Reclassification of *Flexibacter tractuosus* (Lewin 1969) Leadbetter 1974 and '*Microscilla sericea*' Lewin 1969 in the genus *Marivirga* gen. nov. as *Marivirga tractuosa* comb. nov. and *Marivirga sericea* nom. rev., comb. nov.

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The taxonomic position of the misclassified strains [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 was studied using a polyphasic approach. The two strains shared 99.1 % 16S rRNA gene sequence similarity and 28 % DNA–DNA relatedness. On the basis of the phylogenetic evidence supported by genotypic and phenotypic data [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 are classified as two distinct species in a novel genus, *Marivirga*, in the family 'Flammeovirgaceae', as *Marivirga tractuosa* comb. nov. and *Marivirga sericea* nom. rev., comb. nov., with strains KCTC 2958^T (=ATCC 23168^T =CIP 106410^T =DSM 4126^T =NBRC 15989^T =NCIMB 1408^T =VKM B-1430^T) and LMG 13021^T (=ATCC 23182^T =NBRC 15983^T =NCIMB 1403^T), respectively, as the type strains. The type species is *Marivirga tractuosa*.

Detailed analysis of the phylogenetic relationship between members of the genera *Flexibacter*, *Flexithrix* and *Microscilla* based on the comparison of 16S rRNA gene sequences has revealed their considerable heterogeneity (Nakagawa *et al.*, 2002). These authors suggested restricting the genera *Flexibacter* and *Microscilla* to their respective type species, *Flexibacter flexilis* and *Microscilla marina*, while the other species previously allocated to these genera were considered potential members of novel species and genera or families. The name [*Flexibacter*] *aggregans* attributed to strain IAM 14894^T (=IFO 15976^T) was recognized as a later heterotypic synonym of *Flexithrix dorotheae*, and '[*Microscilla*] *furvescens*' LMG 13023^T was reclassified in the novel genus *Marinoscillum* as *Marinoscillum furvescens* comb. nov. (Hosoya & Yokota, 2007; Seo *et al.*, 2009). The genera *Flammeovirga* and *Persicobacter* were created due to reclassification of the species *Cytophaga aprica* and *Cytophaga diffluens*, respectively (Nakagawa *et al.*, 1997). Taxonomic investigations of newly isolated marine bacteria served as a basis for emending the descriptions of these genera (Muramatsu *et al.*, 2010; Takahashi *et al.*, 2006). Moreover, the related genera

Fabibacter, *Fulvivirga*, *Limibacter*, *Perexilibacter*, *Rapidithrix*, *Reichenbachiella*, *Roseivirga* and *Sediminitomix* were described over the past few years due to extensive taxonomic studies (Khan *et al.*, 2007; Lau *et al.*, 2006; Nedashkovskaya *et al.*, 2003, 2005a, b, 2007; Srisukchayakul *et al.*, 2007; Yoon *et al.*, 2007, 2008). The above-mentioned genera have recently been grouped in the family 'Flammeovirgaceae' (Ludwig *et al.*, 2008; Yoon *et al.*, 2007, 2008).

'[*Microscilla*] *tractuosa*' KCTC 2958 and '[*Microscilla*] *sericea*' LMG 13021 were first described by Lewin (1969). Leadbetter (1974) transferred '[*Microscilla*] *tractuosa*' to the genus *Flexibacter* but Reichenbach (1989) restricted this genus to the freshwater and terrestrial species, while its marine representatives were maintained in the genus *Microscilla*. [*Flexibacter*] *tractuosus* was included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) but '[*Microscilla*] *sericea*' was not.

The taxonomic investigation performed in this study on [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021^T by using a polyphasic approach

showed that the strains should be placed in a novel genus as two distinct species, for which the names *Marivirga tractuosa* comb. nov. and *Marivirga sericea* nom. rev., comb. nov. are proposed.

[*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 were routinely cultivated at 28 °C on marine agar 2216 (MA; Difco) and stored at -80 °C in marine broth 2216 (Difco) supplemented with 20 % (v/v) glycerol.

The almost-complete 16S rRNA gene sequences of [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 deposited in GenBank were aligned with those of representative members of selected genera belonging to the phylum *Bacteroidetes* by using PHYDIT version 3.1 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred by using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the model of Jukes & Cantor (1969), and the trees were reconstructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Kluge & Farris, 1969) algorithms. Bootstrap analysis was performed with 1000 resampled datasets, using the SEQBOOT and CONSENSE programs of the PHYLIP package (Fig. 1).

Phylogenetic analysis revealed that [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 were

most closely related to members of the genera *Fabibacter*, *Reichenbachiella* and *Roseivirga*, with 88.4–90.6 % 16S rRNA gene sequence similarity, while the two strains shared 99.1 % sequence similarity.

DNA was isolated following the method of Marmur (1961) and the DNA G + C content was determined by the thermal denaturation method (Marmur & Doty, 1962). The values for [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 were 36.1 and 37.1 mol%, respectively. DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The level of DNA–DNA relatedness between [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 was 28 %, confirming that they represent two distinct species of the same genus (Wayne *et al.*, 1987).

For cellular fatty acid analysis, the two strains were grown at 25 °C for 48 h on MA. The analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (MIDI). The predominant cellular fatty acids of [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 were iso-C_{15:0} (36.8 and 26.4 %, respectively), iso-C_{15:1} (23.0 and 28.6 %, respectively) and iso-C_{17:0} 3-OH (12.2 and 13.6 %, respectively) (Table 1). The two strains could be differentiated by the respective proportions of a number of fatty acids such as C_{15:0} and summed feature 3 (comprising C_{16:1ω7} and/or iso-C_{15:0} 2-OH) (Table 1). Isoprenoid quinones were extracted and analysed by using a standard procedure (Minnikin *et al.*, 1984). The major respiratory lipoquinone of [*Flexibacter*] *tractuosus* KCTC 2958^T and '[*Microscilla*] *sericea*' LMG 13021 was MK-7, in line with all other members of the family 'Flammeovirgaceae' (Lau *et al.*, 2006; Nakagawa *et al.*, 1997; Nedashkovskaya *et al.*, 2007; Yoon *et al.*, 2007).

Physiological and biochemical properties of the two strains were examined by using standard procedures and by using API 20E, API 20NE, API CH50 and API ZYM galleries (bioMérieux) according to the manufacturers' instructions, except that the galleries were incubated at 28 °C.

Gram-staining was done as described by Gerhardt *et al.* (1994). Oxidative or fermentative utilization of glucose was determined on Hugh & Leifson's medium modified for marine bacteria (Lemos *et al.*, 1985). Degradation of agar, starch, casein, gelatin, cellulose (filter paper and CM-cellulose), chitin, DNA, urea and alginic acids, production of flexirubin pigments, growth at different pH, production of acid from carbohydrates, and susceptibility to antibiotics were tested as described previously (Nedashkovskaya *et al.*, 2003). Hydrolysis of Tweens 20, 40 and 80, nitrate reduction, production of hydrogen sulphide, acetoin (Voges–Proskauer reaction) and indole, and presence of β-galactosidase, oxidase, catalase and alkaline phosphatase activities were tested according to the methods of Gerhardt *et al.* (1994). The temperature range for growth was assessed in medium A containing 5 g Bacto peptone

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships of species of the novel genus *Marivirga* and representative members of the family 'Flammeovirgaceae'. Values at nodes are bootstrap supports (>70 %), and asterisks indicate branches also recovered in the maximum-likelihood and maximum-parsimony trees. *Thermonema lapsum* 23/9^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Table 1. Cellular fatty acid composition (%) of species of the novel genus *Marivirga*

Strains: 1, *Marivirga tractuosa* comb. nov. KCTC 2958^T; 2, *Marivirga sericea* nom. rev., comb. nov. LMG 13021^T. Fatty acids amounting to less than 1 % in both strains are not shown. tr, Trace (<1 %). All data are from this study.

Fatty acid	1	2
iso-C _{11:0}	tr	2.7
iso-C _{13:0}	1.2	tr
iso-C _{14:0}	tr	1.8
iso-C _{15:1}	23.0	28.6
iso-C _{15:0}	36.8	26.4
anteiso-C _{15:0}	tr	1.6
C _{15:0}	4.4	2.6
iso-C _{16:1} G	1.4	tr
iso-C _{16:0}	3.7	1.7
iso-C _{15:0} 3-OH	2.8	4.3
iso-C _{16:0} 3-OH	2.8	2.8
iso-C _{17:0}	1.2	1.2
anteiso-C _{17:0}	tr	1.3–2.3
iso-C _{17:0} 3-OH	12.2	13.6
Summed feature 3*	0.8	4.5

*Summed feature 3 contains one or more of the following fatty acids which could not be separated by the Microbial Identification System: C_{16:1}ω7 and/or iso-C_{15:0} 2-OH.

(Difco), 2 g Bacto yeast extract (Difco), 1 g glucose, 0.02 g KH₂PO₄ and 0.05 g MgSO₄·7H₂O per litre of half-strength natural seawater. Tolerance to NaCl was assessed in medium A prepared with distilled water with 0, 0.5, 1, 2, 3, 5, 6, 8, 10 and 12 % (w/v) NaCl. Carbon source utilization was tested (i) using commercial API 20NE identification strips following the instructions of the manufacturer and (ii) using a medium that contained 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract (Difco) and 0.4 % (w/v) carbon source per litre of artificial seawater as described by Suzuki *et al.* (2001). Spreading growth was observed by cultivation of the strains under high moisture conditions on medium B containing 1 g Bacto peptone (Difco), 1 g yeast extract (Difco) and 15 g agar per litre of half-strength natural seawater. Gliding motility was determined as described by Bowman (2000). Susceptibility to antibiotics was tested as described previously (Nedashkovskaya *et al.*, 2004) using additional discs containing chloramphenicol (30 µg), doxycycline (10 µg) and erythromycin (15 µg).

Physiological, morphological and biochemical characteristics of [*Flexibacter*] *tractuosus* KCTC 2958^T and [*Microscilla*] *sericea* LMG 13021 are listed in the genus and species descriptions and in Table 2. The two strains shared most phenotypic features. They were aerobic bacteria motile by gliding, possessing oxidase and catalase activities, decomposing gelatin, DNA and Tween compounds, and utilizing aesculin and arbutin. However, they could be distinguished by several phenotypic traits. [*Flexibacter*] *tractuosus* KCTC

2958^T was able to grow at 40 °C and to utilize L-arabinose, D-glucose, maltose, D-mannose, mannitol, N-acetylglucosamine, adipate, citrate and malate in the API 20NE gallery but was unable to grow in the presence of 12 % NaCl, whereas [*Microscilla*] *sericea* LMG 13021 displayed the opposite properties. The phenotypic features differentiating the two strains from related members of the family 'Flammeovirgaceae' are shown in Table 2.

On the basis of the results of the phylogenetic analysis and of the genotypic and phenotypic examination, [*Flexibacter*] *tractuosus* KCTC 2958^T and [*Microscilla*] *sericea* LMG 13021 are classified as two distinct species in a new genus, *Marivirga* gen. nov., as *Marivirga tractuosa* comb. nov. and *Marivirga sericea* nom. rev., comb. nov.

Interestingly, two pigmentation variants, designated KMM 6275 and KMM 6276, were isolated from a culture of [*Flexibacter*] *tractuosus* KCTC 2958^T on MA. Their colonies were yellow and whitish, respectively, compared to the dark-orange pigmented colonies of strain KCTC 2958^T (Lewin, 1969). The two variants shared most phenotypic features with [*Flexibacter*] *tractuosus* KCTC 2958^T, but they hydrolysed casein and produced hydrogen sulfide in contrast to the type strain and they also displayed slight differences in the proportions of some fatty acids (data not shown). Moreover, variant KMM 6275 produced acid from D-glucose and maltose. [*Flexibacter*] *tractuosus* KCTC 2958^T and variants KMM 6275 and KMM 6276 shared 100 % 16S rRNA gene sequence similarity and 95–99 % DNA–DNA relatedness, confirming that they belong to the same species (Wayne *et al.*, 1987).

Description of *Marivirga* gen. nov.

Marivirga (Ma.ri.vir'ga L. neut. n. *mare* the sea, L. fem. n. *virga* rod, N.L. fem. n. *Marivirga* a rod that inhabits marine environments).

Rod-shaped cells, motile by gliding. Gram-staining-negative. Do not form endospores. Strictly aerobic. Usually produce non-diffusible orange or yellow pigments. Chemo-organotrophic. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. The major respiratory quinone is MK-7. Predominant cellular fatty acids (>12 %) are iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH. DNA G+C content is 36–37 mol%. According to 16S rRNA gene sequence analysis, the genus *Marivirga* is a member of the family 'Flammeovirgaceae' of the phylum *Bacteroidetes*. The type species is *Marivirga tractuosa*.

Description of *Marivirga sericea* nom. rev., comb. nov.

Marivirga sericea (se.ri'ce.a L. fem. adj. *sericea* made from or pertaining to silk; N.L. adj. *sericea* silk-like).

Synonym: '*Microscilla sericea*' Lewin 1969, 200.

The main characteristics are as given for the genus. In addition, cells are long, slender and flexible rods 0.4–

Table 2. Differential phenotypic characteristics of species of the novel genus *Marivirga* and related members of the family 'Flammeovirgaceae'

Taxa: 1, *Marivirga* gen. nov. ($n=2$); 2, *Fabibacter* ($n=1$); 3, *Fulvivirga* ($n=1$); 4, *Marinoscillum* ($n=2$); 5, *Reichenbachella* ($n=1$); 6, *Rosevirga* ($n=3$). n, Number of species; ND, data are not available; v, variable result. Data are from Lau *et al.* (2006), Nedashkovskaya *et al.* (2003, 2005a, b, c, 2007, 2008), Reichenbach (1989), Seo *et al.* (2009) and this study. Data for taxa 1, 3 and 5 and for *Rosevirga ehrenbergii* and *Rosevirga echinicomitans* were obtained using the same methods and growth conditions; some of the data for taxa 2 and 4 and for *Rosevirga spongicola* were obtained using different methods and growth conditions.

Characteristic	1	2	3	4	5	6
Production of flexirubin type pigments	—	—	—	—	+	—
Acetoin production	—	+	ND	v	—	v
Temperature range for growth (°C)	10–40	12–36	14–44	15–45	4–35	4–44
Salinity range for growth (% NaCl)	0.5–12	0–12	0–10	0.5–12	1–6	0–16
Hydrolysis of:						
Agar	—	—	—	—	+	—
Gelatin	+	—	+	v	+	+
Starch	—	+	+	—	+	—
DNA G + C content (mol%)	36–37	42.5	59.9	41–44	44.5	40–44

0.5 µm in diameter and 30–100 µm in length or longer. On marine agar, colonies are circular, dark orange, shiny and 2–4 mm in diameter after 72 h of incubation. Spreading growth may occur on moist media. Growth is observed at 10–38 °C and in the presence of 0.5–12 % NaCl, with optimal growth at 28–30 °C and with 4–6 % NaCl. Flexirubin-type pigments are not produced. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase and tryptophan deaminase activities are absent. Nitrate is not reduced. Indole, H₂S and acetoin (Voges–Proskauer reaction) are not produced. Casein, gelatin, Tweens 20, 40 and 80 and DNA are hydrolysed. Agar, starch, urea, cellulose (CM-cellulose and filter paper) and chitin are not hydrolysed. Acid is not produced from L-arabinose, cellobiose, L-fucose, D-galactose, D-glucose, glycerol, lactose, maltose, melibiose, raffinose, L-rhamnose, L-sorbose, sucrose, trehalose, DL-xylose, N-acetylglucosamine, citrate, acetate, fumarate, malate, adonitol, dulcitol, inositol or mannitol. In the API 50 CH gallery, acid is produced only from aesculin and arbutin. D-Glucose, D-mannose and sucrose are utilized but L-arabinose, lactose, inositol, sorbitol, mannitol, malonate and citrate are not utilized. In the API ZYM gallery, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α- and β-glucosidase activities are present, but lipase (C14), trypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Susceptible to ampicillin (10 µg), benzylpenicillin (10 U), carbenicillin (100 µg), chloramphenicol (30 µg), doxycycline (10 µg), erythromycin (15 µg), lincomycin (15 µg), oleandomycin (15 µg) and tetracycline (30 µg); resistant to gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), polymyxin (300 U) and streptomycin (30 µg). The predominant cellular fatty acids (>4 %) are iso-C_{15:0}, iso-C_{15:1}, iso-

C_{17:0} 3-OH, iso-C_{15:0} 3-OH and summed feature 3 (comprising C_{16:1}ω7 and/or iso-C_{15:0} 2-OH). The detailed fatty acid composition is given in Table 1. The DNA G + C content of the type strain is 37.1 mol%.

The type strain, LMG 13021^T (=ATCC 23182^T =NBRC 15983^T =NCIMB 1403^T), was isolated from marine aquarium outflow, La Jolla, California, USA.

Description of *Marivirga tractuosa* comb. nov.

Marivirga tractuosa (trac.tu.o'sa L. fem. adj. *tractuosa* that draws to itself, gluey, viscous).

Synonyms: '*Microscilla tractuosa*' Lewin 1969, 199; *Flexibacter tractuosus* (Lewin 1969) Leadbetter 1974.

The main characteristics are as given for the genus. In addition, cells are long, slender and flexible rods 0.4–0.5 µm in diameter and 10–50 µm in length or longer. On marine agar, colonies are circular, shiny and 2–4 mm in diameter after 72 h of incubation. Colonies are usually dark-orange-coloured but whitish or yellow-pigmented variants may occur. Spreading growth may occur on moist media. Growth is observed at 10–40 °C and with 0.5–10 % NaCl, with optimal growth at 28–32 °C and with 4–7 % NaCl. Flexirubin-type pigments are not produced. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase and tryptophan deaminase activities are absent. Nitrate is not reduced. Indole and acetoin (Voges–Proskauer reaction) are not produced. Gelatin, Tweens 20, 40 and 80 and DNA are hydrolysed. Agar, starch, urea, cellulose (CM-cellulose and filter paper) and chitin are not hydrolysed. Acid is not produced from L-arabinose, cellobiose, L-fucose, D-galactose, glycerol, lactose, melibiose, raffinose, L-rhamnose, L-sorbose, sucrose, trehalose, DL-xylose, N-acetylglucosamine, citrate, acetate, fumarate, malate, adonitol, dulcitol, inositol or mannitol. In the API 50 CH gallery, acid is produced only from aesculin and arbutin.

Production of hydrogen sulfide, hydrolysis of casein and oxidation of D-glucose and maltose are variable. Citrate is utilized but lactose, inositol, gluconate, caprate, phenylalanine and malonate are not. Utilization of arabinose, D-glucose, D-mannose, sucrose, mannitol, N-acetylglucosamine, maltose, adipate, malate and sorbitol is variable. In the API ZYM gallery, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and α - and β -glucosidase activities are present, but lipase (C14), trypsin, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities are absent. Susceptible to ampicillin (10 μ g), benzylpenicillin (10 U), carbenicillin (100 μ g), chloramphenicol (30 μ g), doxycycline (10 μ g), erythromycin (15 μ g), lincomycin (15 μ g), oleandomycin (15 μ g) and tetracycline (30 μ g); resistant to gentamicin (10 μ g), kanamycin (30 μ g), neomycin (30 μ g), polymixin (300 U) and streptomycin (30 μ g). The predominant cellular fatty acids (>4%) are iso-C_{15:0}, iso-C_{15:1}, iso-C_{17:0} 3-OH and C_{15:0}. The detailed fatty acid composition is given in Table 1. The DNA G + C content of the type strain is 36.1 mol%.

The type strain, KCTC 2958^T (=ATCC 23168^T =CIP 106410^T =DSM 4126^T =NBRC 15989^T =NCIMB 1408^T =VKM B-1430^T), was isolated from a beach sand sample, collected from Nhatrang (South China Sea), Vietnam.

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