

Nation-wide and local diversity of the fish pathogen *Flavobacterium psychrophilum* in Japan revealed by MLST analysis

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Abstract

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome in salmonid fish. In Europe and in North and South America, *F. psychrophilum* usually affects salmonid hosts such as rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*). However, in Japan, ayu (*Plecoglossus altivelis altivelis*) are more frequently affected by BCWD than other fish species. *F. psychrophilum* was isolated for the first time from cultured ayu in Japan in 1987. At present, BCWD occurs not only in aquaculture ponds but also in natural environments in Japan [1–2].

In a previous study, multilocus sequence typing (MLST) was applied to a set of 50 *F. psychrophilum* isolates retrieved from 11 different fish species in 12 different countries all over the world [3]. Thirty-three different sequence types (STs) were identified, some of which were associated with the host fish species, in particular with rainbow trout and coho salmon [3]. The results demonstrated the preponderant role of recombination in genetic diversification whereas the nucleotide diversity as measured in pairwise sequence comparison was limited within the species (approximately 4 differences per Kbp), but the small number of isolates originating from some geographical areas (27 European isolates out of fifty) did not allow the description of population structure at a regional scale.

To investigate the population structure of *F. psychrophilum* in Japan both at the national and local (a model river in Shiga prefecture) scale, 113 bacterial isolates were collected and subjected to MLST analysis. To account for the nation-wide diversity, we genotyped a first set of 33 strains which were isolated between 1993–2005 and provided by different contributors from a variety of locations all over Japan. A second set of 80 isolates were all obtained from the river Chinai (total length, approximately 9.5 km) in Shiga prefecture, which flows into Lake Biwa, the largest lake in Japan. From August 2005 to December 2006, the samples were collected from the downstream area, approximately 0.3 km from the mouth of the river.

Fish from the river Chinai were euthanized by an electric shock and harvested by a fishnet. Bacteriological samples were taken from the gills, internal organs, and possible skin lesions, cultivated on modified cytophaga (MCYT) agar plates and incubated at 15°C for 4 days. Yellow

colonies were subcultivated and identified as *F. psychrophilum* based on the amplification of the 16S rRNA and *gyrB* genes by nested-PCR [4] and PCR [5–6], respectively, according to the guidelines of the council for the control of BCWD in ayu (Japanese Ministry of Agriculture, Forestry and Fisheries). *F. psychrophilum* strains were then inoculated in MCYT broth and incubated on an orbital shaker (140 rpm) for 2 days at 15°C. The genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega) and seven housekeeping genes (*trpB*, *gyrB*, *dnaK*, *fumC*, *murG*, *tuf* and *atpA*) were amplified by PCR. The PCR products were then sequenced and used for MLST analysis.

At the national scale, most Japanese *F. psychrophilum* isolates showed STs different from those previously identified in the U.S and Europe [3]. The Japanese isolates were genetically highly diverse and a clear association between ST and host species was noticed. The STs could be grouped into a rainbow trout group, a coho salmon group, a carp group and three ayu groups. Each group represented one clonal complex (CC; cluster of related STs), with the exception of the carp group and one of the ayu groups that belonged to the same CC. The strains in the three ayu groups showed different virulence levels against ayu in bath infection challenges in our previous study, and the strains in CCs ayu-2 and ayu-3 tended to show higher virulence than those in CC ayu-1 [7]. At the local scale, *F. psychrophilum* strains isolated from various fish hosts and from water of the model river also displayed a high degree of genetic diversity. Co-infections (i.e. infection of the same individual fish by strains belonging to different STs) were also observed.

In conclusion, the CCs were associated with defined host fish species. A large genetic diversity was observed among the Japanese strains, and there was no evidence to suspect that *F. psychrophilum* strains infecting ayu in Japan originated from the U.S or Europe.

References

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