AEM
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Multilocus Sequence Analysis of the Marine Bacterial Genus *Tenacibaculum* Suggests Parallel Evolution of Fish Pathogenicity and Endemic Colonization of Aquaculture Systems

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The genus *Tenacibaculum*, a member of the family *Flavobacteriaceae*, is an abundant component of marine bacterial ecosystems that also hosts several fish pathogens, some of which are of serious concern for marine aquaculture. Here, we applied multilocus sequence analysis (MLSA) to 114 representatives of most known species in the genus and of the worldwide diversity of the major fish pathogen *Tenacibaculum maritimum*. Recombination hampers precise phylogenetic reconstruction, but the data indicate intertwined environmental and pathogenic lineages, which suggests that pathogenicity evolved independently in several species. At lower phylogenetic levels recombination is also important, and the species *T. maritimum* constitutes a cohesive group of isolates. Importantly, the data reveal no trace of long-distance dissemination that could be linked to international fish movements. Instead, the high number of distinct genotypes suggests an endemic distribution of strains. The MLSA scheme and the data described in this study will help in monitoring *Tenacibaculum* infections in marine aquaculture; we show, for instance, that isolates from tenacibaculosis outbreaks in Norwegian salmon farms are related to *T. dicentrarchi*, a recently described species.

The fast development of aquaculture (1) faces an array of sanitary issues, causing important economic losses and with an impact on the environment and animal welfare. As a result, there is growing interest in the analysis of the pathogenic bacteria infecting cultured aquatic organisms. In 2001, the genus *Tenacibaculum* (a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*) was proposed to reclassify *T. maritimum* and *T. ovolyticum*, two species of marine fish-pathogenic bacteria formerly included in the genus *Flexibacter* (2). The number of described species in the genus *Tenacibaculum* has since grown rapidly; it currently contains a total of 21 fish-pathogenic and environmental species (http://www.bacterio.net/tenacibaculum.html).

The best known of the pathogens in this genus, *T. maritimum* (3), has been repeatedly identified as a cause of high levels of mortality and economic losses in many cultured marine fish species worldwide (4). The disease, often referred to as tenacibaculosis, typically consists of external lesions and necrosis that can affect virtually all areas of the body surface (5). *T. ovolyticum* has been described as a bacterium attacking Atlantic halibut (*Hippoglossus hippoglossus*) eggs and larvae (6, 7). The type strains of *T. discolor*, *T. gallaicum*, *T. soleae*, and *T. dicentrarchi* (8–10) were isolated from different species of cultured marine fish or their close environment; evidence of pathogenicity resulted from the isolation source (external lesions) and from the results of experimental infection trials (10–12), but general data on the distribution, degree of pathogenicity, and impact on fish farming are lacking for these species.

Tenacibaculum strains not apparently associated with fish diseases also are widespread in marine environments, where they may decompose organic matter, as suggested by their ability to degrade a variety of biopolymers, such as various cellulose derivatives, xylan, agar, and chitin (2, 13–16). *T. mesophilum*, *T. amylolyticum*, *T. aiptasiae*, *T. adriaticum*, *T. crassostreae*, and "*T. halocynthiae*" (quotation marks denote names that have not been validly published) were isolated from marine organisms (2, 15, 17–19). Also belonging to this category is *T. litopenaei*, a chitinolytic bacterium isolated from the water of a shrimp mariculture pond (20). Other *Tenacibaculum* species were retrieved from inorganic substrates; *T. litoreum*, *T. lutimaris*, *T. aestuarii*, and *T. caenipelagi* were isolated from tidal flat sediments (14, 21–23), while *T. skagerrakense*, *T. jejuense*, *T. geojense*, and *T. xiamenense* were isolated from seawater at various distances from the shore and positions in the water column (13, 16, 24, 25).

Clarification of the taxonomy of the family *Flavobacteriaceae* (26–28) and description of the genus *Tenacibaculum* (2) represented key steps toward a more rational description of the relationships between members of the phylum *Bacteroidetes*. However, our knowledge of the evolutionary relationships between members of the genus *Tenacibaculum* remains very scarce, and no practical molecular technique is available to monitor the diversity and incidence of *Tenacibaculum* infections in marine aquaculture systems worldwide. To bridge this gap, in this work we conducted a multilocus sequence analysis (MLSA) (29, 30) encompassing

Received 10 April 2014 **Accepted** 20 June 2014
Published ahead of print 27 June 2014
Editor: C. R. Lovell
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01177-14.

doi:10.1128/AEM.01177-14

TABLE 1 Primers used for PCR and sequencing of the 11 loci

Locus	Step	Primer sequence (5′–3′)	
		Forward	Reverse
atpA	PCR	ATTGGWGAYCGTCAAACWGG	CCAAAYTTAGCRAAHGCTTC
dnaK	PCR	GGWACYACNAAYTCDTGTGT	TCWATCTTMGCTTTYTCAGC
glyA	PCR	CAYTTAACWCAYGGWTCDCC	ACCATRTTTTTRTTTACHGT
gyrB	PCR	AGTATYCARGCRCTRGAAGG	GTWCCTCCTTCRTGYGTRTT
ileS	PCR	CCWACHTTTGGWGCHGAYGA	GAATCRAACCAWACATCAAT
infB	PCR	ATGCCDCAAACWAAAGARGC	GTAATHGCTCCAACYCCTTT
rlmN	PCR	GCKTGTGTDTCDAGYCARGT	CCRCADGCDGCATCWATRTC
tgt	PCR	GAAACWCCWATWTTYATGCC	TAYAWYTCTTCNGCWGGTTC
trpB	PCR	GTWGCNCGWATGAAAATGYT	CCWGGRTARTCYAATCCTGC
tuf	PCR	AGAGAWTTATTRTCTTTCTA	GTTACCTGACCWGCWCCWAC
yqfO	PCR	GCBGAARRTTTTGAYAAYGT	AYTTCRTARGCDACYTCTTC
All	Sequencing	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT

representatives of the 18 *Tenacibaculum* species published by March 2013 (excluding the environmental species *T. caenipelagi*, *T. halocynthiae*, and *T. xiamenense*) as well as a collection of *T. maritimum* isolates representative of the worldwide diversity of this fish pathogen. In contrast to DNA-DNA hybridization (30), randomly amplified polymorphic DNA (RAPD), and serotyping schemes (31, 32) that already have been used to assess genetic diversity within the genus, MLSA has the advantage of relying on sequence data directly amenable to evolutionary analysis. Furthermore, the data can easily be stored in databases, compared across experiments, and progressively enriched by the addition of new isolates. The generality of MLSA also makes it complementary to more rapid and less expensive detection methods, such as PCR or immunohistochemical assays already proposed for *T. maritimum* (33–36) and *T. soleae* (37). To illustrate the applicability of our approach at the genus level, we include in this report the results of MLSA on a selection of isolates responsible for recent tenacibaculosis outbreaks in Norwegian salmon farms (38) and in Italian sea bass and sea bream farms.

MATERIALS AND METHODS

Loci, strains, and experimental protocol. The 11 loci are located within single-copy protein-coding genes conserved across the family *Flavobacteriaceae* for which it was possible to design generic degenerated PCR primers for the genus *Tenacibaculum*. Data on the genetic polymorphism in the populations of *Tenacibaculum* strains was not used to select these loci, except that we required enough conservation of the 21-bp sequences recognized by the primers. These loci can be considered typical core genome genes whose polymorphism is more likely relatively neutral (i.e., not experiencing frequent adaptive selection). Individually, each of these genes had already been used in MLST studies of other bacterial species and genera. The 16S rRNA sequences of *Tenacibaculum* type strains were retrieved from complete genomes (unpublished data); the sequences of the strains used as outgroups were obtained from GenBank.

Tenacibaculum strains were grown in marine 2216E broth (Difco) for 24 h at 28°C and 70 rpm, and the genomic DNA was extracted from the pellet using the Wizard genomic DNA purification kit (Promega). PCR amplification was performed in a 20-μl reaction volume using GoTaq polymerase (Promega) and the following touchdown protocol: 94°C for 5 min, 24 cycles at 94°C for 0.5 min, 55°C for 0.5 min (−0.4°C/cycle), and 72°C for 1 min (+2 s/cycle); 12 cycles at 94°C for 0.5 min, 45°C for 0.5 min, and 72°C for 2 min (+3 s/cycle); and a final extension step at 72°C for 10 min. The sequences of the primers are listed in Table 1. Five microliters of the PCR products was resolved in a 1% agarose–Tris-borate-EDTA (TBE) gel to check amplification. For sequencing, one microliter of the PCR products was purified by using exonuclease I (Biolabs)-alkaline phosphatase (USB) for 1 h at 37°C, followed by enzyme inactivation for 5 min at 94°C. One-tenth of the purified PCR products was sequenced on both strands using the sequencing primers, the BigDye Terminator version 3.1 sequencing kit (Applied Biosystems), and an Applied Biosystems 3730 automated sequencer.

The 114 *Tenacibaculum* isolates included in this study are listed in Table 2, along with their origins; duplicated stocks of all strains were stored in glycerol at −80°C. This collection encompasses strains originating from five continents since 1976, including the type strains of all *Tenacibaculum* species available by March 2013. European isolates account for 77 strains. A total of 18 host fish species are represented: black sea bream (*Acanthopagrus schlegeli*), white sea bass (*Atractoscion nobilis*), European sea bass (*Dicentrarchus labrax*), sharpsnouted bream (*Diplogus sargus*), northern anchovy (*Engraulis mordax*), cod (*Gadus morhua*), striped trumpeter (*Latris lineata*), Coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), Japanese red sea bream (*Pagrus major*), European red sea bream (*Pagellus bogaraveo*), Japanese flounder (*Paralichthys olivaceus*), Atlantic salmon (*Salmo salar*), turbot (*Scophthalmus maximus*), Japanese amberjack or yellowtail (*Seriola quinqueradiata*), gilthead sea bream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*), and Dover sole (*Solea solea*). Most strains were retrieved from external (i.e., skin, mouth, eye, head, and tail) lesions, but some were collected from the kidney.

Data analysis. The sequences were assembled using Phred/Phrap/Consed (39) and verified manually to ensure high quality. Alignments were generated with a two-step Biopython (40) wrapper: nucleotide sequences first were translated and aligned at the protein level with MUSCLE 5V3.8.31 (41); protein alignment then was back converted into a nucleotide alignment that served for all of the analyses. According to MLST standards (42), arbitrary numbers were used for unambiguous identification of the allele types (ATs; particular alleles at particular loci) and sequence types (STs; unique combinations of ATs at the different loci). Maximum likelihood trees were obtained with PhyML v3.0 (43) using the substitution model selected with Modelgenerator v0.85 (44), which corresponded to GTR+I+G. The gamma distribution was approximated with 4 categories of sites. At the *T. maritimum* species level, recombination challenges exact phylogenetic reconstruction. Nevertheless, a tentative tree was constructed by neighbor joining to graphically represent the sequence divergence between isolates. For this purpose, we used a simple Jukes-Cantor substitution model and the *dnadist* and *neighbor* programs included in Phylip (45) on concatenated nucleotide sequences. All phylogenetic trees were drawn in R using the *ape* package (46).

The detection of recombination within and between loci involved the computation of h and R_{min}. The minimal number of apparent homoplasies (47), designated h, was computed on the most parsimonious tree found with the *dnapars* program included in Phylip (45). The Hudson and Kaplan lower bound on the minimal number of recombination events in

TABLE 2 List of the 114 *Tenacibaculum* isolates included in this study

Strain no. in this study	Strain identifier as received	Country/ state	Origin	Tissue	Yr	ST	Bacterial species	Contributor[a]
1	NCIMB 2154[T]	Japan	*Pagrus major*	Kidney	1977	1	*T. maritimum*	NCIMB
2	ACC13.1	Portugal	*Solea senegalensis*	Kidney	2004	2	*T. maritimum*	AET
3	ACR485.1	Spain	*Solea senegalensis*	Kidney	2011	3	*T. maritimum*	AET
4	ACR488.1	Spain	*Solea senegalensis*	Kidney	2011	3	*T. maritimum*	AET
5	ACR491.1	Spain	*Solea senegalensis*	Kidney	2011	3	*T. maritimum*	AET
6	AF37.1	Spain	*Pagellus bogaraveo*	Kidney	2006	4	*T. maritimum*	AET
7	AF39.1	Spain	*Pagellus bogaraveo*	Tail	2006	5	*T. maritimum*	AET
8	CA42.1	Spain	*Solea senegalensis*	NA[b]	2006	6	*T. maritimum*	AET
9	CA43.1	Spain	*Solea senegalensis*	NA	2006	6	*T. maritimum*	AET
10	COS2.1	Spain	*Solea senegalensis*	Kidney	2011	7	*T. maritimum*	AET
11	COS3.1	Spain	*Solea senegalensis*	Tail	2011	7	*T. maritimum*	AET
12	FS08(1)	Italy	*Sparus aurata*	Skin	2006	8	*T. maritimum*	FS
13	NCIMB 2153	Japan	*Acanthopagrus schlegeli*	Kidney	1976	9	*T. maritimum*	NCIMB
14	NCIMB 2158	Scotland	*Solea solea*	Skin	1981	10	*T. maritimum*	NCIMB
15	PC1012.1	Spain	*Scophthalmus maximus*	Head	2008	11	*T. maritimum*	AET
16	PC424.1	Spain	*Scophthalmus maximus*	Kidney	2000	12	*T. maritimum*	AET
17	PC503.1	Spain	*Solea senegalensis*	Skin	2001	13	*T. maritimum*	AET
18	PC538.1	Spain	*Sparus aurata*	Tail	2002	14	*T. maritimum*	AET
19	PC824.1	Spain	*Sparus aurata*	Kidney	2003	4	*T. maritimum*	AET
20	PC834.1	Spain	*Sparus aurata*	Kidney	2003	5	*T. maritimum*	AET
21	RI93.1	Spain	*Scophthalmus maximus*	Head	2002	12	*T. maritimum*	AET
22	RIM70.1	Spain	*Scophthalmus maximus*	Mouth	2009	15	*T. maritimum*	AET
23	USC RP67.1	Spain	*Scophthalmus maximus*	Mouth	1993	16	*T. maritimum*	AET
24	USC RPM539.1	Spain	*Scophthalmus maximus*	Mouth	1993	17	*T. maritimum*	AET
25	USC SE30.1	Spain	*Oncorhynchus kisutch*	Mouth	1993	18	*T. maritimum*	AET
26	DPIF 90/1445	Tasmania	*Salmo salar*	Skin	1990	19	*T. maritimum*	JC
27	DPIF 89/0239-1	Tasmania	*Salmo salar*	Skin	1989	20	*T. maritimum*	JC
28	DPIF 89/0235-3	Tasmania	*Oncorhynchus mykiss*	Skin	1989	21	*T. maritimum*	JC
29	DPIF 89/0329-11	Tasmania	*Salmo salar*	Skin	1989	22	*T. maritimum*	JC
30	DPIF 89/0329-5	Tasmania	*Salmo salar*	Skin	1989	22	*T. maritimum*	JC
31	DPIF 89/0578-4	Tasmania	*Salmo salar*	Skin	1989	23	*T. maritimum*	JC
32	DPIF 89/0699	Tasmania	*Salmo salar*	Skin	1989	22	*T. maritimum*	JC
33	DPIF 89/1288-8	Tasmania	*Oncorhynchus mykiss*	Skin	1989	22	*T. maritimum*	JC
34	DPIF 89/3001-6.2	Tasmania	*Latris lineata*	Skin	1989	24	*T. maritimum*	JC
35	DPIF 89/0528-1	Tasmania	*Salmo salar*	Skin	1989	21	*T. maritimum*	JC
36	Baxa 1y 1-1	Japan	*Acanthopagrus schlegeli*	Skin	1985	25	*T. maritimum*	RPB
37	JIP 46/00	France	*Scophthalmus maximus*	Skin	2000	26	*T. maritimum*	GG
38	CVI10001048	Holland	*Solea solea*	Skin	2010	27	*T. maritimum*	OH
39	Baxa DBA-4a	Japan	*Seriola quinqueradiata*	Skin	1986	28	*T. maritimum*	RPB
40	FC	Chile	*Scophthalmus maximus*	Eye	1998	29	*T. maritimum*	JM
41	FM1068	France	*Dicentrarchus labrax*	Skin	1993	30	*T. maritimum*	JFP
42	FPC371	Japan	*Pagrus major*	Skin	1977	31	*T. maritimum*	HW
43	FPC386	Japan	*Pagrus major*	Skin	1978	32	*T. maritimum*	HW
44	FPC394	Japan	*Pagrus major*	Skin	1982	32	*T. maritimum*	HW
45	FPC454	Japan	*Pagrus major*	Skin	1983	33	*T. maritimum*	HW
46	Baxa GBF-8601	Japan	*Paralichthys olivaceus*	Skin	1986	34	*T. maritimum*	RPB
47	JIP 05/00(1)	France	*Scophthalmus maximus*	Skin	2000	11	*T. maritimum*	FL
48	JIP 10/97	France	*Scophthalmus maximus*	Skin	1997	12	*T. maritimum*	FL
49	JIP 21/91-1	France	*Dicentrarchus labrax*	Skin	1991	3	*T. maritimum*	JFB
50	JIP 21/91-2	France	*Dicentrarchus labrax*	Skin	1991	3	*T. maritimum*	JFB
51	JIP21/91-3	France	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
52	JIP 24/99	France	*Scophthalmus maximus*	Skin	1999	12	*T. maritimum*	FL
53	JIP 31/99	France	*Scophthalmus maximus*	Skin	1999	12	*T. maritimum*	FL
54	JIP 32/91-1	Corsica	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
55	JIP 32/91-3	Corsica	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
56	JIP 32/91-4	Corsica	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
57	JIP 32/91-5	Corsica	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
58	JIP 32/91-6	Corsica	*Dicentrarchus labrax*	Skin	1991	35	*T. maritimum*	JFB
59	JIP 32/99	France	*Dicentrarchus labrax*	Skin	1991	27	*T. maritimum*	CS
60	LVDH 1577.01	France	*Dicentrarchus labrax*	Skin	2001	36	*T. maritimum*	NK
61	USC RPM522.1	Spain	*Scophthalmus maximus*	Mouth	1992	37	*T. maritimum*	AET
62	UCD SB2	California	*Atractoscion nobilis*	NA	1995	38	*T. maritimum*	RH
63	UCD SD26	California	*Atractoscion nobilis*	NA	1995	39	*T. maritimum*	RH
64	NAC SLCC 101	Malta	*Dicentrarchus labrax*	Skin	1995	40	*T. maritimum*	JT
65	NAC SLCC 105	Malta	*Dicentrarchus labrax*	Skin	1995	41	*T. maritimum*	JT
66	NAC SLCC 109	Malta	*Dicentrarchus labrax*	Skin	1995	42	*T. maritimum*	JT
67	NAC SLCC 115	Malta	*Dicentrarchus labrax*	Skin	1996	42	*T. maritimum*	JT
68	NAC SLCC 120	Malta	*Dicentrarchus labrax*	Skin	1996	42	*T. maritimum*	JT

(Continued on following page)

TABLE 2 (Continued)

Strain no. in this study	Strain identifier as received	Country/ state	Origin	Tissue	Yr	ST	Bacterial species	Contributor[a]
69	NAC SLCC MFF	Malta	*Dicentrarchus labrax*	Skin	NA	43	*T. maritimum*	ALB
70	USC SP9.1	Spain	*Salmo salar*	Skin	1993	44	*T. maritimum*	AET
71	UCD V2b	California	*Atractoscion nobilis*	NA	1993	45	*T. maritimum*	RH
72	UCD V6f	California	*Engraulis mordax*	Skin	1994	46	*T. maritimum*	RH
73	UCD WSB-1b	California	*Atractoscion nobilis*	Skin	1994	47	*T. maritimum*	RH
74	147/ITT	Italy	*Dicentrarchus labrax*	Kidney	1989	NA	*T. discolor*	AM
75	253/ITT-1	Italy	*Sparus aurata*	Kidney	2004	NA	*T. mesophilum*	AM
76	269/ITT	Italy	*Dicentrarchus labrax*	Skin	2010	NA	*T. discolor*	AM
77	43/ITT	Italy	*Dicentrarchus labrax*	Kidney	2010	NA	*T. discolor*	AM
78	FSIXSp1	Italy	*Dicentrarchus labrax*	Eye	1998	NA	*T. discolor*	FS
79	TNO001	Norway	*Salmo salar*	Skin	2011	NA	*Tenacibaculum* sp.	ABO
80	TNO002	Norway	*Salmo salar*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
81	TNO003	Norway	*Salmo salar*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
82	TNO004	Norway	*Salmo salar*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
83	TNO005	Norway	*Salmo salar*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
84	TNO006	Norway	*Salmo salar*	Skin	2011	NA	*Tenacibaculum* sp.	ABO
85	TNO007	Norway	*Salmo salar*	Skin	2011	NA	*Tenacibaculum* sp.	ABO
86	TNO008	Norway	*Salmo salar*	Kidney	2011	NA	*Tenacibaculum* sp.	ABO
87	TNO009	Norway	*Salmo salar*	Skin	1996	NA	*Tenacibaculum* sp.	ABO
88	TNO010	Norway	*Salmo salar*	Skin	1998	NA	*Tenacibaculum* sp.	ABO
89	TNO011	Norway	*Salmo salar*	Skin	1998	NA	*Tenacibaculum* sp.	ABO
90	TNO012	Norway	*Gadus morhua*	Skin	2009	NA	*Tenacibaculum* sp.	ABO
91	TNO013	Norway	*Gadus morhua*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
92	TNO014	Norway	*Gadus morhua*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
93	TNO015	Norway	*Gadus morhua*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
94	TNO018	Norway	*Gadus morhua*	Skin	2010	NA	*Tenacibaculum* sp.	ABO
95	TNO019	Norway	*Salmo salar*	Kidney	1998	NA	*Tenacibaculum* sp.	ABO
96	TNO020	Norway	*Salmo salar*	Skin	1998	NA	*Tenacibaculum* sp.	ABO
97	LL04 12.1.7^T	Spain	*Solea senegalensis*	NA	2004	NA	*T. soleae*	YSR
98	DSM 18961^T	Croatia	*Schizobrachiella sanguinea*	NA	2008	NA	*T. adriaticum*	DSMZ
99	JCM 13491^T	South Korea	Tidal flat sediment	NA	2006	NA	*T. aestuarii*	JCM
100	LMG 24004^T	Taiwan	*Aiptasia pulchella*	NA	2008	NA	*T. aiptasiae*	BCCM/LMG
101	CIP 107214^T	Philippines	*Avrainvilla riukiuensis*	NA	2001	NA	*T. amylolyticum*	CIP
102	JCM 15428^T	South Korea	*Crassostea gigas*	NA	2009	NA	*T. crassostreae*	JCM
103	USC 35/09^T	Spain	*Dicentrarchus labrax*	Skin	2012	NA	*T. dicentrarchi*	YSR
104	YSR-01	Spain	*Solea senegalensis*	NA	2010	NA	*T. discolor*	YSR
105	LL04 11.1.1^T	Spain	*Solea senegalensis*	Kidney	2008	NA	*T. discolor*	YSR
106	A37.1^T	Spain	Seawater from a tank containing turbot	NA	2008	NA	*T. gallaicum*	YSR
107	LMG 23706^T	Taiwan	*Litopenaeus vannamei*	NA	2007	NA	*T. litopenaei*	BCCM/LMG
108	JCM 13039^T	South Korea	Tidal flat sediment	NA	2006	NA	*T. litoreum*	JCM
109	DSM 16505^T	South Korea	Tidal flat sediment	NA	2005	NA	*T. lutimaris*	DSMZ
110	CIP 107215^T	Japan	*Halichondria okadai*	NA	2001	NA	*T. mesophilum*	CIP
111	EKD 002^T	Norway	*Hippoglossus hippoglossus*	Egg	1992	NA	*T. ovolyticum*	GHH
112	DSM 14836^T	Denmark	Seawater	NA	2004	NA	*T. skagerrakense*	DSMZ
113	KCTC 23423^T	South Korea	Seawater	NA	2012	NA	*T. geojense*	KCTC
114	KCTC 22618^T	South Korea	Seawater	NA	2012	NA	*T. jejuense*	KCTC

[a] NCIMB, National Collection of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland); AET, A. Estévez Toranzo (Universidad de Santiago de Compostela, Spain); FS, F. Salati (State Veterinary Institute, Oristano, Italy); JC, J. Carson (Department of Primary Industry and Fisheries, Kings Meadows, Tasmania, Australia); RPB, R. P. Burchard (then at the University of Maryland, Baltimore); GG, G. Gauthier (N.A.T.A., France Turbot, l'Epine, France); OH, O. Haenen (Central Veterinary Institute, Lelystad, the Netherlands); JM, J. Montaña (then at Fundación Chile, Puerto Montt, Chile); JFP, J.-F. Pépin (then at IFREMER, Palavas-les-Flots, France); HW, H. Wakabayashi (then at the University of Tokyo, Japan); FL, F. Leveau (N.A.T.A., France Turbot, l'Epine, France); JFB, J.-F. Bernardet (Institut National de la Recherche Agronomique, Jouy-en-Josas, France); CS, C. Sauvegrain (Aquanord France, Gravelines, France); NK, N. Keck (Laboratoire Départemental Vétérinaire de l'Hérault, Montpellier, France); RH, R. Hedrick (then at the University of California, Davis); JT, J. Tabone (National Aquaculture Center, Marsaxlokk, Malta); ALB, A. Le Breton (VET'EAU Selarl, Grenade-sur-Garonne, France); AM, A. Manfrin (Istituto Zooprofilattico Sperimentale delle Venezie, Adria, Italy); ABO, A.-B. Olsen (National Veterinary Institute, Bergen, Norway); YSR, Y. Santos Rodríguez (Universidad de Santiago de Compostela, Spain); DSMZ, Leibniz Institut Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH (Braunschweig, Germany); JCM, Japan Collection of Microorganisms (Tsukuba, Japan); BCCM/LMG, Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology Gent (Ghent, Belgium); CIP, Collection de l'Institut Pasteur (Paris, France); GHH, G. H. Hansen (University of Bergen, Norway); KCTC, Korean Collection for Type Cultures (Daejeon, South Korea).
[b] NA, no data available.

an infinite site model (48), designated R_{min}, was computed on biallelic sites by using LDhat (49). A quantitative estimate of the contribution of recombination versus that of mutation in short-term nucleotide divergence between strains was obtained using ClonalFrame (50). A total of 150,000 MCMC iterations (including 50,000 for burn-in) were performed for this analysis, and we checked that results from independent runs were comparable. The parameters θ (rate of mutation on the branches of the genealogy) and ν (rate of nucleotide differences in the recombination tracts) were fixed to the average level of pairwise nucleotide diversity of the sequences (π). The ratio of per-nucleotide changes that could be attributed to recombination to those that could be attributed to mutation (r/m ratio) (51) was computed from the parameter estimates provided by

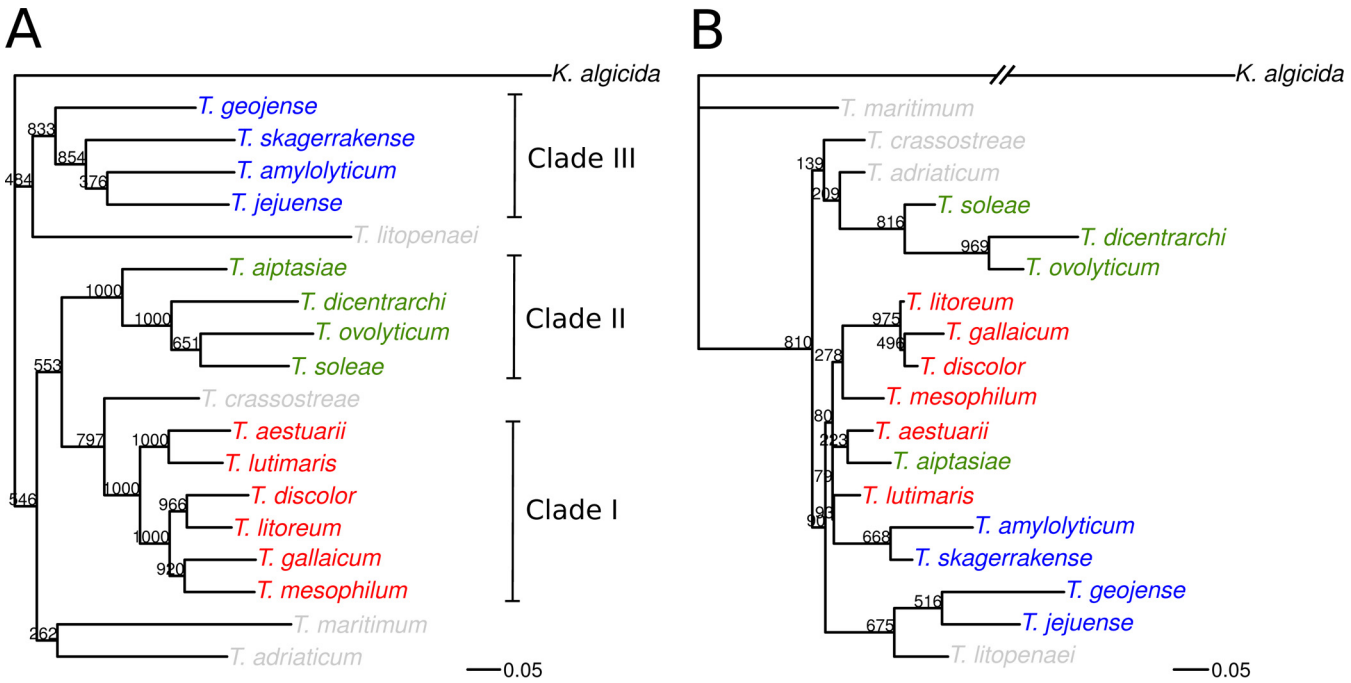

FIG 1 Comparison between the maximum-likelihood phylogenetic trees reconstructed on the 11 loci and on the 16S rRNA locus for the 18 *Tenacibaculum* type strains. (A) Concatenated MLSA tree; (B) 16S rRNA tree. The *Kordia algicida* type strain was included as an outgroup. Bootstrap supports estimated on 1,000 replicate data sets are reported above each internal node. Colors indicate the three clades identified based on high bootstrap support in the concatenated MLSA tree (I in red, II in green, and III in blue); the four isolated lineages that branch more deeply in the three are represented in gray. The same branch-length scale (measured in expected number of nucleotide substitutions per site) is used in both trees. The branch leading to *K. algicida* in the 16S rRNA tree has been shortened by a factor of 3 for the sake of representation.

ClonalFrame as $(R \times \nu \times \delta)/\theta$ (as described in reference 52), where R is the rate of recombination, δ is the average length of a recombination tract, ν is the amount of nucleotide divergence between the two sequences that recombine, and θ is the mutation rate.

Association between genotypes and isolation sources in the *T. maritimum* species were investigated using analysis of molecular variance (AMOVA) (53) based on simple Euclidean distances (d) between STs ($d = \sqrt{n}$, where n is the number of differences between two nucleotide sequences). A nonparametric estimate of the statistical significance was obtained using random permutations of the genotypes with respect to isolation sources. These analyses were conducted in R with the *pegas* package (54).

Nucleotide sequence accession numbers. The nucleotide sequences determined in the course of this work were deposited in GenBank under accession numbers KJ402457 to KJ403732.

RESULTS

Evolutionary relationships within the genus *Tenacibaculum*. The PCR and sequencing protocols proposed in this study allowed sequencing of 11 loci (total length of 5,811 bp) across the whole diversity of the genus *Tenacibaculum*, represented here by 114 isolates. Figure 1A shows an MLSA phylogenetic tree reconstructed by maximum likelihood on the concatenated nucleotide sequences of the 18 *Tenacibaculum* type strains included in our collection and using *Kordia algicida* as an outgroup. The internal nodes of this phylogeny come with much higher bootstrap supports than those in the tree reconstructed on the 16S rRNA locus shown in Fig. 1B: out of 17 internal nodes, 10 reach a bootstrap support of at least 80% in the concatenated MLSA tree, whereas only 3 meet this criterion in the 16S rRNA tree.

On the basis of the most ancestral nodes with bootstrap support above 80%, the 18 *Tenacibaculum* species could be divided into three distinct clades plus four more-distant lineages that root deeper in the tree (*T. adriaticum*, *T. crassostreae*, *T. litopenaei*, and *T. maritimum*). The three clades contain 6, 4, and 4 species, respectively. They will be referred to here as clade I for the group *T. aestuarii*, *T. discolor*, *T. gallaicum*, *T. litoreum*, *T. lutimaris*, and *T. mesophilum*; clade II for *T. aiptasiae*, *T. dicentrarchi*, *T. ovolyticum*, and *T. soleae*; and clade III for *T. amylolyticum*, *T. geojense*, *T. jejuense*, and *T. skagerrakense*. In trees reconstructed on the basis of 16S rRNA (Fig. 1B) and the 11 individual loci (see Fig. S1 in the supplemental material), this three-clade distribution is not visible but is not strongly contradicted, as conflicting nodes never receive bootstrap support above 80%. In contrast, the history of the individual loci often seems to conflict with more recent nodes of the MLSA tree, suggesting recombination, at least between closely related species. For instance, the grouping *T. litoreum*, *T. discolor*, and *T. gallaicum* in the 16S rRNA tree (98% bootstrap support) is incompatible with the grouping *T. gallaicum*-*T. mesophilum* (92% bootstrap support) in the concatenated MLSA tree.

The 18 *Tenacibaculum* type strains included in our study can be divided into four categories according to their origins: 3 were isolated from seawater, 3 from sediments, 5 from diseased fish, and 6 from other marine organisms. The analysis of the distribution of these four categories in the seven different lineages delineated by our examination of the genus *Tenacibaculum* (clades I, II, and III and the four isolated lineages) reveals a statistically significant correlation ($P = 0.045$ by Fisher exact test), suggesting some degree of linkage between the position in the phylogeny of the genus and the ecological niche. In summary, clade I contains a balanced

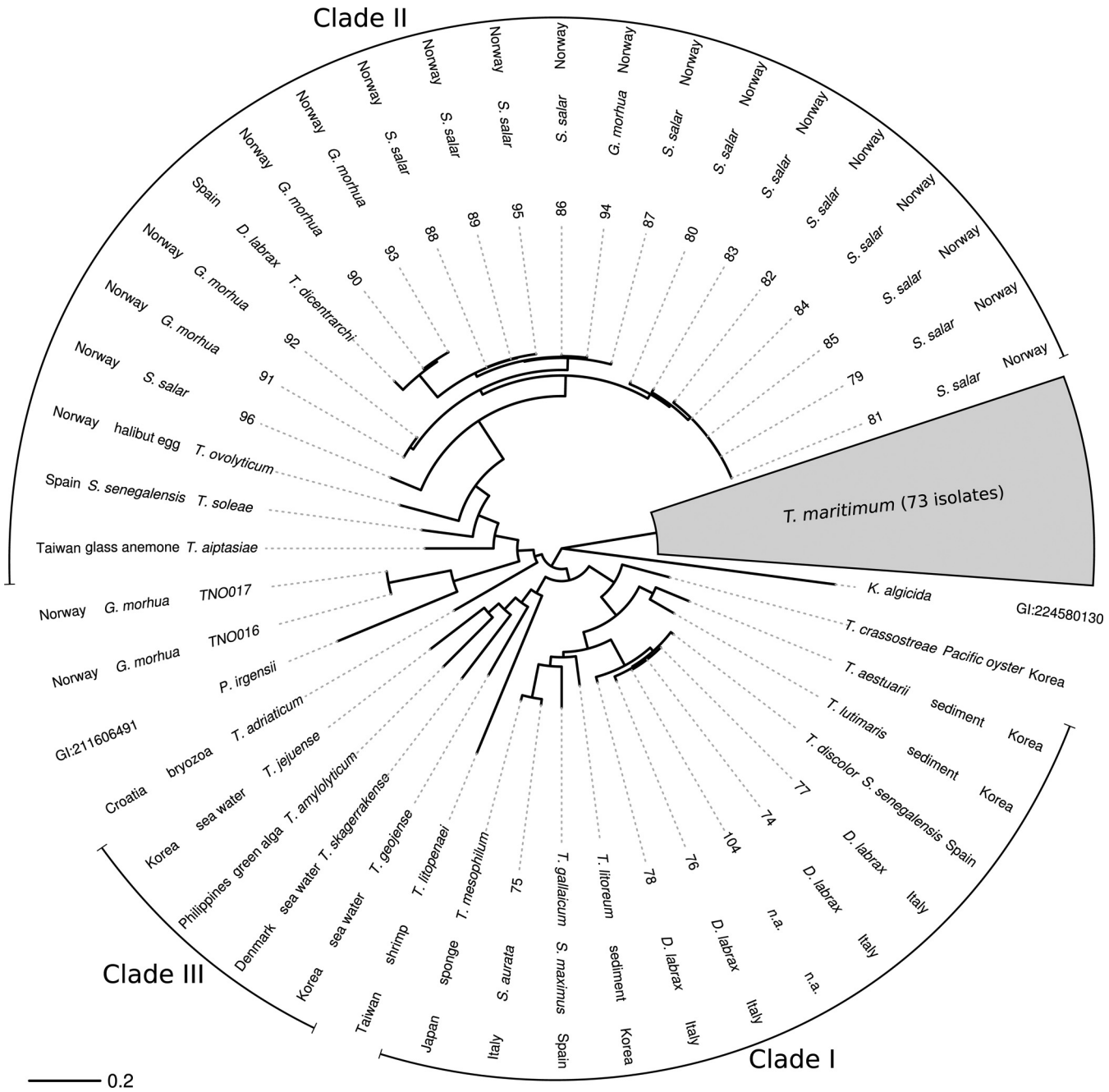

FIG 2 Concatenated MLSA tree reconstructed on the 11 loci by maximum likelihood for the 114 *Tenacibaculum* isolates included in this study. A condensed representation (gray area) is used for the 73 isolates that group with the type strain of *T. maritimum*. For each other *Tenacibaculum* strain, the following information is reported: isolate identifier or bacterial species for type strains, isolation source (binomial names for fish species), and country of origin. The type strains of *Polaribacter irgensii* and *Kordia algicida* (accession numbers NZ_CH724148.1 and NZ_DS544873.1, respectively) were included to help rooting, but only *K. algicida* could easily be used as an outgroup. Branch length is measured as expected number of nucleotide substitutions per site.

mix of strains isolated from sediments and marine organisms (including two fish pathogens), clade II is exclusively composed of strains isolated from marine organisms (primarily from diseased fish), and clade III contains a majority of strains isolated from seawater (all those included in our sample) and none of the fish-pathogenic species. The four species whose lineages root deeper in the genus, including the important fish pathogen *T. maritimum*, all were isolated from marine organisms (fish, oyster, crustacean, or bryozoan). Figure 2 shows the phylogenetic position of the other isolates included in our study. Importantly, all *Tenacibaculum* isolates retrieved from fish that are not *T. maritimum* belong to clades I and II, which corroborates the hypothesis of a nonrandom association between the clades I, II, and III and the ecological niches.

Patterns of polymorphism in the fish-pathogenic species *T. maritimum*. The concatenated MLSA tree of our 114 isolates allowed unambiguous classification of 73 of them as *T. maritimum*

TABLE 3 Summary of statistics on nucleotide polymorphism in *T. maritimum*

Locus	Length (bp)	No. of ATs[a]	S_{nuc}[b] (no.)	S_{prot}[c] (no.)	π[d] (bp^{-1})	R_{min}[e]	h[f] (no.)
atpA	567	20	25 (0/1)	2	0.0082	2	7
dnaK	573	9	9	0	0.0021	2	3
glyA	558	13	13 (1/0)	4	0.0035	2	4
gyrB	597	11	15 (2/0)	3	0.0038	1	1
ileS	546	7	10	2	0.0020	0	0
infB	564	16	18	1	0.0037	1	3
rlmN	549	7	6	0	0.0016	1	1
tgt	486	16	15	4	0.0044	2	5
trpB	369	11	15	4	0.0070	0	0
tuf	555	16	21	1	0.0077	4	9
yqfO	447	16	16 (1/0)	9	0.0060	2	4
Sum[g]	5,811		163 (4/1)	30		17	37
Concatenation[h]	5,811	47 STs	163 (4/1)	30	0.0044		160

[a] Number of allele types (sequence types for the concatenation).
[b] Number of nucleotide polymorphisms, including triallelic and quadriallelic polymorphisms (indicated in parentheses).
[c] Number of amino acid polymorphisms.
[d] Average pairwise nucleotide diversity.
[e] Hudson and Kaplan lower bound on the number of recombination events.
[f] Number of apparent homoplasies.
[g] Sum of the summary statistics over the 11 loci.
[h] Concatenated sequences of the 11 loci.

(Fig. 2). These isolates encompass 16 species of host fish, 5 continents (Europe, Australia, Asia, North America, and South America), and over 30 years of sampling (from 1976 to 2011). Thus, the data provide a broad overview of the genetic diversity in this important fish-pathogenic species.

Sequence comparisons revealed 168 single-nucleotide polymorphisms (SNPs) across the 5,811 bp surveyed in the 73 *T. maritimum* isolates. A summary of the main characteristics of the polymorphisms and their distribution across the 11 loci is presented in Table 3. Overall, 2.9% of the positions showed variations, and the pairs of sequences differed (pairwise nucleotide diversity, π) at 0.44% of the sites on average. The number of SNPs and the π differed between loci, from 6 SNPs and 0.16% nucleotide diversity at locus *rlmN* to 25 SNPs and 0.82% nucleotide diversity at locus *atpA*. As expected given the low level of divergence between the sequences, the vast majority of the SNPs were biallelic; only four were triallelic, and one was quadriallelic. Out of the 168 SNPs, 138 corresponded to synonymous variations, suggesting that most of the polymorphisms examined here are selectively neutral or near neutral, which is a desired property for unbiased analysis of population structure inside species by MLSA.

The presence of intraspecies recombination in the genealogy of the *T. maritimum* sequences was detected by means of two summary statistics, R_{min} and h (Table 3). R_{min} is a lower bound on the minimal number of recombination events when each polymorphism arises from a single mutation, which is a reasonable assumption for most sites given the low divergence between the sequences (48). R_{min} was greater than 0 for 9 loci and summed to 17 over the 11 loci; the 2 loci where recombination could not be detected (*ileS* and *trpB*) also were among the least polymorphic, making recombination more difficult to detect. The second statistic, h, is the minimal number of apparent homoplasies (47). It is obtained as the difference between the number of observed polymorphisms and the minimal number of mutations to obtain the sequences, assuming evolution along the branches of the same tree for all of the polymorphic sites. The value of h is 0 in the absence of recurrent mutations and recombinations. Here, h was 160 for the concatenated sequences of the 11 loci, which is similar to the number of polymorphic sites. The values of h for the loci analyzed separately were consistent with the R_{min} values obtained at the same loci.

The r/m ratio was estimated to be 2.7:1 (95% credibility, 1.7 to 4.0) for *T. maritimum* based on the posterior distribution of the evolutionary parameters obtained from our data set with ClonalFrame (50). We also used the available data for a second group of closely related strains (the 19 *T. dicentrarchi* or *T. dicentrarchi*-like strains shown in Fig. 2) to examine how the values of the r/m ratio could differ across species of the genus *Tenacibaculum*. Our estimate of the r/m ratio for the *T. dicentrarchi* or *T. dicentrarchi*-like strains was 3.4:1 (95% credibility, 2.2 to 4.7), which is quite similar to the value obtained for *T. maritimum*.

Population structure of *T. maritimum*. The number of distinct alleles at a particular locus ranged from 7 for *rlmN* and *ileS* to 20 for *atpA* among the 73 *T. maritimum* isolates (Table 3). The combination of the allele types (ATs) at the 11 loci allowed distinguishing 47 distinct sequence types (STs), which corresponds to an average of 1.6 isolates per ST. None of the ST contained more than 6 isolates, and only three clusters of STs (i.e., clonal complexes) could be identified on the basis of single-locus variation (SLV) links: ST4-ST5, ST16-ST18-ST44, and ST3-ST40. Individually, none of these three clusters accounted for more than 5 isolates. Importantly, strains with the same ST or for which STs are connected by SLV links always originated from the same geographical area. For instance, the small clonal complex ST4-ST5 is composed exclusively of isolates from Spain, and ST3 is composed of isolates sampled 20 years apart in the neighboring countries France and Spain.

For each strain, the ST, ATs, and information on sampling origin are reported in Fig. 3, along with its position in a tentative phylogenetic tree based on concatenated nucleotide sequences. According to this tree, it is tempting to describe our collection of *T. maritimum* isolates as composed of three subgroups, here designated A, B, and C. Subgroup A contains only 9 strains distributed into 6 distinct STs. All of these strains come from south European countries (Spain, Malta, and Italy), and 6 of 9 were retrieved from host fish of the family *Sparidae*. In particular, all gilthead sea bream (*Sparus aurata*) isolates in our collection belong to subgroup A. Subgroup B contains 59 strains; thus, it accounts for the majority of the *T. maritimum* isolates in our collection. Interestingly, the relative positions of the isolates in subgroup B seemed correlated with fish host and geographical origin (which are highly correlated to each other). Subgroup C consists of only 5 strains, but it is indeed far more heterogeneous than the two other subgroups and may justify further delineation in future studies.

The association between the isolation sources and genotypes was statistically confirmed and quantitatively assessed by AMOVA (53). The results are presented in Table 4. The total molecular variance explained by taking each type of information individually was 33.92% for the host fish, 33.52% for the year, and 18.27% for the country; all of these associations were statistically significant at the 5% level. The fraction of variance explained by the tissue also was statistically significant but accounted for only 4.31% of the total variance. Because these values could partly re-

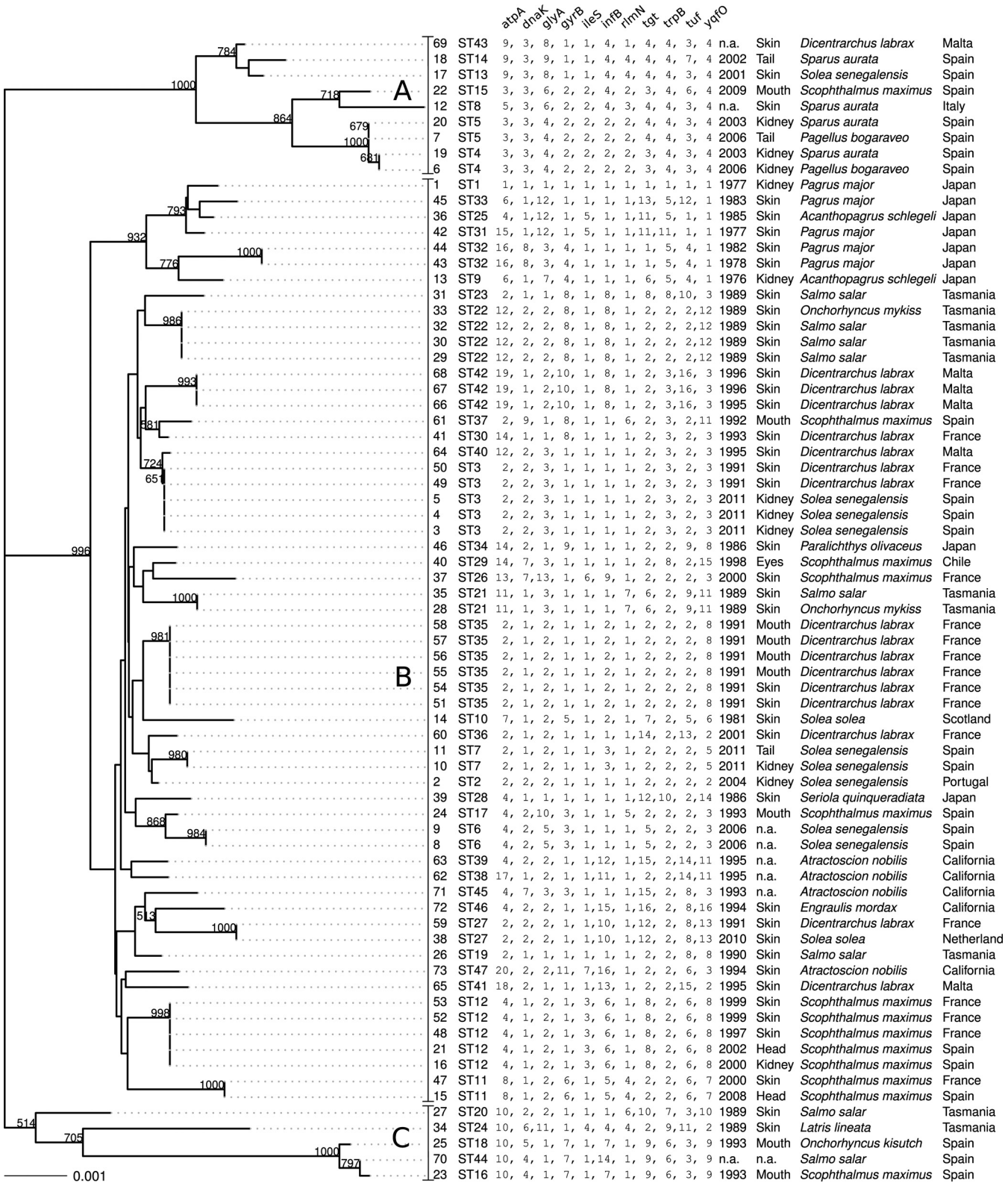

FIG 3 Genotype and background information for the 73 *Tenacibaculum maritimum* isolates. From left to right: tentative phylogenetic tree, isolate identification numbers (see Table S2 in the supplemental material), sequence types, allele types at the 11 loci, and information on the isolation source (year, tissue, host fish, and country; n.a., not available). The tree was obtained by neighbor joining with a simple Jukes-Cantor substitution model on concatenated nucleotide sequences. Branch length is measured in expected number of nucleotide substitutions per site. Bootstrap support was estimated on 1,000 replicate data sets, and only values greater than 500 are shown. The three subgroups of isolates designated A, B, and C are labeled and delineated by vertical bars.

TABLE 4 Analysis of molecular variance on 11 loci in *T. maritimum*[d]

Information type	% molecular variance		
	All[a]	Unique[b]	Subgroup B[c]
Host fish	33.924*	29.635*	27.396*
Country	18.272*	15.763*	17.731*
Yr	33.522*	21.561*	25.943*
Tissue	4.310*	10.415*	−1.850

[a] The whole collection of 73 *T. maritimum* isolates.
[b] The 59 unique isolates obtained by removing replicate genotypes with the same host fish, country, and year.
[c] The unique isolates belonging to subgroup B only.
[d] The fraction of the total variance explained by each individual type of information (host fish, country, year, and tissue) is reported for three sets of isolates. *, permutation-based π value of ≤0.05.

flect sampling biases and correlations between the three types of information, we also applied AMOVA after discarding 15 isolates, including identical genotypes from the same host fish species, year, and country. As expected, removing replicates decreased the fraction of variance explained by each of these three factors. By far, the most important decrease concerned the fraction of variance explained by the year that diminished by 35.7%, whereas the fraction explained by host and country diminished by only 12.6% and 13.7%, respectively. In parallel, removing replicates slightly raised the amount of variance explained by the tissue that reached 10.41%. Importantly, the divergence between the three subgroups of isolates (A, B, and C) was not responsible for these results, since globally similar estimates of the fraction of explained variance were obtained within subgroup B (Table 4), except for tissue that did not seem to correlate with genotype in this subgroup.

MLSA as a tool for monitoring *Tenacibaculum* infections worldwide. MLSA, also known as MLST when focused on a single species, currently is recognized as a reference method for the genotyping of isolates in many bacterial species. In particular, it proved useful to monitor the emergence and prevalence of different strains and to back-trace the contamination routes in a large number of pathogenic species (55). As the result of a balance between cost and resolving power, most MLST schemes rely on seven loci. Thus, for MLST to be effective, the sequences of a few loci have to provide enough information to discriminate a large number of STs. The pattern of nucleotide polymorphism reported in this study shows that this is indeed the case for *T. maritimum*. Our data also allow selecting the most informative loci for this genotyping purpose. We evaluated all of the combinations of loci and propose that future MLSA surveys of *Tenacibaculum* strains should use the 6 loci *atpA*, *dnaK*, *glyA*, *infB*, *rlmN*, and *tgt*, which allow distinguishing all 47 STs identified in the species *T. maritimum* based on the 11 loci, plus the *gyrB* locus, as this gene historically was used to define the genus *Tenacibaculum*. Of note, our *gyrB* sequence lies entirely within the 1,422 bp considered by Suzuki et al. (2). In the supplemental material, we provide additional versions of Fig. 2 and 3 based on the selected 7 loci (see Fig. S2 and S3). It can be seen that these 7 loci capture not only the whole diversity of STs in *T. maritimum* but also the important features of the phylogenetic trees, such as the division into three subgroups in the *T. maritimum* species and the existence of three clades plus four more distant lineages in the genus *Tenacibaculum*. The genotype data of the 114 isolates at these 7 loci has been deposited in a dedicated BIGSdb database (56) available at http://pubmlst.org/tenacibaculum/, which will be enriched progressively with new genotypes.

As a case study, we used the MLSA approach to characterize a number of suspected *Tenacibaculum* sp. isolates retrieved from marine fish. Our data set included strains isolated during recent outbreaks of tenacibaculosis in Norway (38) and Italy. Our MLSA data indicate that 18 Norwegian isolates actually represent *T. dicentrarchi* or *T. dicentrarchi*-like strains, while the two remaining strains were allocated to the genus *Polaribacter* (Fig. 2). The Italian isolates were identified as 5 *T. discolor* strains and 1 *T. mesophilum* strain.

DISCUSSION

Distribution and evolution of fish pathogenicity in the genus *Tenacibaculum*. In light of our MLSA data, the genus appears structured in distinct clades that cannot be observed from the tree reconstructed from the sequence of the 16S rRNA locus. More generally, the lack of resolution and the discrepancies found when analyzing trees based on the individual loci suggest that homologous recombination between species occurred and clearly argue for grounding evolutionary analyses on multilocus data.

The fish-pathogenic strains are distributed into several well-delineated clades, and fish-pathogenic lineages are intertwined with the lineages of strains isolated from other marine organisms and even from sediments. This observation strongly suggests parallel evolution of fish pathogenicity in several lineages of the genus *Tenacibaculum*. An alternative hypothesis is that fish pathogenicity is an ancestral characteristic, but this seems very unlikely given the number of lineages of isolates from a diversity of other sources that root deeply in the genus. Importantly, sampling biases most certainly contribute to the underrepresentation of environmental strains among the described *Tenacibaculum* species, which strengthens our line of reasoning.

Of note, two isolates collected from diseased fish (*Gadus morhua*) included in our collection (TNO016 and TNO017) were found to cluster with the *Polaribacter* representative in the phylogenetic reconstruction (Fig. 2). Therefore, it is tempting to speculate that virulent lineages infecting fish also have evolved in this sister genus, which is currently thought of as grouping with environmental, nonpathogenic bacteria (see reference 57 and references therein). Interestingly, our data also indicate that the *Polaribacter* clade is not clearly distinct from the *Tenacibaculum* clade. Indeed, trees reconstructed from concatenated loci (Fig. 2 shows 11 loci; see Fig. S2 in the supplemental material for 7-locus trees) and from loci taken separately (data not shown) often differ with respect to the position of *Polaribacter* species relative to *Tenacibaculum*. More systematic analyses using complete genome data may shed light on the relationships between the genera *Tenacibaculum* and *Polaribacter* and the genealogical discrepancies between loci.

In this context of parallel evolution of fish pathogenicity, it seems likely that the census of the pathogenic *Tenacibaculum* species is still incomplete. New pathogenic species probably will be described in the future and may be recognized as being responsible for economically important problems as marine aquaculture grows and involves a greater variety of cultured organisms. As an illustration, one of the strains from Italy (isolate 75) that we identified as *T. mesophilum* was retrieved from the kidney of a sea

bream (*Sparus aurata*), suggesting that this bacterial species infects fish, although the type strain was isolated from a sponge.

Genetic diversity of *T. maritimum* colonizing aquaculture systems and comparison to other fish-pathogenic bacteria. Our analysis examined polymorphism within 73 isolates of the fish-pathogenic species *T. maritimum*. With average pairwise nucleotide diversity (π) estimated to be 0.44% and an *r*/*m* ratio estimated to be 2.7 (95% credibility, 1.7 to 4.0), the species can be described as exhibiting moderate levels of nucleotide diversity and recombination (58). Despite recombination, we showed that our collection of *T. maritimum* strains is composed of three subgroups. We also found a statistically significant association between the genotypes and the background information on the isolation source (host fish, year, and geographical origin), but that could account for only a limited amount of the total genetic variance.

After *Flavobacterium psychrophilum* (59), *Yersinia ruckeri* (60), and *Renibacterium salmoninarum* (61), *T. maritimum* becomes the fourth species of fish-pathogenic bacteria for which sequence data are available for a significant number of strains. The niche of *T. maritimum* differs from that of the other three species, as it is the only marine bacterium with broad host range. The three other species have been reported as primarily infecting salmonids, and only *Renibacterium salmoninarum* is regularly isolated from marine fish. Out of the four species, *T. maritimum* is also the one whose attacks have the most marked localization toward fish body surfaces. It is worth attempting a comparison of the patterns of polymorphism and population structures, but we need to have in mind the limited number of loci and the differences between strain sampling schemes.

It is interesting that despite its broader host range and worldwide geographical distribution, *T. maritimum* exhibits a level of diversity comparable to that of *F. psychrophilum* (~0.4%) and *Y. ruckeri* (~0.7%), suggesting that population sizes are of the same order of magnitude, although other factors, such as mutation rate and selective sweeps, also can contribute to shape the level of nucleotide diversity. However, nucleotide diversity is much lower in *R. salmoninarium* (<0.08%). In terms of per-nucleotide *r*/*m* ratio, the rate of recombination in *T. maritimum* may be slightly lower than that in *Y. ruckeri* (~7:1) and is clearly lower than that in the highly recombinogenic bacterium *F. psychrophilum* (~26:1) (see references 58 and 59 for even higher estimates). In contrast, a near absence of recombination was reported for *R. salmoninarum* (61).

The features by which the *T. maritimum* data really stand out are the small number of representatives collected for each ST, the lack of large clonal complexes, and the absence of any trace of transcontinental dissemination. This situation is in sharp contrast to that in *F. psychrophilum* (59, 62, 63), *R. salmoninarium* (61), and *Y. ruckeri* (60), for which the sequence data unambiguously revealed transcontinental dissemination linked to the international trade of broodfish and eggs. Furthermore, as a result of preferential dissemination routes or of adaptive niche specificity, the large clonal complexes detected in *F. psychrophilum* tended to be strongly associated with particular host fish species (59, 62, 64).

Taken together, the population structure described here for *T. maritimum* strongly suggests the endemic colonization of fish farms by local strains with little or no contribution of long-distance contamination linked to fish movements. As most of the marine fish farmers usually buy fry from geographically distant hatcheries, this population structure was not necessarily anticipated. Furthermore, our data indicate that the same ST often is found to infect multiple species of host fish in the same geographical area, which points to the possibility of cross-species contaminations in fish farms by the same bacterial lineage. Interestingly, our results on *T. maritimum* population structure echo the empirical observations that environmental conditions and fish health status are major factors for tenacibaculosis outbreaks (4). Indeed, outbreaks often might correspond to new contaminations from the local environment when conditions are favorable to the pathogen.

ACKNOWLEDGMENTS

We thank the individuals who kindly provided the *Tenacibaculum* isolates included in this study (see Table S2 in the supplemental material). We thank Tatiana Vallaeys for critical reading of the manuscript. We also are grateful to Keith Jolley at the University of Oxford for hosting the *Tenacibaculum* MLSA website, with funding from the Wellcome Trust.

C.H., A.H., A.L., J.-F.B., P.N., and E.D. received support from AIP INRA Bio-Resources 2010 (*Tenacibaculum* genomics), FUI-11 (PathotrackFish), and EU EMIDA ERA-NET (ANR 2010-EMID-006-01 Pathofish); A.B.O. and H.N. received support from the EU EMIDA ERA-NET (RCN 202834/E40 PathoFish) project; and A.E.T. and N.C. received support from the European Project Maximus (FP7-SME-2011-286200).

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