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PII: S1434-4610(14)00081-9
DOI: http://dx.doi.org/doi:10.1016/j.protis.2014.08.001
Reference: PROTIS 25452

To appear in:

Received date: 4-1-2014
Revised date: 7-8-2014
Accepted date: 8-8-2014

Please cite this article as: Duleba, M., Ector, L., Horváth, Z., Kiss, K.T., Molnár, L.F., Pohner, Z., Szilágyi, Z., Tóth, B., Vad, C.F., Várbíró, G., Ács, É.,Biogeography and Phylogenetic Position of a Warm-stenotherm Centric Diatom, Skeletonema potamos (C.I. Weber) Hasle and its Long-term Dynamics in the River Danube, *Protist* (2014), http://dx.doi.org/10.1016/j.protis.2014.08.001

ORIGINAL PAPER

Biogeography and Phylogenetic Position of a Warm-stenotherm Centric Diatom, *Skeletonema potamos* (C.I. Weber) Hasle and its Long-term Dynamics in the River Danube

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Submitted January 4, 2014; Accepted August 8, 2014

Monitoring Editor: Marina Montresor

Running title: Phylogeny and Ecology of *Skeletonema potamos*

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Skeletonema potamos **is a poorly known freshwater species in the ancestrally and predominantly marine genus *Skeletonema*. With phylogenetic analysis of two nuclear {partial *SSU* (18S) and partial *LSU* (28S) rDNA)} and two chloroplast (*rbc*L and *psb*C) genes, we verified its placement within the genus *Skeletonema* and identified the mostly brackish species, *Skeletonema subsalsum*, as its closest known relative. Comparisons of *SSU* and *LSU* rRNA genes from *S. potamos* populations from Europe and North America revealed no intraspecific variation. *Skeletonema potamos* can be a dominant element of the phytoplankton community in various ecosystems, including the River Danube. We tracked phytoplankton composition in the River Danube weekly from 1979 to 2 012, and throughout this period, *S. potamos* exhibited a strong increase in proportion of total phytoplankton abundance and biomass – an increase that was correlated with increasing water temperature over the same time period. Current records indicate a temperate distribution of *S. potamos*, but ecological data predict possible expansion of its geographic range and increase in seasonal duration within existing habitats in response to the warming of surface waters.**

Key words: biogeography; ecology; phylogeny; global warming; *Skeletonema potamos.*

Introduction

The classic taxonomy of diatoms is morphology-based using features of their characteristic silica cell wall called frustule (e.g. Ettl et al. 1986). Medlin et al. (1988) introduced molecular methods into diatom taxonomy. Based on molecular, morphological and cytological results, Medlin and Kaczmarska (2004) proposed major clades of diatoms including Mediophyceae for bipolar centrics and the Thalassiosirales. Phylogenetic relationships of this latter order were reconstructed by Alverson et al. (2007).

Recently, morphological studies are supplemented by molecular investigations on various *Skeletonema* species (Alverson and Kolnick 2005; Kooistra et al. 2008; Sarno et al. 2005, 2007). Medlin et al. (1988) determined the phylogenetic status of the marine diatom *Skeletonema costatum* among the eukaryotes based on 16S-like (=18S) rDNA. Molecular and/or morphological studies investigated *S. costatum or S. costatum*-like diatoms and revealed new species, such as *S. pseudocostatum* (Medlin et al. 1991), *S. grevillei* (Zingone et al. 2005), *S. dohrnii, S. grethae, S. japonicum, S. marinoi* (Sarno et al. 2005) and *S. ardens* (Sarno et al. 2007). *Skeletonema* was found to be monophyletic and ancestrally a marine genus (Alverson et al. 2007). There are only two non-marine species in this genus: *S. subsalsum* occurring mainly in saline and brackish waters and occasionally in freshwater habitats (Aké Castillo et al. 1995; Gibson et al. 1993; Hasle Evensen 1975; Hustedt 1957) and *S. potamos* that is recorded from freshwater and slightly brackish habitats (Kiss et al. 2012). This latter species was not involved in molecular investigations previously.

Microsiphonia potamos C.I. Weber was first collected in 1966 from the Little Miami River, Cincinatti, Ohio and described by Weber (1970). New material from the Little Miami River, 25 May 1973, was observed by Hasle and Evensen (1976) and as they found this species highly similar to species of the genus *Skeletonema*, suggested its transfer to this genus

as *Skeletonema potamos* (C.I. Weber) Hasle in Hasle & Evensen; they found the same taxon in the liquid-preserved sample from the Jensensee, Plön (Germany), 19 August 1922, Hustedt Collection E 4555, which Hustedt (1928) used when preparing the description of the diatom to which he applied erroneously the name *Stephanodiscus subsalsus* (Cleve-Euler) Hustedt. *Skeletonema potamos* is a broadly distributed species (Supplementary Material Table S1) and is considered invasive in several regions, including the Great Lakes of the United States (Mills et al.1993), the River Loire in France (Descy et al. 2012), and the River Elbe in the Czech Republic (Desortová et al. 2011). Investigations of phytoplankton in the River Danube at Göd have been carried out with weekly sampling frequency for more than thirty years. Diatoms in the order Thalassiosirales were among the most abundant in the phytoplankton in this system, with *Skeletonema potamos* (C.I. Weber) Hasle and various *Stephanodiscus* species among the dominant taxa (Kiss 1985; Verasztó et al. 2010). *Skeletonema potamos* was first recorded in the Danube in the late 1950s (Kiss 1986) and subsequently became abundant in the Hungarian stretch of the river by the end of the 1960s (Kiss et al. 1994).

Climate-associated warming has been shown in several freshwater ecosystems, including both lakes (e.g. Lake Baikal, Hampton et al. 2008) and rivers (e.g. the River Loire, Floury et al. 2012). Increased temperature can have cascading ecosystem effects resulting in shifts in the community composition. Sensitive species are replaced by more tolerant species that change the interactions in the community (Anneville et al. 2007; Sommer et al. 2012). As primary producers, phytoplankton plays an important role in the biochemical cycling of both carbon and oxygen and is, therefore, an important regulator of global climate (Winder and Sommer 2012). Climate-mediated changes in phytoplankton communities have the potential to alter ecosystem functioning on both local and global scales. Sommer et al. (2012) summarised known responses of phytoplankton including changes in community biomass and biodiversity, range shifts and alterations in seasonality. One of the best known examples is the

spread of *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju , a cyanobacterium with a tropical origin, which expanded its original range towards the temperate zone both on the northern and the southern hemisphere, where it is now considered invasive in many lakes (Ryan and Hamilton 2003; Stüken et al. 2006). Northward expansion of other thermophilic phytoplankton species can be also enhanced by climate change, such as mild winters that enabled the permanent establishment of some diatom (e.g. *Rhizosolenia indica*) and dinoflagellate (e.g. *Alexadrium minutum*) species in the North Sea and the German Bight (Nehring 1998). Warming can also enhance the frequency of harmful algal blooms (Paerl and Paul 2012).

In the case of the River Danube, Sipkay et al. (2009) predicted a global warming-related increase in total phytoplankton abundance and large inter-annual differences by the end of the century. According to Sipkay et al. (2012), a linear temperature rise leads to drastic changes in phytoplankton biomass only in case of high nutrient load.

We had two major aims in our study. First, we aimed to extend the knowledge on the taxonomy of *S. potamos* and clarify the taxonomic status of the species. To achieve this, we applied both morphological and molecular analyses. Our second aim was to study ecological features of *S. potamos*. We collected all available information on the distribution of *S. potamos*. Based on long-term data (for more than three decades), we aimed to find relationships with possible environmental drivers which enabled this species to become the dominant member of the phytoplankton of River Danube in warm-water periods.

Results

Morphology of *Skeletonema potamos*

Skeletonema potamos is one of the smallest centric diatoms in the River Danube, and its thin frustule and small chloroplasts make it easy to be overlooked (Fig. 1A). It commonly occurs

in chains comprising 2–4 cells, but both single cells and longer chains (6–10 cells) are also somewhat common. Within chains, cells are connected by marginal fultoportulae (Fig. 1B, C) that are barely visible in untreated samples under the light microscope (Fig. 1A). Cell diameter ranges from 3.0 to 6.5 µm (average: 4.5 µm), and the pervalvar axis ranges from 5 to 18 µm (average: 10 µm) in length. The valve face is flat in the centre and slightly rounded near the valve mantle (Fig. 1F). There are fine radial striae on the valve face, consisting of irregular polygonal areolae and branched (2–3 times) interfascicles (Fig. 1D, E). A ring of 4–8 marginal fultoportulae is situated at the valve margin (Fig. 1F, G). The fultoportulae are tubular externally, the cleft at the distal tip and their silica-wall is relatively thick like the valve face with the rib-like elevations extending from the valve surface onto the fultoportulae (Fig. 1E, F, H). At the bases of fultoportulae, external pores cannot be found (a difference compared to *S. subsalsum*). A single rimoportula is visible close to the ring of marginal fultoportulae (Fig. 1F), it can be in a different position in terminal and intercalary valves (similarly to some other *Skeletonema* species). The structure of the valve mantle is the same as that of the valve face with irregular polygonal areolae (Fig. 1D, F). Numerous bands compose the thin girdle. The valve is more heavily silicified than the girdle.

Specificity of Primers and Phylogenetic Analyses of *Skeletonema potamos*

We were able to acquire SSU and LSU rDNA from the River Danube and River Tisza samples, as well as the Missouri River culture. However, plastid genes could be sequenced only from the isolated cells of River Danube and from the Missouri River culture.

Our *Skeletonema*-specific SSU and LSU rDNA primers successfully isolated *S. potamos* sequences from the River Tisza community DNA. The plastid primers were, however, more conserved and therefore not limited to members of *Skeletonema*, so we could only amplify these genes from clonal culture from the River Missouri and isolated cells from the River

Danube. The nuclear SSU rDNA (Supplementary Material Figs S1, S3) and LSU (Supplementary Material Figs S1, S4) sequences were identical among the Missouri culture, Danube and Tisza populations. Moreover, these nuclear rDNA sequences were identical to those of a brackish culture from Japan (GenBank AB728775 and AB728772) identified as *S. subsalsum*. One nucleotide substitution in the *rbc*L gene separated the Danube population and Missouri River culture. This substitution was in the third position of the codon. Phylogenetic analysis of the single-gene (data not shown), the combined two-gene (18S rDNA+28S rDNA and *rbc*L+*psb*C, Supplementary Material Figs S1, S2) and the four-gene dataset (Fig. 2) placed *S. potamos* well within the genus *Skeletonema* supporting the monophyly of this genus and identified *S. subsalsum* as its closest relative. *Skeletonema potamos* therefore represents a recent freshwater coloniser within Thalassiosirales.

Based on the plastid genes, *S. potamos* and *S. subsalsum* formed a lineage distinct from other species within the genus (Supplementary Material Fig. S1). Since 18S and 28S rDNA sequences are available from more *Skeletonema* species than *rbc*L or *psb*C, we were able to investigate the phylogenetic position of *S. potamos* compared to more *Skeletonema* taxa based on the ribosomal genes than on the plastid genes. *S. costatum rbc*L and *psb*C sequences were involved in just the single-gene analyses (data not shown), because these plastid gene sequences are available from different strains. According to both the LSU and SSU rDNA, the *S. potamos*-*S. subsalsum* lineage also involved *S. costatum*, however, the position of this lineage compared to other *Skeletonema* species differed between the 18S and 28S rDNA-phylogeny (Supplementary Material Fig. S2).

In most *Skeletonema* species, intraspecific variability was observed at least in the SSU rRNA gene (this phenomenon occurred also in both ribosomal genes in *S. marinoi-dohrnii, S. menzelii, S. grethae, S. tropicum, S. costatum*; it have to be mentioned that only one SSU

rDNA sequence was available for *S. grevillei*). Invariability in both genes was observed only in *S. potamos* and *S. ardens* (Supplementary Material Figs S3, S4).

Geographical Distribution of *Skeletonema potamos*

Skeletonema potamos is broadly distributed across the temperate zone (Fig. 3). The species is most common in Europe, mainly in large rivers and their reservoirs, slowly flowing tributaries and connected lakes (altogether 91 water bodies). In North America, *S. potamos* was found in some lakes and rivers flowing into the Atlantic and Pacific Oceans and in their estuaries (altogether 61 water bodies). It was detected in South America (Brazil, Argentina) in rivers and lagoons along the coastal region of Atlantic Ocean (altogether 5 water bodies). In Asia it has been found in the River Ob (Russia), Lake Hovsgol (Mongolia), in a channel at Xaimen (China) and it has four occurrences in Japan. From Australia, it was recorded in three water bodies.

Long-term Change of *Skeletonema potamos* and its Response to Environmental Change in the River Danube

Since the abundance and biomass of *S. potamos* showed seasonal peaks in the warmer period (May-October) in the River Danube, we used these data from the years 1979-2012 to investigate environmental factors that affect the quantity of this diatom. The data presented in the following are also referring to this warmer period. The proportion of *S. potamos* in the total phytoplankton abundance (Fig. 4A; R^2=0.035, p<0.001) and biomass (Fig. 4B; R^2= 0. 017, p<0. 0374) showed a significant increase in the last thirty years. Mean values of relative abundance increased from 5.2 to 23.6 %, and its relative biomass from 11.3 to 35.1% from 1979 to 2009. In 2009, its proportion reached higher values (abundance: 87.4; biomass: 91.4%) than at any time in the preceding 30 years. Temperature was the only significant

predictor in the linear mixed-effects model based on relative abundance of *S. potamos* (slope: 0.15, $p<0.001$), while in the case of carbon biomass, temperature (slope: 0.13, $p<0.001$) and water discharge (slope: -0.04, $p<0.01$) were significant predictors. . Water temperature in the River Danube increased during the >30-year period of 1979–2012 (Table 3, Fig. 4C, $R^2=0.023$, $p<0.001$). The mean temperature of May-October ranged from approximately 14.9 to 17.7 ^{o}C until the late 1990s, but temperature did not drop below 16 ^{o}C and often exceeded 18 ^{o}C from 2000 to 2012. Maximum values within a year varied between 19.6–24 ^{o}C during the period of 1979–1999, and between 22–27 ^{o}C during 2000–2012. Years with high and low water discharge alternated in the period of 1979–2012, with a clear decrease in annual mean water discharge from 2003 to 2012 compared to the period of 1979-1999 (Fig. 4D).

Discussion

Morphology of *Skeletonema potamos*

Skeletonema potamos usually occurs in chains comprising 2–4 cells in the River Danube. The length of chains may depend on turbulence of the river; in low water periods when the current is low (turbulence, too) chains of 6–10 cells are much frequent than in high water periods when the current is higher and water is much more turbulent. We investigated the morphological features of *Skeletonema potamos* populations in the River Danube and River Tisza and compared them to *S. subsalsum*. *S. potamos* has 4–8 tubular fultoportulae with cleft tips, without a pore at the bases of them (identical with EM micrographs of Cavalcante et al. 2013), whereas *S. subsalsum* has 7–14 flat, flat-bifurcated, or flat spoon-like marginal fultoportulae with an external pore on the base (Hasle and Evensen 1975; Kiss et al. 2012). Considering these, *Skeletonema potamos* and *S. subsalsum* are clearly distinguishable morphologically based on the presence or absence of an external pore on the base of marginal fultoportulae. The shape and position of the single rimoportula is hardly seen on EM

micrographs. It can be a little bit better seen by TEM if the position of the valve face is optimal (Fig. 1 B, D arrow). It is difficult to find the small external pore of the rimoportula on SEM micrographs.

Phylogenetic Position of *Skeletonema potamos*

Phylogenetic analysis resolved *S. potamos* within the genus *Skeletonema* (Fig. 2, Supplementary Material Figs S1-S4), supporting the transfer of *Microsiphonia potamos* into *Skeletonema* by Hasle and Evensen (1976). Phylogenetic trees also revealed *S. subsalsum* to be the closest known relative of *S. potamos*.

Skeletonema is an ancestrally marine genus (Alverson et al. 2007). *Skeletonema subsalsum* is euryhaline, occurring mainly in saline and brackish waters and occasionally in freshwater habitats (Aké Castillo et al. 1995; Gibson et al. 1993; Hasle Evensen 1975; Hustedt 1957). *Skeletonema potamos* is somewhat more specialised, being restricted to freshwater and slightly brackish habitats (Kiss et al. 2012). Thus, *S. potamos* represents another recent, phylogenetically restricted marine-to-freshwater transition within Thalassiosirales (Alverson et al. 2007).

Unlike previous population-level studies of other *Skeletonema* species that revealed sometimes extensive intraspecific variation at the DNA level (Sarno et al. 2005, 2007, even intragenomic variation as in Alverson and Kolnick 2005), we found little or no sequence variation in populations from Central Europe, the continental United States, and very likely Japan. While the nuclear rDNA sequences from our Hungarian samples and the U.S. culture were identical to one from a brackish culture strain from Japan (Yamada et al. 2013) identified as *Skeletonema subsalsum* (GenBank AB728775 and AB728772), and because *Skeletonema potamos* and *S. subsalsum* are clearly distinguishable morphologically, we

assume that the sequence from the above mentioned Japanese strain could belong to *S. potamos*.

Small and large subunit ribosomal RNA genes are variable within other *Skeletonema* species (Alverson and Kolnick 2005; Sarno et al. 2005, 2007), suggesting relatively recent dispersal of *S. potamos* across the globe. More variable genetic markers and increased population sampling will shed more light on its place of origin and pattern and timing of dispersal.

Geographical Distribution of *Skeletonema potamos*

Krammer and Lange-Bertalot (1991) regarded *Skeletonema potamos* as a relatively rare species. However, more than 160 localities have been published by now. Its distribution is restricted to the temperate zone. The only exception is a recent record in a tropical shallow channel near Itabuna (Brazil) (Cavalcante et al. 2013).

Data compiled from multiple localities show that *S. potamos* occurs in waters ranging from 10 to 29 ^{o}C (see References and Supplementary Material: references). This may represent the full temperature-tolerance range for *S. potamos*. It may survive the unfavourable periods with too low or too high water temperature by forming resting stages (like many other microalgae, McQuoid and Hobson 1995).

Relationship Between *Skeletonema potamos* and Environmental Shifts in the River Danube

The relative abundance and biomass of *S. potamos* exhibited long-term increases in the River Danube and were positively correlated with water temperature over the same period. Moreover, only temperature had a long-term effect on the relative abundance of *S. potamos*. Kiss et al. (1994) demonstrated that seasonal dynamics of *S. potamos* followed the changes of

the water temperature. They classified *S. potamos* as a warm stenotherm species based on a two-year intensive study in the River Danube, which showed that water temperature was one of the main factors influencing the abundance of *S. potamos*. They observed that population growth started in June, with temperature around 15 ^{o}C, and decreased in September–October when temperature was below 14–16 ^{o}C. A similar trend was found in the Little Miami River (USA, Weber 1970) and the River Rott (Germany, Chang and Steinberg 1988). Lehman (2000) found that *S. potamos* commonly occurs in the southern Delta of San Francisco Bay Estuary during summer, when salinity is high from discharge of agricultural return water and the seasonal peak in water temperature is increased by long residence time. Kaeriyama et al. (2011) showed that the occurrence of seven *Skeletonema* species was mainly affected by water temperature and less by irradiance in Dokai Bay (Japan). Under experimental conditions, *Skeletonema* species were able to grow at temperatures ranging from 15 to 25 ^{o}C (Kaeriyama et al. 2011).

Effect of global warming was observed also on other Thalassiosirales species in the River Danube. A shift in the timing of the end-winter centric bloom, in which *Stephanodiscus minutulus* (Kützing) Cleve et Möller is one of the dominant species was reported (Kiss and Genkal 1997; Kiss 2000). It usually occurs 3-4 weeks earlier in the 2000s than in the 1980s. Several papers predict earlier maxima in algal biomass related to climate change, which is generally accompanied by biomass increase (Flanagan et al. 2003; Sipkay et al. 2012), especially in spring (Dokulil et al. 2010; Nõges et al 2010; Sipkay et al. 2009; Thackeray et al. 2008).

S. potamos also occurs in the River Tisza in the region, where it is exposed to similar climatic effects as in the River Danube. One might therefore expect *S. potamos* to be similarly abundant in both rivers. However, the high light demand of *S. potamos* might limit its abundance in the River Tisza, which has higher overall levels of suspended matter throughout

the year compared to the River Danube (Istvánovics et al. 2010). This supposition needs more study, though relationship between light and *S. potamos* abundance had been shown in other aquatic ecosystems. In the Paraná River System, a positive correlation between the abundance and biovolume of the functional group D (containing *S. potamos*) with Secchi transparency was found, which is influenced by water currents through transported suspended material (Devercelli 2006; Devercelli and O'Farrell 2013). Torgan et al. (2009) supposed that the main factors influencing the development of *S. potamos* populations are probably light and/or competition with other chain-forming centric diatoms (*Aulacoseira granulata* and *A. ambigua*) whose species were abundant in the phytoplankton of the Patos lagoon (Brazil). The competition of these *Aulacoseira* taxa is not relevant in the River Danube and Tisza, because they are much less abundant here.

In our earlier studies (Kiss et al. 1994; Sipkay et al. 2012; Verrasztó et al. 2010) we clearly demonstrated that there is an intra-annual effect of water discharge of the River Danube on the quantity of *S. potamos*. In our present study, we focused on long-term changes and found that water discharge was not significantly related to the relative abundance of the species. However, it was significant in the case of relative carbon biomass. The River Danube exhibited a gradual decrease in mean water discharge during the last decades (Fig.4D), parallel to the increase in *S. potamos* dominance. While this trend can have indirect positive influences on the quantity of *S. potamos*, (e.g. via more favourable light conditions), we suggest that its effect is secondary as water temperature was clearly the predominant factor in explaining its dominance (i.e. being significant or showing stronger effect in the linear mixed-effects models).

According to our results, *S. potamos* undoubtedly became the dominant member of phytoplankton of River Danube in warm water period and its seasonal dominance was also observed in other freshwater lakes (e.g. Postmünster Lake, Chang and Steinberg 1988), rivers

(e.g. River Rhine, Friedrich and Pohlman 2009; Ibelings et al. 1998) and estuaries (e.g. Chesapeke Bay, Marshall and Egerton 2009). The consequences of this shift are hard to predict because of the lack of knowledge on the ecology of this species. However, *Skeletonema* spp. are known to be bloom-forming taxa, sometimes contributing to close to monodominant phytoplankton communities (Borkman and Smayda 2009; Rost et al. 2003) due to their high growth potential when silica is not limiting, which can make them superior competitors over other microalgae (Egge and Aksnes 1992). Moreover, *Skeletonema* species were found to negatively affect zooplankton either via mechanical interference with the filtering process of cladocerans (Müller-Solger et al. 2002) or by suppressing hatching success of eggs in some of the copepod consumers (Ban et al. 1997; Miralto et al. 1999). Considering these, the dominance of *S. potamos* in the phytoplankton may have serious consequences on the trophic web, and hence, energy flow in its habitats. Therefore, further studies on its position in nutrient cycling are mandatory. Especially because the species seems to be favoured by the warming of surface waters and can accordingly become dominant in its present habitats and its further expansion can also be expected.

Conclusions

Skeletonema potamos is a species with temperate zone distribution (Fig. 1), where it occurs in a wide variety of low-salinity (mostly freshwater) habitats. *Skeletonema subsalsum*, occurring mainly in brackish waters, proved to be the closest relative of *S. potamos* and marine *Skeletonema* species were more distant relatives. We found little or no sequence variation in *S. potamos* populations from Central Europe, the continental United States, and very likely Japan. Small and large subunit ribosomal RNA genes are variable within other *Skeletonema* species (Alverson and Kolnick 2005; Sarno et al. 2005, 2007), suggesting relatively recent

dispersal of *S. potamos* across the globe. Previous studies reported it as a warm stenotherm species (Devercelli and O'Farrell 2013; Kiss et al. 1994), which is consistent with our 30-year dataset from the River Danube, showing that the species is present from late spring until autumn in the temperate zone (Belcher and Swale 1978; Chang and Steinberg 1988; Kiss and Nausch 1988; Steinberg et al. 1987). Moreover, its proportion in total phytoplankton abundance and biomass increased during our long-term investigation in the River Danube, parallel with a gradual increase of water temperature. In the light of these data, we predict that the geographic and/or seasonal range of *S. potamos* will expand with the warming of surface waters in response to global climate change.

Methods

Environmental sampling and analysis: Since 1979, weekly samples for regular phytoplankton analyses were taken from the River Danube at Göd (riv. km 1669), Central Hungary. Half-litre dipped samples were taken from the main current, 20–30 cm below the water surface and preserved in Lugol's iodine solution. Quantitative investigations were made by inverted microscope (Olympus IX70 and Opton invertoscope D) according to the Utermöhl (1958) method.

To establish the biomass (wet weight) in each sample, the diameter (d) and the length (l) of the pervalvar axis of *S. potamos* specimens (n=50) were measured (to calculate the cell volume: $r2*\pi*l$; r=d/2) and multipled by cell number (Utermöhl 1958). In samples containing less than 1000 ind. mL^{-1}, fewer than 50 specimens were measured. The biovolume was converted into carbon content according to Menden-Deuer and Lessard (2000).

Environmental parameters: Samples were taken from the upper 20 cm of the water. Water temperature (T) was measured *in situ* with a WTW multiline portable meter. Total

suspended solids (TSS) concentration was determined gravimetrically (Eaton et al. 2005). The pore size of the membrane filter was 0.45 μm. Ammonium (NH_4^+) was measured according to the ISO 7150-1:1984. Nitrate (NO_3^-) was measured by the sodium salicylate method (Vijayasarathy 2011). Orthophosphate (SRP) was measured according to the Eaton et al. (2005) by the ammonium molybdate method. Chemical oxygen demand (CODps) was determined with the acidic potassium permanganate method (ISO 8467 1993). Chlorophyll-*a* concentration (chl-*a*) was determined spectrophotometrically, after extraction with hot methanol (Iwamura et al. 1970). Water discharge (WD) data were provided by the General Directorate of Water Management.

Morphological analysis of *Skeletonema potamos*: The samples taken from the River Danube (in parallel with quantitative phytoplankton analysis) were fixed with formaldehyde, and treated with cold H_2O_2 and HCl (CEN 2003), washed in distilled water, filtered through a 3 μm-mesh polycarbonate membrane, fixed on SEM stubs, coated with gold-palladium and investigated with a Hitachi S-2600N scanning electron microscope. Subsamples of the treated material were also used for TEM analysis; a TESLA BS500 transmission electron microscope was used for this purpose.

DNA analysis: For DNA analyses, living samples were collected from three locations: (1) the River Danube at Göd, Hungary (47°40'51"N 19°7'33"E), in June 2011, (2) the River Tisza at Tiszaújváros, Hungary (47°54'32"N 21°4'45"E), in August 2012.

From the River Danube, *S. potamos* cells (around 20 two-celled chains) were isolated using micropipette under inverted microscope (Olympus IX70) and transferred into sterile 100 μL TE buffer (10mM Tris, 1 mM Na_2-EDTA, pH=8). Taking into account the advantage derived from the fragility of the weakly-silicified frustules of *Skeletonema,* the isolated cells were only centrifuged at 20,000 *g* for 5 min that could release DNA from the cells. 50 μL pellet was used for the polymerase chain reaction. Because of the low number of the cells and

the consequently low concentration of extracted DNA, polymerase chain reaction (PCR) was applied in seminested design or a second PCR was performed with the same primers.

Skeletonema potamos was rare in the River Tisza samples, so total community DNA was extracted from a field sample. The sample was mixed with lysis buffer (10mM Tris, 1 mM Na_2-EDTA, 200mM NaCl, 0.02% SDS, pH=8), frozen at –20 °C for 30 min, heated at 95 °C for 10 min, then centrifuged at 14000g for 10 min. Proteins were digested with Proteinase K (recombinant, Fermentas) at 56 °C for 3 h. DNA was then isolated using the DNeasy® Plant Mini Kit (Qiagen).

Publicly available *Skeletonema* sequences were used to design primers for PCR amplification and sequencing of nuclear SSU and partial LSU ribosomal DNA genes as well as plastid *rbc*L and *psb*C genes (Table 1). Priming sites were determined using NCBI Primer-BLAST (Ye et al. 2012), and theoretical melting temperature and the possibility of dimer formation was explored using Integrated DNA Technologies Oligo Analyzer.

The four genes were amplified and sequenced with primers listed in Table 1. For PCR design see Supplementary table 2. For 28S rDNA and *psb*C of the Danube sample, the PCR reaction mixture contained 200 mM of each deoxynucleoside triphosphate (Fermentas), 1 U of Taq DNA Polymerase (Fermentas, Vilnius, Lithuania), 1X Taq buffer with $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.325 μM of each primer, 0.1 mg/μL BSA (Fermentas) and 1-3 μL template in a total volume of 25 μL. For all other reactions the mixture contained 1.25 U DreamTaq™ DNA Polymerase (Thermo Scientific), 200 mM of each deoxynucleoside triphosphate (Fermentas), 1X DreamTaq Buffer (Thermo Scientific), 0.325 μM of each primer, 1-3 μL template in a total volume of 25 μL. PCR amplifications used the following cycles: initial denaturation at 98 °C for 3 min, 32 cycles at 94 °C for 1 min, 52–63 °C (the exact annealing temperature for each primer pair was determined by gradient PCR, see Supplementary Material Table S2) for 30 sec, 72 °C for 1.5 min, and a final

extension at 72 ^{o}C for 10 min. Sequencing reactions and capillary electrophoreses were performed by Biomi Ltd. All nucleotide sequences are available from the DDBJ/EMBL/GenBank databases under accession numbers KF621297–KF621302. Sequences were compared to those ones (accession numbers KJ081744–KJ081747) that were provided by Andrew J. Alverson. These were acquired from a sample taken from Missouri River, United States (38^{o}46'42"N 90^{o}28'48"W) in 2012.

Phylogenetic analysis: The final sequences were assembled from overlapping sequence fragments. Sequences were downloaded from GenBank from the studies of Alverson and Kolnick (2005), Sarno et al. (2005, 2007), Alverson et al. (2007), Kooistra et al. (2008), Alverson (2014) and one 18S rDNA (accession number AB728775.1, Yamada et al. unpublished) and one 28S rDNA sequence (accession number AB728772.1, Yamada et al. (2013). For all accession numbers see Supplementary Table S3 and Supplementary Material Figs S3, S4. Sequences were aligned with ClustalW implemented in MEGA 5.05 (Tamura et al. 2011). "Find best DNA models" option in this software was used to determinate the most appropriate model for DNA sequence evolution of each gene partition (models are summarized in Table 2). Bayesian analyses were run on datasets individually and in combination. Posterior probability of distribution was estimated using Metropolis-coupled Markov Chain Monte Carlo (MCMC) as implemented in MrBayes 3.2 (Ronquist et al. 2012). Default priors were used for all analyses.

Statistical analyses: To investigate the role of local environmental effects in the River Danube, we used only warmer period (May–October) data, because the occurrence of *S. potamos* is restricted to this period in the River Danube. As total phytoplankton abundance and biomass showed a decreasing trend in the last decades (Verasztó et al. 2010) and the same is true for the absolute abundance and biomass of *S. potamos*, we used the relative abundances of the species in our analysis. To normalise residuals, we transformed chlorophyll-*a* data by

cubic root and water discharge, TSS and NH_4^+ by square root. We built a PCA model for the trophic variables of NH_4^+, NO_3^-, SRP, CODps and chl-*a*. We used PCA 1 and PCA 2 axes in the subsequent analyses as proxies for trophic state and nutrient availability. We fitted linear mixed-effects models on relative *S. potamos* abundance and biomass in the package "nlme" in R (Pinheiro et al. 2013; R Development Core Team 2010), wherein water discharge, temperature, TSS, PCA 1 and PCA 2 axes were fixed effects, and year and month were random effects.

The world distribution map of *S. potamos* was prepared by using the ESRI ArcInfo 9.3 GIS program based on sites listed in Supplementary Material Table S1.

Acknowledgements

We thank Angéla Földi for helping in the preparation of the distribution map, Andrew J. Alverson for providing sequences from the Missouri, Bánk Beszteri for his valuable advice on the work. The authors also thank two anonymous reviewers for their useful comments on the manuscript. This study was supported by the Hungarian National Science Foundation (KTIA-OTKA 80140).

Appendix A. Supplementary Data

Supplementary data associated with this article.

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Table 1. Primers used in this study. [1] Primers used in the first PCR. [2] Primers used in the nested PCR. [s] Primers used as sequencing primers.

Name	Marker	Sequence (5' to 3')	Original reference
Sk-50F[1]	18S rRNA gene	CATGTGTAAGTATAAGATACTT	This study
Sk-600F[2,s]	18S rRNA gene	AAATCCCTTATCGAGTATCA	This study
Sk-900F[s]	18S rRNA gene	TTGGTTTGCGAGTCAAAGTA	This study
Sk-1550R[1,2,s]	18S rRNA gene	TCTCGGCCAAGGTTATAT	This study
Sk-1850R[1,2,s]	18S rRNA gene	TACGGAAACCTTGTTACGACTTCA	This study
Sk-28S-15F[1]	28S rRNA gene	CTAGATTTGGTAGGTGCACTT	This study
Sk-28S-120F[2,s]	28S rRNA gene	CCGGAATGAATGTACCTCATCTAT	This study
Sk-28S-860R[s]	28S rRNA gene	CTGTTACTTTCATTACGCATATCAGT	This study
Sk-28S-1250R[1,2,s]	28S rRNA gene	AACCTTCATTCGACGCCAG	This study
psbC_F[1]	*psb*C	ACAGGTTTCGCTTGGTGGAGTGG	Alverson et al. (2007), modified
Sk-psbC90F[2,s]	*psb*C	TTTTGGGCTGGTGCAATGATCTT	This study
Sk-psbC890R[s]	*psb*C	TTGCACCTAAACGTTGATCACG	This study
psbC_R[1,2,s]	*psb*C	CACGACCAGAATGCCACCAGT	Alverson et al. (2007), modified
rbcL66F[1,s]	*rbc*L	TTAAGGAGAAATAAATGTCTCAATCTG	Alverson et al. (2007)
Sk-rbcL90F[1,2,s]	*rbc*L	TGTGATTTATTTGAAGAAGCTT	This study
Sk-rbcL400F[s]	*rbc*L	ATTAACTCTCAACCATTCATGC	This study
Sk-rbcL975R[s]	*rbc*L	CAACATCATCACCTAAATAGTG	This study
Sk-rbcL1210R[1,2,s]	*rbc*L	GCTGTATCTGTAGAAGTATAGTCGA	This study
dp7R[1,s]	*rbc*L	AAGCAACCTTGTGTAAGTCTC	Daugbjerg and Andersen (1997), modified

Table 2. Substitution models suggested by MEGA5 Find best DNA/protein models function. T93 represents Tamura and Nei' 1993 model, K2 Kimura's two-parameter model, GTR the generalised time-reversible model (for more information on these substitution models see references listed in the third column). Letter G means gamma distributions among sites, letter I means that a proportion of invariable sites was assumed.

Marker	Clade	Substitution model	Reference for substitution model
18S rDNA	Thalassiosirales	T92+G+I	Tamura (1992)
28S rDNA	Thalassiosirales	TN93+G+I	Tamura and Nei (1993)
*rbc*L	Thalassiosirales	GTR+G+I	Tavaré (1986)
*psb*C	Thalassiosirales	GTR+G+I	Tavaré (1986)

Table 3. Long-term minimum and maximum values of May-October means of the measured environmental parameters. The corresponding years are given in parentheses.

	Minimum (year)	Maximum (year)
WD (m^3/L)	1375.88 (2011)	2880.41 (1999)
T (^oC)	14.88 (1984)	19.55 (2008)
chl-a (μg/L)	13.58 (2008)	88.52 (1986)
CODps (mg/L)	3.01 (2007)	17.70 (1979)
NH_3-NH_4-N (mg/L)	0.03 (2009)	0.42 (1992)
NO_3-N (mg/L)	1.28 (1992)	2.07 (1987)
PO_4 (μg/L)	8.20 (2005)	88.99 (1989)
TSS (mg/L)	11.72 (2003)	55.76 (1987)

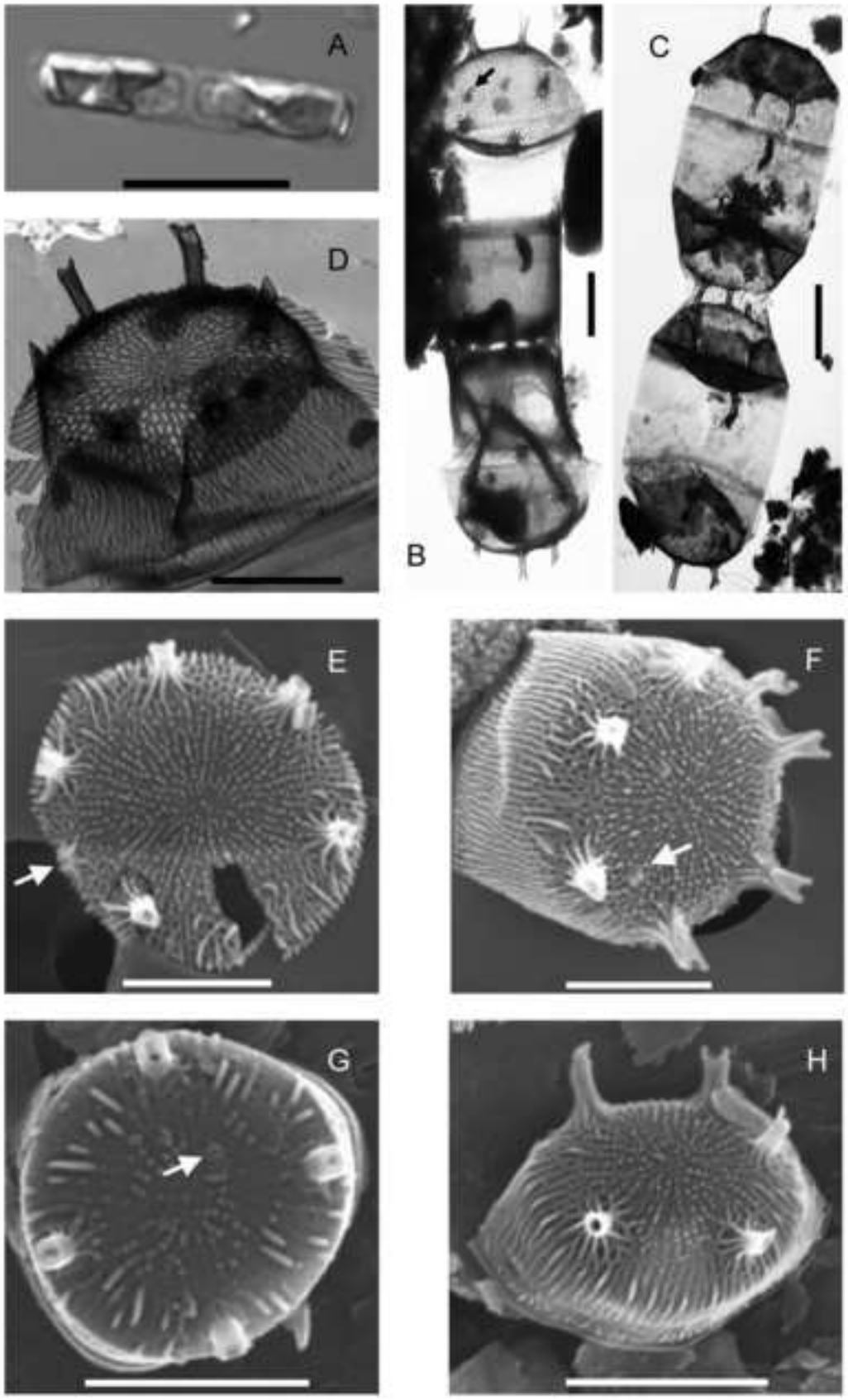
Figure legends

Figure 1. A: LM-, **B-D**: TEM-, E-H: SEM micrographs of *Skeletonema potamos* from River Danube (**A-F**) and from River Tisza (**G,H**); rimoportula arrowed; scale: 2 µm (B-G), 2.5 µm (H) and 10 µm (A).

Figure 2. Bayesian inferred phylogenetic tree based on 18S and 28S rDNA, *rbc*L, *psb*C sequences of species belonging to order Thalassiosirales. *Ditylum brightwellii*, *Bellerochea malleus and Lithiodesmium undulatum* are outgroups. Sequences investigated in this study are indicated by underlining. For substitution models see Table 2. Generic abbreviations are: *Bacterosira* (B.), *Bellerochea* (Be.), *Cyclostephanos* (Cs.), *Cyclotella* (Cy.), *Detonula* (D.), *Discostella* (Di.), *Ditylum* (Dt.), *Lauderia* (La.), *Lithodesmium* (Li.), *Minidiscus* (M.), *Porosira* (P.), *Shionodiscus* (Sh.), *Skeletonema* (Sk.), *Stephanodiscus* (S.), *Thalassiosira* (T.)

Figure 3. Map of *Skeletonema potamos* worldwide distribution (for coordinates and references see Supplementary Material Table S1 and Supplementary Material: references). Arrow indicates the type locality of Weber's (1970) description.

Figure 4. Box plot of long-term distribution in proportion of *Skeletonema potamos* in total phytoplankton abundance (**A**) and in total phytoplankton biomass (**B**), the mean water temperature (**C**) and mean water discharge (**D**) from May to October from 1979 to 2012. Water temperature data from the years 2004 and 2012; relative abundance and biomass data from the year 1999 are missing. The boxes represent 50% of the distribution of the values, the bottom of each boxes shows the first quartile, the top shows the third quartile, the black lines within the boxes show the median values, the dots show the outliers. Grey lines show the fitted linear models.



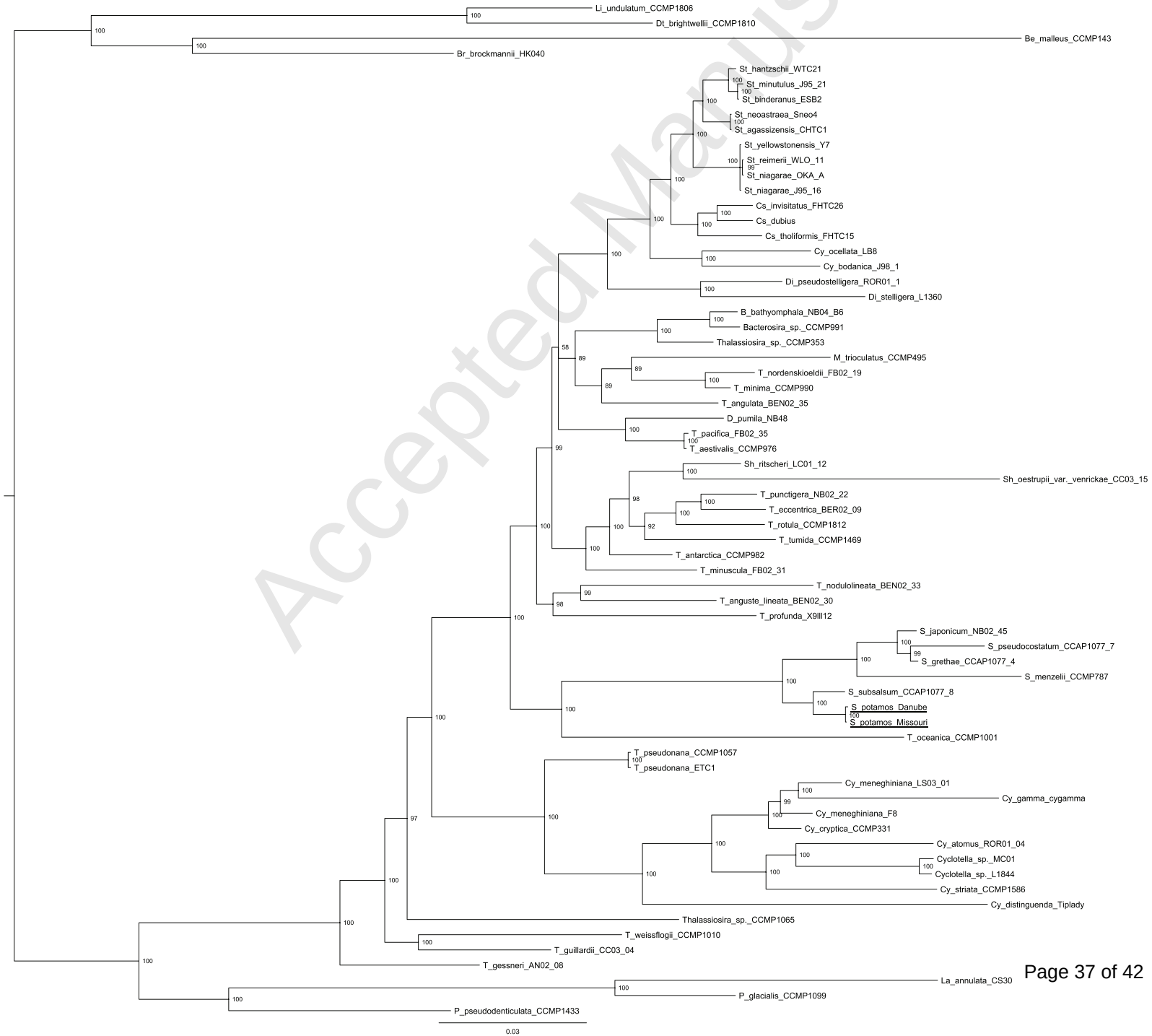


Figure 3