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Morphology, phylogeny and lipid components of an oil-rich microalgal strain

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Summary

Microalgae have attracted much more attentions to their roles in biofuel exploration recently. In this report, one oil-rich microalgal strain (TY02) was isolated from the lawn soil in a park, Taiyuan, Shanxi, China, and the morphology and phylogeny characters of the strain was systematically analyzed. Observed by light microscopy, scanning electron microscopy, transmission electron microscopy and fluorescent microscopy, the lipid bodies were observed clearly. After extracting the total lipid by the chloroform-methanol method, the fatty acid content and composition of the lipid bodies in the strain were detected and analyzed by gas chromatography-mass spectrometry. The results demonstrated that the total lipid content of TY02 was 33.27 ± 1.13%. Among the total seven kinds of fatty acids identified in TY02, the major constituents were C16 and C18 fatty acids, taking up to 88.15%. Moreover, the predominant fatty acids were hexadecanoic acid (C16:0), 9, 12-octadecadienoic acid (C18:2) and 9, 12, 15-octadecatrienoic acid (C18:3). Based on the molecular markers of 18S rDNA, *rbc*L and ITS genes, phylogenetic trees and ITS2 secondary structure analysis all showed that the strain closed to *Parachlorella kessleri*. All results might bring a new view that some microalgae with potential values can be used as raw material for biodiesel production.

Keywords: Oil-rich microalgae. Morphology. Phylogeny. Fatty acids. *Parachlorella kessleri*.

Introduction

The fossil fuel, as the most traditional energy resource, reveals a relevant high efficiency but more obvious disadvantages, such like the related environmental problems, exhaustion problems, and security problems of production. Thus it becomes more and more urgent for us to explore clean and safe new energy sources, such as nuclear power, wind power, solar power, and so on. Based on its advantages, including high combustion efficiency, little pollution, wide adaptability, strong security, sustainable development, non-toxicity and biodegradation, biodiesel is considered as a good replacement of fossil fuel and has a considerable prospect.

Microalgae can only be observed under microscopic levels, and for its properties such as the wide distribution, high photosynthetic efficiency and abundant metabolites, microalgae are thought to have great prospects in biomedicine, for genetic engineering and for biodiesel production (Gong and Jiang, 2011). The amount of lipids in the cells of some microalgae may be the key factors to result in the great potency as raw biodiesel materials. Ryckebosch et al. (2014) detected and analyzed the lipid component of *Isochrysis galbana* Parke, *Nannochloropsis* spp. and *Phaeodactylum tricornutum* Bohlin. Mu et al. (2015) discovered that the biomass and oil production of the heterotrophic oleaginous microalga *Chlorella protothecoides* Shihira and Krauss were enhanced by adding the hydrolysate from sugarcane bagasse.

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However, the distribution and growth of many algal species have their geographic specificities. Moreover, the oil-producing capacity of oleaginous microalgae may be weakened through the cultures of multiple generations. Therefore, it is important to select the excellent biodiesel-producing microalgae with related stable and constant oil-producing capacity. Some oleaginous microalgae have been identified and they belong to Chlorophyta, Chrysophyta, and Bacillariophyta. The studies showed that microalgae can accumulate more nutrients under environmental stress than under control conditions (Griffiths and Harrison, 2009). Illman et al. (2000) discovered that nitrogen deficiency could promote the synthesis of lipids in *Chlorella vulgaris* Stearn. Another study (Liu et al., 2007) reported that the restriction of iron absorption was beneficial to accumulate lipids in *C. vulgaris*. In brief, under various growth environments, biodiesel-producing microalgae may be cultured with different nutrition conditions and then develop diverse oil-producing capacity. So, it is anticipated to find some unique biodiesel-producing microalgae with high oil-producing capacity.

Comparison of microalgae grown in water with microalgae grown in soil may deposit more oil or fat because they undergo dry environmental conditions and variable temperatures. Therefore, we set up a series of experiments to discover some new and unique oleaginous microalgae strains growing in our specific local area, for example, the land soil in the parks or water areas. In the present study, we finally targeted one special oleaginous microalgal strain selected from all the analyzed ones, mainly basing on the determinations of the total lipid content and fatty acid composition. Meanwhile, the morphological and molecular systematical characters of the strain were comprehensively investigated, which may provide a more scientific bases for us to further utilize the strain and explore new other new biodiesel-producing microalgae.

Materials and methods

Microalgal strain collection and processing

The microalgal strain (TY02) was isolated from the lawn soil in a park, Taiyuan, Shanxi, China (Fig. 1). The strain was isolated and purified according to the method reported by Cho et al. (2013), and cultured with Tris-acetate-phosphate medium (TAP) (Deng et al., 2013) in a light incubator (BSG-300, BoXun, Shanghai, China) at 25 °C, 12 h light:12 h dark, 3000 lux. About 10 days later, the purified microalgal strain was settled in the logarithmic growth phase with applicable conditions.

Morphological observations

The algal morphological features were observed and photographed under a light microscope (BX-51, Olympus, Tokyo, Japan). Photographs were taken with a digital camera (CAMEDIA C5060WZ, Olympus, Tokyo, Japan) and a CCD (DP72, Olympus, Tokyo, Japan) mounted on the microscope.

The culture was centrifuged and fixed for 1 h in 2.5% glutaraldehyde at room temperature. A single drop of the cells was placed on each small glass slide. Each small glass slide was coated with 0.1% poly-

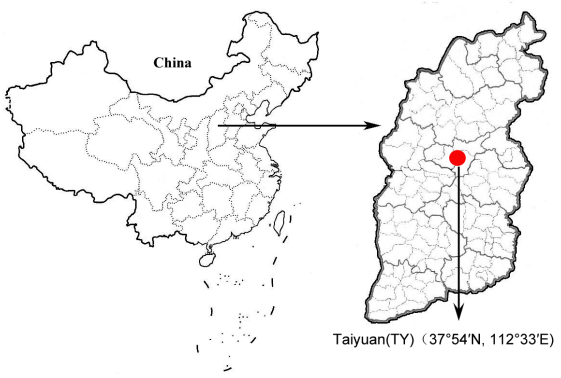

Fig. 1: Location of the study area in Taiyuan (TY), Shanxi, China (filled square).

L-lysine, dried for 30 min, and washed three times for 10 min each in 0.1 M phosphate buffer (pH 7.0). After being washed three times in ultrapure water for 10 min, these sample slides were dehydrated firstly with 50, 70, 80, 90, 95, and 100% ethanol (5 min at each stage) and then dehydrated with 1:1 ethanol:isoamyl acetate for 10 min at room temperature. These slides were critical-point dried with CO_2 in a critical-point dryer (HCP-2, Hitachi, Tokyo, Japan) and coated with gold-palladium (IB-5, Eiko, Tokyo, Japan). The samples were observed by a scanning electron microscope (JSM–35C, Jeol, Tokyo, Japan).

The culture was centrifuged and fixed for 3 h in 2.5% glutaraldehyde followed by 1% cacodylate buffer (pH 7.4) for 2 h. Then the cells of the sample were dehydrated through gradually increasing acetone solutions. After that these samples were embedded in Epon618 (Spurr's resin) at 40 °C for 24 h and polymerized at 60 °C for 48 h. The ultrathin section was cut with the ultramicrotome (PowerTome-PC, LKB, Rochester, USA) and stained with uranyl acetate and lead citrate. All the samples were examined using a transmission electron microscope (JEM–1011, JEOL, Tokyo, Japan) operated at 80 kV.

Lipid observation and determination of fatty acid content

The algal liquid extractions were treated by the cell disruption instrument (SCIENTZ-IID, Scientz, Ningbo, China). Then 240 µL of algal liquid samples were centrifuged at 3000 rpm for 5 min, and the pellet was washed with the TAP medium twice. After that, the pellet was resuspended in 240 µL TAP medium and transferred into the costar flat black 96-well plate. Subsequently, 1 µL Nile red (NR) dye solution (0.5 mg mL^{-1}, Sangon, Shanghai, China) was added and the mixed liquids were incubated in a constant temperature heating incubator (HPX-9882, MBE, Shanghai, China) to dye for 10 min at 37 °C in dark. Afterwards 10 µL of the microalgae suspension was taken out to photograph under 543 nm excitation and 598 nm emission wavelength under the fluorescent microscope (BX-41, Olympus, Tokyo, Japan) (Kimura et al., 2004).

The total lipid content was extracted according to Bligh et al. (1959), and was methyl-esterified (Duong et al., 2015).

The fatty acid content was analyzed by gas chromatography-mass spectrometry instrument (GC-MS) (7890A-5975C, Agilent, Los Angeles, USA). For the GC-MS analysis the column RTW-WAX (30 m × 0.25 mm, 0.5 µm) at a temperature program starting from 50 °C to 150 °C and kept 2 min, raised to 200 °C with the speed of 10 °C min^{-1} and stained 6 min, raised to 230 °C with the speed of 10 °C min^{-1} and stayed 30 min, raised to 240 °C with the speed of 10 °C min^{-1} and stayed 10 min, was used. As carrier gas nitrogen at a flow velocity of 0.35 mL min^{-1} was used and electron ionization (EI) source at anelectron energy of 70 eV. The mass spectrum scanning range was m z^{-1} 20-450 and the sample size0.2 µL. As mass spectral data base NIST 05 was used. The determintion of the molecular structure of content (%), similarity (%) in each component, and peaked areas normalization method was used to obtain relative content of components (Liu et al., 2011).

Determination of DNA sequence and construction of phylogenetic trees

Total DNA was extracted by using the c method withsodium dodecyl sulfate (SDS) as described by Kabir et al. (2012). The 18S rDNA gene region was amplified by using the forward primer MA1 (5'-CGGGATCCGTAGTCATATGCTTGTCTC-3') and the reverse primer MA2 (5'-CGGAATTCCTTCTGCAGGTTCACC-3') (Olmos et al., 2000). The chloroplast *rbc*L gene was amplified through the forward primer 475-497 (5'-CGTGACAAACTAAACAAATATGG -3') and the reverse primer 1181-1160 (5'-AAGATTTCAACTAAAGCTGGCA-3') (Nozaki et al., 1997). The nuclear ITS gene was amplified using the forward primer ITS-F (5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3') and the reverse primer ITS-R (5'-GGGATCCGTTTCCGTAGGTGAACCTGC-3') (Jiang et al., 2013). The PCR (CFX96, Bio-Rad, Hercules, USA) reaction systems of 18S rDNA, *rbc*L and ITS gene were the same and they were conducted in 20 µL volumes containing 7.0 µL ddH_2O (double distilled water), 2.0 µL 10 µM F_W (forward primer) (Sangon, Shanghai, China), 2.0 µL 10 µM R_V (reverse primer) (Sangon), 2.0 µL Taq DNA polymerase (Sangon), 2.0 µL of DNA template, and Taq PCR Master Mix (2x, blue dye) (Sangon). The optimized PCR conditions of 18S rDNA were initial denaturation at 95 °C for 5min, then followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min and saved at 4 °C for 10 min. The optimized PCR conditions of ITS were the same as 18S rDNA. The optimized PCR conditions of *rbc*L were different with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min and saved at 4 °C for 10 min. PCR amplification products were tested by 1% agarose gel electrophoresis and observed in the UV gel imaging system (ZF-268, Jiapeng, Shanghai, China). The respectivefragments were recovered by a small plastic recycling kit (Watson, Shanghai, China), purified and then sequenced by Sangon Biotech Company (Sangon,). Both amplification primers were used to determine the sequences. Sequences were inspected manually with Sequencher ver. 4.14. The bp obtained from PCR of each sequence was 1694 (18S rRNA), 676 (*rbc*L), 686 (ITS), respectively. The sequences were used as query sequences in the National Center for Biotechnology Information (NCBI). Similarsequences were downloaded from GenBank (NCBI) (Tab. S1).

Sequences were aligned through Clustal W (Higgins and Sharp, 1988) and manually checked. The partial error ranking results were corrected and appropriately adjusted. The locus of the sequences was deleted to make all sequences' length tidy. It was carried out to calculate the sequences about the numbers of differential gene loci, p-distance, variable sites and simple information sites, and proportion about the sequences length using MEGA 7.0 (Kumar et al., 2016) based on neighbor-joining (NJ), maximum likelihood (ML) and Bayesian Inference (BI) analyses, respectively. The program ModelTest 3.7 (Bos and Posada, 2005) was used to determine the best-fitting model of sequence evolution for ML and BI methods. Then the phylogenetic trees were constructed through software of MEGA 7.0, PhyML 3.0 (Guindon and Gascuel, 2003) and MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with NJ, ML, and BI methods, respectively. Consensus trees were constructed from all most-parsimonious trees. BI analyses were carried out with 1,000

replicates using a heuristic search with tree bisection and reconnection branch swapping and random taxon addition. Phylogenetic trees were constructed by BI, ML and NJ algorithms and the topologies were generally consistent. Therefore, the BI tree was chosen as the basic one marked with the support rates of ML and NJ at nodes. In the study, *Chloromonas rosae* FR865528 was selected as the outgroup of 18S rDNA and ITS, and *Chloromonas rosae* AB022536 was selected as the outgroup of *rbc*L. Finally, graphic refinement of all trees was done in Adobe Illustrator CS 5. Besides, in order to understand the phylogenetic classification of the microalgal strain (TY02) furthermore, we predicted its ITS2 secondary structure. The ITS2 secondary structure was folded by RNA mfold version 2.3 server (ZUKER, 2003), with a folding temperature set to 37 °C, and other parameters as the defaults. We drew the results of the secondary structure by RNA Viz2.0 (RIJK et al., 2003) and used Adobe illustrator CS5 to modify the final model.

Results and discussion

Morphological characteristics

Under the light microscope, the isolated algal strain presented following features: unicellular, yellow-green, spherical, and 5-15 μm in diameter. The chloroplast was single, irregular laminate, parietal, and occupied the major space of the cell with a big pyrenoid. Some small light globules could be seen in the cells. When cells became mature, 2 or 4 autospores were split by the mother cell. After the spores were released, they might adjacently develop and form palmella. According to its morphological and reproductive characteristics, the strain was ranked to the genus *Chlorella* (Chlorophyta) (BOLD and WYNNE, 1985) (Fig. 2).

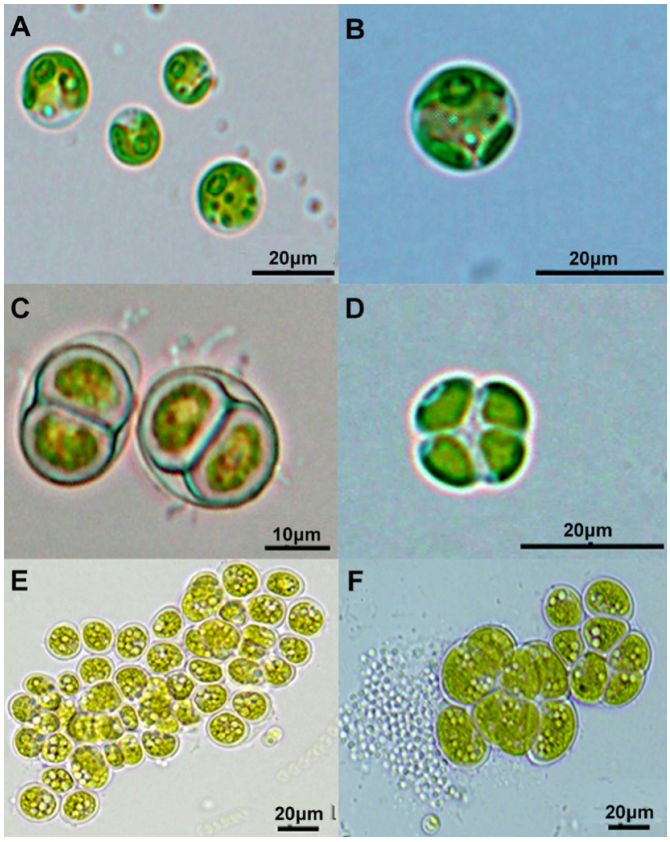

Fig. 2: Light microscopical images of TY02. (A-B) unicellular, (C-D) autospores formed, (E-F) palmella.

Under the scanning electron microscope, the cells were unicellular or in the palmella stage, looked like spherical or ellipsoidal and the cell walls were smooth or with irregular coastal regions (Fig. 3).

Under the transmission electron microscope, the cells exhibited to be spherical or ellipsoidal and the cell walls were transparent. The nucleus was wrapped in each cell and there was an evident nucleolus in it. The irregular chloroplast lamellas were displayed around the inside of the cell walls with an obvious pyrenoid covered by the starch sheath. The starch sheath consisted of several elliptic starch grains and crossed through into one or two thylakoids. In addition, the mitochondria, Golgi apparatus, and the endoplasmic reticulum could be clearly seen (Fig. 4).

Particularly, it was attractive that the light globules were found under the light microscopy. Then the light globules were observed more clearly in the cytoplasm under the transmission electron microscope.

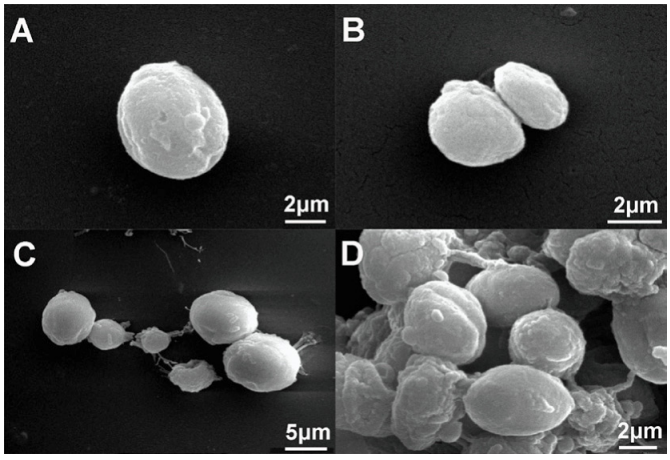

Fig. 3: Scanning electron microscope images of TY02. (A) unicellular, (B-D) palmella.

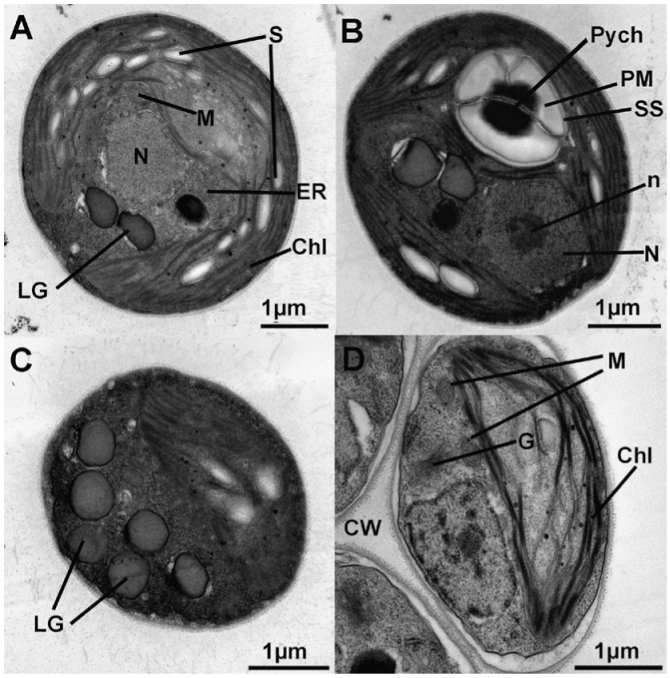

Fig. 4: Transmission electron microscope images of TY02. (S-starch grains, M-mitochondria, N-nucleus, ER-endoplasmic reticula, Chl-chloroplast lamellae, LG-lipid globules, Py-pyrenoid, SS-starch sheath, n-nucleolus, CW-cell wall, G-Golgi apparatus).

Furthermore, in order to verify them as the lipid bodies, the algal cells were dyed by NR and appeared to be bright yellow or orange fluorescence (Fig. 5). It means that lipids were stored in algal cells and the light globules were actually the lipid bodies. Thus, the strain could be the oil-rich microalga.

Although evidences of morphological characters were not enough to delineate the algal taxonomic status, they were useful to observe the oil-rich globules in the algal strain.

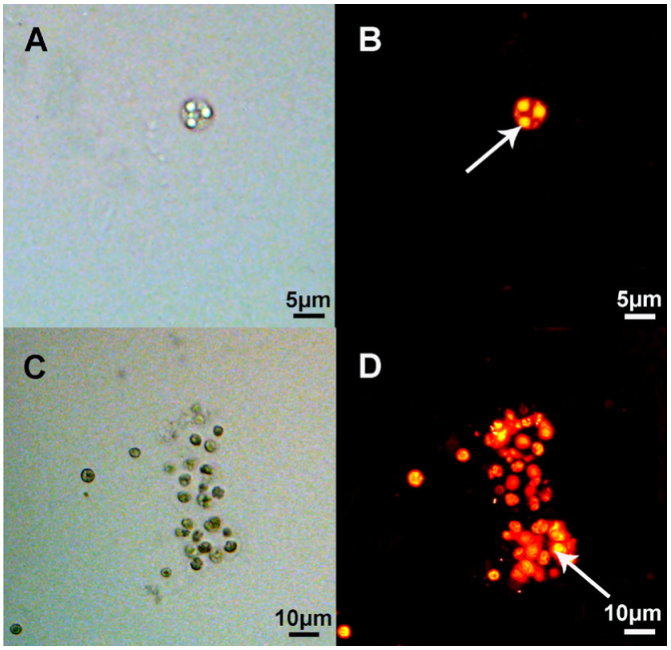

Fig. 5: Fluorescent microscopic images of TY02. (A, C) before staining with by NR, (B, D) after staining with NR (arrow points to lipid bodies). NR, Nile Red.

Total lipid and fatty acid content

To identify the oil-rich algal strains, it is necessary to determine their total lipid and fatty acid compositions. The total lipid content of TY02 was determined to be 33.27 ± 1.13%. According to the relevant references of rich total lipid microalgae, Chlorophyta (Mata et al., 2010), Bacillariophyta (Griffiths and Harrison, 2009), Cyanophyta (Mata et al., 2010), and Chrysophyta (Mansour et al., 2005) have reported. In those microalgae, the members of Chlorophyta (13%-56%) and Bacillariophyta (18%-27%) had higher total lipid than other strains, and *Chlorella* had richer oil than other genera in Chlorophyta. So in terms of above results, TY02 was considered an oil-rich strain.

The fatty acid content of the strain is shown in Fig. 6 and Tab. 1. Fatty acids including 7 categories were identified in TY02. It was mainly composed of C16 and C18 fatty acids, and the content of C16 and C18 in the strain was up to 88.2%. No long-chain fatty acid (more than C20) was determined. The polyunsaturated fatty acids with more than four double bonds were also not found. The predominant fatty acids were 9, 12, 15-octadecatrienoic acid (C18:3), hexadecanoic acid (C16:0), 9, 12-octadecadienoic acid (C18:2) in TY02, and the relative contents of them were 29.8%, 22.3% and 13.9%. According to the relevant references, C16 and C18 reached high contents of fatty acid, up to about 50-95%, 25-80% and 30-40% contents in Chlorophyta (Vello et al., 2014), Bacillariophyta (Fuentes-Grconewald et al., 2009) and Dinophyta (Fuentes-Grconewald et al., 2009), respectively. In those microalgae, Chlorophyta had much more C16 and C18 than the other taxa. So TY02 also contained much more C16 and C18 fatty acids which were the important for biodiesel.

The microalgal strain was isolated from the soil. TY02 was identified to be oil-rich through morphological and chemical methods, which provide an important feasible way to select the oil-rich algal strain from the soil. In the biodiesel, the saturated fatty acids could offer oxidation resistances and the unsaturated fatty acids might provide better stability at low temperature. TY02 contained both saturated and unsaturated fatty acids, meanwhile, the main fatty acid compositions were hexadecanoic acid (C16:0), 9, 12-octadecadienoic acid (C18:2) and 9, 12, 15-octadecatrienoic acid (C18:3), of which the content of C16 and C18 were up to 88.2%. Therefore, the strain had been finally concluded to have the greatest application potential to produce biodiesel in the future.

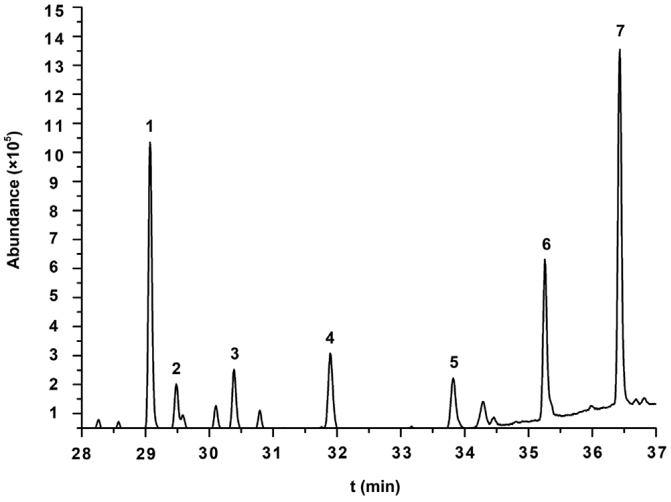

Fig. 6: Total ion chromatogram obtained with the GC-MS of fatty acid methyl esters of TY02. (1) C16:0, (2) C16:1, (3) C16:2, (4) C16:3, (5) C18:0, (6) C18:2, (7) C18:3.

Tab. 1: Fatty acid content of TY02

Sequence number	Similarity (%)	Chemical name	Chemical formula	Content (%)
a	92.8	Hexadecanoic acid	$C_{16}H_{36}O_2$	22.32
b	87.4	9-Hexadecenoic acid (Z)-	$C_{16}H_{30}O_2$	2.35
c	88.6	7, 10-Hexadecadienoic acid	$C_{16}H_{28}O_2$	5.27
d	88.6	7, 10, 13-Hexadecatrienoic acid	$C_{16}H_{26}O_2$	8.81
e	86.8	Octadecanoic acid	$C_{18}H_{36}O_2$	5.75
f	91.6	9, 12-Octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$	13.90
g	87.6	9, 12, 15-Octadecatrienoic acid (Z, Z, Z)-	$C_{18}H_{30}O_2$	29.75

Analysis of molecular phylogeny

For the oil-rich algal strain, it was necessary to d investigate its phylogeny. Therefore, the 18S rDNA, *rbc*L and ITS genes were chosen as molecular markers. The 18S rDNA gene sequence is highly conserved and it was easy to amplify it by general primer; thus it became an important marker for higher ranks such as classes and orders (ANDERSEN et al., 1999). As for *rbc*L gene, due to its relatively slow evolutionary rates, it has been widely applied to the analysis of genera and classification above order systematics (ENTWISLE et al., 2009). ITS sequence mainly composed of ITS1, 5.8S, and ITS2, has a high variation, so it was shown to be a simple and effective method to analysis the phylogenetic relationships of taxa below the level of the genera (LI et al., 2013). Besides, ITS2 rRNA secondary structure had more conserved properties compared to the primary structure. We had found a sufficient amount of information about the phylogenetic analysis and classification in it, so the secondary structure was an important role to complement the classification system. (LI et al., 2013).

The results based on three gene sequences showed that there were only a small number of different bases among TY02 and several *Parachlorella kessleri* Krienitz strains. The phylogenetic comparison of 18S rDNA, *rbc*L and ITS in the strain showed that total of 1697, 676 and 765 aligned sites existed except for some missed ones. 8.07 % of the total aligned sequences were 137 diversity sites and 4.36 % were 74 informative sites for 18S rDNA, 28.99% of the total aligned sequences were 196 variable sites and 23.82% were 161 informative sites for *rbc*L, and 59.87% of the total aligned sequences were 458 variable sites and 39.08% were 299 informative sites for ITS. The appropriate DNA evolutionary models and 11 relevant models were gained, including natural logarithm of a negative number, the frequency of 4 kinds of bases, 6 kinds of alternative models of bases, parameters of gamma shape distribution and proportion of invariable sites. For the 18S rDNA gene, the model was as follows: TIM+I+G distance model, proportion of invariable sites=0.7949, gamma distribution shape parameter=0.5778; base frequencies A=0.2468, C=0.2210, G=0.2762, T=0.2560; and rate matrix A-C=1.0000, A-G=1.7518, A-T=0.5396, C-G=0.5396, C-T=5.9695, G-T=1.0000. For the *rbc*L gene, the model was as follows: GTR+I+G distance model, proportion of invariable sites=0.5925, gamma distribution shape parameter=1.0099; base frequencies A=0.2550, C=0.1666, G=0.2170, T=0.3614; and rate matrix A-C=0.3536, A-G=1.9625, A-T=3.6319, C-G=0.7810, C-T=6.3279, G-T=1.0000. For the ITS gene, the model was as follows: TIM+G distance model, proportion of invariable sites=0, gamma distribution shape parameter=0.4141; base frequencies A=0.2181, C=0.3272, G=0.2537, T=0.2011; and rate matrix A-C=1.0000, A-G=1.2248, A-T=0.7983, C-G=0.7983, C-T=2.5680, G-T=1.0000. And as for the numbers of differences' gene locus based on 18S r DNA, *rbc*L and ITS sequences, they all indicated that TY02 and *P. kessleri* appeared differences about the smaller number of bases sites than other species (Tab. S2, Tab. S4, Tab. S6). By calculated the P-distance values of the 18S r DNA, *rbc*L and ITS sequences, they indicated the same results, TY02 and *P. kessleri* appeared smaller p-distance than other species (Tab. S3, Tab. S5, Tab. S7).

Based on 18S rDNA, phylogenetic trees were constructed by BI, ML and NJ algorithms and the topologies were generally consistent. So the BI tree was chosen as basic one marked with the support rates of ML and NJ at nodes. The strain TY02 gathered with *P. kessleri* KM020114, *P. kessleri* JQ797561, *P. kessleri* AB080309, *P. kessleri* HM744739, *P. kessleri* AB162911, *P. kessleri* KM020113, *P. kessleri* FM205846 and *Chlorella kessleri* Fott and Nováková X56105, which showed better support rates. Therefore, it revealed that there was a close genetic relationship between the TY02 strain and *P. kessleri* (Fig. 7). The BI tree based on *rbc*L gene showed that TY02 was gathered with *P. kessleri* AB260912, which showed the better support rates: 1.00/999/100 (BI/ML/NJ). It showed that TY02 and *P. kessleri* had a close genetic relationship (Fig. 8). Similar to the results of 18S rDNA and *rbc*L gene, the BI tree based on ITS gene was also indicated that TY02 gathered with *P. kessleri* AB162911, *P. kessleri* FM205846, *P. kessleri* KJ676114, *P. kessleri* JQ797561, *P. kessleri* KJ676155, *P. kessleri* KJ676117, *P. kessleri* FR865655, *P. kessleri* KJ676118, *P. kessleri* KJ676116 and *P. kessleri* HM744739, which showed the better support rates: 1.00/983/100 (BI/ML/NJ) (Fig. 9). So, through the results of the three genes' phylogenetic trees, they all showed that TY02 gathered with *P. kesserli*, a member of Chlorophyta.

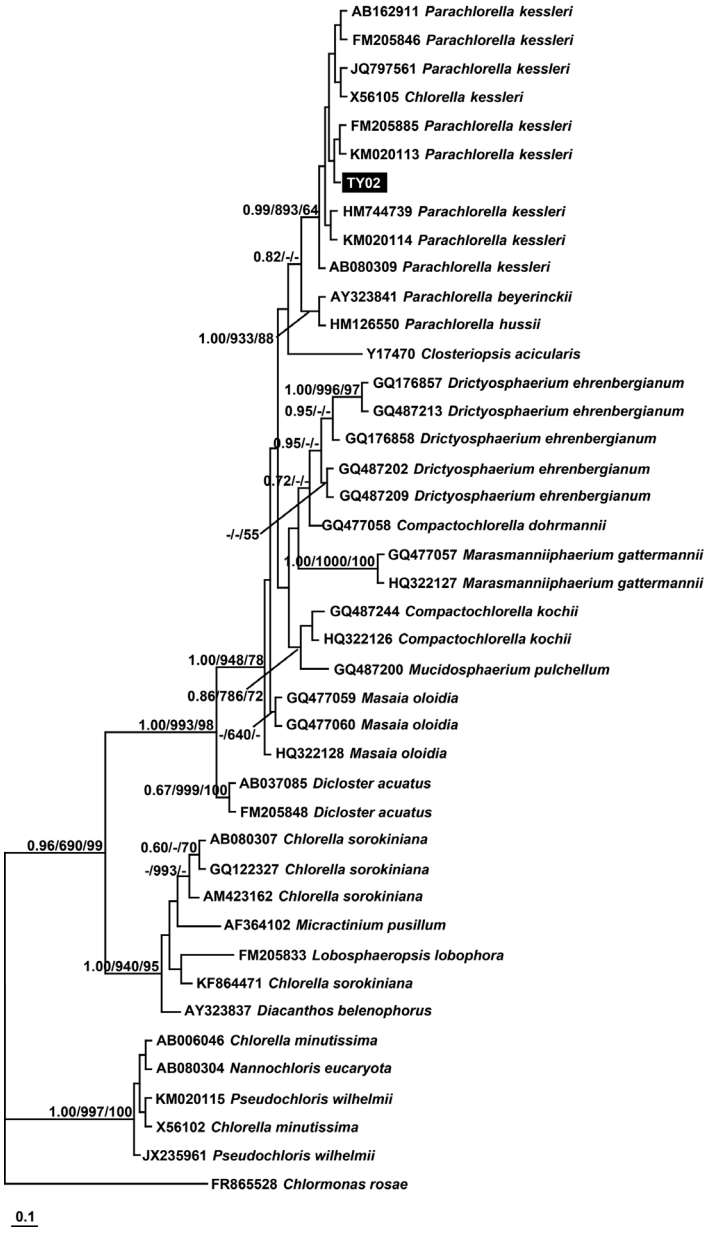

Fig. 7: Bayesian analysis tree based on 18S rDNA gene sequence. The numerical values at the nodes represent the support values of BI bootstrap/ML bootstrap/NJ bootstrap. Values <50% are not shown.

According to the above results, we could find TY02 basically gathered with *P. kesserli*. Meanwhile, we compared its ITS2 secondary structure with the strain *P. kesserli* FM205885 according to BOCK et al. (2011) (Fig. 10). They both have four helices and no Compensatory Base Changes (CBCs) and hemi-CBCs according to LI et al. (2013) and BOCK et al. (2011). Only one bp difference was present

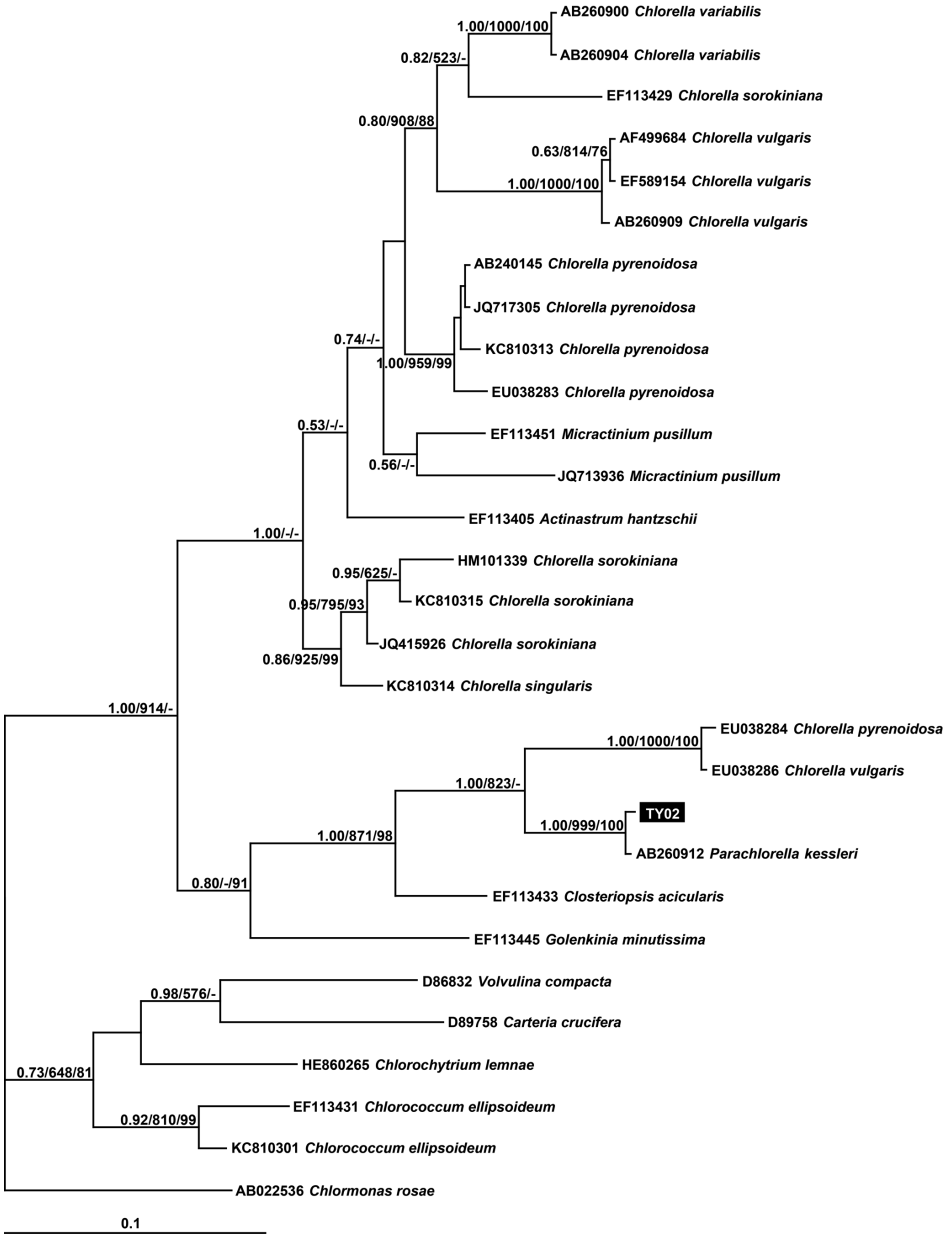

Fig. 8: Bayesian analysis tree based on *rbc*L gene sequence. The numerical values at the nodes represent the support values of BI bootstrap/ML bootstrap/NJ bootstrap. Values <50% are not shown.

and it was U in TY02, while it was C in *P. kessleri* FM205885. So, we could confirm that TY02 followed into the *P. kesserli* taxa.

The genus *Parachlorella* was established based on the 18S rDNA and ITS sequences by KRIENITZ et al. (2004). It is a unicellular planktonic eukaryote, which is solitary or gathered in groups. The morphological characters showed that the cells were spherical or ellipsoidal, the cell walls were smooth, and chromoplast developed the parietal, with a broadly ellipsoidal pyrenoid covered by starch grains. Asexual reproduction was produced 2, 4, 8 or 16 autospores (JIANG et al., 2013). Only three species were reported up to now. The typical species *Parachlorella beijerinckii* Krienitz was collected in the tributary of Tollensesee Lake, Germany. *P Parachlorella kessleri*, which was once named *Chlorella kessleri* collected from Algae Culture Collection of University of Göttingen, Germany (KRIENITZ et al., 2004). *Parachlorella hussii* Bock, Pažoutová et Krienitz was collected from Portugal and it was once named *Coronastrum aestivale* Thompson, *Coenochloris hindakii* Komárek and *Dispora speciosa* Korshikov (BOCK et al., 2011). From the study results, this genus could be presumed to be distributed in the fresh water or moist soil, and be likely to accumulate oils.

Conclusions

The cell structures of the experimental algal strain (TY02) was observed by the light microscope, scanning electron microscope, transmission electron microscope and fluorescence microscope, and some lipid globules were found. It could be confirmed that the algal strain contained oils.

At present, the combination of morphological and molecular data for the identification was necessary. In this study, the phylogenetic trees were constructed by 18S rDNA, *rbc*L, ITS and the ITS2 secondary structure was analyzed. The results showed that the soil microalgal strain (TY02) could be identified as *Parachlorella kessleri*, a member of the Chlorophyta.

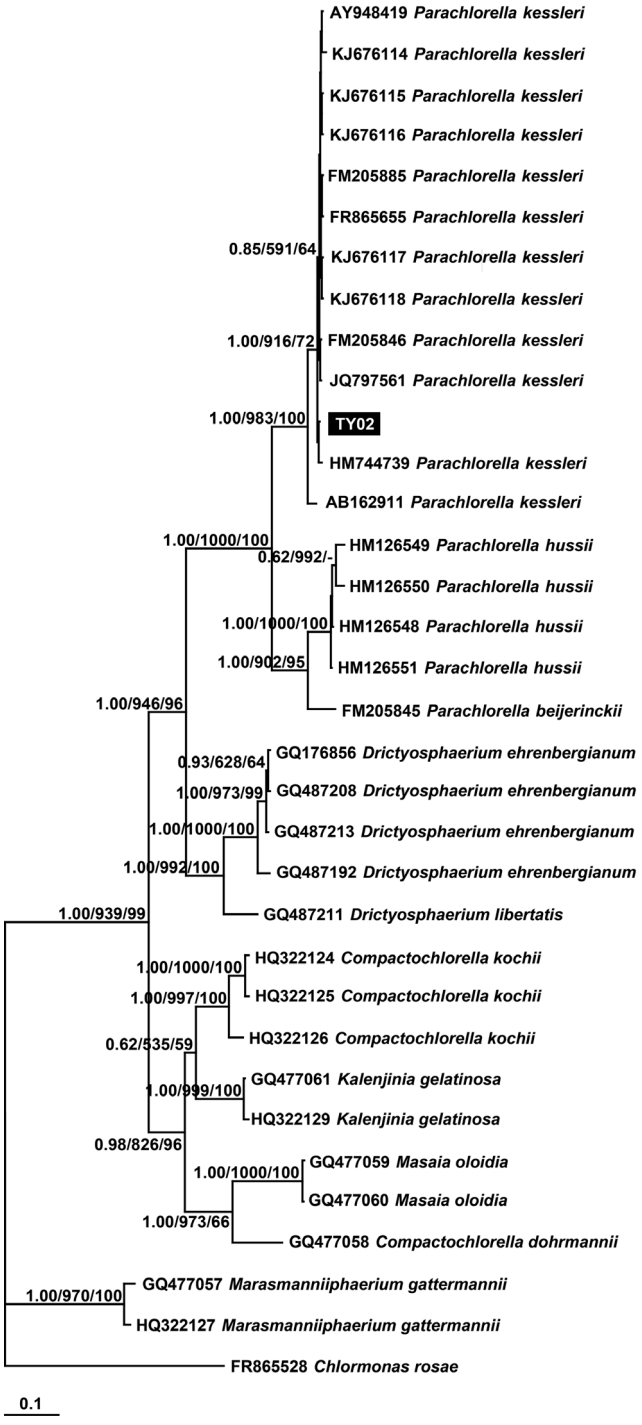

Fig. 9: Bayesian analysis tree based on ITS gene sequences. The numerical values at the nodes represent the support values of BI bootstrap/ML bootstrap/NJ bootstrap. Values <50% are not shown.

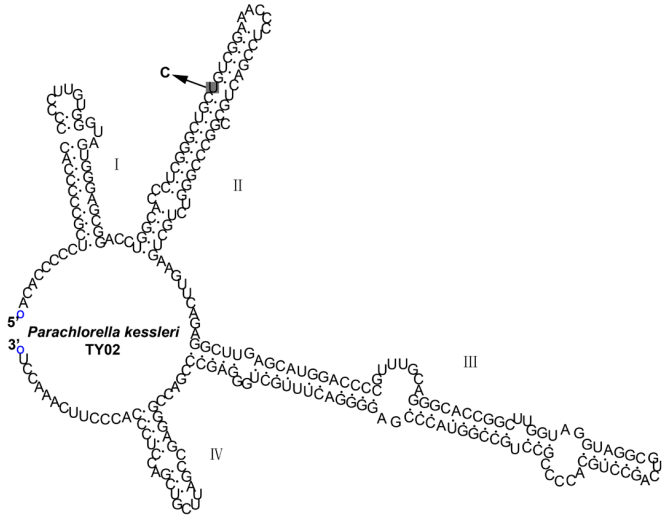

Fig. 10: ITS2 secondary structure prediction for TY02. Compared with *Parachlorella kessleri* FM205885 (Bock et al., 2011), both molecules have basically the same predicted secondary structure, i.e. they both have four helices (I, II, III and IV are presented by four different helices, respectively). The only difference against the closest sequenced relative *P. kessleri* FM205885 is high-lighted in gray. This position in TY02 is U, while in *P. kessleri* FM205885 it is C (position with the arrow point to).

TY02 contained saturated and unsaturated fatty acids, meanwhile, the main fatty acid compositions were hexadecanoic acid (C16:0), 9, 12-octadecadienoic acid (C18:2) and 9, 12, 15-octadecatrienoic acid (C18:3). The content of C16 and C18 were up to 88.15%. Therefore, the strain had application potential to produce biodiesel.

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Morphology, phylogeny and lipid components of an oil-rich microalgal strain

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Tab. S1: GenBank accession number of related gene sequences in this study

Genus	Taxonomic group	18S rDNA	*rbc*L	ITS
Actinastrum	*Actinastrum hantzschii*		EF113405 (VERGHESE, et al., unpublished)	
Cateria	*Cateria crucifera*		D89758 (NOZAKI et al., 2008)	
Chlorella	*Chlorella kessleri*	X56105 (HUSS and SOGIN, 1990)		
	Chlorella minutissima	AB006046 (HANAGAT, unpublished)		
	Chlorella pyrenoidosa		AB240145 (KUMA, et al., unpublished)	
	Chlorella sorokiniama	AB080307 (YAMAMOTO, et al., 2003)	EF113429 (VERGHESE, unpublished)	
Chlorochytrium	*Chlorochytrium lemnae*		HE860265 (SKALOUD, et al., 2013)	
Chlorococcum	*Chlorococcum ellipsoideum*		EF113431 (VERGHESE, et al., unpublished)	
Closteriopsis	*Closteriopsis acicularis*	Y17470 (USTINOVA, et al., 2001)	EF113433 (VERGHESE, unpublished)	
Compactochlorella	*Compactochlorella dohrmannii*	GQ477058 (KRIENITZ, et al., 2012)		GQ477058 (KRIENITZ, et al., 2012)
	Compactochlorella kochii	GQ487244 (KRIENITZ, et al., 2012)		HQ322124 (KRIENITZ, et al., 2012)
Diacanthos	*Diacanthos belenophorus*	AY323837 (KRIENITZ, et al., 2004)		
Dicloster	*Dicloster acuatus*	AB037085 (HEGEWALD, et al., 2000)		
Dictyosphaerium	*Dictyosphaerium ehrenbergianum*	GQ176857 (KRIENITZ, et al., 2010)		GQ176855 (KRIENITZ, et al., 2010)
	Dictyosphaerium libertatis			GQ487211 (BOCK et al., 2011)
Golenkinia	*Golenkinia minutissima*		EF113445 (VERGHESE, et al., unpublished)	GQ477061 (KRIENITZ, et al., 2012)
Kalenjinia	*Kalenjinia gelatinosa*			HQ322129 (KRIENITZ, et al., 2012)
Lobosphaeropsis	*Lobosphaeropsis lobophora*	FM205833 (LUO, et al., 2010)		

Marasphaerium	*Marasphaerium gattermannii*	GQ477057 (KRIENITZ, et al., 2012)		GQ477057 (KRIENITZ, et al., 2012)
Masaia	*Masaia oloidia*	GQ477059 (KRIENITZ, et al., 2012)		GQ477059 (KRIENITZ, et al., 2012)
Micractinium	*Micractinium pusillum*	AF364102 (KRIENITZ, et al., 2004)		
Mucidosphaerium	*Mucidosphaerium pulchellum*	GQ487200 (BOCK et al., 2011)		
Nannochloris	*Nannochloris eucaryota*	AB080304 (YAMAMOTO, et al., 2003)		
Parachlorella	*Parachlorella beyerinckii*	AY323841 (KRIENITZ, et al., 2004)		FM205845 (LUO, et al., 2010)
	Parachlorella hussii	HM126550 (BOCK, et al., 2011)		HM126548 (BOCK, et al., 2011)
	Parachlorella kessleri	AB080309 (YAMAMOTO, et al., 2003)	AB260912 (HOSHINA, et al., unpublished)	AB162911 (HOSHINA, et al., 2004)
	Pseudochloris wilhelmii	KM020115 (Friedl, et al., unpublished)		
	TY02	KX021356	KX021358	KX021360
Chloromonas	**Outgroup** *Chloromonas rosae*	FR865528 (GACHON, et al., 2013)	AB022536 (MORITA, et al., 1999)	FR865528 (GACHON, et al., 2013)

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Tab. S2: Numbers of differences' gene locus based on 18S rDNA sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1	TY02																																									
2	AB006046	43																																								
3	AB037085	9	40																																							
4	AB080304	43	0	40																																						
5	AB080307	22	33	17	33																																					
6	AB080309	0	43	9	43	22																																			
7	AB162911	0	43	9	43	22	0																																		
8	AF364102	25	38	20	38	5	25	25																																	
9	AM423162	21	34	16	34	1	21	21	6																																
10	AY323837	23	36	18	36	3	23	23	8	2																															
11	AY323841	3	40	10	40	23	3	3	26	22	24																														
12	FM205833	27	35	22	35	7	27	27	12	6	8	28																													
13	FM205846	0	43	9	43	22	0	0	25	21	23	3	27																												
14	FM205848	9	40	0	40	17	9	9	20	16	18	10	22	9																											
15	FM205885	0	43	9	43	22	0	0	25	21	23	3	27	0	9																										
16	GQ122327	22	33	17	33	0	22	22	5	1	3	23	7	22	17	22																									
17	GQ176857	7	41	12	41	26	7	7	29	25	27	4	29	7	12	7	26																								
18	GQ176858	4	42	9	42	23	4	4	26	22	24	5	26	4	9	4	23	3																							
19	GQ477057	12	41	19	41	31	12	12	32	30	32	13	36	12	19	12	31	15	12																						
20	GQ477058	3	43	10	43	23	3	3	26	22	24	4	28	3	10	3	23	6	3	11																					
21	GQ477059	1	42	8	42	21	1	1	24	20	22	2	26	1	8	1	21	6	3	11	2																				
22	GQ477060	1	42	8	42	21	1	1	24	20	22	2	26	1	8	1	21	6	3	11	2	0																			
23	GQ487200	3	43	12	43	25	3	3	28	24	26	6	28	3	12	3	25	8	5	13	6	4	4																		
24	GQ487202	3	42	10	42	23	3	3	26	22	24	4	26	3	10	3	23	4	1	11	2	2	2	4																	
25	GQ487209	3	42	10	42	23	3	3	26	22	24	4	26	3	10	3	23	4	1	11	2	2	2	4	0																
26	GQ487213	7	41	12	41	26	7	7	29	25	27	4	29	7	12	7	26	0	3	15	6	6	6	8	4	4															
27	GQ487244	3	42	10	42	23	3	3	26	22	24	4	28	3	10	3	23	6	3	11	2	2	2	4	2	2	6														
28	HM126550	3	40	10	40	23	3	3	26	22	24	0	28	3	10	3	23	4	5	13	4	2	2	6	4	4	4	4													
29	HM744739	0	43	9	43	22	0	0	25	21	23	3	27	0	9	0	22	7	4	12	3	1	1	3	3	3	7	3	3												
30	HQ322126	3	42	10	42	23	3	3	26	22	24	4	28	3	10	3	23	6	3	11	2	2	2	4	2	2	6	0	4	3											
31	HQ322127	12	41	19	41	31	12	12	32	30	32	13	36	12	19	12	31	15	12	0	11	11	11	13	11	11	15	11	13	12	11										
32	HQ322128	1	42	8	42	21	1	1	24	20	22	2	26	1	8	1	21	6	3	11	2	0	0	4	2	2	6	2	2	1	2	11									
33	JQ797561	0	43	9	43	22	0	0	25	21	23	3	27	0	9	0	22	7	4	12	3	1	1	3	3	3	7	3	3	0	3	12	1								
34	JX235961	43	0	40	0	33	43	43	38	34	36	40	35	43	40	43	33	41	42	41	43	42	42	43	42	42	41	42	40	43	42	41	42	43								
35	KF864471	22	33	17	33	0	22	22	5	1	3	23	7	22	17	22	0	26	23	31	23	21	21	25	23	23	26	23	23	22	23	31	21	22	33							
36	KM020113	0	43	9	43	22	0	0	25	21	23	3	27	0	9	0	22	7	4	12	3	1	1	3	3	3	7	3	3	0	3	12	1	0	43	22						
37	KM020114	0	43	9	43	22	0	0	25	21	23	3	27	0	9	0	22	7	4	12	3	1	1	3	3	3	7	3	3	0	3	12	1	0	43	22	0					
38	KM020115	43	0	40	0	33	43	43	38	34	36	40	35	43	40	43	33	41	42	41	43	42	42	43	42	42	41	42	40	43	42	41	42	43	0	33	43	43				
39	X56102	43	0	40	0	33	43	43	38	34	36	40	35	43	40	43	33	41	42	41	43	42	42	43	42	42	41	42	40	43	42	41	42	43	0	33	43	43	0			
40	X56105	0	43	9	43	22	0	0	25	21	23	3	27	0	9	0	22	7	4	12	3	1	1	3	3	3	7	3	3	0	3	12	1	0	43	22	0	0	43	43		
41	Y17470	12	51	17	51	28	12	12	27	29	31	13	35	12	17	12	28	17	14	21	13	11	11	15	13	13	17	13	13	12	13	21	11	12	51	28	12	12	51	51	12	
42	FR865528	93	88	89	88	89	93	93	89	88	88	94	86	93	89	93	89	96	93	88	94	92	92	94	93	93	96	94	94	93	94	88	92	93	88	89	93	93	88	88	93	96

Tab. S3: Pairwise p-distances based on 18S rDNA sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1	TY02																																									
2	AB006046	0.03																																								
3	AB037085	0.01	0.03																																						
4	AB080304	0.03	0.00	0.03																																					
5	AB080307	0.02	0.02	0.01	0.02																																				
6	AB080309	0.00	0.03	0.01	0.03	0.02																																		
7	AB162911	0.00	0.03	0.01	0.03	0.02	0.00																																	
8	AF364102	0.02	0.03	0.01	0.03	0.00	0.02	0.02																																
9	AM423162	0.02	0.02	0.01	0.02	0.00	0.02	0.02	0.00																															
10	AY323837	0.02	0.03	0.01	0.03	0.00	0.02	0.02	0.01	0.00																														
11	AY323841	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02																													
12	FM205833	0.02	0.03	0.02	0.03	0.01	0.02	0.02	0.01	0.00	0.01	0.02																												
13	FM205846	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02																											
14	FM205848	0.01	0.03	0.00	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01																										
15	FM205885	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01																									
16	GQ122327	0.02	0.02	0.01	0.02	0.00	0.02	0.02	0.00	0.00	0.00	0.02	0.01	0.02	0.01	0.02																								
17	GQ176857	0.01	0.03	0.01	0.03	0.02	0.01	0.01	0.02	0.02	0.02	0.00	0.02	0.01	0.01	0.01	0.02																							
18	GQ176858	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00																						
19	GQ477057	0.01	0.03	0.01	0.03	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.03	0.01	0.01	0.01	0.02	0.01	0.01																					
20	GQ477058	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01																				
21	GQ477059	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.01	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00																			
22	GQ477060	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.01	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00																		
23	GQ487200	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00																	
24	GQ487202	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00																
25	GQ487209	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00															
26	GQ487213	0.01	0.03	0.01	0.03	0.02	0.01	0.01	0.02	0.02	0.02	0.00	0.02	0.01	0.01	0.01	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00														
27	GQ487244	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00													
28	HM126550	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00												
29	HM744739	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00											
30	HQ322126	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00										
31	HQ322127	0.01	0.03	0.01	0.03	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.03	0.01	0.01	0.01	0.02	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01									
32	HQ322128	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.01	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01								
33	JQ797561	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00							
34	JX235961	0.03	0.00	0.03	0.00	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03						
35	KF864471	0.02	0.02	0.01	0.02	0.00	0.02	0.02	0.00	0.00	0.00	0.02	0.01	0.02	0.01	0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02					
36	KM020113	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.03	0.02				
37	KM020114	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.03	0.02	0.00			
38	KM020115	0.03	0.00	0.03	0.00	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00	0.02	0.03	0.03		
39	X56102	0.03	0.00	0.03	0.00	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00	0.02	0.03	0.03	0.00	
40	X56105	0.00	0.03	0.01	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.03	0.02	0.00	0.00	0.03	0.03
41	Y17470	0.01	0.04	0.01	0.04	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.03	0.01	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.04	0.02	0.01	0.01	0.04	0.04
42	FR865528	0.07	0.06	0.06	0.06	0.06	0.07	0.07	0.06	0.06	0.06	0.07	0.06	0.07	0.06	0.07	0.06	0.07	0.07	0.06	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.07	0.07	0.06	0.06	0.07	0.07	0.06	0.06

Tab. S4: Numbers of differences' gene locus based on *rbc*L sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	TY02																												
2	AB022536	73																											
3	AB240145	46	62																										
4	AB260900	52	68	19																									
5	AB260904	52	68	19	0																								
6	AB260909	43	69	26	26	26																							
7	AB260912	1	74	47	53	53	42																						
8	AF499684	42	68	27	26	26	1	41																					
9	D86832	56	67	56	64	64	64	57	63																				
10	D89758	61	71	62	63	63	61	62	62	46																			
11	EF113405	60	69	25	30	30	37	61	38	59	62																		
12	EF113429	55	71	27	27	27	32	54	31	63	64	36																	
13	EF113431	57	55	69	68	68	72	58	71	49	54	71	74																
14	EF113433	32	76	50	59	59	51	31	50	60	60	63	53	64															
15	EF113445	50	67	46	44	44	45	51	44	63	57	39	47	65	48														
16	EF113451	50	65	13	25	25	31	51	30	55	62	24	31	66	49	47													
17	EF589154	42	68	27	26	26	1	41	0	63	62	38	31	71	50	44	30												
18	EU038283	49	63	3	22	22	27	48	28	59	63	25	26	68	48	47	13	28											
19	EU038284	31	74	45	53	53	42	30	41	65	65	59	51	74	42	55	51	41	45										
20	EU038286	30	73	44	52	52	41	29	40	64	64	58	50	73	41	54	50	40	44	1									
21	HE860265	64	64	60	58	58	67	65	66	43	44	52	67	38	65	56	54	66	59	73	72								
22	HM101339	56	63	21	25	25	35	57	36	60	61	29	37	67	53	50	23	36	23	54	53	55							
23	JQ415926	56	68	20	22	22	30	57	31	58	59	29	32	65	52	49	21	31	22	57	56	56	8						
24	JQ713936	48	60	21	27	27	27	49	26	53	61	26	26	69	50	44	17	26	22	46	45	59	28	24					
25	JQ717305	46	62	0	19	19	26	47	27	56	62	25	27	69	50	46	13	27	3	45	44	60	21	20	21				
26	KC810301	59	55	56	57	57	64	60	63	46	47	60	65	17	61	56	56	63	55	66	65	31	56	56	59	56			
27	KC810313	48	62	2	21	21	28	49	29	58	64	27	29	71	52	48	15	29	5	47	46	62	23	22	23	2	58		
28	KC810314	55	68	18	25	25	32	56	33	60	61	31	30	70	51	48	21	33	21	58	57	59	11	9	25	18	59	20	
29	KC810315	58	66	21	25	25	33	59	34	58	61	27	35	67	53	50	21	34	23	58	57	57	5	3	26	21	58	23	10

Tab. S5: Pairwise p-distances based on *rbc*L sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	TY02																												
2	AB022536	0.14																											
3	AB240145	0.09	0.12																										
4	AB260900	0.10	0.13	0.04																									
5	AB260904	0.10	0.13	0.04	0.00																								
6	AB260909	0.08	0.13	0.05	0.05	0.05																							
7	AB260912	0.00	0.14	0.09	0.10	0.10	0.08																						
8	AF499684	0.08	0.13	0.05	0.05	0.05	0.00	0.08																					
9	D86832	0.11	0.13	0.11	0.12	0.12	0.12	0.11	0.12																				
10	D89758	0.12	0.14	0.12	0.12	0.12	0.12	0.12	0.12	0.09																			
11	EF113405	0.11	0.13	0.05	0.06	0.06	0.07	0.12	0.07	0.11	0.12																		
12	EF113429	0.10	0.14	0.05	0.05	0.05	0.06	0.10	0.06	0.12	0.12	0.07																	
13	EF113431	0.11	0.10	0.13	0.13	0.13	0.14	0.11	0.14	0.09	0.10	0.14	0.14																
14	EF113433	0.06	0.15	0.10	0.11	0.11	0.10	0.06	0.10	0.11	0.11	0.12	0.10	0.12															
15	EF113445	0.10	0.13	0.09	0.08	0.08	0.09	0.10	0.08	0.12	0.11	0.07	0.09	0.12	0.09														
16	EF113451	0.10	0.12	0.02	0.05	0.05	0.06	0.10	0.06	0.10	0.12	0.05	0.06	0.13	0.09	0.09													
17	EF589154	0.08	0.13	0.05	0.05	0.05	0.00	0.08	0.00	0.12	0.12	0.07	0.06	0.14	0.10	0.08	0.06												
18	EU038283	0.09	0.12	0.01	0.04	0.04	0.05	0.09	0.05	0.11	0.12	0.05	0.05	0.13	0.09	0.09	0.02	0.05											
19	EU038284	0.06	0.14	0.09	0.10	0.10	0.08	0.06	0.08	0.12	0.12	0.11	0.10	0.14	0.08	0.10	0.10	0.08	0.09										
20	EU038286	0.06	0.14	0.08	0.10	0.10	0.08	0.06	0.08	0.12	0.12	0.11	0.10	0.14	0.08	0.10	0.10	0.08	0.08	0.00									
21	HE860265	0.12	0.12	0.11	0.11	0.11	0.13	0.12	0.13	0.08	0.08	0.10	0.13	0.07	0.12	0.11	0.10	0.13	0.11	0.14	0.14								
22	HM101339	0.11	0.12	0.04	0.05	0.05	0.07	0.11	0.07	0.11	0.12	0.06	0.07	0.13	0.10	0.10	0.04	0.07	0.04	0.10	0.10	0.10							
23	JQ415926	0.11	0.13	0.04	0.04	0.04	0.06	0.11	0.06	0.11	0.11	0.06	0.06	0.12	0.10	0.09	0.04	0.06	0.04	0.11	0.11	0.11	0.02						
24	JQ713936	0.09	0.11	0.04	0.05	0.05	0.05	0.09	0.05	0.10	0.12	0.05	0.05	0.13	0.10	0.08	0.03	0.05	0.04	0.09	0.09	0.11	0.05	0.05					
25	JQ717305	0.09	0.12	0.00	0.04	0.04	0.05	0.09	0.05	0.11	0.12	0.05	0.05	0.13	0.10	0.09	0.02	0.05	0.01	0.09	0.08	0.11	0.04	0.04	0.04				
26	KC810301	0.11	0.10	0.11	0.11	0.11	0.12	0.11	0.12	0.09	0.09	0.11	0.12	0.03	0.12	0.11	0.11	0.12	0.10	0.13	0.12	0.06	0.11	0.11	0.11	0.11			
27	KC810313	0.09	0.12	0.00	0.04	0.04	0.05	0.09	0.06	0.11	0.12	0.05	0.06	0.14	0.10	0.09	0.03	0.06	0.01	0.09	0.09	0.12	0.04	0.04	0.04	0.00	0.11		
28	KC810314	0.10	0.13	0.03	0.05	0.05	0.06	0.11	0.06	0.11	0.12	0.06	0.06	0.13	0.10	0.09	0.04	0.06	0.04	0.11	0.11	0.11	0.02	0.02	0.05	0.03	0.11	0.04	
29	KC810315	0.11	0.13	0.04	0.05	0.05	0.06	0.11	0.06	0.11	0.12	0.05	0.07	0.13	0.10	0.10	0.04	0.06	0.04	0.11	0.11	0.11	0.01	0.01	0.05	0.04	0.11	0.04	0.02

Tab. S6: Numbers of differences' gene locus based on ITS sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	TY02																																	
2	AB162911	11																																
3	AY948419	0	11																															
4	FM205845	43	39	43																														
5	FM205846	0	11	0	43																													
6	FM205885	0	11	0	43	0																												
7	FR865655	0	11	0	43	0	0																											
8	GQ176856	76	80	76	73	76	76	76																										
9	GQ477057	112	110	112	106	112	112	112	112																									
10	GQ477058	94	94	94	85	94	94	94	91	100																								
11	GQ477059	92	93	92	96	92	92	92	95	107	59																							
12	GQ477060	92	93	92	96	92	92	92	95	107	59	0																						
13	GQ477061	85	87	85	88	85	85	85	74	90	66	69	69																					
14	GQ487192	77	79	77	72	77	77	77	12	110	85	91	91	66																				
15	GQ487208	76	80	76	73	76	76	76	1	111	90	96	96	75	13																			
16	GQ487211	75	73	75	69	75	75	75	38	103	81	87	87	67	35	38																		
17	GQ487213	76	80	76	73	76	76	76	2	114	91	95	95	74	12	3	38																	
18	HM126548	38	38	38	24	38	38	38	71	106	93	101	101	90	72	71	69	71																
19	HM126549	38	38	38	24	38	38	38	71	106	93	101	101	90	72	71	69	71	0															
20	HM126550	38	38	38	24	38	38	38	71	106	93	101	101	90	72	71	69	71	0	0														
21	HM126551	38	38	38	24	38	38	38	71	106	93	101	101	90	72	71	69	71	0	0	0													
22	HM744739	1	12	1	43	1	1	1	76	112	94	92	92	85	77	76	75	76	38	38	38	38												
23	HQ322124	89	90	89	81	89	89	89	74	92	51	68	68	43	70	73	68	74	85	85	85	85	89											
24	HQ322125	90	91	90	82	90	90	90	75	93	52	69	69	44	71	74	69	75	86	86	86	86	90	1										
25	HQ322126	85	89	85	80	85	85	85	76	93	51	65	65	47	71	75	67	76	86	86	86	86	85	17	18									
26	HQ322127	115	113	115	106	115	115	115	112	7	98	110	110	90	109	111	102	114	105	105	105	105	115	91	92	91								
27	HQ322129	87	89	87	90	87	87	87	76	92	68	71	71	2	68	77	69	76	92	92	92	92	87	45	46	49	92							
28	JQ797561	0	11	0	43	0	0	0	76	112	94	92	92	85	77	76	75	76	38	38	38	38	1	89	90	85	115	87						
29	KJ676114	3	12	3	46	3	3	3	79	112	97	95	95	87	80	79	78	79	41	41	41	41	4	92	93	88	115	89	3					
30	KJ676115	0	11	0	43	0	0	0	76	112	94	92	92	85	77	76	75	76	38	38	38	38	1	89	90	85	115	87	0	3				
31	KJ676116	0	11	0	43	0	0	0	76	112	94	92	92	85	77	76	75	76	38	38	38	38	1	89	90	85	115	87	0	3	0			
32	KJ676117	0	11	0	43	0	0	0	76	112	94	92	92	85	77	76	75	76	38	38	38	38	1	89	90	85	115	87	0	3	0	0		
33	KJ676118	0	11	0	43	0	0	0	76	112	94	92	92	85	77	76	75	76	38	38	38	38	1	89	90	85	115	87	0	3	0	0	0	
34	FR865528	218	216	218	221	218	218	218	212	195	212	221	221	203	209	213	210	212	219	219	219	219	217	207	206	209	194	204	218	217	218	218	218	218

Tab. S7: Pairwise p-distances based on ITS sequences of TY02

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	TY02																																	
2	AB162911	0.02																																
3	AY948419	0.00	0.02																															
4	FM205845	0.08	0.07	0.08																														
5	FM205846	0.00	0.02	0.00	0.08																													
6	FM205885	0.00	0.02	0.00	0.08	0.00																												
7	FR865655	0.00	0.02	0.00	0.08	0.00	0.00																											
8	GQ176856	0.14	0.15	0.14	0.13	0.14	0.14	0.14																										
9	GQ477057	0.21	0.20	0.21	0.20	0.21	0.21	0.21	0.21																									
10	GQ477058	0.17	0.17	0.17	0.16	0.17	0.17	0.17	0.17	0.18																								
11	GQ477059	0.17	0.17	0.17	0.18	0.17	0.17	0.17	0.17	0.20	0.11																							
12	GQ477060	0.17	0.17	0.17	0.18	0.17	0.17	0.17	0.17	0.20	0.11	0.00																						
13	GQ477061	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.14	0.17	0.12	0.13	0.13																					
14	GQ487192	0.14	0.15	0.14	0.13	0.14	0.14	0.14	0.02	0.20	0.16	0.17	0.17	0.12																				
15	GQ487208	0.14	0.15	0.14	0.13	0.14	0.14	0.14	0.00	0.20	0.17	0.18	0.18	0.14	0.02																			
16	GQ487211	0.14	0.13	0.14	0.13	0.14	0.14	0.14	0.07	0.19	0.15	0.16	0.16	0.12	0.06	0.07																		
17	GQ487213	0.14	0.15	0.14	0.13	0.14	0.14	0.14	0.00	0.21	0.17	0.17	0.17	0.14	0.02	0.01	0.07																	
18	HM126548	0.07	0.07	0.07	0.04	0.07	0.07	0.07	0.13	0.20	0.17	0.19	0.19	0.17	0.13	0.13	0.13	0.13																
19	HM126549	0.07	0.07	0.07	0.04	0.07	0.07	0.07	0.13	0.20	0.17	0.19	0.19	0.17	0.13	0.13	0.13	0.13	0.00															
20	HM126550	0.07	0.07	0.07	0.04	0.07	0.07	0.07	0.13	0.20	0.17	0.19	0.19	0.17	0.13	0.13	0.13	0.13	0.00	0.00														
21	HM126551	0.07	0.07	0.07	0.04	0.07	0.07	0.07	0.13	0.20	0.17	0.19	0.19	0.17	0.13	0.13	0.13	0.13	0.00	0.00	0.00													
22	HM744739	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07												
23	HQ322124	0.16	0.17	0.16	0.15	0.16	0.16	0.16	0.14	0.17	0.09	0.13	0.13	0.08	0.13	0.13	0.13	0.14	0.16	0.16	0.16	0.16	0.16											
24	HQ322125	0.17	0.17	0.17	0.15	0.17	0.17	0.17	0.14	0.17	0.10	0.13	0.13	0.08	0.13	0.14	0.13	0.14	0.16	0.16	0.16	0.16	0.17	0.00										
25	HQ322126	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.14	0.17	0.09	0.12	0.12	0.09	0.13	0.14	0.12	0.14	0.16	0.16	0.16	0.16	0.16	0.03	0.03									
26	HQ322127	0.21	0.21	0.21	0.20	0.21	0.21	0.21	0.21	0.01	0.18	0.20	0.20	0.17	0.20	0.20	0.19	0.21	0.19	0.19	0.19	0.19	0.21	0.17	0.17	0.17								
27	HQ322129	0.16	0.16	0.16	0.17	0.16	0.16	0.16	0.14	0.17	0.13	0.13	0.13	0.00	0.13	0.14	0.13	0.14	0.17	0.17	0.17	0.17	0.16	0.08	0.08	0.09	0.17							
28	JQ797561	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07	0.00	0.16	0.17	0.16	0.21	0.16						
29	KJ676114	0.01	0.02	0.01	0.08	0.01	0.01	0.01	0.15	0.21	0.18	0.17	0.17	0.16	0.15	0.15	0.14	0.15	0.08	0.08	0.08	0.08	0.01	0.17	0.17	0.16	0.21	0.16	0.01					
30	KJ676115	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07	0.00	0.16	0.17	0.16	0.21	0.16	0.00	0.01				
31	KJ676116	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07	0.00	0.16	0.17	0.16	0.21	0.16	0.00	0.01	0.00			
32	KJ676117	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07	0.00	0.16	0.17	0.16	0.21	0.16	0.00	0.01	0.00	0.00		
33	KJ676118	0.00	0.02	0.00	0.08	0.00	0.00	0.00	0.14	0.21	0.17	0.17	0.17	0.16	0.14	0.14	0.14	0.14	0.07	0.07	0.07	0.07	0.00	0.16	0.17	0.16	0.21	0.16	0.00	0.01	0.00	0.00	0.00	
34	FR865528	0.40	0.40	0.40	0.41	0.40	0.40	0.40	0.39	0.36	0.39	0.41	0.41	0.37	0.38	0.39	0.39	0.39	0.40	0.40	0.40	0.40	0.40	0.38	0.38	0.38	0.36	0.38	0.40	0.40	0.40	0.40	0.40	0.40