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Elucidating the phylogeny and taxonomic position of the genus *Chrysodidymus* Prowse (Chrysophyceae, Synurales)

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Abstract – *Chrysodidymus* represents a monotypic genus of silica-scaled chrysophytes, with well characterised morphology and ultrastructure, as well as pretty known ecology. However, the taxonomic status of this genus remains ambiguous due to the absence of relevant sequence data. In this study, we have aimed to genetically characterize a newly established *C. synuroideus* culture to elucidate the taxonomy of *Chrysodidymus*. Our multigene SSU rDNA + LSU rDNA + rbcL phylogenetic analysis inferred *C. synuroideus* to be significantly nested deeply inside the genus *Synura*. This convincing evidence led us to propose a new combination for this taxon – *Synura synuroidea* (Prowse) Pusztai *et al.*, comb. nov.

***Chrysodidymus synuroideus* / Chrysophyceae / multigene phylogeny / Silica-scaled chrysophytes / *Synura synuroidea* comb. nov. / Synurales / taxonomy**

INTRODUCTION

Chrysophytes (Chrysophyceae, Stramenopiles) represent a monophyletic group (Yang *et al.*, 2012) of predominantly freshwater microalgae. They often dominate the phytoplankton of the oligotrophic temperate lentic ecosystems (Nicholls, 1995). However, the evidence for undiscovered diversity and important role of especially picoplanktonic chrysophytes in marine ecosystems has been shown recently (del Campo & Massana, 2011; Kirkham *et al.*, 2013).

Chrysophyte taxa bearing silica scales constitute a group possessing relatively good species concept based on the ultrastructure of scales and bristles (Kristiansen & Preisig, 2007). These complex structures, which are formed of amorphous silica in silica deposition vesicles (SDVs), represent resistant imprints in time and space. Therefore, silica-scaled chrysophytes are among the most explored groups of chrysophytes in terms of their ecology and species richness. Interestingly, the production of silica scales evolved at least three times during the evolution of Chrysophyceae, with the vast of scale-bearing organisms occurring in two unrelated orders: Paraphysomonadida and Synurales (Škaloud *et al.*, 2013a; Scoble & Cavalier-Smith, 2014).

In a recent taxonomic treatment (Kristiansen & Preisig, 2007), the Synurales comprise five well-recognized genera as well as the highly dubious *Jaoniella*

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Skvortzov and *Pseudosyncrypta* Kisselev. Besides *Mallomonas* Perty, *Synura* Ehrenberg and *Neotessella* (Playfair) Jo *et al.* belonging to molecularly well-defined genera, taxonomic position of *Chrysodidymus* Prowse and *Conradiella* Pascher remains unresolved (Škaloud *et al.*, 2013a). In the case of the enigmatic genus *Conradiella* there is suspicion that it represents a species of *Mallomonas* (Kristiansen, 1988; Kristiansen & Preisig, 2007). Conversely, *Chrysodidymus* has a well characterised morphology and ultrastructure (Wujek & Wee, 1983; Graham *et al.*, 1993), a distinctive ecology, and a wide distribution (Kristiansen & Preisig, 2007; Škaloud *et al.*, 2013b). This flagellate is free-living and autotrophic. Typically it forms two-celled "stretched out sausages-like" colonies where the cells are united at their broad posterior bases. The colony swims back and worth along its longitudinal axis, yielding a very characteristic swimming behavior. Cells are covered with a number of small imbricate plate-shaped scales each with a short apical spine. Each cell contains two golden brown plastids without pyrenoids and bears two unequal flagella covered by linear or clavate scales. *Chrysodidymus* is an acidophilic alga with a cosmopolitan, but scattered distribution.

The genus was erected by Prowse from Malayan acid swamps as "*Chrysodidyma*" (Prowse, 1960) and validly described two years later (Prowse, 1962). However, the description was made without any illustrations of the siliceous scales. Originally, *Chrysodidymus* encompassed two distinct species – *C. synuroideus* Prowse and *C. gracilis* Prowse differing in cell shape and size. Later, these two taxa were synonymized based on the high degree of phenotypic plasticity observed within a single *C. synuroideus* colony covering morphological characteristics of both species (Wujek & Wee, 1983; Kristiansen & Preisig, 2007). The first electron micrographs of *C. synuroideus* scales and scale-case were published just ten years after its original description, based on the collections from Canada (Puytorac *et al.*, 1972). In 2000, *Chrysodidymus synuroideus* became the first photosynthetic stramenopile where a complete mitochondrial genome sequence was recovered (Chesnick *et al.*, 2000). Unfortunately, the original culture deposited in the UTEX Algal Culture Collection, Austin TX, USA (LB 2713) has not survived. Moreover, no sequences of the generally used nuclear- or plastid-encoded molecular markers have been obtained to date, and consequently the taxonomy of this "golden-twins" microalga has not been elucidated correctly.

The general goal of this study was to establish a culture of *Chrysodidymus synuroideus*, characterize its genetic makeup, and resolve its taxonomic status within the Chrysophyceae.

MATERIAL AND METHODS

Collection, isolation and cultivation of a *Chrysodidymus* strain

On September 19th 2012 the samples of phytoplankton containing *Chrysodidymus* were collected from a small, unnamed lake near the Loch Garten in the Grampian Mountains, Scotland (57° 13' 32.55" N, 3° 43' 20.71" W). A plankton net with 20μm mesh was used. Standard measurement of abiotic factors on the sampling site encompassing water temperature: 11.0°C, pH: 5.9 and specific conductivity: 27 μS cm^{-1} was carried out using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Collected samples were kept in a polystyrene box containing cooling gel pad during the sampling day. Samples were

examined with an Olympus CX 31 light microscope and *Chrysodidymus* colonies were isolated immediately after returning to a research base. In the effort to establish uni-algal cultures, the individual colonies were isolated by micropipetting. Each colony was washed 3-5 times with distilled water to minimize the risk of contamination. Finally, the colony was placed into a separate well of a 96-well polypropylene plate. Each well was filled with approximately 400 µl of MES-buffered DY IV liquid medium (pH ≈ 6; Andersen *et al.*, 1997). The well plates were transported to the lab safely stored in a fridge bag (TK 51, Ardes SpA, Ponte Nossa, Italy). Climatic conditions in a fridge bag were maintained as approximately 15°C and constant illumination of 50-200 µmol m^{-2} s^{-1} provided by 6 W LED diodes (LB115A-6W-X, Yuyao Lianliang Electric Appliance Co Ltd, Ningbo, China). In the laboratory, the uni-algal "pre-cultures" were transferred from wells into 50 ml Erlenmeyer flasks filled with the same MES-buffered DY IV liquid medium (pH ≈ 6). Thereafter they were cultivated in cooling box (C5G, Helkama Oy, Helsinki, Finland) at 15°C, under the permanent illumination of 40 µmol m^{-2} s^{-1} (TLD 18W/33 fluorescent lamps, Philips, Amsterdam, the Netherlands). The cultures were periodically checked and reinoculated into fresh medium as necessary.

Morphological investigations

Chrysodidymus strains were determined based on the species specific scales using transmission electron microscopy (TEM). Samples from each culture were dropped onto Formvar-coated copper grids. Grids were dried, rinsed in 5 drops of distilled water, dried, and examined with a JEOL 1011 transmission electron microscope equipped by CCD camera Veleta with acquisition software (Olympus Soft Imaging Solution GmbH, Müenster, Germany). Morphological diversity of two-celled colonies from cultures with different age and condition was investigated in detail using Olympus BX 51 light microscope equipped by Nomarski interference contrast.

Sequencing and phylogenetic analysis

DNA isolations were carried out as described in Škaloudová & Škaloud (2013). Three molecular markers were amplified by PCR: nuclear SSU rDNA, nuclear LSU rDNA and plastidial rbcL. The amplification of SSU rDNA was performed as described by Škaloud *et al.* (2013a), using the primers 18S-F and 18S-R (Katana *et al.* 2001) and 528F (Montresor *et al.*, 2004). The amplification of LSU rDNA was performed as described by Jo *et al.* (2011), using the primers 28S_25F, 28S_861R and 28S_2160R (Jo *et al.*, 2011). Additionally, new primers 28S_732F2 (5′-CCC GAA AGA TGG TGA ACT-3′) and 28S_1435R (5′-GTT CAC ATG GAA CCT TTC TCT AC-3′) were designed for this study using the Primer3 software (Untergasser *et al.* 2007). The amplification of the rbcL marker was performed using newly designed primers S_IF (5′-GTT TAT GAA GGA TTA AAA GGT GG-3′) and S_IR (5′-GAC ATT CTC ATC CAT TTA CAA AT-3′). The PCR products were purified and sequenced at Macrogen Inc. in Seoul, Korea.

The newly determined sequences were aligned to other sequences from the GenBank database. The GenBank accession numbers of all strains used in this study are provided in Table 1. A concatenated SSU rDNA, LSU rDNA, and rbcL alignment was produced, including a total of 43 sequences of Synurales taxa. The sequences were aligned using MAFFT v. 6 software (Katoh *et al.* 2002) under the Q-INS-I

Table 1. Specific names, strain numbers and GenBank accession numbers of the Synurales taxa used in this study

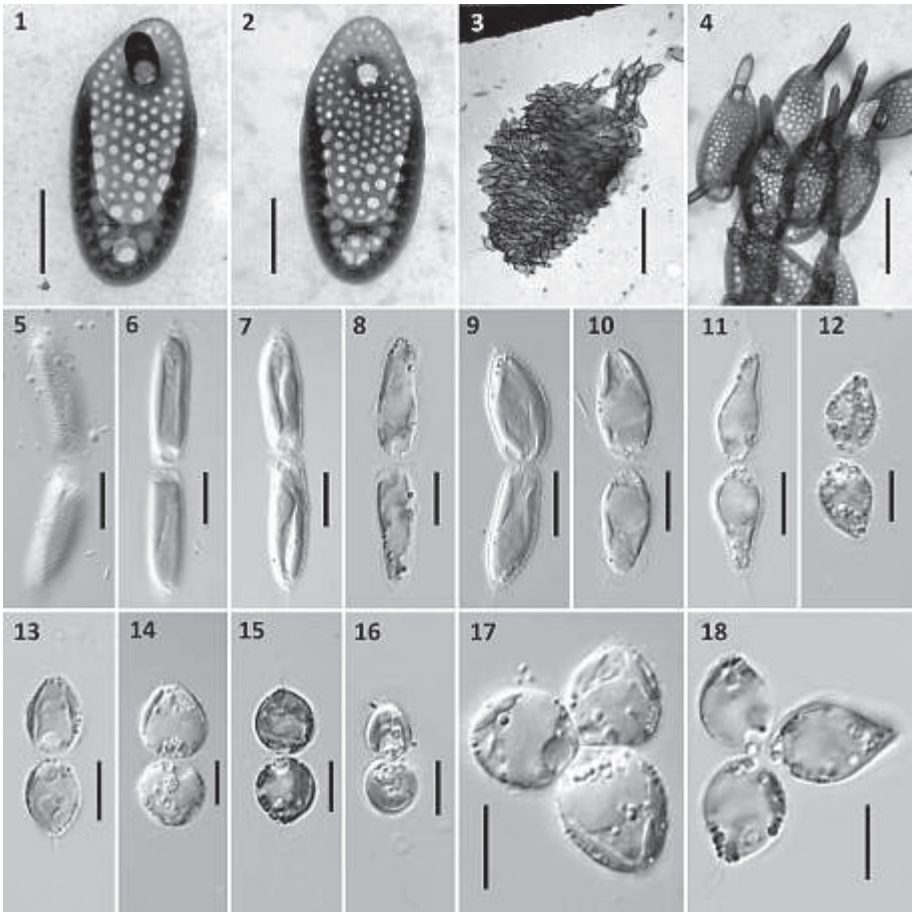
Taxon	*Strain*	*GenBank accession numbers*		
		SSU rDNA	*LSU rDNA*	*rbcL*
Chrysodidymus synuroideus Prowse	S 95.E5	KX815882	KX815883	KX815884
Mallomonas acaroides Perty	SYJMAc	JX946333	JX946341	JX946349
Mallomonas akrokomos Ruttner	Posan012608J	GU935625	GU935647	GU935667
Mallomonas caudata Ivanov	Dangje060207A	GU935629	GU935651	GU935671
Mallomonas heterospina Lund	Posan012608A	GU935617	GU935639	GU935659
Mallomonas insignis Penard	Beopsu033107D	GU935634	GU935656	GU935676
Mallomonas matvienkoae Asmund & Kristiansen	Muryeong112807B	GU935628	GU935650	GU935670
Mallomonas punctifera Korshikov	Angumal032010C	JQ955667	JQ955672	JQ955662
Neotessella lapponica (Skuja) Jo *et al.*	S 59.C4	HF549063	–	HF549074
Neotessella volvocina (Playfair) Jo *et al.*	CCMP 1782	EF165119	–	EF165199
Synura americana Kynčlová & Škaloud	CCMP 862	GU325582	–	GU325485
Synura americana Kynčlová & Škaloud	Johae010508F	JX455151	JX455155	JX455147
Synura asmundiae (Cronberg & Kristiansen) Škaloud *et al.*	S 90.D10	HF549069	–	HF549079
Synura bjoerkii (Cronberg & Kristiansen) Škaloud *et al.*	SC 57.A6	HF549070	–	HF549080
Synura conopea Kynčlová & Škaloud	NIES 1007	GU325578	–	GU325479
Synura conopea Kynčlová & Škaloud	CCMP 859	GU325580	–	GU325482
Synura curtispina (Petersen & Hansen) Asmund	SAG 29.92	GU325515	–	GU325415
Synura echinulata Korshikov	SAG 15.92	GU325513	–	GU325414
Synura glabra Korshikov	NIES 233	GU325577	–	GU325480
Synura glabra Korshikov	Dohak111107C	JX455149	JX455153	JX455145
Synura heteropora Škaloud *et al.*	WA18K_U	GU325597	–	GU325499
Synura heteropora Škaloud *et al.*	CCMP 2898	GU325596	–	GU325498
Synura longitubularis Jo *et al.*	Jeongsan070607A	KM590580	KM590646	KM590867
Synura macracantha (Petersen & Hansen) Asmund	S 90.B5	HF549064	–	HF549075
Synura mammillosa Takahashi	S 89.C3	HF549066	KM590652	–
Synura mammillosa Takahashi	SIE105A	HF549065	KM590654	HF549076
Synura mollispina (Petersen & Hansen) Péterfi & Momeu	S 71.C10	HF549067	–	HF549077
Synura multidentata (Balonov & Kuzmin) Péterfi & Momeu	S 90.C11	HF549068	–	HF549078
Synura petersenii Korshikov	KNU 09	GU325525	–	GU325426
Synura petersenii Korshikov	Youngji101407A	JX455150	JX455154	JX455146
Synura soroconopea Jo *et al.*	CNU 01	GU325530	–	GU325431
Synura sp.	CCMP 847	EF165128	–	EF165196
Synura sp.	CCAC 0052	GU325606	–	GU325508
Synura sp.	CCMP 869	GU325587	–	GU325489
Synura sp.	UTEX LB 239	GU325591	–	GU325493
Synura sphagnicola (Korshikov) Korshikov	CCMP 1705	U73221	–	EF165197
Synura sphagnicola (Korshikov) Korshikov	JYS001	DQ980485	DQ980475	–
Synura spinosa Korshikov	S 74.D2	–	–	HF549081
Synura splendida Korshikov	S 90.E4	HF549071	–	HF549082
Synura truttae (Siver) Škaloud & Kynčlová	Nemcova 2	GU325598	–	GU325500
Synura truttae (Siver) Škaloud & Kynčlová	Nemcova D5	GU325600	–	GU325502
Synura uvella Ehrenberg	CNU 53	GU325514	–	GU325416
Synura uvella Ehrenberg	CCMP 871	U73222	–	EF165192

strategy, and checked for obvious sequencing errors. For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al.* 2012). This BIC-based model selection procedure selected the following models: (1) TIM2 + I + Γ for SSU rDNA, (2) GTR + I + Γ for LSU rDNA and the first codon position of the rbcL gene, (3) TPM3 + I for the second codon position of the rbcL gene, and (4) TIM3 + I + Γ for the third codon position of the rbcL gene. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al.* 2012). The analysis was carried out on partitioned datasets using the substitution models best matching those selected by jModelTest 2.1.4. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for eight million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value was 0.0013. Finally, the burn-in value was determined using the "sump" command. Bootstrap analyses were performed by maximum likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl 2006) and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The analysis was performed on partitioned datasets using the different substitution models selected by jModelTest 2.1.4. The wMP bootstrapping (1,000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1,000. New weights were based on the mean fit values for each character over all trees in the memory.

RESULTS

Morphology, ultrastructure and molecular systematics

A novel strain of *Chrysodidymus* S95.E4 was successfully isolated from a sampled material, and a uni-algal culture for long-term cultivation was established. The strain was determined as *C. synuroideus* based on ultrastructure of scales and the scale-case (Figs 1-4). Small elliptical scales bearing an apical spine (0.4-1.5 μm) were 1.5-2.0 μm long and 0.6-0.9 μm broad. Two-celled colonies exhibited high degree of phenotypic plasticity (Figs 5-16). There were colonies consisted of bigger elongated cells through trapezoid, pyriform, ellipsoidal or ovoid cells to smaller almost spherical cells presented in the cultures of different age and condition. Cells were 10.0-27.0 μm long and 6.5-16.0 μm broad. Three-celled colonies were exceptionally presented (Figs 17, 18). Multigene phylogenetic analysis based on three molecular markers (nuclear SSU rDNA, nuclear LSU rDNA, plastidial rbcL) clearly demonstrated the phylogenetic position of *C. synuroideus* lying deeply inside the genus *Synura* (Fig. 19). *Chrysodidymus synuroideus* was inferred in a sister position to the clade composed of two *Synura sphagnicola* (Korshikov) Korshikov strains, within the statistically well supported monophyletic clade additionally including *S. curtispina* (Petersen & Hansen) Asmund, *S. longitubularis Chrysodidymus*



Figs 1-18. Morphology and ultrastructure of *Synura synuroidea* ("Chrysodidymus synuroideus") – Figs 1-4: TEM; Figs 5-18: LM. **1-2.** Body scales with different pattern of base-plate pores. **3.** Whole scale case. **4.** Apical scales with distinctly longer spines. **5.** Arrangement of scales on the cell surface. **6-16.** Phenotypic plasticity of two-celled colonies in the cultures of different age and condition. **17-18.** Rare three-celled colonies. Scale bars represent: 0.5 μm (Figs 1-2), 5 μm (Fig. 3), 1 μm (Fig. 4), 10 μm (Figs 5-18).

synuroideus, *S. mollispina* (Petersen & Hansen) Péterfi & Momeu and *S. spinosa* Korshikov. We therefore propose a new combination for this taxon – *Synura synuroidea* (Prowse) Pusztai *et al*., comb. nov.

Taxonomic conclusion

Synura synuroidea (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud, comb. nov. Figs 1-18

Basionym: *Chrysodidymus synuroideus* Prowse 1962, in Garden Bull., Singapore, 19: 128-129, Plate IV, fig. n. Type locality: Malacca - in acid swamps, Malaya.

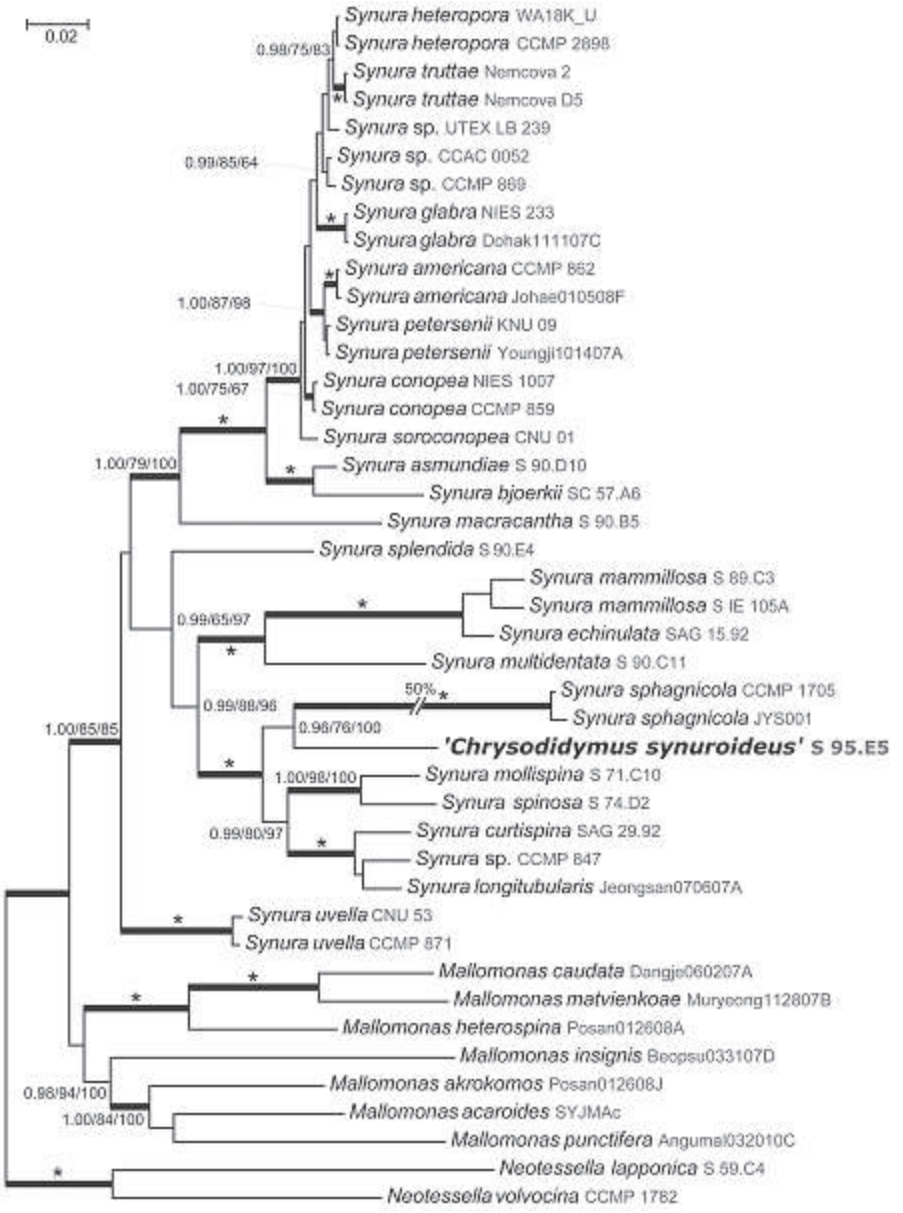


Fig. 19. Phylogeny of the Synurales obtained by Bayesian inference of the concatenated SSU rDNA, LSU rDNA and rbcL dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Only statistical supports higher than 0.95/60/60 are shown. Thick branches highlight nodes receiving the highest posterior probability (PP) support (1.00). Nodes receiving the absolute statistical support 1.00/100/100 are marked by asterisks. *C. synuroideus* strain is marked in bold. Scale bar represents the expected number of substitutions per site.

Synonyms: *Chrysodidymus gracilis* Prowse (1962: 128); *Synura microcrepis* Nygaard (1978: 200).
Reference strain: The live culture of strain S95.E4 has been deposited as CAUP B712 in the Culture Collection of Algae of Charles University in Prague, Czech Republic (http://botany.natur.cuni.cz/algo/caup.html).

DISCUSSION

On the basis of colony character, cell morphology and scale ultrastructure provided by Puytorac *et al.* (1972) our isolated strain distinctly belongs to the description of the *Chrysodidymus synuroideus* Prowse. High degree of phenotypic plasticity exhibited by our strain (e.g. from bigger elongated cells to smaller almost spherical cells) is in agreement with previous observations (Wujek & Wee, 1983; Graham *et al.*, 1993; Khondker *et al.*, 2007). This plasticity can be detected in a natural sample as well within a cultured strain. Stress conditions and maturity of cells seem to be the common denominator that makes the plasticity noticeable. In the same sample from Bangladesh, Khondker *et al.* (2007) observed *C. synuroideus* colonies composed of typically elongated cells as well as of smaller ellipsoidal ones corresponding to description of already synonymized *C. gracilis*. It resembles the situation of the original simultaneous erection of both species by Prowse from the same locality, "Malacca – in acid swamps". Wujek & Wee (1983) reported the same morphological divergence as a product of stress caused progressive changes in *Chrysodidymus* cells shape during microscopic observation. They suggested merging the two former species into a single valid species *C. synuroideus* on the basis of priority. Moreover, Graham *et al.* (1993) revealed that colonies of typically elongated cells are more mature, while colonies of smaller oval cells represent more recently divided cells. They further reported a smooth transition between these two frequently mentioned morphotypes which is in concordance with our findings. We therefore agree with the presumption, that Prowse observed only phenotypic plasticity within the single species. Notwithstanding the above mentioned, on the basis of subtle variations in scales ultrastructure between temperate and (sub)tropic populations of *C. synuroideus*, Kapustin a Gusev (2016) suggested that there could be more than one species within the genus *Chrysodidymus*. However, a comparative molecular and morphological investigation of several isolated strains is needed to decipher the real species diversity within this genus.

The ultrastructural analogy of *Synura* and *Chrysodidymus* silica scales led several authors to consider their close taxonomic relationship (Bourrelly, 1968; Nicholls & Gerrath, 1985; Graham *et al.*, 1993). Nygaard (1978) even described *C. synuroideus* as a distinct species of *Synura*, *S. microcrepis*, although in "appendix" he mentioned a question regarding the synonymy of these taxa. According to Nicholls & Gerrath (1985), the principal differences between *Chrysodidymus* and *Synura* comprise colony formation and movement characteristics. In *Synura*, the colonies are generally multi-celled, although two-celled young stages or colony fragments with irregular tumbling can be seen, as well. On the contrary, *Chrysodidymus* consistently forms two-celled colonies in both natural conditions and laboratory cultures, rarely forming three-celled, probably mitotic stages (Norris & Munch, 1970; Gerrath, 1974; Graham *et al.*, 1993; own observation). Therefore, the main distinguishing feature remains a little bit peculiar back and worth moving along colony longitudinal axis in *C. synuroideus*.

Nevertheless, our multigene phylogenetic analysis clearly demonstrated that the *C. synuroideus* is a member of the genus *Synura*, forming a distinct clade together with *S. sphagnicola* (Fig. 19). This position is furthermore supported by the fact that these two taxa share several common features (Hibberd, 1978; Graham *et al.*, 1993). First, both species bear relatively loose scale case consisting of scales with very similar simple perforated baseplate, although the scales are distinct in the number and size. Second, both species form characteristic apical scales with distinctly longer spines. Furthermore, *C. synuroideus* and *S. sphagnicola* share the presence of numerous linear or clavate scales on both flagella (Hibberd, 1978; Graham *et al.*, 1993). Another common feature is the frequent accumulation of red droplets in the cytoplasm. Finally, both species exhibit a similar ecology are often found together, and are reported from freshwater sites with low pH, including sphagnum ponds and bogs (Nygaard, 1979; Škaloud *et al.*, 2013b). We have also noticed that *S. sphagnicola* scales were present altogether with *C. synuroideus* scales within the same sample of our collections from Scotland.

The above-mentioned, well supported observations warrant to place the genus *Chrysodidymus* into synonymy with the genus *Synura*. Therefore, we propose new combination *Synura synuroidea* (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud, comb. nov. We clearly demonstrated that this two-celled taxon is not an intermediate evolutionary step between the single-celled *Mallomonas* and multi-celled colonies of *Synura*. Instead, we revealed the interesting case of evolutionary simplification in colonial organisms. Coloniality in microalgae is usually perceived as an evolutionary innovation that increases protection from predation or improve acquisition of resources (Lürling & Van Donk, 1996; Siver & Trainor, 1981). Independent origins of coloniality have been revealed in many algal lineages, including green algae (Herron *et al.*, 2009), chrysophytes (Němcová & Pichrtová, 2009), diatoms (Yamaoka *et al.*, 2016) or dinoflagellates (Matsuoka & Fukuyo, 1986). However, colony simplification is much less common, usually associated with a significant ecological shift, e.g., a transition from the aquatic to the terrestrial biotope (Lewis & Flechtner, 2004). We may only speculate on evolutionary drivers of colony simplification in *S. synuroidea*. For example, acidic conditions of sites this species strictly inhabits may steer the evolution towards less colonial forms due to shift in nutrients availability and predation pressure.

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