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A new species of *Thiohalocapsa*, *Thiohalocapsa marina*, sp. nov., from an Indian marine aquaculture pond

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA142^T is **AM491592**.

A spherical-shaped phototrophic purple sulfur bacterium was isolated in pure culture from anoxic sediment in a marine aquaculture pond near Bheemli (India). Strain JA142^T is Gram-negative and non-motile. It has an obligate requirement for NaCl (optimum of 2% and maximum of 6% NaCl (w/v)). Intracellular photosynthetic membranes were of the vesicular type. Spectral properties of *in vivo* absorption spectra indicate the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. Phylogenetic analysis on the basis of 16S rRNA gene sequence analysis showed that strain JA142^T is related to halophilic purple sulfur bacteria of the genera *Thiohalocapsa* and *Halochromatium* with the highest (97.5%) sequence similarity to the type strain of *Thiohalocapsa halophila* DSM 6210^T. Morphological and physiological characteristics discriminate strain JA142^T from other species of the genera *Halochromatium* and *Thiohalocapsa*. Strain JA142^T is sufficiently different from *Thiohalocapsa halophila* based on the 16S rRNA gene sequence analysis, morphological and physiological characteristics and therefore is described as a novel species, *Thiohalocapsa marina* sp. nov. (= JCM 14780^T = DSM 19078^T).

The genus *Thiohalocapsa* was established to separate species of purple sulfur bacteria based on their halophilic growth response, the lack of gas vesicles, the large phylogenetic distance and clustering with the marine and halophilic strains from other species of the genus *Thiocapsa* (Imhoff *et al*., 1998). At present the genus *Thiohalocapsa* comprises only one species, *Thiohalocapsa halophila* (Imhoff *et al*., 1998; originally described as *Thiocapsa halophila* by Caumette *et al*., 1991).

Strain JA142^T was isolated from photolithoautotrophic enrichments with 2% NaCl (w/v) of anoxic sediment and water (sample properties: pH of 7.0, salinity of 2% and

temperature of 30°C) from a marine aquaculture pond near Bheemli, Visakhapatnam, India (17° 54’ N, 83° 27’ E). Purification was achieved by repeated agar-shake dilution series (Pfennig & Trüper, 1992; Imhoff, 1988; Trüper, 1970). Polyphasic taxonomic studies and spectral analysis were carried out as described earlier (Anil Kumar *et al.*, 2007a and 2008). The utilization of organic compounds as carbon sources/electron donors for phototrophic growth was tested in the presence of yeast extract (0.03%, w/v) without any additional carbon source/electron donor. The concentrations of these compounds were 0.1% (v/v) (for formic acid, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol) and 0.3% (w/v) (for the other organic compounds tested), 1 mM benzoate.

Cells of strain JA142^T were spherical, non-motile, 1.5-2.0 μm in diameter and multiplied by binary fission (Supplementary Fig. 1). Electron microphotographs of ultrathin sections of the cells revealed a vesicular type of internal membrane structures. Strain JA142^T was able to grow photolithoautotrophically [anaerobic, light (30 μE x m^{-2} x s^{-1}), $Na_2S \cdot\cdot 9H_2O$ (2 mM)/ $Na_2S_2O_3 \cdot\cdot 5H_2O$ (5 mM) and $NaHCO_3$ (12 mM)]. Phototrophic growth (Table 1) in the presence of bicarbonate (12 mM) (photomixotrophy) and $Na_2S \cdot\cdot 9H_2O$ (0.5 mM) was observed with acetate, pyruvate, lactate, fumarate, succinate, glucose and casamino acids. Substrates not utilized include formate, propionate, butyrate, malate, fructose, ethanol, propanol, glycerol and crotonate. Photoorganoheterotrophy [anaerobic, light (30 μE x m^{-2} x s^{-1}), pyruvate (27 mM)], chemolithoautotrophy [aerobic, dark, $Na_2S_2O_3 \cdot\cdot 5H_2O$ (5 mM) and $NaHCO_3$ (12 mM)], chemoorganoheterotrophy [aerobic, dark, and pyruvate (27 mM)] and fermentative growth [anaerobic, dark with pyruvate (27 mM)] could not be demonstrated. $Na_2S \cdot\cdot 9H_2O$ and $Na_2S_2O_3 \cdot\cdot 5H_2O$ were utilized as electron

donors under photolithoautotrophic conditions with a minimum concentration of 0.5 mM $Na_2S \cdot\cdot 9H_2O$ and a tolerance of up to 4 mM, while sulfite, elemental sulfur and hydrogen did not support growth. During oxidation of sulfide, elemental sulfur droplets were stored inside the cells. $Na_2S \cdot\cdot 9H_2O$ and $Na_2S_2O_3 \cdot\cdot 5H_2O$ were utilized as sulfur sources by strain JA142^T, while sulfate, sulfite, thioglycolate, and cysteine did not support growth. Ammonium chloride was utilized as nitrogen source by strain JA142^T, while nitrate, nitrite, glutamate, glutamine, urea and dinitrogen did not support growth. Strain JA142^T is a true marine strain and growth occurs from 1.0-6.0% NaCl (w/v) with an optimum at 2.0% (w/v). The pH range of strain JA142^T is 6.5-8.5 with an optimum at pH 7.5. The temperature optimum of growth is 25-30 °C (range 25-35 °C). Strain JA142^T does not require vitamins for growth. The color of the phototrophically grown cell suspension is purple-red. The whole cell absorption spectrum of strain JA142^T exhibited absorption maxima at 395, 509, 584, 803, 845 nm and a shoulder at 878 nm confirming the presence of bacteriochlorophyll *a* (Supplementary Fig. 2a) and the absorption spectrum for pigments extracted with acetone exhibited absorption maxima at 462, 488 and 516 nm indicating the presence of carotenoid okenone (Supplementary Fig. 2b).

The DNA base composition of strain JA142^T was 64.8 mol% of G+C (by HPLC). DNA was extracted and purified by using the Qiagen genomic DNA extraction kit. PCR amplification and 16S rRNA gene sequencing were performed as described previously (Imhoff *et al.*, 1998). Recombinant Taq polymerase was used for PCR, which was started with the primers 5'–GTTTGATCCTGGCTCAG–3' and 5'–TACCTTGTTACGACTTCA–3' (*E. coli* positions 11-27 and 1489-1506, respectively). Sequences were obtained by cycle sequencing with the SequiTherm sequencing kit

(Biozym) and the chain termination reaction (Sanger *et al*., 1977) using an automated laser fluorescence sequencer (Pharmacia). Next relatives and sequence similarities were determined by BLAST search (Altschul *et al*., 1990) and BLAST 2 SEQUENCES alignment (Tatusova and Madden, 1999). 16S rRNA gene sequences of the closest related type strains belonging to the genera *Halochromatium* and *Thiohalocapsa* were newly determined and the corresponding EMBL database entry were updated prior to phylogenetic analysis. 16S rRNA gene sequences of representative type strains of *Chromatiaceae* species and of strain JA142^T were aligned using the FastAlign function of the alignment editor implemented in the ARB software package (http://www.arb-home.de) (Ludwig *et al*., 2004) and refined manually employing secondary structure information. For phylogenetic calculations the PhyML online version (Guindon *et al*., 2005), MEGA version 4.0 (Kumar *et al*., 2004) as well as the Phylip DNAPARS program implemented in ARB (Ludwig *et al*., 2004) was used. For tree calculation a character-based method (maximum-likelihood (Felsenstein, 1981), two distance-based methods (neighbor-joining (Saitou and Nei, 1987) and minimum evolution (Rzhetsky and Nei 1993) as well as a maximum parsimony (Eck and Dayhoff 1966, Fitch 1971, 1977) method was employed. The Tamura-Nei model was determined as the model best suited for phylogenetic calculation using the program ModelGenerator (Keane et al. 2006). The ML-tree was calculated using the TN93 model, 6 rate categories, gamma distribution parameter alpha = 0.31 and proportion of invariable sites value =0.43 as determined by ModelGenerator. For the ML bootstrap analysis, the non-bootstrapped ML-tree was used as starting tree. The neighbor-joining tree was calculated based on distances corrected by the Tamura-Nei nucleotide substitution model, using sites corresponding to the pairwise

deletion option, including transitions and transversions substitutions, assuming a heterogeneous pattern among lineages and a gamma distributed substitution rates (alpha = 0.31). The MP-tree was calculated using the "more thorough search" option and a randomized sequence order.

The 16S rRNA gene sequence analysis revealed that the new isolate belongs to the family *Chromatiaceae* and is affiliated to the group of marine and halophilic genera such as *Halochromatium, Marichromatium, Thiorhodovibrio, Rhabdochromatium* and *Thiohalocapsa*. Highest 16S rRNA gene sequence similarity was shared with *Thiohalocapsa halophila* DSM 6210^T (97.5%) and *Halochromatium glycolicum* DSM 11080^T (97.2%). The similarity values of <98.7% indicate a separation on the species level according to Stackebrandt and Ebers (2006). Phylogenetic analyses (Fig. 1) confirmed a close relationship between strain JA142^T and both *Halochromatium roseum* JA134^T and *Thiohalocapsa halophila* DSM 6210^T. In all cases *Halochromatium* spp. and *Thiohalocapsa halophila* and JA142^T clustered monophyletically. Additionally, in all trees (Fig. 1, Supplementary Fig 3a,b,c) *Halochromatium* spp. formed a tight cluster highly supported by bootstrap analysis, not including strain JA142^T. The distance based trees further indicate a separate clustering of *Thiohalocapsa halophila* and strain JA142^T. Detailed comparison of 16S rRNA gene sequences revealed particular sequence differences in a number of characteristic nucleotide positions of strain JA142^T to both *Halochromatium* species and *Thiohalocapsa halophila* DSM 6210^T (Table 2). Overall sequence similarity as well as signature nucleotides demonstrate a closer relationship of strain JA142^T to *Thiohalocapsa halophila* as compared to the *Halochromatium* species (12 nucleotides identical to *Thiohalocapsa* compared to 8 identical nucleotides to

Halochromatium; Table 2). However, 9 characteristic nucleotides were different to both *Thiohalocapsa halophila* and *Halochromatium* species, which clearly indicates an intermediate or borderline position between known representatives of both genera. This view is supported by the phylogenetic relationship as demonstrated by phylogenetic trees constructed by a variety of different methods. All methods used (neighbor-joining, minimum evolution, maximum likelihood and maximum parsimony; Fig. 1; supplementary Fig 3a ,b, c) demonstrate the clustering of JA142^T with *Halochromatium* and *Thiohalocapsa halophila*. Furthermore, all phylogenetic methods highly support a subcluster of the three known *Halochromatium* type strain species not including strain JA142^T nor *Thiohalocapsa halophila*.

Sequences of *pufLM* support the association of the new isolate with the *Halochromatium*/*Thiohalocapsa* cluster. More specifically they demonstrate a clear relationship to the *pufLM* sequence of *Thiohalocapsa halophila*, but not of *Halochromatium* sequences (Tank and Imhoff, unpublished results). Similarities of *pufLM* nucleotide sequences (approx. 1390 bp) to those from the type strain of *Thiohalocapsa halophila* were 88%, to several sequences from *Halochromatium* species were 84-85% and to sequences from *Thiorhodovibrio* species were below 80%).

A value of 70% DNA homology has been used as a benchmark for the separation on the species level during the past years and a 16S rRNA gene sequence similarity of 97% was regarded as borderline for demanding DNA-DNA hybridization data, assuming that this value more or less coincides with the 70% DNA-DNA homology. In their critical analysis Stackebrandt & Ebers (2006) carefully compared 16S rRNA gene sequence similarities with DNA-DNA reassociation values of a great number of

publications. Their convincing result was that below 98.5% sequence similarity, there was not a single case where DNA-DNA reassociation was more than 70% and these authors argued that with high quality sequences (as used in this study) a 99% sequence similarity almost excludes reassociation values of 70% and more. They recommended a 16S rRNA gene sequence similarity threshold range of 98.7-99% as the point at which DNA-DNA reassociation experiments should be mandatory for testing the genomic uniqueness of new isolates.

Therefore, the 97.5% 16S rRNA gene sequence similarity between strain JA142^T and *Thiohalocapsa halophila* DSM 6210^T clearly indicate their separation into different species. This is also supported by differences in the G+C content of both bacteria by 1.1-1.8%, by different salt responses and a number of differences in substrate and electron donor utilization including the ability to grow chemolithotrophically (Table 1).

Due to the closer association of the new bacterium to *Thiohalocapsa halophila* both by sequence information and by phenotypic properties, strain JA142^T is recognized as a new species of the genus *Thiohalocapsa*, for which the name *Thiohalocapsa marina* sp. nov. is proposed.

Description of *Thiohalocapsa marina* sp. nov. *marina* (ma.rin'na. L. fem. adj. *marina* pertaining to the marine environment). Cells are spherical in shape, 1.5-2.0 μm in diameter, non-motile and divide by binary fission. Growth occurs under anaerobic conditions in the light under photolithoautotrophic conditions. In addition, several organic substrates can be photoassimilated. Internal photosynthetic membranes are of the vesicular type. Color of the phototrophically grown cell suspension is purple-red. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 395, 509, 584, 803,

845 nm, indicating the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. The type strain is mesophilic (30°C), with a pH optimum at 7.5 (range 6.5-8.5). Salt is obligatory for the growth of strain JA142^T and growth occurs from 1.0-6.0% NaCl (w/v) with an optimum at 2.0% (w/v). Photolithotrophic growth in the presence of bicarbonate (12 mM) and $Na_2S\cdot\cdot 9H_2O$ (0.5 mM) is possible. A few organic substrates can be photoassimilated in the presence of sulfide and bicarbonate: acetate, pyruvate, lactate, fumarate, succinate, glucose and casamino acids. Photoorganoheterotrophy and chemotrophy was not detected. No growth factors are required. DNA base composition of the type strain is 64.8 mol% of G+C (by HPLC). The type strain JA142^T (= JCM 14780^T = DSM 19078^T), was isolated from a marine aquaculture pond near Bheemli, Visakhapatnam, India.

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Table 1. Differential characteristics between strain JA142^T and species of the genera *Thiohalocapsa* and *Halochromatium.*

Strain/species: 1, JA142^T; 2, *Thiohalocapsa halophila*; 3, *Halochromatium roseum*; 4, *Halochromatium salexigens*; 5, *Halochromatium glycolicum*. Data for reference species 2, 4 & 5 were taken from Imhoff (2005) and data for reference species 3 was taken from Anil Kumar *et al*., 2007b. Cells of all taxa studied divide by binary fission. All strains are having vesicular type of internal membrane structures. Na_2S and thiosulfate are utilized by all the strains. Organic substrate utilization was tested in the presence of sulfide and bicarbonate. Propionate, butyrate, ethanol, propanol were not utilized by any of the strains. +, substrate utilized or present; –, substrate not utilized or absent; (+), weak growth; NR, not reported.

Characteristic	1	2	3	4	5
Cell shape	sphere	sphere	Rod	Rod	Rod
Motility	-	-	-	+	+
Cell diameter (μm)	1.5-2.0	1.5-2.5	2.0-3.0 x 3.0-5.0	2.0-2.5 x 4.0-7.5	0.8-1.0 x 2.0-4.0
Gas vesicles	-	-	+	-	-
Color of cell suspensions	Purple-red	Purple-red	Purple-pink	Pink, Rose-red	Pink, Pinkish red
Carotenoid group	(Okenone*)	Okenone	Okenone	Spirilloxanthin	Spirilloxanthin
Mol% G+C of DNA	64.8	65.9-66.6	64	64.6	66.1-66.5
B_{12} requirement	-	-	+	+	-
Chemolithotrophic growth	-	+	-	+	+
pH optimum	7.5	7.0	7.5	7.4-7.6	7.2-7.4
(range)	(6.5-8.5)	(6.0-8.0)	(7-8)	(7.0-8.0)	(6.2-9.0)
Temperature optimum (°C)	25-30	20-30	27	20-30	25-35
NaCl optimum (%, w/v)	2	4-8	1.5-2.5	8-11	4-6
(range)	(1-6)	(3-20)	(1-3)	(4-20)	(2-20)
Substrates photo assimilated:					
Hydrogen	-	+	NR	+	+
Sulfur	-	+	-	+	+
Sulfite	-	+	-	+	+
Formate	-	-	-	-	(+)
Acetate	+	+	-	+	(+)
Pyruvate	+	+	+	+	(+)
Lactate	+	+	-	-	-
Fumarate	+	-	+	-	+
Succinct	+	-	+	-	+
Malate	-	-	+	-	-
Fructose	-	+	-	-	NR
Glucose	+	(+)	-	-	-
Glycerol	-	(+)	-	-	+
Glycolate	-	-	-	-	+
Crotonate	-	-	-	-	-
Valerate	-	-	-	-	NR
Casamino acids	+	-	+	-	(+)

* according to absorption spectra the presence of okenone as major carotenoid is likely.

***E. coli* Position**	***Halochromatium* spp. (N=7)**	**Strain JA142^T**	***Thiohalocapsa* spp. (N=5)**
144	G	G	A
148	A	G	G
223	G	A	G
250	M = U/G	A	A
269	U	C	U
381	A	A	C
444	G	A	G
454	A	U	A
457	C	U	C
473	U	U	C
490	C	U	C
589	U	C	C
590	G	U	G
653	U	U	C
658	A	C	C
660	G	A	G
745	C	U	C
748	U	G	G
838	U	U	C
839	C	C	U
1001	U	C	C
1007	-	U	U
1010	U	G	G
1021	A	U	U
1022	-	U	U
1256	U	U	C
1257	C	U	U
1265	C	C	A
1424	C	U	U

Table 2. Base composition of certain 16S rRNA gene signature nucleotide positions for *Thiohalocapsa* spp. (obtained from five databank sequences), *Halochromatium* spp. (from seven databank sequences) and the new strain JA142^T.

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences showing the relationship of strain JA142T within the family *Chromatiaceae*. Phylogenetic trees were calculated by the minimum evolution and the neighbor-joining method as well as by maximum parsimony and maximum likelihood method. Tree topology of all four methods was compared and shared knots marked in the minimum evolution tree as follows: knots supported by all four methods were marked by an open circle (o); knots shared by all four methods, supported by bootstrap values >95 or 100 were marked with a filled circle (• /(100)•, respectively); one knot only supported by the distance based methods was indicated by NJ, ME. The bar represents 0.1 substitutions per alignment position.

Supplementary Fig. 1. Phase-contrast micrograph of strain JA142^T. Bar, 5 μm.

Supplementary Fig. S2. Whole-cell absorption spectrum (a) of strain JA142^T and acetone spectrum (b) of extracted pigments.

Supplementary Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain JA142T within the family *Chromatiaceae* calculated by the neighbor-joining method (A), the maximum likelihood method (B), the Minimum Evolution method (C) and the Maximum Parsimony method (D). Numbers at nodes represent percent bootstrap values of 100 replicates done. The bar represents 0.1 substitutions per alignment position.

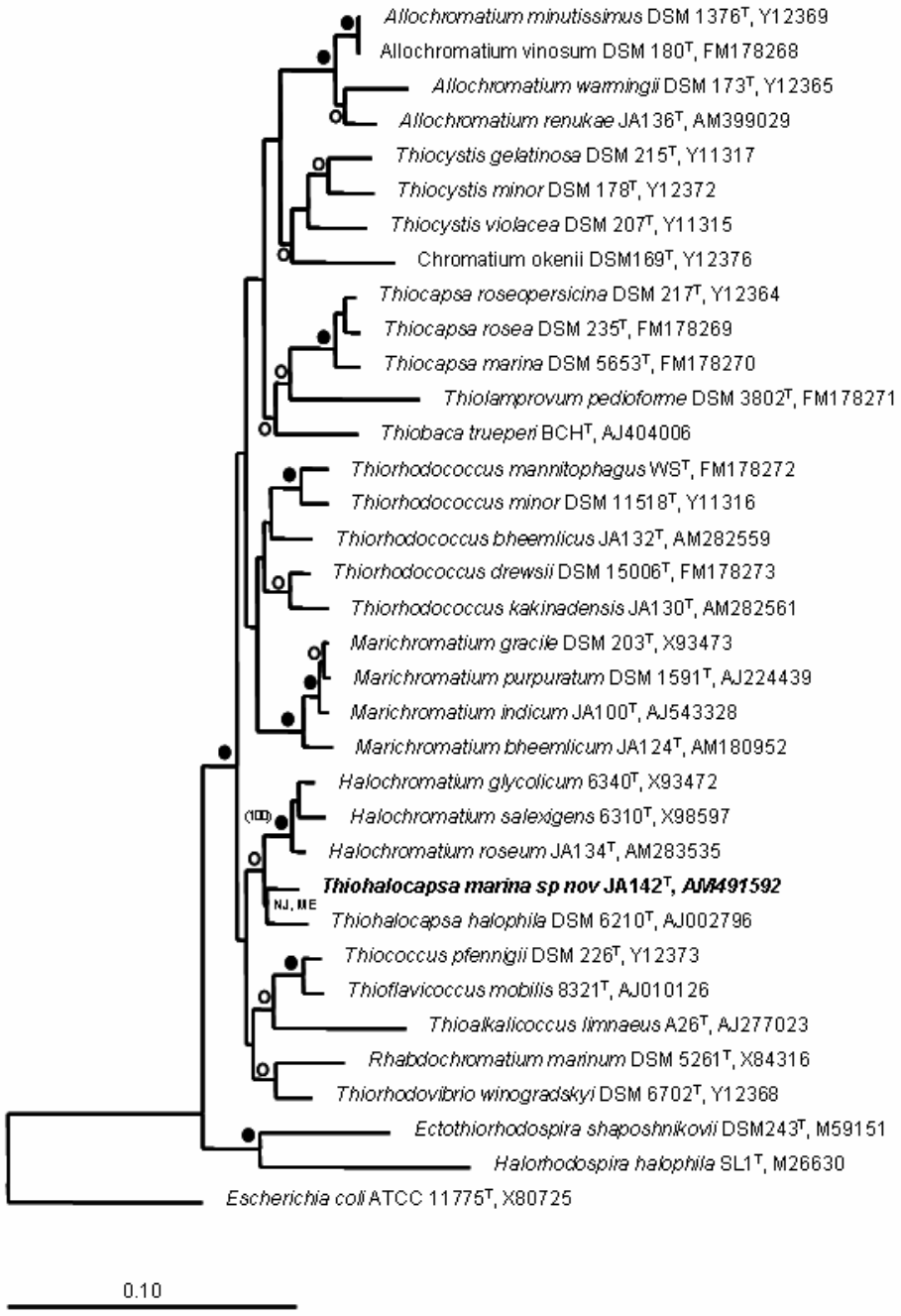

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences showing the relationship of strain JA142^T within the family *Chromatiaceae*. Phylogenetic trees were calculated by the minimum evolution and the neighbor-joining method as well as by maximum parsimony and maximum likelihood method. Tree topology of all four methods was compared and shared knots marked in the minimum evolution tree as follows: knots supported by all four methods were marked by an open circle (○); knots shared by all four methods, supported by bootstrap values >95 or 100 were marked with a filled circle (● / (100)● , respectively); one knot only supported by the distance based methods was indicated by NJ, ME. The bar represents 0.1 substitutions per alignment position.

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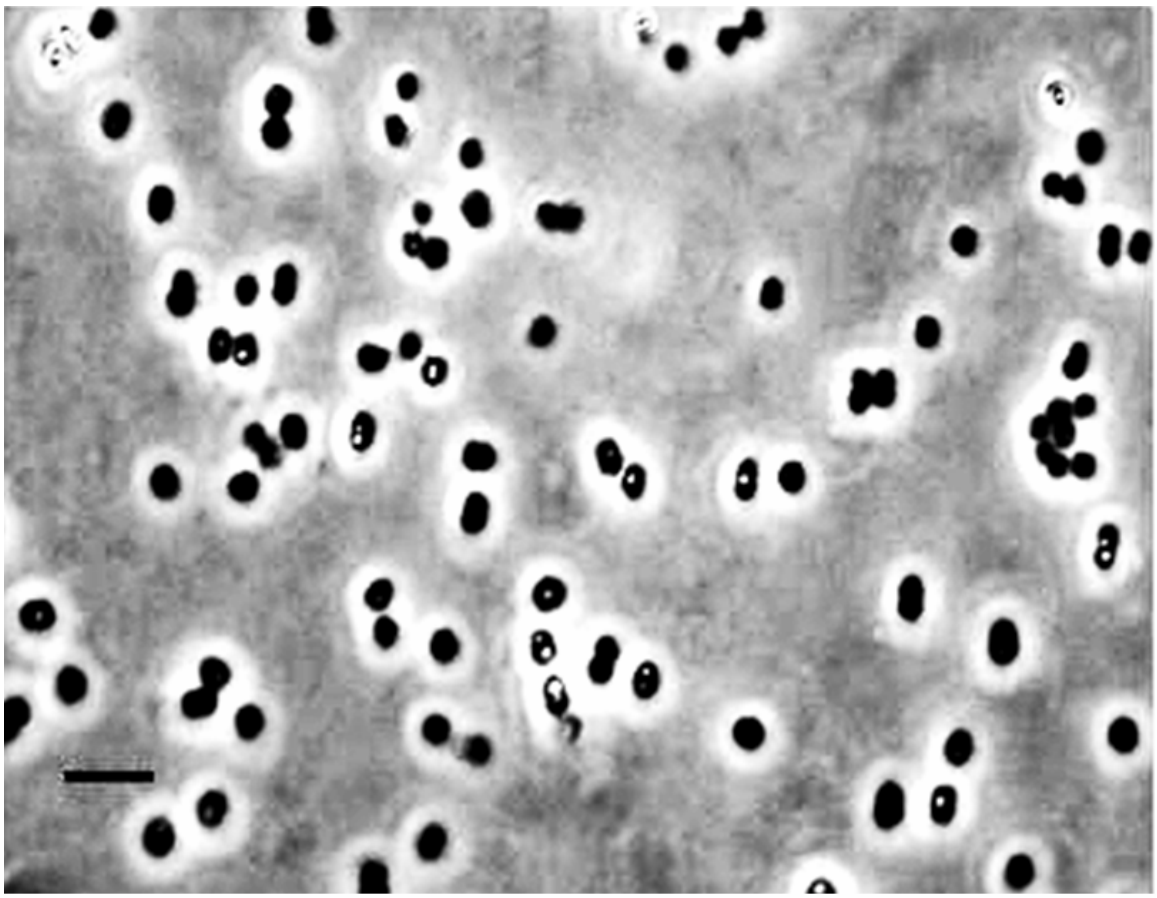
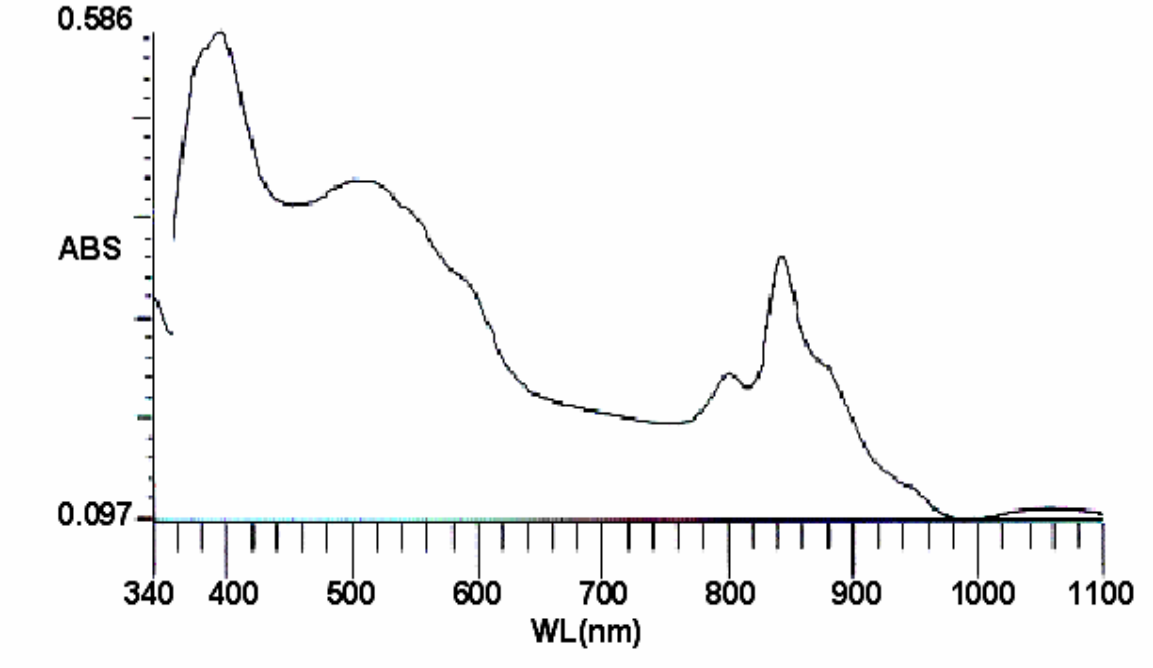
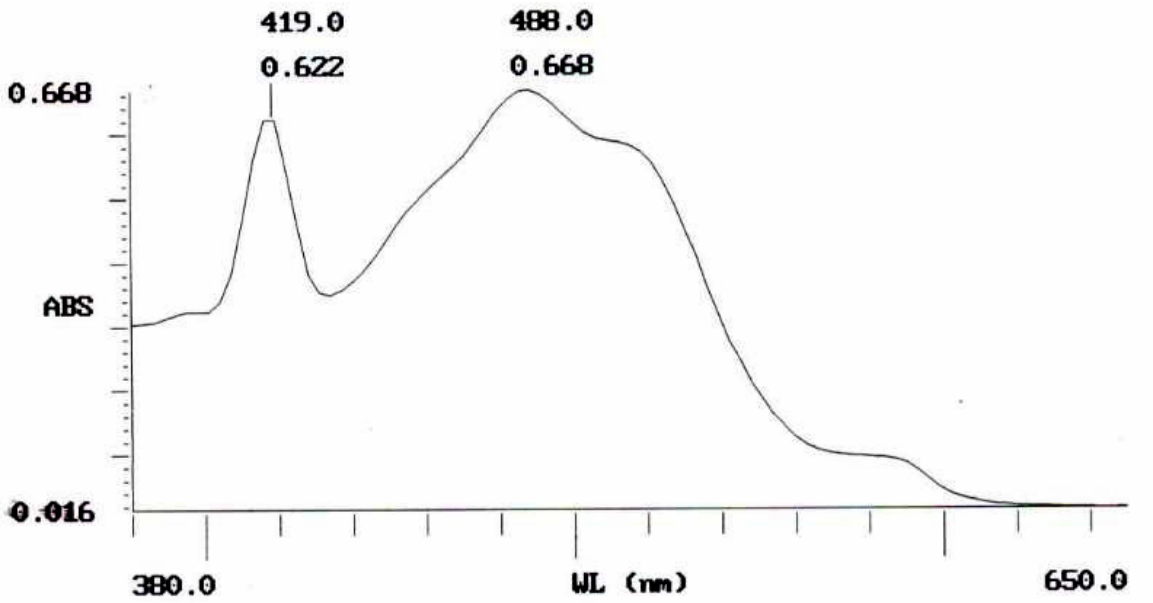


Supplementary Fig 1



Supplementary Fig. 2a



Supplementary Fig. 2b

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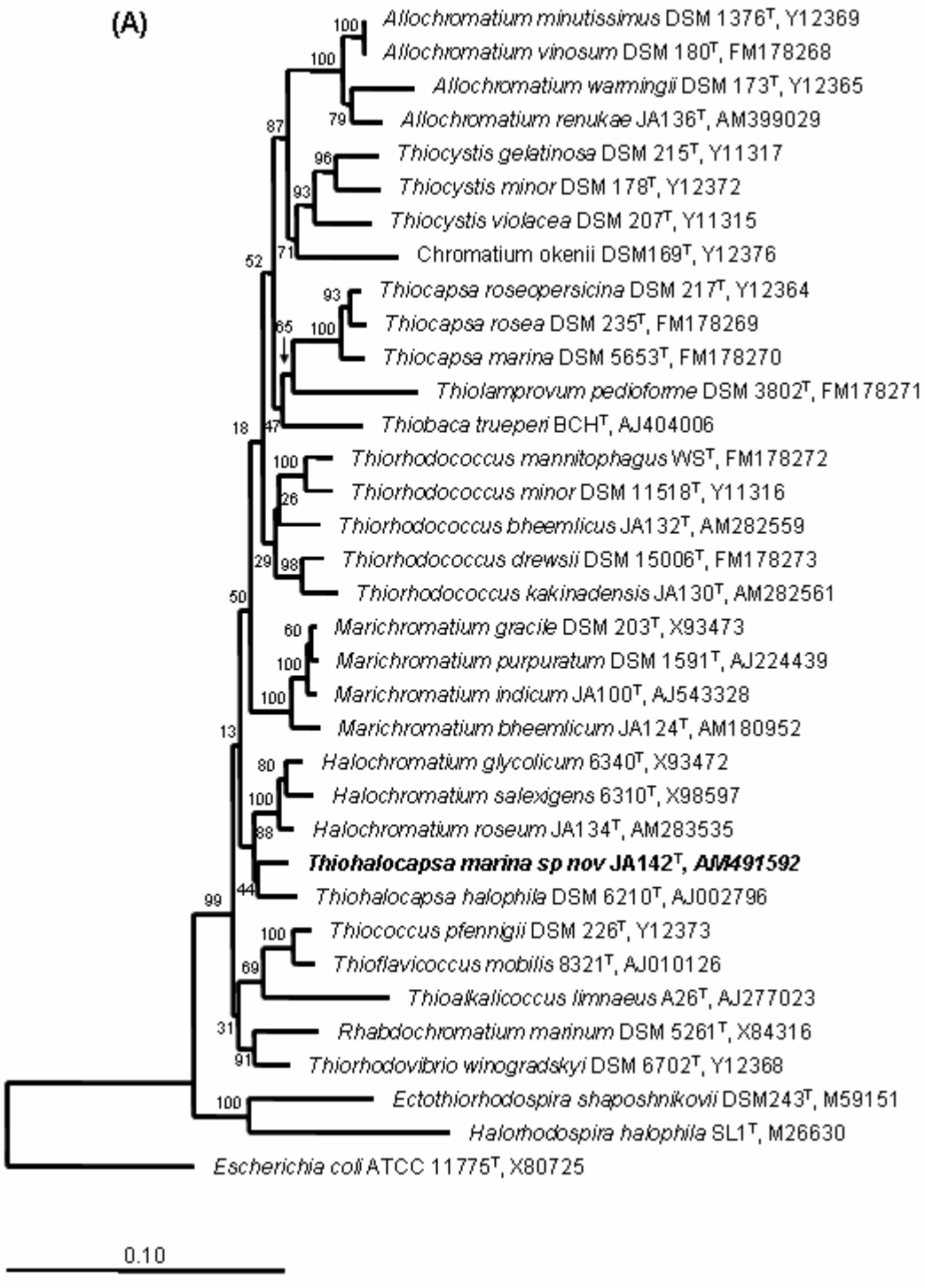
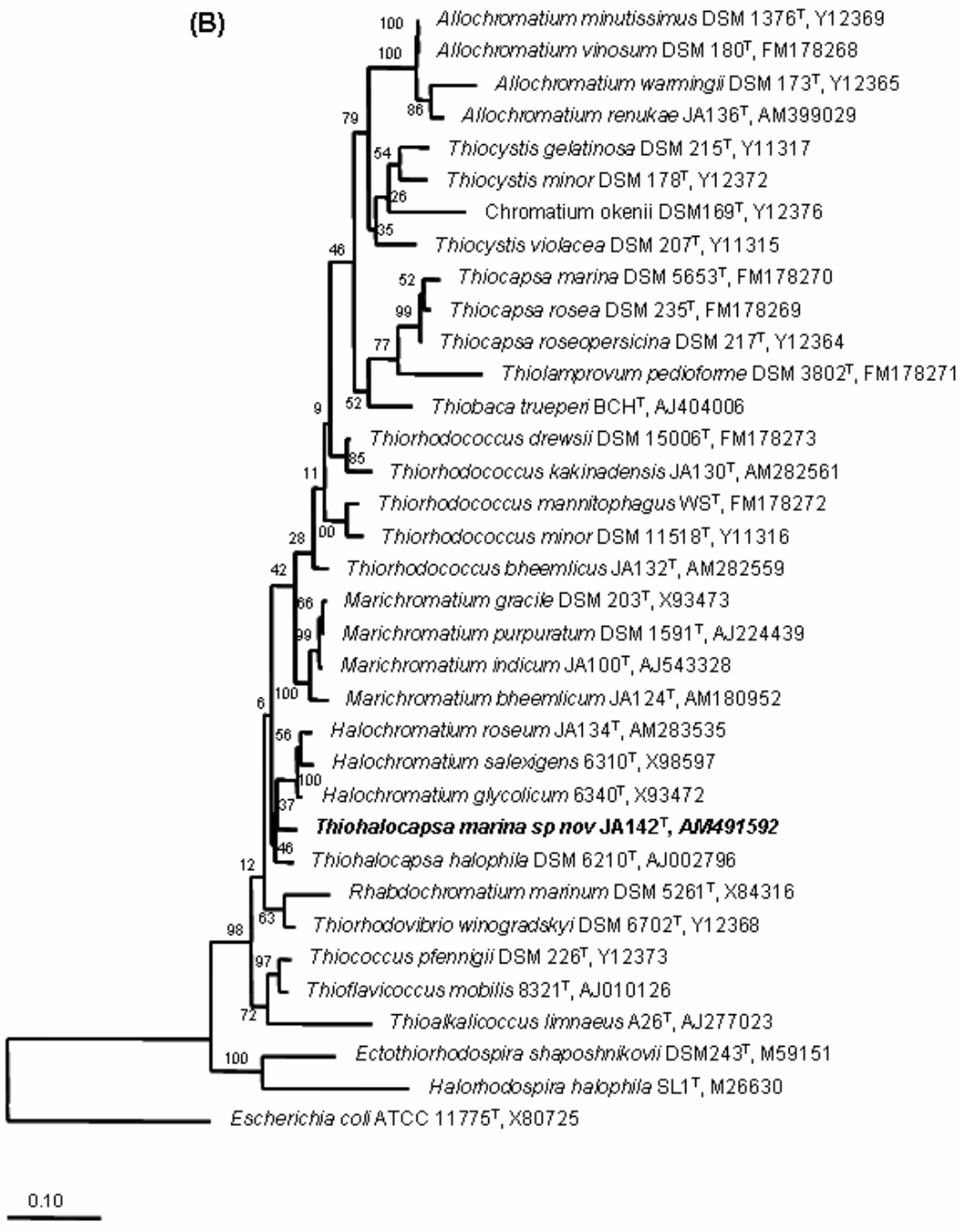


Supplementary Fig. 3A

354

355

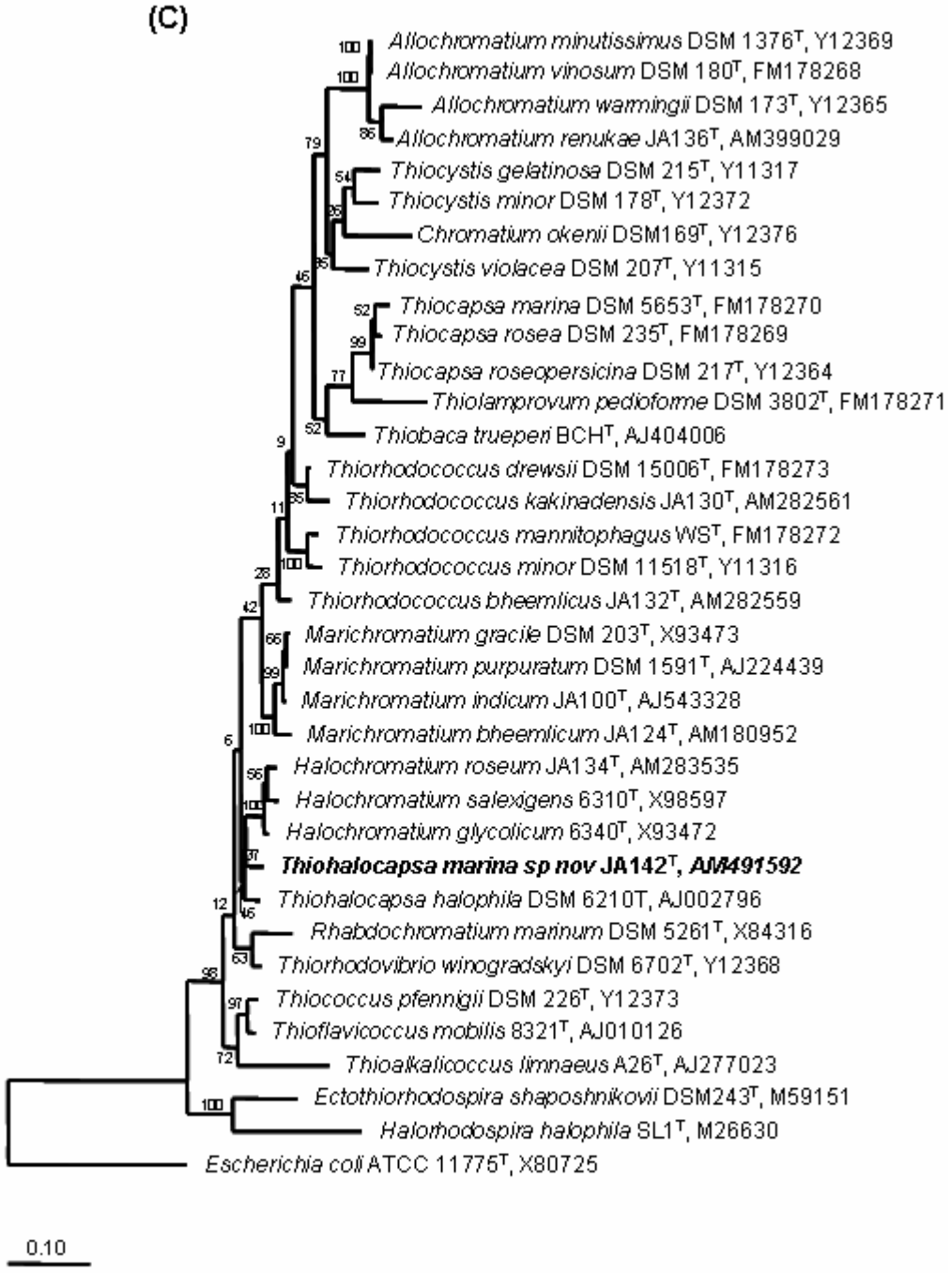


Supplementary Fig. 3B

356

357

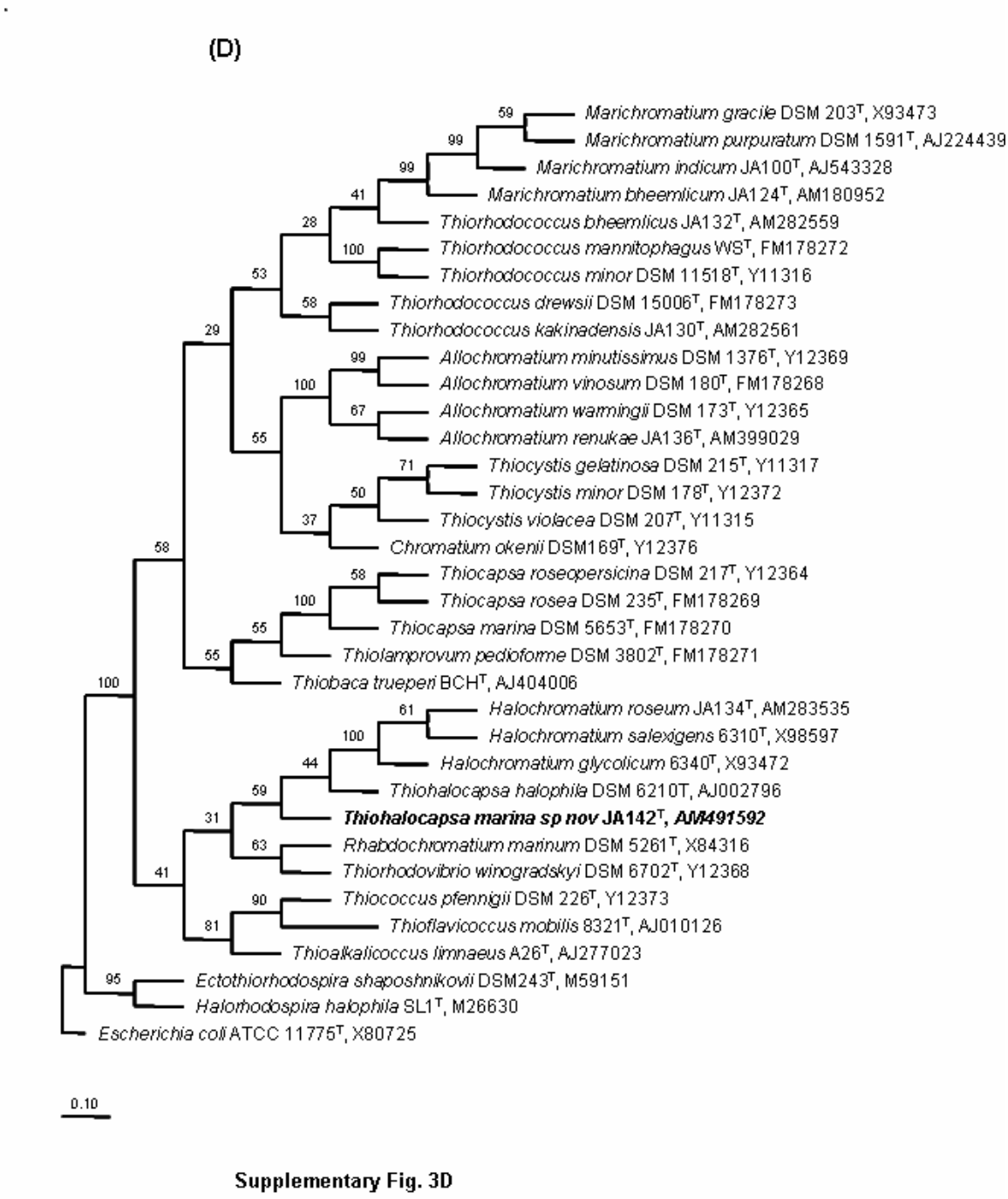
358



359 **Supplementary Fig. 3C**

360

361



362 **Supplementary Fig. 3D**