACA GCG	839–3,822
FC-R2	5′-CGA CAA GGA ATT TCG CTA CC	747–3,728
FC-R5	5′-TCG CTC GCC ACT ACT ATG GG	54–2,035
FC-R1	5′-TCA CTC CGT GGT AAA CGC C	432–1,414
FopA forward		
FMP-F1	5′-GTC TCA ATG TAC TAA GGT TTG CCC	Outside
FMP-F2	5′-CAA GAT AGA ACT GGY CAG TGG	393–413
Reverse		
FMP-R1	5′-RSC ACC AAT CAT RTT AGT ACC	outside
FMP-R2	5′-CAC CYA AAC CAG CAA ATA CTC	561–541

Inc.). Sequences obtained in the present study were submitted to the GenBank.

Phylogenetic analysis

The Vector NTI Suite software package was used for the multiple alignments of nucleotide sequences. To perform pair wise comparisons between the different sequences of the 16S rRNA gene, the multiple sequence alignment editor GeneDoc was used (Nicholas 1997). Sequences already available on the EMBL nucleotide database were also included in the comparisons.

Phylogenetic trees were constructed using maximum likelihood analysis to describe the phylogenetic history of the bacteria based on the 16S nucleotide sequence. Analyses were performed using TREE-PUZZLE 5.0 (Available at: http://www.tree-puzzle.de). Parallel trees were constructed using PAUP v4.0 (Swofford 1998) with maximum likelihood as optimality criterion and the heuristic search option, in order to verify that the two methods produced a similar tree topology. For the PAUP analysis, Modeltest 3.6 (Posada and Crandall 1998) was used to identify the models best suited for the datasets. The PAUP tree was not bootstrapped due to lack of computer capacity. Phylogenetic trees were drawn using TreeView (Page 1996).

Challenge experiment

Bacteria, *Francisella* sp., *Vibrio logei* and *Chryseobacter* sp., grown on blood agar were used in a challenge experiment on Atlantic cod (*Gadus morhua*). The bacteria were scraped of blood agar plates and suspended in a phosphate buffer solution (PBS) to a concentration of about 1.0×10^8.

The cod was supplied by a hatchery close to Bergen. Initial weight and length of fish were about 15 g and 12 cm. The fish were acclimatised for 7 days to particle (20 μm) and UV-filtered (Katadyn J1/P, effect: 50 mWs $cm^{(2)}$) seawater (34‰), kept at 10°C in 0.15 m^3 tanks (flow rate = 5 l $min^{(1)}$; 50 specimens in each tank), and fed commercial pellets twice a day.

The challenge experiment included five different groups kept in separate tanks, (30 cod in each tank). The control group was injected intraperitoneal (i.p.) with 0.2 ml PBS. The second group received an i.p. injection of filtered (0.45 μm) supernatant from tissue homogenate (spleen) of cod with internal white granulomas. The last three groups were challenged by an i.p. injection (0.2 ml) of *V. logei, Chryseobacter* sp. and *Francisella* sp., respectively. The fish were challenged on 4 February 2005. The experiment was terminated after 122 days (6 June 2005). To determine if *Francisella* sp. could be transmitted from cod challenged by i.p. injections to disease-free cod, 20 fish were added to the tank, 19 days after challenge. These co-habitants were tagged by marking the pectoral fins.

Mortality was registered in all tanks and all fish were examined bacteriologically by inoculation of kidney tissues on: (a) BCG-plates which were incubated for 30 days at 10°C, and (b) on blood agar plates containing 1.5% NaCl which were incubated for 30 days at 15°C. Tissues were sampled from the skin, gills, heart, kidney, liver and spleen from all fish and; (a) stored at (80°C for later RNA extraction and (b) fixed in a modified Karnovsky fixative for histological and transmission electronmicroscopical (TEM) studies.

Results

Clinical signs and histopathology

Atlantic cod (*Gadus morhua*) suffering from infection with *Francisella* sp. shows loss of appetite, reduced swimming performance, and dark pigmentation. There

are few other external signs of disease, but white granulomas can be found in the skin, gills and in the mouth cavity. Internal signs may range from just slightly swollen spleen and kidney to most *viscera* covered with white granulomas. In the terminal stages of the disease, the spleen may have grown to three times the normal size being completely covered with and penetrated by granulomas (Fig. 1). Heart, kidney and liver are also among the organs/tissues most strongly affected by degeneration and presence of granulomas. At the terminal stages, there are few bacteria present within the granulomas which may be filled with a transparent liquid. The cell types, organization, and ultrastructural features were similar in granulomas from spleen, kidney, skin and heart tissues.

The target cells for *Francisella* sp. seem to be phagocytes and cells with phagocyte functions (reticuloendothelial system, RES) (Fig. 2). In the early stages of the disease, bacteria are always present in phagocytes in the spleen and kidney, but can also be found in endothelial cells lining the heart chambers and in leucocytes attached to the blood vessel walls in the liver, pseudobranch and gills. In the later stages of the disease, the granulomas consist mainly of host cells (phagocytes, fibroblasts and lymphocytes) organized in concentric cellular layers and with little or no bacteria present (Fig. 3). In the terminal stages of granulomas, there is a prominent development of necrosis in the core, i.e. the cells in the center dies and is replaced by a transparent liquid, and no bacteria can be detected by microscopy in the core vacuole.

Growth temperature and morphology

Francisella sp. grows on BCG-plates at 6, 10, 15 and 19°C. The growth at 22°C is slow compared to the growth at 15 and 19°C. No growth was observed at 29 and 37°C.

Francisella sp. grown on blood agar, are coccoid with a diameter of about 500 nm (range from 300 to 700 nm) (Fig. 4). More elongated forms, as long as 1.0 µm, may also be present. *Francisella* sp. present in cells and tissues have more or less the same size and morphology as that seen on blood agar plates (Fig. 4). However, they are slightly more polymorphic and less coccoid shaped, and may be as long as 1.5 µm.

Francisella sp. grows in both SHK-1 and ASK cells at 10°C, but the growth is best in SHK-1 cells. A visible production of *Francisella* sp. in SHK-1 cells could be observed 11 days after inoculation. Most of the SHK-1 cells were lost 26 days after inoculation with *Francisella* sp. due to replication of the bacterium.

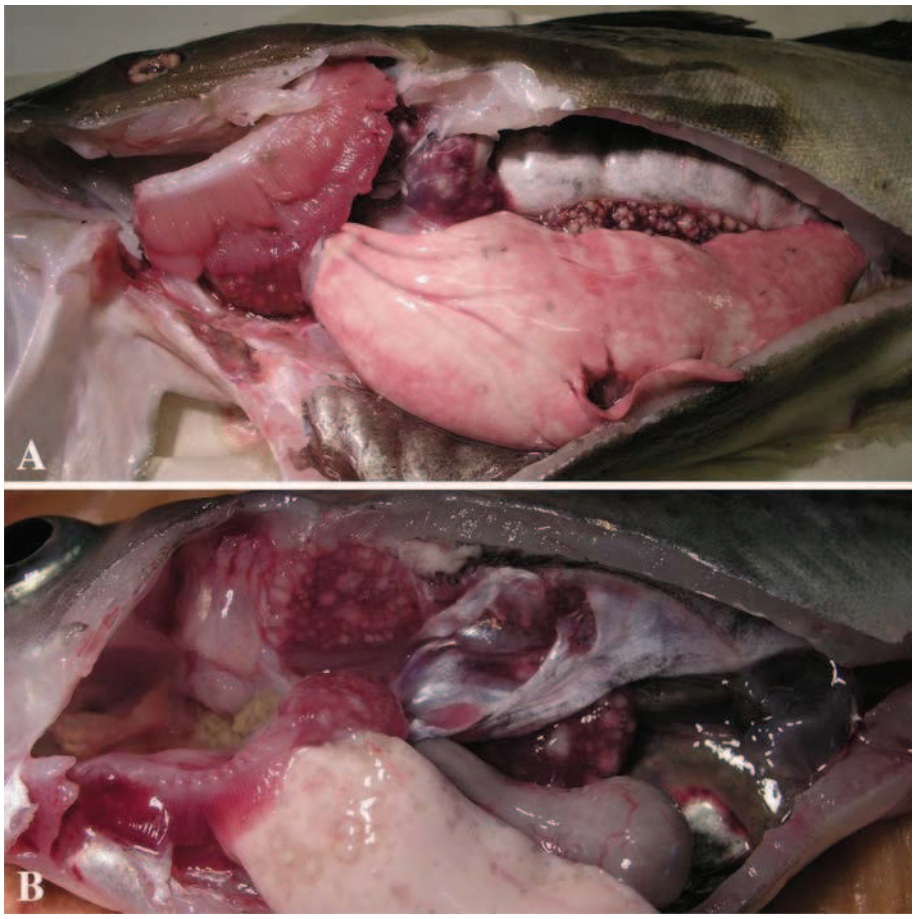

Fig. 1 **a** Atlantic cod (*Gadhus morhua*), from a fish farm, showing the typical signs of francisellosis, including white granulomas on the spleen, kidney, and heart. **b** Atlantic cod challenged with *Francisella* sp. Note the presence of white granulomas in kidney, liver and spleen

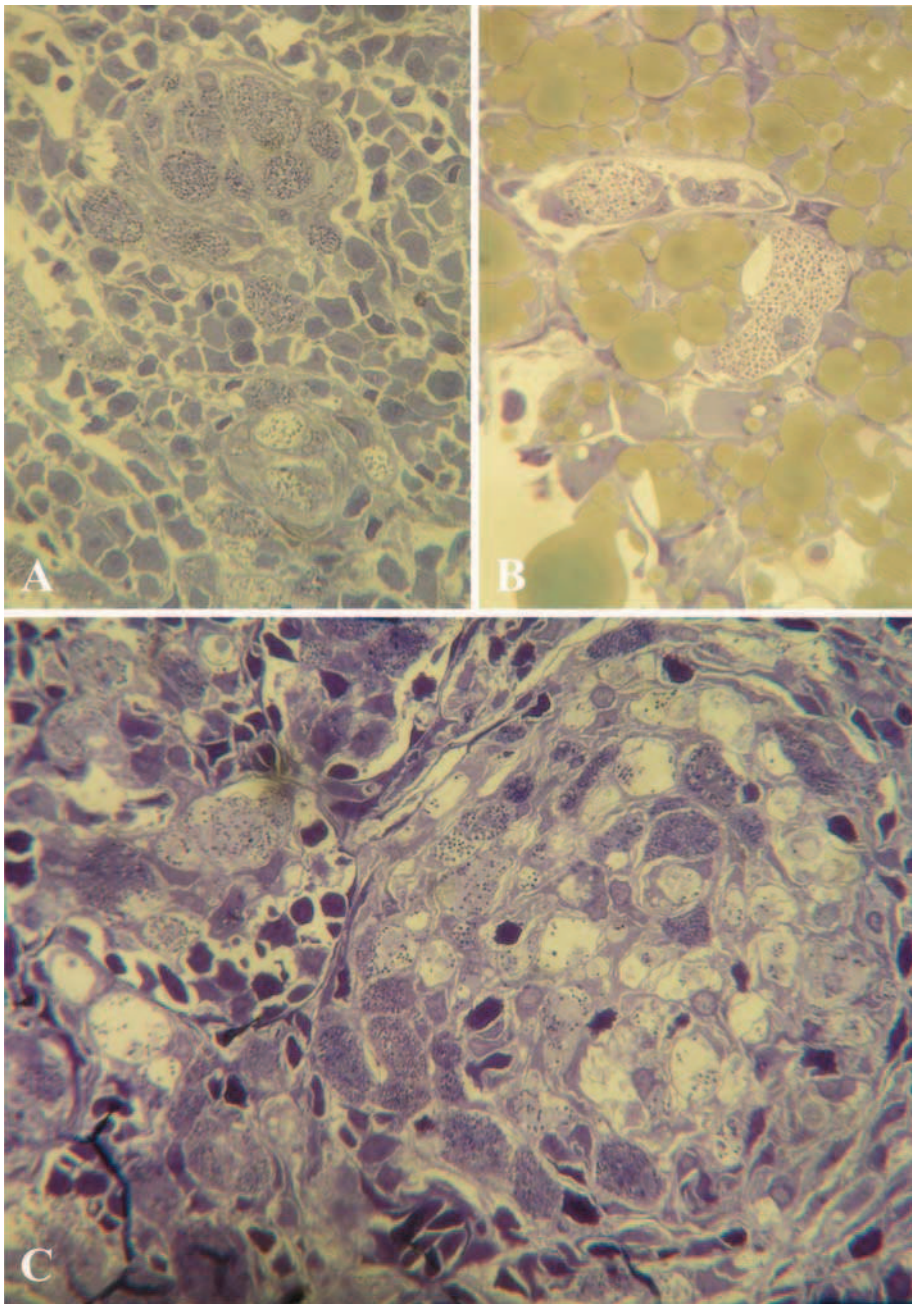

Fig. 2 Sections of kidney (**a**), liver (**b**), and spleen (**c**) tissues from Atlantic cod challenged with *Francisella* sp. Note the cells infected with bacteria and the starting development of granulomas

DNA sequences

The 16S rRNA gene was sequenced from *Francisella* sp. obtained from 20 different specimens of cod collected in four different farms (DQ309246). The 16S was also sequenced from cod challenged by *Francisella* sp. and cod challenged by tissue homogenate from cod with white granulomas on the internal organs. No variation was observed in the 16S when a partial sequence of 1,430 nt was compared with *Francisella* sp. in the different individual hosts

Comparison of the partial 16S, the complete 16S–23S ribosomal RNA intergenic spacer, and the partial 23S (a total of 3,850 nt) from the cod isolate and a *Francisella* isolate from tilapia (Accession no: AF385857) shows a similarity of 97.4%. A comparison of the partial 16S sequence from the Atlantic cod isolate and *F. philomiragia* (AJ698862), and the isaki isolate (AB194068) from Asia gives the following similarities, 99.17 and 99.25%, respectively. Comparison of the 16S–23S ribosomal RNA intergenic spacer, from *Francisella* sp. (249 nt) with that from the tilapia isolate (AF385857) and *F. tularensis* (NC006570) give similarities of 96.8 and 35.9%, respectively. The 23S from *Francisella* sp. show a similarity of 97.7% (2,862 nt) and 96.8% (2,131 nt), respectively, with that from *F. tularensis* (AJ749949) and tilapia (AF385857).

Comparison of the partial putative outer membrane protein (FopA) sequence (781 nucleotides, Accession no.: DQ333226) from *Francisella* sp. with that from *F.*

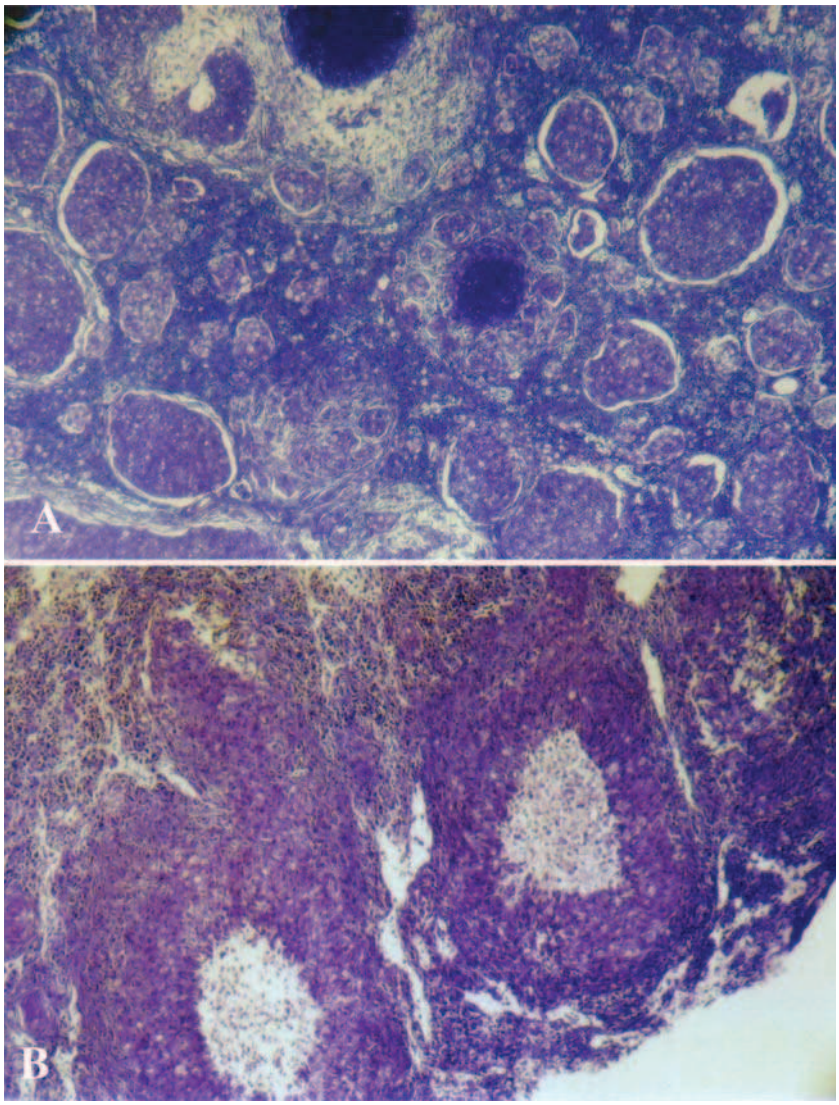

Fig. 3 **a** and **b**) Sections through the spleen of cod infected with *Francisella* sp. Note the different stages of development of granulomas. In **b**, the center of the granulomas contains dead cells

tularensis (M93695) and *F. philomiragia* (AF097543) give the following similarities; 77.3 and 98.2%, respectively.

Phylogeny

The genus *Francisella* consists of only two recognized species, *F. tularensis* and *F. philomiragia*, where the former consists of several subspecies. In addition to these recognized species, isolates from ticks and Asian fish (tilapia and isaki) seem to constitute at least two other species (Fig. 5). The cod isolate *Francisella* sp. is positioned between *F. philomiragia* and the Asian fish isolates in the 16S phylogeny. The support values are high and support the distinctness of the Norwegian cod isolate based on the partial 16S sequence.

Challenge experiment

During the experimental period of 122 days, 23 cod died after i.p. injection of *Francisella* sp. and 10 co-habitants died during a period of 103 days. The mortality, in the i.p. challenged cod, started after day 7. Twelve fish died during the period from day 7 until day 12. The rest of the mortality occurred during the period from day 12 until the termination of the experiment. The co-habitants died during the last 30 days of the experimental period. There were no mortalities in the control group and only a low mortality in the groups challenged by *V. logei*, *Chryseobacter* sp. and tissue homogenate from cod with white granulomas, i.e. 2, 3 and 4 specimens, respectively.

Francisella sp. was present in both the i.p. challenged and the cohabitant fish in the tank challenged by *Francisella* sp.. No other bacteria were detected by PCR or isolated from the moribund fish in this tank. The four fish that died in the group challenged by tissue homogenate were all positive for *Francisella* sp., tested by on-oculation on blood agar and by PCR on kidney and spleen. *V. logei* and *Chryseobacter* sp. were present in the fish that died in the groups challenged by *V. logei* and *Chryseobacter* sp., respectively.

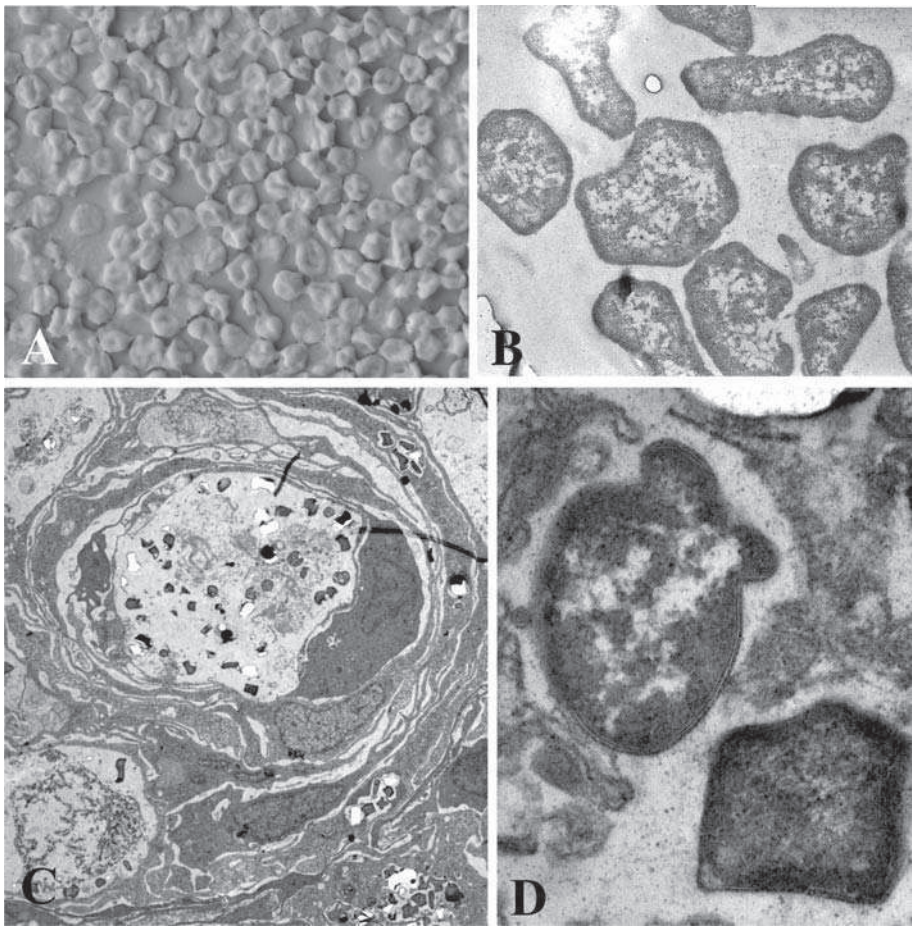

Fig. 4 Electronmicroscopic pictures of *Francisella* sp. grown on blood agar plates (A and B) and from infected cells in the kidney tissue (C and D). The diameter of *Francisella* sp. is about 0.5 μm

The cod that died during the first period after infection with *Francisella* sp., acute mortality, showed few gross clinical signs of disease and no macroscopical granulomas. However, sections of tissues showed that most organs and tissues were degenerating containing a high number of cells infected by bacteria. The most prominent sign was a swollen and liquified kidney. Cod suffering from chronic infections with *Francisella* sp., i.e. mortality late in the experimental period, had swollen spleen and kidney and, these and heart were covered with and penetrated by white granulomas (Figs. 1 and 3). Sections of these tissues showed degeneration, inflammation and proliferation of cells (Fig. 3), but few intracellular bacteria were observed in cells from these tissues. Granulomas could also be found on and in the liver. The fish that were killed at the termination of the experiment all showed signs of chronic infection with *Francisella* sp., with white granulomas in heart, spleen and kidney.

Discussion

The induction of chronic granulomatous infections in fish by rickettsia-like organisms (RLO) has been previously described in the literature (Chen et al. 1994; Chen et al. 2000; Khoo et al. 1995; Chern and Chao 1994; Mauel et al. 2003; Timur et al. 2005). It has not always been possible to detect any members of *Piscirickketsia* by PCR or other specific diagnostic methods (Mauel et al. 2003). However, in 2002 a new *Francisella* isolate was detected in cultured three-line Grunt (*Parapristipoma trilineatum*) suffering from a granulomatous disease and it was shown that this bacterium was the causative agent (Kamaishi et al. 2005). The disease had been present in Japanese aquaculture since 1999. The granulomatous disease detected in Atlantic cod (*Gadus morhua*) culture in Norway in 2004 share much of the same histopathology as that described from tilapia in Hawaii and isaki in Japan, and based on 16S sequences, the causative agent of the cod disease is yet another new isolate of *Francisella* related to *F. philomiragia* and the *Francisella* species from isaki.

The family Francisellaceae, which belongs to the gamma subclass of the class Proteobacteria includes closely related organisms within the genus *Francisella* (Sjöstedt 2005a). There are only two recognized species, *F. philomiragia* and *F. tularensis*, where the former is often associated with the aquatic environment and less virulent for humans compared to *F. tularensis*. However, there is evidence for the existence of several species of

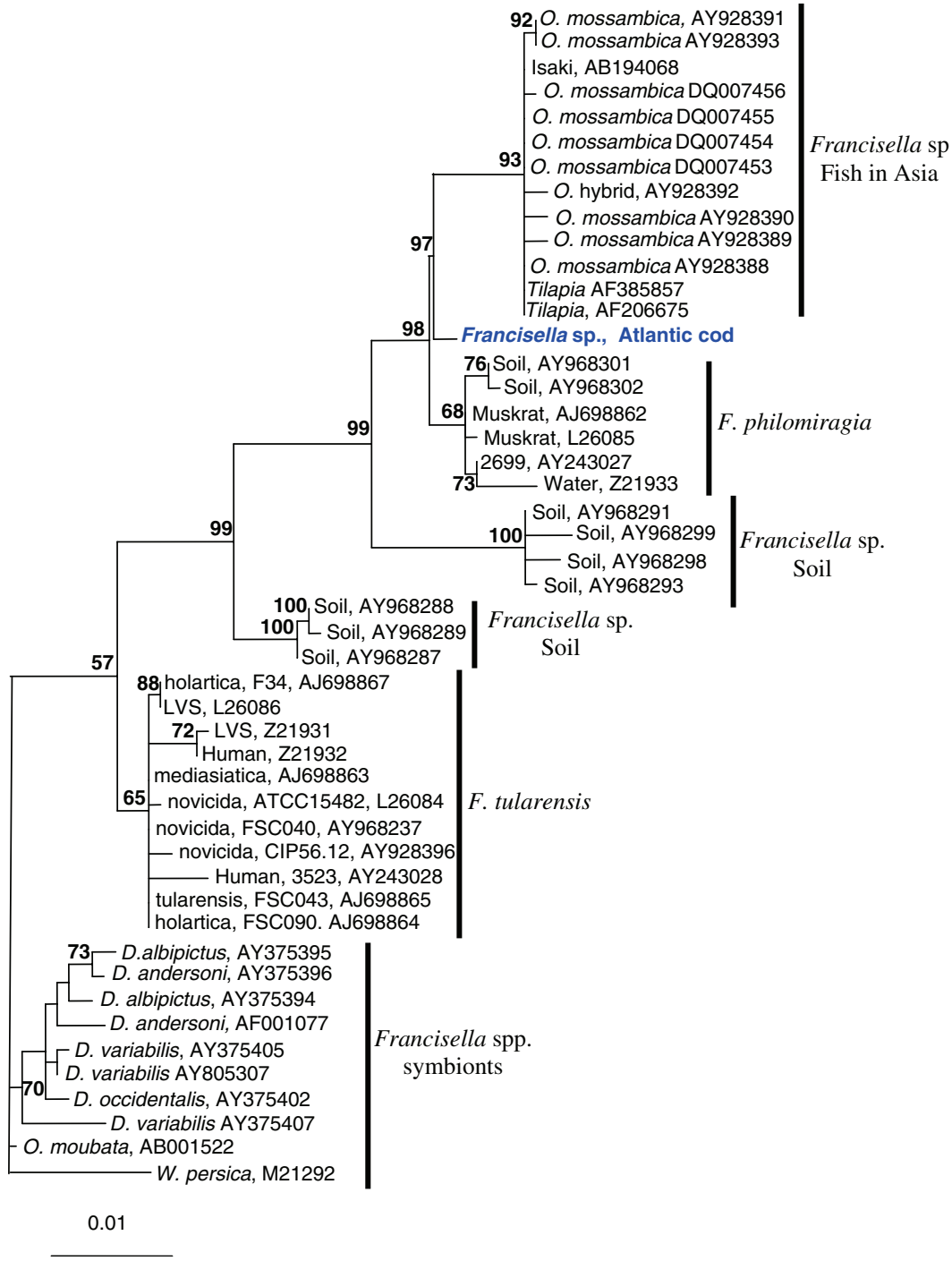

Fig. 5 The phylogenetic relationship of the *Francisella* isolate (DQ309246) from Atlantic cod, *Gadus morhua,* in Norway. The isaki (*Parapristipoma trilineatum*) *Francisella* isolate (AB194068) comes from Japan. *Francisella* spp. isolated from *Tilapia* in Taiwan; AF206675, AF385857, AY928388, AY928389, AY928390, AT928391, AY928392, AY928393, DQ007453, DQ007454, DQ007455, DQ007456, from the environment in USA; AY968287, AY968288, AY968289, AY968291, AY968293,AY968298, AY968299, AY968301, AY968302, AJ698867, Z21933, AY928396, L26084, AY968237, from mammalian hosts; AJ698862, L26085, AJ698863, AY243027, AJ698864, AJ698865, AY243028, Z21931, L26086, Z21932, as endosymbionts of ticks; AY375405, AY805307, AY375394, AY375395, AY375396, AY375402, AY375407, AF001077, AB001522, M21292

Francisella in the environment, or in association with organisms like ticks, amoeba and fish (Barns et al. 2005). These are yet to be described as full species or members of the genus *Francisella*.

The causative agent of francisellosis in farmed cod, in Norway, differs distinctly, in the rDNA gene sequences, the 16S–23S ribosomal RNA intergenic spacer, the putative outer membrane protein (FopA), and the

optimal culture temperature, compared to the two described species of *Francisella*. It is also distinctly different, in most of the same characters, compared to the *Francisella* isolate from isaki in Japan. Based on phylogenetic analysis of the 16S sequences, the cod *Francisella* is positioned between *F. philomiragia* and the isaki strain. Comparing the 16S–23S ribosomal RNA intergenic spacer and the FopA result in a much closer relationship to the isaki strain and *F. philomiragia*, respectively, compared to *F. tularensis*. However, the 23S sequence from the cod *Francisella* is more similar to *F. tularensis* compared to the isaki isolate. Unfortunately, the 23S rDNA gene from *F. philomiragia* is not available in the Genbank preventing a comparison to this species. Based on this, it is not possible to suggest the correct phylogenetic position of the cod *Francisella* since different genes may result in different phylogenetic positions.

Based on the rDNA genes, the 16S–23S ribosomal RNA intergenic spacer, the partial outer membrane protein (FopA) gene sequence, the growth temperatures and the phylogenetic position of this new *Francisella* isolate, it could be that this bacterium should be given status as a new and distinct species in the genus *Francisella*, family Francisellaceae.

Little is known about the virulence mechanisms of *Francisella* species in general (Larsson et al. 2005; Sjöstedt 2005b), but the challenge experiment clearly shows that when given a high enough dosage, the *Francisella* isolate from cod may cause a high and acute mortality among infected cod. It is also demonstrated that the bacterium can be transmitted from host to host over short distances. In the present experiment, all challenged fish were strongly affected by *Francisella* sp. which shows that this bacterium, when present, has a high potential to cause problems in cod farms. It is too early to have any strong opinions about how this *Francisella* isolate may enter cod farms, but there are several possibilities. The bacterium may arrive when the fry is delivered from brood fish companies and, so far, the brood fish companies are using wild caught fish as brood fish, which could be naturally infected. Another possibility is natural reservoirs in the vicinity of farms. These reservoirs could be amoeba, wild fish and a long range of marine species (Abd et al. 2003; Barnes et al. 2005). The potential for horizontal transmission between fish farms may also be largely due to the possibility of naturally occurring vectors and due to a long survival time for bacteria in the genus *Francisella* which may allow long distance transmission via sea currents. It has been shown in other *Francisella* species that they may survive for as long as a year in the aquatic environment. Vector transmission and long time survival combined with the fact that the infectious dose necessary to cause a fatal disease can be low ($<$ 10 CFU) in an individual (Oyston et al. 2005; Sjöstedt 2005a, b), point towards this new *Francisella* isolate as a very serious danger for future culture of cod in Norway.

Clinical signs and histopathology indicate that this new *Francisella* isolate may infect and kill a large range of fish species (Chen et al. 1994; Chen et al. 2000; Khoo et al. 1995; Mauel et al. 2003). This, and the fact that other *Francisella* isolates are causing mortality among tilapia and isaki in Taiwan and Japan, suggests that *Francisella* species constitutes a future threat, not only to cod production in Norway, but to all fish farming in the marine environment including salmonids. Future fish farming in Norway and other countries may depend upon the development of an effective control strategy that may involve everything from vaccination to stamping out.

References

Abd H, Johansson T, Golovliov I, Sanström G, Forsman M (2003) Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl Envirion Microbiol 69(1):600–606

Agnalt AL, Ervik A, Kristiansen TS, Oppedal F (2004) Havbruksrapporten 2004 Fisken og Havet, 3 Bergen, Norway

Anda P, del Pozo JS, Garcia JMD et al (2001) Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis 7(3):575–582

Barns SM, Grow CC, Okinaka RT, Keim P, Kuske CR (2005) Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl Environ Microbiol 71(9):5494–5500

Brown JA, Minkoff G, Puvanendran V (2003) Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. Aquaculture 227:357–372

Chen SC, Tung MC, Chen SP, Tsai JF, Wang PC, Chen RS, Lin SC, Adams A (1994) Sytematic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochromis niloticus* (L.), from southern Taiwan. J Fish Dis 17:591–599

Chen MF, Yun S, Marty GD, McDowell TS, House ML, Appersen JA, Guenther TA, Arkush KD, Hedrick RP (2000) A *Piscirickettsia salmonis*-like bacterium associated with mortality of white seabass *Astractoscion nobilis*. Dis Aquat Org 43:117–126

Chern RS, Chao CB (1994) Outbreaks of a disease caused by rickettsia-like organism in cultured tilapias in Taiwan. Fish Pathol 29:61–71

Dannevig BH, Brudeseth BE, Gjøen T, Rode M, Wergeland HI, Evensen Ø, Press CM (1997) Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 7:213–226

Devold M, Krossøy B, Aspehaug V, Nylund A (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Dis Aquat Organ 40:9–18

Giovannoni S (1991). The polymerase chain reaction. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 77–201

Hollis DG, Weaver RE, Steigerwalt AG, Wenger JD, Moss CW, Brenner DJ (1989) *Francisella philomiragia* comb. nov. (Formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (Formerly *Francisella novicida*) associated with human disease. J Clin Microbiol 27(7):1601–1608

Jensen WI, Owen CR, Jellison WL (1969) *Yersinia philomiragia* sp.n., a new member of the *Pasturella* group of bacteria, naturally pathogenic for the muskrat (*Ondatra zibethica*). J Bacteriol 100(3):1237–1241

Kamaishi T, Fukuda Y, Nishiyama M, Kawakami H, Matsuyama T, Yoshinaga T, Oseko N (2005) Identification and pathogenicity of intracellular *Francisella* bacterium in three-line grunt *Parapristipoma trilineatum*. Fish Pathol 40(2):67–71

Khoo L, Dennis PM, Lewbart GA (1995) Rickettsia-like organisms in the blue-eyed plecostomus, *Panaque suttoni* (Eigenmann & Eigenmann). J Fish Dis 18:157–164

Larsson P, Oyston PCF, Chain P, Chu MC et al (2005) The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet 37(2):153–159

Mauel MJ, Miller DL, Frazier K, Liggett AD, Styer L, Montgomery-Brock D, Brock J (2003) Characterization of piscirickettsiosis-like disease in Hawaiian tilapia. Dis Aquat Org 53:249–255

Nicholas KB, Nicholas HBJ, Deerfield DWI (1997) GeneDoc: Analysis and visualization of genetic variation. Embnew News 4:14

Niebylski ML, Peacock MG, Fisher ER, Porcella SF, Schwan TG (1997). Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. Appl Environ Microbiol 63(19):3933–3940

Noda H, Munderloh UG, Kurtti TJ (1997). Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. Appl Environ Microbiol 63(19):3926–3932

Nylund A, Hovland T, Watanabe K, Endresen C (1995). Presence of infectious salmon anaemia virus (ISAV) in tissues of Atlantic salmon, *Salmo salar* L., collected during three separate outbreaks of the disease. J Fish Dis 18:135–145

Oyston PCF, Sjøstedt A, Titball RW (2005) Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol 3:967–978

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Scoles GA (2004) Phylogenetic analysis of the *Francisella*-like endosymbionts of *Dermacentor* ticks. J Med Entomol 41(3):277–286

Sjöstedt AB (2005a) Family III. Francisellaceae fam.nov. In: Garrity GM (ed) Bergeys manual of systematic bacteriology, 2nd edn. Springer, USA pp 199–210

Sjöstedt A (2005b) Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. Microbes Infect 8:561–567

Suitor EC, Weiss E (1961). Isolation of a rickettsialike microorganism (*Wolbachia persica*, n.sp.) from *Argas persicus* (Oken). J Infect Dis 108:95–106

Swofford DL (1998) Phylogenetic analysis using parsimony and other methods. Sinauer Associates, Sunderland

Timur G, Timur M, Akayli T, Korin J, Thompson KD (2005) First observation of rickettsia-like organisms in cultured sea bass (*Dicentrarchus labrax*) in Turkey. Bull Eur Assoc Fish Pathol 25(5):196–202

Tomaso H, Dahouk SA, Hofer E, Splettstoesser WD, Treu TM, Dierich MP, Neubauer H (2005) Antimicrobial susceptibilities of Austrian *Francisella tularensis holartica* biovar II strains. Int J Antimicrob Agents 26:279–284

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ORIGINAL PAPER

Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*)

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Abstract A *Francisella* sp., isolate GM2212^T, previously isolated from diseased farmed Atlantic cod *Gadus morhua* in Norway is characterized. The complete 16S rDNA, 16S–23S intergenic spacer, 23S rDNA, 23S–5S intergenic spacer, 5S rDNA, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase and a hypothetical lipoprotein (LpnB) is sequenced and compared with *Francisella tularensis* and *Francisella philomiragia*. All these sequences support a close relationship between GM2212^T and *F. philomiragia*. The bacterium grows at 10–25°C with an optimum at about 20°C, a temperature range clearly different from *F. tularensis* and *F. philomiragia*. GM2212^T is catalase-positive, indole positive, oxidase-negative, do not produce H_2S in Triple Sugar Iron agar, and does not hydrolyze gelatin, is resistant to erythromycin and susceptible to ceftazidime, the latter five characteristics separating it from *F. philomiragia*. Cysteine enhances growth. Acid is produced from D-glucose, maltose, sucrose (weak) but not from lactose or glycerol. GM2212^T grows on both MacConkey agar and in nutrient broth (6% NaCl). The bacterium is resistant to trimethoprim-sulfamethoxazole, penicillines, cefuroxime and erythromycin; but is susceptible to ceftazidime, tetracycline, gentamicin, ciprofloxacin. Based on the molecular and phenotypical characteristics, we suggest that this GM2212 isolate, may represent a new species of *Francisella*. Isolate GM2212^T (=CNCM I-3481^T = CNCM I-3511^T = DSM 18777^T).

Keywords *Francisella* sp. · GM2212 · rRNA genes · FopA · Lipoprotein TUL4 · LpnA · LpnB · Malate dehydrogenase · Antibiotics · Fatty acids

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Introduction

The family Francisellaceae contains one genus, *Francisella*, and two recognized species, *F. tularensis* and *F. philomiragia* (Sjøstedt 2005). These have been shown to be widespread in some terrestrial and aquatic environments (Forsman et al. 1994; Anda et al. 2001; Barns et al. 2005; Petersen and Schriefer 2005; Sjøstedt 2005). However, based on similarities in the 16S rRNA gene sequence, several other potential new species of *Francisella* from ticks (Suitor and Weiss 1961; Niebylski et al. 1997; Noda et al. 1997; Scoles 2004), environmental samples (Barns et al. 2005) and fish (Kamaishi et al. 2005; Nylund et al. 2006; Olsen et al. 2006) have been reported. From diseased farmed Atlantic cod (*Gadus morhua*) in the county of Rogaland, western Norway, a *Francisella* sp. was isolated in 2004 (Nylund et al. 2006). Challenge experiments showed that this *Francisella* sp. was the cause for the new disease. The 16S rRNA gene sequence from the bacterium showed highest similarity to *Francisella* strains isolated from other fish species and to *F. philomiragia* strains, and based on

SSU sequence data the *Francisella* sp. from cod was considered a likely new species (Nylund et al. 2006).

The objectives of this study were to compare the characteristics and a selection of genes from this *Francisella* sp. from cod (Nylund et al. 2006) with that of the recognized members of genus *Francisella*; *F. philomiragia* and *F. tularensis*. Based on these comparisons it is proposed that the *Francisella* isolate from cod could represent a new species within genus *Francisella*.

Materials and methods

In 2004 a *Francisella* sp. (GM2212^T) was isolated from the kidney of farmed Atlantic cod (*G. morhua*) in the county Rogaland, western Norway. GM2212^T was first isolated on blood agar-plates containing 0.1% cysteine and 1% D-glucose (BCG agar-plates) incubated at 15°C for 30 days (Nylund et al. 2006). The isolate has been deposited in the Collection Nationale de Cultures de Microorganismes, Institute Pasteur, Paris, France (Identification reference: *Francisella* n.sp. GM2212^T, Registration number: CNCM I-3481 and I-3511) and has been sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Identification reference: *Francisella* sp., GM2212^T, Registration number: DSM 18777).

Culture media and growth conditions

Two different liquid media have been successfully used for culturing of GM2212^T: (a) Bacto™ Eugon Broth (Becton, Dickinson and Company) was made following the manufacturers recommendation, and added $FeCl_3 \cdot 6H_2O$ (Merck™) to a final concentration of 2 mM as described by Kamaishi et al. (2005). (b) Growth medium B1817 consists of 900 ml of Marine Broth 2216 (Difco), added 100 ml of Fetal Calf Serum (Gibco/BRL™), 60 ml of Yeastolate Ultrafiltrate (Gibco/BRL), 40 ml L-cysteine·HCl (Merck) sol 6.3 g/l dH_2O (distilled water, filter sterilized) and 40 ml D-glucose (Merck) sol 200 g/l dH_2O. Medium B1817 were also made as agar plates where 900 ml Marine Broth 2216 was added 15 g Agar–agar (Mikrobiologie) and boiled until the agar was dissolved. The mixture was cooled to 60°C and added the same amount of Fetal Calf Serum, Yeastolate Ultrafiltrate, L-cysteine and D-glucose as mentioned above. Portions of 25 ml were dispensed into sterile petridishes.

Tubes with 10 ml aliquots of the liquid medium Bacto Eugon Broth or B1817 were inoculated with GM2212^T, from BCG agar-plates. The tubes were incubated in Infors Unitron incubators at 10, 15, 20, 25, 29 and 37°C and with 250 rpm shaking for testing of optimal growth temperatures. Growth was monitored using optical density measurements at 550 nm (OD_{550}). After the optimal growth temperature was determined GM2212^T were grown to the mid-logarithmic phase ($OD_{550} = 0.6$), pelleted at 2,500×*g* for 30 min, before the growth media was removed and the bacteria eluted in sterile filtered sea-water. The eluted bacteria were progressively frozen in liquid nitrogen in 500 µl aliquots and stored at −80°C.

If not otherwise stated, all inoculations with GM2212^T were performed with the isolate grown in B1817 to the mid-logarithmic phase, or with the isolate grown on B1817 agar-plates. All liquid media were incubated at 20°C with shaking at 250 rpm in an Infors Unitron incubator and the agar-plates and agar-tubes were incubated at 20°C in a Memmert GTR0214 incubator.

GM2212^T was also grown on Trypton Soy Agar (TSA)- and Brain Heart Infusion Agar (BHIA)-plates without any supplement of L-cysteine. These were made following the manufacturers recommendation (Difco). Plates were inoculated with GM2212^T grown in liquid medium or on agar-plates, incubation as mentioned above.

Growth on MacConkey (MAC) agar was also tested. MAC agar-plates were prepared following the manufacturers recommendation (Difco), inoculated and incubated as mentioned above.

To test if CO_2 enhanced the growth of GM2212^T, B1817, chocolate and MAC agar-plates were incubated in an ASSAB Medicine CO_2−incubator in an atmosphere supplemented with 5% CO_2.

Growth was also tested in nutrient broth both with and without 6% NaCl. Nutrient broth was prepared following the manufacturers recommendation (Difco). Nutrient broth with 6% NaCl was added 60 g/l of NaCl. Tubes containing 10 ml of nutrient broth were inoculated and incubated as mentioned above.

Oxygen requirements of GM2212^T, was tested in thioglycollate medium. This medium was prepared following the manufacturers recommendation (Difco) and 5 ml aliquots were made in glass tubes. Tubes were stab inoculated with GM2212^T and incubated as mentioned above.

Phenotyping

The size and morphology was described by Nylund et al. (2006). Gram staining and studies on motility was performed on the bacterium grown on B1817 agar-plates. The Gram-staining procedure was performed using the crystal-violet, iodine, ethanol, safranin

method. Motility tests were performed using wet mounts. The same procedures were also performed with *F. philomiragia* (DSM 7535, ATCC 25015^T).

Agglutination tests with two polyclonal rabbit antisera were performed: (a) antiserum against *F. tularensis* (Becton, Dickinson and Company) and, (b) rabbit antiserum against GM2212^T. The suspension used in immunization of the rabbits consisted of formaline-inactivated bacteria. These bacteria had been grown on BCG agar-plates, suspended in PBS to a final concentration of 5.0×10^9 bacteria/ml, and inactivated by addition of formalin to a final concentration of 0.5%. Undiluted anti *F. tularensis* rabbit antiserum 50 µl was placed on a micro-slide and added 50 µl GM2212^T (grown in B1817). The solution was mixed well and the slide was gently agitated for 5–10 min before investigated under a microscope. The same procedure was also performed with antiserum against GM2212^T. Antisera against both *F. tularensis* and GM2212^T were also tested in an agglutination-test on the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T) and on ethanol-inactivated *F. philomiragia* (strain 1951) and GM2212^T.

Biochemical characterization

Bergey's Manual give the characteristics for members of the genus *Francisella*, its species and subspecies (Sjøstedt 2005). Tests to determine these characteristics were performed with GM2212^T, along side with the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T). All inoculations with *F. philomiragia* (DSM 7535, ATCC 25015^T) were performed with the bacterium grown in B1817 or on B1817 agar-plates as described for GM2212^T.

The presence of the enzymes catalase and oxidase were tested on GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) grown on B1817 agar-plates using a 3% H_2O_2 solution and an oxidase-reagent (bioMérieux®sa), respectively. Indole tests were performed on the bacteria grown in TSB or on TSA agar-plates using Kovacs test reagent (Merck).

Tests to determine the nitrate reduction, urease activity and gelatin hydrolysis were performed as described by Smibert and Krieg (1994) with some modifications. In the gelatin hydrolysis test, B1817 was used as the growth medium. H_2S production was tested on Triple Sugar Iron (TSI) agar (Difco) and in B1817 using lead acetate paper strips (Fluka). Acidification of carbohydrates was tested in B1817 without D-glucose. The different substrates were added to a final concentration of 1%. Each tube was added 50 µl of a pH indicator, Bromocresol-purple [0.2% Bromocresol-purple (Sigma) in 50% ethanol]. Tubes were inoculated and incubated as mentioned above together with control tubes without any substrate.

The antibiotic susceptibility of GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) was tested on B1817 agar-plates. Plates were inoculated with 200 µl of bacteria and incubated at 20°C. Tablets of trimethoprim-sulfamethoxazole (23.75–1.25 µg), penicillin (10 µg), ampicillin (10 µg), cefuroxime (30 µg), ceftazidime (30 µg), erythromycin (15 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) was added to the inoculated B1817-plates. The plates were incubated as mentioned above. Growth in liquid B1817 added ampicillin and oxolinic acid was also tested. Tubes with 10 ml B1817 containing 100 µg/ml ampicillin or 15 µg/ml oxolinic acid were inoculated with the bacteria and incubated as mentioned above.

For fatty acid analysis, 500 µl of GM2212^T were inoculated in 80 ml B1817 and incubated at 20°C with 250 rpm for 7 days. After the incubation period the bacterial cultures were harvested at 2,500×*g* for 50 min followed by removal of the growth media. The bacterial pellets were weighted and stored at −80°C prior to fatty acid analysis at NIFES (National Institute of Nutrition and Seafood Research). The fatty acids were analyzed using a gas liquid chromatography flame ionization detector as described by Lie and Lambertsen (1991). No hydroxy fatty acids were determined using this procedure.

DNA extraction and gene sequencing

DNA from GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) was extracted as described by Nylund et al. (2006). The following genes and spacers in GM2212^T, and both of the *F. philomiragia* strain genomes were sequenced: 16S, 16S–23S intergenic spacer, 23S rRNA, 23S–5S intergenic spacer, 5S, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase (mdh) and a hypothetical lipoprotein (LpnB). The PCR reaction mixture was as described by Nylund et al. (2006). Primers used to amplify the rRNA and FopA genes from the *F. philomiragia* strains are the same as previously described by Nylund et al. (2006). New primers used to amplify additional protein genes are presented in Table 1. Amplification was performed in a GeneAmp PCR system 9700 [Applied Biosystems (ABI)] at 95°C for 5 min; 40 cycles of 94°C for 30 s, *X*°C (50°C for the protein primers, and 55°C for the rRNA primers) for 45 s, 72°C for 1 min and 30 s followed by extension at 72°C for 10 min and a short storage at 4°C.

PCR products were purified using QIAquick PCR purification kit (Qiagen) as described by the manufac-

Table 1 Overview of primers used in amplification and sequencing of the 17-kDa lipoprotein TUL4 (LpnA), hypothetical lipoprotein (LpnB), malate dehydrogenase (mdh) and outer membrane protein (FopA) from GM2212[1] and *F. philomiragia* (DSM 7535, ATCC25015^T, strain 1951)

Primer	Sequence	Location
17 kDa lipoprotein gene TUL4 (LpnA)		
Lipo-F3	5′-AGT CTT TTA TCT TTG TCA ATC GCA G	22–47
Lipo-F4	5′-TCT TGC TAG TTG TTC TAC AGT	48–69
Lipo-R3	5′-GGA GCT TGC CAT TGT AAT CT	267–247
Lipo-R4	5′-CAC TTC CTT GAG GAT TAT TA	245–225
Lipo-R5	5′-AKT KAT TGA ATC AGA AGC RAT TAC	436–412
Hypothetical lipoprotein gene (LpnB)		
Lipo-F2	5′-GTT AAT CCT GAT GAT AAC YTT G	121–143
Lipo-R1	5′-GCT GTC CAT GTC CCA TTA CA	426–406
Malate dehydrogenase gene (mdh)		
mdh-F1	5′-GCT TRT TGG TGC TGG TAA TA	21–41
mdh-R1	5′-RCT TTC WGC CAT TTG RAT WC	736–716
Outer membrane protein gene (FopA)		
FMP-F1	5′-GCT TCA ATG TAC TAA GGT TTG CCC	Outside
FMP-F2	5′-CAA GAT AGA ACT GGY CAG TGG	202–223
FMP-R4	5′-ATA KAT MTC AAA YTC GCT WCC AG	985–962

The location of the primers is given in relation to the open reading frame of the protein encoding genes from *F. philomiragia* (DSM 7535, ATCC 25015^T)

turer. Sequencing was then performed with ABI PRISM BigDye terminator chemistry (version 2) according to ABI. All sequences were assembled using the Vector NTI Suite 7.0 program (InforMax Inc.). The different genes were identified by BLASTn search. Sequences obtained in the present study were submitted to the GenBank.

Phylogenetic analysis

The Vector NTI Suite software package was used for the multiple alignments of nucleotide sequences. To perform pair wise comparisons between the different gene-sequences the multiple sequence alignment editor GeneDoc was used (Nicholas et al. 1997). Sequences of *F. tularensis* subsp. *tularensis* (accession no.: NC_006570) already available on the EMBL nucleotide database were included in the comparisons.

Results

Culture media and growth conditions

Growth of GM2212^T, in the two liquid media Eugon Broth and B1817 was tested at 10, 15, 20, 25, 29 and 37°C. Growth was observed in the temperature range 10–25°C in both liquid media. The growth was very slow at 10°C and even less evident at 25°C, while no growth was observed at 29 and 37°C. The best growth was obtained at 20°C.

The bacterium grew best in B1817 medium and the mid-logarithmic phase was reached within 2–3 days. Growth in Eugon Broth was slower and the mid-logarithmic phase was first reached 4–5 days after inoculation, consequently, B1817 was used in the further growth-studies of GM2212^T, at 20°C.

When inoculated onto B1817, and chocolate agar-plates (20°C) colonies were produced that were distinct, punctiform, convex, slightly transparent to grayish-white after 2–3 days. Colonies grew in size to 1–2 mm within 4–5 days. Growth of the isolate on TSA- and BHIA-plates was also observed, but was very slow compared to growth on media supplemented with L-cysteine. The colonies described above were not observed, instead, a very thin transparent layer was evident at the rim of the inoculated area after 7–10 days.

No difference in growth on B1817 or chocolate agar-plates could be observed between those incubated in an atmosphere supplemented with 5% CO_2 and those incubated in normal atmosphere. Growth on MAC agar, however, was evident after 14 days (colorless colonies <1 mm) only in an atmosphere supplemented with 5% CO_2, while incubation without CO_2 did not support growth of GM2212^T on MAC agar.

Growth was evident on top of and beneath the uppermost layer in the thioglycollate medium. Weak growth was evident after 7–14 days of incubation in nutrient broth added 6% NaCl, while no growth was evident in nutrient broth without NaCl.

Phenotypic and biochemical characteristics

Cells were Gram-negative and non-motile. Antiserum against *F. tularensis* subsp. *tularensis* did not agglutinate GM2212^T, in contrast to antiserum against GM2212^T. *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) was slightly agglutinated by the GM2212^T antiserum, but not by the *F. tularensis* subsp. *tularensis* antiserum (Table 2).

H_2S production was detected by the lead acetate paper strips in GM2212^T cultures supplemented with L-cysteine grown for 7–14 days (B1817), but not on TSI-agar. H_2S production was detected by both methods after 5–7 days in cultures with the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T).

GM2212^T was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, erythromycin and susceptible to ceftazidime, tetracycline, gentamicin and ciprofloxacin. Reduced growth in B1817 tubes containing oxolinic acid was observed, while growth in B1817 tubes with ampicillin was similar to the growth in tubes without any supplement of antibiotics. *F. philomiragia* (DSM 7535, ATCC 25015^T) was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, ceftazidime and susceptible to erythromycin, tetracycline, gentamicin and ciprofloxacin. Growth of *F. philomiragia* in B1817 tubes containing ampicillin was similar to the growth in tubes without any supplement of antibiotics, while growth in B1817 with oxolinic acid was reduced.

An overview of the relative fatty acid composition (except the hydroxy fatty acids) of GM2212^T is given in Table 3. Of the total fatty acids examined in GM2212^T 52.6% was completely identified and 47.4% partly determined. The identified fatty acids were predominantly made up of long-chain saturated and mono-saturated acids ranging from C_{18} to C_{24}, and the saturated even-chain acids $C_{14:0}$ and $C_{16:0}$. The partly determined

Table 2 Differential physiological and biochemical characteristics at 20°C of strains 1; *Francisella* sp., GM2212^T and 2; *F. philomiragia* (DSM 7535, ATCC 25015^T)

Characteristic	1	2	3	4	5	6
Size	<1.5 μm	<1.5 μm	<0.5 μm	<0.5 μm	<0.5 μm	<1.5 μm
Gram stain	+	+	w+	w+	w+	w+
Cysteine required for growth	−[a]	−	+	+	+	−
H_2S production in cysteine-supplemented medium	w+ (7–14 days)	+ (7 days)	+	+	+	+
β-Lactamase	+	+	+	+	−	+
Acid production from:						
Maltose	+	+	+	+	−	w
Lactose	−	−	−	−	−	−
Sucrose	w+	+	−	−	−	+
D-Glucose	+	+	+	+	−	+
Glycerol	−	−	+	−	+	w
Agglutination of *F. tularensis* antiserum	−	−	+	+	+	w
Agglutination of strain GM2212T antiserum	+	−[b]	_[c]	_[c]	_[c]	_[c]
Presence of *F. tularensis* 17-kDa lipoprotein	+	+	+	+	+	+
Aerobic, microaerophilic	+	+	+	+	+	+
Growth in nutrient broth 0% NaCl	−	−	−	−	−	−
Growth in nutrient broth 6% NaCl	w+	+	−	−	−	w
Catalase	+	+	w	w	w	w
Oxidase	−	+	−	−	−	−
Indole	+	+	−	−	−	−
Urease	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−
H_2S slant, TSI	−	+ (7 days)	−	−	−	−
Gelatin hydrolysis	−	+ (9 days)	−	−	−	−
Motility	−	−	−	−	−	−

Literature data from Sjøstedt (2005), strains: 3, *F. tularensis* subsp. *tularensis*; 4, *F. tularensis* subsp. *holarctica*; 5, *F. tularensis* subsp. *mediasiatica*; 6, *F. tularensis* subsp. *Novicida*

\+ stands for positive

− stands for negative

w stands for weak

[a] Grows very slowly on TSA- and BHIA-agars without added cysteine

[b] Negative or slight agglutination of *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951)

[c] Not tested, since these are literature data

Table 3 Relative fatty acid composition of *Francisella* sp., GM2212^T

Fatty acid	Abbreviation	*Francisella* sp., GM2212^T
Unidentified	$<C_{14}$	17.8
Unidentified	$<C_{14}$	1.3
Tetradecanoic acid	14:0	10.3
Pentadecanoic acid	15:0	0.1
Hexadecanoic acid	16:0	4.0
9-Hexadecenoic acid	16:1$n-7$	1.0
Heptadecanoic acid	17:0	0.1
Octadecanoic acid	18:0	4.5
9-Octadecenoic acid	18:1$n-9$	9.9
11-Octadecenoic acid	18:1$n-7$	0.1
9,12-Octadecadienoic acid	18:2$n-6$	0.1
Eicosanoic acid	20:0	3.4
9-Eicosenoic acid	20:1$n-11$	2.4
Docosanoic acid	22:0	4.9
5,8,11, 14-Eicosatetraenoic acid	20:4$n-6$	0.1
11-Docosaenoic acid	22:1$n-11$	4.7
Tetracosanoic acid	24:0	6.0
15-Tetracosaenoic acid	24:1$n-9$	0.5
7,10,13,16, 19-Docosapentaenoic acid	22:5$n-3$	0.1
4,7,10,13,16, 19-Docosahexaenoic acid	22:6$n-3$	0.2
Unidentified	$>C_{20}$	22.7
Unidentified	$>C_{22}$	1.1
Unidentified	$>C_{22}$	4.5
SUM		100

The amount of each fatty acid is shown as a percentage of the total content of fatty acids

fatty acids were seen in the gas chromatographic profile as five distinct peaks, of which peaks 1 and 2 represented saturated fatty acids shorter than C_{14} in length (probably $C_{10:0}$ and $C_{12:0}$). Peaks 3–5 were observed late in the chromatogram and represented fatty acids longer than C_{20} in length.

Gene sequences

The rRNA genes from GM2212^T have been sequenced by Nylund et al. (2006). This study provides the same genes (4,841 nt) from *F. philomiragia* (strain 1951, accession no.: DQ813266) and (4,512 nt) from the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T, accession no.: EF153479). The 16S and 23S sequence from GM2212^T is more similar to the *F. philomiragia* strains than to *F. tularensis*. In the more variable 16S–23S spacer region, the similarity is lower (<98%) between GM2212^T and the two *F. philomiragia* strains, but differ markedly from *F. tularensis* (Table 4).

Results from the comparisons of the putative outer membrane protein (FopA), mdh, lipoprotein LpnA (TUL4) and the conserved hypothetical lipoprotein (LpnB) gene-sequences from GM2212^T, *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) and *F. tularensis* subsp. *tularensis* (SHCU4) is also listed in Table 4. In all proteins the nucleotide sequence similarity between the two *F. philomiragia* strains and GM2212^T (89–99%) are clearly higher than to *F. tularensis* (70–98%). The maximum divergence of GM2212^T from the *F. philomiragia* strains is seen in the LpnB gene (11%).

Discussion

Genus *Francisella* contains two recognized species, *F. tularensis* and *F. philomiragia* (Sjøstedt 2005). The most closely related bacterium to these is *Wolbachia persica*, an obligate intracellular bacterium from a tick that should be transferred to *Francisella* (Niebylski et al. 1997; Noda et al. 1997). Several other bacteria, clearly related to or members of *Francisella*, have also

Table 4 Nucleotide similarities in the rRNA- and protein-genes between *Francisella* sp., GM2212^T, *F. philomiragia* (DSM 7535, ATCC 25015^T), *F. philomiragia* (strain 1951) and *F. tularensis* (SCHU4)

	16S rRNA	16S–23S rRNA spacer	23S rRNA	FopA	Malate dehydrogenase	TUL4 (LpnA)	Lipoprotein (LpnB)
F. philomiragia (DSM 7535, ATCC 25015^T)	99.1/1,498nt	98/328nt	99.3/2,862nt	98.5/1,098nt	96/653nt	98/420nt	89/272nt
F. philomiragia (strain 1951)	99.1/1,498nt	98/328nt	99.3/2,862nt	98.5/955nt	97/653nt	97/373nt	89/272nt
F. tularensis (SCHU4)	97.5/1,498nt	91/328nt	97.8/2,862nt	81.9/955nt	83/653nt	70/382nt	78/272nt
Francisella sp., GM2212^T	1,498nt	328nt	2,862nt	955nt	653nt	383nt	272nt

Similarities are shown in percent and in number of nucleotide (nt). rRNA genes [*Francisella* sp., GM2212^T , accession no.: DQ309246, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153479, *F. philomiragia* (strain 1951) accession no.: DQ813266], FopA [*Francisella* sp., GM2212^T , accession no.: DQ333226, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153476, *F. philomiragia* (strain 1951) accession no.: DQ825766], mdh [*Francisella* sp., GM2212^T , accession no.: DQ825768, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153478, *F. philomiragia* (strain 1951) accession no.: DQ813265], TUL4 [*Francisella* sp., GM2212^T , accession no.: DQ825765, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153475, *F. philomiragia* (strain 1951) accession no.: DQ813267], LpnB [*Francisella* sp., GM2212^T , accession no.: DQ825767, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153477, *F. philomiragia* (strain 1951) accession no.: DQ813268]

been detected as endosymbionts in a wide range of tick species (Niebylski et al. 1997; Noda et al. 1997; Scoles 2004) and in environmental samples (Barns et al. 2005). Several strains of *Francisella* have also been isolated from diseased fish in culture, tilapia (*Oreochromis niloticus*), three-line grunt (*Parapristipoma trilineatum*) and from cod in Norway (Kamaishi et al. 2005; Kay et al. 2006; Nylund et al. 2006; Olsen et al. 2006), but no validly published descriptions or names are yet available for these. As shown by Nylund et al. (2006), it also appears that *Francisella* sp. from cod in Norway is distinct from the *Francisella* sp. causing disease in Asian fish culture.

The present study confirm a high 16S rRNA and FopA gene sequence similarity between GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951), found by Nylund et al. (2006). Our previous study (Nylund et al. 2006) was hampered by the lack of spacer and 23S sequence information for *F. philomiragia*, obtained in the present study from strain 1951 and DSM 7535, ATCC 25015^T. These comparisons support a closer relation of GM2212^T to *F. philomiragia* than to *F. tularensis*. However, the differences suggest that GM2212^T could be a new species. This is supported by comparison of the additional protein genes sequenced (TUL4 = LpnA, LpnB and mdh).

In the biochemical characters there are similarities between GM2212^T and *F. philomiragia* (DSM 7535, ATCC25015^T). However, the most striking differences are that GM2212^T is oxidase-negative, do not produce H_2S in TSI agar, and lack the ability to hydrolyze gelatin (present study). GM2212^T also grows slower than *F. philomiragia* and clearly differ by its low optimal growth temperature and growth temperature range. The susceptibility of GM2212^T to some antibiotics is also different from that of *F. philomiragia*.

The differentiating biochemical characters of GM2212^T generally differ from literature data of *F. tularensis*, the highest similarity evident is to *F. tularensis* subsp. *novicida*. However, the most striking differences between GM2212^T and literature data on *F. tularensis* subsp. *novicida* (Sjøstedt 2005), is that the former show tryptophanase activity (indole positive), do not degrade glycerol, is not agglutinated by antisera against *F. tularensis* subsp. *tularensis* and grows best at lower temperatures. On the basis of 16S rRNA, 16S–23S spacer, 23S rRNA, TUL4 = LpnA, LpnB and mdh gene sequence analysis and phenotypic characteristics, GM2212^T could indeed represent a novel species within the genus *Francisella*.

Due to the high similarity in the rRNA genes, a DNA–DNA hybridization between GM2212^T and the type strain of *F. philomiragia* (DSM 7535, ATCC25015^T) is currently being performed. This should clarify if *Francisella* sp., GM2212^T, isolate from cod represent a species distinct from *F. philomiragia*. DNA–DNA hybridization has previously been used to delineate *F. philomiragia* from *F. tularensis* in studies by Hollis et al. (1989). In these studies *F. philomiragia* showed 39% average relatedness to the type strain of *F. tularensis* and four other *F. tularensis* strains.

A characteristic shared by *Francisella* sp., GM2212^T, *F. philomiragia* and *F. tularensis* is an association with or presence in water. Strains of *F. tularensis* have been associated with lakes, streams and rivers and there are cases where people have become infected after direct contact with or by ingestion of contaminated water (Karphoff and Antonoff 1936; Greco et al. 1987; Whipp et al. 2003). In most of the cases where such waterborne infections with *F. tularensis* has occurred, carcasses of rodents or lagomorphs have been found in or near the water source (Anda et al. 2001; Reintjes et al. 2000; Hoel et al. 1991). Strains of *F. philomiragia* have so far only been found in saline-, brackish- and seawater (Jensen et al. 1969; Hollis et al. 1989; Friis-Møller et al. 2004), where infection in humans may occur in immunocompromised individuals and in near drowning victims (Wenger et al. 1989; Friis-Møller et al. 2004). In contrast to *F. tularensis* there is no evidence that animal carcasses were involved in cases where *F. philomiragia* have caused disease. So far, *Francisella* sp., GM2212^T, has been detected in tissues of cod, and with a specific real-time PCR also in saithe (*Pollachius virens*), blue mussels (*Mytilus edulis*) and crabs (*Cancer pagurus*) from the environment of affected cod farms (pers. obs.). In contrast to the related *F. tularensis* and *F. philomiragia*, *Francisella* sp., GM2212^T, have not been reported to cause disease in humans despite that people working in the cod-farming industry have been in contact with infected cod, and despite that infected cod frequently must have been used for human consumption. It is unlikely that humans can acquire *Francisella* sp., GM2212^T, infections due to fact that the bacterium does not grow at 37°C. Nevertheless, if disease is suspected in immunocompromised patients who have been in contact with infected cod, diagnostic laboratories should use appropriate growth media, incubation temperature and time which could support growth of the bacterium to rule out infection with *Francisella* sp., GM2212^T.

Description of *Francisella* sp., GM2212^T

Aerobic, microaerophilic; Gram-negative, coccoid to short rods, single cells, typically 0.5 µm, maximum 1.5 µm. Growth at 10–25°C, optimal at c. 20°C.

Oxidase-negative, catalase-positive. Do not produce H_2S in TSI agar, does not hydrolyze gelatin. Cysteine enhances growth. Acid production from D-glucose, maltose, sucrose (weak) but not from lactose or glycerol. Indole positive. Growth on MAC agar and in nutrient broth (6% NaCl). Resistant to trimethoprim-sulfamethoxazole, penicillines, cefuroxime, erythromycin; susceptible to ceftazidime, tetracycline, gentamicin and ciprofloxacin. The predominant non-hydroxy fatty acids are $C_{<14}$ unidentified (17.8%), C_{14} (10.3%), $C_{18:1n-9}$ (9.9%), C_{24} (6.0%), C_{20+} unidentified (22.7%).

Isolate GM2212^T (=CNCM I-3481^T = CNCM I-3511^T = DSMZ 18777^T) was isolated from the kidney of farmed Atlantic cod (*G. morhua*) from Rogaland County, western Norway.

References

Anda P, del Pozo JS, Garcia JMD et al (2001) Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis 7:575–582

Barns SM, Grow CC, Okinaka RT, Keim P, Kuske CR (2005) Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl Environ Microbiol 71:5494–5500

Forsman M, Sandstrøm G, Sjøstedt A (1994) Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int J Syst Bacteriol 44:38–46

Friis-Møller A, Lemming LE, Valerius NH, Bruun B (2004) Problems in identification of *Francisella philomiragia* associated with fatal bacteremia in a patient with chronic granulomatous disease. J Clin Microbiol 43:1840–1842

Greco D, Allegrini G, Tizzi T, Ninu E, Lamanna A, Luzi S (1987) A waterborne tularemia outbreak. Eur J Epidemiol 3:35–38

Hoel T, Scheel O, Nordahl SHG, Sandvik T (1991) Water- and airborne *Francisella tularensis* biovar palaearctica isolated from human blood. Infection 5:348–350

Hollis DG, Weaver RE, Steigerwalt AG, Wenger JD, Moss CW, Brenner DJ (1989) *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. J Clin Microbiol 27:1601–1608

Jensen WI, Owen CR, Jellison WL (1969) *Yersinia philomiragia* sp.n., a new member of the *Pasturella* group of bacteria, naturally pathogenic for the muskrat (*Ondatra zibethica*). J Bacteriol 100:1237–1241

Kamaishi T, Fukuda Y, Nishiyama M, Kawakami H, Matsuyama T, Yoshinaga T, Oseko N (2005) Identification and pathogenicity of intracellular *Francisella* bacterium in three-line Grunt *Parapristipoma trilineatum*. Fish Pathol 40:67–71

Karphoff SP, Antonoff NE (1936) The spread of tularemia through water, as a new factor in its epidemiology. J Bacteriol 32:243–258

Kay W, Petersen BO, Duus JØ, Perry MB, Vinogradov E (2006) Characterization of the lipopolysaccharide and β-glucan of the fish pathogen *Francisella victoria*. FEMS J 273:3002–3013

Lie O, Lambertsen G (1991) Fatty acid composition of glycerophospholipids in seven tissues of cod (*Gadus morhua*), determined by combined high-performance liquid chromatography and gas chromatography. J Chromatogr 565:119–129

Nicholas KB, Nicholas HBJ, Deerfield DWI (1997) GeneDoc: analysis and visualization of genetic variation. Embnew.news 4:14

Niebylski ML, Peacock MG, Fisher ER, Porcella SF, Schwan TG (1997) Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the Genus *Francisella*. Appl Environ Microbiol 63:3933–3940

Noda H, Munderloh UG, Kurtti TJ (1997) Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. Appl Environ Microbiol 63:3926–3932

Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossøy B (2006) *Francisella* sp. (Family Francisellaeceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol 185:383–392

Olsen AB, Mikalsen J, Rode M, Alfjorden A, Hoel E, Straum-Lie K, Haldorsen R, Colquhoun DJ (2006) A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. J Fish Dis 29:307–311

Petersen JM, Schriefer ME (2005) Tularemia: emergence/re-emergence. Vet Res 36:455–467

Reintjes R, Dedushaj I, Gjini A, Jorgensen TR, Cotter B, Lieftucht A, D'Ancona F, Dennis DT, Kosoy MA, Mulliqi-Osmani G, Grunow R, Kalaveshi A, Gashi L, Humolli I (2000) Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerg Infect Dis 8:69–73

Scoles GA (2004) Phylogenetic analysis of the *Francisella*-like endosymbionts of *Dermacentor* ticks. J Med Entomol 41:277–286

Sjøstedt AB (2005) Family III. Francisellaceae fam. nov. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, 2nd edn, vol II, Part B. The gamma-proteobacteria. Springer, Berlin Heidelberg New York, pp 199–210

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, pp 607–654

Suitor EC, Weiss E (1961) Isolation of a rickettsia like microorganism (*Wolbachia persica*, n.sp.) from *Argas persicus* (Oken). J Infect Dis 108:95–106

Wenger JD, Hollis DG, Weaver RE, Baker CN, Brown GR, Brenner DJ, Broome CV (1989) Infection caused by *Francisella philomiragia* (formerly *Yersinia philomiragia*). A newly recognized human pathogen. Ann Intern Med 110:888–892

Whipp MJ, Davis JM, Lum G, de Boer J, Zhou Y, Bearden SW, Petersen JM, Chu MC, Hogg G (2003) Characterization of a novicida-like subspecies of *Francisella tularensis* isolated in Australia. J Med Microbiol 52:839–842

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SHORT COMMUNICATION

New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*)

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Abstract A *Francisella* strain, GM2212, previously isolated from moribund farmed Atlantic cod (*Gadus morhua*) in Norway, is closely related to *Francisella philomiragia* among *Francisella* spp. according to its complete 16S rDNA, 16S-23S intergenic spacer, 23S rDNA, 23S–5S intergenic spacer, 5S rDNA, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase and hypothetical lipoprotein (LpnB) sequences. A comparison between GM2212 and the type strain of *Francisella philomiragia* were performed by DNA–DNA hybridization and fatty acid analysis. The DNA–DNA hybridization showed a 70% similarity. The fatty acid analysis showed only minor differences between the *Francisella* isolates. Due to the inconclusive result from the DNA–DNA hybridisation, major emphasis concerning the status of this isolate is made on previously published molecular, phenotypic and biochemical characters. All characteristics taken together support the establishment of GM2212 as a novel species, for which the name *Francisella piscicida* sp. nov. is proposed (=CNCM I-3511^T = DSM 18777^T = LMG registration number not yet available).

Keywords *Francisella piscicida* sp. nov. · DNA–DNA hybridization · Fatty acids

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Introduction

The family Francisellaceae contains one genus, *Francisella*, and only two recognized species, *F. tularensis* and *F. philomiragia* (Sjøstedt 2005). From diseased farmed Atlantic cod (*Gadus morhua*) in the county of Rogaland, western Norway, a *Francisella* sp. (GM2212) was isolated in 2004 (Nylund et al. 2006). Based on complete 16S rDNA, 16S–23S intergenic spacer, 23SrDNA, 23S–5S intergenic spacer, 5S rDNA, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase and hypothetical lipoprotein (LpnB) sequences, GM2212^T was generally clearly separated from *F. tularensis*, and was closest to *F. philomiragia*. Based on the above-listed molecular data and comparative biochemical and phenotypic characters GM2212^T was also considered different from *F. philomiragia* and a likely new species (Nylund et al. 2006; Ottem et al. 2006). To further elucidate the relationship between GM2212^T from cod and *F. philomiragia* (DSM 7535, ATCC25015^T), a DNA–DNA hybridization and comparative fatty acid analysis were performed. Based on the polyphasic taxonomic data presented in our previous studies (Nylund et al. 2006; Ottem et al. 2006) and in the present study we propose that strain GM2212^T should be given the status as a novel species, *F. piscicida* sp. nov.

Materials and methods

Bacterial strain

In 2004, a *Francisella* sp. strain (GM2212) was isolated from farmed Atlantic cod (*Gadus morhua*) in Norway. This strain has been previously deposited in the Collection Nationale de Cultures de Microorganismes, Institute Pasteur,

Paris, France (identification reference: *Francisella* n.sp. GM2212^T, registration number: I-3511^T), in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (identification reference: *Francisella* sp., GM2212^T, registration number: DSM 18777^T) and in the BCCM/LMG Bacteria Collection, Ghent University (identification reference: *Francisella* sp., GM2212^T, LMG registration number not yet available). Phylogenetic position of GM2212^T based on rRNA genes, together with phenotypic and biochemical characteristics has been previously published by Nylund et al. (2006) and Ottem et al. (2006).

16S rRNA sequence variation

In addition to GM2212^T, we obtained partial 16S rDNA sequences using primers and PCR as previously published by Nylund et al. (2006), from *Francisella* sp., diseased farmed cod from Bodø area, northern Norway (accession no: EF685354), Møre and Romsdal, mid Norway (accession no: EF685355), Sotra, Hordaland, W Norway (accession no: EF685353), and Denmark (accession no: EF685349) (cod of Norwegian origin); and from wild cod from Karmøy (accession no: EF685350) and Ryfylkebassenget (accession no: EF685351), Rogaland. We also obtained a partial 16S rDNA sequence from moribund Atlantic salmon from Rogaland (accession no: EF685352), and included a *Francisella* sp. 16S rDNA sequence from farmed cod from W Norway available from GenBank (accession no: DQ295795). The sequence variation among these was examined using GenDoc (Nicholas et al. 1997) and compared with that of *F. philomiragia* as revealed by sequences deposited in GenBank: EF153479 (strain DSM 7535, ATCC 25015T), AY928395 (strain 25017), DQ813266 (strain 1951), AY243027 (strain 2669), AY968239 (strain FSC 144) and DQ314206 (no strain number).

DNA–DNA hybridization

GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) were grown in Eugon broth at 20°C with 150 rpm shaking for 4–7 days. The bacteria were centrifuged at 5,000 rpm for 40–60 min, the growth media removed and the pellets re-suspended in 1:1 isopropanol/dH_2O. The process was repeated, before storage at 4°C. Tubes (50 ml) with bacteria in 1:1 isopropanol/dH_2O were sent to DSM for DNA–DNA hybridization. At DSM, 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 hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1982) using a model Cary 100 Bio UV/Vis-spectrophotometer equipped with Peltier-thermostated 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian).

Fatty acid analysis

GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) were grown in B1817, incubated, harvested and analyzed as previously described (Ottem et al. 2006) with a slightly modified protocol. The modification was the use of a fatty acid standard making it possible to detect fatty acids with a chain length ranging from four carbons.

Results

The result of the DNA–DNA hybridization between GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) showed 69.5 and 70.8% similarity in 2 × SSC at 61°C. The relative fatty acid composition (nonhydroxy) of GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) is compared in Table 1. The fatty acid profile was similar in both bacteria, but there were differences in the amounts of some fatty acids between the bacteria. Of the total fatty acids examined 93.5 and 92.3% were completely identified in GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) respectively, while the remaining 6.5 and 7.7% fatty acids were only partly determined. The fatty acids were predominantly made up of long-chain saturated and mono-saturated acids ranging from C_{18} to C_{24}, and the saturated even-chain acids $C_{10:0}$, $C_{14:0}$ and $C_{16:0}$. The largest differences between the bacteria were found in the fatty acids: $C_{10:0}$ (2.9%), $C_{14:0}$ (3.4%), $C_{18:0}$ (2.2%), $C_{20:0}$ (2.3%), $C_{22:0}$ (3.4%), $C_{22:1n-11}$ (3.2%) and $C_{24:0}$ (4.6%). Of the partly determined fatty acids, a peak representing 4.1 and 3.8% was observed late in the chromatogram for GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T), respectively. These peaks probably represented fatty acids longer than C_{22} in length.

The eight additional partial sequences of *Francisella* sp. from farmed cod from Norway and Denmark, farmed salmon from Norway and wild cod from Norway showed 100.0% identity at 16S rDNA with GM2212^T. All *F. philomiragia* sequences from GenBank were virtually identical with the type strain in 1443 nt (99.9% identity). The percentage identity of GM2212^T with *F. philomiragia* is 99.2%. (1404 nt) GM2212^T and the other *Francisella* sp. sequence isolates from fish in the NE Atlantic also share a common signature in the v2 region (nt 163–174 with reference to DQ309246) which clearly distinguish them from the *F. philomiragia* strains. All distinguishing characteristics between GM2212^T and *F. philomiragia* are shown in Table 2.

Table 1 Relative fatty acid composition (non hydroxy) of GM2212^T, and *F. philomiragia* (DSM 7535, ATCC 25015^T)

Fatty acid	Abbreviation	GM2212^T	*F. philomiragia* (DSM 7535, ATCC 25015^T)
Octanoic acid	8:0	0.1	0.1
Decanoic acid	10:0	15.2	18.1
Dodecanoic acid	12:0	0.9	0.9
Tetradecanoic acid	14:0	7.9	11.3
5-tetradecenoic acid	14:1n-9	0	0.1
Pentadecanoic acid	15:0	0	0.2
Hexadecanoic acid	16:0	4.4	3.6
7-Hexadecenoic acid	16:1n-9	0.1	0.1
9-Hexadecenoic acid	16:1n-7	1.0	1.9
Heptadecanoic acid	17:0	0	0.2
Octadecanoic acid	18:0	3.4	1.2
9-Octadecenoic acid	18:1n-9	11.8	11.3
11-Octadecenoic acid	18:1n-7	0.1	0.1
9, 12-Octadecadienoic acid	18:2n-6	0.3	0.2
9, 12, 15-Octadecatrienoic acid	18:3n-3	0.2	0.1
6, 9, 12, 15-Octadecatetraenoic acid	18:4n-3	0	0.1
Eicosanoic acid	20:0	3.0	1.3
9-Eicosenoic acid	20:1n-11	2.9	3.3
11, 14-Docoseadienoic acid	20:2n-6	0	0.2
5, 8, 11-Docoseatrienoic acid	20:3n-9	0	0.2
6, 9, 12, 15, 18-Eieicosapentaenoic acid	21:5n-3	24.7	23.2
Docosanoic acid	22:0	5.6	2.1
5, 8, 11, 14 -Eicosatetraenoic acid	20:4n-6	0.3	0
5, 8, 11, 14, s17-Docoseapentaenoic acid	20:5n-3	0.2	1.2
11-Docosaenoic acid	22:1n-11	5.7	8.9
9-Docosaenoic acid	22:1n-9	0.1	0
7, 10, 13, 16, 19-Docosapentaenoic acid	22:5n-3	0	0.4
Tetracosanoic acid	24:0	5.1	1.7
15-Tetracosaenoic acid	24:1n-9	0.7	0.3
Unidentified	>C22	4.1	3.8
Unidentified		2.4	3.9
Sum		100	100

The amount of each fatty acid is shown as a percentage of the total content of fatty acids

Discussion

A threshold value of 70% similarity in DNA–DNA hybridization studies to delineate bacterial species was recommended by the ad hoc committee on reconciliation of approaches to bacterial systematics (Wayne et al. 1987). GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) share 70% DNA binding (i.e. the treshold value), and hence, the hybridization data do not refute our previous claims (Nylund et al. 2006, Ottem et al. 2006) for a specific status of GM2212^T. The comparative fatty acid analysis does not provide clear distinguishing characters between GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T). *Francisella* sp. sequence isolates have now been obtained from fish in a large geographic area in the NE Atlantic, and these show absolute 16S identity with GM2212^T. This new information supports the 16S rRNA gene as a conservative specific character, and the existence of a distinct fish parasitic *Francisella* species typified by GM2212^T. The 16S rRNA gene of *F. philomiragia* strains or sequence isolates obtained worldwide appears to show comparable 16S rRNA gene sequence stability. Based on the biochemical, phenotypic and molecular characteristics previously published (Nylund et al. 2006; Ottem et al. 2006), new data on 16S rDNA sequence variation and the different ecological niches (notably hosts and temperature

Table 2 Differential characters between *Francisella* sp., GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015 ^{T})

Characteristic	*Francisella* sp. GM2212^T	*F. philomiragia* DSM 7535, ATCC 25015^T
Cysteine required for growth	–[a]	–
Growth at 37 °C	–	+
H_2S production in cysteine-supplemented medium	+ w (7–14 days)	+ (7 days)
Agglutination in strain GM2212^T antiserum	+	–[b]
Oxidase	–	+
H_2S slant, triple sugar iron	–	+ (7 days)
Gelantine hydrolysis	–	+ (9 days)
16S rDNA sequence	DQ309246	EF153479
16S rDNA signature at site 163–174[c]	5′-GGCCTTTGTGCT-3′	5′-GGC– – TTAGGCT-3′

+ Positive, – negative, *w* weak

[a] Negative or slight growth on TSA and BHIA agars without added cysteine

[b] Negative or slight agglutination of *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951)

[c] With reference to DQ309246

optima) of *F. philomiragia* and *Francisella* sp. from cod, it is proposed that GM2212^T is a novel species within the genus *Francisella*.

Description of *Francisella piscicida* sp. nov

(*piscicida* L. f.; from L. *pisces* m. fish, suff. –cida from L. f. *caedere*, to kill, 'fish-killer' i.e. causing fish mortality)

Aerobic, microaerophilic; Gram-negative, coccoid to short rods, single cells, typically 0.5 μm, max. 1.5 μm. Growth at 10–25°C, optimal at ca. 20°C. Oxidase-negative, catalase-positive. Does not produce H_2S in triple sugar iron agar and does not hydrolyze gelatine. Cysteine enhances growth. Acid production from sucrose (weak), D-glucose, maltose but not from lactose or glycerol. Indole positive. Growth on MacConkey agar (weak) and in nutrient broth (6% NaCl). Resistant to trimethoprim-sulfamethoxazole, penicillines, cefuroxime, erythromycin and susceptible to ceftazidime, tetracycline, gentamicin, ciprofloxacin. The predominant non-hydroxy fatty acids are C_{10} (15.2%), C_{14} (7.9%), $C_{18:1n-9}$ (11.8%), C_{22} (5.6%), $C_{22:1n-11}$ (5.7%), C_{24} (5.1%) and $C_{21:5n-3}$ (24.7%).

The type strain GM2212^T (=CNCM I-3511^T = DSM 18777^T = LMG registration number not yet available) was isolated from the kidney of farmed Atlantic cod (*Gadus morhua*) from Rogaland County, W Norway.

References

Cashion P, Hodler-Franklin MA, McCully J, Franklin M (1977) A rapid method for base ratio determination of bacterial DNA. Anal Biochem 81:461–466

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12:133–142

Huss VAR, Festl H, Schleifer KH (1982) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol 4:184–192

Nicholas KB, Nicholas HBJ, Deerfield DWI (1997) GeneDoc: analysis and visualization of genetic variation EMBNEW News 4:1–14

Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossøy B (2006) *Francisella* sp. (Family Francisellaeceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol 185:383–392

Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Krossøy B (2006) Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). Arch Microbiol 187:343–350

Sjøstedt AB (2005) Family III. Francisellaceae fam. nov. In: Brenner DJ, Krieg NR, Staley JT, (eds) Bergey's manual of systematic bacteriology, 2nd edn., vol II. Part B. The gammaproteobacteria. Springer, USA, pp 199–210

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky L, Moore LH, Moore WC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

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Occurrence of *Francisella piscicida* in farmed and wild Atlantic cod, *Gadus morhua* L., in Norway

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Abstract

Francisellosis, caused by the bacterium *Francisella piscicida*, has become one of the most serious diseases in Atlantic cod production in Norway. The major aim of this study was to determine the distribution of *F. piscicida* in farmed and wild fish in areas with cod farming along the Norwegian coast, and its occurrence in cod from areas without cod farming. Two real-time PCR assays, targeting the 16S rRNA gene and the FopA gene of *F. piscicida*, were developed since sensitive and specific diagnostic tools are required for detecting asymptomatic carriers of the bacterium. A total of 422 wild cod from 13 sampling areas and 955 farmed cod from 10 areas along the coast of Norway were examined. Using the real-time polymerase chain reaction (PCR) assays, *F. piscicida* was detected in wild populations of cod from all counties examined south of Sogn og Fjordane in southern Norway (overall prevalence 13%, $n = 221$). Wild cod north of Sogn og Fjordane were negative for the bacterium ($n = 201$). Farmed cod from most parts of Norway were *F. piscicida* positive. The apparent absence of the bacterium in wild populations of cod in the northern parts of Norway and its widespread occurrence in wild cod from southern parts of Norway is believed to relate to differences in seawater temperatures.

Keywords: Atlantic cod, *Francisella piscicida, Gadus morhua,* Norway, occurrence, PCR.

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Introduction

In the autumn of 2004, a previously unknown bacterium, *Francisella piscicida*, was isolated from Atlantic cod, *Gadus morhua* L., in a farm in Rogaland County, western Norway (Nylund, Ottem, Watanabe, Karlsbakk & Krossøy 2006; Ottem, Nylund, Karlsbakk, Friis-Møller & Krossøy 2006; Ottem, Nylund, Karlsbakk, Knappskoy & Krossøy 2007b). The fish showed loss of appetite, reduced swimming performance and dark pigmentation. The most prominent internal signs were a swollen spleen, kidney and heart, which were covered with, and penetrated by, white granulomas. The closest relative to *F. piscicida* is *Francisella philomiragia* (Nylund *et al.* 2006; Ottem *et al.* 2006). Other isolates of the genus *Francisella* had previously been isolated from tilapia, *Oreochromis* spp., in Taiwan and three-line grunt, *Parapristipoma trilineatum* L., in Japan (Fukuda, Okamura, Nishiyama, Kawakami, Kamaishi & Yoshinago 2000; Kamaishi, Fukuda, Nishiyama, Kawakami, Matsuyama, Yoshinaga & Oseko 2005). After the reports of these isolates, other *Francisella* isolates have been obtained from several fish species worldwide (Hsieh, Wu, Tung & Tsui 2007; Kay, Peterson, Duus, Perry & Vinogradev 2006; Ostland, Stannard, Creek, Hedrick, Ferguson, Carlberg & Westerman 2006). The unknown Agent 2 (UA2) described from Atlantic salmon, *Salmo salar* L., parr in fresh water in Chile (Cvitanich, Garate, Silva, Andrave, Figueroa & Smith 1995) was also recently identified as a member of the genus *Francisella* (Birkbeck, Bordevik, Frøystad & Baklien 2007).

Since the first isolation of *F. piscicida*, several new cases of francisellosis have been recorded in Norway, mainly in the southwestern counties

(Ottem, Nylund & Karlsbakk 2007a). *Francisella piscicida* has caused large losses and in some farms all fish have been culled to reduce production losses and limit the spread of the disease agent. Hence, the bacterium is of growing concern among cod farmers in Norway, and as a result of this the research has focused on its distribution in farmed and wild populations of Atlantic cod.

Screening for *F. piscicida* carriers in farmed and wild fish populations requires specific and sensitive diagnostic tools. Culture systems for the bacterium have been developed and polyclonal antibodies have been tested (Nylund *et al.* 2006; Ottem *et al.* 2006). However, none of these methods are sensitive enough to detect asymptomatic carriers, and hence, PCR based methods, including real-time PCR, have been developed as they provide both high sensitivity and specificity (Andersen, Bratland, Hodneland & Nylund 2007; Hodneland & Endresen 2006; Plarre, Devold, Snow & Nylund 2005). A major aim in this study was to develop sensitive and specific real-time PCR assays targeting the 16S rRNA gene and the outer membrane protein gene (FopA) of *F. piscicida*. These assays have been constructed, optimized and used for screening of selected tissues from farmed and wild fish along the Norwegian coast with a major focus on Atlantic cod.

Materials and methods

Samples

A total of 1474 fish were sampled from October 2004 to early winter 2007 (Table 1). These included farmed Atlantic cod from most Norwegian counties with cod farms (Fig. 1), including the core area for francisellosis, Rogaland County. The disease and the bacterium, *F. piscicida,* have also been reported from three other counties, Hordaland, Møre og Romsdal and Nordland (Ottem *et al.* 2007a). Fish from two counties, with no known history of francisellosis, Sogn og Fjordane and Troms, were also included in the study together with two farmed cod from Denmark (Norwegian origin). Fish were sampled from farms representing ongrowing sites, brood fish farms and fry production sites. Wild Atlantic cod were collected from all the above counties except for Troms County (Fig. 1). Wild cod were also collected from two counties, Aust-Agder and Vest-Agder where there are no cod-farms. A few wild fish of other species including mackerel, S*comber scombrus* L., saithe,

Table 1 Fish species sampled and their geographical origin

Samples	Aust-Agder		Vest-Agder		Rogaland		Hordaland		Sogn og Fjordane		Møre og Romsdal		Nordland	
	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild
Atlantic cod (*Gadus morhua*)	_*	53	_*	60	414	37	162	71	33	61	201	100	141	40
Atlantic salmon (*Salmo salar*)	–	–	–	–	32	–	–	–	–	–	–	–	–	–
Saithe (*Pollachius virens*)	–	–	–	–	–	4	–	8	–	–	–	–	–	1
Pollock (*P. pollachius*)	–	–	–	–	–	–	–	2	–	1	–	–	–	–
Poor cod (*Trisopterus minutus*)	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Mackerel (*Scomber scombrus*)	–	–	–	–	–	5	–	4	–	–	–	–	–	–
European plaice (*Pleuronectes platessa*)	–	–	–	–	–	–	–	4	–	–	–	–	–	–
Megrim (*Lepidorhombus whiffiagonis*)	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Angler–fish (*Lophius piscatorius*)	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Flounder (*Platichthys flesus*)	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Blue Mussels (*Mytilus edulis*)	–	–	–	–	–	10	–	10	–	–	–	10	–	
Edible crab (*Cancer pagurus*)	–	–	–	–	–	–	–	6	–	–	–	–	–	–
Total	–	53	–	60	446	56	162	109	33	62	201	110	141	41

*There is no cod-farming in these two counties.

Figure 1 Map of Norway showing sampling counties (AA = Aust-Agder, VA = Vest-Agder, R = Rogaland, H = Hordaland, SF = Sogn og Fjordane, MR = Møre og Romsdal, N = Nordland, T = Troms).

Pollachius virens (L.), and pollock, *Pollachius pollachius* (L.), were collected from areas close to cod farms between Rogaland and Møre og Romsdal (Table 1). Atlantic salmon were collected from a farm located approximately 500 m away from a site containing cod suffering from francisellosis in Rogaland. Two species of invertebrates, blue mussel, *Mytilus edulis* L., and edible crabs, *Cancer*

pagurus L., were collected close to one farm site in Hordaland containing cod infected with *F. piscicida*. Blue mussels were also collected from two farming sites in Rogaland and Møre og Romsdal.

All fish were investigated for external and internal clinical signs of disease, except for a few cases where tissue samples and the clinical history of the fish were received. Tissue samples from gills, heart, kidney, spleen and brain were placed in cryo-tubes (NUNC™, A/S Kamstrupsvej 90, Roskilde, Denmark) and stored at −80 °C, while samples for RNA and DNA extraction were placed in Eppendorf-tubes (PLASTIBRAND®, Brand, Germany) at −20 °C. Preliminary results from screening of infected farmed cod had shown that kidney and spleen were the most suitable tissues for screening of carrier fish. However, kidney tissue performed slightly better than the spleen in the real-time PCR analysis; hence, the kidney from sampled fish was chosen for screening in this study. Samples from the gills of blue mussels and hearts from edible crabs were processed and stored as described above.

DNA and RNA extraction

Total DNA from collected samples was extracted using a DNeasy kit as recommended by the manufacturer (Qiagen Inc., Valencia, CA, USA), and total RNA was extracted using TRIZOL agent according to standard protocols (Devold, Krossøy, Aspehaug & Nylund 2000). Reverse transcription of RNA was performed using M-MLV reverse transcriptase (Promega Corp, Madison, WI, USA) with random hexamer primers $pd(N)_6$ as previously described (Devold, Falk, Dale, Krossøy Biering, Aspehaug, Nilsen & Nylund 2001).

Real-time PCR

Two different real-time PCR assays were designed using the PrimerExpress software package (PE Applied Biosystems, Foster City, CA, USA) based on sequences in GenBank (Nylund *et al.* 2006). One assay, Fc50, was specific for the 16S rDNA from *F. piscicida*. The second assay targeted the outer membrane protein (FopA) gene and detects both *F. piscicida* and *F. philomiragia*. The two assays, Fc50 and FopA, amplify 101 bp and 85 bp regions, respectively. Primers and probes are listed in Table 2.

To test the specificity of the real-time PCR assays, DNA from several bacteria was used as a template (Table 3). *Francisella piscicida* and *Fran-*

Table 3 Bacterial isolates used in testing the specificity of the real-time PCR assays Fc50 and FopA

Bacterial isolates/strains	Fc50	FopA
Francisella piscicida (DSM 18777, CNCM-3511, LMG 24256)*	+	+
Francisella sp. (isolate UA2660, *S. salar*, Chile)	+	+
Francisella sp. (Ehime-1, *P. trilineatum*, Japan)	+	–
F. philomiragia (DSM 7535, ATCC25015^T)	–	+
F. philomiragia (CCUG 19701, ATCC25017)	–	+
F. philomiragia (strain 1951)	–	+
F. philomiragia (CCUG 12603)	–	–
F. philomiragia (CCUG 13404)	–	+
Aeromonas salmonicida subsp. *achromogenes* (AH010906, kidney, *G. morhua*)	–	–
Vibrio fischeri (NCIMB 2262^T)	–	–
V. salmonicida (NCIMB 1281^T)	–	–
V. wodanis (ATCC BAA-104^T)	–	–
V. logei (NCIMB 2252^T)	–	–
V. logei (K25-F1, kidney, *G. morhua*)	–	–
Vibrio sp. aff *logei* (H109 mid-gut, *G. morhua*)	–	–
Vibrio sp. aff *logei* (H109 hind-gut, *G. morhua*)	–	–
Vibrio sp. aff *logei* (HMY T4-2, kidney, *G. morhua*)	–	–
Vibrio sp. (VA361-07, kidney, *G. morhua*)	–	–
Vibrio sp. (SF302-07, kidney, *G. morhua*)	–	–
Moritella marina (SF203-07, kidney, *G. morhua*)	–	–
Listonella anguillarum (HA270207-5, kidney, *G. morhua*)	–	–

*All isolates/sequence-isolates from diagnosed cases of francisellosis in Norway are identical to *F. piscicida* (CNCM-3511, DSM 18777) in their partial 16S and FopA gene-sequences.

Table 2 Primers and probes used in real-time PCR assays

Oligonucleotide	Sequence	Amplicon length	Position	Position no.
FcF50	5'-AACGACTGTTAATACCGCATAATATCTG-3'	101	123–151	DQ309246
FcR50	5'-CCTTACCCTACCAACTAGCTAATCCA-3'		224–198	
Fc50-probe	FAM-5'-GTGGCCTTTGTGCTGC-3'-MGB		161–177	
FopA F	5'-GGTGCGAACATGACTATTGGTTAT-3'	85	471–494	DQ333226
FopA R	5'-AACCTGCAAATACTCTACCCACTAACT-3'		555–529	
FopA-probe	FAM-5'-TTTGCAGTTCAGTATAAC-3'-MGB		510–527	

Primers Fc-F50, Fc-R50 and the Fc-50 probe target the 16S rDNA of *Francisella piscicida*. Primers FopA F, FopA R and the FopA probe target the outer membrane protein (FopA) from *F. piscicida* and *F. philomiragia* (DSM 7535, ATCC 25015^T).

cisella sp. (isolate UA-2260 from Chile) were used as positive controls for the two assays. In addition, *F. philomiragia* (DSM 7535) was used as an additional positive control for the FopA assay.

The reaction mix for the real-time PCR was 1 μL of total DNA or cDNA (RNA) as template, 6.25 μL Absolute QPCR Rox Mix (Abgene), 600 nM of each primer, and 175 nM of the probe. The total volume was adjusted to 12.5 μL using DEPC H_2O. The reaction was performed in an ABI 7000 sequence detection system (Applied Biosystems), where the reaction cycle was 15 min at 95 °C, 45 cycles of 95°C for 15 s and 60 °C for 1 min. Both negative and positive controls were included in each real-time PCR run. The elongation factor from cod (EF1A) was used as an internal control for cod, while the elongation factor from salmon (EF1AA) was used as an internal control for salmon (Olsvik, Kristensen, Waagbø, Tollefsen, Rosseland & Toften 2006; Olsvik, Lie, Jordal, Nilsen & Hordvik 2005).

Standard curves and detection limits

Francisella piscicida was counted using the protocol of Patel *et al.* (2007) with slight modification. The bacteria were grown in growth medium B1817 (Ottem *et al.* 2006) for 7 days and inactivated by addition of a 1 : 1 isopropanol : dH_2O mixture. Total DNA and RNA were extracted from the counted bacteria (10^{10} bacteria mL^{-1}) as previously described. The amount of extracted DNA and RNA was determined using a NanoDrop® ND-1000 Spectrophotometer, and serial logarithmic dilutions were made. The DNA dilution series (100 ng–1 fg) was made in AE buffer (Qiagen), while the cDNA dilution series (corresponding to 30 ng–0.3 fg RNA) was made in dH_2O. Triplicates from each dilution were analyzed using the real-time PCR assays for Fc50 and FopA.

Using Q-gene (Simon 2003), the cycle threshold values (C_t-values) were plotted against the serial logarithmic dilutions of DNA and cDNA (RNA) to obtain standard curves. The linear dynamic range for each assay was obtained, and the amplification efficiencies E ($E = [10^{1/(-\text{slope})}] - 1$) calculated. Standard curves were also made using DNA and cDNA (RNA) from fish tissue infected with *F. piscicida*. This was performed in order to test whether host DNA or cDNA (RNA) had an effect on the real-time PCR assay amplification efficiencies. The sensitivities of the assays were determined using twofold dilution series (100 fg–10 ag) of *F. pisicida* DNA and cDNA (RNA) analyzed in 10 replicates. A cut-off C_t-value of approximately 37.5 was set based on the highest dilution at which all 10 replicates were positive.

PCR and sequencing

DNA from field material positive for *F. piscicida* was amplified and sequenced using the same primers as previously described by Nylund *et al.* (2006) and Ottem *et al.* (2006).

PCR products were purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. Sequencing was then performed in both directions using ABI PRISM BigDye terminator chemistry (version 2) [Applied Biosystems (ABI, Foster City, CA, USA)]. All sequences were assembled using the Vector NTI Suite 7.0 program (InforMax Inc., Carlsbad, CA, USA). The different genes were identified by alignment to the respective gene sequences from the type-strain of *F. piscicida*.

Results

Specificity

The FopA assay was designed to detect both *F. philomiragia* and *F. piscicida*, whereas the Fc50 assay was designed to detect *F. piscicida* only. Both the assays gave a positive result for *F. piscicida* and *Francisella* sp. (isolate UA-2660) from Atlantic salmon in Chile, while DNA from *Francisella* sp. (Ehime-1) from three-line grunt, *P. trilineatum,* in Japan gave a positive signal with the Fc50 assay only. All but one (CCUG 12603) *F. philomiragia* isolates tested positive with the FopA assay while all were negative using the Fc50 assay. Both assays gave negative results when DNA from a range of bacteria infecting cod was used as a template (Table 3).

Amplification efficiencies and sensitivity

Linearity was observed between 100 ng and 100 fg of bacterial DNA and 30 ng–30 fg bacterial RNA (cDNA) (Fig. 2). A summary of standard curve slopes (s), regression coefficients (R^2) and amplification efficiencies (E) are presented in Table 4. Standard curve slopes and amplification efficiencies obtained using the Fc50 and FopA assays on cDNA obtained from RNA extracted from *F. piscicida*-

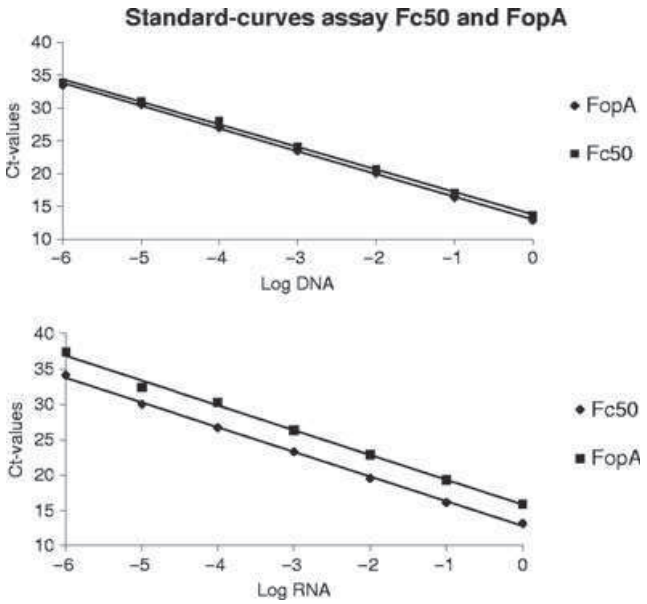

Figure 2 Regression analysis and standard-curves for Fc50 and FopA assays with DNA and RNA (cDNA) from *Francisella piscicida*, grown in B1817. A 10-fold dilution series of DNA (100 ng–100 fg) is plotted against the mean C_t-value obtained for each triplicate using the Fc50 and FopA assays. A 10-fold dilution series from RNA (30 ng–30 fg) is plotted against the mean C_t-value obtained for each triplicate using the Fc50 and FopA assays.

Table 4 Standard curve slopes (s), regression coefficients (R^2) and amplification efficiencies (E) for the Fc50 and FopA real-time PCR assays detecting *F. piscicida* grown *in vitro* (liquid culture) and *in vivo* (cod tissue)

	Fc50			FopA		
Origin	s	R^2	E	s	R^2	E
DNA						
Liquid culture	−3.43	0.998	0.954	−3.46	0.999	0.942
Cod tissue	−3.70	0.999	0.863	−3.67	0.998	0.872
RNA (cDNA)						
Liquid culture	−3.48	0.998	0.936	−3.50	0.996	0.930
Cod tissue	−3.42	0.999	0.960	−3.36	0.998	0.984

infected cod tissue were similar to cDNA obtained from RNA extracted from bacterial culture. When analyzing DNA from the same cod tissue using the Fc50 and FopA assays lower amplification efficiencies were observed than when using DNA from bacterial culture as the template (Table 4). The amplification efficiencies (E), however, were near or within the accepted range of 0.9–1.1. The sensitivity of the Fc50 and FopA assays for bacterial genomic DNA was approximately 25 and 40 fg, respectively, which corresponds to 12–13 and 20 genomic equivalents (GE), assuming that the size of the *F. piscicida* genome is approximately 2 Mbp (genome size of *F. tularensis* ~1.8 Mbp and *F. philomiragia* ~2 Mbp). The sensitivity with RNA (cDNA) was approximately 5 and 40 fg (corresponding to 2–3 and 20 GE), respectively, for the two assays.

Screening

Samples were analyzed as described earlier using the Fc50 assay and cDNA (RNA) as this combination was the most sensitive. The assay was not used for quantification, but to test whether samples were positive or negative for *F. piscicida*. The FopA assay was only used to test whether cod negative for *F. piscicida* were positive for *F. philomiragia.*

The results of the screening of farmed cod showed that 26% of the fish were positive for *F. piscicida* (Table 5). Most of the positive fish came from Rogaland and Møre og Romsdal. In Møre og Romsdal, 48% of farmed cod were positive for *F. piscicida*, while 25% of farmed cod from Rogaland and only 10% from Hordaland were positive. In Nordland, 25% of farmed cod were positive, while only 1 of 33 cod from Sogn og Fjordane was positive. None of the farmed cod in Troms were positive. The two farmed cod of Norwegian origin from Denmark had clinical signs of francisellosis and were positive for *F. piscicida* with C_t-values of 19.4 and 17.4.

Overall, only a few farmed cod had C_t-values lower than 25. Almost half of the positive farmed cod had C_t-values ranging from 25 to 37 and were clinically diseased as demonstrated by swollen kidneys and spleens containing granulomas. The remainder of the positive farmed cod had C_t-values higher than 37 and few or no clinical signs of disease. High C_t-values (above 37.5) are not always

Table 5 Screening of farmed Atlantic cod for *Francisella piscicida* in 2004–2007

Counties	Rogaland	Hordaland	Sogn og Fjordane	Møre og Romsdal	Nordland	Troms	Total	Total %
N	414	162	33	201	141	4	955	–
Positive	104	16	1	96	35	–	252	26.3
C_t-value range								
13–19	5	–	–	1	1	–	7	0.7
19–25	14	–	–	6	–	–	20	2.0
25–31	14	3	–	24	3	–	44	4.6
31–37	23	2	–	28	6	–	59	6.2
>37	48	11	1	37	25	–	122	12.8

The results are presented as the number of *F. piscicida*-positive fish together with the C_t-values obtained using the Fc50 assay.

Table 6 Screening of wild Atlantic cod for *Francisella piscicida* in 2004–2007

	Aust-Agder	Vest-Agder	Rogaland	Hordaland	Sogn og Fjordane	Møre og Romsdal	Nordland	Total	Total %
n	53	60	37	71	61	100	40	422	–
Positive	6	4	8	11	–	–	–	29	6.8
<25	1	1	2	–	–	–	–	4	0.9
25–30	1	–	3	–	–	–	–	4	0.9
30–35	3	–	–	–	–	–	–	3	0.7
>35	1	3	3	11	–	–	–	18	4.3

Results are presented as the number of *F. piscicida* positive-fish together with C_t-values obtained using the Fc50 assay.

Table 7 Screening of fish species from Rogaland and Hordaland for *Francisella piscicida* in 2004–2007

	Rogaland					Hordaland				
Ct-values	<25	25–30	30–35	>35	*n*	<25	25–30	30–35	>35	*n*
Saithe (*Pollachius virens*)	–	–	–	–	4	–	–	–	2	8
Pollock (*Pollachius pollachius*)	–	–	–	–	–	–	–	–	2	2
Poor cod (*Trisopterus minutus*)	–	–	–	–	–	–	–	–	–	1
Mackerel (S*comber scombrus*)	–	–	–	–	5	–	–	–	1	4
European plaice (*Pleuronectes platessa*)	–	–	–	–	–	–	–	–	2	4
Megrim (*Lepidorhombus whiffiagonis*)	–	–	–	–	–	–	–	–	1	1
Angler-fish (*Lophius piscatorius*)	–	–	–	–	–	–	–	–	–	1
Flounder (*Platichthys flesus*)	–	–	–	–	–	–	–	–	–	1
Blue mussels (*Mytilus edulis*)	–	–	–	1	10	–	–	–	4	10
Edible crab (*Cancer pagurus*)	–	–	–	–	–	–	–	–	3	6
Farmed Atlantic salmon (*Salmo salar*)	1	–	–	1	32	–	–	–	–	–

Results are presented as the number of *F. piscicida*-positive individuals together with C_t-values obtained using the Fc50 assay.

reproducible; nevertheless, they represented positive samples and were taken into account. Samples that were negative when tested by using the Fc50 assay were also negative with the FopA assay.

Clinical signs of francisellosis were seen in only a few wild cod from Aust-Agder to Hordland, but several wild cod were positive for *F. piscicida* (Table 6). Of a total of 422 wild cod, 6.8% were positive. In Aust-Agder 6 out of 53 (11%) wild cod were positive, in Vest-Agder 4 out of 60 (7%), in Rogaland 8 out of 37 (22%) and in Hordaland 11 out of 71 (15%) wild cod were positive. No wild fish from Nordland, Møre og Romsdal or Sogn og Fjordane were positive for *F. piscicida*. None of these latter fish had clinical signs of disease. In Rogaland and Hordaland, several other species of fish were positive for the bacterium (Table 7) without clinical signs of disease. All fish that were negative using the Fc50 assay were also negative using the FopA assay.

Gene sequencing

Partial 16S rRNA gene sequences from bacterial isolates from farmed cod collected in Rogaland, Hordaland, Møre og Romsdal and Nordland, from Denmark, from wild caught cod from Aust-Agder to Hordaland, and from farmed salmon collected in Rogaland were compared. No differences were found between these isolates and *F. piscicida*.

Accession numbers for the 16S rRNA genes from Aust-Agder and Vest-Agder isolates are EU219397 and EU219398, respectively, while the accession numbers for the 16S rRNA genes from the rest of the counties are given in Ottem *et al.* (2007b).

Discussion

In this study, the real-time PCR assays used for detection of *F. piscicida* have primarily been used as a diagnostic assay and not for quantification of the bacterium. However, the results from these assays on farmed and wild cod, combined with the results from the use of the real-time PCR assay for detection of the elongation factor from cod (EL1A) using the same DNA/RNA, gives a semi-quantitative estimate of the amount of bacteria present in the cod tissues studied. Hence, the C_t-values roughly reflect the amount of bacteria present in the analysed tissues.

Real-time PCR assays for detection of *F. piscicida* were designed because they are less labour-consuming and more reliable diagnostic tests compared to culture, antibody staining (IFAT and immunohistochemistry) or ordinary PCR.

Both assays, primers and probes are designed to match 100% of all known isolates of *F. piscicida* from Norway. The Fc50 assay seems to detect *F. piscicida* isolates only, while the FopA assay also detects the majority of *F. philomiragia* isolates. Both the Fc50 and FopA assays are also able to detect other isolates of fish *Francisella* not present in Norway, including *Francisella* sp. from tilapia and three-line grunt and *Francisella* sp. (isolate UA-2660) from Atlantic salmon in Chile. Hence, these assays should be able to detect all known *Francisella* species causing disease in fish. The FopA assay can be used as an additional test, targeting a different gene. Positive results, using the FopA assay, should be checked by sequencing of rRNA-genes.

A wide range of RNA and DNA template concentrations were amplified using both the assays with amplification efficiency within or near the accepted range of 0.9–1.1. The Fc50 assay was able to detect as few as ~2–3 GE and the observed linearity in the wide range of GE numbers allows detection of *F. piscicida* from tissues of cod in all stages of disease development. Hence, this assay can also be used for screening of brood fish to prevent possible vertical or trans-generational transmission of *F. piscicida*, and for screening of fry populations, to prevent transport of the bacterium. Fry from infected sites might be a major route of horizontal transmission to areas where the bacterium has not been detected.

Differences in sensitivity between the two assays may be because only one copy of the FopA gene occurs in *Francisella* spp. genomes (according to the complete genomes of *F. tularensis* and *F. philomiragia*), while the 16S rRNA gene targeted by the Fc50 assay is present as three copies in the genome and by numerous copies of ribosomes (RNA). This may also explain why the Fc50 assay is more sensitive with cDNA (RNA) than DNA. The results of this study show a similar pattern to other studies; real-time PCR assays targeting genes with multiple copies in the genome have been shown to be more sensitive than assays targeting genes present as only one copy (Versage, Severin, Chu & Petersen 2003). An assay targeting the IS*Ftu2* element, which is present as numerous copies in *F. tularensis* subsp. *tularensis* and subsp. *holarctica,* detected less than 1 GE, while in the same study the detection limit for the assay targeting the FopA gene was 44 GE (Versage *et al.* 2003).

Francisella spp. have been found in a wide range of farmed or ornamental fish species (Hsieh *et al.* 2007), as also in wild Atlantic cod (Alfjord, Jansson & Johansson 2006; Ottem *et al.* 2007a). Preliminary studies have indicated that *F. piscicida* could be introduced into farmed populations through collection of brood fish from wild or farmed populations (Kamaishi *et al.* 2005). Hence, an important mechanism of *F. piscicida* transfer in farmed populations could be through vertical transmission from brood fish to offspring (Nylund *et al.* 2006). Screening of cod from Norway shows that *F. piscicida* is present in farmed cod in most counties where cod farming occurs and is also present in wild cod populations in areas where there is no cod farming, i.e. Aust-Agder and Vest-Agder. The bacterium has also been detected in other wild species in Rogaland and Hordland. However, the distribution of *F. piscicida* in wild species seems to be limited to areas south of Sogn og Fjordane county. Transport of infected farmed cod from the SW coast of Norway to counties further north may therefore explain the current distribution of *F. piscicida* in farms in these areas.

With the exception of a few fish the amount of *F. piscicida* present in wild cod is very low judging from the high C_t-values obtained. It should be noted that the wild cod with low C_t-values (< 25) in this study came from the most southern part of

Norway, i.e. Aust-Agder to Rogaland. While relatively few fish have been examined from northern Norway, it is clear that there is a significant difference in *F. piscicida*-prevalence in cod from the North and the South of Norway. Challenge experiments have shown that the bacterium is transmitted from fish to fish over short distances (Nylund *et al.* 2006). Horizontal transmission probably accounts for the serious francisellosis problems in some 'hot-spots' such as in south-western Norway. However, both *F. piscicida*-positive and diseased cod were detected in Agder County in the current study as well as from the Swedish West coast (Alfjord *et al.* 2006), areas free from cod farming. Hence, it seems likely that the presence of the bacterium in wild cod is little influenced by farming activities. Either the bacterium is a recent introduction to the Skagerrak area of Sweden and Norway, or it is enzootic in the area and has emerged as a serious disease agent recently, perhaps due to environmental change.

Since 1998, the sea surface temperatures along the Norwegian coast during the summer and early autumn period have increased significantly as seen in data available at The Bundesamt für Seeschifffahrt und Hydrographie (BSH) (Federal Maritime and Hydrographic Agency) http://www.bsh.de/en/Marine%20data/Observations/Sea%20surface%20temperatures/anom.jsp#SSTJ. In this period, both farmed and wild cod in south-western Norway have experienced summer temperatures close to 20 °C, which is close to the optimal *in vitro* growth temperature of *F. piscicida* (Ottem *et al.* 2006), while the temperatures in the northern parts of Norway have seldom exceeded 14 °C in summer/autumn during this period. This difference in temperature between the northern and southern areas seems to be an important factor in the current distribution of *F. piscicida* in Norway in both farmed and wild cod.

Temperature may play an important role in *F. piscicida* virulence since cod immunocompetence is probably influenced by prolonged exposure to high sea temperatures. This hypothesis is currently being investigated and the information regarding this will be of vital importance for the industry and future management of francisellosis.

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References

Alfjord A., Jansson E. & Johansson K.E. (2006) A systematic granulomatous inflammatory disease in wild Atlantic cod, *Gadus morhua*, associated with a bacterium of the genus *Francisella*. *DipNet Newsletter* **44**.

Andersen L., Bratland A., Hodneland K. & Nylund A. (2007) Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (*Salmo salar*). *Archives of Virology* **152,** 1871–1883.

Birkbeck T.H., Bordevik M., Frøystad M.K. & Baklien A. (2007) Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. *Journal of Fish Diseases* **30,** 505–507.

Cvitanich J.D., Garate N.O., Silva P.C., Andrave V.M., Figueroa P. & Smith C.E. (1995) Isolation of a new rickettsia-like organism from Atlantic salmon in Chile. *FHS Newsletter, Fish Health Section American Fisheries Society* **23,** 1–3.

Devold M., Krossøy B., Aspehaug V. & Nylund A. (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. *Diseases of Aquatic Organisms* **40,** 9–18.

Devold M., Falk K., Dale B., Krossøy B., Biering E., Aspehaug V., Nilsen F. & Nylund A. (2001) Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. *Diseases of Aquatic Organisms* **47,** 119–128.

Fukuda Y., Okamura A., Nishiyama M., Kawakami H., Kamaishi T. & Yoshinaga T. (2000) Granulomatosis of cultured three-line grunt *Parapristipoma trilineatum* caused by an intracellular bacterium. *Fish Pathology* **37,** 119–124.

Hodneland K. & Endresen C. (2006) Sensitive and specific detection of salmonid alphavirus using real-time PCR (TaqMan). *Journal of Virological Methods* **131,** 184–192.

Hsieh C.Y., Wu Z.B., Tung M.C. & Tsai S.S. (2007) PCR and *in situ* hybridization for the detection and localization of a new pathogen *Francisella*-like bacterium (FLB) in ornamental cichlids. *Diseases of Aquatic Organisms* **75,** 29–36.

Kamaishi T., Fukuda Y., Nishiyama M., Kawakami H., Matsuyama T., Yoshinaga T. & Oseko N. (2005) Identification and pathogenicity of intracellular *Francisella* bacterium in three-line grunt *Parapristipoma trilineatum*. *Fish Pathology* **40,** 67–71.

Kay W., Petersen B.O., Duus J.Ø., Perry M.B. & Vinogradev E. (2006) Characterization of the lipopolysaccharide and *b*-glucan of the fish pathogen *Francisella victoria*. *Federation of European Biochemical Societies Journal* **273,** 3002–3013.

Nylund A., Ottem K.F., Watanabe K., Karlsbakk E. & Krossøy B. (2006) *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. *Archives of Microbiology* **185,** 383–392.

Olsvik P.A., Lie K.K., Jordal A.E., Nilsen T.O. & Hordvik I. (2005) Evaluation of potential reference genes in real-time

RT-PCR studies of Atlantic salmon. *BMC Molecular Biology* **17,** 6–21.

Olsvik P.A., Kristensen T., Waagbø R., Tollefsen K.E., Rosseland B.O. & Toften H. (2006) Effects of hypo- and hyperoxia on transcription levels of five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*. *The Journal of Experimental Biology* **209,** 2893–2901.

Ostland V.E., Stannard J.A., Creek J.J., Hedrick R.P., Ferguson H.W., Carlberg J.M. & Westerman M.E. (2006) *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*. *Diseases of Aquatic Organisms* **72,** 135–145.

Ottem K.F., Nylund A., Karlsbakk E., Friis-Møller A. & Krossøy B. (2006) Characterization of *Francisella* sp. GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). *Archives of Microbiology* **187,** 343–350 (Epub 2006 Dec.).

Ottem K.F., Nylund A. & Karlsbakk E. (2007a) Utbredelse og status av *Francisella* på torsk i Norge. *Kyst og Havbruk*, 164–165 (in Norwegian).

Ottem K.F., Nylund A., Karlsbakk E., Knappskog D. & Krossøy B. (2007b) New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Archives of Microbiology* **188,** 547–550.

Patel A., Noble R.T., Steele J.A., Schwalbach M.S., Hewson I. & Fuhrman J.A. (2007) Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. *Nature Protocols* **2,** 269–276.

Plarre H., Devold M., Snow M. & Nylund A. (2005) Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Diseases of Aquatic Organisms* **66,** 71–79.

Simon P. (2003) Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* **19,** 1439–1440.

Versage J.L., Severin D.D., Chu M.C. & Petersen J.M. (2003) Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *Journal of Clinical Microbiology* **41,** 5492–5499.

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ORIGINAL ARTICLE

Elevation of *Francisella philomiragia* subsp. *noatunensis* Mikalsen *et al.* (2007) to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem *et al.* (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens

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16S rRNA-genes, fish pathogens, *Francisella noatunensis* comb. nov., *Francisella noatunensis* subsp. *orientalis* subsp. nov., *Francisella piscicida*, housekeeping genes, synonymy.

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Abstract

Aims: This study was conducted to clarify the taxonomic status of *Francisella* sp. strain Ehime-1, a fish pathogen, in relation to the fish pathogens *F. piscicida* and *F. philomiragia* subsp. *noatunensis* and to *F. philomiragia* subsp. *philomiragia*.

Methods and Results: *Francisella* sp. Ehime-1 was compared to *F. piscicida*, *F. philomiragia* subsp. *noatunensis* and several *F. philomiragia* subsp. *philomiragia* isolates through sequencing of the 16S rRNA-gene and several housekeeping genes and determination of biochemical and phenotypic properties. Results show that *F. piscicida* is indistinguishable from *F. philomiragia* subsp. *noatunensis* by sequence and phenotypic traits. *Francisella* sp. Ehime-1 and *F. philomiragia* subsp. *noatunensis* are clearly separated from *F. philomiragia*. *Francisella* sp. Ehime-1 is biochemically, phenotypically and genetically different from *F. philomiragia* subsp. *noatunensis* (=*F. piscicida*), but DNA–DNA hybridization does not clearly support establishment as a separate species (level of relatedness 64% and 73·4%, mean 68·7%).

Conclusions: We propose to elevate *F. philomiragia* subsp. *noatunensis* to species rank as *F. noatunensis* comb. nov., while *F. piscicida* is considered a heterotypic synonym of *F. noatunensis* comb. nov. Evidence suggests that *Francisella* sp. Ehime-1 represents a novel subspecies of *F. noatunensis*, for which the name *F. noatunensis* subsp. *orientalis* subsp. nov. is proposed (=DSM21254^T, =LMG24544^T).

Significance and Impact of the Study: This study contributes to the taxonomy and characteristics of fish-pathogenic *Francisella* spp.

Introduction

Genus *Francisella* (Francisellaceae) consists of facultative intracellular, Gram-negative, pleomorphic coccobacilli (size ~0·5–1·7 μm) with somewhat fastidious nature, particularly a common dependency on the amino-acid cysteine for growth. Historically, the genus contained two recognized species; *Francisella tularensis* and *F. philomiragia* (Sjøstedt 2005). In addition, several *Francisella* species have been detected in environmental samples, as symbionts in organisms such as ticks and amoeba, from human infections (Niebylski *et al.* 1997; Barns *et al.* 2005; Kugeler *et al.* 2008) and as fish pathogens. The latter infect a wide range of fish-hosts; cultured three-line grunt

(*Parapristipoma trilineatum*) in Japan (Fukuda *et al.* 2002; Kamaishi *et al.* 2005), tilapia (*Orechromis* spp.) in America and Asia (Hsieh *et al.* 2006; Kay *et al.* 2006; Mauel *et al.* 2007), hybrid striped bass (*Morone saxatillis*) from America (Ostland *et al.* 2006), Atlantic salmon parr from Chile (Birkbeck *et al.* 2007) and Atlantic cod from Norway (Nylund *et al.* 2006; Olsen *et al.* 2006). *Francisella* spp. infections in fish occur in both marine and freshwater environments and generally induce chronic granulomatous infections in the kidney, spleen, liver and heart of infected fish. Their 16S rRNA-gene sequences show highest similarities to each other and to *F. philomiragia* (Kamaishi *et al.* 2005; Hsieh *et al.* 2006; Nylund *et al.* 2006; Olsen *et al.* 2006; Ostland *et al.* 2006; Birkbeck *et al.* 2007; Mikalsen *et al.* 2007; Mauel *et al.* 2007).

The *Francisella*-bacterium infecting cod in Norway was recently characterized and has two validly published names; one as a subspecies of *F. philomiragia,* subsp. *noatunensis* (Mikalsen *et al.* 2007) and one as a separate species, *F. piscicida* (Ottem *et al.* 2007; Euzeby 2008). Except for the *Francisella* spp. from cod in Norway, taxonomic information of *Francisella* isolates from fish is scarce. The aim of this study was therefore to characterize and clarify the taxonomic status of *Francisella* sp. strain Ehime-1, the first isolated representative of a growing 16S rRNA-gene clade with fish pathogens. *Francisella* sp. strain Ehime-1 is compared to *Francisella* spp. from cod and several *F. philomiragia* subsp. *philomiragia* isolates. *Francisella philomiragia* subsp. *noatunensis* and *F. piscicida* are conspecific, leading us to propose elevation of the former as *F. noatunensis* comb. nov. Based on results from this study, we propose that *Francisella* sp. Ehime-1 strain from Japan represents a novel subspecies, *Francisella noatunensis* subsp. *orientalis* subsp. nov.

Materials and methods

Bacterial strains

In 2001, a *Francisella* sp. strain (Ehime-1) was isolated from three-line grunt (*Parapristipoma trilineatum*) in Japan. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (identification reference: *Francisella* sp. Ehime-1 = DSM21254^T) and in the BCCM/LMG Bacteria Collection, Ghent University (identification reference: *Francisella* sp. Ehime-1 = LMG24544^T). Other *Francisella* strains used in this study are *F. piscicida* (=CNCM-3511^T, =DSM18777^T, =LMG24256^T) and *F. philomiragia* subsp. *noatunensis* (=NCIMB14265^T, =LMG23800^T) from cod in Norway in addition to several strains of *F. philomiragia* subsp. *philomiragia* CCUG12603, CCUG13404, CCUG19701 from Culture Collection University of Göteborg (CCUG), ATCC25015^T (=DSM7535^T) and strain 1951 from a human clinical infection (cf. Friis-Møller *et al.* 2004).

In addition, DNA from a *Francisella* sp. isolate from a tilapia (*Oreochromis mossambicus*) from Indonesia in 2004 was kindly supplied by Intervet Singapore (designated *Francisella* sp. Ind04 throughout).

Phenotypic and biochemical characteristics

Growth was tested at 6°C, 10°C, 15°C, 20°C, 22°C, 25°C, 30°C, 35°C and 37°C on Cysteine Heart Agar and Eugon Broth agar-plates, both supplemented with 5% defibrinated sheep-blood (CHAB and EB), and B1817 agar-plates. For determination of biochemical characteristics, all strains were grown at 22°C on CHAB agar-plates. Phenotypic and biochemical characteristics were determined for all isolates as previously described (Ottem *et al.* 2006); in addition, the API rapid ID 32A, API rapid ID 32E and API ZYM kits were used (all from bioMérieux). The kit-strips were inoculated as described by the manufacturer and incubated at 22°C before they were read visually after 4 and 24 h and the results interpreted.

DNA extraction and gene sequencing

DNA from the different *Francisella* isolates was extracted as previously described (Nylund *et al.* 2006). The following housekeeping genes were amplified; Chromosomal replication initiator protein alpha subunit (dnaA), DNA mismatch repair protein (mutS), phospho-glucomutase (pgm), peptide chain release factor 2 beta subunit (prfB), bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase alpha subunit (putA), DNA-directed RNA polymerase alpha subunit (rpoA), DNA-directed RNA polymerase beta subunit (rpoB) and triosephosphate-isomerase alpha subunit (tpiA). Oligonucleotide primers used in amplification of the malate-dehydrogenase genes (mdh) were the same as previously described (Ottem *et al.* 2006). New oligonucletide primers used in amplification of the housekeeping genes (Table S1) were designed on the basis of the complete genome sequence of *F. philomiragia* subsp. *philomiragia* (ATCC25017). Amplification was performed in a 50-μl reaction mixture containing 10 × buffer (Promega) including 1·5 mmol l^{-1} $MgCl_2$, 2·5 mmol l^{-1} dNTP (Promega), 10 μmol l^{-1} of each primer (Invitrogen), 2 μl DNA and 0·6 U Thermal Ace™ DNA polymerase (Invitrogen). Amplification was carried out in a Mastercycler gradient (Eppendorf) at 95°C for 5 min; 40 cycles of 94°C for 30 s, *X*°C (Gradient ranging from 46·8°C to 55·2°C) for 45 s, 72°C for 1 min and 30 s followed by extension at 72°C for 10 min and a short storage at 4°C. All PCR-products were visualized

using gel-electrophoresis. PCR-products were then purified using the E.Z.N.A Cycle-Pure Kit (OMEGA) as described by the manufacturer. Sequencing was performed in both directions using PCR-primers and the ABI PRISM BigDye terminator chemistry (ver. 3.1) according to Applied Biosystems (ABI). All sequences were assembled using the Vector NTI Suite 9·0 program (InforMax Inc.). Sequences obtained in the present study were submitted to the GenBank (Fig. 2).

Phylogenetic analysis

Multiple alignments of nucleotide sequences were made using AlignX (Vector NTI) software, followed by manual adjustments using the multiple sequence alignment editor GeneDoc (Nicholas and Deerfield 1997). Sequence similarity (%) was calculated as identical residues/aligned residues (PID2, cf. Raghava and Barton 2006). To examine the suitability of the chosen housekeeping genes for use in phylogenetic analysis, the mean ratio of nonsynonymous to synonymous substitutions d_N/d_S was calculated according to the algorithm of Yang and Nielsen (2000) included in the PAML package (Yang 2007).

Phylogenetic analyses were based on alignments of 16S rRNA sequences or concatenated sequences including all the nine studied housekeeping genes from the examined isolates and homologous gene-sequences from strains of *F. tularensis* present in the GenBank. The mdh-gene sequences of *Francisella philomiragia* subsp. *philomiragia* ATCC25015^T (EF153478), strain 1951 (DQ813285) and *F. piscicida* DSM18777^T (DQ825768) were also obtained from GenBank.

Maximum likelihood trees based on 16S rRNA-gene sequences already available in the GenBank in addition to the rRNA-gene sequence from Ehime-1 (EU683030) and Ind04 (FJ217163), the housekeeping genes and the concatenated gene-sequences were constructed in Tree Puzzle 5·0 (available at: http://www.tree-puzzle.de), with 10 000 replicates. Optimal models were determined using Modeltest 3.7 (Posada and Crandall 1998), and were as follows: 16S rRNA and pgm (TrN + I + G), dnaA and rpoA (GTR + G), mdh (GTR + I), tpiA (HKY + G), mutS (TrN + G), prfB, putA, rpoB and the concatenated sequences (GTR + I + G). Phylogenetic trees were drawn using TreeView (Page 1996).

DNA–DNA hybridization

Francisella philomiragia subsp. *philomiragia* (ATCC25015^T), *Francisella* sp. Ehime-1 and *Francisella piscicida* (DSM18777^T) were grown on CHAB agar-plates at 22°C and incubated for 4–7 days. After 1 week of growth, the bacteria were suspended in 1 : 1 isopropanol/dH_2O before storage at 4°C. Tubes (50 ml) with bacteria were sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) for DNA–DNA hybridization. At DSMZ, DNA–DNA hybridization was performed as previously described (Ottem *et al.* 2007).

Results

Phenotypic and biochemical characteristics

Cells of *Francisella* sp. Ehime-1 are aerobic, Gram-negative, single, coccoid to short rods 0·5–1·5 μm. Best growth occurred in the temperature range 18–30°C with weak to almost no growth at 15°C and 34°C. No growth was observed at 37°C. Optimal growth at 25°C. Growth was observed for all *F. philomiragia* subsp. *philomiragia* isolates from 20°C to 37°C. Characteristics for the *Francisella* isolates are shown in Table 1. *Francisella* sp. Ehime-1 was negative for Histidine arylamidase and Tyrosine arylamidase unlike the other *Francisella* isolates. In addition, *Francisella* sp. Ehime-1 was positive for InDoxyl phosphate and Phenylalanine arylamidase unlike *F. piscicida* (DSM18777^T) and *F. philomiragia* subsp. *noatunensis* (=NCIMB14265^T, =LMG23800^T). *Francisella piscicida* (DSM18777^T) and *F. philomiragia* subsp. *noatunensis* (=NCIMB14265^T, =LMG23800^T) showed an identical biochemical profile. The *F. philomiragia* subsp. *philomiragia* isolates showed an almost identical biochemical profile and grew in triple sugar iron agar and were oxidase and gelatinase positive. Also, the *F. philomiragia* subsp. *philomiragia* isolates did not agglutinate in *F. piscicida* antisera, while *F. piscicida* (DSM18777^T), *F. philomiragia* subsp. *noatunensis* (=NCIMB14265^T, =LMG23800^T) and *Francisella* sp. Ehime-1 were agglutinated.

Sequence analyses

Results from comparisons of the 16S rRNA-gene, housekeeping genes dnaA, mdh, mutS, pgm, prfB, putA, rpoA, rpoB, tpiA and the concatenated gene-sequences from the *Francisella*-isolates included in this study in addition to homologues gene-sequences from *F. tularensis* in the GenBank are listed in Table S2.

In the 16S rRNA-gene (1054 bp), *Francisella* sp. Ehime-1 and Ind04 were identical and showed similarities of 98·9–99·1% to the isolates of *F. philomiragia* subsp. *philomiragia*. The highest similarity of Ehime-1 and Ind04 to *F. tularensis* was 97·3%. Ehime-1 and Ind04 showed 99·3% similarity to *F. piscicida* (DSM18777^T)/*F. philomiragia* subsp. *noatunensis* (NCIMB14256^T) and *Francisella* sp. UA2660 from Atlantic salmon in Chile. Similarities of 99·8–100% were seen between Ehime-1 (EU683030) and Ind04 (FJ217163) to the other fish isolates from Asia

Table 1 Differential biochemical and phenotypic characteristics at 22°C; 1 *Francisella* sp. Ehime-1^T, 2 *F. philomiragia* subsp. *noatunensis* 2005/50/F292-6C^T/*F. piscicida* GM2212^T and *F. philomiragia* subsp *philomiragia* (3 strain 1951, 4 CCUG12603, 5 CCUG13404, 6 CCUG19701, 7 ATCC25015^T)

	1	2	3	4	5	6	7
α-Glucosidase	–	–	–	–	–	–	+
Mannose and raffinose fermentation	–	–	+	+	+	+	+
Arylamidases; Leucyl glycine, Leucine, Pyroglutamic acid, Glutamyl glutamic acid, Glycine, Valine, Cystine, Serine	–	–	+	+	+	+	+
Phenylalanine arylamidase	+	–	+	+	+	+	+
Tyrosine arylamidase	–	+	+	+	+	+	+
Histidine arylamidase	–	+	+	+	+	+	+
D-maltose, D-trehalose, Colistin, Coumarate, O-Nitrophenyl *N*-Acetyl-BD-Glucosaminide	–	–	+	+	+	+	+
P-Nitrophenyl-BD-galactopyranoside	–	–	–	+	–	–	+
InDoxyl phosphate	+	–	+	+	+	+	+
Napthol-AS-BI-phosphohydrolase	+	–	+	+	+	+	+
β-Galactosidase	–	–	–	+	–	–	+
N-acetyl-β-glucosaminidase, oxidase, H_2S slant, Triple Sugar Iron, gelatine hydrolysis	–	–	+	+	+	+	+
Agglutination with *F. piscicida* antiserum	+	+	–	–	–	–	–
Cysteine required for growth	+	+	–	–	–	–	–
16S rRNA-signature v2 region (CT)	+	+	–	–	–	–	–

All isolates were positive for catalase, tryptophanase, D-glucose, D-saccharose, alkaline phosphatase, esterase, esterase lipase, acid phosphatase, arginine arylamidase, alanine arylamidase, proline arylamidase and H_2S-production. All isolates were negative for motility, urease, arginine dehydrolase, α-arabinose, β-N-acetyl-β-glucosaminidase, glutamic acid decarboxylase, α-fucosidase, nitrate reduction, lysine decarboxylase, ornithine decarboxylase, esculin, L-arabinose, adonitol, L-rhamnose, D-mannitol, D-sorbitol, D-cellobiose, D-melibiose, sodium glucuronate, para-phenylalanine deaminase, 5-ketogluconate, palatinose, galacturonate, tetra-thionate reductase, raffinose, lipase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase and α-mannosidase.
+, Positive; –, negative.

(AY928391, AY928393, AY928389, AY928388, AY928390, DQ473646, DQ007456, EU672884, AY928392, DQ007455, DQ007453 and AF385857) and the *Francisella* sp. isolates LA1 (DQ473646) and LADL07-285A (EU672884) from tilapia in America. A common signature-sequence (CT cf. Ottem *et al.* 2007) in the V2 region was shared by all the fish *Francisella*, which is absent in all investigated *F. philomiragia* subsp. *philomiragia*.

The housekeeping genes showed a mean d_N/d_S ratio (Supporting information) from 0·043 in the prfB to 0·079 in the mutS gene, respectively, being <1 for all genes indicating strong purifying selection in all loci investigated and thereby demonstrating suitability of selected genes for phylogenetic analysis (Viscidi and Demma 2003). *Francisella piscicida* (DSM18777^T) and *F. philomiragia* subsp. *noatunensis* (NCIMB14265^T) were identical in all the sequenced genes. Ehime-1 and Ind04 was also identical in all sequenced genes except in the putA gene where they differed at two positions. In the housekeeping genes sequenced, *Francisella* sp. Ehime-1 and Ind04 strains showed similarities of 92·5–96·6% and 92·6–99·2% to that of the isolates of *F. philomiragia* subsp. *philomiragia* and to *F. piscicida* (DSM18777^T)/*F. philomiragia* subsp. *noatunensis* (NCIMB14265^T), respectively. In comparison, the corresponding similarity within *F. philomiragia* subsp. *philomiragia* was 94·9–99·9%. The concatenated gene-sequence (15687 nt) of *Francisella* sp. Ehime-1 and Ind04 showed similarities to *F. philomiragia* subsp. *philomiragia* of 94·2–94·4%, while the similarity to *F. piscicida*/*F. philomiragia* subsp. *noatunensis* was 95·4%. In comparison, the similarity of the concatenated gene-sequences within *F. philomiragia* subsp. *philomiragia* was 98·4–98·9%. Ehime-1 and Ind04 showed a genetic distance of 0·046 to *F. piscicida/F. philomiragia* subsp. *noatunensis* while the distance (Table S3) to *F. philomiragia* subsp *philomiragia* (ATCC25015^T) was 0·0598.

Phylogenetic analyses

The maximum likelihood tree based on partial 16S rRNA-gene sequences is shown in Fig. 1. *Francisella* sp. Ehime-1and Ind04 occurs in a well-supported clade (support value 97) together with *Francisella* isolates from Asian and American fishes. *Francisella philomiragia* subsp. *noatunensis/F. piscicida* is placed in a sister clade to *Francisella* sp. Ehime-1/Ind04 together with an isolate from Chilean salmon and a sequence-isolate MB33 (Accesion number: EU503153) from seawater in Massachusetts, USA. These two sister clades (SV 74) with fish parasitic *Francisella* spp. represent a sister group to a poorly supported (SV 58) clade with *Francisella philomiragia* subsp. *philomiragia* isolates. This topological pattern is strongly

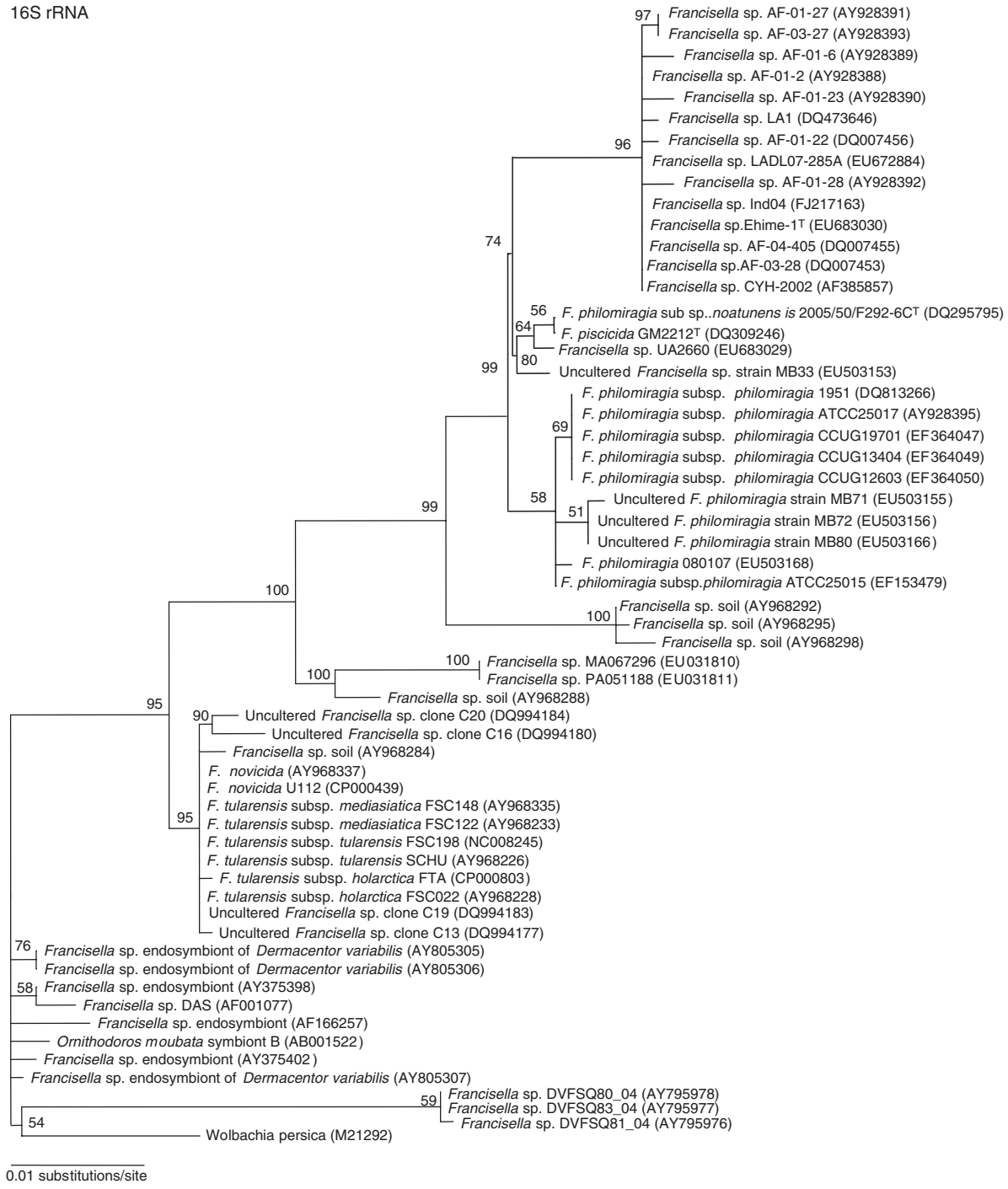

Figure 1 Maximum-likelihood trees (TreePuzzle) for the all the housekeeping gene-sequences; dnaA (1305 nt), mdh (576 nt), mutS (2328 nt), pgm (1515 nt), prfB (882 nt), putA (3789 nt), rpoA (870 nt), rpoB (3918 nt), tpiA (507) and the concatenated gene-sequences (15687 nt) from several *Francisella* spp., rooted with *F. tularensis* subsp. *tularensis* SCHU. values at nodes = Quartet puzzling support values. Small indels show substitutions per site.

supported by phylogenetic analyses based on the dnaA, mdh, pgm and rpoA genes (Fig. 2). However, analyses based on the mutS, putA, rpoB genes place *Francisella* sp. Ehime-1 and Ind04 basal to *F. piscicida/F. philomiragia* subsp. *noatunensis* + *F. philomiragia* subsp. *philomiragia*, and this topology is shared by the concatenated three

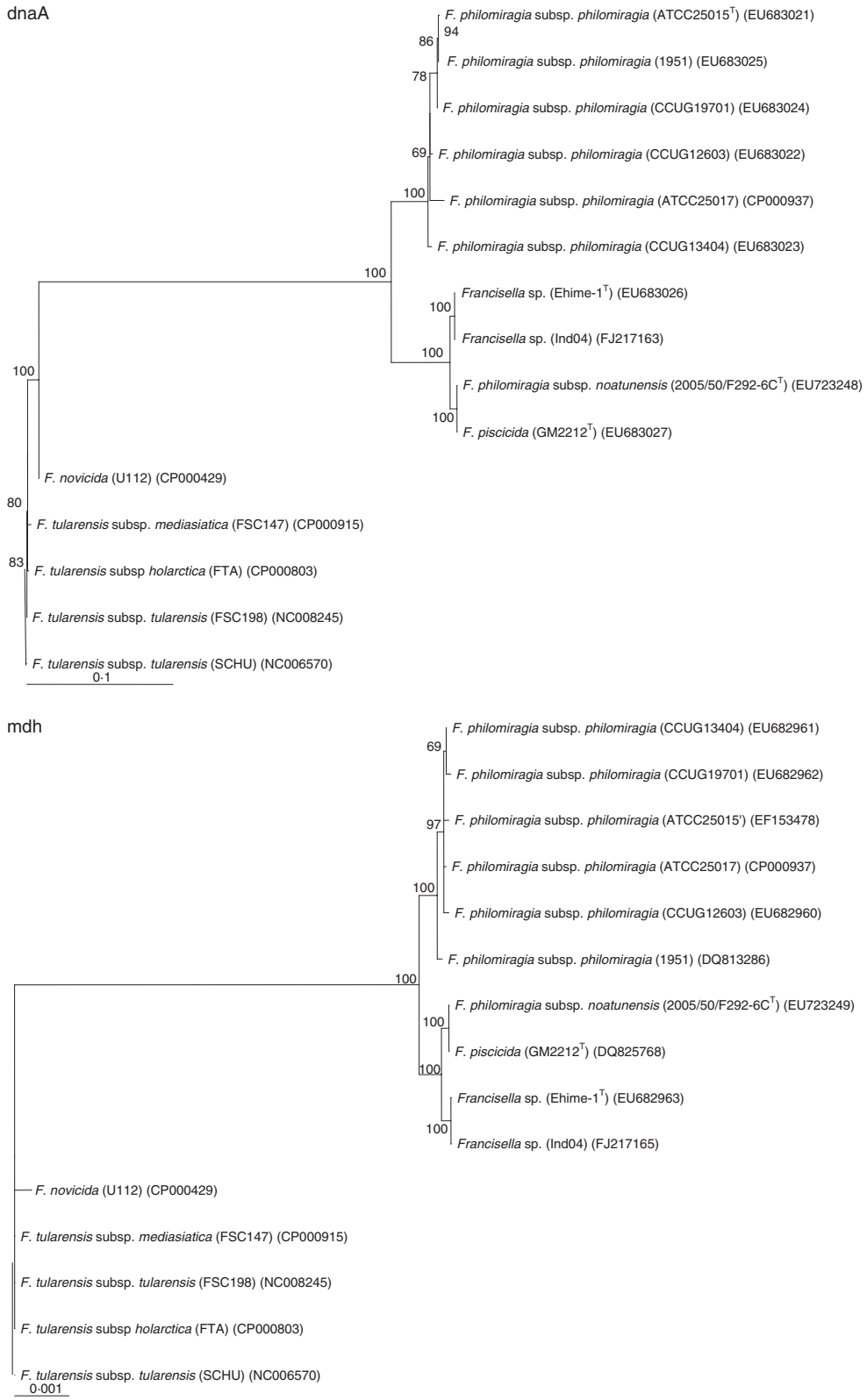

Figure 2 Maximum-likelihood tree (TreePuzzle) based on the 16S rRNA-gene sequences (1054 bp) from *Francisella* spp., rooted with *F. tularensis* subsp. *tularensis* SCHU. Values at nodes = Quartet puzzling support values.

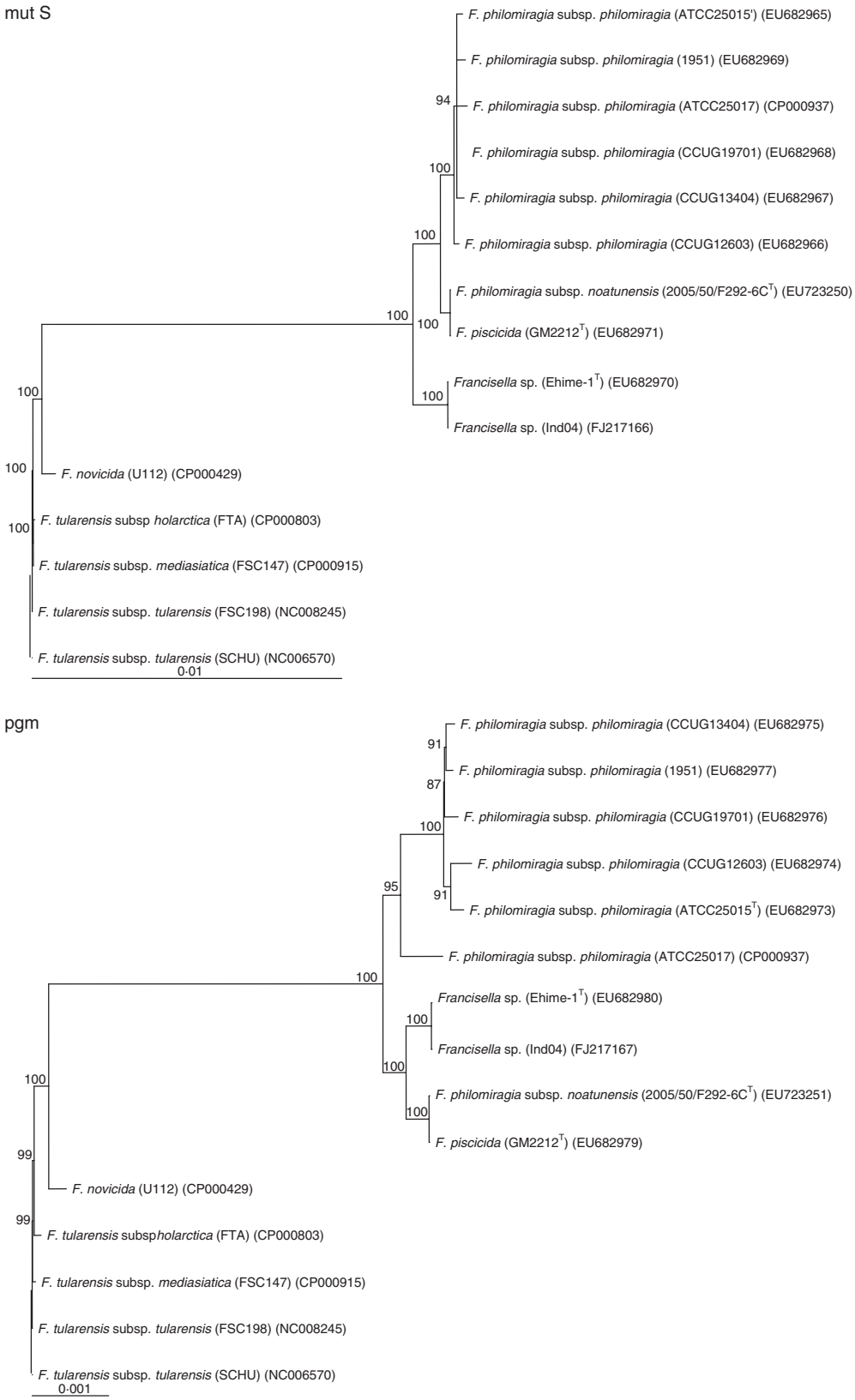

Figure 2 (*Continued*)

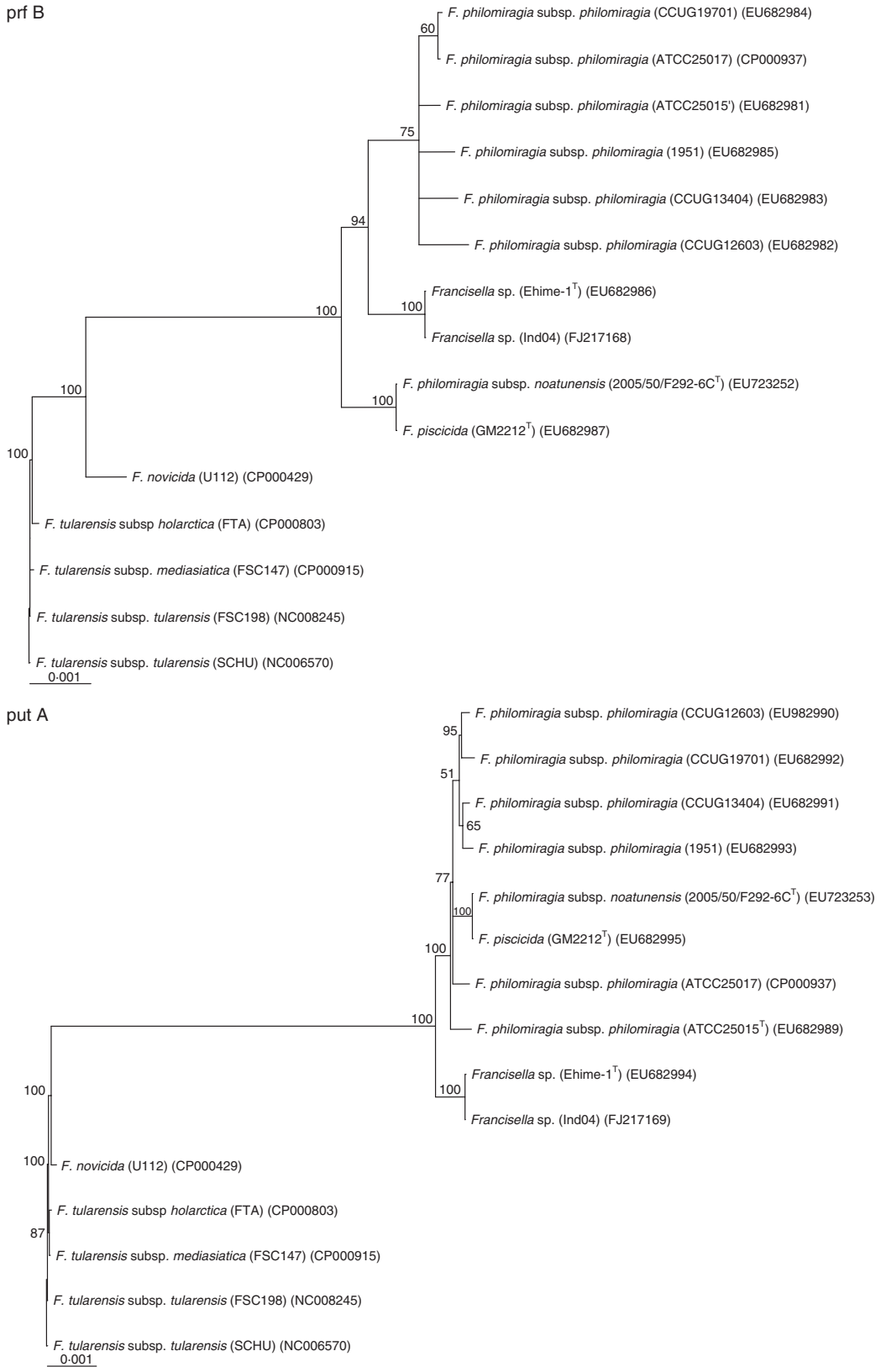

Figure 2 (*Continued*)

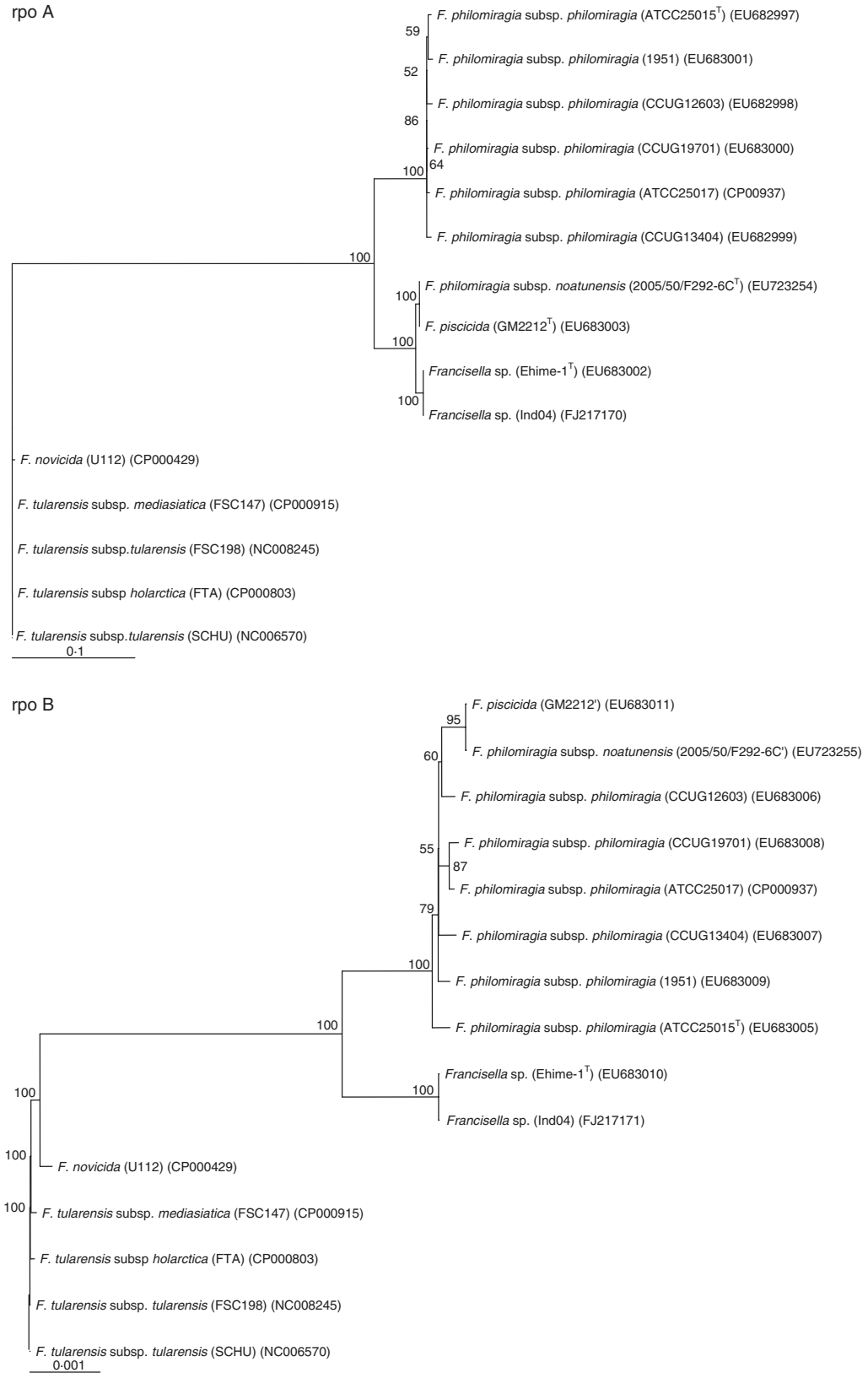

Figure 2 (*Continued*)

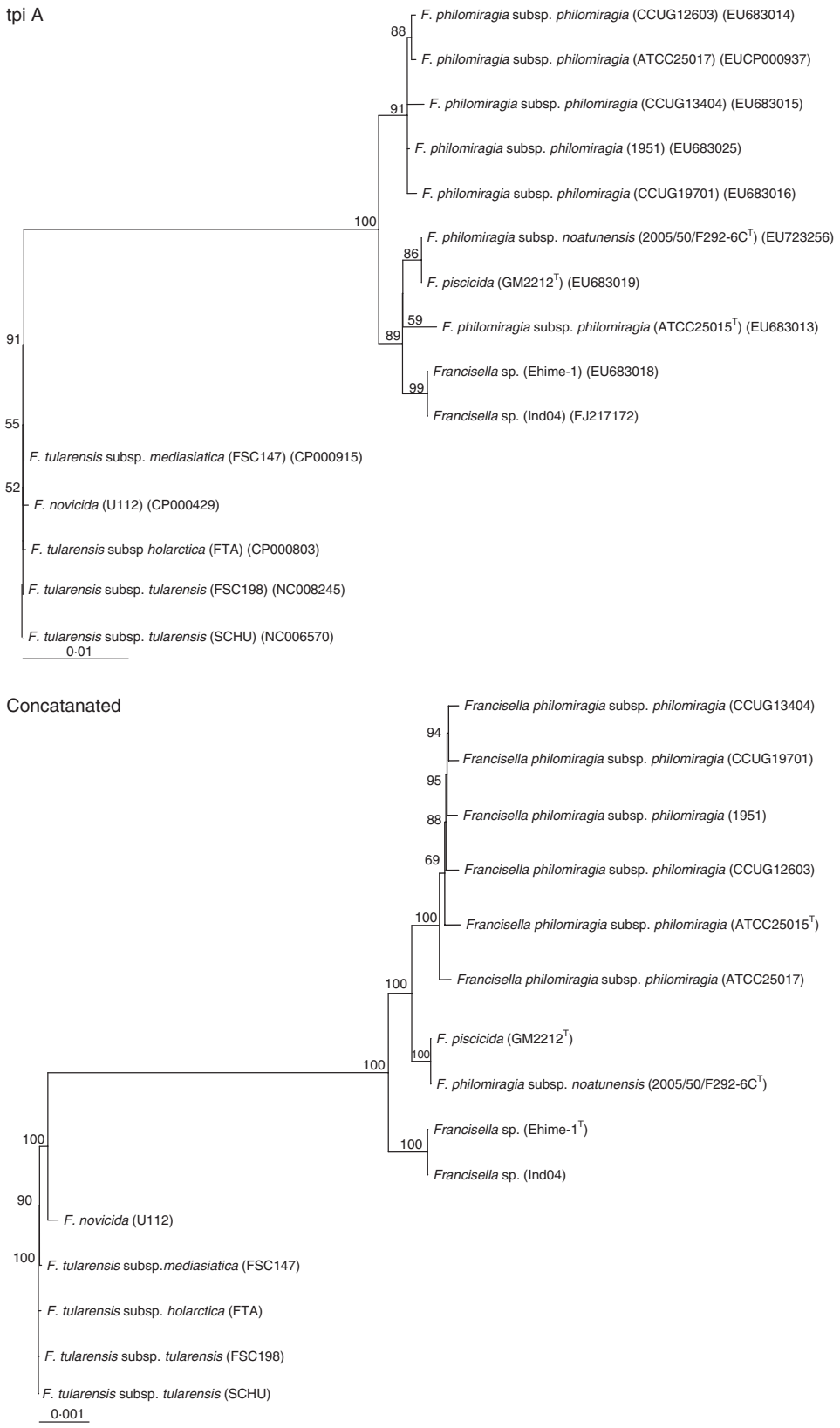

Figure 2 (*Continued*)

(Fig. 2). The prfB tree places *F. piscicida/F. philomiragia* subsp. *noatunensis* as a sister group to *F. philomiragia* subsp. *Philomiragia* + *Francisella* sp. Ehime-1 and Ind04. The tpiA tree groups the fish *Francisella* isolates with the *F. philomiragia* type strain in the same clade, representing a sister group to the other *F. philomiragia* subsp. *philomiragia* isolates (Fig. 2).

DNA–DNA hybridization

The levels of relatedness as determined by DNA–DNA hybridization at 63°C between *Francisella* sp. Ehime-1 and *F. piscicida* (DSM18777^T) averaged 68·7% (64% and 73·4%). Corresponding DNA–DNA hybridization parallels of *Francisella* sp. Ehime-1 to *F. philomiragia* subsp. *philomiragia* (ATCC25015^T) gave hybridization values averaging 60·8% (57·3% and 64·3%).

Discussion

The fish-pathogenic *Francisella* bacteria cause disease and losses in aquaculture worldwide in several unrelated fish species. These infections occur in both fresh- and seawater environments. All these fish pathogens have previously been found to show high 16S rRNA-gene sequence similarities to each other (Hsieh *et al.* 2006; Nylund *et al.* 2006; Olsen *et al.* 2006; Ostland *et al.* 2006; Birkbeck *et al.* 2007) and to *F. philomiragia* (Kamaishi *et al.* 2005). Most of these fish-pathogenic *Francisella* types have not been characterized, however, but the more detailed analyses of the *Francisella* species infecting cod in Norway have led to the proposal of a separate species *F. piscicida* (Ottem *et al.* 2007). Another cod-parasitic *Francisella* isolate was simultaneously named *F. philomiragia* subsp. *noatunensis* (Mikalsen *et al.* 2007), but validated prior to the former. Results presented in the present study show that *F. piscicida* and *F. philomiragia* subsp. *noatunensis* are identical in all sequenced genes and in biochemical properties. Therefore, *Francisella piscicida* is in fact a later heterotypic synonym of *F. philomiragia* subsp. *noatunensis* as suggested (Euzeby 2008), and consequently we propose to establish *Francisella noatunensis* (Mikalsen *et al.* 2007) comb. nov. (syn. *F. piscicida* Ottem *et al.* 2007; syn. nov.). The results presented here suggest a higher similarity of *Francisella* sp. Ehime-1 to *F. noatunensis* than to *F. philomiragia* subsp. *philomiragia,* since they share important biochemical characteristics, notably the lack of gelatinase and oxidase activity, no growth in Triple Sugar Iron (TSI) agar and agglutination using *F. piscicida* antisera, the insert in the V2 region of the 16S rRNA-gene sequence and similar ecology (growth temperature and their pathogenicity to fish). There is also a higher similarity of *Francisella* sp. Ehime-1 to *F. noatunensis* in the DNA–DNA hybridization (64% and 73·4%, mean 68·7%), which is near the threshold value of 70% proposed by Wayne *et al.* (1987) to delineate bacterial species. Both hybridization data, sequence data and phenotypic traits clearly discern *Francisella* sp. Ehime-1 from *F. philomiragia* subsp. *philomiragia* as a separate specific entity. Since *F. noatunensis* and *Francisella* sp. Ehime-1/Ind04 at present cannot be unambiguously discerned as specific entities, we suggest the establishment of *Francisella noatunensis* subsp. *orientalis* subsp. nov. to encompass the latter with *Francisella* sp. Ehime-1 as type strain. Upon validation according to the Bacterial Code rule 40b, the valid publication of *Francisella noatunensis* subsp. *orientalis* subsp. nov. will automatically create the subspecies *Francisella noatunensis* subsp. *noatunensis* subsp. nov.

Emendation of *Francisella noatunensis* Mikalsen *et al.* (2007) comb. nov.

Type strain 2005/50/F292-6C^T (=NCIMB14265^T = LMG 23800^T), was isolated from Atlantic cod. Description by (Mikalsen *et al.* 2007), with the addition that isolates lack gelatinase activity, do not grow in Triple Sugar Iron agar, and is agglutinated using *F. noatunensis* GM2212 antisera unlike all *F. philomiragia*. Heterotypic synonym: *Francisella piscicida* (Ottem *et al.* 2007).

Description of *Francisella noatunensis* subsp. *orientalis* subsp. nov.

Francisella **subsp.** *orientalis* [o.ri.en.ta'lis. L. fem. adj. ***orientalis*** from the East, referring to where the type strain was first isolated].

Cells are aerobic, Gram-negative, coccoid to short rods 0·5–1·5 μm. Growth observed at 15–34°C, no growth at 37°C, optimal at *c.* 25°C. Colonies on CHAB are convex, pale-yellow and mucoid in appearance. Single colonies ~1 mm in diameter are observed after 4–6 days at 25°C. Positive for InDoxyl phosphate, phenylalanine arylamidase and Napthol-AS-BI-phosphohydrolase, do not show histidine arylamidase or tyrosine arylamidase activity unlike *F. noatunensis* comb. nov. The type strain, *Francisella* sp. Ehime-1 (=DSM21254^T = LMG24544^T), was isolated from a marine fish, Three-line Grunt (*Parapristipoma trilineatum*), in Uwajima, Ehime-prefecture, Japan.

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References

Barns, S.M., Grow, C.C., Okinaka, R.T., Keim, P. and Kuske, C.R. (2005) Detection of diverse new *Francisella*-like bacteria in environmental samples. *Appl Environ Microbiol* **71**, 5494–5500.

Birkbeck, T.H., Bordevik, M., Frøystad, M.K. and Baklien, A. (2007) Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. *J Fish Dis* **30**, 505–507.

Euzeby, J. (2008) List of new names and new combinations previously effectively, but not validly, published. *Int J Syst Evol Microbiol* **58**, 1–2.

Friis-Møller, A., Lemming, L.E., Valerius, N.H. and Bruun, B. (2004) Problems in identification of *Francisella philomiragia* associated with fatal bacteremia in a patient with chronic granulomatous disease. *J Clin Microbiol* **43**, 1840–1842.

Fukuda, Y., Okamura, A., Nishiyama, M., Kawakami, H., Kamaishi, T. and Yoshinga, T. (2002) Granulomatosis of cultured three-line grunt *Parapristipoma trilineatum* caused by an intracellular bacterium. *Fish Pathol* **37**, 119–124.

Hsieh, C.Y., Tung, M.C., Tu, C., Chang, C.D. and Tsai, S.S. (2006) Enzootics of visceral granulomas associated with *Francisella*-like organism infection in tilapia (*Oreochromis* spp.). *Aquaculture* **254**, 129–138.

Kamaishi, T., Fukuda, Y., Nishiyama, M., Kawakami, H., Matsuyama, T., Yoshinaga, T. and Oseko, N. (2005) Identification and pathogenicity of intracellular *Francisella* bacterium in three-line Grunt *Parapristipoma trilineatum*. *Fish Pathol* **40**, 67–71.

Kay, W., Petersen, B.O., Duus, J.O., Perry, M.B. and Vinogradov, E. (2006) Characterization of the lipopolysaccharide and beta-glucan of the fish pathogen *Francisella victoria*. *FEBS J* **273**, 3002–3013.

Kugeler, K.J., Mead, P.S., McGowan, K.L., Burnham, J.M., Hogarty, M.D., Ruchelli, E., Pollard, K., Husband, B. *et al.* (2008) Isolation and characterization of a novel *Francisella* sp. from human CSF and Blood. *J Clin Microbiol* **46**, 2428–2431.

Mauel, M.J., Soto, E., Moralis, J.A. and Hawke, J. (2007) A piscirickettsiosis-like syndrome in cultured Nile tilapia in Latin America with *Francisella* spp. as the pathogenic agent. *J Aquat Anim Health* **19**, 27–34.

Mikalsen, J., Olsen, A.B., Tengs, T. and Colquhoun, D.J. (2007) *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua,* L.). *Int J Syst Evol Microbiol* **57**, 1960–1965.

Nicholas, K.B., Nicholas, H.B.J. and Deerfield, D.W.I. (1997) GeneDoc: analysis and visualization of genetic variation. *Embnew. News* **4**, 14.

Niebylski, M.L., Peacock, M.G., Fisher, E.R., Porcella, S.F. and Schwan, T.G. (1997) Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the Genus *Francisella*. *Appl Environ Microbiol* **63**, 3933–3940.

Nylund, A, Ottem, K.F., Watanabe, K., Karlsbakk, E. and Krossøy, B. (2006) *Francisella* sp. (Family Francisellaeceae) causing mortality in Norwegian cod (Gadus morhua) farming. *Arch Microbiol* **185**, 383–392.

Olsen, A.B., Mikalsen, J., Rode, M., Alfjorden, A., Hoel, E., Straum-Lie, K., Haldorsen, R. and Colquhoun, D.J. (2006) A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. *J Fish Dis* **29**, 307–311.

Ostland, V.E., Stannard, J.A., Creek, J.J., Hedrick, R.P., Ferguson, H.W., Carlberg, J.M. and Westerman, M.E. (2006) Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* x *M. saxatilis*. *Dis Aquat Organ* **72**, 135–45.

Ottem, K.F., Nylund, A., Karlsbakk, E., Friis-Møller, A. and Krossøy, B. (2006) Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). *Arch Microbiol* **187**, 343–350.

Ottem, K.F., Nylund, A., Karlsbakk, E., Friis-Møller, A., Krossøy, B. and Knappskog, D. (2007) New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Arch Microbiol* **188**, 547–550.

Page, R.D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Posada, D. and Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Raghava, G.P.S. and Barton, G.J. (2006) Quantification of the variation in percentage identity for protein sequence alignments. *BMC Bioinformatics* **7**, 1–4.

Sjøstedt, A.B. (2005) Family III. Francisellaceae fam. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Vol. II, Part B *The Gammaproteobacteria* ed. Brenner, D.J., Krieg, N.R. and Staley, J.T. pp. 199–210. USA: Springer.

Viscidi, R.P. and Demma, J.C. (2003) Genetic diversity of *Neisseria gonorrhoeae* housekeeping genes. *J Clin Microbiol* **41**, 197–204.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D, Kandler, O., Krichevsky, L., Moore, L.H., Moore, W.C. *et al.* (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yang, Z. (2007) PAML 4: a program package for phylogenetic analysis by maximum likelihood. *Mol Biol Evol* **24**, 1586–1591.

Yang, Z. and Nielsen, R. (2000) Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Mol Biol Evol* **1**, 32–43.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Overview of primers used in amplification and sequencing of the housekeeping genes from *Francisella* spp. The location of the primers is given in relation to the open reading frame (ORF) of the protein encoding genes from *F. philomiragia* subsp. *philomiragia* ATCC 25017 (CP000937).

Table S2 Table show gene-sequence similarity in percent of *Francisella* sp. Ehime-1^{T} and Ind04 to *Francisella philomiragia* subsp. *philomiragia* (1 = ATCC25015^{T}, 2 = CCUG12603, 3 = CCUG13404, 4 = CCUG19701, 5 = strain 1951, 6 = ATCC25017), 7 = *F. philomiragia* subsp. *noatunensis* 2005/50/F292-6C^{T}/*F. piscicida* GM2212^{T}, 8 = *F. novicida* U112, 9 = *F. tularensis* subsp. *holarctica*, FTA 10 = *F. tularensis* subsp. *tularensis* FSC 198, 11 = *F. tularensis* subsp. *tularensis* SCHU, 12 = *F. tularensis* subsp. *mediasiatica* FSC 148. In-frame fragments of 1054, 1305, 576, 2328, 1515, 882, 3789, 870, 3918, 507 and 15687 for the 16S rRNA, dnaA, mdh, mutS, pgm, prfB, putA, rpoA, rpoB, tpiA-genes and the concatenated gene-sequences, respectively.

Table S3 Kimura 2-parameter distance for the concatenated gene sequences (dnaA, mdh, mutS, pgm, prfB, putA, rpoA, rpoB and tpiA) from *Francisella philomiragia* subsp. *philomiragia* (1 = ATCC25015^{T}, 2 = CCUG12603, 3 = CCUG13404, 4 = CCUG19701, 5 = strain 1951, 6 = ATCC25017), 7 = *Francisella* sp. Ehime-1^{T} and Ind04, 9 = *F. philomiragia* subsp. *noatunensis* 2005/50/F292-6C^{T}/*F. piscicida* GM2212^{T}, 10 = *F. novicida* U112, 11 = *F. tularensis* subsp. *holarctica* FTA, 12 = *F. tularensis* subsp. *tularensis* FSC198, 13 = *F. tularensis* subsp. *tularensis* SCHU, 14 = *F. tularensis* subsp. *mediasiatica* FSC148.

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