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Zhihengliuella somnathii sp. nov., a halotolerant actinobacterium from the rhizosphere of a halophyte Salicornia brachiata

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Abstract:	Two novel Gram- positive, rod shaped, halotolerent bacteria, strains JG 03T and JG 05 were isolated from the rhizosphere of Salicornia brachiata, an extreme halophyte. Comparative analyses of 16S rRNA gene sequence showed that they were closely related to members of the genus Zhihengliuella with sequence similarity of 96.9-99.1 %. Sequence similarity of strains JG 03T and JG 05 was 99.4 % with each other. DNA-DNA hybridization of JG 03T and JG 05 with other known species of Zhihengliuella showed reassociation value of 19.8% -53.4% and 91.4% in between themselves. The peptidoglycan type of both strains was A4α and MK-9 and MK-10 were predominant menaquinones. The predominant fatty acid in JG 03T was anteiso-C15:0 and anteiso-C17:0. However, iso-C15:0, anteiso-C15:0 and anteiso-C17:0 were the major fatty acid in strain JG 05. The DNA G+C content of strains JG 03T and JG 05 was 70.0 and 70.1 mol%, respectively. In nutrient broth medium both strains grew at NaCl concentrations up to 15% (w/v). On the basis of chemotaxonomic characteristics and phylogenetic analyses, strains JG 03T and JG 05 should be affiliated to the genus Zhihengliuella. Strains JG 03T and JG 05 represent a novel species of the genus Zhihengliuella, for which the names Zhihengliuella somnathii sp. nov. is proposed. The type strain is JG 03T (=DSM 23187T =IMCC 253T).

Zhihengliuella somnathii sp. nov., a halotolerant actinobacterium from the rhizosphere of a halophyte *Salicornia brachiata*

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The NCBI GenBank accession number for the 16S rRNA gene sequence of strain JG 03[T] is EU937748 and strain JG 05 is EU937749

Summary

Two novel Gram- positive, rod shaped, halotolerent bacteria, strains JG 03^T and JG 05 were isolated from the rhizosphere of *Salicornia brachiata*, an extreme halophyte. Comparative analyses of 16S rRNA gene sequence showed that they were closely related to members of the genus *Zhihengliuella* with sequence similarity of 96.9-99.1 %. Sequence similarity of strains JG 03^T and JG 05 was 99.4 % with each other. DNA–DNA hybridization of JG 03^T and JG 05 with other known species of *Zhihengliuella* showed reassociation value of 19.8% -53.4% and 91.4% in between themselves. The peptidoglycan type of both strains was A4α and MK-9 and MK-10 were predominant menaquinones. The predominant fatty acid in JG 03^T was anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. However, iso-$C_{15:0}$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ were the major fatty acid in strain JG 05. The DNA G+C content of strains JG 03^T and JG 05 was 70.0 and 70.1 mol%, respectively. In nutrient broth medium both strains grew at NaCl concentrations up to 15% (w/v). On the basis of chemotaxonomic characteristics and phylogenetic analyses, strains JG 03^T and JG 05 should be affiliated to the genus *Zhihengliuella*. Strains JG 03^T and JG 05 represent a novel species of the genus *Zhihengliuella*, for which the names *Zhihengliuella somnathii* sp. nov. is proposed. The type strain is JG 03^T (=DSM 23187^T =IMCC 253^T).

The genus *Zhihengliuella* was proposed by Zhang *et al* (2007). At the time of writing, the genus *Zhihengliuella* contains five recognized species namely *Zhihengliuella halotolerans* (Zhang *et al*., 2007), *Zhihengliuella alba* (Tang *et al*., 2009), *Zhihengliuella salsuginis* (Chen *et al*., 2010), *Zhihengliuella aestuarii* (Baik *et al*., 2011) and *Zhihengliuella flava* (Hamada *et al*., 2013).

The roots of *Salicornia brachiata*, an extreme halophytic plant is a potential source of novel bacteria (Schmid *et al.,* 2009; Jha *et al*., 2012). Strains JG 03^T and JG 05 were isolated from roots of *S. brachiata,* collected from coastal marshy swamps, Bhavnagar, Gujarat (21° 45’ N 72°

14’ E), India. The bacteria were isolated using nitrogen-free semisolid NFb and DYGS medium following the method described earlier (Gontia *et al*., 2011). Strains JG 03^T and JG 05 grew up to 4 % (w/v) NaCl concentration on semisolid nitrogen free NFb medium. Tolerance to NaCl was tested using nutrient broth supplemented with NaCl (ranging from 1-15%, w/v) with increment of 1% (w/v). Growth of both strains (JG 03^T and JG 05) was observed up to 15 % (w/v), whereas the optimum concentration of NaCl for growth was 4 % (w/v). The temperature range for growth was determined by keeping the bacterial culture (in nutrient broth) at different temperature in incubator shaker. The optimum growth temperature of the novel strains was 30°C. However, these strains grew at temperature range of 10-37°C. The pH range and optimum pH for growth were tested using nutrient broth with pH adjusted from 4-12 (one pH unit interval) using the following buffer systems, pH 4.0-5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0-8.0: 0.1 M KH_2PO_4/0.1 M NaOH; pH 9.0-12.0: 0.1 M $NaHCO_3$/0.1 M Na_2CO_3. The bacteria grew at pH ranges from 6-10 with optimum growth at pH 8.

Cell morphology was observed using scanning electron microscopy according to Yumoto *et al*. (2001). The presence or absence of flagella was visualised using transmission electron microscopy according to Näther *et al*. (2006). The genomic DNA of strains was isolated by following the method of Sambrook & Russell (2001). The 16S rRNA genes of each strain were amplified according to method described by Weisburg *et al*. (1991). The purified PCR products were sequenced by Macrogen Inc (Seoul, South Korea). Phylogenetic analysis was performed using MEGA version 6 (Tamura *et al*., 2013) and neighbour-joining and maximum-likelihood methods were applied to infer the phylogenetic tree (Saitou & Nei, 1987). Bootstrap analysis was done (Felsenstein, 1985) and maximum composite likelihood algorithms were used for the determination of the evolutionary distances (Tamura *et al.*, 2004). The highest sequence

similarity for both strains JG 03^T and JG 05 were observed 99.1 % with *Z. flava* DSM 26152^T. The strains JG 03^T and JG 05 had 98.4 % and 98.3 % 16S rRNA gene sequence similarity with *Z. salsuginis* DSM 21149^T and 98.2 % and 98.1 % sequence similarity with *Z. alba* DSM 21143^T , respectively. Both strains showed 98.0 % and 96.9 % sequence similarity to *Z. halotolerans* DSM 17364^T and to *Z. aestuarii* KCTC 19557^T , respectively. Strains JG 03^T and JG 05 had high 16S rRNA gene similarity of 99.4 % with each other. The 16S rRNA gene sequence-based tree showing the position of strains JG 03^T and JG 05 within the genus *Zhihengliuella* is presented in Fig. 1 (constructed by neighbour-joining method) and Supplementary Fig. S3 (constructed by maximum-likelihood method).

Chemotaxonomic analyses such as respiratory quinones (menaquinones), the peptidoglycan types, cell-wall sugars and polar lipids of strains JG 03^T and JG 05 were performed by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Analyses of respiratory quinones and polar lipids were carried out by previously described procedures (Tindall, 1990a, 1990b, Tindall *et al.*, 2007). Determination of the peptidoglycan structure was carried out as described by Schumann (2011). Cell-wall sugar was determined according to Staneck & Roberts (1974). The menaquinone types for JG 03^T were MK-10 (50 %), MK-9 (43 %) and MK-8 (6 %), whereas for JG 05, MK-9 (48 %), MK-10 (46 %) and MK-8 (6 %). Menaquinones MK-10 and MK-9 are predominant in all other species of the genus *Zhihengliuella.* The peptidoglycan type of both strains was A4α (Molar ratio of amino acids are strain JG 03^T: 1.9 Ala: 1.9 Glu : 1.0 Lys: 1.0 Mur: 0.1 Asp and strain JG 05: 1.6 Ala: 1.9 Glu : 1.0 Lys: 1.0 Mur: 0.1 Asp with an interpeptide bridge of L-Lys–L-Ala–L-Glu) that is consistent with those determined for the other members of the genus *Zhihengliuella* (Hamada *et al*., 2013). The major component of cell wall sugar was galactose and minor amounts of glucose, mannose

and rhamnose were also detected in both strains (JG 03^T and JG 05). Glucose and tyvelose are major cell wall sugars in *Z. halotolerans* and *Z. aestuarii* (Zhang *et al*., 200; Baik *et al*., 2011) whereas mannose and tyvelose were the major cell wall sugars in Z. *alba* and *Z. salsuginis* (Tang *et al*., 2009; Chen *et a*l., 2010). In case of *Z. flava*, however, galactose was also present as major constituent (Hamada *et al*., 2013). The polar lipids diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI) were present in both strains. In addition one unidentified polar lipids two unidentified phospholipid and one unidentified glycolipid were also present (Supplementary Fig. S1). The presence of polar lipids DPG and PG is consistent with other species of the genus *Zhihengliuella.*

For cellular fatty acid analysis strains JG 03^T, JG 05, and reference strains (*Z. salsuginis* DSM 21149^T, *Z. halotolerans* DSM 17364^T, *Z. alba* DSM 21143^T, *Z. aestuarii* KCTC 19557^T and *Z. flava* DSM 26152^T) were grown in tryptic soy yeast agar for 24 h at 30 °C. Fatty acid methyl esters (FAME) were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Microbial ID) (Sasser, 1990; Whittaker *et al*., 2005). Peaks were identified using the RTSBA6 6.10 database. The major fatty acids anteiso-$C_{15:0}$ (55.1 %) and anteiso-$C_{17:0}$ (14.3 %) were present in JG 03^T and iso-$C_{15:0}$ (35.4 %), anteiso-$C_{15:0}$ (34.8 %), and anteiso-$C_{17:0}$ (10.3 %) in strain JG 05 (Table1). Strain JG 05 has highest iso-$C_{15:0}$ instead of anteiso-$C_{15:0,}$ which is commonly high in all other known species of *Zhihengliuella* as well as strain JG 03^T.

Utilization of different substrates by strain JG 03^T and JG 05 and reference strains of *Zhihengliuella* was tested using Biolog GEN III microplates of the Microlog system (Biolog). The results were summarised in supplementary Table S1. Additionally, activity of some important enzymes, such as amylase, catalase, oxidase, pectinase, gelatinase , protease, lipase

and cellulase were tested for strains JG 03^T and JG 05 (Gontia *et al*., 2011). Both strains were tested positive for the production of amylase, catalase, pectinase, gelatinase, protease and lipase enzyme, but negative for oxidase and cellulase. All species of the genus *Zhihengliuella* reported so far are catalase positive and oxidase negative. Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs. The strains were tested for antibiotic sensitivity to ampicillin (10 µg), azireonam (10 µg), bactracin (10 U), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), co-trimoxazole (25 µg), erythromycin (10 µg), gatifloxacin (10 µg), gentamicin (10 µg), levofloxacin (5 µg), neomycin (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), oflaxacin (5 µg), penicillin G (1 U), polymyxin (300 U), sulphamethoxazole (23.75 µg) and tetracycline (25 µg) using standard disc diffusion protocols, whereas aztreonam, fusidic acid, guanidine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin SV, 1% sodium lactate, sodium butyrate, sodium bromated, tetrazolium violet, tetrazolium blue, troleandomycin and vancomycinare were tested using chemical sensitivity assay using Biolog GEN III MicroPlate. Strain JG 03^T was sensitive to erythromycin, fusidic acid, troleandomycin, rifamycine SV, minocycline, lincomycine, niaproof 4 and vancomycin whereas JG 05 was sensitive to fusidic acid, troleandomycin, minocycline, lincomycine, niaproof 4 and vancomycin.

Scanning electron micrograph showed that strains JG 03^T and JG 05 were rod shaped (Supplementary Fig. S2 (a), (c)), which is similar to those observed for *Z. halotolerans*, Z. *alba*, *Z. aestuarii* and *Z. flava*. However Z. *salsuginis* has coccoid morphology. Transmission electron microscopy of strains JG 03^T and JG 05 revealed absence of any flagella (Supplementary fig. S2 (b), (d)), which is in consistent with all other members of the genus *Zhihengliuella*. Strains JG 03^T and JG 05 differed from the other related species of the genus *Zhihengliuella* with respect to the

following major biochemical and physiological characteristics. The results are summarised in Table 2.

The determination of the G+C content of the DNA and DNA–DNA hybridization experiments were performed by the DSMZ, Braunschweig, Germany. The G+C content was determined by DNA isolation (Cashion *et al*., 1977), followed by DNA degradation (Mesbah *et al.,* 1989), HPLC (Tamaoka & Komagata, 1984) and finally calculation of GC content (Mesbah *et al.,* 1989). For DNA-DNA hybridization, cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments, Germany). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.,* (1977). DNA-DNA hybridization was carried out as described by Ley *et al*. (1970) under consideration of the modifications described by Huss *et al*. (1983). The G+C content of strains JG 03^T and JG 05 was 70.0 mol % and 70.1 mol % , respectively, which are similar to the values (59.1-70.3 %) reported for other species of the genus *Zhihengliuella* (Zhang *et al.,* 2007; Tang *et al.,* 2009; Chen *et al.,* 2010; Baik *et al*., 2011; Hamada *et al*., 2013). DNA-DNA hybridization of JG 03^T with *Zhihengliuella alba* DSM 21143^T, *Zhihengliuella halotolerans* DSM 17364^T, *Zhihengliuella salsuginis* DSM 21149^T, *Zhihengliuella flava* DSM 26152^T and *Zhihengliuella aestuarii* KCTC 19557^T showed reassociation values of 53.4 % , 49.6 %, 37.2 %, 25.9 % and 19.8 %, respectively. In case of strain JG 05 the reassociation values with *Z. salsuginis* DSM 21149^T, *Z. halotolerans* DSM 17364^T, *Zhihengliuella flava* DSM 26152^T, *Z. alba* DSM 21143^T and *Z. aestuarii* KCTC 19557^T were 35.6 %, 35 %, 26.4 %, 25 % and 24.6 %, respectively. DNA-DNA hybridization between JG 03^T and JG 05 resulted in reassociation value of 91.4 %. DNA-DNA relatedness value has been used as a genotypic parameter to delineate species (Wayne *et al*., 1987). DNA–DNA hybridization percentage values <70% are considered to show that organisms belong to different

164 species (Stackebrandt & Goebel, 1994). Thus, according to accepted criteria for novel species,
165 these two strains belong to same species, which represent a new species of the genus
166 *Zhihengliuella*.
167 From the results of phylogenetic analysis based on 16S rRNA gene sequences, differences in
168 biochemical characteristics, the polar lipid profile, the fatty acid composition and the low
169 reassociation values of DNA–DNA hybridization with its closest relatives, it is evident that
170 strains JG 03^T and JG 05 represent a novel species of the genus *Zhihengliuella*. The name
171 *Zhihengliuella somnathii* sp. nov. is proposed for this novel species.
172
173 **Description of *Zhihengliuella somnathii* sp. nov.**
174 *Zhihengliuella somnathii* (som.nath´i.i. N.L. gen. n *somnathii* of somnath, the presiding deity,
175 dating back to prehistoric era of the area Saurashtra, Gujarat, India, from where the type strain
176 was isolated).
177 Cells are Gram-positive-staining, rod shaped and have a diameter 1.1-1.9 × 0.3-0.5 µm. Aerobic
178 and non-motile. Colonies are pale yellow and white, circular, have an entire margin and are
179 opaque within 24 h with diameter of approximately 2 mm on nutrient agar. Mesophilic, with an
180 optimum temperature of 30 °C, but able to grow between 10 and 37 °C and at pH 6 -10
181 (optimum at pH 8.0). Able to tolerate concentration of NaCl up to 15 % (w/v) with optimal
182 growth at 4 %. Positive results in tests for amylase, catalase, pectinase, gelatinase, protease, and
183 lipase but negative for oxidase and cellulase. Carbon source utilization are indicated in
184 supplementary Table S1. The peptidoglycan type is A4α and major menaquinones are MK-10
185 and MK-9. The predominant cell-wall sugar is galactose and minor amount of glucose, mannose
186 and rhamnose are also present. Polar lipids profile consist of DPG, PG, PI, one unidentified polar

lipid, two unidentified phospholipid and one unidentified glycolipid. The predominent fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ or iso-$C_{15:0}$, anteiso-$C_{15:0}$.and anteiso-$C_{17:0}$.

The type strain, JG 03^T (=DSM 23187^T=IMCC 253^T) and the strain JG 05 (=DSM 23191=IMCC 254), were isolated from roots of an extreme halophyte *Salicornia brachiata* from coastal region of Bhavnagar district, Gujarat, India. The DNA G+C content of the type strain JG 03^T and strain JG 05 is 70.0 mol % and 70.1 mol %, respectively.

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Figure Legend

Fig.1. The phylogenetic tree of stains JG 03^T and JG 05 with taxonomic neighbours. The phylogenetic tree was reconstructed by neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). Numbers at nodes are percentage bootstrap values. Circles at the node indicate that the corresponding nodes were also recovered in the trees generated by maximum-likelihood algorithms. The evolutionary distances were computed using the maximum composite likelihood

286 method and are in the units of the number of base substitutions per site. All positions containing
287 gaps and missing data were eliminated by complete deletion option. Phylogenetic analyses were
288 conducted in MEGA version 6.
289

290 Table 1. Whole cell fatty acid profiles of JG 03^T and JG 05 with type strains of *Zhihengliuella*
291 species. Taxa: 1, strain JG 03^T; 2, strain JG 05; 3, *Z. aestuarii* KCTC 19557^T; 4, *Z. alba* DSM
292 21143^T; 5 *Z. salsuginis* DSM 21149^T; 6, *Z. halotolerans* DSM 17364^T; *Z. Flava* DSM 26152^T.
293 Symbol "–" represents not detected.
294

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**
$C_{10:0}$	–	–	–	–	–	0.2	–
$C_{12:0}$	0.1	0.2	0.2	0.4	0.1	0.2	0.2
$C_{14:0}$	1.6	0.5	1.6	2.5	1.0	1.0	1.2
$C_{16:0}$	6.0	2.4	4.3	6.5	4.9	4.0	3.8
$C_{17:0}$	0.2	–	–	0.5	0.2	0.5	0.2
$C_{18:0}$	0.4	–	–	0.5	0.4	5.7	0.3
$C_{19:0}$	–	–	–	–	–	0.3	–
$C_{20:0}$	–	–	–	–	–	2.7	–
iso-$C_{13:0}$	–	0.3	–	–	0.1	0.4	0.2
iso-$C_{14:0}$	2.0	1.4	1.2	2.6	0.5	2.8	1.4
iso-$C_{15:0}$	8.2	35.4	9.0	4.2	7.2	15.0	14.3
iso-$C_{16:0}$	9.8	6.9	7.7	8.0	3.0	2.1	6.0
iso-$C_{17:0}$	1.1	7.0	0.7	0.4	1.0	3.3	1.6
iso-$C_{18:0}$	0.1	–	–	–	–	0.5	–
iso-$C_{19:0}$	–	–	–	–	–	0.6	–
anteiso-$C_{13:0}$	0.2	0.2	–	0.4	–	0.2	0.2
anteiso-$C_{15:0}$	55.1	34.8	64.9	63.5	58.4	52.1	58.1
anteiso-$C_{17:0}$	14.3	10.3	9.3	9.2	16.6	7.6	12.3
anteiso-$C_{19:0}$	–	–	–	–	–	0.7	–
$C_{18:1}\omega 9c$	0.3	–	0.4	0.4	0.3	0.2	0.2
$C_{15:0}$ 2-OH	–	–	0.2	0.3	–	–	–
$C_{17:0}$ 2-OH	0.3	0.1	0.2	0.2	–	–	0.1

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296

297 **Table 2.** Differential phenotypic characteristics of strains JG 03^T, JG 05 and type strains of
298 *Zhihengliuella* species. Taxa: 1, strain JG 03^T; 2, strain JG 05^T; 3, *Z. aestuarii* KCTC 19557^T; 4,
299 *Z. alba* DSM 21143^T; 5, *Z. salsuginis* DSM 21149^T; 6, *Z. halotolerans* DSM 17364^T; 7, *Z. flava*
300 DSM 26152^T

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Colony color	Pale yellow	White	Yellow	White	Light yellow	Pale yellow	Pale yellow
Morphology	Short rod	Short rod	Ovoid to short rod[a]	Short rod[b]	Cocci[c]	Short rod[d]	Rod shaped[e]
NaCl range (%, w/v)	0.5-15	0.5-15	0–7[a]	0–15[b]	0.5–20[c]	0–25[d]	0–10[e]
pH range	6-10	6-10	6-10[a]	5-9[b]	6.5-11.5[c]	6-10[d]	6-11[e]
Temperature range (℃)	10-37	10-37	4-37[a]	4-45[b]	10-40[c]	4-45[d]	10-37[e]
Carbon source utilization							
Acetic acid	+	±	+	+	±	+	+
D-Arabitol	+	-	+	+	-	+	±
Citric acid	+	+	±	±	-	-	±
Formic acid	±	-	+	+	±	±	±
Gentiobiose	±	+	±	±	±	+	±
L-Histidine	+	-	+	+	-	+	±
α-Hydroxy-butyric acid	+	±	+	±	-	+	±
D-Malic acid	±	-	-	±	-	+	±
D-Mannose	±	+	+	±	±	±	±
D-Mannitol	±	+	+	+	-	±	±
Mucic acid	±	-	-	±	-	+	±
Propionic acid	+	±	±	+	±	±	+
Quinic acid	±	+	±	+	-	+	±
Tween 40	±	±	+	±	±	-	±
Cell wall sugar	Gal	Gal	Tyv, Glc[a]	Tyv,Man[b]	Tyv,Man[c]	Tyv, Glc[d]	Gal[e]
DNA G+C content (mol%)	70.0	70.1	59.1[a]	70.3[b]	67.8[c]	66.5[d]	70.3[e]

+, positive; -, negative; ±, weakly positive.

Gal, Galactose; Glc, Glucose; Man, Mannose; Tyv, Tyvelose.

Data from [a]Baik *et al*. (2011). [b] Tang *et al*. (2009). [c] Chen *et al.* (2010). [d]Zhang *et al*.(2007). [e] Hamada *et al*.2013

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Figure

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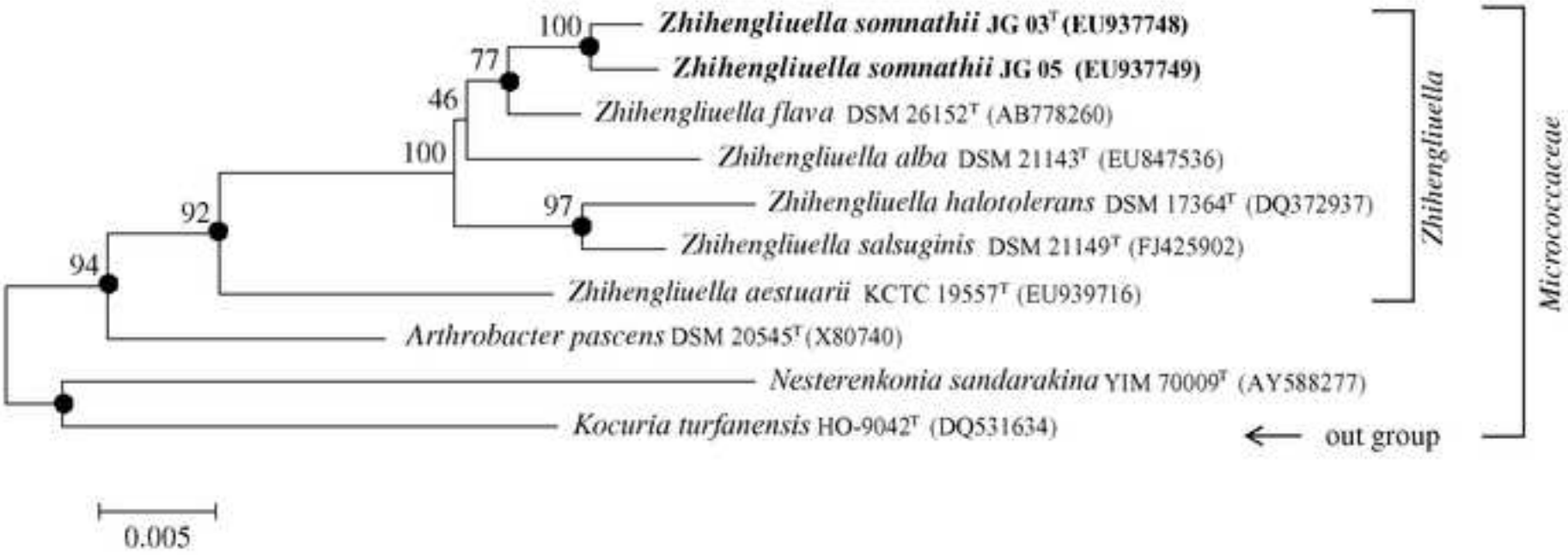

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