

Morganella psychrotolerans sp. nov., a histamine-producing bacterium isolated from various seafoods

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Mesophilic *Morganella morganii* ($n=6$) and psychrotolerant *M. morganii*-like isolates from various seafoods ($n=13$), as well as clinical *M. morganii* isolates ($n=3$), were characterized by using a polyphasic approach including multi-locus sequencing. Based on the phylogenetic analysis, the 22 strains were divided into two distinct groups comprising mesophilic and psychrotolerant isolates, respectively. This classification was supported by DNA–DNA hybridization studies, whereby a psychrotolerant isolate (strain U2/3^T) showed 41.0 and 17.8% relatedness to the type strains of the mesophilic species *Morganella morganii* subsp. *morganii* (strain LMG 7874^T) and *Morganella morganii* subsp. *sibonii* (strain DSM 14850^T), respectively. Analysis of the 16S rRNA gene sequences showed a similarity of 98.6% between mesophilic and psychrotolerant isolates. However, fragments of seven protein-encoding housekeeping genes (*atpD*, *dnaN*, *gyrB*, *hdc*, *infB*, *rpoB* and *tuf*) all showed less than 90.9% sequence similarity between the two groups. The psychrotolerant isolates grew at 0–2 °C and also differed from the mesophilic *M. morganii* isolates with respect to growth at 37 °C and in 8.5% (w/v) NaCl and fermentation of D-galactose. The psychrotolerant strains appear to represent a novel species, for which the name *Morganella psychrotolerans* sp. nov. is proposed. The type strain is U2/3^T (=LMG 23374^T=DSM 17886^T).

Morganella was described by Fulton (1943) and in 1978 it was established as a genus within the *Enterobacteriaceae* (Brenner *et al.*, 1978). Strains previously known as *Proteus morganii* now belong to the genus *Morganella*; the two species have the same type strain, with *Morganella morganii* having priority. The genus *Morganella* comprises a single species (*Morganella morganii*) that includes two subspecies (*Morganella morganii* subsp. *morganii* and *Morganella morganii* subsp. *sibonii*), which can be distinguished from one another on the basis of trehalose fermentation (Jensen *et al.*, 1992). In addition, *M. morganii* isolates have been divided into biogroups depending on glycerol fermentation, lysine- and ornithine-decarboxylase activities and tetracycline sensitivity (Jensen *et al.*, 1992; Janda & Abbott, 2005). *M. morganii* is often found

in the intestines of humans and animals. It grows between 4 and 45 °C and has the ability to produce toxic concentrations of histamine in seafood, but only at storage temperatures above 7–10 °C (Lehane & Olley, 2000; Janda & Abbott, 2005). Recently, psychrotolerant *M. morganii*-like bacteria have been isolated from fresh tuna, cold-smoked tuna and garfish. The psychrotolerant isolates produce toxic concentrations of histamine at 0–5 °C and appear to be important in histamine formation in chilled seafood. In fact, these strains have been isolated from seafood implicated in incidents of histamine fish poisoning (Emborg *et al.*, 2005; Emborg & Dalgaard, 2006; Dalgaard *et al.*, 2006). The present study includes a polyphasic characterization with multi-locus sequencing of psychrotolerant *Morganella* isolates.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *atpD*, *dnaN*, *gyrB*, *hdc*, *infB*, *rpoB* and *tuf* gene fragments determined in this study are given in Fig. 1 and Supplementary Figs S1–S7.

Neighbour-joining trees based on *atpD*, *dnaN*, *gyrB*, *hdc*, *infB*, *rpoB* and *tuf* genes and tables detailing primer sequences and annealing temperatures used in PCR and whole-cell fatty acid content and characteristics of isolates of *Morganella psychrotolerans* sp. nov. and *Morganella morganii* are available as supplementary material in IJSEM Online.

Nine mesophilic *M. morganii* and 13 psychrotolerant *M. morganii*-like isolates were studied including *M. morganii* subsp. *morganii* LMG 7874^T and *M. morganii* subsp. *sibonii* DSM 14850^T (Table 1). As shown in Table 1, the isolates originated from different geographical locations, date of isolation and habitats, including different seafoods. Prior to sequence analysis all strains were cultured overnight at 25 °C on blood agar and prior to phenotypical testing on Long and Hammer agar (van Spreekens, 1974) with 1% NaCl.

Table 1. Source and geographical origin of strains used in this study

Isolate	Source	Geographical origin	Year	Reference
<i>Morganella morganii</i> subsp. <i>morganii</i>*				
25a32	Tuna	Japan	2000/2001	Takahashi <i>et al.</i> (2003)
M04090	Clinical isolate	Belgium	–†	Wauters <i>et al.</i> (2004)
LMG 7874 ^T	Human stool	England	1920	Winslow <i>et al.</i> (1919)
U6/1	Tuna	–	2004	This study
<i>Morganella morganii</i> subsp. <i>sibonii</i>				
NCIMB 865‡	Spoiled fish	Japan	–	Kimata & Kawai (1953), Kimata <i>et al.</i> (1958)
DSM 14850 ^T	Human sputum	Denmark	–	Jensen <i>et al.</i> (1992)
AP28	Yellowtail	Japan	2000/2001	Takahashi <i>et al.</i> (2003)
03A11	Smoked fish	New Zealand	1996	Fletcher <i>et al.</i> (1998)
03B10	Smoked fish	New Zealand	1996	Fletcher <i>et al.</i> (1998)
<i>Morganella psychrotolerans</i> sp. nov.				
F39-1	Lumpfish roe	Canada/Denmark	1998	Basby <i>et al.</i> (1998)
F39-3	Lumpfish roe	Canada/Denmark	1998	Basby <i>et al.</i> (1998)
JB-T11	Fresh vacuum-packed tuna	Sri Lanka	2000	Emborg <i>et al.</i> (2005)
JB-T12	Fresh vacuum-packed tuna	Sri Lanka	2000	Emborg <i>et al.</i> (2005)
JB-T16	Fresh vacuum-packed tuna	Sri Lanka	2000	Emborg <i>et al.</i> (2005)
U2/3 ^T	Cold-smoked tuna	–	2004	Emborg & Dalgaard (2006)
U2/4	Cold-smoked tuna	–	2004	Emborg & Dalgaard (2006)
U2/5	Cold-smoked tuna	–	2004	Emborg & Dalgaard (2006)
U2/6	Cold-smoked tuna	–	2004	Emborg & Dalgaard (2006)
U2/7	Cold-smoked tuna	–	2004	Emborg & Dalgaard (2006)
FD24	Fresh vacuum-packed tuna	Indonesia	2003	Emborg <i>et al.</i> (2005)
1F10	Garfish	Denmark	2003	Dalgaard <i>et al.</i> (2006)
2F6	Garfish	Denmark	2004	Dalgaard <i>et al.</i> (2006)

*The strains are divided into subspecies according to their fermentation of trehalose, where *M. morganii* subsp. *morganii* is negative.

†Unknown.

‡Deposited as *Providencia rettgeri*, but identified as *Morganella morganii*.

A partial sequence of the 16S rRNA gene (1356 bp) and fragments from seven protein-encoding housekeeping genes: *atpD* (β -subunit of ATP synthase; 509 bp), *dnaN* (DNA polymerase III β -subunit; 582 bp), *gyrB* (DNA gyrase; 443 bp), *hdc* (histidine decarboxylase; 576 bp), *infB* (translational initiation factor; 467 bp), *rpoB* (β -subunit of DNA-dependent RNA polymerase; 454 bp) and *tuf* (elongation factor; 526 bp), were selected for use in classification and identification of the isolates. Gene fragments were amplified by PCR from chromosomal DNA, with denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 1 min at the appropriate annealing temperature, 72 °C for 1.5 min and finally an extension step at 72 °C for 10 min, in a reaction volume of 50 μ l. The primers used were designed for the present study, except the *hdc* and *dnaN* primers, which were used previously by Takahashi *et al.* (2003) and Torpdahl *et al.* (2005), respectively. Primer sequences and annealing temperatures are given in Supplementary Table S1 in ISJEM Online. Amplified gene fragments were purified using QIAquick spin columns (Qiagen) and sequenced on an ABI377 automatic sequencer using a Prism BigDye Terminator Cycle sequencing kit (Applied Biosystems). The 16S rRNA gene was sequenced by using standard

methods, as described by L'Abée-Lund *et al.* (2003). DNA base composition (G + C content) and DNA–DNA hybridization studies using strains U2/3^T, LMG 7874^T, DSM 14850^T, FD24, JB-T16, 03A11 and 1F10 were performed at Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany. DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) and Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 \times 6 multi-cell changer and a temperature controller with an *in situ* temperature probe (Varian). Alignments and phylogenetic analysis were made using the software MEGA version 3.1 (Kumar *et al.*, 2004). Distances and clustering were determined using parsimony maximum and neighbour-joining methods. Distance matrices were calculated by using Kimura's two-parameter distances (Gascuel, 1997). Reliability of topologies was assessed by bootstrap analysis with 1000 replicates. Reference 16S rRNA gene sequences for *Proteus* and *Providencia* species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>).

Phenotypic characterization of the strains was conducted by using commercially available kits: API 20E and API 50 CH-E

(bioMérieux), according to the manufacturer's protocols. Other standard tests were performed as described previously (Emborg *et al.*, 2005) and by Barrow & Feltham (1999), but were conducted at 25 °C instead of 30–37 °C to obtain growth of the psychrotolerant isolates. Growth at various temperatures (0, 2, 4, 25, 30, 35 and 37 °C) was determined using a nutrient-rich broth with 1.0 % NaCl (Dalgaard *et al.*, 1994). The same broth with Tris/HCl instead of phosphate buffer was used to determine growth at various pH values (pH 4.1–9.6). Growth at pH values above 8 was determined using Hungate tubes with butyl-rubber stoppers to prevent CO₂ from air reducing the pH of the medium during the experiment. Growth with 0–10 % (w/v) NaCl was tested in broth containing 1 % tryptone and 0.5 % yeast extract. The presence of tyrosine lyase was tested at 25 and 30 °C instead of 37 °C as suggested by Sheth & Kurup (1975). Formation of a reddish-brown pigment from tryptophan was tested according to Muller (1985). The test was performed at 25 °C. The antibiotic sensitivity of the isolates was tested using methods from the Clinical and Laboratory Standards Institute with commercially prepared, dry-form broth micro-dilution panels (Sensititre; TREK Diagnostics) and Mueller–Hinton broth (National Committee on Clinical Laboratory Standards, 2002, 2003; Clinical and Laboratory Standards Institute, 2005). The whole-cell fatty acid composition was determined for strains U2/3^T, LMG 7874^T, DSM 14850^T, 1F10 and 03A11 at DSMZ, using gas chromatography according to MIDI Inc. Histamine formation by the isolates in broth at 10 °C was tested as described previously (Emborg *et al.*, 2005).

Phylogenetic analysis of almost-complete 16S rRNA gene sequences (1356 bp) differentiated psychrotolerant *M. morganii*-like isolates from mesophilic *M. morganii*, *Proteus* and *Providencia* isolates (Fig. 1). The similarity between group means of the psychrotolerant and mesophilic *Morganella* isolates was 98.6 %, which is above the 97 % similarity generally used to distinguish two groups of isolates as representing separate species (Stackebrandt & Goebel, 1994). However, among some taxa, such as the *Enterobacteriaceae*, 16S rRNA gene sequence similarity well beyond 97 % is common (Cilia *et al.*, 1996). Protein-encoding genes frequently show more sequence variation than 16S rRNA genes and multi-locus sequencing of such housekeeping genes can be valuable for classification and identification of isolates (Enright & Spratt, 1999; Giraffa, 2001; Stackebrandt *et al.*, 2002). Phylogenetic analysis of merged sequences of all seven housekeeping gene fragments showed 88.7 % similarity between group means of the psychrotolerant and mesophilic *Morganella* isolates (Fig. 2). Furthermore, the gene fragments from each of the seven studied housekeeping genes clearly separated the psychrotolerant and mesophilic *Morganella* isolates (Supplementary Figs S1–S7 in IJSEM Online). The sequence similarity of each of the housekeeping gene fragments was less than 90.9 % and bootstrap values of 100 for all of the seven housekeeping gene fragments as well as for the merged sequences supported the separate clustering of psychrotolerant and mesophilic *Morganella* isolates. The degree of sequence similarity for psychrotolerant *M. morganii*-like isolates corresponded to the degree

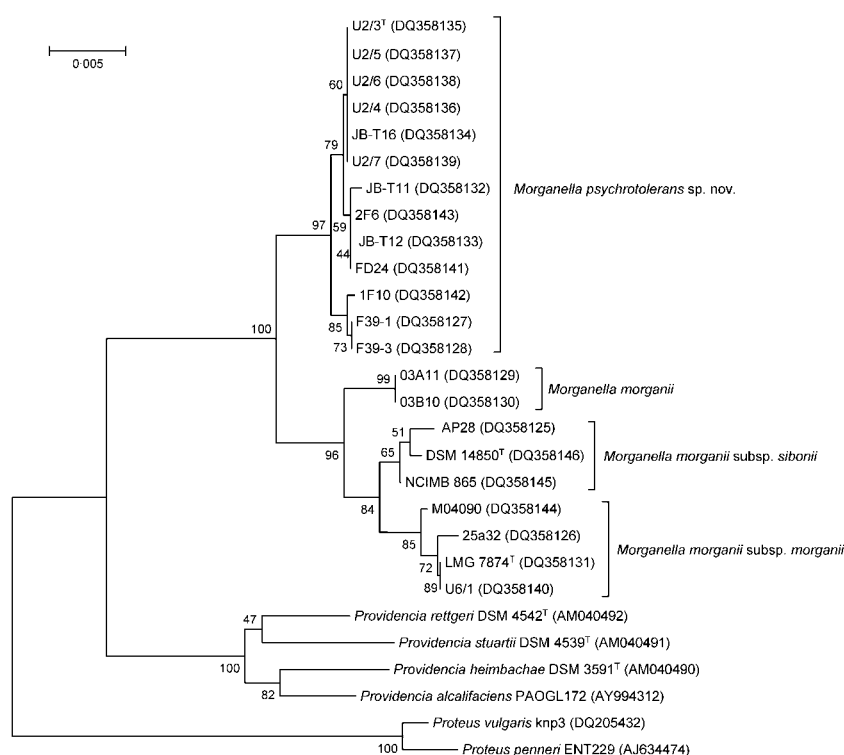


Fig. 1. Neighbour-joining tree (Kimura two-parameter) showing the relationship between 16S rRNA gene sequences (1356 bp) of 22 *Morganella* strains and selected strains from the nearest neighbours (*Proteus* and *Providencia*). Bootstrap values from 1000 replicates appear next to the corresponding branch. GenBank accession numbers are given in parentheses. Bar, 0.005 nucleotide substitutions per site.

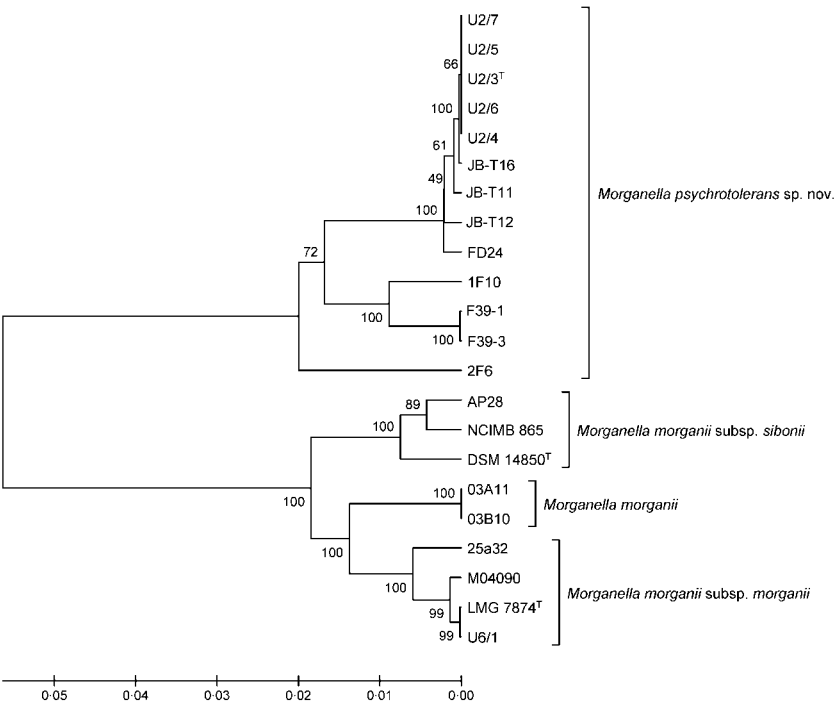


Fig. 2. Neighbour-joining tree (Kimura two-parameter) showing the relationship between merged sequences (3557 bp) of seven housekeeping gene fragments from 22 *Morganella* strains. Bootstrap values from 1000 replicates appear next to the corresponding branch. Bar, nucleotide substitutions per site.

of similarity observed between the mesophilic *Morganella* isolates studied (Fig. 2).

DNA–DNA hybridization showed a low relatedness between the psychrotolerant isolate U2/3^T and the type strains of both *M. morganii* subsp. *morganii* (LMG 7874^T; 41.0 %) and *M. morganii* subsp. *sibonii* (DSM 14850^T; 17.8 %). The DNA–DNA relatedness was lower than that of 51.0 % found between *M. morganii* subsp. *morganii* LMG 7874^T and *M. morganii* subsp. *sibonii* DSM 14850^T (Table 2). This low level of DNA–DNA relatedness between the psychrotolerant isolate and *M. morganii* supports that the strains represent separate species (Wayne *et al.*, 1987). The relatedness values between strain U2/3^T and the psychrotolerant isolates FD24, JB-T16 and 1F10 were 85.3, 97.0 and 67.7 %, respectively (Table 2). The mesophilic strain 03A11 showed significantly higher DNA–DNA relatedness to strain LMG 7874^T than to strain DSM 14850^T (Table 2). This is in conflict with the strain’s fermentation of trehalose, indicating that strain 03A11 represents *M. morganii* subsp. *sibonii*. Strains 03A11 and 03B10 from smoked fish from New Zealand thus appear to represent a separate cluster of *M. morganii*. This was supported by the phylogenetic analysis of 16S rRNA and housekeeping genes (Fig. 2 and Supplementary Figs S1–S7 in IJSEM Online). The other mesophilic *M. morganii* isolates were divided into two clusters corresponding to *M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii*. However, the *tuf* gene fragment resulted in a slightly different clustering for *M. morganii* subsp. *sibonii* DSM 14850^T (Fig. 2 and Supplementary Figs S1–S7 in IJSEM Online).

The DNA G + C contents for *M. morganii* subsp. *morganii* LMG 7874^T and *M. morganii* subsp. *sibonii* DSM 14850^T

were identical (49.6 mol%) and similar to that of strain 03A11 (50.7 mol%), whereas slightly lower values of 46.5 and 47.1 mol% were found for the psychrotolerant isolates U2/3^T and 1F10, respectively.

Phenotypic characterization confirmed that all the isolates studied belonged to the genus *Morganella* (Janda & Abbott, 2005). Cells were Gram-negative, fermentative, motile rods, with negative oxidase and positive catalase reactions. The isolates were phenylalanine deaminase-positive, gelatin-negative, urease-positive (except strain 25a32), indole-positive (except strain JB-T11) and citrate-negative (except strain 1F10). They all degraded tyrosine crystals after 72 h at 25 and 30 °C (except strains 2F6 and JB-T11) and produced a reddish-brown pigment from tryptophan after 48 h at 25 °C (except strain 2F6). With regard to the whole-cell fatty acids, the *Morganella* strains all contained

Table 2. DNA–DNA hybridization between selected *Morganella* isolates

Values are means ± SD (*n* = 2). –, Not tested.

Strain	Hybridization (%) with ³ H-labelled DNA from:		
	U2/3 ^T	LMG 7874 ^T	DSM 14850 ^T
FD24	85.3 ± 1.3	–	–
JB-T16	97.0 ± 1.8	–	–
1F10	67.7 ± 2.3	–	–
LMG 7874 ^T	41.0 ± 3.6	–	–
DSM 14850 ^T	17.8 ± 3.1	51.0 ± 5.9	–
03A11	27.0 ± 3.5	92.4 ± 0.0	47.6 ± 1.6

higher concentrations of 12:0 and lower concentrations of 18:1 ω 7c (Supplementary Table S2 in IJSEM Online) than usually observed for *Proteus* and *Providencia* (Vasyurenko & Chernyavskaya, 1990). This further supported their allocation to the genus *Morganella*.

As observed previously (Emborg *et al.*, 2005; Emborg & Dalgaard, 2006; Dalgaard *et al.*, 2006), the psychrotolerant *M. morganii*-like isolates differed from mesophilic *M. morganii* by their ability to grow at 0–2 °C, but not at 37 °C. In addition, fermentation of D-galactose and growth in 8.5 % (w/v) NaCl support this differentiation (Table 3). At 10 °C, high concentrations of histamine were produced by all the psychrotolerant *M. morganii*-like isolates (6420–10 980 p.p.m.) as well as by the *M. morganii* isolates (5000–9120 p.p.m.). It has been shown previously that various mixtures of psychrotolerant *M. morganii*-like isolates produced 7400 ± 1050 p.p.m. histamine in vacuum-packed tuna steaks after storage at 2 °C. At 5 °C, 3500–4000 p.p.m. histamine was formed in vacuum-packed, cold-smoked tuna and 1190–3310 p.p.m. histamine in broth (Emborg *et al.*, 2005; Emborg & Dalgaard, 2006; Dalgaard *et al.*, 2006). Such a pronounced histamine formation at 2–5 °C has not been reported for mesophilic *M. morganii* isolates.

In the present study, all *M. morganii* subsp. *morganii* isolates tested were sensitive to tetracycline, whereas *M. morganii* subsp. *sibonii* isolates were all resistant. This is in agreement with earlier findings by Janda & Abbott (2005). Jensen *et al.* (1992) used tetracycline resistance among other criteria

to divide *Morganella* into biogroups. Interestingly, we observed the same pattern for the psychrotolerant *M. morganii*-like isolates and the mesophilic *M. morganii* isolates, with D-trehalose-fermenting isolates being resistant to tetracycline. Only strain 1F10 was resistant to tetracycline and unable to ferment D-trehalose. Janda & Abbott (2005) reported that adonitol and D-arabitol fermentation performed using the conventional test differed between *M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii*, but this was not confirmed in the present study; however, in the present study, the test was performed using API 50 CH-E with results being recorded after 24 and 48 h at 25 °C (Supplementary Table S3 in IJSEM Online). Consequently, API 50 CH-E can be used for identification of *M. morganii*, but not for separation into subspecies, where conventional tests are needed.

In combination with the sequence data (Fig. 2), the phenotypic characterization suggests that strain U2/3^T and the other psychrotolerant *M. morganii*-like isolates studied represent a novel *Morganella* species, for which the name *Morganella psychrotolerans* sp. nov. is proposed.

Similar to *M. morganii*, the psychrotolerant isolates studied varied with respect to D-trehalose fermentation, L-ornithine decarboxylase activity and tetracycline sensitivity (Table 3 and Supplementary Table S3 in IJSEM Online). The nine psychrotolerant isolates from fresh and cold-smoked tuna were D-trehalose-negative and L-ornithine decarboxylase-positive and were able to grow at 0 °C. They differed from the two isolates from lumpfish roe (F39-1 and F39-3) in these phenotypic characteristics, as well as in the gene fragments studied (Fig. 2 and Supplementary Figs S1–S7 in IJSEM Online). The two ‘garfish’ isolates also differed from the ‘tuna’ isolates in the gene fragments studied (Fig. 2 and Supplementary Figs S1–S7 in IJSEM Online). In addition, strain 1F10 was the only psychrotolerant isolate that was able to grow in 8 % NaCl; it was also citrate-negative and showed only 67.7 ± 2.3 % DNA–DNA relatedness with strain U2/3^T. Strain 2F6 differed from the ‘tuna’ isolates with respect to formation of pigment from tryptophan and tyrosine degradation at 30 °C. From the present study, it appears that a future division of *M. psychrotolerans* may be relevant, but analysis of a larger number of isolates would be required to determine whether a novel subspecies is appropriate or whether a novel species can be separated from *M. morganii* and *M. psychrotolerans*.

In this study, the usefulness of multi-locus sequencing to elucidate the genomic relatedness at inter- and intraspecific levels is illustrated. The sequence information obtained has improved the taxonomy of the genus *Morganella* and made data available for future studies of molecular identification and detection methods. The recognition and acknowledgement of *M. psychrotolerans* sp. nov. and its growth conditions will most probably be of significant importance for the investigation of future outbreaks of histamine fish poisoning.

Table 3. Characteristics that distinguish *Morganella psychrotolerans* sp. nov. and *M. morganii* isolates

Taxa: 1, *M. psychrotolerans* sp. nov. (13 strains); 2, *M. morganii* subsp. *morganii* (4 strains including LMG 7874^T); 3, *M. morganii* subsp. *sibonii* (3 strains including DSM 14850^T); 4, *M. morganii* (strains 03A11 and 03B10). Cells of all strains are Gram-negative, oxidase-negative, catalase-positive, rod-shaped, motile and produce H₂S from cysteine but not from thiosulfate. All are fermentative with production of gas from glucose, but do not hydrolyse ONPG or degrade gelatin. All ferment D-ribose, D-fructose, D-mannose, N-acetylglucosamine, xylitol and potassium gluconate. Values are the percentage of strains that are positive for the characteristic concerned.

Characteristic	1	2	3	4
Growth at/in:				
2 °C	100	0	0	0
37 °C	0	100	100	100
8.5 % NaCl	0	100	100	100
Fermentation of:				
D-Trehalose (48 h)	21	0	100	100
D-Galactose (48 h)	0	100	100	100

Description of *Morganella psychrotolerans* sp. nov.

Morganella psychrotolerans (psy.chro.to'le.rans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

Cells are Gram-negative, straight rods, 1 µm wide and 2–3 µm long with rounded ends, occurring singly and in short chains. Motile with aerobic and facultatively anaerobic growth. Growth occurs at 2–35 °C (77 % grew at 0 °C), in zero to close to 7.5 % (w/v) NaCl (46 % grew in 7.5 % NaCl) and at pH 4.6–9.2 (77 % grew at pH 4.6). Glucose is fermented with gas formation and phenylalanine is deaminated. D-Ribose, D-fructose, D-mannose, xylitol, N-acetylglucosamine, xylitol and potassium gluconate are fermented. Does not ferment glycerol, erythritol, arabinose, xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glycopyranoside, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, gentiobiose, D-turanose, D-lyxose, D-tagatose, fucose, arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. The major cellular fatty acids are 16:0 (31 %) and 17:0 cyclo (30 %). Sensitive to apramycin, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole and trimethoprim.

The type strain, U2/3^T (= LMG 23374^T = DSM 17886^T), was isolated by the Danish Institute for Fisheries Research from cold-smoked tuna involved in an outbreak of histamine poisoning in Denmark in 2004.

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