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Bacillus endophyticus: Symbiotic Bacterium in Subterranean Termites Intestine (Blattodea: Termitoidae) from Bogor, Indonesia

(*Bacillus endophyticus*: Bakteri Simbion pada Saluran Cerna Rayap Tanah (Blattodea: Termitoidae) dari Bogor, Indonesia)

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ABSTRACT

Termites are social insects that play an essential role in the nutritional cycle. In the termite digestive system, some symbionts help the process of cellulose degradation. This study aims to isolate symbiont bacteria in the digestion tract of subterranean termites. The research began with a collection of the termites at IPB University Campus, followed by isolating the symbiotic bacteria from the proctodeum (hindgut), which was then identified based on morphology, physiology, and molecular using the 16S rRNA gene. The six termites found are *Macrotermes gilvus, Odontotermes javanicus, Microtermes insperatus*, and *Capritermes mohri* (Family Termitidae); *Schedorhinotermes javanicus* and *Coptotermes curvignathus* (Family Rhinotermitidae). Of the six termites, we obtained 43 isolates and one isolate with a general character. The 8A_27F isolate has a white, like-elevation button with slippery edges. From physiological tests, these isolates are Gram-positive, have spores, and are aerobic bacteria. 16S rRNA gene identification showed a similarity level of 98% with the *Bacillus endophyticus* species.

Keywords: bacteria, subteranean termites, 16S rRNA, symbionts, proctodeum

ABSTRAK

Rayap merupakan serangga sosial yang berperan penting dalam perputaran siklus nutrisi. Di dalam sistem pencernaan rayap, terdapat simbion yang membantu proses degradasi selulosa. Penelitian ini bertujuan mengisolasi bakteri simbion yang terdapat di dalam saluran cerna rayap tanah. Penelitian diawali dengan koleksi rayap tanah di Kampus IPB University, diikuti isolasi bakteri simbion dari saluran cerna belakang (proktodeum) yang kemudian diidentifikasi berdasarkan morfologi, fisiologi, dan molekuler menggunakan gen 16S rRNA. Enam rayap tanah yang diperoleh adalah *Macrotermes gilvus, Odontotermes javanicus, Microtermes insperatus*, dan *Capritermes mohri* (Famili Termitidae); *Schedorhinotermes javanicus* dan *Coptotermes curvignathus* (Famili Rhinotermitidae). Dari enam rayap diperoleh 43 isolat dan satu isolat yang memiliki karakter umum. Isolat bakteri kode 8A_27F berwarna putih, elevasi seperti tombol dengan tepian licin. Dari uji fisiologis, isolat ini termasuk ke dalam bakteri Gram positif, berspora, dan bersifat aerob. Identifikasi dengan gen 16S rRNA menunjukkan bahwa isolat bakteri tersebut memiliki tingkat kemiripan sebesar 98% dengan spesies *Bacillus endophyticus*.

Kata kunci: bakteri, rayap, 16S rRNA, simbion, proktodeum

INTRODUCTION

Termites are social insects that are beneficial as a decomposer in nature. This animal becomes a good biodegradation agent because it can degrade woodpiles in nature faster than insects or other organisms such as worms, fungi, and bacteria. Its role is vital in the turnover of nutrients that other organisms can utilize (Krishna 1969; Freymann *et al.* 2008; Paul *et al.* 2018).

Termites can degrade lignocellulosic materials such as wood. However, these insects cannot produce their cellulolytic enzymes to destroy wood. Termites are aided by prokaryotic organisms such as bacteria. Bacteria at the end of the digestive tract (proctodeum) associate and produce cellulolytic enzymes to destroy the cellulose they eat. Termites and bacteria have a very beneficial or mutualistic relationship. This mutually beneficial relationship is used to carry out their life.

The diversity of bacterial symbionts has been widely analyzed. Salunke *et al.* (2010) found *Wolbachia* diversity in *Odontotermes* spp. And *Coptotermes heimi*. Mackenzie *et al.*(2007) reported bacteria in the digestive tract of termites *Macrotermes michaelseni* that were amplified with the 16S rRNA gene and obtained IM_4B sequence (DQ312469) that formed a cluster with *Bacillus licheniformis* (X68416). *Paracoccus yeei* is one of the symbionts bacteria found in the digestion tract of worker termite *M. gilvus* that is amplified with the 16S rRNA gene (Febriyanto 2013). *Bacillus* is a minor endosymbiont found in *Coptotermes* sp*., C. acinaciformis, Odontotermes* sp., and *Schedorhinotermes intermedius*. However, the

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presence of this minor bacteria is also influential in aiding metabolic processes (Eutick *et al.*1978, Djunaid *et al.* 2019).

From bacteria in the digestive tract of *O. formosanus* that have been found using amplification of the 16S rRNA gene and RFLP cutting sites, four phylogenetic groups were obtained, namely Firmicutes, Bacteriodates/Chlorobi, Proteobacteria, and Actinobacteria from the bacteria domain (Shinzato *et al.* 2005). The results indicated that the specific bacteria in termites present implications for the co-evolutionary relationship between microbes' indigestion and termites as their host (Shinzato *et al.* 2007).

Bacteria in the termite digestive tract have been widely characterized but only limited to the process of characterization and molecular identification. The conventional characterization process combined with molecular techniques to obtain the results of aerobic bacterial isolation has not been carried out because it is considered very simple and less profitable. This study aims to isolate and characterize the symbiotic bacterial isolates from the termite intestine.

MATERIALS AND METHOD

Termite Sampling and Identification

Termite samples were taken in the rubber plantation behind the IPB University Library (S: 06°33.443' E: 106°43,579') and the oil palm plantation in Cikabayan (S: 06°33.119' E: 106°43.997') using a transect area of 10 m ×10 m. Termites found were sorted by caste, i.e., workers and soldiers were later identified using the identification books Ahmad (1958) and Tho (1992) in the Insect Taxonomy Laboratory of the Department of Plant Protection, Faculty of Agriculture, IPB University. In addition, soldier's caste termites were identified with characteristics easily distinguished by the size and shape of the mandible.

Isolation of Symbiotic Bacteria from Termite Gut

The worker termites from the field (live) were sterilized using 70% alcohol and washed with sterile water. The worker termite cuticles were peeled off with sterile tweezers. The digestive tract will be visible then the proctodeum part was taken and inserted into a 1.5 mL Eppendorf tube. The proctodeum was crushed with micropistiles until smooth, then 1 mL of sterile water was added. Afterwards, serial dilution was performed by flushing on NA (Nutrient Agar) media. The isolation results were incubated at room temperature for 24–48 hours.

Morphological Characterization and Physiology Testing of the Symbiotic Bacteria

The bacteria were purified based on different visualizations (colors, shapes, and elevations). The type of bacteria was tested for Gram type with Gram staining method and KOH 3%. Spores and anaerobic growths were tested using the Schaad *et al.* method (2001).

DNA Extraction of the Symbiotic Bacteria

To the bacterial pellets obtained from culture on NB (nutrient broth) media, 250 uL buffer TE (containing 5 mg/mL lysozyme) was added, then vortexed until homogeneous and incubated at 37°C for 30 minutes. After adding 50 uL SDS 10%, incubation is resumed for 1 hour. Next was the addition of 65 uL NaCl 5 M and 80 uL CTAB-NaCl and incubated at 65 °C for 20 minutes, then added 450 uL chloroform:isoamyl alcohol (24:1) as an organic solvent, mixed and shaken for 30 minutes. The sample was centrifuged at a speed of 11,000 rpm for 15 minutes at 25°C. The supernatant formed was then transferred to a new 1.5 tube of ± 400 uL. Next, the transferred supernation was added with 400 uL isopropanol and incubated for 30 minutes or overnight at 20°C. After incubation, the mixture is centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was removed, then the precipitate formed was washed using 70% alcohol (800 uL), recentrifuged at a speed of 12,000 rpm for 3 minutes at 4 °C. The supernatant was removed, and the DNA pellets were drained in the laminar flow. The obtained DNA pellets were dissolved in 0.5 mM TE, then stored at −4°C (Sambrook *et al.* 1989).

Bacterial DNA Amplification

The DNA fragments were amplified using the polymerase chain reaction (PCR) method. The primers for the 16S rRNA gene were the 27F universal primary (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and 1492R (5'-TTACCTTGTTACGACTT-3') (Turner *et al*. 1999). The PCR conditions were predenaturation at 95°C for 5 minutes, 35 cycles (denaturation at 95 °C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes), and final elongation at 72°C for 10 minutes. The PCR results were electrophoresis at 1% agarose at 75 V for 30 minutes and visualized using an ultraviolet transilluminator. The visualized band were then analyzed for each DNA fragment.

Analysis and Construction of the Phylogenetic Tree

Sequencing was done at the First Base Asia Laboratory; Homology was analyzed on the 16S rRNA gene sequence with GenBank (www.ncbi.nlm.nih.gov) data. The homoloy analysis uses the *Basic Local Alignment Search Tool-Nucleotide* (BLAST-N) program from the *National Center for Biotechnology Information* (NCBI) website (www.ncbi.nlm.nih.gov/Blast.cgi).

The nucleotide sequences of the analysis from the sequencing company were aligned using Clustal-X. The alignment results were sought for homology with the Genbank database using the BLAST-N program (www.ncbi.mlm.niv.gov) and re-aligned using Clustal-X. Furthermore, phylogenetic trees were constructed using the Neighbor-Joining Tree Method program with

1000× bootstrap values in the MEGA 5 program (Tamura *et al.* 2011).

RESULTS AND DISCUSSION

Subterranean Termites at the IPB University Campus

A total of 6 termites found in the IPB University Campus and identified based on the head of soldier caste (Table 1) were *Macrotermes gilvus, Odontotermes javanicus, Microtermes insperatus,* and *Capritermes mohri* (family Termitidae) (Figure 1), and *Schedorhinotermes javanicus* and *Coptotermes curvignathus* (family Rhinotermitidae) (Figure 2). All termite colonies were found in both areas (rubber and oil palm plantations), the termite feed sources. The termites feed on cellulose from wood, including rubber and oil palm trees.

Tarumingkeng (1971) describes the types of termites based on their nesting location. The family of Termitidae was a type of termite that nested in the soil, especially closed to a source of organic material that contains cellulose. Examples of termites from the Termitidae family that commonly attack buildings were *M. gilvus*, *O. javanicus*, and *M. insperatus*. These types of termites could attack objects that were 200 m away from the nest. The Rhinotermitidae family was a subterranean termite that generally lives in soils containing many dead or decomposed organic matter, dead wood, or living wood. The subterranean termites that damage the buildings are *C. curvignathus* and *S. javanicus*.

The species found in the field were types that can damage buildings except for *C. mohri,* a termite species with little potential to damage it. *C. mohri* is humus-eating termite that is very influential on habitat. Pribadi *et al.* (2011) found no humus-eating termites in a residential area on Mount Selamet, Central Java. It was caused by the low rate of spread of termites so that the ability to colonize the surrounding habitat is not extensive. Humus-eating termites were very sensitive to disturbed areas. Ideal habitat conditions for humus-eating termites were tropical forests with dense canopy closure (Eggleton *et al.* 2002).

Isolates and Characters of the Symbiotic Bacteria in the Termite Intestine

A total of 43 isolates were obtained from the results of bacterial isolation from the six termite species. Then the morphological and physiological characterization based on color, elevation, periphery, Gram test, spore test, and an-aerobic test gives one commonly found isolate, namely 8A_27F. This isolate has a white character, elevation-like button, smooth edges, Gram-positive, spore-producing, and aerobic (Figure 3).

Amplified of 16S rRNA Gene and Homology of Isolate 8A_27F

Amplicon target in the 16S rRNA gene using universal primers in isolates 8A_27F showed DNA bands of ± 1500 bp. Wells number 1 and 2 (Figure 4) are replication treatments to determine the universal primary consistency in amplifying 8A_27F isolates.

The alignment of the 16S rRNA 8A_27F gene in this study with other bacteria in GenBank (Table 2) showed the similarity of nucleotide sequences with *Bacillus endophyticus* haplotype (KC237279) with an identity value of 98%. *Bacillus* sp. is one of the symbiotic bacteria that live in plant tissues (Misaghi and Donndelinger 1990). Reva and Priest (2002) found this *B. endophyticus* from cotton plant tissue (*Gossypium* sp.). Symbiotic microorganisms have an important role in helping the metabolic process, namely protecting plants and triggering growth. In addition, symbiotic microbes protect plants from fungi and pathogenic bacteria because they could produce antibiotics for protection, such as *B. cereus* (Pleban and Sørensen 1996).

Genetic Distance and Phylogeny of 8A_27F Isolate

Genetic distance analysis and phylogeny were performed between 8A_27F isolates and *L. cassei* as an out-group. The genetic distance between 8A_27F and *L. cassei* isolates is 0.149. Furthermore, the highest genetic distance between in-groups (*B. endophyticus*) is 0.007 (KC237279_India).

The smaller the value given, the closer (similar) it is to the sequence of species referred to. Likewise, the greater the value, the closer it will be. The 8A_27F bacterial sequence gives a small value to Baen_ KC237279_India and Baen_KF311082_ Pakistan with 0.007 and 0.009 (Table 3). It can also be seen from the location of the second sequence. Indonesia's 8A_27F as *B. endophyticus* sequence was geographically close to India and Pakistan. Whereas the out-group (*L. cassei*_ AB008205) gives a high value of 0.149 (Table 3), the proximity of the 8A_27F sequence was very far compared to the sequence of the other isolates.

The phylogenetic tree analysis results show that the sequence of 8A_27F isolates is in the *Bacillus* group. The 8A_27F sequence is separated from *L. cassei*, the out-group (Figure 5). The sequence of isolates 8A_27F has proximity to *B. endophyticus* sequences from India and Pakistan. In contrast, those from Egypt, China, and Tajikistan sequences are still in one clade (group *B. endophyticus*) but have different geographical regions (Figure 3). Therefore, it is possible to have different base arrangements. Compared with the sequence *B. carboniphilus* and *B. licheniformis*, the 8A_27F isolates are separated because they have different species even though they were still in the genus *Bacillus*.

Table 1 Characteristics of subterranean termites based on head capsule and mandible

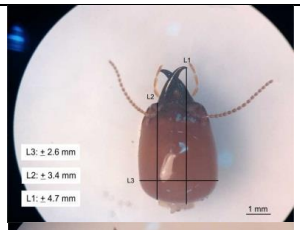
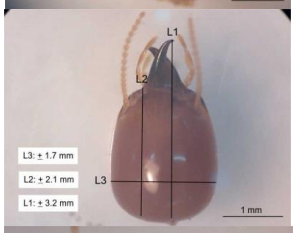
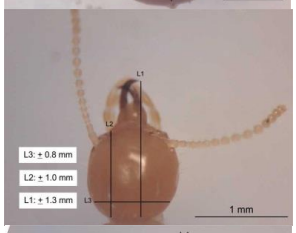
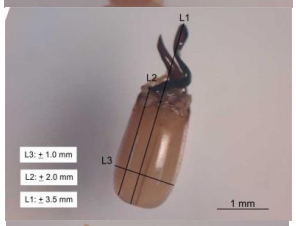
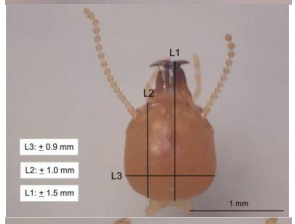
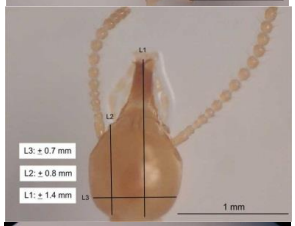
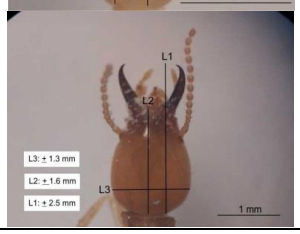
Species	Description	Illustration
M. gilvus (major)	Reddish brown head, sparsely hairy, head length with mandible 4.80–5.48 mm, without mandible 3,40–3.65 mm, width 2,30–2.68 mm; 17-segment antenna.	
M. gilvus (minor)	Reddish-brown head, 17- antenna, third antennal segment somewhat longer than the second as long as the fourth; head length with mandible 3.07–3.43 mm, without mandible 1.84–2.08 mm, width 1.18–1.40 mm.	
O. javanicus	The labrum extends up to the tooth of the left mandible, the inner edge of left mandible below the middle; reddish-brown head; head length with mandible 3.27–3.36 mm, without mandible 2.19–2.49 mm, width 1.80–1.94 mm; 17 -segment antenna.	
M. insperatus	Round head, yellowish, sparsely hairy; mandible with denticle, hook-like; head length with mandible 1.14–1.52 mm, without mandible 0.80–0.95 mm; 14-segment antenna.	
C. mohri	Head length is twice the width, a few scattered bristles; asymmetrical mandible; head lenght with left mandible 3.36–3.65 mm, without mandible 1.84–2.18 mm, width 0.77–1.00 mm; 14-segment antenna.	
S. javanicus (major)	Yellowish head, 16-segment antenna, the third is longer than the second; head length with mandible 1.10–1.95 mm, width 0.89–1.15 mm.	
S. javanicus (minor)	Yellowish head, a few long bristles; 15-segment antenna, the third slightly shorter than the fourth; head length with mandible 1.41–1.52 mm, without mandible 0.85–0.90 mm, width 0.74–0.82 mm.	
C. cuvignathus	Broadly oval head, fontanelle at the front; 14 to 16-segment antenna, the second segment length is twice the third segment; head length with mandible 2,34–2.65 mm, without mandible 1,59–1.62 mm, width 1,34–1.43 mm.	

Figure 1 Soldiers of subterranean termites Family Termitidae, *M. gilvus* [major] (a)*, M. gilvus* [minor] (b), *O. javanicus* (c)*, M. insperatus* (d)*,* and *C. mohri* (e) (scale bar 1 mm).

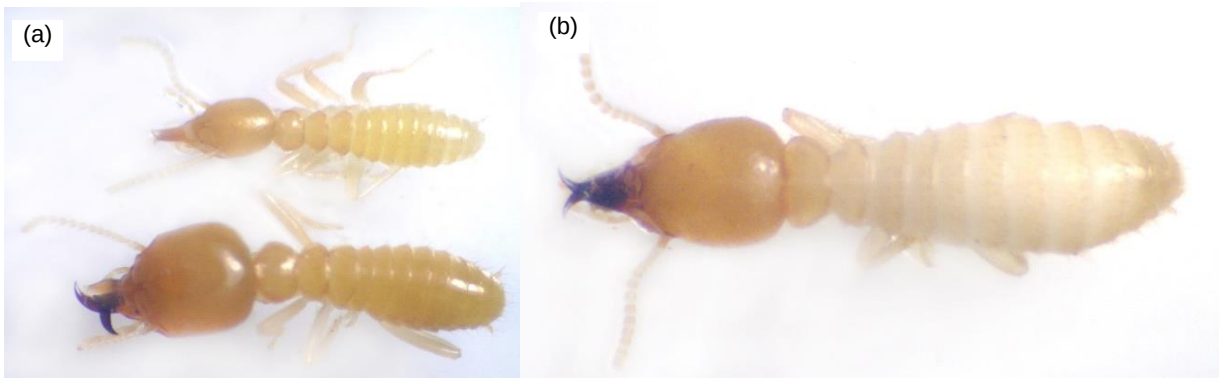


Figure 2 Soldiers of subterranean termites Family Rhinotermitidae, *S. javanicus* [above: minor, below: major] (a), and *C. curvignathus* (b) (scale bar 1 mm).

CONCLUSION

The six termites at IPB University Campus are *M. gilvus, O. javanicus, M. insperatus, C. mohri, S. javanicus,* and *C. curvignathus.* Isolation and characterization from worker castes of the six termites resulted in 43 isolates and one isolate with a general character and was often found in all types of termites. The morphological characteristics are white, with elevation-like buttons, and smooth edges. The physiological test indicates Gram-positive, has spores during spore staining, and is aerobic. The DNA extraction and amplification using the 16S rRNA gene exhibit sequencing results with a 98% similarity level, referring to *Bacillus endophyticus* species.

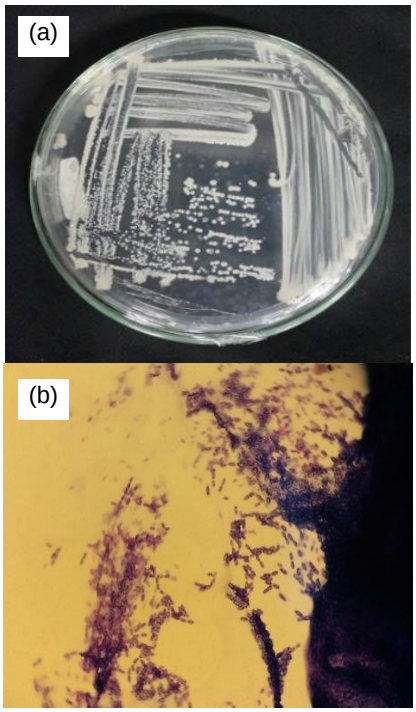


Figure 3 Isolated colony of 8A_27F in NA medium (a) and rod shape (400× magnification) (b).

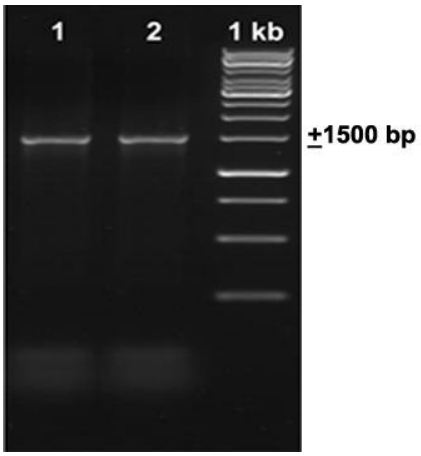


Figure 4 Visualisation on 16S rRNA gene amplification of isolat 8A_27F.
Remarks: 1: 8A_27F (replication 1), 2: 8A_27F (replication 2), and 1 kb: marker 1kb or 1000 bp

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Table 2 The results of BLAST from sequencing 8A_27F isolate

Description	Max score	Total score	Query cover	E-value	Ident	Acc. No
Bacillus endophyticus strain MDSR34 16S ribosomal RNA gene, partial sequence	1801	1801	99%	0.0	98%	KC237279.1
Bacillus sp. HJB001 16S ribosomal RNA gene, partial sequence	1801	1801	99%	0.0	98%	HQ331100.1
Bacillus sp. HJB002 16S ribosomal RNA gene, partial sequence	1801	1801	99%	0.0	98%	HQ331101.1
Bacillus endophyticus strain BAB-2484 16S ribosomal RNA gene, partial sequence	1797	1797	99%	0.0	98%	KC443078.1
Bacillus endophyticus strain C2-2 16S ribosomal RNA gene, partial sequence	1797	1797	99%	0.0	98%	HM770880.1

Table 3 Genetic distance of 8A-27F isolate

1	2	**3**	4	5	6	7	8	9
ID								
0.002	ID							
0.007	0.009	ID						
0.015	0.017	0.022	ID					
0.006	0.008	0.014	0.015	ID				
0.006	0.008	0.014	0.015	0.000	ID			
0.059	0.060	0.060	0.069	0.057	0.057	ID		
0.063	0.063	0.069	0.071	0.061	0.061	0.045	ID	
0.148	0.147	0.149	0.153	0.145	0.145	0.142	0.135	ID

Remarks: 1: Baen_KC237279_India; 2: Baen_KF311082_Pakistan; 3: 8A_27F; 4: Baen_KF011545_Mesir; 5: Baen_KJ542778_China; 6: Baen_AF295302_Tajikistan; 7: Baca_AB021182_Japan; 8: Bali_X68416_Jerman; and 9: Laca_AB008205_Japan.

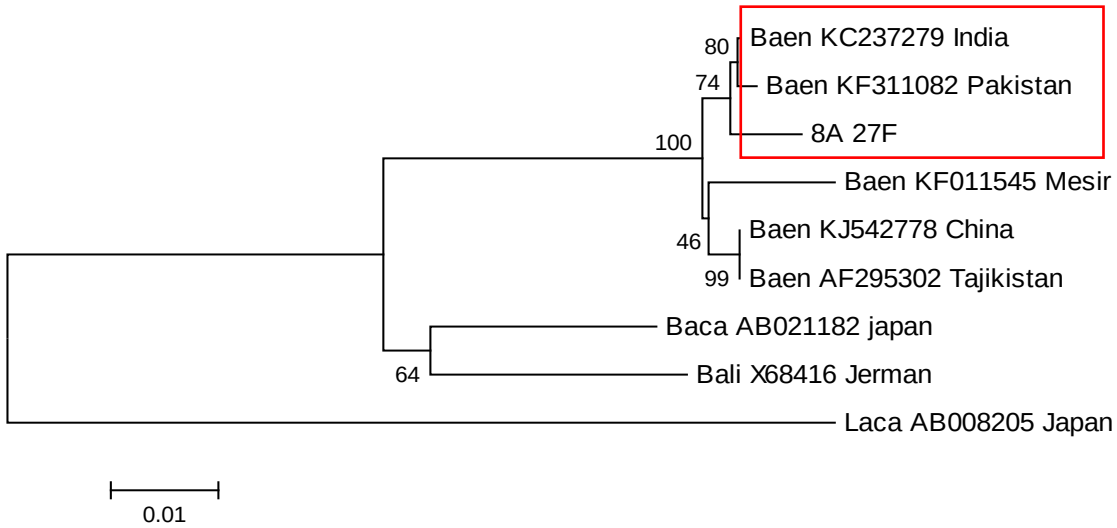

Figure 5 Construction of phylogenetic tree of 8A_27F isolate colony using Neighbour-Joining method (bootstrap 1000×).

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