R e s e a r c h   a r t i c l e

A new species of *Cryptomonas* (Cryptophyceae) from the Western Urals (Russia)

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Abstract. A new species, *Cryptomonas uralensis* Martynenko, Gusev, Kulizin & Guseva sp. nov., is described from western slopes of the Ural Mountains (Russia) based on morphological and molecular data. Phylogenetic relationships inferred from nuclear SSU and LSU rDNA sequences show that the new species forms a clade with *C. tetrapyrenoidosa* Skuja emend. Hoef-Emden & Melkonian. Comparison of secondary structures of nuclear rDNA ITS2, including analysis of Compensatory Base Changes (CBC), confirms the separation between *C. uralensis* sp. nov. and *C. tetrapyrenoidosa*. Cell morphology and sizes of *C. uralensis* sp. nov. are very similar to *C. tetrapyrenoidosa* and *C. pyrenoidifera*, and *C. uralensis* sp. nov. may thus represent a species that can only be reliably identified using molecular data.

Keywords. *Cryptomonas*, new species, SSU rDNA, LSU and ITS2 rDNA markers, nuclear ITS2 secondary structure, Compensatory Base Changes approach, Urals.

Introduction

Cryptophytes (also known as cryptomonads) are mostly photoautotrophic, unicellular, biflagellate, soft-bodied protists distributed in diverse freshwater, brackish, and marine habitats. They are characterized by a number of morphological and ultrastructural features, including a distinct cellular asymmetry, presence of extrusive organelles (termed ejectosomes), cell-covering from organic compounds (termed

periplast), and a remnant nucleus of endosymbiotic origin in close association with the plastids (Hoef-Emden & Archibald 2016).

Recent research has shown that even in well-sampled geographical regions and habitats, the true diversity of cryptomonads is often unknown (Hoef-Emden 2007; Lane & Archibald 2008; Shalchian-Tabrizi *et al.* 2008; Choi *et al.* 2013). *Cryptomonas* Ehrenb. (Ehrenberg 1831) is a diverse genus of cryptophytes, with about 70 morphospecies currently accepted (Hoef-Emden & Melkonian 2003). Species in the genus are restricted to freshwater, contain chloroplasts with the biliprotein phycoerythrin 566 or leucoplasts, and are characterized by a dimorphic life cycle (Hoef-Emden & Melkonian 2003). Species display two alternative cell morphotypes, which are related to the life cycle: 1) cryptomorph with an inner periplast composed of hexagonal to polygonal organic plates and a non-keeled posteriorly directed microtubular flagellar root, and 2) campylomorph with a sheet-like inner organic periplast component and keeled rhizostyle (Hill 1991; Hoef-Emden & Melkonian 2003).

Molecular phylogenetic analyses based on nuclear and nucleomorph ribosomal RNA gene sequences demonstrated inconsistencies with traditional *Cryptomonas* taxonomy based on cell morphology studies by light microscopy (LM). Hoef-Emden & Melkonian (2003) revised the genus by combining phylogenetic analyses with morphological data, emended several species by including molecular data, and described two new species. DNA sequences of additional strains resulted in emendation of five species including one new combination (Hoef-Emden 2007). Identification of most *Cryptomonas* species may be possible only with molecular data. A total of 14 *Cryptomonas* species can now be identified by nuclear ITS2/partial LSU rDNA or by nucleomorph SSU rDNA sequences in molecular ecological surveys (Hoef-Emden & Melkonian 2003; Hoef-Emden 2007). It is worth mentioning that these taxa are phylogenetic species, which may not correspond to biological species limits.

Cryptomonads from some freshwater habitats in Russia have been studied morphologically, but molecular data is currently lacking. Data on taxonomic composition and ecology of this group are available mainly from phytoplankton studies. *Cryptomonas marssonii* Skuja, *C. ovata* Ehrenb. and *C. erosa* Ehrenb. have been reported from the Urals based on morphological data (Tretyakova *et al.* 1988; Tretyakova

Fig. 1. Map of the study area; created using SASPlanet (http://www.sasgis.org).

1989; Belyaeva 2013, 2015). A new species of *Cryptomonas* from Russia is here described based on combination of morphological and molecular data.

Material and methods

Study area

Samples were collected from an unnamed small lake in the Usolka River flood plain, located in Solikamsk town, Permskiy Krai, Russia (59°38′59″ N, 56°44′57″ E, Fig. 1). Permskiy Krai is located on the western slope of the Ural Mountains, in upper and middle reaches of Kama River basin. The climate is cold temperate, with an average January temperature of -18.9°C, and varies from +14.8°C in the north to +18.7°C in the south during July. The annual precipitation also varies, from 450–600 mm in the western plain to 700–1000 mm in eastern mountainous territories of the Northern Urals, with snow cover usually 160–170 days/year (Nazarov 2006).

Samples and collections

Planktonic samples were collected by the first author using a plankton net with 20 μm mesh size. Water mineralization and temperature measurements were performed using the Hanna device (HI 9828, Hanna Instruments, Inc., Woonsocket, RI, USA). Strains were isolated and cultures perpetually transferred to IPPAS (Culture Collection of Microalgae at the K.A. Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences).

Culturing

Monoclonal strains were established by examination of micropipetted single cells under an inverted microscope. Non-axenic unialgal cultures were maintained in WC and Waris-H liquid medium (Andersen 2005; McFadden & Melkonian 1986) at 10°C in a growth chamber with a 12:12 h light:dark photoperiod.

Light microscopy

For light microscopy, living cells were immobilized by embedment in ultra-low gelling agarose (Sigma-Aldrich, A4018, USA) and examined by Nomarski differential interference contrast (DIC) with an oil immersion lens (Plan-Apochromat 100x/1.40 Oil DIC M27; microscope Zeiss AxioScope A1; Carl Zeiss AG, Oberkochem, Germany). Cell shape, size, shape of the furrow–gullet system and cell plastids, and presence, number and position of pyrenoids were examined (88 cells). The morphological description follows the nomenclature of Hoef-Emden & Melkonian (2003) (ventral side corresponds to the opening side of the furrow–gullet system). Calibration of magnification was done with a grated micrometer. Light micrographs were taken with an AxioCam ERc 5s Rev.2.

DNA extraction and amplification

Total DNA was extracted from monoclonal cultures using InstaGeneTM Matrix according to manufacturer's protocol. Fragments of SSU (length 1533 bp) and LSU rDNA (length 946 bp) and ITS2 rDNA (402 bp) were amplified using primers from Choi *et al.* (2013): 18S_CrN1F, 18S_826F, 18S_956R, 18S_BRK for nuclear SSU rDNA, crLSU_29F and crLSU_942R for partial nuclear LSU rDNA, and crITS_03F and crITS_05R for nuclear ITS2 rDNA. Amplification of all studied fragments was carried out using the premade mix ScreenMix (Evrogen, Russia) for the polymerase chain reaction (PCR). The conditions of amplification for SSU rDNA, LSU rDNA and ITS2 rDNA fragments were: an initial denaturation of 5 min at 95°C, followed by 35 cycles at 94°C for denaturation (30 s), 52°C for annealing (30 s) and 72°C for extension (50–80 s), and a final extension step of 10 min at 72°C. The resulting amplicons were visualized by horizontal agarose gel electrophoresis (1.5%), colored with SYBR Safe (Life Technologies, Carlsbad, CA, USA). Purification of DNA fragments was performed

with the ExoSAP-IT kit (Affymetrix, Santa Clara, CA, USA) according to manufacturer's protocol. SSU rDNA, LSU rDNA and ITS2 rDNA fragments were decoded from two sides using forward and reverse PCR primers and the Big Dye system (Applied Biosystems, Foster City, CA, USA), followed by electrophoresis using a Genetic Analyzer 3500 sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Electropherograms were verified by eye and sequences were assembled using BioEdit ver. 7.1.3 and MegaX (Kumar *et al.* 2018). Newly determined sequences and GenBank sequences of 45 other cryptomonads from different morphological groups were included in the alignments. The species *Rhodomonas* sp. strain M1480 and *Chroomonas* sp. strain SAG 980-1 were chosen as outgroup taxa. Sequences were aligned using MAFFT ver. 7 with model EINS-i (Katoh & Toh 2010), refined by eye and difficult to align regions were excluded from the analysis. jModelTest ver. 2.1.1 indicated that the GTR model of nucleotide substitution, with Gamma (G) distributed rates across sites and a proportion of invariable sites (I), was the most appropriate evolutionary model for the SSU rDNA and LSU rDNA alignments separately and combined (Posada 2006). Finally, we constructed the concatenated SSU + LSU rDNA alignment of 47 taxa. The SSU and LSU sequences were combined from the same strain. Phylogenies were constructed based on this model for the concatenated alignment using a single partition Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. BI analysis was conducted with MrBayes ver. 3.2.5 (Ronquist & Huelsenbeck 2003). Three "hot" and one "cold" Markov chains were run for 1×10^6 cycles in two repetitions with the selection of each 200th generated tree. Phylogenetic tree and posterior probabilities were obtained after discarding the first 25% to produce estimate parameter models of nucleotide substitutions and likelihood. ML-analysis was performed using MegaX (Kumar *et al.* 2018). Branch support was estimated using bootstrap analysis with 1000 replicas. Viewing and editing of trees were performed in FigTree ver. 1.4.2 (FigTree 2014) and Adobe Photoshop CC (ver. 19.0).

RNA secondary structure prediction

Nuclear ITS2 sequences were folded using the mfold server (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker 2003) with default values. Hoef-Emden (2007) provided a complete RNA secondary structure graph of the nuclear ITS2 rDNA of *Cryptomonas* sp. M1634, which was used as a template and assisted in inference of common stems, loops, and bulges.

Results

Molecular phylogeny

The new species, *Cryptomonas uralensis* sp. nov., represents a separate clade with high statistical support, sister to *Cryptomonas tetrapyrenoidosa* Skuja emend. Hoef-Emden & Melkonian (Fig. 13). *Cryptomonas uralensis* sp. nov. differs from the type strain of *C. tetrapyrenoidosa* by the SSU rDNA, LSU rDNA and ITS2 rDNA sequences as well as in the secondary structure of ITS2 (Fig. 14). For ITS2 sequence of *C. uralensis* sp. nov. four-helix structures could be inferred with long third helices (Fig. 15). An unpaired U-U was found in Helix II. The length of the helices was different between two taxa (Fig. 15). For the new species, length of ITS2 was 402 nt, while for *C. tetrapyrenoidosa* was 370 nt. Three Compensatory Base Changes (CBCs) between *C. uralensis* sp. nov. and *C. tetrapyrenoidosa* were observed in Helix III (Fig. 15).

Phylum Cryptophyta Cavalier-Smith
Class Cryptophyceae F.E.Fritsch
Order Cryptomonadales Pascher
Genus *Cryptomonas* Ehrenb.

Cryptomonas uralensis Martynenko, Gusev, Kulizin & Guseva sp. nov.
Figs 2–12

Diagnosis

Cells (absolute minimal and maximal values of strain UR168) 15–26 µm long, 8–17 µm wide, 8–12 µm thick. Cells more or less flattened in dorso-ventral plane, in ventral view elliptical to ovoid. In lateral view with oblique or rounded apex and rounded antapex. One plastid with two to four pyrenoids. Species differs from other species of the genus by the order of nucleotides in nuclear ITS2, LSU and SSU rDNA gene sequences.

Etymology

The species name derived from "Ural", the region from which this taxon was described.

Type material

    Holotype

RUSSIA • Solikamsk town, water-body in the Usolka River flood plain; 59°38′59″ N, 56°44′57″ E; 2014; collected by N.A. Martynenko; strain UR168; GenBank MN509781, MN509779; MHA[*Cryptomonas uralensis* Russia Martynenko 20-2 MHA, strain UR168].

The holotype is a large drop of unfixed dried cells of the strain UR168 (=IPPAS H-2046) on water colour paper (*hic designatus*), deposited at MHA (Herbarium, Main Botanical Garden, Botanicheskaya Str. 4, Moscow, 127276, Russia) under the designation *Cryptomonas uralensis* Russia Martynenko 20-2 MHA, strain UR168. Representative living strain and DNA sample are kept at IPPAS.

Representative DNA sequences

GenBank accession numbers MN509781 (nuclear ITS2 and partial nuclear LSU rDNA), and MN509779 (nuclear SSU rDNA).

Authentic strain

UR168 (=IPPAS H-2046).

Type locality

A water-body in the Usolka River flood plain, Solikamsk town, Russia. Latitude/Longitude 59°38′59″ N, 56°44′57″ E, collected by N.A. Martynenko in 2014.

Other representative culture

UR167, GenBank accession numbers MN509780 (nuclear ITS2 and partial nuclear LSU rDNA), and MN509778 (nuclear SSU rDNA).

Geographical distribution

To date, *Cryptomonas uralensis* sp. nov. has only been observed at the type locality. At the time of collection, the pH was 6.5, temperature 18°C.
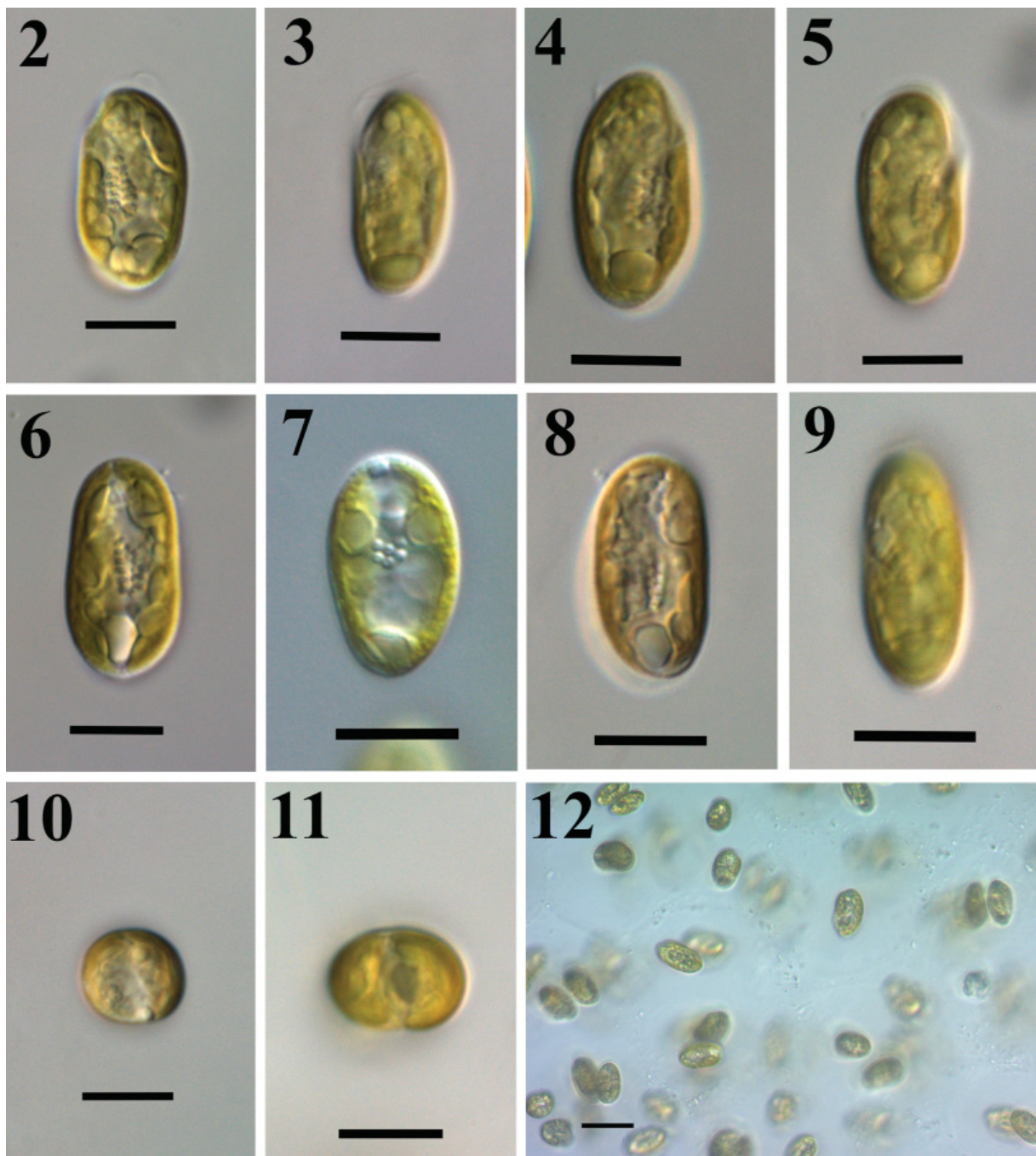
Figs 2–12. Light micrographs of *Cryptomonas uralensis* Martynenko, Gusev, Kulizin & Guseva sp. nov. (strain UR168). **2–3**. Lateral view (left side). **4–5**. Lateral view (right side). **6–8**. Ventral view. **9**. Dorsal view. **10–11**. Apical view. **12**. Cells of the strain UR168 in mucilage. Scale bars: 10 μm.

Discussion

Cryptomonas uralensis sp. nov. represents a distinct clade with high statistical support, sister to *C. tetrapyrenoidosa*, but no obvious morphological characters distinguishing the two taxa were found. Initially *C. tetrapyrenoidosa* was described based on LM studies of populations from Sweden and Latvia (Skuja 1948). Later, the strain M1092 from Germany (Remscheid) was chosen as the epitype and sequenced (Hoef-Emden & Melkonian 2003). To date, *C. tetrapyrenoidosa* has not been recorded in the Urals, thus areals of this species and *C. uralensis* sp. nov. do not overlap. Mention should be made that there were no previous intensive molecular studies of cryptophytes in the Urals. Recent studies of cryptomonads of South Korea, region remote from Europe, revealed several strains from the *Cryptomonas tetrapyrenoidosa* clade that showed SSU rDNA, LSU rDNA and ITS2 rDNA nucleotide differences from the type specimen and formed two subclades (Choi *et al.* 2013). However, no formal taxonomic descriptions have been made. In our opinion, these are very likely a new species. Thus, the *C. tetrapyrenoidosa* clade requires further study.
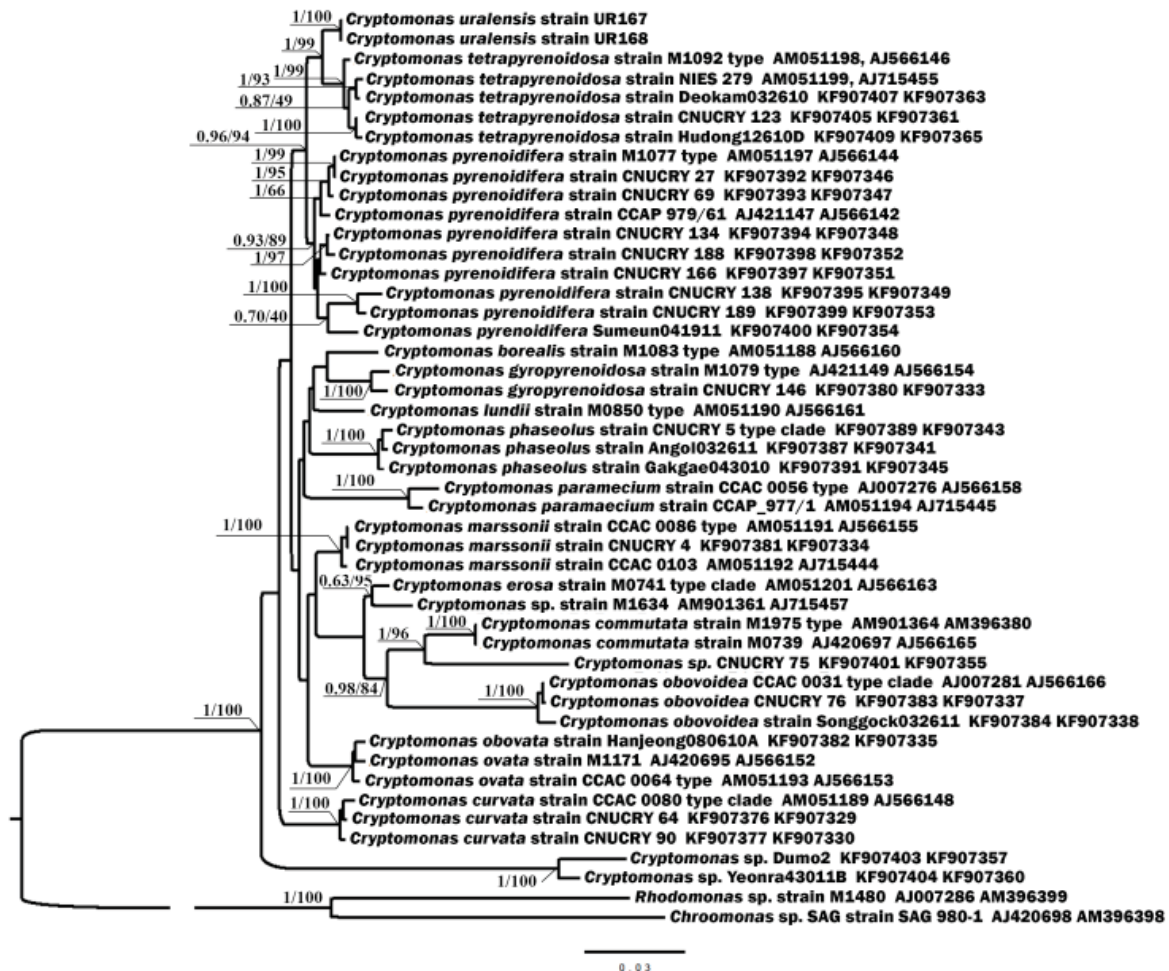

Fig. 13. Bayesian phylogenetic tree of the partial small subunit rDNA (SSU rDNA) and large subunit ribosomal DNA (LSU rDNA) combined data set. The Bayesian posterior probability (left) and maximum likelihood bootstrap value (right) are shown. Scale bar represents estimated number of substitution per site.

Previous studies have suggested that the nuclear internal transcribed spacer 2 (ITS2) is a good marker to identify biological species using degree of conservation. Coleman (2000) and Müller *et al.* (2007) have proposed that CBCs in the ITS2 correlate with speciation and thus can be used to distinguish species. The CBC species concept states that two organisms/strains whose ITS2 sequences differ by even a single CBC in the conserved regions of Helix II and Helix III represent two different biological species (Coleman 2000). According to Müller *et al.* (2007) and Wolf *et al.* (2013) the presence of any CBC in the whole ITS2 molecule is sufficient for distinguishing species. Later, Caisová *et al.* (2013) identified consensus structure model of the ITS2 molecule that can be applied to two classes of green algae. Also was reported that CBCs in the ITS2 of four orders of the green algal class Chlorophyceae Wille in Warming were mostly associated with higher order taxonomic levels like genera, families and orders, and CBC concept for species delimitation have some constraints in chlorophycean algae (Caisová *et al.* 2013). Dimorphic strains and a few reports of sexual reproduction indicate the presence of biological species also in cryptophyte taxa (Hoef-Emden 2007). The nuclear ITS2 was successfully used to predict putative biological species in the last revision of *Cryptomonas* (Hoef-Emden 2007). Here, comparison of the secondary structures of ITS2 confirms separation between *C. uralensis* sp. nov. and *C. tetrapyrenoidosa*.
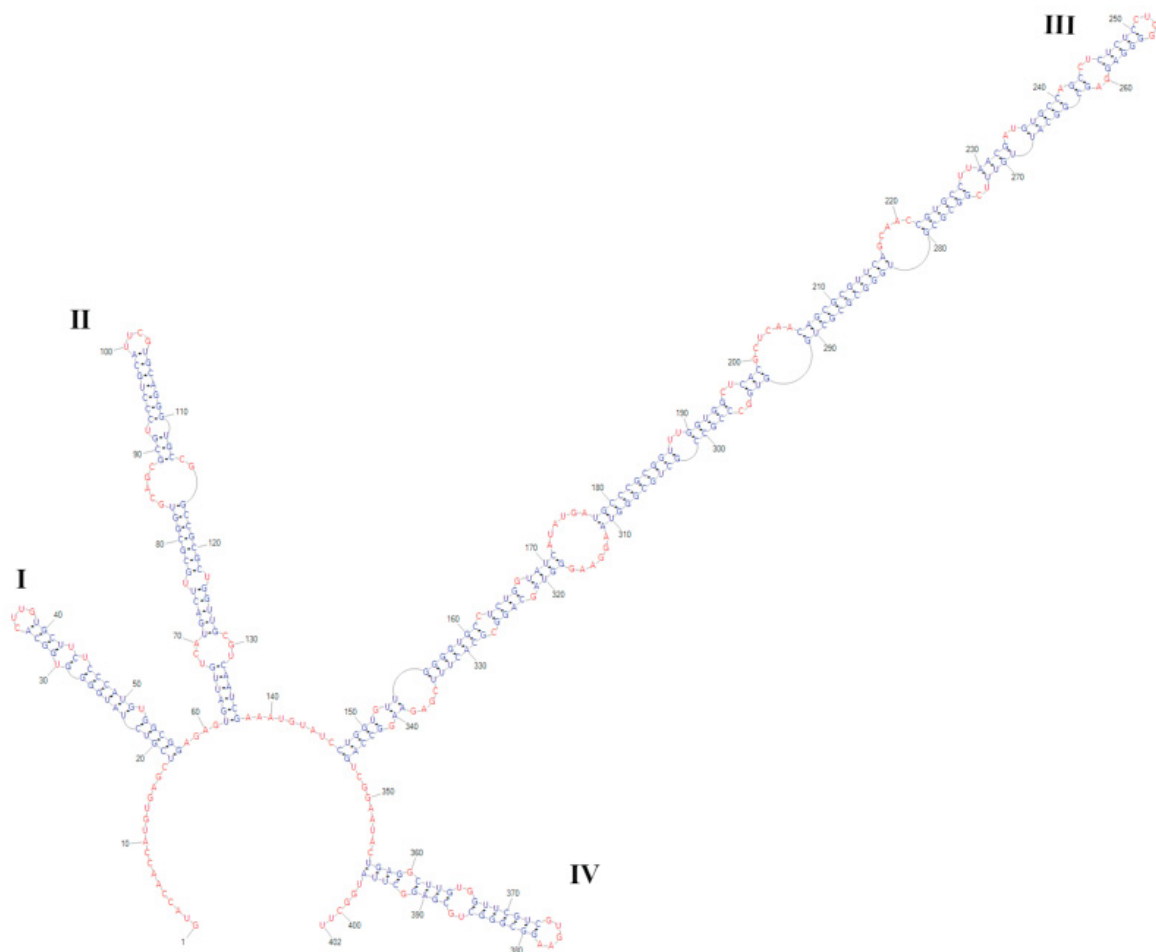

Fig. 14. Predicted secondary structure of the nuclear internal transcribed spacer 2 of the strain UR168 (from 5' to 3' terminus in clockwise direction).
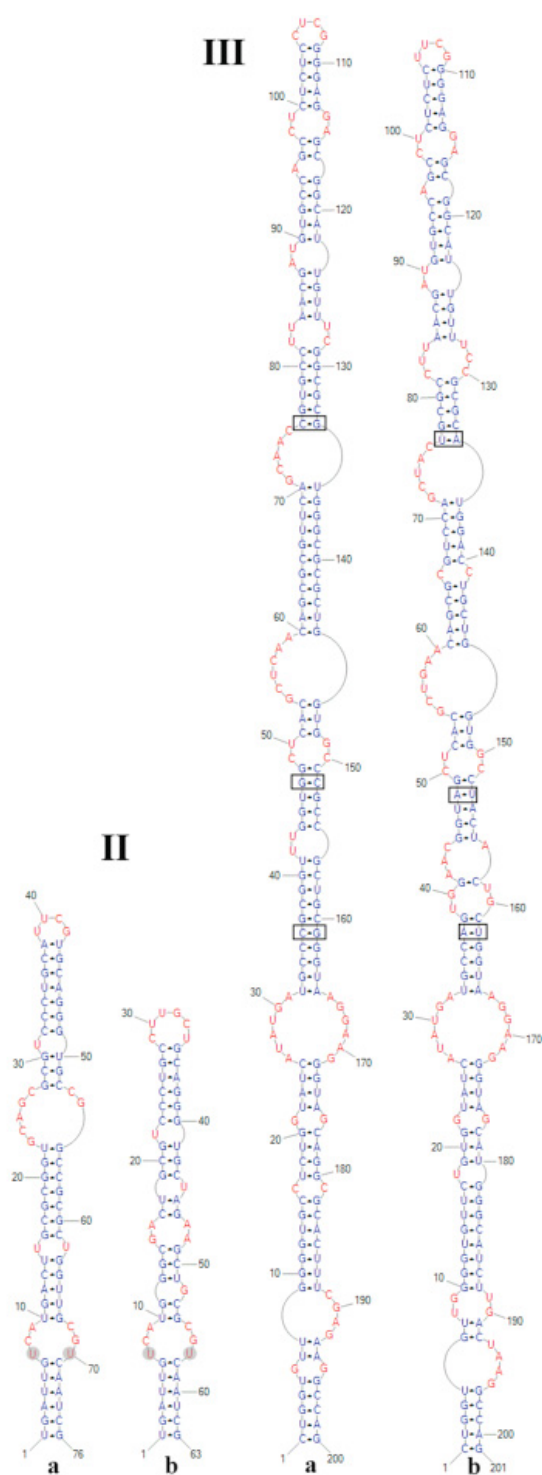
Fig. 15. Predicted secondary structures of the nuclear internal transcribed spacer 2 helix II and helix III (clockwise from 5' to 3' termini) of *Cryptomonas uralensis* Martynenko, Gusev, Kulizin & Guseva sp. nov. (**a**, strain UR168) and *C. tetrapyrenoidosa* Skuja emend. Hoef-Emden & Melkonian (**b**, strain M1092, GenBank accession number AJ566146). CBCs are marked by rectangular boxes. In both helices II, the conserved unpaired U-U motif was found.

Cryptomonas pyrenoidifera Geitler emend. Hoef-Emden & Melkonian was found to be sister to the clade containing *C. uralensis* sp. nov. and *C. tetrapyrenoidosa*. *Cryptomonas pyrenoidifera* is known from Germany, United Kingdom, Czech Republic and Australia based on molecular data. The morphology and cell sizes of *C. uralensis* sp. nov. are very similar to cryptomorphs of *C. pyrenoidifera*, as well as *C. tetrapyrenoidosa*. *Cryptomonas curvata* Ehrenberg emend. Hoef-Emden & Melkonian is one more species which has cryptomorph cells with the same morphological features (shape, size, number of pyrenoids), but placed distantly on phylogenetic tree from *C. uralensis* sp. nov. Incongruences between classical morphospecies and molecular phylogenetic trees have previously been reported for many species in the genus *Cryptomonas* (Hoef-Emden 2007). Morphological species descriptions of this genus are often ambiguous because of microscopic cells and only few visible characters. The most recent revisions of the genus show that correct identification of most *Cryptomonas* species may be possible only with molecular data (Hoef-Emden & Melkonian 2003, Hoef-Emden 2007). In the case of *C. uralensis* sp. nov., no sufficiently distinct morphological characters could be identified. The species can only be distinguished by molecular characters.

Acknowledgements

References

Andersen R.A. (ed.) 2005. *Algal Culturing Techniques*. Elsevier Academic Press, Oxford.

Belyaeva P.G. 2013. Species composition and phytoplankton structure of the Kama reservoir. *Bulletin of Perm University. Series: Biology* 3: 4–12.

Belyaeva P.G. 2015. Spatio-temporal changes in the phytoplankton of the Kama reservoir *Bulletin of the Samara Scientific Center of the Russian Academy of Sciences* 17: 733–738.

Caisová L., Marin B., Melkonian M. 2013. A consensus secondary structure of ITS2 in the Chlorophyta identified by phylogenetic reconstruction. *Protist* 164: 482–496. https://doi.org/10.1016/j.protis.2013.04.005

Choi B., Son M., Jong Im Kim J.I. & Shin W. 2013. Taxonomy and phylogeny of the genus *Cryptomonas* (Cryptophyceae, Cryptophyta) from Korea. *Algae* 28: 307–330. https://doi.org/10.4490/algae.2013.28.4.307

Coleman A.W. 2000. The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. *Protist* 151: 1–9. https://doi.org/10.1078/1434-4610-00002

Ehrenberg C.G. 1831. *Symbolae physicae seu icones et descriptiones animalium evertebratorum sepositis insectis quae ex itinere per Africanum Borealem et Asiam Occidentalem Friderici Guilelmi Hemprich et Christiani Godofredi Ehrenberg medicinae et chirurgiae doctorum studio novae aut illustratae redierunt*. Berlin, Mittler.

FigTree 2014. FigTree version 1.3.2. Available from http://tree.bio.ed.ac.uk/software/figtree/ [accessed 20 Apr. 2020].

Hill D.R.A. 1991. A revised circumscription of *Cryptomonas* (Cryptophyceae) based on examinations of Australian strains. *Phycologia* 30 (2): 170–188. https://doi.org/10.2216/i0031-8884-30-2-170.1

Hoef-Emden K. 2007. Revision of the genus *Cryptomonas* (Cryptophyceae) II: Incongruences between classical morphospecies concept and molecular phylogeny in smaller pyrenoid-less cells. *Phycologia* 46 (4): 402–428.

Hoef-Emden K. & Archibald J.M. 2016. Cryptophyta (Cryptomonads). *In*: Archibald J.M., Simpson A.G.B., Slamovits C.H., Margulis L., Melkonian M., Chapman D.J. & Corliss J.O. (eds) *Handbook of the Protists*. Springer International Publishing, Cham, Switzerland.
https://doi.org/10.1007/978-3-319-28149-0_35

Hoef-Emden K. & Melkonian M. 2003. Revision of the genus *Cryptomonas* (Cryptophyceae): A combination of molecular phylogeny and morphology provides insights into a long-hidden dimorphism. *Protist* 154: 371–409. https://doi.org/10.1078/143446103322454130

Katoh K. & Toh H. 2010. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* 26: 1899–1900. https://doi.org/10.1093/bioinformatics/btq224

Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547–1549.
https://doi.org/10.1093/molbev/msy096

Lane C.E. & Archibald J.M. 2008. New marine members of the genus *Hemiselmis* (Cryptomonadales, Cryptophyceae). *Journal of Phycology* 44 (2): 439–450.
https://doi.org/10.1111/j.1529-8817.2008.00486.x

McFadden G.I. & Melkonian M. 1986. Use of Hepes buffer for microalgal culture media and fixation for electron microscopy. *Phycologia* 25: 551–557. https://doi.org/10.2216/i0031-8884-25-4-551.1

Müller T., Philippi N., Dandekar T., Schultz J. & Wolf M. 2007. Distinguishing species. *RNA* 13: 1469–1472. https://doi.org/10.1261/rna.617107

Nazarov N.N. 2006. *Geography of the Perm region. Part I. Natural (physical) geography*. Publishing House of Perm State University, Perm.

Posada D. 2006. Modeltest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. *Nucleic Acids Research* 34: 700–703. https://doi.org/10.1093/nar/gkl042

Ronquist F. & Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Shalchian-Tabrizi K., Brate J., Logares R., Klaveness D., Berney C. & Jakobsen K.S. 2008. Diversification of unicellular eukaryotes: Cryptomonad colonizations of marine and fresh waters inferred from revised 18S rRNA phylogeny. *Environmental Microbiology* 10 (10): 2635–2644.
https://doi.org/10.1111/j.1462-2920.2008.01685.x

Skuja H. 1948. Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symbolae Botanicae Upsaliensis* 9 (1): 1–399.

Tretyakova S.A. 1989. Phytoplankton of the Kama reservoirs. *In*: Yarushina M.I. (ed.) *Hydrobiological characteristics of the Urals*. Ural Branch of USSR Academy of Sciences, Sverdlovsk.

Tretyakova S.A., Golovacheva S.I. & Batova E.M. 1988. Phytoplankton. *In*: Aleksevnina M.S. (ed.) *Biology of the Votkinsk reservoir*. Publishing of Irkutsk University, Irkutsk.

Wolf M., Chen S., Song J., Ankenbrand M. & Müller T. 2013. Compensatory Base Changes in ITS2 Secondary Structures Correlate with the Biological Species Concept Despite Intragenomic Variability in ITS2 Sequences – A Proof of Concept. *PLoS ONE* 8: e66726.
https://doi.org/10.1371/journal.pone.0066726

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31: 3406–3415. https://doi.org/10.1093/nar/gkg595