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Substantial intraspecific genome size variation in golden-brown algae and its phenotypic consequences

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• **Background and Aims** While nuclear DNA content variation and its phenotypic consequences have been well described for animals, vascular plants and macroalgae, much less about this topic is known regarding unicellular algae and protists in general. The dearth of data is especially pronounced when it comes to intraspecific genome size variation. This study attempts to investigate the extent of intraspecific variability in genome size and its adaptive consequences in a microalgal species.
• **Methods** Propidium iodide flow cytometry was used to estimate the absolute genome size of 131 strains (isolates) of the golden-brown alga *Synura petersenii* (Chrysophyceae, Stramenopiles), identified by identical internal transcribed spacer (ITS) rDNA barcodes. Cell size, growth rate and genomic GC content were further assessed on a sub-set of strains. Geographic location of 67 sampling sites across the Northern hemisphere was used to extract climatic database data and to evaluate the ecogeographical distribution of genome size diversity.
• **Key Results** Genome size ranged continuously from 0.97 to 2.02 pg of DNA across the investigated strains. The genome size was positively associated with cell size and negatively associated with growth rate. Bioclim variables were not correlated with genome size variation. No clear trends in the geographical distribution of strains of a particular genome size were detected, and strains of different genome size occasionally coexisted at the same locality. Genomic GC content was significantly associated only with genome size via a quadratic relationship.
• **Conclusions** Genome size variability in *S. petersenii* was probably triggered by an evolutionary mechanism operating via gradual changes in genome size accompanied by changes in genomic GC content, such as, for example, proliferation of transposable elements. The variation was reflected in cell size and relative growth rate, possibly with adaptive consequences.

Key words: Intraspecific DNA content variation, genome size, flow cytometry, golden-brown algae, *Synura petersenii*, GC content, biovolume, growth rate, environmental conditions, ITS.

INTRODUCTION

The nuclear genome constitutes an essential cell component. While the quality of nuclear DNA (expressed by nucleotide sequences or presence of specific alleles) has been intensively in the focus of biologists for more than a half of century, its quantity per cell has received far less attention. Nonetheless, genomes contain orders of magnitude more DNA than required to sustain the basic cell functioning (a phenomenon called the 'C-value enigma'; Gregory, 2001*a*) and this may suggest that even the overall quantity of nuclear DNA has an adaptive potential (Mirsky and Ris, 1951; Thomas, 1971; Cavalier-Smith, 2005). Our knowledge of the genome size variation and its evolutionary consequences mainly comes from plant and animal studies (e.g. Leitch *et al.*, 1998; Gregory, 2005; Beaulieu *et al.*, 2008; Liedtke *et al.*, 2018; Trávníček *et al.*, 2019). Much less of this topic is known from single-celled eukaryotes, despite the fact that protist genome size ranges >28 600-fold compared with 6600-fold variation among plants and animals (Veldhuis *et al.*, 1997; Gregory, 2005; Keeling and Slamovits, 2005; Pellicer and Leitch, 2020).

There are several evolutionary mechanisms responsible for variation of the amount of nuclear DNA . The genome size can either increase or decrease by chromosomal aberrations (aneuploidy), non-homologous recombination and changes in the relative genome-wide frequency of insertions to deletions (Devos *et al.*, 2002; Roux *et al.*, 2003; Lynch and Conery, 2003; Wu *et al.*, 2018). Conversely, only an increase in genome size is possible via higher activity of transposable elements and, more abruptly, by whole-genome doubling (polyploidization; Soltis and Soltis, 1999; Kidwell, 2002; Cavalier-Smith, 2005; Sun *et al.*, 2012). Recent or past hybridization events between closely related but separate species can also contribute to differentiation of the amount of nuclear DNA (Baack *et al.*, 2005).

Variation in nuclear DNA content is usually accompanied by phenotypic consequences. The genome size directly affects the size of the nucleus (Sparrow and Evans, 1961; Baetcke *et al.*, 1967; Bennett, 1972; Gregory, 2001*b*; Zubáčová *et al.*, 2008) and through it also fundamentally relates to the cell size (Cavalier-Smith and Beaton, 1999; Gregory, 2001*a*, *b*; Cavalier-Smith, 2005). The genome size–cell size correlation, also known as the karyoplasmic ratio, has been observed across the eukaryotic tree of life, including many protist lineages (Wilson, 1925; Bennett, 1972; Suzuki *et al.*, 1982; Shuter *et al.*, 1983; Veldhuis *et al.*, 1997; LaJeunesse *et al.*, 2005;

Connolly *et al.*, 2008; von Dassow *et al.*, 2008). Although the exact cause of this relationship is still debated, evolutionary changes in cell size may either cause or be caused by changes in genome size (Gregory, 2001*a*). The cell size is a particularly important trait in single-celled organisms as it inversely correlates with metabolic rate and growth rate, and directly correlates with generation time (Van't Hof and Sparrow, 1963; Bennett, 1972; Shuter *et al.*, 1983; Gregory, 2001*a*; Cavalier-Smith, 2005). Changes in cell size may also be reflected in protist ecology, for example by altering the grazing pressure, efficiency of nutrient acquisition and/or light harvesting (Garcia-Pichel, 1994; Finkel *et al.*, 2001; Smetacek *et al.*, 2004; Irwin *et al.*, 2006). Therefore, genome size variation could be (via cell size) subject to natural selection in populations of protists (Cavalier-Smith, 2005).

Another understudied genomic parameter with possibly adaptive nature is the relative genome-wide frequency of AT to GC base pairs, often expressed as a %GC content (Bennett and Leitch, 2011; Šmarda and Bureš, 2012). The higher genomic GC content is sometimes associated with extreme climatic conditions such as pronounced cold, drought or temperature fluctuations, probably due to the increased thermal stability of the DNA double helix (Šmarda and Bureš, 2012; Šmarda *et al.*, 2014; Trávníček *et al.*, 2019). Additionally, the GC content variation may be linked to changes in genome size, since the genomic nucleobase composition might be altered by high activity of transposable elements (TEs) or chromosomal aberrations (Wichman *et al.*, 1993; Armbrust, 2004; Derelle *et al.*, 2006). However, only little is known about the evolutionary impact of GC content variation and, what is known, comes almost exclusively from studies on prokaryotes, plants and animals (Goodsell and Dickerson, 1994; Hildebrand *et al.*, 2010; Šmarda *et al.*, 2014; Mugal *et al.*, 2015; Veleba *et al.*, 2017; Trávníček *et al.*, 2019).

Both genome size and GC content may be estimated using flow cytometry (FCM). This technique is based on measuring the properties of fluorescent-stained particles (e.g. cells or isolated nuclei) in a stream of fluid and allows rapid and precise nuclear DNA analysis (Doležel *et al.*, 2007). While FCM has found a wide spectrum of applications in genomic surveys on plants and animals (Dionisio Pires *et al.*, 2004; Kron *et al.*, 2007; Galbraith, 2012; Pellicer and Leitch, 2014), its potential has not yet been explored in depth in protist studies (but see Figueroa *et al.*, 2010), and robust methodological protocols allowing work with diverse protist material are missing.

The dearth of data is especially pronounced when it comes to intraspecific genome size variation in unicellular eukaryotes. This can be attributed to analysing only a single strain per species in most studies (Veldhuis *et al.*, 1997; Mazalová *et al.*, 2011) and, possibly, to the fact that in contrast to, for example, plant studies, best-practice protocols preventing false reports based on methodological and instrumental errors (Greilhuber, 2005) are not routinely applied.

To study the evolution of genome size and its phenotypic and physiological consequences, we chose the golden-brown algal species *Synura petersenii* (Chrysophyceae, Stramenopiles) as our model system. In general, protist species have a short generation time and huge population size, which allows them to respond quickly to environmental change (Lynch and Conery, 2003; Foissner, 2007; Ribeiro *et al.*, 2013). *Synura petersenii* is an autotrophic flagellate with assumed worldwide distribution. It creates colonies of cells covered by siliceous scales with species-specific ornamentation and a characteristic pronounced central keel (Kristiansen and Preisig, 2007). The species has recently undergone thorough taxonomic revision supported by molecular markers and morphometric analysis of its siliceous scales, which revealed 15 separate species in the formerly recognized *S. petersenii* species complex (Wee *et al.*, 2001; Boo *et al.*, 2010; Kynčlová *et al.*, 2010; Škaloud *et al.*, 2012, 2014; Jo *et al.*, 2016). One of these taxonomically revised species, *S. petersenii sensu stricto* (*s.s.*) is used as a model species in this study. During our pilot FCM analysis, we detected intraspecific genome size variation among the strains of *S. petersenii*. The general aims of the study are to prove the existence of intraspecific genome size variation in *S. petersenii* and investigate in detail, for the first time, the extent of intraspecific variability in genome size in a microalgal species. Additionally, we ask the following questions. (1) Is the variability in DNA content linked to GC content variation? (2) Are there any phenotypic and physiological consequences of varying genome size? (3) Is genome size variation among strains reflected in their ecogeographical distribution?

MATERIALS AND METHODS

Origin, cultivation and identification of the investigated strains

Altogether, 131 isolates of the species *Synura petersenii* were obtained from various freshwater localities across the Northern hemisphere. Sampling details are listed in Supplementary data Table S1. To establish new cultures, water samples were taken using a 25 µm mesh plankton net and single *Synura* colonies were captured by micro-pipetting and transferred into separate culture wells filled with WC medium (Guillard and Lorenzen, 1972). All cultures were maintained at 17 °C (cooling box Pol-Eko Aparatura Sp.J., model ST 1, Wodzisław Śląski, Poland) with 24 h light mode under illumination of 30 µmol m^{-2} s^{-1} (TLD 18 W/33 fluorescent lamps, Philips, Amsterdam, the Netherlands).

All strains were identified based on their internal transcribed spacer sequence of nuclear ribosomal DNA (ITS1, 5.8S and ITS2 rDNA) since this is the most variable of the commonly used molecular markers in this group (Jo *et al.*, 2016). For this purpose, genomic DNA was extracted from a centrifuged pellet of cells by InstaGene Matrix (Bio-Rad, USA) and the resulting supernatant was directly used as a PCR template. The amplifications were performed using the universal primer ITS4 (White, 1990) and a lineage-specific primer Kn1.1 (Wee *et al.*, 2001). The PCRs were carried out in a total volume of 20 µL with a PCR mix containing 0.2 µL of MyTaqHS DNA polymerase (Bioline), 4 µL of MyTaqHS buffer (Bioline), 0.4 µL of each primer, 14 µL of double-distilled water and 1 µL of template DNA (not quantified). The amplifications were performed in Eppendorf Mastercycler ep Gradient 5341 (Eppendorf GmbH, Hamburg, Germany) using the following program: 1 min of denaturation at 95 °C; followed by 35 cycles of denaturation at 95 °C (15 s), annealing at 52 °C (30 s) and elongation at 72 °C (40 s), concluded with a final extension at 72 °C (7 min) and held at 10 °C. The PCR products were sized on a 1 % agarose gel and then purified using AMPure XP

magnetic beads (Agencourt). The purified DNA templates were sequenced by the Sanger Sequencing method at Macrogen, Inc. (Seoul, Korea, http://dna.macrogen.com). Finally, the obtained sequences were identified using BLAST in the National Center for Biotechnology Information (NCBI) Search database and our personal ITS database created during previous studies (Škaloud *et al.*, 2012, 2014). The strains with ITS rDNA sequence identical to that of *S. petersenii* were transferred into Erlenmeyer flasks with 30 mL of WC medium and kept for longer cultivation. The collection was further supplemented with five strains from previous studies (Kynčlová *et al.*, 2010; Kim *et al.*, 2019).

DNA content estimation

To estimate the nuclear genome size of our strains, we employed propidium iodide FCM. Approximately 2 weeks before the planned FCM analyses, cultures were inoculated into fresh medium. For sample preparation, 1 mL of well-grown culture was centrifuged (5500 rpm for 5 min) and the superfluous medium was removed by pipetting. Subsequently, 350 µL of ice-cold nuclei isolation buffer Otto I (0.1 M citric acid, 0.5 % Tween-20; Otto, 1990) was added to the algal pellet, causing the release of the sample nuclei. The resulting suspension was thoroughly shaken and kept on ice. *Solanum pseudocapsicum* (2C = 2.59 pg; Temsch *et al.*, 2010) was used as an internal standard. To release nuclei of the standard, an approx. 20 mg piece of fresh leaf tissue was chopped with a razor blade in a plastic Petri dish with 250 µL of ice-cold Otto I buffer. Both suspensions (with algal and standard nuclei) were thoroughly mixed and filtered through a 42 µm nylon mesh into a special 3.5 mL cuvette for direct use with the flow cytometer. Following a 20 min incubation at room temperature, the sample was mixed with 1 mL of staining solution consisting of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$; Otto, 1990), 50 µg mL^{-1} propidium iodide, 50 µg mL^{-1} RNase IIA and 2 µL mL^{-1} β-mercaptoethanol. The stained sample was immediately analysed using a Partec CyFlow SL cytometer (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). Measurements on each sample were taken for up to 5000 particles, and the resulting FCM histograms analysed using FloMax ver. 2.4d (Partec, Münster, Germany). Since there is no knowledge of the ploidy level in the genus *Synura* (Olefeld *et al.*, 2018), we identified the first sample peak on the FCM histogram as G_1 (vegetative cells) and the second peak as G_2 (dividing cells). The absolute nuclear DNA amount (C-value) was calculated as sample G_1 peak mean fluorescence/standard G_1 peak mean fluorescence × standard 2C DNA content (according to Doležel, 2005).

In the case of low quality measurement, i.e. G_1 sample coefficient of variation (CV) >5 %, the sample preparation and analysis was repeated. To minimize the effect of random instrumental shift, each *S. petersenii* strain was analysed at least three times on separate days. Whenever the three independent genome size estimates differed by >3 %, the most outlying measurement was discarded and a new measurement conducted, until this condition was fulfilled. In order to corroborate genome size differences among strains, simultaneous analysis of multiple selected strains (i.e. A64, D55 and G61) was performed. We also tested for strain genome size stability during its cultivation. Following inoculation into a fresh medium, three strains exhibiting the highest variation among repeated measurements (i.e. F19, G16 and H11) were analysed once a week for the period of 6–7 weeks. Two other strains (961 and S63.B3) were then re-analysed 2 years following the first measurements.

GC content estimation

To assess variation in genomic GC content, we analysed 38 strains of *S. petersenii* using FCM with the AT-selective dye DAPI (4',6-diamidino-2-phenylindole) and compared the results with propidium iodide FCM. The strains for GC content estimation, cell size measurements and growth rate analysis (see below) were selected representatively across the whole range of genome size diversity; however, a different set of strains was used for each assessment (Supplementary data Table S2). This was due to unavailability of some strains at the time of particular assessments (e.g. the cultured strains did not survive, provided limited biomass or a contamination occurred); replacement strains with similar genome size were then randomly selected. We employed the same sample preparation as for propidium iodide FCM, except that the staining solution consisted of 1 mL of Otto II buffer, 4 µg mL^{-1} DAPI and 2 µL mL^{-1} β-mercaptoethanol. Samples were immediately analysed using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488 nm UV LED as a source of excitation light. Analyses were run up to 5000 particles and the resulting FCM histograms were analysed using FloMax ver. 2.4d (Partec, Münster, Germany). Computation of the base content was done according to Šmarda *et al.* (2008) via a publicly available Excel spreadsheet (http://sci.muni.cz/botany/systemgr/download/Festuca/ATGCFlow.xls).

Cell size measurements

After 2 weeks of cultivation in fresh medium, the cell size of 39 selected *S. petersenii* strains was analysed by imaging FCM using Benchtop B3 Series FlowCAM (Fluid Imaging Technologies, Yarmouth, ME, USA). The FlowCAM settings were AutoImage mode, 50 µm flow cell, ×20 objective and flow rate 0.020 mL min^{-1}. The mean biovolume of 100 cells per strain was calculated on manually selected images using VisualSpreadsheet® Particle Analysis Software ver. 4.11.12 as the volume of a sphere of the area-based radius measured automatically by the FlowCAM for each cell.

Growth rate

Test of growth rate was performed on eight replicate cultures of each of the 31 selected *S. petersenii* strains. The chlorophyll fluorescence, i.e. the effective quantum yield of photochemical energy conversion in photosystem II (Φ_{PSII}), of cultures starting with the same initial concentration was measured daily at the same hour over 15 d using a PAM 2500 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The variable

Φ_{PSII} is a relative parameter calculated as $(F_M' - F)/F_M'$, where F is the steady-state fluorescence in the light-adapted state and F_M' is the maximum fluorescence in the light-adapted state measured after the application of a saturation pulse (Roháček and Barták, 1999). The growth rate was subsequently derived as an inverse value of the median time at which the population density reaches half the carrying capacity, i.e. the inflection point (t-mid value^{-1}) in R software ver. 3.4.3 (R Development Core Team, 2017) using the package growthcurver ver. 0.3.0 (Sprouffske and Wagner, 2016).

Ecogeographical patterns

Geographical distribution of the genome size diversity in *Synura* was visualized on a map using ArcGIS 10.0 (ESRI, Redlands, CA, USA). In order to assess putative ecogeographical trends in the distribution of genome size diversity well beyond any obvious spatial patterns, we also tested for associations between the genome size of *Synura* and database-derived climatic variables. We used ArcGIS to extract climate data from 19 Bioclim variables of the WorldClim database ver. 2 (http://worldclim.org/; Fick and Hijmans, 2017) downloaded in the highest available resolution (30 arc seconds). Climatic conditions may affect aquatic microalgae e.g. via temperature-regulated onset and duration of their seasonal blooms or precipitation frequently being associated with input of nutrients into aquatic ecosystems (Baek *et al.*, 2009). Each of 67 sampling sites was assigned values of the climatic variables and geographic latitude, included as an additional ecogeographically relevant parameter. When multiple strains were collected at a site, we only retained those with genome size estimates differing by at least 5 % (i.e. an arbitrarily selected threshold to prevent pseudoreplication of data) and such strains were then treated as independent observations. If two or more strains with a similar genome size (<5 % difference) originated from the same site, all except one randomly selected strain were excluded from the dataset. The resulting dataset consisting of 82 strains was analysed using a redundancy analysis (RDA) implemented in Canoco 5 (Lepš and Šmilauer, 2014); the genome size of *Synura* was used as an explanatory variable. All response variables were standardized prior to the RDA and statistical significance was tested using a Monte Carlo test with 999 permutations. The RDA was then also used to test the effect of GC content.

Statistical analysis

Unless stated otherwise, statistical data analysis was conducted in R. Separate linear regression models were applied to test whether the variation in cell size and growth rate can be explained by genome size (log transformed). These analyses were conducted on a sub-set of 39 and 31 strains, respectively, for which data were available. Both models were also re-run using the GC content as an explanatory variable.

We then employed a regression model to assess the relationship between GC content (response variable) and genome size (explanatory variable, log transformed). Due to previous reports of a quadratic relationship between the two genomic parameters in plants (e.g. Šmarda *et al.*, 2014), we used manual Akaike information criterion (AIC)-based forward selection with the function 'addterm' from the R package MASS ver. 7.3-50 (Venables and Ripley, 2002) to test whether incorporating the logarithm of genome size either in linear (approx. log.GS) or quadratic form [approx. log.GS + I(log.GS^2)] will significantly improve the model performance.

RESULTS

Intraspecific variability in genome size

We successfully determined the absolute nuclear DNA amount in 131 strains of *S. petersenii* with identical ITS rDNA sequence from 67 localities across the Northern hemisphere (Fig. 1). The sampled *S. petersenii* strains exhibited a 2.1-fold variation in their genome size, ranging from 0.971 to 2.022 pg of DNA (Supplementary data Table S2). The frequency distribution of the genome size values was conspicuously positively skewed (median = 1.170 pg, mean = 1.296 pg; Fig. 2). Sufficient precision of flow cytometric measurements was ensured by relatively low CVs for both sample and standard G_1 nuclei peaks (mean CV = 3.29 and 2.25 %, respectively); see Fig. 3A for a representative analysis. The intraspecific variability in genome size was also verified in a simultaneous analysis of three *S. petersenii* strains with contrasting nuclear DNA amounts that resulted in three clearly differentiated peaks in the flow cytometric histogram (Fig. 3B).

To corroborate the longer term stability of genome size differences among strains in cultivation, two selected strains were re-analysed 2 years following the first measurements: strain 961 (1.071 and 1.077 pg of DNA, respectively) and strain S63.B3 (1.516 and 1.492 pg of DNA, respectively). In both cases, the deviation between repeated estimates fell within the limits of instrumental precision (i.e. mean CV). Similarly, three other strains investigated for genome size stability via regular weekly measurements (F19, G16 and H11) did not show any substantial deviations from the original estimates (Fig. 4).

Phenotypic consequences and ecogeographical distribution of genome size diversity

Genome size was significantly associated with both cell size ($F_{1,37} = 6.25$, $P = 0.017$, $R^2 = 0.145$; Fig. 5A) and growth rate ($F_{1,29} = 5.03$, $P = 0.033$, $R^2 = 0.148$; Fig. 5B); an increase in genome size led to an increase in cell size and a decrease in growth rate. However, the two observed relationships were not affected by a putative strong correlation between the cell size and growth rate ($t_{23} = –1.32$, $P = 0.201$, $r = –0.265$).

Synura strains with smaller or larger genome size did not display any apparent spatial trends in their geographic distribution (Fig. 1). This was supported by the lack of a significant association between spatial distribution of genome size diversity and climatic variables or latitude in the RDA ($P = 0.505$; 999 permutations). The first, constrained RDA axis explained only 1.0 % of overall variation, whereas the second, unconstrained axis explained 41.6 % of variation (Supplementary data Fig. S1).

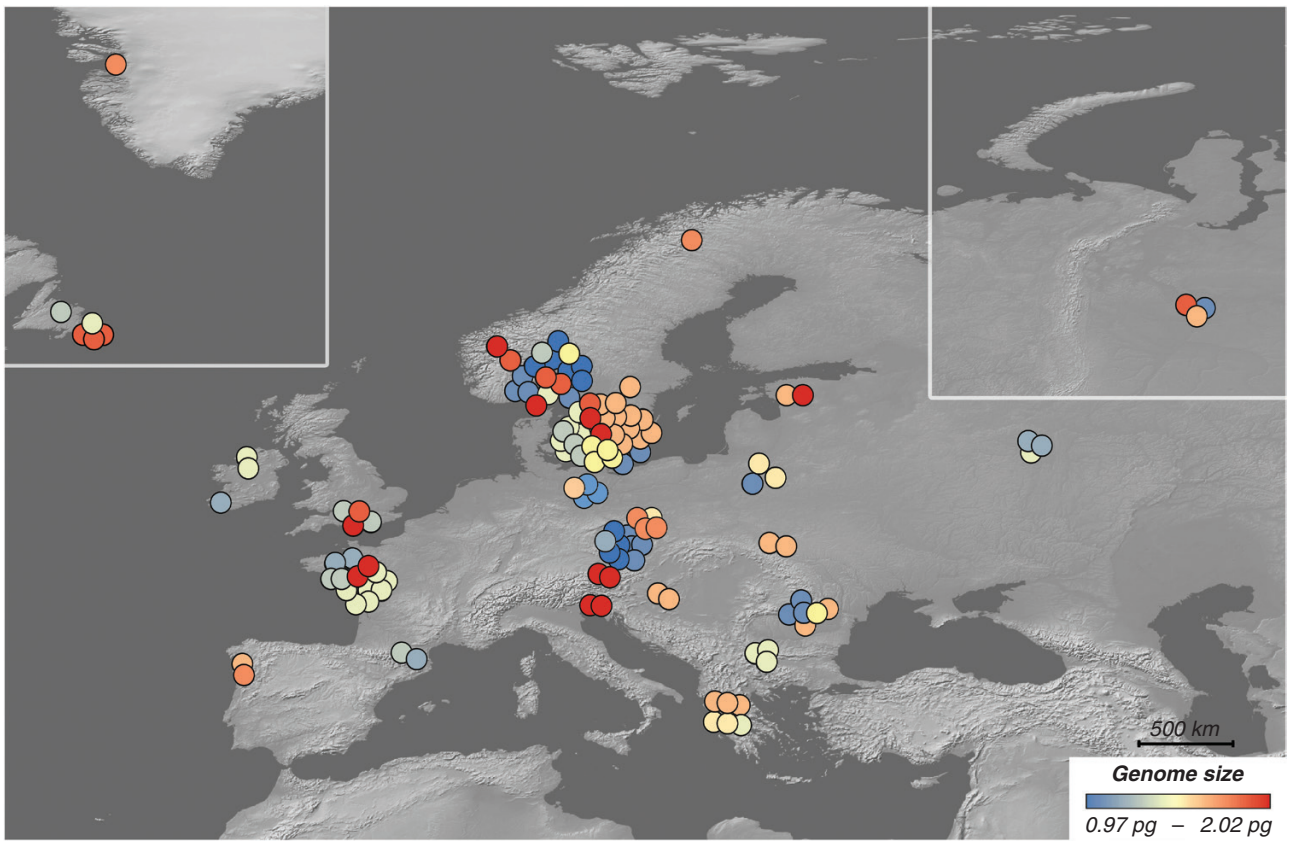

FIG. 1. Distribution of 131 strains of *Synura petersenii* under study and their estimated genome size. Symbol colour refers to different genome size categories.

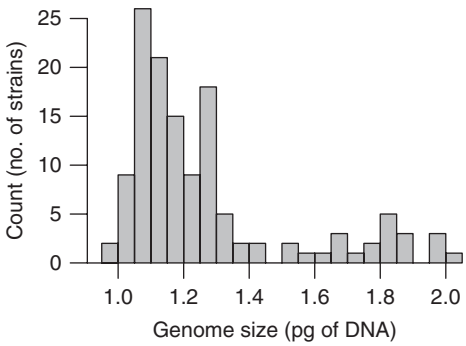

FIG. 2. Frequency distribution of genome size categories among investigated *Synura petersenii* strains.

Diversity in genomic GC content

The other genomic parameter, GC content, varied from 37.1 to 41.2 %, with a mean value of 39.5 % (Supplementary data Table S2). The GC content had no significant effect on either cell size ($F_{1,15}$ = 0.29, P = 0.598, R^2 = 0.019) or growth rate ($F_{1,11}$ = 1.96, P = 0.189, R^2 = 0.151). No significant association between GC content diversity and climatic variables or latitude was detected in RDA (P = 0.890; 999 permutations). The first, constrained RDA axis explained only 0.8 % of the overall variation, whereas the second, unconstrained axis explained 42.2 % of the variation (Supplementary data Fig. S1). On the other hand, a significant quadratic relationship was detected between GC content and genome size ($F_{2,35}$ = 6.95, P = 0.003, R^2 = 0.284; Fig. 5C). The appropriateness of including the predictor in a quadratic form was corroborated using manual forward selection, as such a model significantly outperformed both the linear relationship ($F_{1,36}$ = 0.11, P = 0.743, R^2 = 0.003) and the null model without any predictors.

DISCUSSION

Genome size variability and its evolutionary sources

Currently, there is an apparent dearth of genome size estimates from protists, especially regarding the degree of intraspecific genome size variation. This might be due to the fact that the use of FCM, an efficient and widely applied technique of nuclear DNA content estimation (Doležel *et al.*, 2007), is often methodologically challenging in protists as a result of difficulties in obtaining sufficient amounts of biomass, protoplast extraction or the presence of a wide variety of pigments and secondary metabolites interfering with fluorescent staining (Veldhuis *et al.*, 1997; Kapraun, 2007; Mazalová *et al.*, 2011; Poulíčková *et al.*, 2014). Considering the above, we adopted an FCM protocol with several steps improving the robustness of our estimates that included, inferring each genome size estimate from mean value of three analyses on different days, simultaneous analysis of strains with different genome size

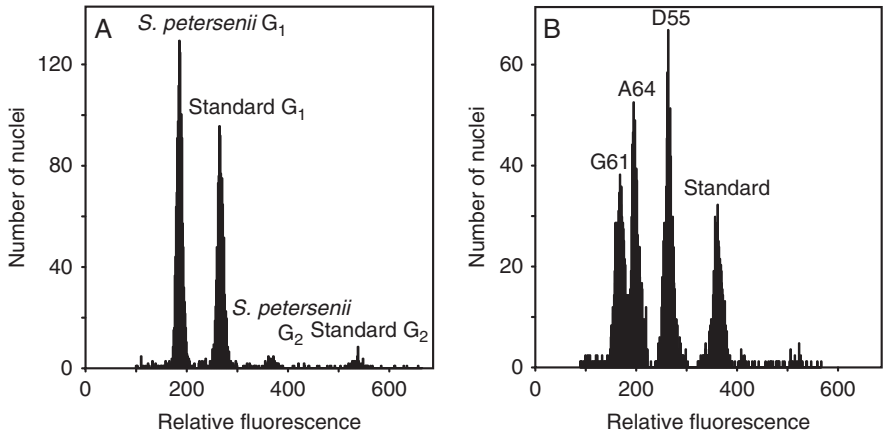

FIG. 3. Flow cytometric histograms showing relative fluorescence of propidium iodide-stained nuclei of *Synura petersenii* samples and *Solanum pseudocapsicum* (Standard). (A) A representative analysis of a single *S. petersenii* strain with G_1 and G_2 phase nuclei apparent for both the analysed sample and standard. (B) A simultaneous analysis of three strains with distinct genome size (G61, A64 and D55) to confirm the existing differences.

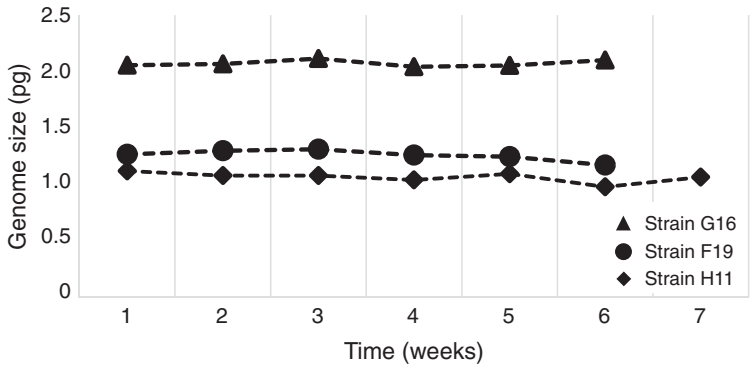

FIG. 4. Temporal stability of the genome size of three selected *Synura petersenii* strains.

to confirm the existing differences (multiple peaks in an FCM histogram) and re-analysing strains after a period of time to account for genome size stability under cultivation. In our study, we successfully estimated genome size of >130 strains belonging to a single microalgal species and, to our knowledge, this is the most comprehensive intraspecific genome size screening conducted on protists so far. We revealed considerable variability in genome size of *Synura petersenii*, ranging 2-fold across the analysed strains, from 0.97 to 2.02 pg of DNA. Our estimates (median value = 1.17 pg) are not consistent with an earlier estimate of *S. petersenii* genome size of 0.78 pg made by Olefeld *et al.* (2018). However, the published data belonged to the strain WA18K-A (with other designation CCMP 2892) that in the taxonomic revision of the *S. petersenii* species complex was assigned to a different species, *S. heteropora* (Škaloud *et al.*, 2014). Therefore, we present the first genome size data for *S. petersenii s.s.*

There are several possible scenarios for what could be the source of genome size diversity observed among *S. petersenii* strains. First, owing to the robust FCM protocol, consistent methodology and generally high precision of our analyses, we are convinced that the error of measurement has not substantially contributed to the genome size variation. Taking into account the 2-fold difference between the lowest and highest genome size estimates, alternating life cycle stages or whole-genome doubling (polyploidization) events would seem to be likely explanations. However, none of these mechanisms can be the sole source of the diversity observed in *Synura* as there were no discrete genome size categories that would reflect the inherent ploidy shifts (Fig. 2). Another argument against the alternating life cycle stages is that strains re-analysed after weeks (or 2 years) exhibited more or less stable genome size estimates (Fig. 4). This contrasts with the genome size differentiation that emerged within a long-term cultivated strain of *Thalassiosira weissflogii* belonging to diatoms, a more intensively studied group of Stramenopiles, implying the ability to rapidly change the amount of DNA (von Dassow *et al.*, 2008), possibly in the context of sexual reproduction or (theoretically) local adaptation. On the other hand, genome size appeared to be stable in cultivation of another diatom, *Ditylum brightwellii*, where intraspecific variation among strains was also previously detected (Koester *et al.*, 2010). While we are unable to exclude the possibility that strains at both extreme ends of the observed genome size continuum are in fact different ploidy cytotypes or distinct stages of the *S. petersenii* life cycle, other evolutionary mechanisms operating with more gradual increases or decreases in genome size were most probably involved.

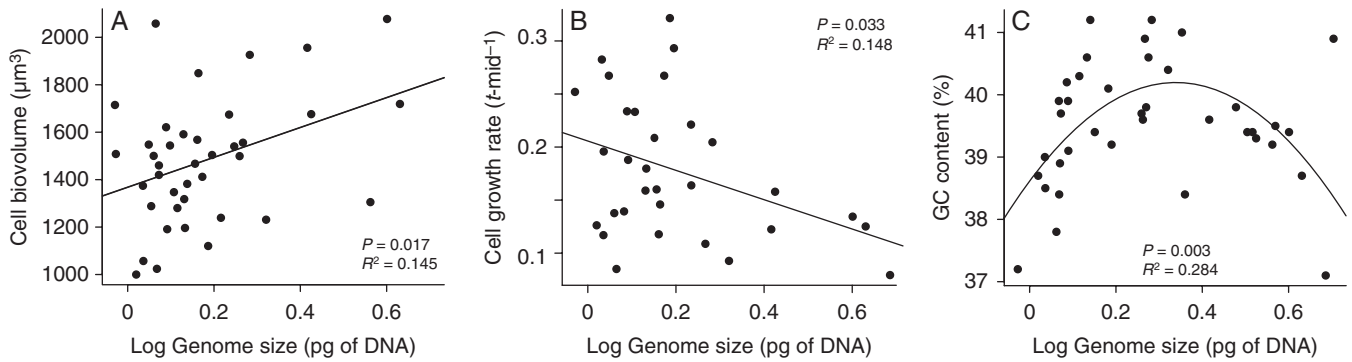

FIG. 5. Three genomic and phenotypic traits associated with intraspecific genome size diversity in *Synura petersenii*: cell size (A), relative growth rate (B) and genomic GC content (C). Model predictions are depicted using lines in (A and B) and the curve of the quadratic function in (C).

Alternative explanations may be provided by proliferation of TEs, unequal frequency of insertions to deletions and multiplication of larger genomic segments or even whole chromosomes (supernumerary chromosomes or aneuploidy; Jones *et al.*, 2008; Šmarda and Bureš, 2010; Ruiz-Ruano *et al.*, 2011; Stelzer *et al.*, 2019). For example, genome size diversity in the diatom *T. weissflogii* was attributed to polyploidization, aneuploidization and gene duplications (von Dassow *et al.*, 2008). A recent chromosome doubling was also detected in the diatom *T. pseudonana* (Armbrust, 2004). Unfortunately, despite a considerable effort, we did not succeed with karyotyping of *S. petersenii* strains and neither chromosome counts nor complete genomic sequences are available for any representative of the class Chrysophyceae (including the genus *Synura*). It is thus unclear whether prompt karyotype evolution or chromosomal aberrations could be responsible for the observed intraspecific genome size variation. Genome size changes caused by chromosomal aberrations or increased TE activity may be accompanied by significant alterations of genomic GC content (i.e. the relative proportion of GC base pairs; Wichman *et al.*, 1993; Armbrust, 2004; Derelle *et al.*, 2006). Interestingly, we found a significant relationship between the genome size of *Synura* strains and their genomic GC content, which had a quadratic nature and predicted highest GC content in medium-sized genomes (Fig. 5C). A similar quadratic relationship between the two variables was previously documented in monocot plants (Veselý *et al.*, 2012; Šmarda *et al.*, 2014), where it was explained by involvement of GC-rich long terminal repeat (LTR) retrotransposons in genome size expansion in combination with a mechanism responsible for decreasing GC content in large genomes (e.g. lower energetic cost of synthesis of dATPs and dTTPs leading to their misincorporation into the newly synthesized DNA as a mutational bias toward an AT-rich genome; Rocha and Danchin, 2002; Grover and Wendel, 2010). It is worth emphasizing that in contrast to a 207-fold genome size variation among monocot plants in the dataset analysed by Šmarda *et al.* (2014), we were able to detect the significant quadratic relationship with GC content on a very fine scale of 2-fold genome size variation. This might suggest that the observed GC content variation is a mere by-product of the mechanism governing genome size evolution in *S. petersenii*, which is in line with the absence of any biological or environmental correlates of GC content diversity in our dataset.

Intraspecific variability vs. cryptic diversity

As a general rule in multicellular organisms, individuals belonging to the same species share a constant nuclear DNA content (Swift, 1950). Nonetheless, intraspecific genome size variation manifested either via multiple ploidy cytotypes or at a homoploid level is occasionally observed among both plants and animals (Jeffery *et al.*, 2016; Kolář *et al.*, 2017; Stelzer *et al.*, 2019). There is also some evidence of intraspecific genome size variation among microalgae coming from diatoms (von Dassow *et al.*, 2008; Koester *et al.*, 2010), desmids (Poulíčková *et al.*, 2014) and haptophytes (Medlin *et al.*, 1996; Veldhuis *et al.*, 1997; Read *et al.*, 2013). Nonetheless, the frequency of this phenomenon and its prevalence across various groups of protists is still poorly documented, and the evolutionary mechanisms involved are only exceptionally addressed. Theoretically, there are two mutually non-exclusive evolutionary scenarios that would result in intraspecific genome size variation. First, the mechanisms of genome size change might act recurrently with high enough frequency to compensate for only a transient character of induced changes (e.g. via aneuploidy or presence of supernumerary chromosomes). Secondly, intraspecific genome size variation could be maintained in populations when it is coupled with a reproductive barrier that prevents crosses between conspecific individuals with different genome sizes. The reproductive barrier in the latter scenario could either directly result from the mechanism inducing genome size differences (e.g. polyploidization) or arise independently (e.g. spatial or temporal isolation or a specific mate recognition systems).

A unique insight into mechanisms maintaining intraspecific genome size variation was recently documented in one species of rotifer (Stelzer *et al.*, 2019). The variation, apparent already at the within-population level, was possibly due to independently segregating large genomic elements present in males. Regardless of the genome size difference, individuals were able to interbreed and produce viable offspring, stressing their identity to one species (Stelzer *et al.*, 2019). However, under natural conditions, the species relies predominantly on asexual reproduction mediated by parthenogenetic females. Similar to rotifers, *S. petersenii* also reproduces mainly clonally (by a cell division), though sexual reproduction has been documented (Sandgren and Flanagin, 1986). Despite our great efforts, we were unable to experimentally interbreed *S. petersenii* strains. Neither crosses between contrasting genome size categories

nor those performed between strains with similar-sized genomes were successful, possibly suggesting inadequate conditions for mating. It thus remains unclear whether the different genome size categories of *S. petersenii* strains are coupled with a reproductive barrier or not. Another similarity between our studied *S. petersenii* populations and the case study on rotifers is that many strains of different genome size categories co-occurred contemporarily at the same locality. We detected the common presence of two or more strains differing in their genome size (up to 1.8-fold difference) in 14 out of 67 localities (21 %). However, the actual rate may be even higher as our sampling strategy was primarily focused at between-locality comparisons and there seem to be no clear trends in geographical distribution of genome size categories. It is likely that the prevalence of clonal reproduction contributes to the maintenance of strains of different genome size categories and their coexistence in *S. petersenii* populations. *Synura petersenii* is a colonial species and it is generally unknown whether the colonies are composed of genetically identical cells or may combine multiple genotypes (strains). Since the cultures for this study were established from one colony of cells each and always had uniform genome size, we hypothesize that strains of different genome size coexist at a locality in well-separated colonies.

Our results cannot rule out the scenario that various genome size categories in *S. petersenii* are coupled with reproductive barriers and thus reflect cryptic diversity within the taxon. Were this the case, it would mean that the nuclear ITS rDNA region is not always a sufficient molecular marker to separate microalgal species, even though this marker (and rDNA in general) is widely used as a barcode for species identification in many algal studies (e.g. Helms *et al.*, 2001; Connell, 2002; von Dassow *et al.*, 2008; Whittaker *et al.*, 2012; Jo *et al.*, 2016). Genome size estimation using FCM might then serve as a useful tool for identifying potential cryptic diversity in protists. Such an approach has already been applied in some diatoms and harmful dinoflagellates (Figueroa *et al.*, 2010; Koester *et al.*, 2010).

Adaptive role of genome size variation

An important aspect of intraspecific genome size diversity and its evolutionary maintenance is its putative adaptive potential, i.e. whether strains with a certain genome size have a fitness (dis-)advantage in some environmental or evolutionary context. Among *S. petersenii* strains, an increase in genome size resulted in a significant increase in cell size and a significant decrease in relative growth rate. The genome size–cell size correlation has been previously documented in many other protists (LaJeunesse *et al.*, 2005; Connolly *et al.*, 2008; von Dassow *et al.*, 2008; Poulíčková *et al.*, 2014; Olefeld *et al.*, 2018), though in our study the relationship was not as tight as presumed, explaining 14.5 % of the overall variation (Fig. 5A). There are two likely explanations for this discrepancy. First, in the other studies, the relationship was tested across different species, thus with a much broader range of both genome sizes and cell sizes, increasing the chance of finding a general trend. Secondly, the cells of *Synura* lack a cell wall and flexibly adjust their volume under varying temperature, nutrient composition, etc. (Němcová *et al.*, 2010; Řezáčová-Škaloudová *et al.*, 2010; Pichrtová and Němcová, 2011), which could have contributed to residual model variance.

The genome size was further associated with the relative growth rate of particular strains (14.8 % of overall variation explained), leading to up to a 3-fold difference in growth rates between strains from the lowest and highest genome size categories (Fig. 5B). As a result, strains with larger genomes could not grow and divide as quickly as their counterparts with a smaller genome size, a feature that should be reflected in their relative fitness at least under specific environmental conditions. In aquatic micro-organisms, rapid population growth is a key factor for successful colonization of a new site and effective monopolization of local resources (i.e. the monopolization hypothesis; De Meester *et al.*, 2002). Once a population is well established and possibly also locally adapted, the existence of a large bank of resting propagules (in this case *Synura* cysts) provides a powerful buffer against newly invading genotypes. Under this scenario, *S. petersenii* strains with larger genomes should be inferior colonizers of new sites, possibly sometimes outcompeted at the existing localities by other strains with smaller genomes. This is in line with the frequency distribution of genome size categories across *S. petersenii* populations, which was strongly skewed towards smaller genomes (Fig. 2). The strains with larger genomes could then be maintained in populations either due to their recurrent *in situ* origin from smaller genome progenitors or because of other compensatory adaptive traits that were not included in our study, possibly stemming from their larger cell size, e.g. more efficient nutrient uptake and/or photosynthesis (Finkel *et al.*, 2001; Irwin *et al.*, 2006).

The question is whether the identified phenotypic consequences of genome size variation could also have translated into contrasting ecogeographical distributions of *Synura* strains.

Based on our results, this does not seem to be the case. It was already suggested by the lack of any clear geographical trends in distribution of strains from particular genome size categories (Fig. 1) and was further strengthened by the occasional co-occurrence of strains with different genome size at the sampled localities. The RDA on a dataset consisting of 19 database-derived climatic variables and geographical latitude characterizing the *Synura* sampling sites provided a more comprehensive assessment (Supplementary data Fig. S1). The non-significant effect of genome size in the RDA indicated that the current spatial distribution of different genome size categories in *Synura* is not a result of large-scale environmental filtering. To our knowledge, our study was the first attempt to evaluate intraspecific genome size variation in protists in an ecogeographical context.

Conclusions

Genome size variation and its evolutionary consequences are highly understudied among protists, particularly on the intraspecific level, with nearly no data available. We present the most comprehensive intraspecific genome size screening conducted to date, revealing a gradient of continuous genome size variation among *S. petersenii* strains.

Even though we were unable to identify the main evolutionary mechanism responsible for genome size variation in this species, it probably operates via gradual changes in genome size which are accompanied by changes in genomic GC content. We hypothesize that proliferation of TEs and multiplication of larger genomic segments or even whole chromosomes are the most likely scenarios. Interestingly, the genome size variability was reflected in cell size and relative growth rate but not in distinct ecogeographical distribution of strains. Occasionally, we even detected strains with different genome size coexisting at the same locality. Whether these strains are associated with reproductive barriers (suggesting cryptic diversity within *S. petersenii*) remained unresolved, though prevailing clonal reproduction of the species could substantially contribute to the maintenance of local genome size diversity even in their absence.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: collection details for *Synura petersenii* strains used in this study. Table S2: genomic and physiological parameters recorded on investigated strains of *Synura petersenii*. Figure S1: redundancy analyses testing associations between genomic parameters and climatic conditions across the collection sites.

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