Actinomadura apis sp. nov., isolated from a honey bee (*Apis mellifera*) hive, and the reclassification of *Actinomadura cremea* subsp. *rifamycini* Gauze *et al.* 1987 as *Actinomadura rifamycini* (Gauze *et al.* 1987) sp. nov., comb. nov.

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A Gram-reaction-positive aerobic actinomycete, designated strain IM17-1^T, was isolated from a honey bee (*Apis mellifera*) hive in Chiang Mai Province, Thailand. The strain formed a branched substrate mycelium and mature aerial mycelium bore short chains of arthrospores with warty surfaces. The cell wall contained *meso*-2,6-diaminopimelic acid (cell-wall type III) and the whole cell sugars were fucose, galactose, glucose, madurose, mannose and ribose. The major isoprenoid quinone was hexahydrogenated menaquinone with nine isoprene units and the predominant cellular fatty acids were C_{16:0} (33.8%), C_{18:1 ω 9c} (32.7%), summed feature 3 (C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH) (8.7%) and 10-methyl C_{18:0} (8.2%). The phospholipids were diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. These morphological and chemotaxonomic characteristics were consistent with the classification of IM17-1^T within the genus *Actinomadura*. Based on 16S rRNA gene sequence analysis, strain IM17-1^T was closely related to the type strains of *Actinomadura cremea* subsp. *cremea* (98.1%) and *Actinomadura cremea* subsp. *rifamycini* (98.6%); however, it represented a distinct phylogenetic lineage from the other species within this genus. The unique genetic characteristics were reaffirmed by low levels of DNA–DNA relatedness between strain IM17-1^T and the two most closely related type strains, *A. cremea* subsp. *cremea* JCM 3308^T (56.5 $\pm$ 4.9%) and *A. cremea* subsp. *rifamycini* JCM 3309^T (31.0 $\pm$ 22.6%), and further supported the proposal of IM17-1^T as a novel species. Strain IM17-1^T (=JCM 16576^T =TISTR 1980^T) thus represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura apis* sp. nov. is proposed. In addition, the genotypic and phenotypic data suggested the reclassification of *Actinomadura cremea* subsp. *rifamycini* Gauze *et al.* 1987 as a separate species, *Actinomadura rifamycini* sp. nov., comb. nov.

The genus *Actinomadura* was established by Lechevalier & Lechevalier (1968) and, at the time of writing, encompassed 42 species with validly published names including two subspecies, *Actinomadura cremea* subsp. *cremea* (Preobrazhenskaya *et al.*, 1975) and *Actinomadura cremea* subsp. *rifamycini* (Gauze *et al.*, 1975, 1987) (<http://www.bacterio.cict.fr/>). Members of this genus are aerobic Gram-positive actinomycetes that can grow at 10–60 °C and form non-fragmenting branched substrate mycelia, which give

rise to aerial hyphae bearing spore chains up to 15 spores long with various shapes and ornamentations. Other characteristics of the genus are as follows. The cell-wall peptidoglycan is type III *sensu* Lechevalier & Lechevalier (1970) containing *meso*-diaminopimelic acid (A₂pm) without glycine; the major isoprenoid quinone is hexahydrogenated menaquinone with nine isoprene units; whole-cell hydrolysates mostly contain madurose; the fatty acid profiles are type 3a *sensu* Kroppenstedt (1985) containing saturated, unsaturated and branched fatty acids plus tuberculostearic acid, with the exception of the thermophilic actinomadurae, which differ in their fatty acid composition; and the phospholipid pattern is type PI *sensu* Lechevalier *et al.* (1981), in which no nitrogenous

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of strain IM17-1^T and *Actinomadura rifamycini* JCM 3309^T are AB557596 and AB557595, respectively.

A supplementary table is available with the online version of this paper.

phospholipids are present (Kroppenstedt *et al.*, 1990; Kroppenstedt & Goodfellow, 2006).

Most actinomadurae are widespread in soil (Cook *et al.*, 2005; Kroppenstedt & Goodfellow, 2006; Ara *et al.*, 2008), a few are found in clinical materials (Yassin *et al.*, 2010) and one was isolated as an endophyte from the leaves of a medicinal plant in China (Qin *et al.*, 2009). During the course of a study on the microflora of the European honey bee (*Apis mellifera* Linnaeus, 1758) in Thailand, actinomycetes were isolated from honey bee hive materials. Most of the isolates belonged to the genus *Streptomyces* and some less common isolates were classified as belonging to the genera *Nonomuraea* and *Nocardiopsis*. The remaining isolate, strain IM17-1^T, was identified as belonging to the genus *Actinomadura* but could not be classified in any known species (Promnuan *et al.*, 2009). Here, we describe the characterization and classification of strain IM17-1^T, which is, to our knowledge, the first member of the genus *Actinomadura* isolated from a hive of the European honey bee.

The bacterial strain was isolated using the standard dilution plate method on starch-casein agar (Küster & Williams, 1964) supplemented with 25 µg ml⁻¹ each of nystatin and nalidixic acid. After being incubated at 30 °C for 21 days, the isolate was transferred to, and purified and maintained on, oatmeal agar (International *Streptomyces* Project Medium 3; ISP 3) (Shirling & Gottlieb, 1966) supplemented with 0.1 % yeast extract.

The morphological and physiological characteristics of strain IM17-1^T were determined and compared with those of phylogenetically closely related type strains, *A. crenea* subsp. *crenea* JCM 3308^T and *A. crenea* subsp. *rifamycinii* JCM 3309^T. The temperature range for growth and NaCl tolerance were determined by culturing on ISP 3 medium and incubating for 14 days. Carbon utilization and melanoid pigment production were tested using the methods described by Shirling & Gottlieb (1966). Biomass for chemotaxonomic studies was prepared from cultures grown in yeast extract–malt extract (ISP 2) broth on a rotary shaker for 2–4 weeks at 30 °C. The isomers of A₂pm were determined by TLC of whole-cell hydrolysates according to the method of Stanek & Roberts (1974). Reducing sugars in whole-cell hydrolysates were analysed by using the HPLC method of Mikami & Ishida (1983). Phospholipids were extracted and identified following the methods of Minnikin *et al.* (1984). Methyl esters of cellular fatty acids were prepared and analysed according to the instructions for the Sherlock Microbial Identification System (version 2.998, MIDI) using GC (model HP6890; Hewlett Packard) with the TSBA40 method (Sasser, 1990). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977, 1984) and were analysed in an HPLC system (model LC-10AD VP; Shimadzu) equipped with a Cosmosil 5C₁₈ column (Nacalai Tesque) (Tamaoka *et al.*, 1983).

Cultural characteristics of strain IM17-1^T were determined after incubation at 30 °C for 21 days on various media as described by Shirling & Gottlieb (1966) and Asano &

Kawamoto (1986). Determination of colony colours was based on Jacobson *et al.* (1958). After incubation on ISP 2 and ISP 3 agar at 30 °C for 21 days, morphological properties were examined using a light microscope and a scanning electron microscope (model S-2400; Hitachi). Samples for scanning electron microscopy were prepared as described by Itoh *et al.* (1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima *et al.* (1999). CLUSTAL_X (Thompson *et al.*, 1997) was used to perform a multiple alignment of sequences downloaded from GenBank and the sequences obtained in this study. The alignment was verified manually and adjusted prior to the reconstruction of a phylogenetic tree. For reconstruction of phylogenetic trees, the software packages MEGA version 4 (Tamura *et al.*, 2007) and CLUSTAL_X were used. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony methods (Takahashi & Nei, 2000), based on a comparison of 1172 nt present in all of the strains after elimination of gaps and ambiguous nucleotides from the sequences corresponding to positions 34 and 1491 in *Escherichia coli*. *Streptosporangium album* DSM 43023^T was used as an outgroup. Confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). Sequence similarities between strains of the genus *Actinomadura* were calculated manually after pairwise alignment was performed using CLUSTAL_X.

For preparation of chromosomal DNA for G+C content analysis and DNA–DNA hybridization experiments, cells were freeze-dried and mechanically ground as described by Reader & Broda (1985). The G+C content of the DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of the DNA base composition (Yamasa Shoyu) was digested by bacterial alkaline phosphatase and used as the quantitative standard. Levels of DNA–DNA relatedness were measured fluorometrically using the microplate hybridization method devised by Ezaki *et al.* (1989). Hybridization was carried out at 60 °C for 12 h.

Strain IM17-1^T was found to be a Gram-positive aerobic actinomycete that produced well-developed and branched substrate hyphae. Spore chains were short, curved and consisted of ~3–5 non-motile spores with warty surfaces. The spores were borne on aerial hyphae as shown in Fig. 1a. The spore morphology was similar to that of *A. crenea* subsp. *rifamycinii*, the most closely related species, phylogenetically (Fig. 1b).

The cultural characteristics of strain IM17-1^T were also examined. The isolate showed good growth on glucose–yeast extract agar and moderate growth on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 7 media, Bennett agar, glucose–asparagine agar, Hickey–Tresner agar, nutrient agar and oatmeal–nitrate agar. Strain IM17-1^T formed white to grey aerial

Fig. 1. Scanning electron micrographs showing short chains of arthrospores in strains IM17-1^T (a) and *Actinomadura cremea* subsp. *rifamycinii* JCM 3309^T (b). The cultures were grown on oatmeal agar (ISP 3) with 0.1 % yeast extract at 30 °C for 3 weeks. Bar, 5 µm.

mycelia on the various media tested and the substrate mycelia showed different colours on different media (Supplementary Table S1, available in IJSEM Online). A greenish-black pigment was produced on ISP 2 and ISP 7 media. Strain IM17-1^T grew at 20–45 °C, at pH 6.0–12.0 and in the presence of 4 % (w/v) NaCl.

Chemotaxonomic analyses revealed that strain IM17-1^T exhibited characteristics that were typical of members of the genus *Actinomadura*. The cell wall contained meso-A₂pm and whole-cell sugars including fucose, galactose, glucose, madurose, mannose and ribose. The phospholipids were diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The predominant menaquinones were MK-9(H₆) (53 %) and MK-9(H₈) (26 %); MK-9(H₄) (8 %) was detected as a minor component. The major cellular fatty acids were C_{16:0} (33.8 %), C_{18:1}ω₉c (32.7 %), summed feature 3 (C_{16:1}ω₇c and/or iso-C_{15:0} 2-OH) (8.7 %) and 10-methyl C_{18:0} (8.2 %); minor amounts of C_{18:0} (6.9 %), iso-C_{16:0} (1.9 %), C_{14:0} (1.7 %), C_{17:0} (<1 %) and C_{16:1}ω₉c (<1 %) were also present (Table 1).

The 16S rRNA gene sequence of strain IM17-1^T (1495 nt) was compared with sequences of all species with validly published names within the genus *Actinomadura* and related taxa in the family *Thermomonosporaceae* (Rainey

et al., 1997 in Stackebrandt *et al.*, 1997; emend. Zhi *et al.*, 2009). Based on the comparative study of 16S rRNA gene sequences, strain IM17-1^T shared the closest sequence similarity values with *A. cremea* subsp. *cremea* (98.1 %) and *A. cremea* subsp. *rifamycinii* (98.6 %). The neighbour-joining tree (Fig. 2) showed that strain IM17-1^T was a member of the genus *Actinomadura* and was most closely related to *A. cremea* subsp. *cremea* and *A. cremea* subsp. *rifamycinii* but it was also readily distinguishable from the two subspecies (95 % bootstrap support). To confirm the taxonomic position of the isolate, DNA–DNA hybridization studies were carried out. The mean of DNA–DNA relatedness values of strain IM17-1^T with *A. cremea* subsp. *cremea* JCM 3308^T and *A. cremea* subsp. *rifamycinii* JCM 3309^T were 56.5 ± 4.9 and 31.0 ± 22.6 %, respectively. According to the 70 % DNA–DNA relatedness cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987), strain IM17-1^T represents a novel species. This assumption was also confirmed by differences in phenotypic characteristics observed between strains IM17-1^T, *A. cremea* subsp. *cremea* JCM 3308^T and *A. cremea* subsp. *rifamycinii* JCM 3309^T, such as the production of melanoid pigments, the composition of polar lipids, menaquinones, whole-cell sugars and fatty acids, and carbon utilization patterns (Table 1).

On the basis of the cultural, morphological, physiological and chemotaxonomic characteristics, 16S rRNA gene sequence similarity and DNA–DNA hybridization results, strain IM17-1^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura apis* sp. nov. is proposed.

A. cremea subsp. *rifamycinii* appeared on validation list no. 23 (Gauze *et al.*, 1987) as a new subspecies and *A. cremea* subsp. *cremea* was automatically created by Rule 46 (Howey *et al.*, 1990). Our findings suggested that taxonomic revision of these subspecies is necessary. We found that the level of DNA–DNA relatedness between *A. cremea* subsp. *cremea* JCM 3308^T and *A. cremea* subsp. *rifamycinii* JCM 3309^T was 34 ± 4.2 %, which was much lower than the threshold recommended for members of a single genomic species (Wayne *et al.*, 1987). The partial 16S rRNA gene sequence of *A. cremea* subsp. *rifamycinii* JCM 3309^T was also analysed to confirm the previously determined data and to obtain a longer 16S rRNA gene sequence. *A. cremea* subsp. *rifamycinii* JCM 3309^T (accession number AB557595) exhibited 98.5 % 16S rRNA gene sequence similarity to *A. cremea* subsp. *cremea* NBRC 14182^T (accession number AB462291). These values supported the elevation of *A. cremea* subsp. *rifamycinii* to an independent species (Stackebrandt & Ebers, 2006). Some phenotypic differences, such as differences in growth at 45 °C and menaquinone and fatty acid compositions, between the two subspecies are listed in Table 1. These differences were previously documented in a taxonomic revision of the genus *Actinomadura* based on chemotaxonomic properties (Kroppenstedt *et al.*, 1990). It is evident from the present and previous studies that *A. cremea* subsp. *rifamycinii* JCM 3309^T merits status as the type strain of a

Table 1. Characteristics of strain IM17-1^T and the type strains *Actinomadura cremea* subsp. *cremea* JCM 3308^T and *Actinomadura cremea* subsp. *rifamycinini* JCM 3309^T

All data are from this study, except where indicated otherwise. Strains: 1, IM17-1^T; 2, *A. cremea* subsp. *cremea* JCM 3308^T; 3, *A. cremea* subsp. *rifamycinini* JCM 3309^T. +, Positive; (+), moderate; ±, poor; –, negative; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; Fuc, fucose; Gal, galactose; Glu, glucose; Mad, madurose; Man, mannose and moderate Rib, ribose.

Characteristics	1	2	3
Number of spores per chain	3–5	3–8	3–8
Diffusible pigment production in ISP 7 medium	+	–	–
Melanoid pigment production in ISP 6 medium	–	–	+
Growth at 45 °C	+	–	+
Polar lipids	DPG, PI, PIMs	DPG, PI, PG*	DPG, PI, PIMs*
Menaquinones	MK-9 (H ₆) (53 %), MK-9 (H ₈) (26 %), MK-9 (H ₄) (8 %)	MK-9 (H ₆) (83 %), MK-9 (H ₄) (11 %), MK-9 (H ₈) (6 %)*	MK-9 (H ₈) (41 %), MK-9 (H ₆) (39 %), MK-9 (H ₁₀) (5 %)
Fatty acids (%):			
C _{14:0}	1.7	3.9	2.5
C _{16:0}	33.8	41.2	40.9
C _{17:0}	<1	<1	1.3
C _{18:0}	6.9	4.3	4.0
C _{16:1ω9c}	<1	1.1	<1
C _{18:1ω9c}	32.7	14.6	25.6
iso-C _{16:0}	1.9	1.8	2.3
10-Methyl C _{18:0}	8.2	13.4	9.4
Summed feature 3†	8.7	16.3	9.3
Cell-wall sugars	Fuc, Gal, Glu, Mad, Man, Rib	Gal, Mad, Man, Rib*	Gal, Mad, Man, Rib*
DNA G + C content (mol%)	73.7	75.1*	74.5
Utilization of:			
L-Arabinose	±	–	(+)
Sucrose	–	±	±
Inositol	–	±	±
Rhamnose	±	±	±
D-Xylose	–	±/–	±/–

*Data from Kroppenstedt *et al.* (1990).

†Summed feature 3 comprises C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

novel species of the genus *Actinomadura*, for which the name *Actinomadura rifamycinini* sp. nov., comb. nov. is proposed.

Description of *Actinomadura apis* sp. nov.

Actinomadura apis (a'pis. L. gen. *apis* of/from a honey bee, the genus name of the true honey bee *Apis mellifera* (Linnaeus, 1758), a hive of which was the source of the type strain).

Cells are aerobic Gram-reaction-positive actinomycetes that form a branched substrate mycelium. Aerial mycelium at maturity forms short branched spore chains with 3–5 spores. Spore shape is oval and the spore surface is warty at maturity. On ISP 2 agar, colonies are lacking in aerial mycelia and have a cartilaginous appearance. Good growth occurs on glucose–yeast extract agar and moderate growth occurs on ISP 2, 3, 4, 5 and 7 media, Bennett agar, glucose–asparagine agar,

Hickey–Tresner agar, oatmeal–nitrate agar and nutrient agar. Substrate mycelia are dark-olive to light-brown on various agar media. Aerial mycelia and sporulation are found on ISP3, 4, 5 and 7 media, Hickey–Tresner agar and oatmeal–nitrate agar. A soluble greenish-black pigment is produced on ISP 2 and ISP 7 media. Growth occurs at 20–45 °C and pH 6–12 and moderate growth occurs in the presence of 4 % (w/v) NaCl. D-Glucose, L-arabinose and rhamnose are utilized as sole carbon sources but sucrose, D-xylose, inositol, D-mannitol, D-fructose and raffinose are not. Negative for the production of melanoid pigments. The dominant diaminopimelic acid isomer in the cell wall is *meso*-2,6-diaminopimelic acid. Fucose, galactose, glucose, madurose, mannose and ribose occur in whole-cell hydrolysates. Polar lipids detected include diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major menaquinones are MK-9(H₆) and MK-9(H₈); minor amounts of MK-9(H₄) are also present. The fatty acid profile

Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1172 nt) showing the relationship between strain IM17-1^T and the type strains of closely related species of the genus *Actinomadura*. *Streptosporangium album* DSM 43023^T was used as an outgroup. Bootstrap values >50% (based on 1000 replications) are given at nodes. Asterisks indicate the clades that were conserved when the maximum-parsimony and minimum-evolution methods were used to construct phylogenetic trees. Bar, 0.005 substitutions per nucleotide position.

is characterized by the presence of significant amounts (>7% of the total) of C_{16:0}, C_{18:1ω9c}, summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) and 10-methyl C_{18:0}.

The type strain, IM17-1^T (=JCM 16576^T =TISTR 1980^T), was isolated from a honey bee (*Apis mellifera*) hive in Chiang Mai Province, Thailand. The DNA G+C content of the type strain is 73.7 mol%.

Description of *Actinomadura rifamycini* (Gauze *et al.* 1987) sp. nov., comb. nov.

Actinomadura rifamycini (ri.fa.my.ci'ni. N.L. gen. n. *rifamycini* of rifamycin, referring to an antibiotic, rifamycin).

Basonym: *Actinomadura crenea* subsp. *rifamycini*. Gauze *et al.* 1987.

Forms branched substrate mycelia. Aerial mycelia at maturity form short spore chains with 3–8 spores per chain. Spore shape is oval and the spore surface is warty.

Good growth occurs on ISP 2 medium; poor growth on ISP 3 and 4 media. Substrate mycelia are brown on ISP 2

medium and colourless on ISP 3 and 4 media. Aerial mycelia are white to pale-pink and sporulation occurs in ISP 2, 3 and 4 media. A soluble brown pigment is produced in ISP 2 medium. Growth occurs at 20–50 °C and pH 6–12; moderate growth occurs in the presence of 4% (w/v) NaCl. D-Glucose, L-arabinose, sucrose, inositol and rhamnose are utilized as sole carbon sources but D-mannitol, D-fructose, D-xylose and raffinose are not. Production of melanoid pigments is positive only in ISP 6 medium after 4 days of growth. The dominant diaminopimelic acid isomer in the cell wall is *meso*-2,6-diaminopimelic acid. Galactose, madurose, mannose and ribose occur in whole-cell hydrolysates. Polar lipids detected include diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major menaquinones are MK-9(H₈) and MK-9(H₆); minor amounts of MK-9(H₁₀) are also found. The fatty acid profile is characterized by the presence of significant amounts (>7% of the total) of C_{16:0}, C_{18:1ω9c}, 10-methyl C_{18:0} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH).

The type strain is JCM 3309^T (=ATCC 33264^T =DSM 43936^T =INA 1349^T =KCTC 9248^T =NBRC 14183^T

=NCIMB 12768^T =NRRL B-16122^T =VKM Ac-1085^T). The G+C content of the DNA of the type strain is 74.5 mol%. The type strain produces rifamycin.

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