

Taxonomic studies of *Cryptomonas lundii* clade (Cryptophyta: Cryptophyceae) with description of a new species from Vietnam

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Abstract: A new species from Vietnam, *Cryptomonas vietnamica* sp. nov., is described based on morphological and molecular data. Six strains from different habitats were analyzed. *Cryptomonas vietnamica* is characterized by large cells up to 54 µm long, 30 µm wide and 20 µm thick, flattened in dorso–ventral plane and often twisted. Phylogenetic relationships inferred from nuclear small and large subunit ribosomal DNA sequences show that the new species forms a clade with *Cryptomonas lundii*, a rare taxon, described from Europe. *Cryptomonas lundii* is reported for the first time from Russia and second time since its initial description.

Key words: *Cryptomonas*, new species, nuclear SSU and LSU rDNA phylogeny, ITS2 secondary structure, Vietnam, Russia

INTRODUCTION

Cryptophytes (= cryptomonads) are mostly photoautotrophic, unicellular, biflagellate protists distributed in diverse freshwater, brackish, and marine habitats (HOEF–EMDEN & ARCHIBALD 2016). Cryptomonads tend to be quite fragile due to their organic periplast; their numbers may therefore be underestimated in field collections. This feature also makes it difficult to isolate species in a culture. Recent research has shown that even in well-sampled regions and habitats, the true diversity of cryptomonads has not yet been uncovered (VON DER HEYDEN et al. 2004; CERINO & ZINGONE 2006; LANE & ARCHIBALD 2008; SHALCHIAN–TABRIZI et al. 2008; BOENIGK et al. 2018).

Cryptomonas Ehrenberg (EHRENBERG 1831) is a genus of cryptomonads with about 70 currently accepted morphospecies which are restricted to freshwater (HOEF–EMDEN & MELKONIAN 2003). The dimorphic life history of *Cryptomonas* have resulted in inconsistent systematics, and a lack of species-specific character hampers the identification of species by morphology (HOEF–EMDEN & MELKONIAN 2003). Correct species identification of most *Cryptomonas* taxa is possible only with molecular characters (HOEF–EMDEN 2007).

The most extensive studies of *Cryptomonas* diversity and taxonomy have been conducted in Europe,

resulting in two comprehensive revisions (HOEF–EMDEN & MELKONIAN 2003; HOEF–EMDEN 2007). Studies in South Korea have revealed the rich flora of the genus *Cryptomonas*, including four new lineages (unidentified species), but no taxonomic revisions have been made (CHOI et al. 2013). Two species were also reported from China during molecular studies of algae (XIA et al. 2013). No specialized molecular studies of freshwater cryptomonads have been conducted in the tropics. Here, we describe a new species from Vietnam, *Cryptomonas vietnamica* sp. nov., and also report the presence of a rare species, *C. lundii* Hoef–Emden et Melkonian, in Russia.

MATERIAL AND METHODS

Samples were collected in Russia and Vietnam. Sample collection in Vietnam took place in the Khanh Hoa and Dong Nai provinces in 2015 and 2018. Samples in Khanh Hoa province were collected from an unnamed sand pit with standing water and a bog pool situated on the Cam Ranh Peninsula in April 2018 (Table 1). In Dong Nai province, three samples were collected in the area of Cat Tien National Park in October 2015, and one was collected from the Ba Hao reservoir, Vinh Cuu district, in May 2018 (Table 1). This area has a tropical monsoon climate. The average annual temperature ranges between 25 and 28 °C, the relative humidity between 80 and

94%, the annual precipitation is 1800–2100 mm and the annual evaporation is 1000–1200 mm. The sample from Russia was collected in Meshchyora National Park in Vladimirskaya oblast in October 2016 (Table 1).

Planktonic samples were collected using a plankton net with a 20- μ m mesh. Water mineralization and temperature measurements were performed using the Hanna device (HI 9828, Hanna Instruments, Inc., Woonsocket, RI, USA). Strains were isolated and cultures were transferred to the Collection of Algae at Laboratory of Molecular Systematics of Aquatic Plants of K.A. Timiryazev Institute of Plant Physiology Russian Academy of Sciences.

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 (McFADDEN & MELKONIAN 1986; ANDERSEN 2005) at 10 °C in a growth chamber with a 12:12 h light:dark photoperiod.

For light microscopical examinations, live cells were immobilized by embedding 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, Oberkochen, Germany). Cell shape, size, shape of the furrow–gullet system and cell plastids were examined (55 cells). For description, the terminology introduced in HOEF–EMDEN & MELKONIAN (2003) was used (ventral side corresponds to the opening side of the furrow–gullet system). Light micrographs were taken with an AxioCamERc 5s Rev.2.

Total DNA was extracted from monoclonal cultures using InstaGene™ Matrix according to the manufacturer's

protocol. Fragments of nuclear SSU and LSU rDNA, and ITS2 rDNA 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, LSU 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 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 the manufacturer's protocol. SSU rDNA and LSU 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).

Sequences were checked manually and assembled using BioEdit v7.1.3 and MegaX (KUMAR et al. 2018). Newly determined sequences and GenBank sequences of 46 other cryptomonads from different morphological groups were included in the alignments. Two species (*Rhodomonas* sp. strain M1480 and *Chroomonas* sp. strain SAG 980–1) were chosen as outgroup taxa. Sequences were aligned using MAFFT v7 with model EINS-i (KATO & TOH 2010), refined by eye and nonalignable sites were excluded from the analysis. jModelTest 2.1.1 indicated that the GTR model

Table 1. Basic characteristics of the studied localities (Cond. – specific conductance (μ S.cm⁻¹), T – temperature (°C)) and GenBank accession numbers of the strains.

Strains	Localities	Coordinates	pH	Cond.	T	ITS2–LSU rDNA	SSU rDNA
Vietnam							
VN873	Old irrigation pond, Cat Tien National Park, Dong Nai Province	11°24.386' N 107°22.463' E	6.2	20	31	MT216866	MT216859
VN874	Grass wetlands, Cat Tien National Park, Dong Nai Province	11°24.217' N 107°22.451' E	5.5	11	29	MT232230	MT232228
VN877	Stream, Cat Tien National Park, Dong Nai Province	11°24.342' N 107°22.442' E	5.8	20	29	MT232231	MT232229
VN881	Ba Hao reservoir, Dong Nai Province	11°15.390' N 107°04.493' E	5.9	26	33	MT216868	MT216861
VN882	Bog pool in Cam Ranh Peninsula, Khanh Hoa Province	12°4.674' N 109°11.220' E	5.2	80	30	MT216869	MT216862
VN880	Unnamed sandpit in Cam Ranh Peninsula, Khanh Hoa Province	12°04.785' N 109°11.130' E	6.7	127	35	MT216867	MT216860
Russia							
R179	Peat bog in Meshchyora National Park, Vladimirskaya oblast	55°35.077' N 40°26.485' E	6.3	42	4.2	MT216865	MT216858

of nucleotide substitution, with Gamma (G) distributed rates across sites and a proportion of invariable sites (I), was the most appropriate evolutionary model for SSU rDNA and LSU rDNA alignments individually and in general (POSADA 2006). Finally, we constructed the concatenated SSU + LSU rDNA alignment (2416 nt) of 52 taxa. Phylogenies were constructed based on this model for complete alignment using Bayesian Inference (BI) and Maximum Likelihood (ML) analysis. BI analysis was conducted with MrBayes–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 branching probabilities were obtained after discarding the first 25% to produce estimate parameter models of nucleotide substitutions and likelihood. ML–analysis was performed using the program MegaX (KUMAR et al. 2018). The bootstrap analysis with 1,000 replicas was used. Viewing and editing of trees were carried out in the programs FigTree (ver. 1.4.2) and Adobe Photoshop CC (19.0).

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. The ITS2 sequences were aligned according to secondary structure and were checked for compensatory base changes (CBCs) using 4SALE (SEIBEL et al. 2008).

RESULTS

Seven strains from the *Cryptomonas lundii* clade were revealed during studies of freshwater cryptomonads in Russia and Vietnam. Phylogenetic relationships inferred from nuclear SSU and LSU rDNA show the strains are grouped in two clades. The strain from Russia (R179, Fig. 8–9) was grouped with the type *C. lundii* strain CCAC 0107 (=M0850). Strains from Vietnam formed another clade with high statistical support. The cell size and morphology differ from that of *Cryptomonas lundii*. The Vietnamese strains come from two distant areas. Strains VN873, VN874, VN877 and VN881 were isolated from different localities in Dong Nai Province. The other two strains, VN880 and VN882, were isolated in Khanh Hoa Province, several hundred kilometres to the north. All six have identical LSU, SSU and ITS2 rDNA nucleotide sequences. Comparison of ITS2 secondary structures between strains from Vietnam and *C. lundii* revealed some differences. For ITS2 sequences of *C. vietnamica* (340 nt) and *C. lundii* (355 nt) four–helix structures could be inferred with long third helices. An unpaired U–U was found in Helix II. Three Compensatory Base Changes (CBCs) between two taxa were observed in Helix III, one CBC was found in Helix I and Helix II (Fig. 11). Below, we describe a new species based on morphology and molecular data.

***Cryptomonas vietnamica* Gusev, Podunay, Martynenko, Shkurina et Kulikovskiy sp. nov. (Figs 1–7)**

Diagnosis: Cells (absolute minimal and maximal values of strain VN873) 35–54 µm long, 21–30 µm wide, 14–20 µm thick, variable cell shapes. Cells flattened in dorso–ventral plane, often twisted. In ventral view, apices notched, with a nose–like protrusion. In lateral view, antapices dorsal reflex. One massive plastid. Species differs from others of the genus by the order of the nucleotides in nuclear ITS2, LSU and SSU rDNA gene sequences.

Holotype: a large drop of unfixed dried cells of the strain VN873 on watercolour paper (hic designatus), deposited at MHA (Herbarium, Main Botanical Garden, Botanicheskaya Str. 4, Moscow, 127276, Russia) under the designation ***Cryptomonas vietnamica* Vietnam Gusev 20–1 MHA, strain VN873.**

Type strain: Representative living strain VN873 and DNA sample maintained at the Laboratory of Molecular Systematics of Aquatic Plants, IPPAS. The strain is also conserved as a formaldehyde–fixed sample.

Representative DNA sequences for VN873: GenBank accession numbers MT216866 (nuclear ITS2 and partial nuclear LSU rDNA), and MT216859 (nuclear SSU rDNA).

Type locality: An old irrigation pond, Cat Tien National Park, Dong Nai Province, Vietnam. Latitude/Longitude 11°24.386' N, 107°22.463' E, collected by E.S. Gusev in 2015.

Habitat: plankton

Etymology: The species name derived from “Vietnam”, country from which this taxon was described.

Other representative cultures: VN874, VN877, VN880, VN881 and VN882. GenBank accession numbers see in Table 1.

Geographical distribution: To date, *Cryptomonas vietnamica* has been observed in six localities in Vietnam (Table 1). The new species was found in acidic to slightly acidic conditions (pH from 5.2 to 6.7) with a specific conductance range of 11–127 µS.cm^{–1}, and temperature 29–35 °C (Table 1).

New record for *Cryptomonas lundii*. *C. lundii* is reported for the first time from Russia (Figs 8–9). This species was found in a peat bog in Meshchyora National Park, Vladimirskaia oblast (55°35.077' N 40°26.485' E), with pH of 6.3, specific conductance of 42 µS.cm^{–1}, and temperature of 4.2 °C.

DISCUSSION

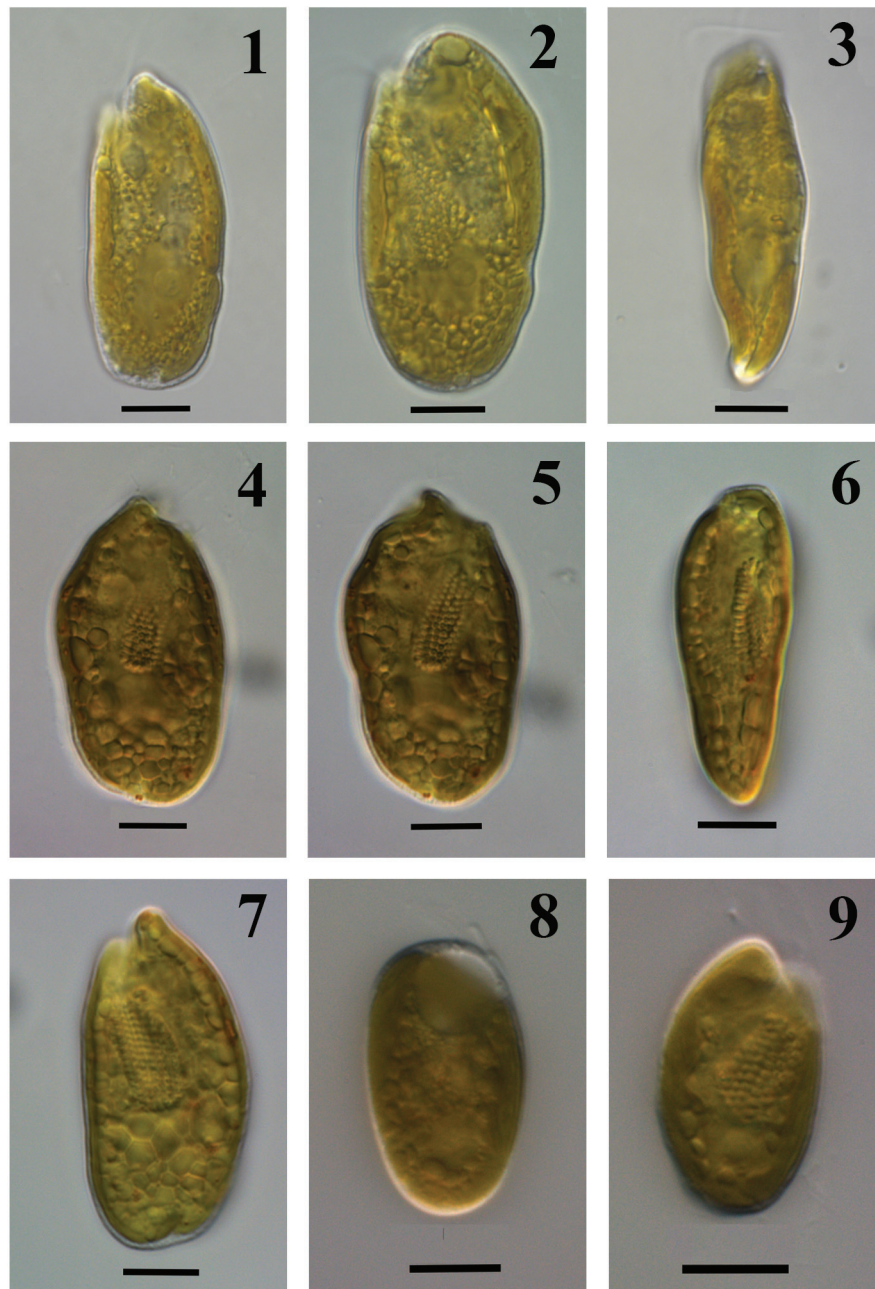
Cryptomonas lundii was described from a pond in Cologne (Germany) by HOEF–EMDEN & MELKONIAN (2003), and previously known only from this habitat. A similar morphotype was previously reported by Lund from the British Isles (HOEF–EMDEN & MELKONIAN 2003). One more sequence of partial LSU rDNA (strain

M2587, GenBank accession number HE820910, not included in analysis due to the absence of nuclear SSU rDNA sequences) attributed to *C. lundii* is available in GenBank. But it is not identical to the sequence of *C. lundii* and represents a separate branch in the clade on the phylogenetic tree. Thus, our finding of this taxon is the second confirmed by molecular data, and the first report of *C. lundii* in Russia.

Molecular data confirm separation of *Cryptomonas vietnamica* and *C. lundii* both on the SSU and LSU rDNA phylogeny (Fig. 10) and secondary structure of the ITS2 molecule (Fig. 11). The CBC concept was previously used for species delimitation in the genus *Cryptomonas*

(HOEF–EMDEN 2007). 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. *Cryptomonas vietnamica* have five CBCs in the whole ITS2 molecule comparing to *C. lundii* in helices I, II and III (Fig. 11).

The strains from Vietnam clearly differ in morphology from cells of *C. lundii*. All strains represent cells with campylomorph-like morphotypes. Cells of



Figs 1–7. Light micrographs of *Cryptomonas vietnamica* sp. nov.: (1–3) light micrographs of the type strain VN873, (1–2) ventral view, (3) lateral view (left side); (4–7) light micrographs of the strain VN881, (4–5, 7) ventral view, (6) lateral view (right side). Figs 8–9. Light micrographs of *Cryptomonas lundii* (strain R179): (8) dorsal view; (9) lateral view (right side). Scale bar 10 µm.

the new species are larger, $35\text{--}54 \times 16\text{--}30 \times 13\text{--}20 \mu\text{m}$ (based on measurements of all strains of *C. vietnamica*) instead of $19\text{--}30 \times 12\text{--}16 \times 11\text{--}15 \mu\text{m}$ in *C. lundii*. The shape and sizes of *C. vietnamica* cells are more similar to campylomorphs of *Cryptomonas curvata* Ehrenberg emend. Hoef–Emden et Melkonian, which is in a different clade on the phylogenetic tree. Among taxa not revised with molecular data, the most morphologically similar species is *Cryptomonas platyuris* Skuja, described from Sweden (SKUJA 1948). It has large cells, $31\text{--}50 \times 15\text{--}24 \times 9\text{--}14 \mu\text{m}$. It differs from *C. vietnamica* by the presence of a convex lateral edge and a sigmoid cell shape in broader view. Moreover, it was described in areas with a cold climate and has a northern distribution. Strains of *Cryptomonas vietnamica* were isolated in various, fairly remote areas of Vietnam, which may indicate the wide distribution of this taxon.

Up to now, only five taxa of the genus *Cryptomonas* were reported from Vietnam: *C. erosa* Ehrenberg, *C. commutata* (Pascher) Hoef–Emden (as *Cryptochrysis commutata* Pascher), *C. ovata* Ehrenberg, *C. paramaecium* (Ehrenberg) Hoef–Emden et Melkonian (as *Chilomonas*

paramaecium Ehrenberg), and *C. curvata* Ehrenberg (SHIROTA 1966; LE TRONG CUC 2001; DANG et al. 2002). All were identified in fixed samples during phytoplankton studies and lack illustrations and molecular data. There is no doubt that the real diversity of species of the genus *Cryptomonas*, as well as that of other cryptomonads, is much higher in Vietnam.

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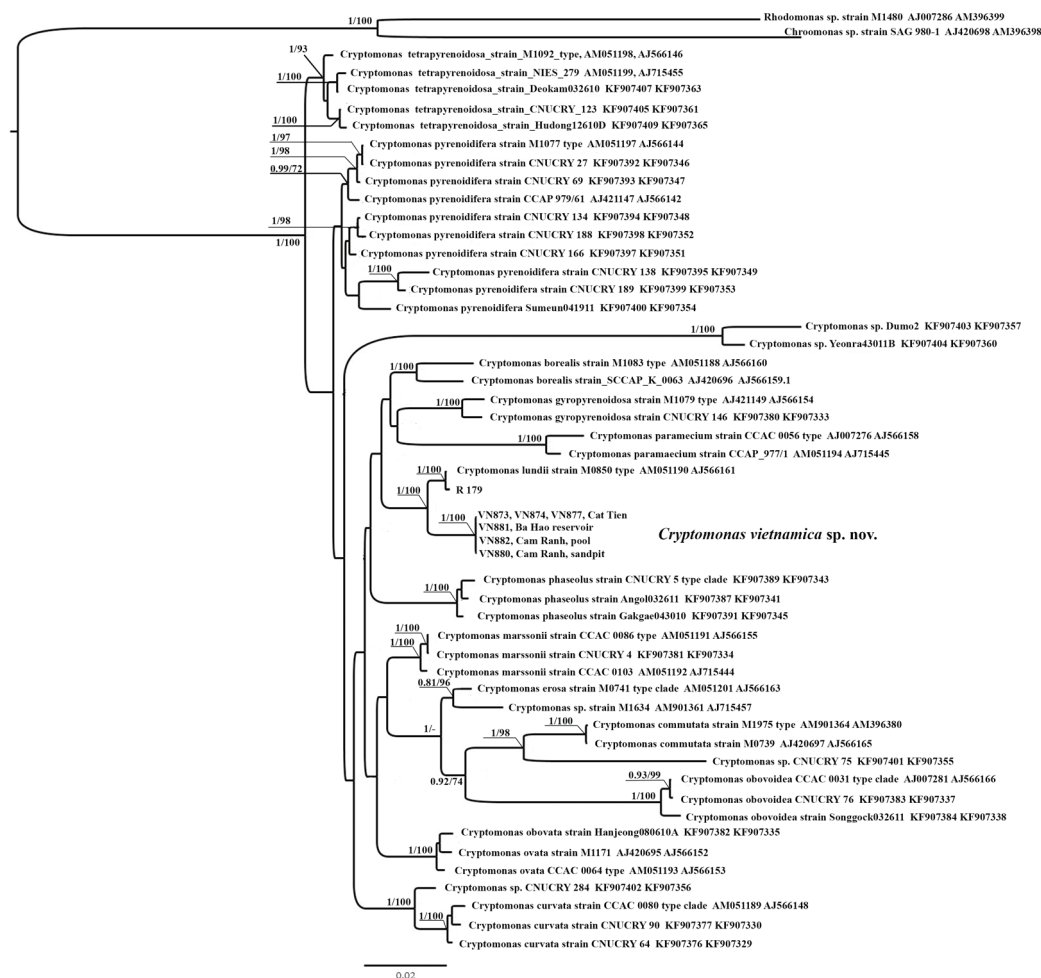


Fig. 10. Bayesian tree of the partial small subunit nuclear DNA (SSU rDNA) and large subunit nuclear DNA (LSU rDNA) combined data set. The Bayesian posterior probability and maximum likelihood bootstrap value are shown left and right of the fraction line respectively. *Cryptomonas vietnamica* sp. nov. is highlighted in bold. Scale bar represents: substitution per site.

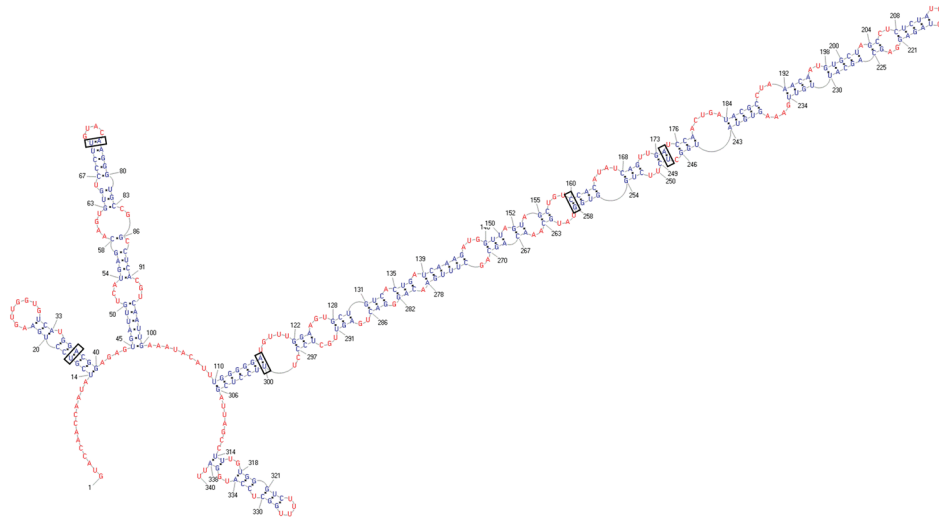


Fig. 11. Predicted secondary structure of the nuclear internal transcribed spacer 2 of *Cryptomonas vietnamica* (strain VN873). Black rectangular boxes – positions of Compensatory Base Changes (CBC) between *Cryptomonas vietnamica* and *Cryptomonas lundii* (strain R179).

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