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Euglenophycin is produced in at least six species of euglenoid algae and six of seven strains of *Euglena sanguinea*

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Abstract

Euglena sanguinea is known to produce the alkaloid toxin euglenophycin and is known to cause fish kills and inhibit mammalian tissue and microalgal culture growth. An analysis of over 30 species of euglenoids for accumulation of euglenophycin identified six additional species producing the toxin; and six of the seven *E. sanguinea* strains produced the toxin. A phylogenetic assessment of these species confirmed most taxa were in the Euglenaceae, whereas synthesis capability apparently has been lost in the *Phacus, Eutreptiella, and Discoplastis* branches.

Keywords

algae; euglena; euglenophycin; harmful algal bloom; toxin

1. Introduction

At present, known freshwater toxin-producing algae include cyanobacteria (reviewed in Smith et al. 2008), haptophytes (Ulitzer and Shilo, 1966; Skelbred et al., 2011), Dinophyceans (Hashimoto et al., 1968; Rengefors and Legrand, 2001) and at least one species of Euglenophyceae(Zimba et al., 2010). The discovery of a new-to-science toxin

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produced by euglenoids was surprising: the species was first described in 1830, and while no previous reports have identified euglenoid toxins there is a single report of some fish mortality when co-cultured with *E. sanguinea* (Xavier et al., 1991).

The class Euglenophyceae contains a diverse assembly of photosynthetic and non-photosynthetic taxa (Buetow, 1968; Wolowski, 2002). Distinctive characteristics include the storage of carbohydrate as paramylon (Ciugulea and Triemer, 2010), which has β-1,3 linkages that differ from the alpha linkages found in higher plant starch, and motility in the water column with flagella and on and within freshwater and estuarine sediments via metaboly (Kingston, 1999 a, b; Arroyo et al. 2012). Identification of euglenoid taxa requires observation of the pellicle strip configuration, chloroplast shape, location, and number, presence and shape of pyrenoids, in addition to length/width and shape determination. Specific family-level differences include the absence of a cell wall and pigmentation, and a dietary requirement for vitamins (Buetow, 1968; Ciugulea and Triemer, 2010). Euglenoid occur throughout estuaries, with greatest abundance in temperate waters during Fall (Marshall et al., 2005, Manna et al. 2010).

Euglenophycin is very similar structurally to the alkaloid solenopsin, the venom found in fire ants (Fig. 1). Fire ant venom causes necrotic activity at the site of a sting (Adrouny et al., 1959), has hemolytic properties, is not degraded after 1 h at 100°C (Jouvenaz et al., 1972), inhibits the growth of a wide range of bacteria (Blum et al. 1958 1960), and is phytotoxic/inhibitory against bean leaves (Blum and Callahan, 1960). Zimba et al. (2004) reported fish mortalities in freshwater aquaculture systems, including catfish, striped bass, tilapia, and sheepshead from euglenophycin exposure. Euglenophycin inhibited tissue cell growth in mammalian cancerous cell lines; it also inhibitsphytoplankton growth (Wahome et al., 2015; Zimba et al., 2010, 2016). The toxin is stable for more than 30 days at -80C (Gutierrez et al., 2013) and is an inhibitor of enzymes associated with blood vessel formation (angiogenesis-VEGF, Ang-2) at millimolar concentrations (Zimba et al., 2016).

Strain variation in many traits, including toxin production and toxicity is evident in all cyanobacteria and algae (Rapala, 1998; Doblin et al., 2000; Burkholder and Glibert, 2006; Zapomapomêv et al., 2008; Bachvaroff et al., 2009; Skjelbred et al., 2011). Toxin variation in terms of production rates, potency of different isoforms of the toxin, and the effect of environmental conditions are perhaps best known in the cyanobacterium *Microcystis aeruginosa* (with both nontoxic and toxic strains that can produce 10-fold higher toxin concentrations under selected temperature, nutrient, or light conditions). There are over 90 isoforms of microcystin known, with toxin LD_{50} potencies differing by 6-fold (Chorus, 2001). Laboratory culture typically decreases secondary product formation, including off-flavor compounds (Zimba, pers. obs.) and toxin accumulation. Mutations or responses to antibiotic treatment may explain the loss of PSP toxin production in one *Alexandrium* strain (Martins et al., 2004), with culture conditions likely selecting away from toxin formation in unialgal culture.

This research evaluated whether multiple *E. sanguinea* strains and other species of photosynthetic euglenoids produced euglenophycin toxin. Our goal was to better understand whether toxin production in euglenoids is widespread amongst all families of euglenoids, or

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clustered within specific families. Toward assessing the biological importance of this toxin accumulation, cell counts of cultures were used to normalize toxin concentration. Multiple reaction monitoring (MRM) ratios of qualifier to quantifier ions and cell concentration of toxin were used as a conservative method to discern euglenophycin-producing strains and species.

2. Materials and Methods

E. sanguinea and other euglenoid clonal isolates (Table 1) were obtained from culture collections and other phycologists, with additional strains isolated from water samples collected by the authors, and from samples provided by aquaculture farms and water quality personnel; all cultures were grown in AF6 media (Andersen, 2005). Culture conditions included 22°C, and a 14:10 L:D cycle using 45 μmol PAR using “cool-white” and aquarium (far-red) fluorescent lights. Cultures were grown to mid-exponential phase and harvested by filtration (<15 psi) using 1.2 μm porosity GF/C filters (Whatman Corporation, Maidestone, England). Filters were stored at -80°C until toxin extraction (holding time <7 days). Samples used to quantify cells were preserved using buffered formalin (2% final v/v).

Cell counts were performed on a Wild M5 inverted microscope at 150-600× magnification as originally described by Utermöhl (1958). Prior to counting, samples were settled for >2 hr in chambers and then counted using ≥10 random fields and a minimum count of 200 cells as recommended by Venrick (1978). Culture purity was confirmed during cell counts and settling chambers were scanned prior to counting to assure settling and random distribution.

Euglenophycin was identified and quantified by liquid chromatography tandem mass spectrometry (LC-MSMS) using an Agilent 1200 series HPLC in line with an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Wilmington DE) equipped with Peak nitrogen generator #NM30LA (Peak Scientific, Inc. Billerica, MA). Samples were initially extracted using 80% MeOH for 6 hr at 4°C, clarified using 0.2 μm filters, then maintained at 8°C prior to 10 μL being injected via the autosampler onto a Luna C18(2) 150 × 3 mm, 3 μm particle size column (Phenomenex Corporation, Torrance, California) maintained at 40°C. The analytes were separated using a flow rate of 400 μL/min over a 17-minute gradient. Conditions were maintained at 10% mobile phase B (100% acetonitrile, 0.1% formic acid), 90% mobile phase A (90% water, 10% acetonitrile, 0.1% formic acid) for 2 minutes, then ramped from 10 to 90% B in 6 minutes, held at 90% B for 3 minutes, and returned to 10% B in 2 minutes, after which initial conditions were maintained for 4 minutes prior to the next injection. The triple quadrupole was operated with an electrospray ionization source in positive ion, MRM mode (Gutierrez et al. 2013). Euglenophycin was detected as the $[M+H-H_2O]^+$ ion at m/z 288.3. The product ions monitored upon collision-induced dissociation were m/z 97.2, 110.2 and 136.2. The most abundant product ion, 110.2, was used for quantitation.

Mass spectrometry data were analyzed with Agilent MassHunter Qualitative Analysis software (version B.03.01). For each sample, the peak areas of the product ions were determined using the MS/MS integrator option. The amount of euglenophycin in each sample was determined by comparing the peak area of the m/z 110.2 ion to a standard curve

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produced by the analysis of 1-50 ng/uL of purified euglenophycin. The ratio of the peak area of qualifier ion to quantifier ion (97.2/110.2 and 136.2/110.2) was also determined. For each culture, euglenophycin production was normalized to cell number to account for culture density differences.

Phylogenetic analyses were used to evaluate toxin production within Euglenophyceae. A total of 30 species were analyzed for toxin accumulation by harvesting late-exponential cultures (centrifugation at 1000 RPM) as described previously (Zimba et al. 2016). The phylogenetic analysis was performed using SSU rDNA (18S) DNA sequences available from GenBank. Sequences were aligned against each other, and 1741 conserved sites between the taxa were analyzed. A Bayesian analysis was performed with Mr. Bayes 3.2.3 (Ronquist and Huelsenbeck, 2003), with the model (GTR+I+Γ) and priors determined with jModeltest 2.1.3 (Darriba et al., 2012): Prset statefreqpr= dirichlet(0.2134,0.2894,0.2784,0.2188) shapepr=exponential(0.7430) pinvarpr=uniform(0,1). Four Markov chains of 1,000,000 generations per chain were used, and trees were saved every 100 generations with the first 2,000 trees discarded. The majority-rule consensus tree was generated from the remaining trees and convergence was confirmed by utilizing the ‘sump’ command.

3. Results

A total of seven *E. sanguinea* isolates were acquired/cultured; however, one of the Denmark isolates did not survive. For all strains, the average toxin accumulation was 57 fg euglenophycin/cell (range 1.8-397 fg/cell-see Fig. 2). Cultures have had a reduction in toxin production since euglenophycin monitoring began in 2010 by about 25% (data not shown). The ratios of qualifier to quantifier ions for euglenophycin in *E. sanguinea* were 0.257 (SD=0.02) for the 136.2/110.2 and 0.859 (SD = 0.05) for the 97.2/110.2 ions. As this represents the first species in which toxin production was determined, these ratios were used to screen other euglenoid cultures for toxin presence assuming values had to fall within the 95% confidence band and exceed 5 fg/cell. This value was chosen as identifying strains that both produced the correct structure and produced the toxin in large enough concentration to have biological impact. Species producing >5 fg/cell euglenophycin included single members of the genera *Lepocinclus, Trachelomonas, Stombomonas,* two species of *Euglenaria,* and three species of *Euglena* (including *sanguinea*)-see Fig. 3. Seven species produced euglenophycin at levels exceeding 5 fg/cell in addition to *E. sanguinea*. Accumulated toxin ranged from 1.8- 39700 fg/cell. Two taxa, *Euglena socialis* and *E. sanguinea,* accumulated euglenophycin at levels 100- and 1000-fold higher than other Euglenaceae. Most toxin producing taxa were found in the Euglenaceae, with none found in ancestrial Eutreptiales or Phacaceae.

4. Discussion

This research should be considered a minimum toxin content estimate for the eight taxa identified as accumulating euglenophycin (Fig. 3) as the dissolved fraction was not included in estimates. Typically, less than 20% of the intracellular toxin is released extracellularly in *E. sanguinea* until late exponential/senescence compared to early exponential cultures

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(Zimba, pers. obs.). The two top toxin-producing euglenoid species (*E. socialis* and *sanguinea*) accumulated 100- and 1000-fold greater quantities of euglenophycin than the other taxa, with concentrations similar to the range reported for microcystin-producing taxa (Vezie et al., 1998; Kardinaal and Visser, 2005). We have not separated the potential four isomers of this compound at positions C2 and C6 –these compounds likely have differing toxicity (see Figure 1, Zimba et al. 2010).

Our decision to report levels of toxin on a per-cell basis is critical to begin definition of threshold limits (legal and biological) associated with toxic euglenoid blooms, and to develop relationships for toxin-producing organisms specific to this plant line which currently has no specific biomarker aiding its identification (*e.g.,* diagnostic carotenoids or synthesis pathway), a problem also found in separating other toxic and non-toxic strains (Jähnichen et al., 2008; Salmasco et al., 2014). Further, the use of multiple qualifier-to-quantifier ion ratios is critical to assure correct identification of the toxin, as observed with microcystin ELISA analyses identifying portions of the microcystin molecule or when cleaving microcystin to measure the Adda portion for bound toxin and assuming maximal toxicities of the highest toxicity isoform (Kurmayer et al., 2004). As synthesis of euglenophycin would require ~11 concerted steps including piperidine ring synthesis and NPKS involvement (Fig. 1, Zimba et al. 2010), mutations to the genes responsible for enzymes necessary for such a complicated synthesis pathway involving polyene formation and multiple isomerizations could account for widespread loss of toxin synthesis capability.

The addition of euglenophycin to the euglenoid genome occurred long after the family Eutreptiaceae formed, and has been lost entirely in the Phacaceae (Fig. 3). While none of the taxa in the Phacaceae had the toxin, almost every genus within the Euglenaceae have at least one taxon with the toxin present. The only exceptions are *Euglenaformis proxima* and *Colacium mucronatum*. In both cases, the genera are taxon-poor and only a single species was measured. It is possible both genera lost capacity to synthesize this compound. *Euglenaformis* is also interesting because it is basal to the rest of the Euglenaceae, and may indicate that the toxin gene complex was acquired after the split of this genus from the rest of the Euglenaceae. The loss of synthesis ability is otherwise widespread throughout the Euglenaceae. No evaluations have been made of colorless euglenoids, pointing the need for further phylogenetic work to more fully characterize toxin production in this enigmatic group of microalgae.

We have identified euglenophycin occurrence during euglenoid blooms that have included mixed assemblages of euglenoids (*E. sanguinea, E. deses,* and *Lepocinclus acus*). Blooms have been identified in 17 US states, with bloom occurrence in ponds, lakes, rivers, and estuaries. The proportion of toxin production attributable to each toxin was not determined in these bloom events, however fish mortality occurred in these blooms when dissolved euglenophycin concentrations exceeded 70 ng/mL. These levels are similar to those first identified in striped bass and catfish mortality events in NC and MS (Zimba et al. 2004).

Previously, reviewers asked how it was possible that toxin production had escaped detection in the Euglenophyceae for over 100 years (Zimba et al. 2010). After the authors'11+ years work examining field and laboratory cultures, it seems likely that an over-generalized

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perception of euglenoids as predominatantly planktonic has caused toxin production by benthic populations to be missed. Cells form resting temporary cysts by loss of flagella and then sink to the sediment surface (Graham et al. 2016). Cells can actively grow in the palmelloid stage at rates equivalent to planktonic cells for significant portions of their lifetime, suggesting a tycoplanktonic existence (Zimba, unpubl.). Cells lose flagella under turbulent conditions (*e.g.* windy days, rain storms,) which could be similar to results from vortex experiments with *Heterosigma akashiwo* (Durham et al. 2013). The authors have observed numerous euglenoid blooms "disappear" from the plankton by loss of flagella and sinking. The lack of unique chemical biomarkers and limited unique visual identification methods for cysts may also help explain why fish kill events involving euglenoids have not been commonly reported.

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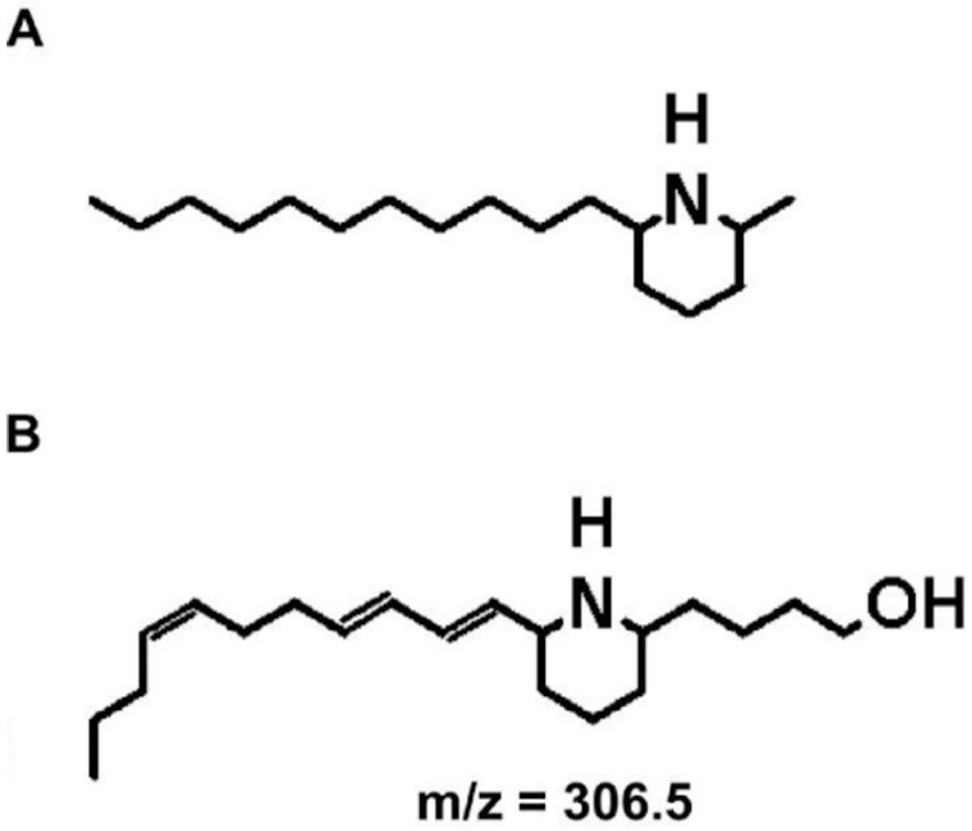

Figure 1.
Structure of solenopsin (fire ant venom, top) and euglenophycin (bottom).

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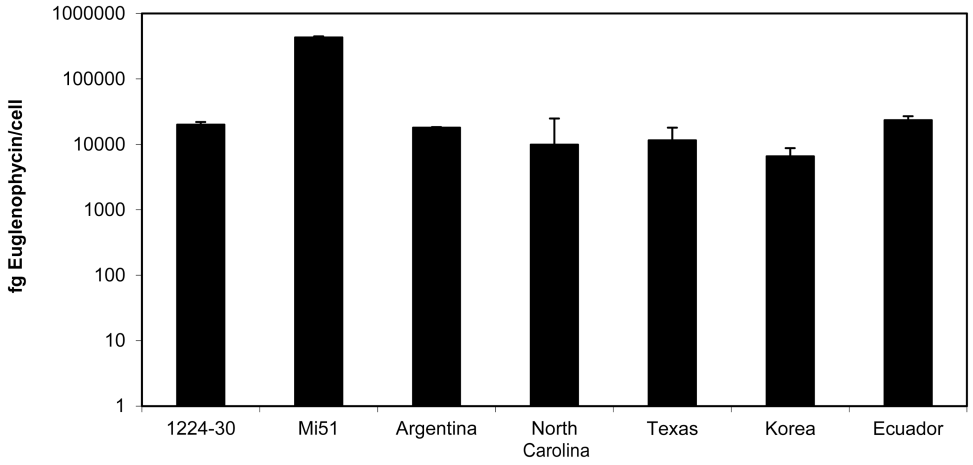

Figure 2.

Toxin accumulation in 7 *Euglena sanguinea* strains. Euglenophycin concentration normalized to a per cell basis (fg/cell). Values are mean (n≥3, standard deviation indicated.

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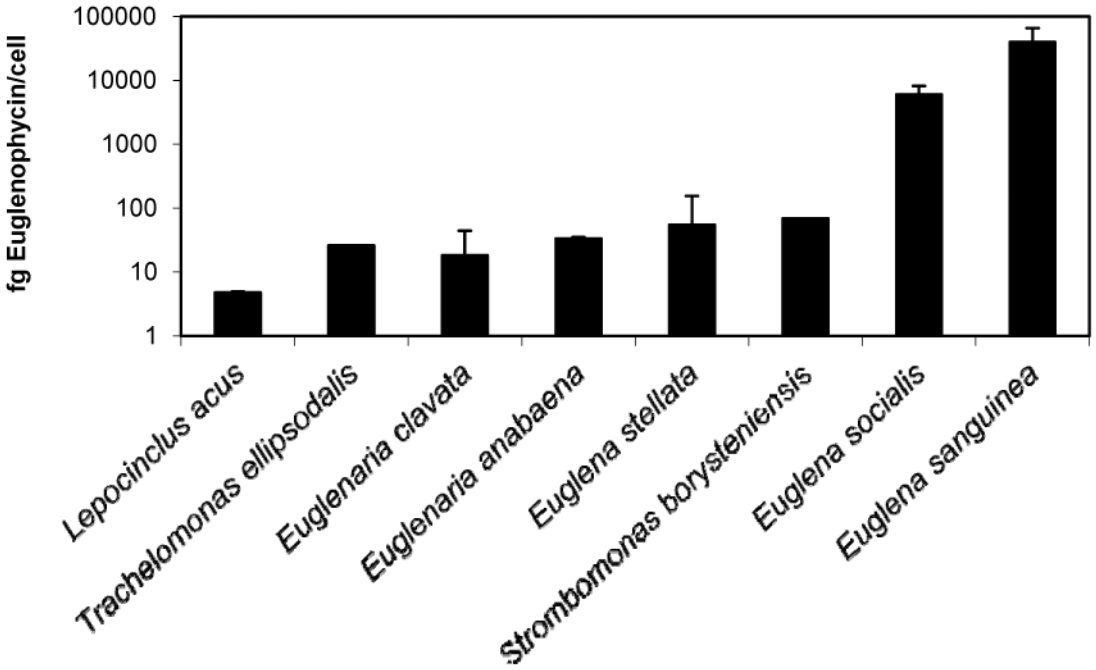

Figure 3.

Concentration of cell bound euglenophycin in species having correct qualifier to quantifier ion ratios. Euglenophycin concentration in fg/cell (n>3), standard deviation indicated.

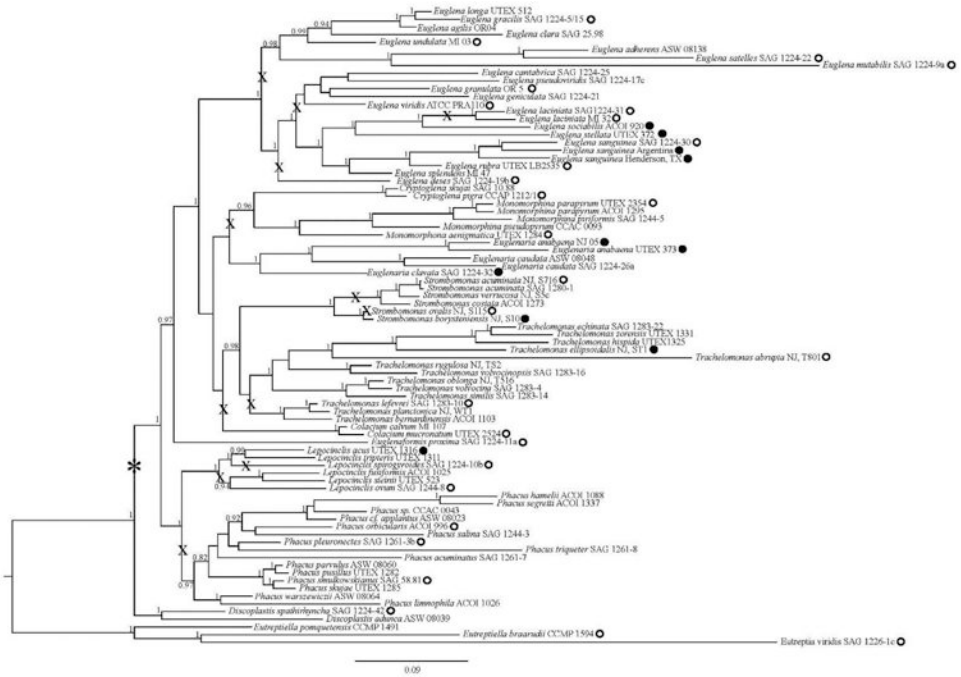

Figure 4.

Phylogenetic tree of tested euglenophyte taxa, clustered by 18s similarity of 1741 bases. Toxin production indicated by open (none) and closed (positive) circles indicating euglenophycin production. An asterisk denotes presence of toxin within a clade, an X indicates loss of this capability.

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Table 1

List of euglenoid species evaluated for euglenophycin production.

Species Tested		Authority	Strain ID	GenBank ID
Colacium	*mucronatum*	Bourrelly et Chadefaud	UTEX 2524	AJ532440
Cryptoglena	*pigra*	Ehrenberg	CCAP 1212/1	AJ532437
Discoplastis	*spathiryncha*	(Skuja) Triemer	SAG 1221-12	AJ532454
Euglena	*deses*	Ehrenberg	SAG 1224-19b	AJ532409
Euglena	*gracilis*	Klebs	SAG 1224-5/15	M12677
Euglena	*granulata*	(Klebs) Schmidtz	OR 5	AJ532422
Euglena	*laciniata*	Pringsheim	SAG 1224-31	AJ532420
Euglena	*laciniata*	Pringsheim	MI 32	DQ140158
Euglena	*mutabilis*	Schmitz	CCMP 2916	AJ532404
Euglena	*rubra*	Hardy	MI 103	none
Euglena	*satelles*	Braslavska-Spectorova	SAG 1224-22	AJ532406
Euglena	*sanguinea*	Ehrenberg	SAG 1224-30	JQ281806
Euglena	*sanguinea*	Ehrenberg	Zimba- NC	None
Euglena	*sanguinea*	Ehrenberg	Triemer- Argentina	JQ281804
Euglena	*sanguinea*	Ehrenberg	Zimba- Henderson, TX	JQ281805
Euglena	*sociabilis*	Dangeard	ACOI 920	EU750715
Euglena	*stellata*	Mainx	UTEX 372	AF150936
Euglena	*undulata*	S.Kato	MI 03	DQ140148
Euglena	*viridis*	(O.F.Müller) Ehrenberg	ATCC PRA110	AY523036
Euglenaformis	*proxima*	Bennett et Triemer	SAG 1224-11a	AJ532440
Euglenaria	*anabaena*	(Mainx) Karnkowski et Linton	UTEX 373	AF242548
Euglenaria	*anabaena*	(Mainx) Karnkowski et Linton	Triemer NJ 05	DQ140155
Euglenaria	*clavata*	Skuja (Karnkowski et Linton)	SAG 1224-32	AJ532436
Eutreptia	*viridis*	Perty	SAG 1226-1c	AJ532395
Eutreptiella	*braarudii*	Throndsen	CCMP 1594	DQ249879
Lepocinclis	*acus*	(OF Muller) Maren et Melkonian	UTEX LB 1316	AF152104
Lepocinclis	*ovum*	(Ehrenberg) Lemmermann	SAG 1244-8	AF110419
Lepocinclis	*spirogyroides*	(Ehrenberg) Marin et Melkonian	SAG 1224-10b	AJ532464
Monomorphina	*aenigmatica*	(Drézepolski) Nudelman et Triemer	UTEX 1284	AF190814
Monomorphina	*parapyrum*	Kim, Triemer et Shin	UTEX 2354	AF112874
Phacus	*orbicularis*	Hübner emend Zakryś et Kosmala	ACOI 996	DQ397670
Phacus	*pleuronectes*	(OF Müller) Dujardin	SAG1261-3b	DQ397669
Phacus	*smulkowskianus*	(Zakryś) W.-H.Kusber	SAG 58.81	AJ532467
Strombomonas	*acuminata*	Deflandre	Triemer NJ, S716	EU624029
Strombomonas	*borystheniensis*	(Roll) Papova	Triemer NJ, S10	DQ140131
Strombomonas	*ovalis*	(Playfair) Deflandre	Triemer NJ, S115	DQ140133
Trachelomonas	*abrupta*	Sdwirenko emend. Delfandre	Triemer NJ, 801	DQ140134
Trachelomonas	*ellipsodalis*	Singh	Triemer NJ, ST1	DQ140135
Trachelomonas	*lefévrei*	Deflandre	SAG 1283-10	DQ140136