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Genotyping of *Tenacibaculum maritimum* isolates from farmed Atlantic salmon in Western Canada

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Abstract

Mouthrot infections (bacterial stomatitis) have a significant impact on the Atlantic salmon aquaculture industry in Western Canada due to economic losses and fish welfare. Bacteria isolated from lesions in the field have been identified as *Tenacibaculum maritimum*. Mouthrot is different to classical tenacibaculosis, which is most commonly associated with ulcerative lesions, frayed fins and tail rot. The marine fish pathogen *T. maritimum* is found worldwide; however, in Western Canada, the knowledge of the genetic profile of *T. maritimum* is limited. This study looked at increasing this knowledge by genotyping *T. maritimum* isolates collected from Atlantic salmon from farms in Western Canada. These genotypes were compared to other species of the genus *Tenacibaculum*, as well as other known sequence types within the species. The Western Canadian isolates belong to two new sequence types within the *T. maritimum* species. Phylogenetic analysis shows that the isolates form a distinct branch together with *T. maritimum* NCIMB 2154^T separate from other *Tenacibaculum* type strains, and they are most closely related to strains from Norway and Chile.

KEYWORDS
mouthrot, multilocus sequence analysis, phylogenetic, *Salmo salar*

1 | INTRODUCTION

Tenacibaculum maritimum is a marine Gram-negative bacterium in the family Flavobacteriaceae (Suzuki, Nakagawa, Harayama, & Yamamoto, 2001; Wakabayashi, Hikida, & Masumura, 1986). It is a pathogen found worldwide causing infections in a wide variety of farmed marine fish. The disease, tenacibaculosis, is described as causing mouth erosion, ulcerative skin lesions, frayed fins and tail rot (Toranzo, Magariños, & Romalde, 2005). However, in British Columbia (BC), Canada, the bacterium is most commonly associated with yellow plaques in the mouth (mouthrot) and is a significant issue for Atlantic salmon, *Salmo salar* (L.), smolts newly transferred into saltwater (Ostland, Morrison, & Ferguson, 1999). Mouthrot outbreaks are associated with major economic losses in the Western Canadian aquaculture industry and cause significant fish welfare problems (Hewison & Ness, 2015). There are also reports of mouthrot affecting farmed Atlantic salmon in Washington State (Frelier, Elston, Loy, & Mincher, 1994).

On the west coast of North America, the bacterium has also been isolated in California from white seabass, *Atractoscion nobilis* (Ayres), northern anchovy, *Engraulis mordax* (Girard), Pacific sardine, *Sardinops sagax* (Jenyns) and farmed Chinook salmon, *Oncorhynchus tschawytscha* (Walbaum) (Chen, Henry-Ford, & Groff, 1995), and in these fish species, the pathogen has been associated with gill lesions and skin ulceration. Mouthrot has been recorded since the 1990s in BC, however little research has been conducted into the disease and

the role of *T. maritimum* as a causative agent. There are no publications confirming, through genetic identification, that the bacteria isolated from mouthrot lesions are *T. maritimum*.

Molecular typing methods such as multilocus sequence typing (MLST) have allowed the development of uniform and reproducible nomenclature schemes for bacterial pathogens (Maiden, 2006). MLST characterizes prokaryotes within a species using the allelic mismatches of a small number of housekeeping (HK) genes (Gevers et al., 2005) and allows researchers to perform epidemiological studies and define strains within named species (Gevers et al., 2005). Because MLST schemes are based on nucleotide sequences, they are intrinsically reproducible and portable between laboratories (Maiden, 2006). The sequence data from MLST can be used for phylogenetic analyses, called multilocus sequence analysis (MLSA) and defined as the sequence analysis of multiple protein-coding genes for the genotypic characterization of a diverse group of prokaryotes including entire genera (Gevers et al., 2005). The usual approach to MLSA is to concatenate the sequences of several housekeeping genes and then use these to assess clustering patterns. MLSA is the current method of choice to explore phylogenetic relationships at the genera and species levels (Glaeser & Kämpfer, 2015). Habib et al. (2014) used MLST and MLSA to describe 47 sequence types (STs) of 73 *T. maritimum* strains from a variety of host species worldwide. The genetic data from their study were uploaded into the *Tenacibaculum* MLST database (http://pubmlst.org/tenacibaculum/) and allow others to use and add to this work as was suggested by (Maiden, 2006).

Although a lot of genetic research has been completed on *T. maritimum* strains from European, Asian and Australian waters (Habib et al., 2014), nothing is known about the genetic profile of *T. maritimum* from Western Canada. This study provides this knowledge and adds to the genotyping of *T. maritimum* worldwide.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolation

The *T. maritimum* isolates included in this study were collected from Atlantic salmon smolts showing clinical signs of mouthrot (yellow plaques in the mouth) from saltwater farms in BC, Canada from 2011 to 2016 as shown in Figure 1. A marine agar (Difco 2216) was modified to include 50 μg/ml kanamycin (MKA) to improve the recovery of *T. maritimum* from primary cultures. MKA was used for the primary isolation from diseased fish, and the plates were incubated for a minimum of 5 days at 16°C. Cultures that matched the phenotypic description of the *Tenacibaculum* genus (Suzuki et al., 2001) were subcultured on marine agar (MA) and incubated at 16°C.

2.2 | PCR and sequencing

Genomic DNA was extracted from isolates of interest using an E.Z.N.A Tissue DNA Kit (Omega Bio-tek). A PCR was performed using the 16S rRNA primers 27F and 1518R (Giovannoni, Rappé, Vergin, & Adair, 1996). Amplification was based on a standard reaction mixture containing 2.5 μl Extra buffer, 1.25 mM deoxyribonucleotide triphosphates, 0.75 units (0.15 μl) Taq DNA polymerase (VWR), 5 μM (1 μl) of forward and reverse primers, and then DNase-RNase free water was added to a final volume of 25 μl (16.85 μl H_2O). The amplification was performed at 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 90 s, followed by 72°C for 10 min in a Veriti Thermal Cycler (Thermo Fisher Scientific). The PCR product was confirmed using gel electrophoresis and then enzymatically purified using ExoSAP-IT PCR Product Cleanup (Thermo Fisher Scientific) in a Veriti Thermal Cycler at 37°C for 15 min then heated to 80°C for 15 min. Sequencing was performed on PCR products using both the forward and reverse PCR primers. Analyses of the sequences were carried out by the Sequencing Facility at the University of Bergen (http://www.uib.no/seqlab) using big dye termination chemistry. Vector NTI (Invitrogen) software suite was used to assemble and align the obtained sequences. A BLAST search was performed to determine the identity of each isolate. Pure cultures of *T. maritimum* were cryopreserved at −80°C.

PCRs were performed on the *T. maritimum* isolates using primers designed by Habib et al. (2014) for 11 HK genes. Amplifications were performed at 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C (50°C for primers: *glyA*, *infB*, *tgt*, *tuf* and *yqfO*) for 30 s and 72°C for 1 min, followed by 72°C for 10 min using the same reaction mixture and machine as above. The PCR products were confirmed and sequenced as above.

2.3 | Genetic analysis

To determine whether the Canadian *T. maritimum* isolates from this study belonged to a known ST of *T. maritimum*, the MLST profiles that consisted of seven HK gene sequences (*atpA*, *gyrB*, *dnaK*, *glyA*, *infB*, *rlmN* and *tgt*) were uploaded and analysed in the *Tenacibaculum* MLST database. The Canadian isolates found to be genetically identical were assigned one unique ST name (e.g., STCan1, STCan2) and a unique strain name (e.g., TmarCan1, TmarCan2) for the phylogenetic analyses.

In this study, one 16S rRNA and two concatenated HK gene sequence alignments were constructed for phylogenetic analysis. All alignments were constructed in AlignX in Vector NTI before sequences were adjusted to equal length and correct reading frames in GeneDoc (Nicholas, Nicholas, & Deerfield, 1997). The 16S rRNA gene sequence alignment included the Canadian *T. maritimum* strains from this study and sequences from all known type strains in genus *Tenacibaculum*. The length of the 16S rRNA gene alignment was 1351 bp. Concatenation of the HK gene alignments was performed using Kakusan4 (Tanabe, 2011). The length of the concatenated HK gene alignment was 5811 bp (*atpA* 1-567, *dnaK* 568-1140, *glyA* 1141-1698, *gyrB* 1699-2295, *ileS* 2299-2841, *infB* 2842-3405, *rlmN* 3406-3954, *tgt* 3955-4440, *trpB* 4441-4809, *tuf* 4810-5364 and *yqfO* 5365-5811). The first HK gene sequence alignment included concatenated sequences of the Canadian strains found in this study and 19 type strains in genus *Tenacibaculum*. The second HK gene sequence alignment included concatenated sequences of the Canadian strains found

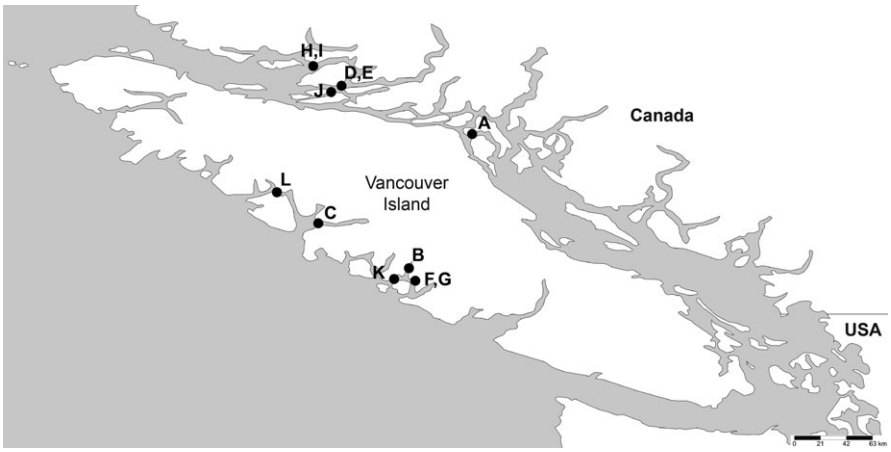

FIGURE 1 Location of the origin of each isolate listed in Table 1

TABLE 1 Canadian *Tenacibaculum maritimum* isolates used in this study

Isolate	Map reference (Figure 1)	Sampling date	Strain (sequence type)	Seawater temperature (°C)
TmarCan11-1	A	Jun. 2011	TmarCan1 (STCan1)	8.9
TmarCan15-1	B	Sep. 2015	TmarCan2 (STCan2)	14.7
TmarCan16-1	C	Feb. 2016	TmarCan1 (STCan1)	8.7
TmarCan16-2	D	Apr. 2016	TmarCan1 (STCan1)	9.0
TmarCan16-3	E	Apr. 2016	TmarCan1 (STCan1)	9.0
TmarCan16-4	F	Apr. 2016	TmarCan1 (STCan1)	12.0
TmarCan16-5	G	Apr. 2016	TmarCan2 (STCan2)	12.0
TmarCan16-6	H	May 2016	TmarCan1 (STCan1)	11.3
TmarCan16-7	I	May 2016	TmarCan1 (STCan1)	11.3
TmarCan16-8	J	May 2006	TmarCan2 (STCan2)	10.7
TmarCan16-9	K	Jun. 2016	TmarCan1 (STCan1)	11.1
TmarCan16-10	L	Oct. 2016	TmarCan2 (STCan2)	12.1

in this study (Table 1), the Norwegian *T. maritimum* strain NLF-15 isolated from lumpsuckers, *Cyclopterus lumpus* (L.) (Småge, Frisch, Brevik, Watanabe, & Nylund, 2016), the Chilean *T. maritimum* strain Ch-2402 isolated from Atlantic salmon (Apablaza et al., 2017), as well as a single strain from each of the 47 *T. maritimum* STs (identified by their ST number) described in Habib et al. (2014).

All gene sequences, except the isolates from this study and the *Tenacibaculum finnmarkense*^{T} HK genes sequences, were obtained from GenBank (Table S1). The *T. finnmarkense*^{T} HK gene sequences were obtained as described for the Canadian *T. maritimum* isolates.

For the 16S rRNA gene data set, the best fitted evolutionary model was calculated using Mega 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The BEAST package v1.8 (Drummond, Suchard, Xie, & Rambaut, 2012) was used for Bayesian analysis using the K2 + G + I model, a relaxed lognormal molecular clock and a mcmc of 100,000,000 generations. *Kordia algicida*^{T} was used as the outgroup.

For the MLSA of the two concatenated HK alignments, Kakusan4 was used to calculate the substitution rate and best fit model for the individual loci and codon positions for the Bayesian analysis performed in MrBayes (Ronquist et al., 2012) using the data block with the proportional codon proportional model from Kakusan4 and a mcmc of 100,000,000 generations. The effective sample size (ESS) values in the Bayesian analysis were inspected using Tracer ver. 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). Due to the size of the output file of the *T. maritimum* HK analysis, the sump command in MrBayes was used to summarize the ESS values.

A maximum clade credibility tree was obtained for each analysis using a 10% burn-in in Tree-Annotator and viewed using FigTree (Drummond et al., 2012). Posterior probability values above 95% were regarded as accurate (Huelsenbeck & Rannala, 2004). All sequences obtained in the current study are available in GenBank with accession numbers KY428880 to KY428914 and MF421902 to MF422021.

3 | RESULTS

The MKA media supported growth for all Canadian isolates from this study, as well as the Norwegian *T. maritimum* strain NLF-15, the Chilean *T. maritimum* strain Ch-2402 and *T. maritimum*^{T}. Field experience generated during the isolation of the Canadian *T. maritimum* showed that MKA improved the success of isolating the bacterium when compared to MA. The added kanamycin decreased the amount of overgrowth by other bacteria.

The Western Canadian isolates collected in this study were found to belong to two distinct ST (STCan1 and STCan2) based on the *Tenacibaculum* MLST database (Table 1). This was supported by the fact that the alignment of the 11 HK gene sequences for the MLSA showed only two distinct genetic strains. The two strains were isolated on both the east and west coast of Vancouver Island, and for one outbreak, they co-occurred (isolates F and G) (Figure 1). The results of the 16S rRNA phylogenetic analysis (Figure 2) show that the *T. maritimum* isolates found in Western Canada form a distinct branch together with *T. maritimum*^{T} separate from other *Tenacibaculum* type strains. This is further supported by the *Tenacibaculum* HK phylogenetic analysis (Figure 3). Figure 4 shows that the *T. maritimum* strains found in this study form a branch with strains NLF-15 and Ch-2402.

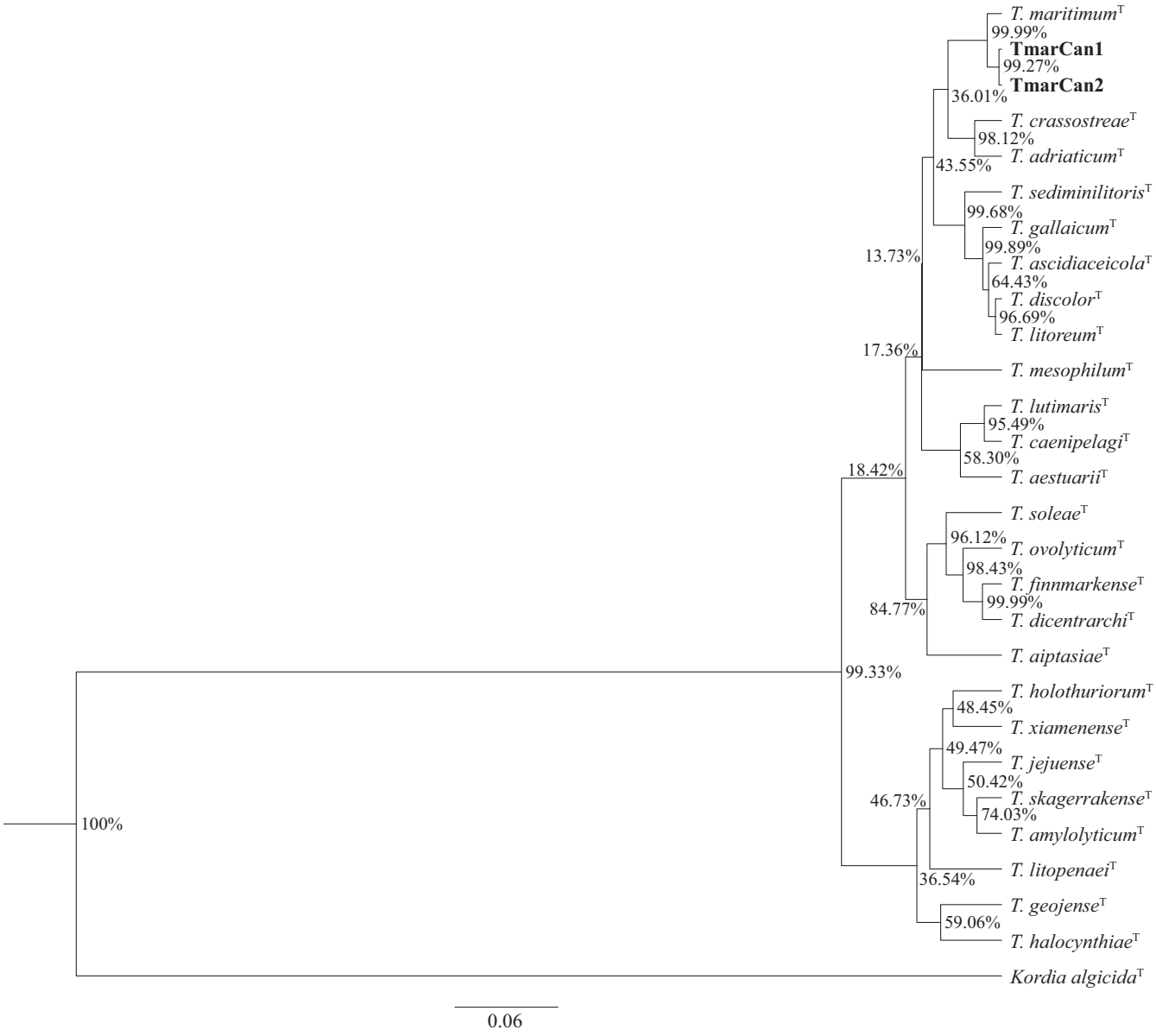

FIGURE 2 The 16S rRNA phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains and all type strains in genus *Tenacibaculum*. *Kordia algicida*^{T} was used as an outgroup. The Bayesian analysis was performed on 1351 bp using the K2 + G + I model. The posterior probability is presented next to each node. Scale bar = 0.06 substitutions per site

4 | DISCUSSION

The use of MKA improved the success of isolating *T. maritimum* from the field when compared to only using MA. MKA inhibited the growth of other faster growing bacteria that routinely outcompete *T. maritimum* on primary cultures from skin or ulcers grown on MA. The idea of adding kanamycin to the growth medium was based on previous work with Flavobacteria that showed that adding kanamycin to growth media, such as TYS or SYL agar, allowed for selective isolation of certain genera due to growth inhibition of others (Hahnke & Harder, 2013; Rahman, Suga, Kanai, & Sugihara, 2014).

The *T. maritimum* isolates described in this study belonged to two distinct STs (STCan1 and STCan2) different from all other previously published STs (Habib et al., 2014), and the MLSA shows that the Western Canadian strains are most closely related with strain NLF-15 isolated from lumpsuckers in Norway (Småge et al., 2016) and strain Ch-2402 isolated from Atlantic salmon in Chile (Apablaza et al., 2017). These four strains are grouped together in subgroup C (designated C in Habib et al. (2014)) with three strains from Spain (salmonids and turbot, *Scophthalmus maximus*, L.) and two from Tasmania (Atlantic salmon and striped trumpeter, *Latris lineata*, Forster). Strains NLF-15 and Ch-2402 were isolated from fish reared in waters of approximately 12°C and 14 °C, respectively (Apablaza et al., 2017; Småge et al., 2016), which is in the same range as the Canadian isolates (Table 1). Temperatures from the other strains in subgroup C are unknown to the authors of this study, but thought

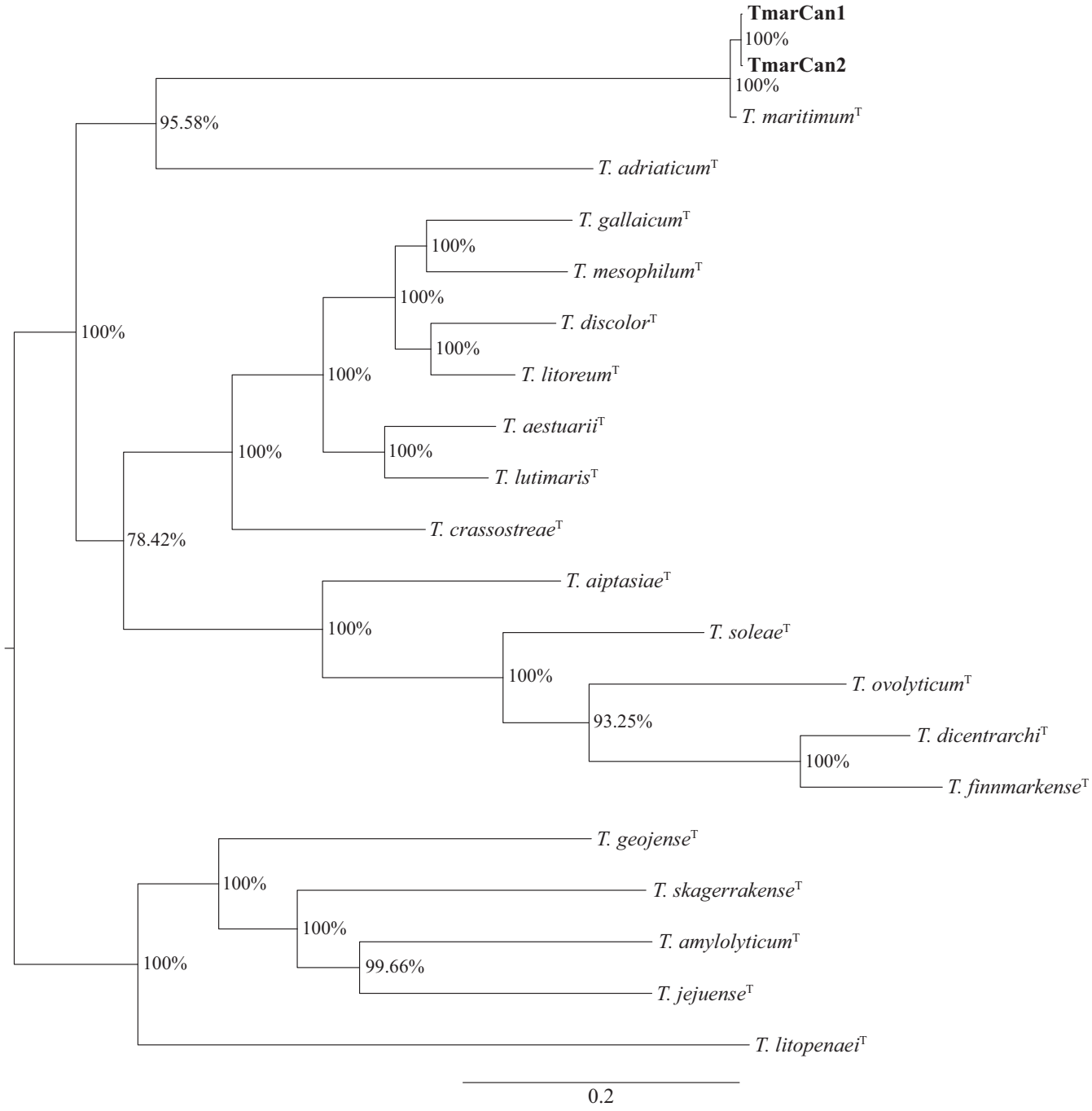

FIGURE 3 The phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains and 19 type strains in genus *Tenacibaculum* based on the concatenated HK gene sequences. The Bayesian analysis was performed on 5811 bp. The posterior probability is presented next to each node. Scale bar = 0.2 substitutions per site

to be in the same temperature range (9–16°C), which is in the lower range of *T. maritimum* growth (Suzuki et al., 2001). This suggests that there may be a temperature distribution, which would fit with the geographic relationship suggested by Habib et al. (2014). Increased data regarding the host environment of the other strains in subgroup C are required to test this hypothesis. The isolation of different *T. maritimum* strains from the same mouthrot outbreak at the same site is likely due to the bacterium being present in the marine environment.

Habib et al. (2014) found that the relative positions of the strains in subgroup B seemed correlated with fish host and geographic origin. Based on support values in the MLSA, the authors of this study decided to divide subgroup B into two (Figure 4), one of which consists solely of strains from Japanese waters (B1) including the type strain, which would fit with the proposed geographic distribution. A possible hypothesis as to the global distribution of closely related strains is the movement of organisms between locations; for example, the transference of ballast waters

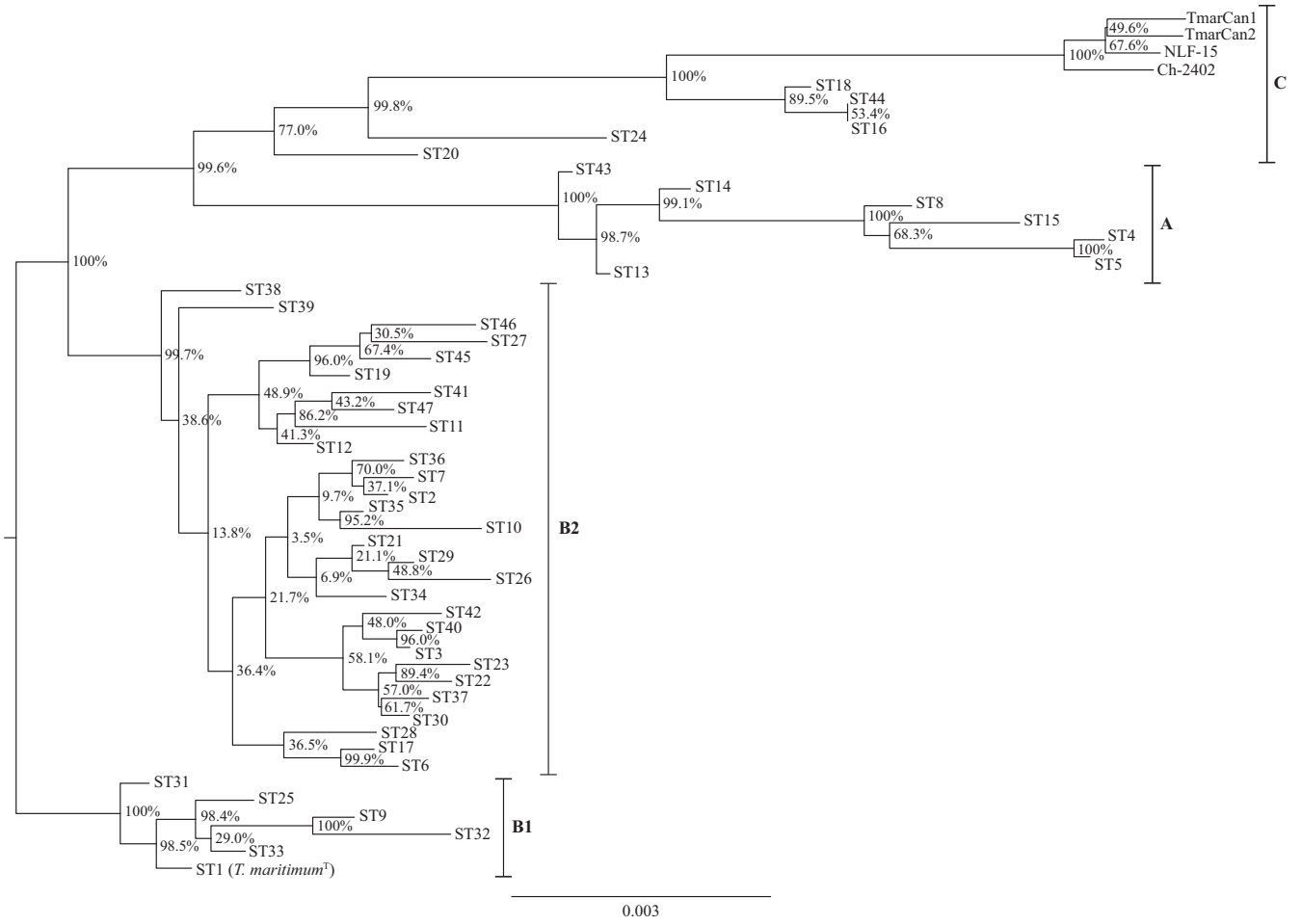

FIGURE 4 The phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains, the Norwegian *T. maritimum* strain NLF-15 (Småge et al., 2016), the Chilean *T. maritimum* strain Ch-2402 (Apablaza et al., 2017), as well as a single strain from each of the 47 *T. maritimum* STs (identified by their ST number) (Habib et al., 2014) based on the concatenated HK gene sequences. The Bayesian analysis was performed on 5811 bp. The posterior probability is presented next to each node. Scale bar = 0.003 substitutions per site

when ships travel from one region to another. A wide range of bacteria have been shown to be present in these waters including *Tenacbaculum* spp. and more specifically *T. maritimum* (Brinkmeyer, 2016).

Other information, not looked at by Habib et al. (2014), such as environmental conditions (e.g., temperature, salinity), clinical signs and tissue tropism of the different *T. maritimum* strains would enable further investigations into the pathogenic differences seen worldwide, even within one host species. This variation is particularly noticeable in Atlantic salmon, where typical tenacibaculosis infections have been described in Tasmania, Australia (Handlinger, Soltani, & Percival, 1997) and Spain (Toranzo, 2015), whereas the infections seen in Western Canada present as mouthrot (small yellow plaques in the mouth) (Ostland et al., 1999). Understanding the pathogenic differences between strains could allow for better management of the disease, and studies of genetic variation, as presented in this paper, may provide the needed genetic markers.

The strain most closely related to the two Canadian ones is the one found in lumpsuckers in Norway. This finding should be a concern to the Norwegian Atlantic salmon aquaculture industry as the use of lumpsuckers as biological lice controls has increased in recent years (Bornø et al., 2016). However, the average rearing water temperatures for Atlantic salmon in Norway are lower than the ones seen in Western Canada, which may decrease the risk of the development of mouthrot or *T. maritimum* tenacibaculosis.

Most of the research conducted on *T. maritimum* has been on warmer water (more than 16°C) strains. The authors suggest that more research is needed on strains from Atlantic salmon in colder regions. Furthering the knowledge is essential for the development of diagnostic tools for the pathogen such as real-time PCR and preventative measures against the disease such as vaccine development.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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