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ISOLATION, CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF MYXOBACTERIA FROM TWO OUTERMOST ISLANDS OF INDONESIA

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ABSTRACT

Myxobacteria are Gram negative bacteria commonly found in soil, tree bark, and decay wood. These bacteria have unique social behaviors by forming fruiting bodies, moving by gliding motility and preying on other microorganisms. The research was conducted to isolate, characterize, and identify indigenous myxobacteria from Sumba and Papua Islands of Indonesia as a preliminary step to utilize their potential in the pharmaceutical industry. Myxobacteria were isolated using filter paper and baiting with *Escherichia coli* to obtain cellulolytic and bacteriolytic myxobacteria, respectively. Characterization of myxobacteria was performed with Gram staining, observation on pigmentation, morphology of vegetative cells, fruiting bodies, and myxospores. Molecular identification was conducted based on 16S rRNA gene sequence analysis. A total of 10 myxobacterial strains were successfully isolated and purified. All isolates obtained were Gram negative, rod shaped with yellow or orange pigmentation. Fruiting bodies observed contained spherical myxospores. Molecular identification of these bacterial strains showed that they belong to myxobacteria from suborder Cystobacterineae, namely *Myxococcus fulvus*, *Myxococcus stipitatus*, and *Melittangium lichenicola*. To our knowledge, this is the first record of their occurrence in Indonesia.

Keywords: characterization, identification, isolation, myxobacteria

INTRODUCTION

In 1809, a German botanist Heinrich Friederich Link reported his observation on myxobacterial fruiting bodies of *Polyangium vitellinum* and described them as "gasteromycete", member of fungi Basidiomycota (Link 1809). Two more species, *Stigmatella aurantiaca* and *Chondromyces crocatus,* were described 48 years later (Berkeley 1857). They were classified as hyphomycetes, a fungi imperfecti. They continued to be mistaken for fungi until a United State botanist Roland Thaxter introduced them as myxobacteria in 1892 (Thaxter 1892). It took about 20 years before Thaxter's work became widely accepted. Until 2006, there were approximately 50 species of recognized myxobacteria which were grouped into 3 suborders, 6 families, and 17 genera (Shimkets *et al.* 2006).

Myxobacteria are Gram negative bacteria predominantly found in terrestrial habitats, such as soil, decaying plant materials, and bark of living or dead trees. However, some reports revealed that myxobacteria can also be isolated from marine samples (Zhang *et al.* 2005; Brinkhoff *et al.* 2012). Among the reported myxobacteria collected, those from marine samples were novel, including *Haliangium ochraceum, H. tepidum* (Fudou *et al.* 2002), *Enhygromyxa salina* (Iizuka *et al.* 2003a), *Plesiocystis pacifica* (Iizuka *et al.* 2003b) and *Pseudenhygromyxa salsuginis* (Iizuka *et al.* 2013). Myxobacteria in general are characterized by their ability to form fruiting bodies and their gliding motility on solid surface. The fruiting bodies are formed after exhaustion of the food supply and contain dry resistant myxospores. Colonies can spread into an unoccupied area. This spreading

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movement is called swarming behavior. The shape, size, color, or arrangement of vegetative cells, swarms, fruiting bodies, and myxospores are important in determining genus of myxobacteria. In contrast to most bacteria, myxobacteria are also capable of lysing living cells. For this reason, they are called predators. Based on the specialization of myxobacteria in degrading biomacromolecules, they are divided into bacteriolytic myxobacteria that lyse whole cells of other microorganisms and cellulolytic myxobacteria that efficiently decompose cellulose instead of living cells (Singh 1947; Hou *et al.* 2006).

Myxobacteria are known for their enormous potential in producing secondary metabolites with various biological activities. For certain reasons, their utilization as potential secondary metabolite producers is limited. They are relatively difficult to isolate and purify. In addition, only some of their species are easily grown in liquid culture during fermentation. Thus, they are often overlooked by industrial sectors. However, due to the emerging of multiple drugs resistance in pathogenic microorganisms, the need to find new alternatives of potential bioactive compounds from natural resources, including myxobacteria is inevitable. Myxosporin, myxovalargin, and myxothiazol are some of the secondary metabolites produced by myxobacteria with antimicrobial activity. These compounds are extracted from typical bacteriolytic myxobacteria, *Myxococcus fulvus* (Dawid 2000). The genus *Myxococcus* is also reported to produce myxovirescin (Williams & McGill 1990) and myxalamides (Gerth *et al.* 1983; Konovalova *et al.* 2010). Thuggacin antibiotics active against *Mycobacterium tuberculosis;* the causative agent of tuberculosis, were reported to be *Chondromyces crocatus* (Buntin *et al.* 2010). Another secondary metabolite, epothilone that acts upon cancer cells was extracted from cellulolytic myxobacteria, *Sorangium cellulosum* (Gerth *et al.* 1996).

The myxobacteria were isolated from various samples and places, such as seawater and sediment samples from Shandong province in China (Li *et al.* 2002); marine sediment from Santa Barbara in US, Texel in Netherlands, and Bokum in Germany (Schäberle *et al.* 2010); and also soil samples from Yunnan, Qinghai, Hebei and Yuenan in China (Zhang *et al.* 2003); India (Singh & Singh 1971); and Kiritimati Island, Republic of Kiribati (Mohr *et al.* 2016). There is no sufficient record of this bacterial group that has been isolated in Indonesia despite their medical importance, except a report on a comparative analysis of predation of *Myxococcus xanthus* isolated from Sulawesi in 2010 (Morgan *et al.* 2010). However, studies on diversity and composition of prokaryotic communities in Sumatera using culture independent methods revealed that fungi-like myxobacteria (*Sorangium* and *Haliangium*) were detected in the soils samples and were slightly more abundant in the managed soils than in rainforest soils (Schneider *et al.* 2015).

Sumba and Papua islands are some of the outermost islands of Indonesia. The geography and ecology of these islands are different from each other. However, like most Indonesian areas, they are assumed to be a biodiversity rich habitat, particularly for microbes. Therefore, this research is conducted to isolate, characterize, and identify indigenous myxobacteria in order to record their occurrence in Indonesia and is a preliminary step to exploit their potential as a natural producer of anti-infective.

MATERIALS AND METHODS

Materials and Sampling Methods

Materials used in this research are listed in Table 1. These samples were collected from two different locations of Indonesia in April 2016. Soils were sampled using composite method. Soil and limestone samples were air dried overnight to reduce the growth of untargeted micro-organisms. In total, the number of samples used in this study was 37.

Isolation of Cellulolytic and Bacteriolytic Myxobacteria

The isolation of cellulolytic and bacteriolytic myxobacteria was conducted using methods described by Reichenbach and Dworkin (1992) with some modifications. Cellulolytic myxobacteria were isolated by placing a drop of soil sample, limestone sample, and a fragment of decay wood onto a piece of Whatman No. 1 filter paper sized 1 cm^2 on Stan 21 agar media (a mix of A solution: 1 g K_2HPO_4, 0.02 g yeast extract, 10 g agar in 700 mL distilled water; and B solution: 1 g KNO_3, 1 g $MgSO_4.7H_2O$, 1 g $CaCl_2.2H_2O$, 0.2 g $FeCl_3$, 0.1 g $MnSO_4.7H_2O$ in 300 mL distilled

Table 1 Sampling locations and materials used to isolate myxobacteria

Sampling location	Altitude (masl)	pH	T (°C)	Samples	Number of samples
Wanggameti National Park, Sumba, East Nusa Tenggara					
E 120° 15.360' S 10° 04.696' – E 120° 16.703' S 10° 03.496'	983-1164	7	23-36	Soil Limestone Decay wood	13 3 10
Tambraw, West Papua					
E 132° 15' 05.2" S 00° 45' 0.99" – E 132° 44' 05.8" S 00° 52' 0.94"	443-891	6.5	26.5-32	Soil Decay wood	7 4

water). These solutions were autoclaved separately. Solution A and B were combined and then supplemented with 25 µg/mL cycloheximide. This medium was designated as ST21CX. The samples in ST21CX media were incubated at 30°C for 2-4 weeks.

Bacteriolytic myxobacteria were isolated using baiting technique with *Escherichia coli*. *E. coli* cells were cultured on Luria Broth (LB) medium (10 g/L Tryptone, 10 g/L NaCl, 5 g/L yeast extract suspended in 1L distilled water) for 24 hours. *E. coli* suspension was then centrifuged for 10 minutes at 10,000 rpm. The bacterial pellet was resuspended with cycloheximide solution 25 µg/mL enough to make a thick slurry of bacterial cells. *E. coli* cells were then cross striked on a water agar (WCX) medium (1 g/L $CaCl_2.2H_2O$, 15 g/L agar, supplemented with 25 µg/mL cycloheximide). In the center of the cross, a pea-sized amount of soil and limestone sample was inoculated. The samples in WCX media were then incubated at 30°C for 2-4 weeks.

Purification of Myxobacteria

Myxobacteria obtained from both methods were transferred using sterilized syringe needle to a fresh WCX medium cross striked with autoclaved *E. coli* to purify them. Dissecting microscope was used to recognize the myxobacteria so that this direct purification technique can be done. The bacteria were then incubated at 30°C for 1-3 weeks.

The fruiting bodies or swarm cells that produce clear zones around the dead *E. coli* were transferred to a modified VY/2CX medium consisting of 5 g/L Baker's yeast Fermipan, 1 g/L $CaCl_2.2H_2O$, 15 g/L agar, 0.5 µg/mL cyanocobalamin, supplemented with 25 µg/mL cycloheximide using sterilized syringe needle. The transferring process was conducted several times until pure cultures of myxobacteria were obtained. Pure cultures were stored in 10% gycerol stock solution supplemented with 0.1% $CaCl_2.2H_2O$ at -80°C.

Phenotypic Characterization

The isolates obtained were morphologically observed with the help of dissecting (Olympus SZ) and binocular (Olympus BX43) microscopes. Several characters were observed including fruiting bodies that emerge days after incubation, vegetative cells, and also the pattern and the color of swarm colonies. The fruiting bodies were crushed to examine the myxospores. Each of the pure isolates was subjected to Gram staining using crystal violet, iodine, and safranin reagents.

Molecular Identification based on 16S rRNA Gene Analysis

Molecular identification was conducted based on 16S rRNA gene analysis. Genomic DNA was extracted using a set of processes started by rinsing bacterial cells with 500 µL TE buffer pH 8.0. After centrifugation at 13,000 rpm for 5 minutes, pellet obtained was resuspended with 50 µL TE buffer pH 8.0 and 300 µL extraction buffer consist of Tris-HCl, EDTA, sodium dodecyl sulfate, and NaCl. This suspension was homogenized using vortex mixer for 5 minutes. An amount of 150 µL 3M sodium acetate was added to the suspension and incubated for 10 minutes at room temperature. After incubation, the suspension was centrifuged at 13,000 rpm for

another 5 minutes. The supernatant obtained was transferred to a new microtube and gently mixed with isopropanol in the same volume. This mixture was centrifuged for 10 minutes at 13,000 rpm. The pellet obtained was suspended with 70% ethanol and centrifuged for 1 minute at 13,000 rpm. The DNA or pellet was air dried and resuspended with 50 µL TE buffer pH 8.0. The quality and quantity of genomic DNA was examined by BioSpec-nano micro-volume UV-Vis spectrophotometer (Shimadzu).

Universal eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify 16S rRNA gene (Lane 1991). Gene amplification was performed under the following conditions: pre denaturation at 94 °C for 2 minutes, subsequently followed by 35 cycles of denaturing at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, elongation at 72 °C for 1 minute, and final extension at 72 °C for 10 minutes in Mastercycler Gradient (Eppendorf). PCR products were checked on 1% agarose gel stained with ethidium bromide solution and observed under UV transilluminator. These DNA fragments were sequenced by Macrogen Inc. (South Korea) using 27F and 1492R primers in ABI 3730xl DNA Analyzer.

The sequences of the isolates obtained were analyzed using BioEdit program (Hall 1999). The identification of phylogenetic neighbors was initially carried out by the BLASTN (Altschul *et al.* 1997) program against the database containing type strains with validly published prokaryotic names and representatives of uncultured phylotypes (Kim *et al.* 2012). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.ezbiocloud.net/eztaxon). The 16S rRNA gene sequences in this study were submitted to GenBank NCBI under these following accession numbers; MG561397 (SMDw06.2), MG561394 (SMCv05.1), MG561395 (SMCv05.2), MG561396 (SMCv05.3), MG561388 (PS3.1), MG561389 (PS3.2), MG561390 (PS3.3), MG561398 (SMS05.1), MG561399 (SMS05.2) and MG561400 (SMS05.3). All the identified isolates were also deposited in Indonesian Culture Collection (InaCC) using the number SMDw06.2 (InaCC B1220), SMCv05.1 (InaCC B1221), SMCv05.2 (InaCC B1222), SMCv05.3 (InaCC B1223), PS3.1 (InaCC B1224), PS3.2 (InaCC B1225), PS3.3 (InaCC B1226), SMS05.1 (InaCC B1227), SMS05.2 (InaCC B1228) and SMS05.3 (InaCC B1229).

Phylogenetic Analysis

Multiple alignment of all the DNA sequences were performed by MUSCLE program (Edgar 2004). Type strains and their sequences were collected from a list of prokaryotic names with standing in nomenclature (LPSN) website (www.bacterio.net) and GenBank (www.ncbi.nlm.niv.gov). Kimura 2-parameter model was selected to calculate the distance matrices between sequences (Kimura 1980). Phylogenetic tree was constructed using neighbor-joining method (Saitou & Nei 1987) with 1,000 replicates of bootstrap. All these programs are implemented in MEGA version 6 (Tamura *et al.* 2013). *Desulfovibrio desulfuricans* Acc. No. M34113 which belongs to delta-Proteobacteria, was used as out-group in constructing the phylogenetic tree.

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RESULTS AND DISCUSSION

Taxonomically, the fruiting gliding myxobacteria belong to the phylum of Proteobacteria, subphylum delta-Proteobacteria, order Myxococalles, and consist of 3 suborders (Cystobacterineae, Nannocystineae, Sorangiineae). They are characterized by rod and yellow, orange, or red pigmented cells. In this study, yellow and orange pigmented swarms and fruiting bodies emerged on Whatman No. 1 filter paper on ST21CX and on streaked *E. coli* on WCX agar media, after 2-3 weeks of incubation. Visible clear zones also appeared around the streaked *E. coli* on WCX media after 5-7 days of incubation. It is known that bacteriolytic myxobacteria consume other living bacteria or yeasts as their nutrient source. Hence, these clear zones were an indication of predatory activity of bacteriolytic cells. The predatory activity of myxobacteria on other bacteria and yeasts is supported by the production of extracellular lytic enzymes and antibiotics (Xiao *et al.* 2011). This mechanism also plays an important role in competing with their natural opponents in a habitat.

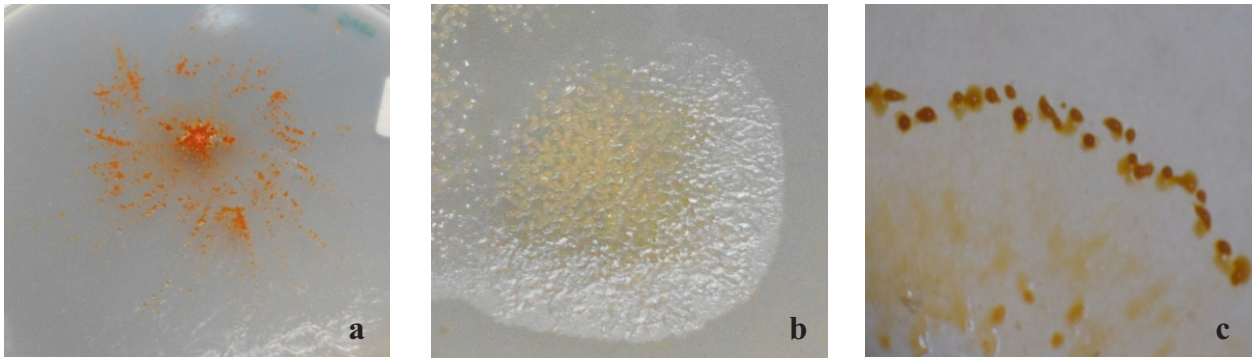

Figure 1 Morphological appearance of purified myxobacterium collected from Sumba SMS05.1 (a), SMDw06.2 (b), and Papua PS3.1 (c) on VY/2CX agar medium after incubation for 5-10 days. Fruiting bodies appeared as yellow/orange cells aggregate.

Under a dissecting microscope, swarms and fruiting bodies grown on samples were transferred to a new WCX media with dead *E. coli* as a nutrient source and subsequently transferred to VY/2CX agar using a sterile syringe needle. This purification method was able to produce a more uniform colony appearance (Fig. 1). From a total of 37 samples using ST21CX and WCX medium as isolation media, only 10 myxobacterial isolates were successfully recovered and purified. Compared to common bacteria, purification of myxobacteria is relatively tricky. They easily carry contaminates, such as fungi, other bacteria, and soil amoeba, because they produce slime to help them move on a solid medium. A number of purification techniques have been developed to increase proportion of pure cultures. Improved methods, such as purification with crystal violet and second baiting technique subjected to fruiting body can improve the proportion of pure culture obtained up to 42.5% and 69.7%, respectively (Zhang *et al.* 2003).

All the 10 bacterial isolates were Gram negative bacteria. They were rod shaped with slight differences in size. Morphologically, the isolates obtained vary in color, shape and swarming pattern. These isolates showed conspicuous characteristic of myxobacteria by producing fruiting bodies. Round or spherical myxospores were observed under the microscope from crushed fruiting bodies. These characteristics were identical to morphology of myxobacteria described in Bergey's Manual (Reichenbach 2005). Based on their morphology, these isolates were grouped into three morphological groups (Table 2).

Morphological identification based on vegetative cells, swarms, fruiting bodies, and myxospores is still valid for some myxobacteria genera. However, these morphological characters are not stable and may change or be lost under artificial growth condition despite expressed by their genotype. Hence, identification and classification of myxobacteria remains challenging for most of the genera and species (Garcia *et al.* 2010). This is also partly due to the fact that some recently isolated myxobacteria strains do not usually have typical myxobacterial morphological characteristics. Therefore, molecular identification is needed to confirm the identity of the isolates.

Analysis on 16S rRNA gene sequences of 10 isolated bacteria revealed that these isolates were member of myxobacteria group. They belong to the genera *Myxococcus* and *Melittangium* with more than 99% similarities (Table 3). *Myxococcus* and *Melittangium* genera were often found in soil samples throughout the world, from tropical rain forests, central European forests, to Mediterranean regions and so far, to our

Table 2 Isolates grouping based on morphological appearance

Morphological characteristics of colony	Myxospores	Total isolates
Orange, centrifugal pattern, orange fruiting body	Spherical	3
Yellow swarms, orange fruiting body	Spherical	4
Orange swarms, short stalked orange fruiting body	Spherical	3

Table 3 Molecular identification of isolated myxobacteria

Strain (accession number)	Next relative (Ez Taxon)	Sequence similarity (%)	Sequence length (bp)	Sample material	Location
InaCC B1227 (MG561398)	*Melittangium lichenicola* (AM930269)	99.2	1363	Soil	Sumba
InaCC B1228 (MG561399)	*Melittangium lichenicola* (AM930269)	99.3	1351	Soil	Sumba
InaCC B1229 (MG561400)	*Melittangium lichenicola* (AM930269)	99.7	1354	Soil	Sumba
InaCC B1221 (MG561394)	*Myxococcus fulvus* (DQ768117)	99.2	1365	Limestone	Sumba
InaCC B1222 (MG561395)	*Myxococcus fulvus* (DQ768117)	99.5	1333	Limestone	Sumba
InaCC B1223 (MG561396)	*Myxococcus fulvus* (DQ768117)	99.7	1329	Limestone	Sumba
InaCC B1220 (MG561397)	*Myxococcus fulvus* (DQ768117)	99.3	1370	Decay wood	Sumba
InaCC B1224 (MG561388)	*Myxococcus stipitatus* (CP004025)	99.1	1360	Soil	Papua
InaCC B1225 (MG561389)	*Myxococcus stipitatus* (CP004025)	99.3	1343	Soil	Papua
InaCC B1226 (MG561390)	*Myxococcus stipitatus* (CP004025)	99.1	1345	Soil	Papua

knowledge this is the first record of the three species occurrence in Indonesia.

Based on morphological observation supported by molecular identification, diversity of myxobacteria obtained from this work was not numerous. Some isolates might not form typical fruiting bodies at the time of observation, hence overlooked during transferring process. Isolation techniques, incubation time, and media used to isolate myxobacteria still unable to culture various species occur in a habitat in this study. Every method would be a compromise, as different myxobacteria species would grow well on one medium, others would grow less well and show little tendency to form fruiting bodies (Dawid 2000). Hence, the variation on isolation technique, purification technique, and enrichment media are expected to increase the probability to obtain more diverse myxobacteria.

Phylogenetic analysis of the 10 myxobacteria sequences showed that all the isolates are placed in suborder Cystobacterineae (Fig. 2). Taxonomically, genus *Myxococcus* along with *Aggregicoccus, Corallococcus* and *Pyxidicoccus* are classified into the family Myxococcaceae in the suborder Cystobacterineae. On the other hand, the genus *Melittangium* along with *Angiococcus*, *Archangium*, *Cystobacter*, *Hyalangium*, *Stigmatella* and newly described genus Vitiosangium (Awal *et al.* 2017) are classified into the family Cystobacteraceae also in the suborder Cystobacterineae.

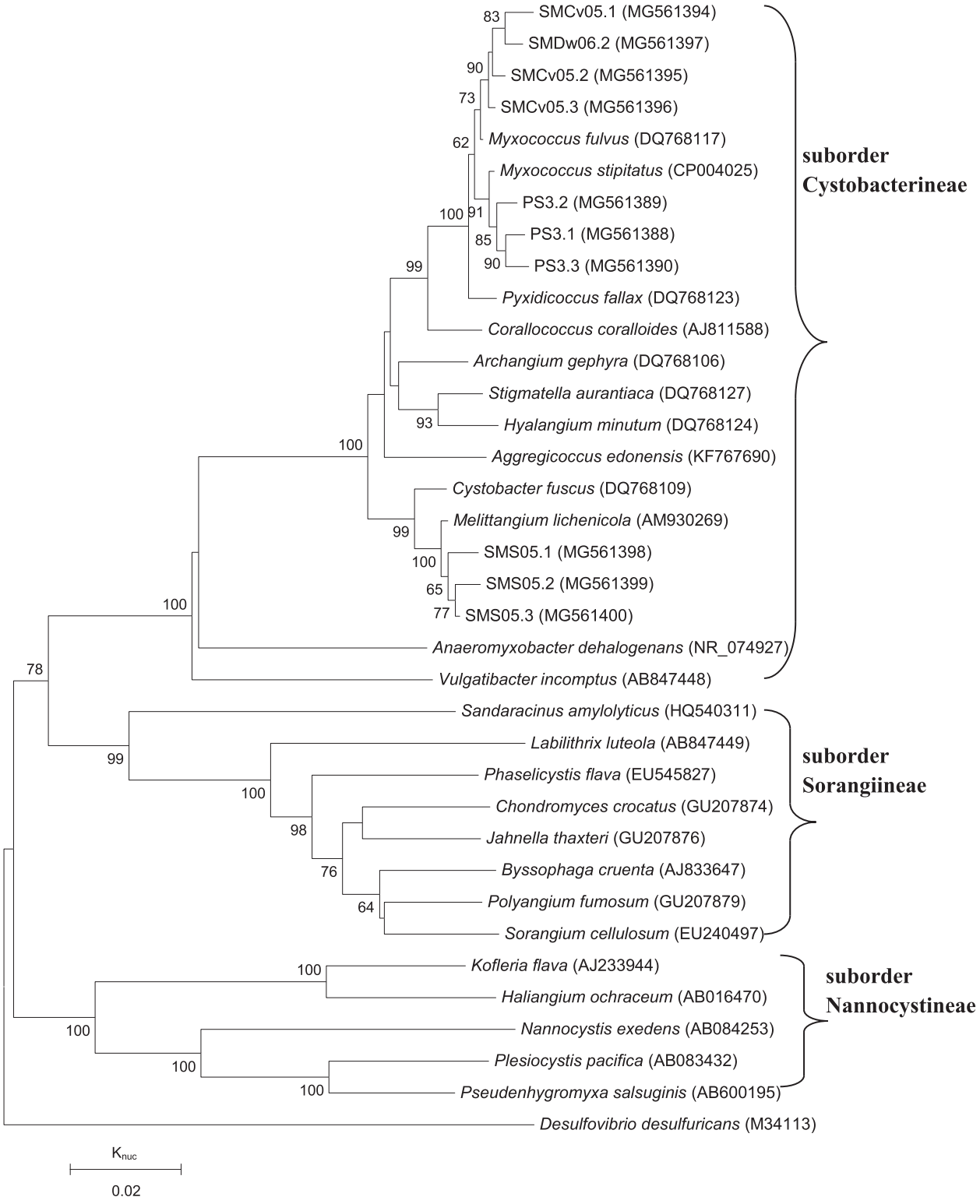

Figure 2 Phylogenetic tree constructed on the basis of 16S rRNA gene sequences using neighbor-joining method. Isolates from Sumba (SM) and Papua (PS) Islands were placed in the suborder Cystobacterineae among myxobacteria species. Bar means 1 substitution per 200 nucleotides. Numerals at branch points indicate the bootstrap value as percentages derived from 1000 replications. Only values greater than 60% are shown.

CONCLUSION

A total of 10 myxobacterial isolates was successfully isolated from soil, limestone and decay wood samples collected from two outermost islands of Indonesia, Sumba and Papua. They were isolated using filter paper and baiting with *E. coli* methods. Characterization of Sumba and Papua isolates revealed that they were Gram negative and rod shaped. Their colonies were yellow and orange in pigmentation, and produce fruiting bodies. Molecular identification showed that they are members of myxobacterial species *Myxococcus fulvus*, *Myxococcus stipitatus* and *Melittangium lichenicola*. To our knowledge, this is the first record of the occurrence of the three species in Indonesia. All the isolates were deposited in Indonesian Culture Collection (InaCC) under the name *Myxococcus fulvus* InaCC B1220, *Myxococcus fulvus* InaCC B1221, *Myxococcus fulvus* InaCC B1222, *Myxococcus fulvus* InaCC B1223, *Myxococcus stipitatus* InaCC B1224, *Myxococcus stipitatus* InaCC B1225, *Myxococcus stipitatus* InaCC B1226, *Melittangium lichenicola* InaCC B1227, *Melittangium lichenicola* InaCC B1228 and *Melittangium lichenicola* InaCC B1229.

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