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ORIGINAL ARTICLE

Exploring Cryptic Diversity and Distribution Patterns in the *Mallomonas kalinae/rasilis* Species Complex with a Description of a New Taxon—*Mallomonas furtiva* sp. nov.

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Keywords
Molecular analysis; new species; scale ultrastructure; section Papillosae; Synurales.

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ABSTRACT

A complex of closely related *Mallomonas* taxa belonging to the section Papillosae, *M. kalinae* Řezáčová and *M. rasilis* Dürrschmidt, has been studied in detail by molecular and morphometric methods. Our investigations uncovered the existence of a new species found in water bodies in Vietnam, which we describe here as *Mallomonas furtiva* sp. nov. This taxon is morphologically very similar to *M. kalinae*, from which it differs by minute, but statistically significant morphological differences on the structure of silica scales. Indeed, the principal component analysis of morphological traits measured on silica scales significantly separates all three species in the complex. *Mallomonas kalinae* and *M. furtiva* differ by number of papillae on the shield and the dome, as well as by the scale sizes. Likewise, *Mallomonas rasilis* and *M. furtiva* are primarily differentiated by the absence of submarginal anterior ribs on silica scales of the former species. Phylogenetic analyses showed that *Mallomonas furtiva* is closely related to *M. kalinae*, with which it formed a highly supported lineage. Distribution patterns of all three studied taxa are further discussed.

MALLOMONAS (Stramenopiles, Chrysophyceae, order Synurales) taxonomy is based on the ultrastructure of siliceous scales and bristles (Asmund and Kristiansen 1986; Siver 1991). The genus, which contains more than 180 taxa, is divided into 19 sections due to the large number of species and great variability of scale ultrastructure (Kristiansen and Preisig 2007). The main diagnostic characters of scales used to separate species into sections are the presence/absence of a V-rib and dome, the nature of secondary siliceous structures such as papillae, ribs, and reticulations, and the morphology and position of different scale types on the cell covering. Nine of the sections are rare and contain only one or two species each. The remaining 10 sections are more speciose, each with between 5 and over 40 taxa (Siver et al. 2015). Molecular studies, based on the analysis of several nuclear and plastid genes, corroborated most of the sections with a few exceptions (Jo et al. 2011, 2013). According to the most recent multigene phylogeny of synuralean algae, the genus *Mallomonas* is divided into two large major lineages. The A1 lineage comprises species with scales possessing a V-rib, whereas the A2 lineage includes species lacking this structure (Siver et al. 2015). The clade A2 thus comprises only two *Mallomonas* sections (Planae and Heterospinae), whereas all the remaining ones belong in the clade A1. The section Papillosae was established by Asmund and Kristiansen (1986) to comprise taxa producing papillae on the scale surface, and having a shallow dome on all scale types. According to Kristiansen and Preisig (2007), the section Papillosae consists of 11 species and three varieties. Recently, four additional species were described from the tropics (Gusev 2013; Gusev et al. 2016; Piątek 2015). However, the genetic diversity as well as the relationship among the taxa within the section are poorly known, as only two species (*Mallomonas kalinae* Řezáčová and *Mallomonas papillosa* Harris & Bradley) have been molecularly characterized up to now.

Silica-scaled chrysophytes are often used as model objects to study the biogeography and dispersal of protists, as the scales of these organisms have distinct and stable characters, which make exact identifications possible (Kristiansen 2001; Kristiansen and Vigna 1996;

Řezáčová and Neustupa 2007; Siver and Lott 2012). Up to now, about one-third of the species of silica-scaled chrysophytes are considered as endemic taxa (Kristiansen 2008). However, these estimates are largely based on morphological investigations of silica scales only. In fact, recently published molecular phylogenetic studies have revealed a much greater proportion of endemic taxa, emphasizing the fact that geographically distant populations of traditionally defined taxa frequently represent distinct cryptic species (Boo et al. 2010; Škaloud et al. 2014).

The main aim of this study was to improve our knowledge of the diversity within the *Mallomonas rasilis/kalinae* species complex, by analyzing three newly cultured *Mallomonas* strains isolated from water bodies in Vietnam. We used a combination of molecular and morphometric approaches to characterize distinct species entities within this complex, and further morphometrically analyzed all published silica scales of these taxa to trace their distributional patterns.

MATERIALS AND METHODS

Sampling and morphological observations

Strains VN802 and VN828 were isolated from the bog pool located in Cam Ranh Peninsula, Khanh Hoa Province, Central Vietnam (N12°04′42″N, 109°11′12″E) on June 25, 2012. The pool had a temperature of 30.2 °C, pH 5.2, and low values of conductivity (49 μS/cm). VN841 (*M. rasilis*) was isolated from a sample taken on March 1, 2014 in a small pond near Da Ban reservoir in Khanh Hoa Province (12°37′43″N, 109°06′40″E) with pH 6.3, specific conductivity 54 μS/cm, and temperature 32 °C.

Plankton samples were taken using a plankton net (mesh size 20 μm). For electron microscopy studies, an aliquot of each sample was washed by repeated centrifugation in deionized water. Drops of the washed sample were dried or digested in sulfuric acid with potassium dichromate. For scanning electron microscope (SEM) studies, samples were placed on the SEM stub and coated with gold for 10 min. SEM observations were carried out with a JEOL 6510 LV SEM. For transmission electron microscope (TEM) studies, formvar-coated grids (EMS FF200-Cu-50, Electron Microscopy Sciences) were used and observations were made on a JEM-1011. Water mineralization, pH, and temperature measurements were performed using a Hanna Combo (HI 98129) device, Hanna Instruments, Inc. (Woonsocket, RI, USA).

Statistical analyses

For the purpose of a detailed morphological investigation of *Mallomonas rasilis* VN841, *M. kalinae* CAUP B 601 (the authentic strain), and a newly isolated *Mallomonas* strain VN802, the following features were determined for at least 40 randomly selected body scales: (i) scale length, (ii) scale width, (iii) number of papillae on the shield, (iv) number of papillae on the dome, (v) number of papillae between the end of V-rib and the shield pore, and (vi) the absence/presence of anterior flanges. In addition, the above-mentioned morphological features were determined for a number of published *M. kalinae* (Janatková and Němcová 2009; Gusev 2013) and *M. rasilis* (Cronberg 1989; Dürrschmidt and Cronberg 1989; Dürrschmidt and Croome 1985; Hansen 1996; Kim et al. 2009; Lavau et al. 1997; Pichrtová et al. 2011; Škaloud et al. 2013; Vyverman and Cronberg 1993; Wei and Kristiansen 1998; Wei and Yuan 2013; Wei et al. 2014) scales, including the iconotype of *M. rasilis* published by Dürrschmidt (1983). The measurements were performed using the program ImageJ 1.45 s (Schneider et al. 2012). Principal component analysis of the measured data was performed using the R statistical software (http://www.r-project.org/).

Molecular and phylogenetic analyses

For the purpose of molecular analysis, 200 μl of grown cultures of four *Mallomonas* strains (*M.* strain VN802, *M.* strain VN828, *M. rasilis* VN841, and *M. kalinae* CAUP B 601) were transferred into PCR tubes, centrifuged at 2,600 *g* for 3 min, and 30 μl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) was added to pellets. Genomic DNA extraction followed the manufacturer's instructions and the outcomes were directly used as PCR templates. One, two, and three molecular markers were amplified for *M. kalinae* CAUP B 601, *M. rasilis* VN841 and *M.* strain VN802, and *M.* strain VN828, respectively (Table 1). One new primer combination was designed for this study using Primer 3 software (Untergasser et al. 2007). The PCR amplifications were performed in a total volume of 20 μl (0.2 μl of MyTaqHS DNA polymerase [for nuclear LSU rDNA and *rbc*L gene region; Bioline] or Gold DNA polymerase [for nuclear SSU rDNA region; Applied Biosystems, Foster City, CA, USA], 14 or 13.1 μl of sterile Milli-Q Water, 0.25 μl of each primer [25 pmol/ml], 4 μl of MyTaqHS buffer [Bioline] or 0.4 μl of dNTP [10 μM], 2.2 μl of $MgCl_2$, 0.6 μl of enhancer, 2 μl of Gold buffer and 1 μl of DNA [not quantified]). The nuclear SSU and LSU rDNA and the *rbc*L genes were amplified using an Eppendorf Mastercycler ep Gradient 5341 thermocycler (Eppendorf GmbH, Hamburg, Germany) with the following program: 35 cycles of denaturing at 94 °C for 5 min, annealing at 52 °C/40 °C/38 °C for 1 min for the nuclear SSU, LSU rDNA, and the *rbc*L genes, respectively, and elongation at 72 °C for 2/4/2 min 30 s for the nuclear SSU, LSU rDNA, and the *rbc*L genes, respectively, with a final extension at 72 °C for 10 min. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and purified either with the Sigma PCR Purification Kit (Sigma-Aldrich, Darmstadt, Germany) or with Qiaex II Gel Extraction Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocols. Purified amplification products were sequenced with the PCR primers at Macrogen, Inc. (Seoul, Korea, http://dna.macrogen.com).

Along with the sequenced strains, 61 representatives of *Mallomonas*, *Synura*, and *Neotessella* were added to the phylogenetic analysis. The strain information and accession numbers are listed in Table S1. A concatenated dataset was aligned manually using MEGA ver. 5.0 (Tamura et al.

Table 1. Primers used for amplifying and sequencing of the nuclear SSU and LSU rDNA and the plastid-encoded *rbc*L gene

Designation	Sequence (5′–3′)	References
Nuclear SSU rDNA		
1122F	GGC TGA AAC TTA AAG GAA TTG	Thüs et al. (2011)
1263R	GAA CGG CCA TGC ACC ACC	T. Friedl, unpubl.
18L	CAC CTA CGG AAA CCT TGT TAC GAC TT	Hamby et al. (1988)
34F	GTC TCA AAG ATT AAG CCA TGC	Thüs et al. (2011)
370R	AGG CTC CCT CTC CGG AAT CRA ACC C	T. Friedl, unpubl.
Nuclear LSU rDNA		
28S_25F	ACC CGC TGA ATT TAA GCA TAT A	Jo et al. (2011)
28S_1228F	CCT GAA AAT GGA TGG CGC	Jo et al. (2011)
28S_861R	GTT CGA TTA GTC TTT GCG CCC T	Jo et al. (2011)
28S_2160R	CCG CGC TTG GTG GAA TCC	Jo et al. (2011)
28S_736F2	CCC GAA AGA TGG TGA ACT	Pusztai et al. (2016)
28S_1435R	GTT CAC ATG GAA CCT TTC TCT AC	Pusztai et al. (2016)
*rbc*L gene		
rbcL_2F	AAA AGT GAC CGT TAT GAA TC	Daugbjerg and Andersen (1997)
rbcL_R3	GTA ATA TCT TTC TTC CAT AAA T	Jo et al. (2011)
Synura_rbcLR	CTG CTC TTT CAT ACA TAT CTT CCA	This study

2011). To improve the quality of alignment, the positions with deletions prevailing in a majority of sequences were removed from the alignment. The resulting dataset comprised of 1,711 nucleotide sites of the nuclear SSU, 2,516 sites of the LSU rDNA, and 1,048 sites of the *rbc*L gene. The dataset was analyzed using Bayesian inference (BI) method implemented in MrBayes ver. 3.2.5. (Ronquist et al. 2012). A likelihood-ratio test was performed using jModeltest ver. 2.1.4 (Posada 2008) to determine the best model for each molecular marker using the Bayesian information criterion (BIC), with GTR + Γ + I chosen as best model for all markers. In the BI analysis, two runs of four Markov chains over 6,000,000 generations were used, sampling was performed every 1,000 generations. The burn-in was determined using the "sump" command, and the remaining trees were used to infer the Bayesian posterior probabilities (PP). The robustness of the tree topologies was assessed by bootstrapping the dataset with maximum likelihood analysis (ML) and also with weighted maximum parsimony analysis (wMP). The ML was performed by a heuristic search with 1,000 random sequence addition replicates, stepwise addition, using a Tree Bisection Reconnection branch-swapping algorithm. The reliability of the resulting topology was tested by bootstrapping (100 replications) consisting of a heuristic search with 10 random sequence addition replicates, Tree bisection reconnection swapping, and rearrangement limit of 5,000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequences (the upper limit of 10,000 for each replicate) and gap characters were treated as the fifth character state.

RESULTS

We morphometrically and molecularly analyzed four strains belonging to the section Papillosae, characterized by the presence of evenly spaced papillae on the shield. Three strains were isolated from Vietnam: VN802 and VN828 close in morphology to *M. kalinae*; and VN841, similar in ultrastructure to *M. rasilis*. The fourth analyzed strain represented the authentic strain of *M. kalinae* deposited in the CAUP culture collection (CAUP B601). In addition, we compared these strains with two already sequenced cultures MUCC 292 (isolated from Australia and determined as *Mallomonas* cf. *rasilis*) and CCMP 479 (isolated from the U.S. and identified as *M. rasilis*). Morphology and SSU sequences of these strains were studied by Škaloud et al. (2013), who attributed them to *M. kalinae*. Molecular and morphological comparison of all above-mentioned strains suggests that VN802 and VN828 should be recognized as a new species. Its description is given below.

Description

Stramenopiles Patterson 1989
Chrysophyceae Pascher 1914
Synurales Andersen 1987
Mallomonadaceae Diesing 1866
Mallomonas Perty 1852
Mallomonas furtiva Gusev, Čertnerová, Škaloudová, Škaloud (Fig. 1A–M, 2A–E).

Cells ellipsoidal, elongated, 18–25 × 8–15 μm, covered by scales with bristles. Body scales oval, often slightly asymmetrical, 3.6–4.3 × 2.2–2.5 μm, tripartite, with a dome and a V-rib (Fig. 1C–L). Half of the dome is covered by papillae (Fig. 1D, G, H). Shield with densely and regularly spaced papillae. Distinct base plate pore is situated in the proximal area of the shield at the base of the V-rib and often surrounded by papillae (Fig. 1F, H, L). The V-rib is conspicuous, rounded. Distal ends of arms of the V-rib curve and become continuous with anterior submarginal ribs. Anterior and posterior flanges are smooth. Shield and posterior flanges with perforations. Posterior rim smooth. Rear scales are smaller in size, 2–3.2 × 1.2–1.6 μm (Fig. 1I). Bristles serrated, 6–11 μm, curved and pointed (Fig. 1M). Cysts were not observed.

Holotype specimen. Portion of a single gathering of cells on SEM stub number VN802 deposited at the Herbarium of the I.D. Papanin Institute for Biology of Inland Waters RAS, Borok (IBIW). Material is from the culture BOROK VN802 established from sample CR100 made by E.S.

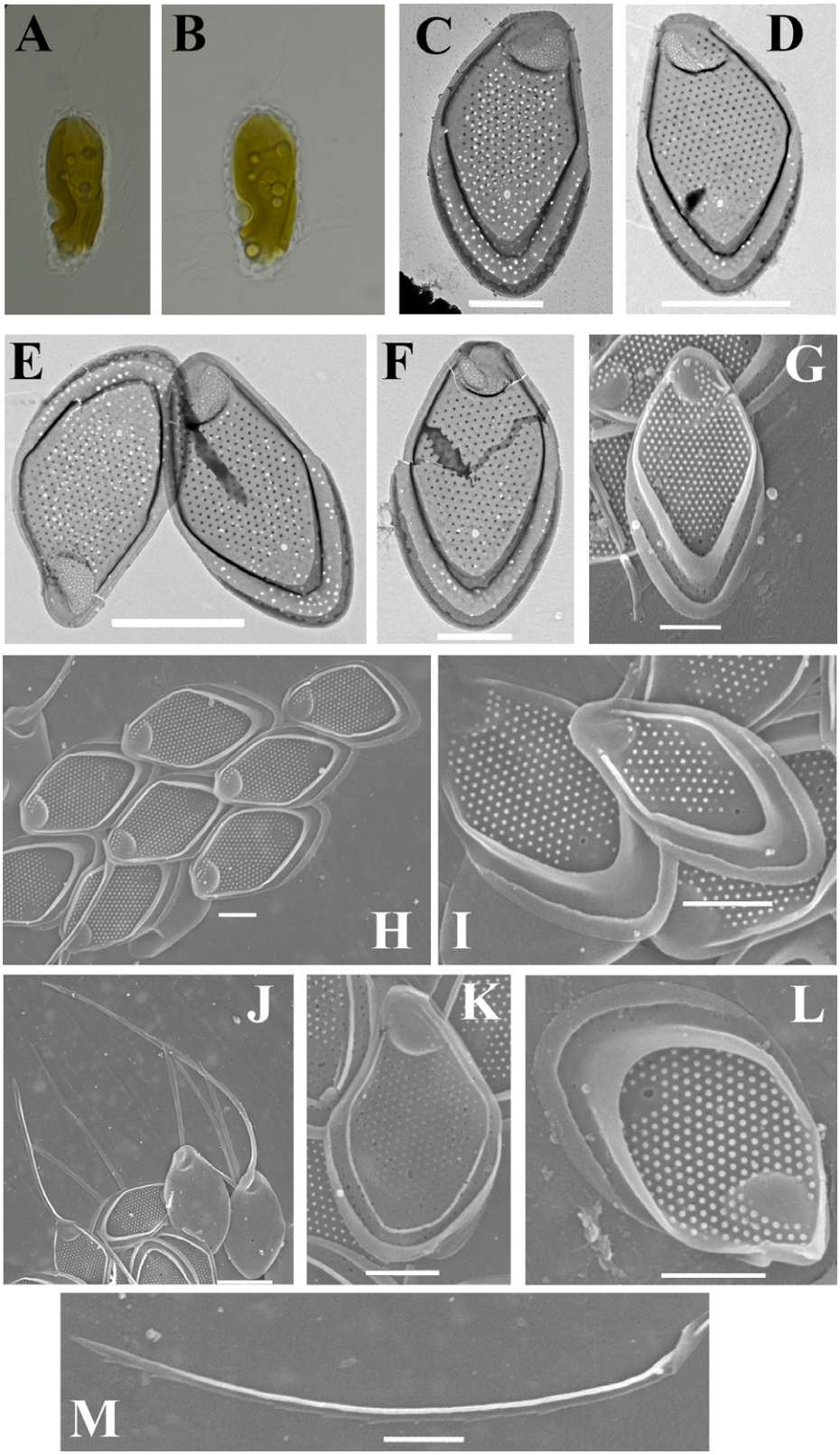

Figure 1 *Mallomonas furtiva* sp. nov. (**A–M**). Strain BOROK VN802 (A–K, M). (A, B) Whole cells, LM. (C–F) Body scales, TEM. (G–K) Body scales, SEM. (G) is a representative scale from the specimen. (L) Body scale in natural population from Cam Ranh Peninsula. (M) Bristle, SEM, strain BOROK VN802. Scale bars = 2 μm (D–E, J). Scale bars = 1 μm (C, F–I, K–M).

Gusev on June 25, 2012. Figure 1G is a representative scale from the specimen. New taxon is registered to the Zoobank under the number: 8259DE27-C3E9-444C-857A-7AAEAE1D5527.

Type locality. VIETNAM. Khanh Hoa province: unnamed pool in Cam Ranh Peninsula, N12°04′42″N, 109°11′12″E, 25.06.2012.

Etymology. The epithet "***furtiva***" (hidden, invisible, cryptic) refers to its morphological similarity to *M. kalinae* and difficulty in distinguishing the two taxa by morphological features.

Distribution and habitat. This species was found in the type locality and other water bodies in Khanh Hoa and Dong Nai provinces in Vietnam. In Vietnam, scales of *M. furtiva* have been previously found in mangrove wetlands in Cam Ranh Peninsula (Gusev 2013), in Cat Tien National park (Fig. 2A–C and Gusev et al. 2017) and Dzua river near Nha Trang City (Fig. 2E).

Morphological observations

Mallomonas furtiva belongs to the section Papillosae Asmund and Kristiansen (1986) and within that section it is morphologically highly similar to *M. kalinae* Řezáčová. Comparison of the type material of these two taxa shows that *M. furtiva* differs from *M. kalinae* by the serrated bristles, perforations on the base plate, and papillae arrangement (Řezáčová 2006). In *M. furtiva*, the papillae usually surround the base plate pore, whereas in *M. kalinae*, they do not pass over the pore. However, the considerable variability of the scale ultrastructure has been demonstrated for *M. kalinae*, including serration of bristles and changes in scale shape and size (Řezáčová-Škaloudová et al. 2010). During cultivation of the *M. furtiva* strain, an increase in the number of base plate pores was observed (Fig. 1C), possibly due to silica depletion.

Study of fixed material (natural populations) shows that the base plate pores, scattered on the shield and flanges, are present on all observed scales from Cam Ranh Peninsula and Dzua river. Some scales (Fig. 2C) from Cat Tien National park (Dong Nai Province) have no base plate pores (except a big one in the angle of the V-rib) like *M. kalinae*, others have few perforations (Fig. 2A, B, E). Thus, number and presence of base plate pores is very variable in natural populations and during cultivation and cannot be considered as a reliable taxonomic character. On the other hand, serrated bristles were observed in all natural populations, and their morphology was not altered by the artificial cultivation conditions (Fig. 2E). Almost all scales of *M. furtiva* have domes half-covered with papillae.

A second taxon, isolated from Vietnam, was attributed to *M. rasilis* Dürrschmidt, although it differs slightly in morphology from the type, described from Chile by Dürrschmidt (1983). Our strain has smooth bristles, curved to a higher degree, whereas the bristles of *M. rasilis* are serrated. In addition, *M. rasilis* has scales with smooth domes (without papillae), while the specimens from Vietnam (natural and isolated into culture, with few exceptions in culture) have scales with domes fully covered with papillae (Fig. 2F–K). Similar scales and bristles were reported from Korea (Kim et al. 2009, *Mallomonas* sp. 4, fig. 52–53). The taxonomic significance of these differences is not clear. Nevertheless, in this paper, we consider strain VN841 from Vietnam to be *M. rasilis*.

For better delineation of *M. furtiva*, *M. kalinae*, and *M. rasilis*, we performed a PCA based on silica scale morphometric parameters, such as scale length, scale width, number of papillae on the shield, number of papillae on the dome, number of papillae between the end of V-rib and the shield pore, and the absence/presence of anterior flanges. In addition to the three cultured strains, we included in the analysis a number of published scales collected from different regions. These include scales similar to those of *M. furtiva* and *M. kalinae* previously reported (as *M.* cf. *rasilis* or *M.* cf. *kalinae*) from Papua New Guinea (Vyverman and Cronberg 1993), Malaysia (Dürrschmidt and Croome 1985) and Vietnam (Gusev 2013); as well as scales determined as *M. rasilis* reported from Chile (Dürrschmidt 1983), Sri-Lanka (Dürrschmidt and Cronberg 1989), India (Wujek and Saha 1996), Madagascar (Hansen 1996), China (Wei and Kristiansen 1998; Wei et al. 2014), and Korea (Kim et al. 2009).

The PCA significantly separated all three species (Fig. 3). *Mallomonas kalinae* and *M. furtiva* differed by number of papillae on the shield and the dome (Fig. S1A, B). *Mallomonas furtiva* has domes with numerous papillae (18.9 ± 9.0), while domes on *M. kalinae* scales are smooth or with a few papillae (0.6 ± 0.9). Papillae on the shield of scales of *M. furtiva* are more numerous (268 ± 48) than on *M. kalinae* scales (100 ± 24). Generally, scales of *M. furtiva* are slightly longer than those of *M. kalinae* (Fig. S1C). However, the extreme values of these parameters overlapped, so we were unable to define exact borders between these two taxa. Concerning the analysis of previously published scales, those reported from Malaysia and Vietnam (Dürrschmidt and Croome 1985; Gusev 2013) were assigned to the newly proposed species *M. furtiva* (Fig. 3). Surprisingly, the analysis assigned to this species one scale reported from Czech Republic, as well (Janatková and Němcová 2009). The remaining scales were assigned to either *M. kalinae* or *M. rasilis*, with the single exception of a *M. rasilis* scale found in Jamaica (Cronberg 1989) forming a distinct position on the ordination plot. The iconotype of *M. rasilis* (Dürrschmidt 1983) plotted near the cluster of scales produced by the strain VN841 (Fig. 3), corroborating the determination of this strain as *M. rasilis*.

Phylogenetic analysis

Bayesian inference, maximum likelihood, and maximum parsimony analyses were conducted on the concatenated SSU rDNA, LSU rDNA, and *rbc*L dataset consisting of 65 Synuralean taxa (Fig. 4 and Table S1). All major lineages had significant bootstrap support from both ML and wMP analyses, with the exception of the lineage consisting of *Mallomonas morrisonensis*, *M. muskokana*, *M. crassisquama*,

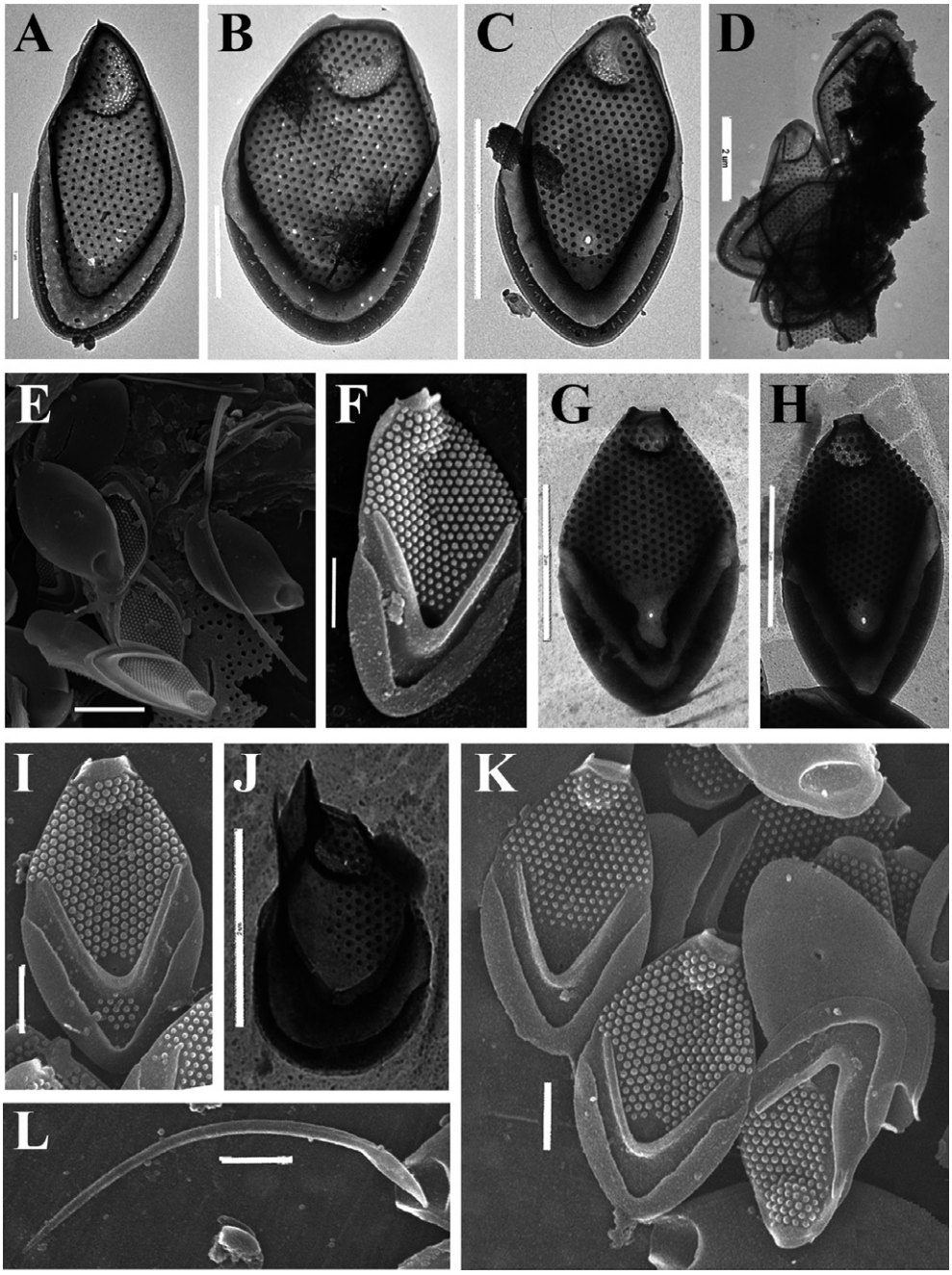

Figure 2 *Mallomonas furtiva* sp. nov. (**A–E**) and *Mallomonas rasilis* (**F–L**). (A–E) *Mallomonas furtiva* sp. nov. Scales in natural population. (A–C) Cat Tien National park, Dak Lua swamp, Dong Nai Province, TEM. (D) River Dzua, Khanh Hoa Province, TEM. (E) Cat Tien National park, Lake Đâu Ca, Dong Nai Province, SEM. (F–L) *Mallomonas rasilis*. (F) Body scale, natural population, reservoir Câu Đôi, Khanh Hoa Province, SEM. (G–L) Strain BOROK VN841. (G, H) Body scales, TEM. (I) Body scale, SEM with papillae on the posterior flange as a consequence of culturing. (J) Apical scale, TEM. (K) Group of body scales, SEM. (L) Bristle, SEM. Scale bars = 2 μm (C–E, G, H, J). Scale bars = 1 μm (A, B, F, I, K, L).

M. acaroides, *M. alpina*, *M. elongata*, and *M. areolata*. The resolved phylogeny was in accordance with previously published phylogenetic analyses of Synurales (e.g. Kim et al. 2014; Siver et al. 2015). The genus *Mallomonas* was divided into two major lineages (A1 and A2 according to Siver et al. 2015) with high bootstrap support. *Mallomonas furtiva* was recovered as a member of the section Papillosae, along with *M. kalinae*, *M. rasilis*, and *M. papillosa*. It was closely related to *M. kalinae*, with which it formed a highly supported lineage (1.00/100/99). Pairwise genetic distances (p-distances) of the SSU rDNA and *rbc*L sequences were calculated for *M. furtiva*, *M. kalinae*, and *M. rasilis*. Distances between *M. kalinae* and *M. rasilis* (0.011 for the SSU rDNA, 0.058 for the *rbc*L) were slightly

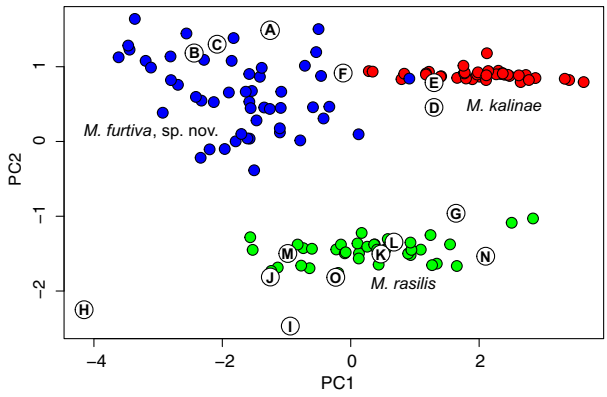

Figure 3 Principal component analysis (PCA) of morphological traits measured on silica scales of three *Mallomonas* species, *M. kalinae* (CAUP B 601), *M. rasilis* (VN841), and *M. furtiva*, sp. nov. (VN802), as well as several previously published scales. The position of published scales is denoted by letters enclosed in circles: A: Dürrschmidt and Croome (1985); B: Janatková and Němcová (2009); C: Gusev (2013); D: Vyverman and Cronberg (1993); E: Lavau et al. (1997), strain MUCC 292; F: Škaloud et al. (2013), strain CCMP 479; G: Dürrschmidt (1983), type of *M. rasilis*; H: Cronberg (1989); I: Dürrschmidt and Cronberg (1989); J: Hansen (1996); K: Wei and Kristiansen (1998); L: Kim et al. (2009); M: Pichrtová et al. (2011); N: Wei and Yuan (2013); O: Wei et al. (2014).

lower or similar to those calculated for *M. furtiva–M. kalinae* (0.018, 0.061) and *M. furtiva–M. rasilis* (0.015, 0.051), corroborating the separation of all three taxa.

DISCUSSION

Recent molecular studies of synuralean algae revealed a considerable hidden diversity in both major genera, *Synura* and *Mallomonas* (Boo et al. 2010; Jo et al. 2011; Kynčlová et al. 2010; Siver et al. 2015). However, detailed studies of the closely related taxa generally revealed the existence of molecular and morphological markers useful for their separation. Accordingly, new species have been described within the *Synura petersenii* Korshikov (Škaloud et al. 2012, 2014) and *Mallomonas matvienkoae* Asmund & Kristiansen species complexes (Gusev 2015; Jo et al. 2013). These studies questioned the choice of taxonomically relevant morphological features for distinguishing species on the basis of siliceous scale ultrastructure. In some cases, closely related taxa can be separated using qualitative features like form of basal pores, papilla arrangement, and bristle morphology, as we can see in the *M. matvienkoae* species complex (Jo et al. 2013). One of the other promising ways to separate morphologically similar taxa is the use of quantitative traits (canonical discriminant analysis or other multidimensional statistical methods). This approach was successfully applied to distinguish closely related species in the *S. petersenii* complex (Jo et al. 2016; Škaloud et al. 2012, 2014). In this paper, we deal with another synuralean complex of closely related, cryptic taxa, which requires the application of detailed morphometric approaches for the correct identification of particular species.

The taxonomy of *Mallomonas* species from the section Papillosae is built mainly on papilla arrangement, form of the V-rib, and the presence/absence of anterior ribs. *Mallomonas rasilis*, which was described from Chile, has tripartite scales with a shield covered with papillae and without obvious anterior ribs (Dürrschmidt 1983). Later on, some authors found scales with anterior ribs and marked them as *M.* cf. *rasilis* (Dürrschmidt and Croome 1985; Vyverman and Cronberg 1993). In 2006, a newly described taxon *M. kalinae* was distinguished from the morphologically similar *M. rasilis* primarily by the presence of anterior ribs (Řezáčová 2006). Our molecular data confirm the separation of *M. rasilis* and *M. kalinae* as distinct taxa, and prove the presence of anterior ribs as a taxonomically important character. Moreover, we describe here a new species, *M. furtiva*, morphologically almost identical to the closely related *M. kalinae*. At first glance, when only traditional morphological features were used, these species were virtually unrecognizable, though some minute differences were uncovered, such as the presence of bristle serration or papilla pattern on the scale's shield and dome. However, the combination of molecular approaches and morphometric analyses clearly distinguished these taxa from each other. The most important separating features are as follows: the number of papillae on the shield, the presence of papillae on the dome, and the scale length. In addition, PCA of several morphometric features allowed us to trace the distribution patterns of morphologically cryptic, yet genetically distinct taxa within the complex, analyzing the published microphotographs of siliceous scales. For example, *M. kalinae* is considered to be a cosmopolitan species, as molecularly it has been detected in the Czech Republic, U.S., and Australia (Škaloud et al. 2013). Our PCA confirmed a broad distribution of this species, as scales reported from Russia (Siver et al. 2005; under the name *M. paxillata*, fig. 5C) and Papua New Guinea (Vyverman and Cronberg 1993, see Fig. 3) were also assigned to *M. kalinae*. Similarly, two already published scales can be assigned to the newly described *M. furtiva* (Fig. 3). These were found in Malaysia (Dürrschmidt and Croome 1985) and the Czech Republic (Janatková and Němcová 2009). It should be noted however that the scale from the Czech Republic has some specific morphological features, such as a disrupted papilla arrangement where rows of papillae change their direction by 90°, and the presence of a smooth dome. The assignment of this scale to *M. furtiva* is therefore rather doubtful. Nevertheless, it seems that both *M. furtiva* and *M. kalinae* represent widely distributed taxa with rather overlapping areas. *Mallomonas furtiva* seems to prefer warmer areas while *M. kalinae* was revealed mainly in temperate and subarctic habitats.

We expect that further molecular studies of *M. rasilis* will detect an additional hidden diversity, primarily between the populations originated from different habitats and climatic zones. Although the PCA did not show significant differences between the scales of *M. rasilis* sampled

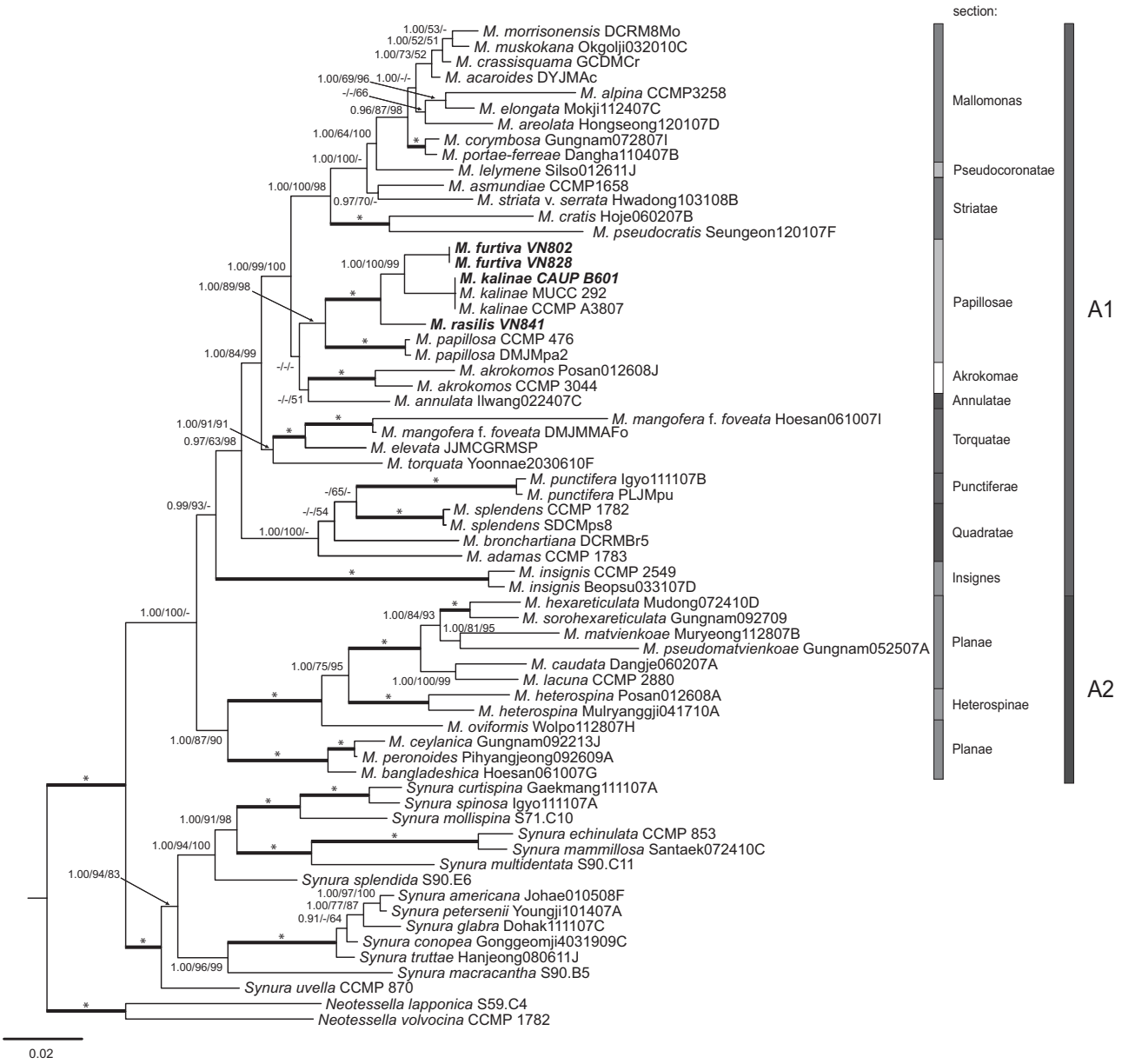

Figure 4 Bayesian analysis of Synurales based on the combined SSU rDNA, LSU rDNA, and *rbc*L dataset with GTR + Γ + I nucleotide substitution model for all molecular markers. Values at the nodes indicate statistical support estimated by three methods—MrBayes posterior-node probability (left), maximum likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Thick branches represent nodes receiving the highest support (1.00/100/100). The scale bar shows the estimated number of substitutions per site.

in distinct geographical areas, scales from the type specimen are slightly dissimilar to those of our strain isolated in Vietnam. The type specimen from Chile has scales with serrated bristles and smooth dome without papillae (Dürrschmidt 1983), and morphologically identical scales were found in Argentina, as well (Kristiansen and Vigna 2002). It seems that the scales completely fitting the original description have been so far reported only from South America. The culture VN843, as well as the other populations from Vietnam, produced smooth bristles and scales whose domes were covered with papillae. Such scales and bristles were additionally reported from South Korea (Kim et al. 2009), Sri-Lanka (Dürrschmidt and Cronberg 1989), China (Wei and Kristiansen 1998; Wei et al. 2014), Madagascar (Hansen 1996)), and Europe (Barreto 2005; Němcová 2010). However, the taxonomic relevance of these characters is still unclear. In addition, a number of different unusual morphotypes identified as *M. rasilis* were reported from the tropics. The scale found in Jamaica has a larger size than usually observed for *M. rasilis* (Cronberg 1989). Scales reported from Bali (Indonesia) have strong ribs on the dome (Cronberg and

Hickel 1985). Recently, two of such morphologically unusual *M. rasilis* morphotypes were described as new species. The first is *M. camerunensis* Piątek, found in Cameroon, Africa (Piątek 2015). Main distinctive features of this taxon are ribs on the dome and numerous papillae on the shield of scales. The second newly described taxon is *M. skvortsovii* Gusev et al. described from Vietnam, which has large scales with internal reticulation and a row of ribs on the dome (Gusev et al. 2016). Certainly, a more detailed comparison of populations sampled in different geographical areas is definitely needed to better understand the real diversity in the *M. rasilis* group.

As a conclusion, we would like to mention that, in spite of the well-developed species concept of synuralean algae, diversity in the genus *Mallomonas* is strongly underestimated. Many described species very likely represent complexes of closely related taxa (cryptic or pseudocryptic), weakly differentiated by standard morphological approaches. It is necessary to intensify molecular studies of the genus and perform more detailed morphological observations, especially for comparison of populations sampled in remote habitats and different climatic regions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. PCA of *Mallomonas* scales as pictured on Fig. 3, overlaid with a smooth fitted surface of estimated number of papillae on shield (A), number of papillae on dome (B), and scale length (C).
Table S1. Strains of the synuralean algae used in this study and the GenBank accession numbers for their nr SSU, nr LSU, and *rbc*L gene sequences.
Table S2. The eigenvector coefficients of six measured morphological characters for the first two principal components.
Table S3. Comparison of morphological features of three investigated *Mallomonas* species.

SUPPORTING INFORMATION

Exploring Cryptic Diversity and Distribution Patterns in the *Mallomonas kalinae/rasilis* Species Complex with a Description of a New Taxon – *Mallomonas furtiva* sp. nov. by Evgeniy S. Gusev, Dora Čertnerová, Magda Škaloudová, & Pavel Škaloud

Figure S1. PCA of *Mallomonas* scales as pictured on Fig. 3, overlaid with a smooth fitted surface of estimated number of papillae on shield (A), number of papillae on dome (B), and scale length (C).

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Table S3. Comparison of morphological features of three investigated *Mallomonas* species. Mean and standard deviation values are provided.

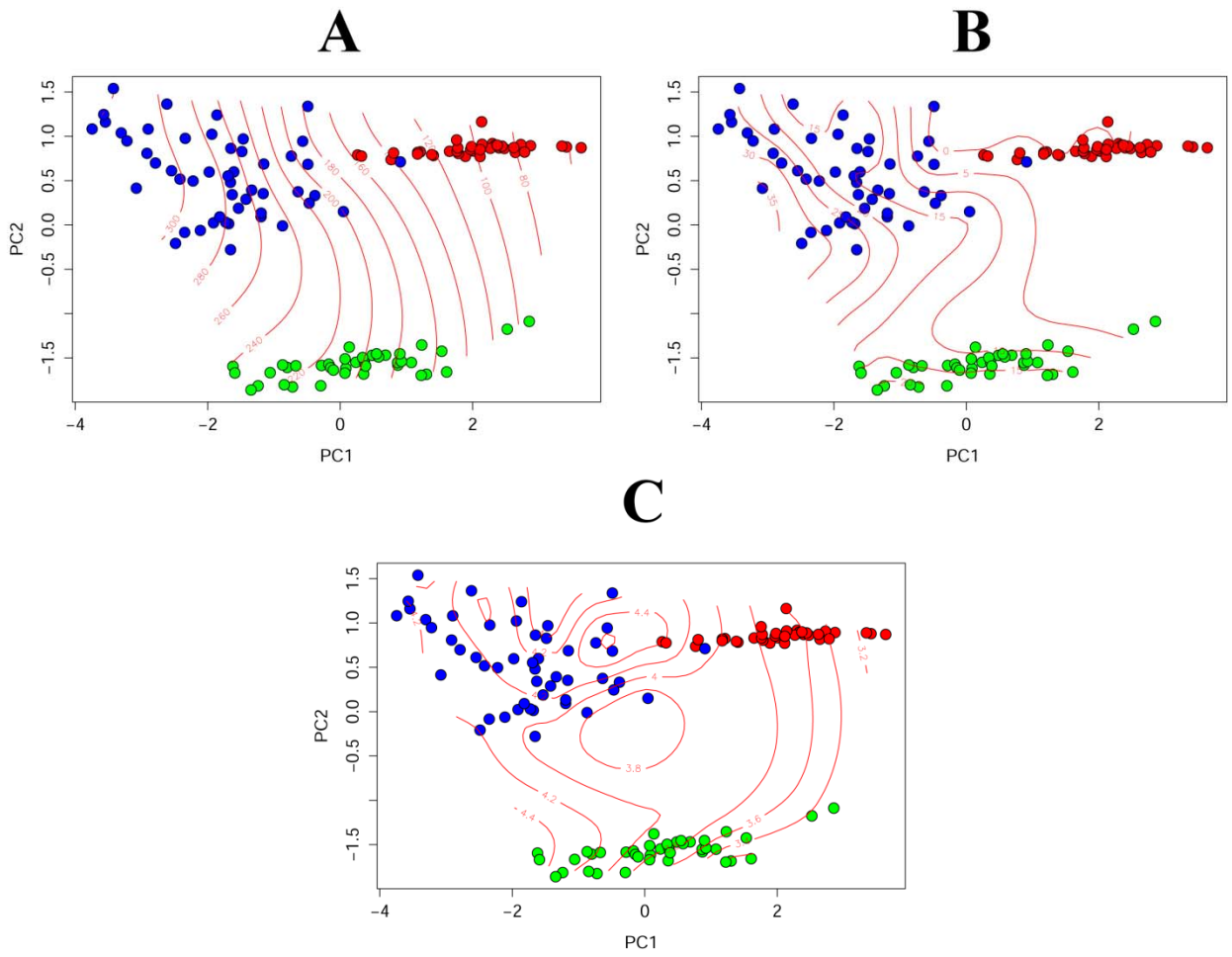

Table S1. Strains of the Synuralean algae used in this study and the GenBank accession numbers for their nr SSU, nr LSU and *rbc*L gene sequences.

Taxon	Strain	GenBank accesion		
		Nuclear SSU	Nuclear LSU	Plastid *rbc*L
Mallomonas				
M. acaroides	DYJMAc	JX946333	JX946341	JX946349
M. adamas	CCMP 1783	JQ281515	JQ281508	JQ281502
M. akrokomos	Posan012608J	GU935625	GU935647	GU935667
M. akrokomos	CCMP 3044	JQ281520	JQ281509	JQ281503
M. alpina	CCMP 3258	KM817860	KM817895	KM818086
M. annulata	Ilwang022407C	GU935626	GU935648	GU935668
M. areolata	Hongseong120107D	GU935619	GU935641	GU935661
M. asmundiae	CCMP1658	M87333	AF409122	AF015585
M. bangladeshica	Hoesan061007G	GU935630	GU935652	GU935672
M. bronchartiana	DCRMBr5	JQ955670	JQ955675	JQ955665
M. caudata	Dangje060207A	GU935629	GU93565	GU935671
M. ceylanica	Gungnam092213J	KM817864	KM817899	KM818090
M. corymbosa	GungnamI072807I	GU935620	GU935642	GU935662
M. crassisquama	GCDMCr	KM817865	KM817900	KM818091
M. cratis	Hoje060207B	GU935623	GU935645	GU935665
M. elevata	JJMCGRMSP	JX946339	JX946347	JX946355
M. elongata	Mokji112407C	GU935621	GU935643	GU935663
M. furtiva sp. nov.	VN802	**KY942077**	**-**	**KY942074**
M. furtiva sp. nov.	VN828	**KY942078**	**KY942071**	**KY942075**
M. heterospina	Posan012608A	GU935617	GU935639	GU935659
M. heterospina	Mulryanggji041710A	JN991179	JN991188	JN991170
M. hexareticulata	Mudong072410D	JN991182	JN991191	JN991173
M. insignis	CCMP 2549	EF165118	-	EF165198
M. insignis	Beopsu033107D	GU935634	GU935656	GU935676
M. kalinae	CAUP B601	HF549061	**KY942072**	HF549073
M. kalinae	MUCC 292	U73231	-	-
M. kalinae	CCMP A3807	M55285	-	-
M. lacuna	CCMP 2880	JN991177	JN991186	JN991168

M. lelymene	Silso012611J	KM817874	KM817909	KM818100
M. mangofera var. *foveata*	Hoesan061007I	GU935633	GU935655	GU935675
M. mangofera var. *foveata*	DMJMMAFo	JX946338	JX946346	JX946354
M. matvienkoae	Muryeong112807B	GU935628	GU935650	GU935670
M. morrisonensis	DCRM8Mo	KM817875	KM817910	KM818101
M. muskokana	Okgolji032010C	KM817877	KM817912	KM818103
M. oviformis	Wolpo112807H	GU935631	GU935653	GU935673
M. papillosa	CCMP 476	HF549062	-	-
M. papillosa	DMJMpa2	JX946337	JX946345	JX946353
M. peronoides	Pihyangjeong092609A	JN991180	JN991189	JN991171
M. portae-ferreae	Dangha110407B	GU935618	GU935640	GU935660
M. pseudocratis	Seungeon120107F	GU935624	GU935646	GU935666
M. pseudomatvienkoae	Gungnam052507A	GU935627	GU935649	GU935669
M. punctifera	Igyo111107B	GU935632	GU935654	GU935674
M. punctifera	PLJMpu	JX946340	JX946348	JX946356
M. rasilis	VN841	**KY942076**	-	**KY942073**
M. sorohexareticulata	Gungnam092709	JN991183	JN991192	JN991174
M. splendens	CCMP 1782	JQ955668	JQ955673	JQ955663
M. splendens	SDCMps8	JQ955669	JQ955674	JQ955664
M. striata var. *serrata*	Hwadong103108B	GU935622	GU935644	GU935664
M. torquata	Yoonnae2030610F	KM817887	KM817922	KM818113
Synura				
Synura americana	Johae010508F	JX455151	JX455155	JX455147
Synura conopea	Gonggeomji4031909C	KM590555	KM590621	KM590842
Synura curtispina	Gaekmang111107A	KM590559	KM590625	KM590846
Synura echinulata	CCMP 853	KM590563	KM590629	KM590850
Synura glabra	Dohak111107C	JX455149	JX455153	JX455145
Synura macracantha	S90.B5	HF549064	KM590648	HF549075

Synura mammillosa	Santaek072410C	KM590583	KM590649	KM590870
Synura mollispina	S71.C10	HF549067	KM590655	HF549077
Synura multidentata	S90.C11	HF549068	KM590656	HF549078
Synura petersenii	Youngji101407A	JX455150	JX455154	JX455146
Synura spinosa	Igyo111107A	JX455148	JX455152	JX455144
Synura splendida	S90.E6	KM590603	KM590674	KM590890
Synura truttae	Hanjeong080611J	KM590609	KM590680	KM590896
Synura uvella	CCMP 870	KM590615	KM590686	AF015586
Neotesella				
Neotesella lapponica	S59.C4	HF549063	KM590690	HF549074
Neotesella volvocina	CCMP 1781	EF165119	KM590691	EF165199

New sequences are indicated in **bold type**.

Table S2. The eigenvector coefficients of six measured morphological characters for the first two principal components.

	PCA1	PCA2
Length	0.393171	0.093562
Width	0.488686	-0.119183
Papillae on the shield	0.515091	-0.102337
Papillae on the dome	0.452826	-0.240026
Papillae window	0.366139	0.388554
Anterior flanges	0.046628	0.870622

Table S3. Comparison of morphological features of three investigated *Mallomonas* species. Mean and standard deviation values are provided.

	Length of scale	Width of scale	Papillae on the shield	Papillae on the dome	Papillae window	Anterior flanges
M. furtiva						
Mean	4.16	2.36	268.14	18.86	8.76	1.00
Std	0.26	0.10	47.70	8.97	8.67	0.00
M. kalinae						
Mean	3.70	1.87	100.38	0.58	0.20	1.00
Std	0.30	0.16	24.13	0.90	0.99	0.00
M. rasilis						
Mean	3.82	2.15	195.43	13.58	0.00	0.00
Std	0.36	0.21	35.80	4.96	0.00	0.00