

Genetic and morphological characteristics of two ecotypes of *Eustigmatos calaminaris* sp. nov. (Eustigmatophyceae) inhabiting Zn- and Pb-loaded calamine mine spoils

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Abstract: Knowledge on species diversity and distribution of eustigmatophycean algae (Stramenopiles) in the environment is currently very limited. Some of them are considered promising organisms for industrial application. Phylogenetic analysis of two strains (E120, E5) of a coccoid microalga isolated from highly Zn- and Pb-polluted calamine mine spoils revealed that they belong to the genus *Eustigmatos*. Cells of both strains were morphologically identical. Some features of their vegetative cells and zoospores differed from other described nominal *Eustigmatos* species. 18S rDNA sequences of E120 and E5 were identical but also differed in two or three nucleotide substitutions from the other *Eustigmatos* species (*E. vischeri*, *E. magnus*, *E. polyphem*). Only 1 – 3 nucleotide substitutions in 18S rDNA sequence were found among all the examined *Eustigmatos* species. ITS1–5.8S rDNA–ITS2 region was more variable (15 – 22 differences) than 18S rDNA, except for E120 and E5 strains which differed in only one nucleotide substitution (in 5.8S rDNA). Genetic differences found between the isolated strains (E120, E5) and the other *Eustigmatos* species were comparable to those observed among all nominal *Eustigmatos* species studied. This suggests that the isolates represent a new species. The name *Eustigmatos calaminaris* sp. nov. (referred to the locality) has been proposed. The E120 strain from the mine spoil with higher heavy metal contents was more Zn- and Pb-resistant than E5 from the less polluted ground. Their high, though varied resistance to Zn/Pb stress, together with the very slight genetic divergence between them, indicate that they represent two ecotypes of the same new species.

Key words: *Eustigmatos*, 18S rDNA, ITS1–5.8S rDNA–ITS2, lead, soil algae, zinc

INTRODUCTION

Eustigmatophycean algae are considered promising microorganisms for industrial applications due to their ability to produce hydrocarbons (that may be useful in biofuel production), and accumulation of high levels of polyunsaturated fatty acids (KHOZIN–GOLDBERG et al. 2002; PRIOR et al. 2009; LUKAVSKÝ 2012). Although aeroterrestrial *Eustigmatos* (Eustigmatophyceae) species have been increasingly reported in recent years (PRIOR et al. 2009; GÄRTNER et al. 2012; TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2013) the knowledge on their diversity and environmental distribution is still in its infancy. The genus *Eustigmatos* was established by HIBBERD (1981) when he removed several unicellular, coccoid species of *Pleurochloris*

PASCHER (with unique ultrastructural features) from Pleurochloridaceae (Xanthophyceae) and placed them into the class Eustigmatophyceae. The name of the class (gr. *eu* – well developed; gr. *stigma* – eyespot) refers to the orange–red extraplastidial eyespot. This is the most conspicuous morphological feature present in the anterior part of their flagellate zoospores (HIBBERD & LEEDALE 1972; SANTOS et al. 1996). The features distinguishing Eustigmatophyceae from Xanthophyceae include also photosynthetic pigment composition (WHITTLE & CASSELTON 1975), chloroplast thylakoids occurring in stacks of three without a thylakoidal girdle lamella, polyhedral stalked pyrenoid projected on the inner side of the chloroplast (only in *Eustigmatos*, *Vischeria*, *Chlorobotrys*, *Pseudocharaciopsis minutus*), and a prominent reddish

globule present within the cytoplasm of vegetative cells (HIBBERD & LEEDALE 1971; SANTOS et al. 1996; GÄRTNER et al. 2012). Nevertheless, taxonomic identification of eustigmatophycean algae found in the field is difficult by means of light microscopy alone. Due to their simple morphology, cell size and colour, chloroplast structure and shape as well as reproduction type, they can be easily misidentified as coccoid green algae (GRAHAM & WILCOX 2000) or some Xanthophyceae from which they were excluded by HIBBERD & LEEDALE (1970, 1971). As suggested by ANDERSEN (1992) the class Eustigmatophyceae is poorly known and even 1 000–10 000 species still remained to be described. So far, the genus *Eustigmatos* (Stramenopiles; ADL et al. 2012) comprised only three species: a type (holotype) – *E. vischeri* HIBBERD (1981; syn. *Pleurochloris commutata* PASCHER sensu VISCHER), *E. magnus* (J.B. PETERSEN) HIBBERD (1981; syn. *Pleurochloris magna* J.B. PETERSEN) and *E. polyphem* (PITSCHMANN) HIBBERD (1981; syn. *Pleurochloris polyphem* PITSCHMANN). It is probable that hitherto unknown *Eustigmatos* species may also exist. *Eustigmatos* strains (identified and non-identified) have been frequently reported from both natural soils and those degraded by brown and lignite coal mining (LUKEŠOVÁ & HOFFMANN 1996; LUKEŠOVÁ 2001; ZANCAN et al. 2006; KHAYBULLINA et al. 2010). Recently, we have found two strains (E120, E5) of *Eustigmatos* sp. in soils of two post-mining calamine spoils, extremely polluted with heavy metals such as zinc and lead (TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2008). Previously, only *Eustigmatos* cf. *magnus* was reported in Cu-polluted soils (ELSTER et al. 1999).

Post-industrial areas highly polluted with heavy metals such as Zn, Pb, Cu, Cd, etc., create harsh conditions for algae (MAXWELL 1991; SHUBERT et al. 2001; NAGY et al. 2005; TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2008). Nevertheless, in this extreme environment, microalgae (including *Eustigmatos*) may exist (TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2008). Resistant or even tolerant algal species or ecotypes with specific intrinsic features were found (PAWLIK-SKOWROŃSKA 2003a, b; KALINOWSKA & PAWLIK-SKOWROŃSKA 2010; TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2013). For example, the ecotype of the filamentous green alga *Stigeoclonium tenue* (C. AGARDH) KÜTZING occurring in a Zn/Pb/Cd-polluted ditch was more Zn-tolerant due to its higher efficiency of intracellular metal detoxification than the ecotype inhabiting unpolluted waters (PAWLIK-SKOWROŃSKA 2003b). Several species of green microalgae (*Chlamydomonas boldii* H. ETTL, *Dictyococcus* cf. *varians* R. GERNECK, *Stichococcus minor* NÄGELI) inhabiting grounds of heavy metal polluted post-flotation tailing ponds exhibited higher Zn/Pb/Cu-resistance than did *Geminella terricola* J.B. PETERSEN from an unpolluted area (KALINOWSKA & PAWLIK-SKOWROŃSKA 2008).

The aim of this study was to carry out (i) a morphological and molecular comparison of two

strains (E120, E5) of *Eustigmatos* inhabiting heavy metal polluted soils, described here as a new species; (ii) an ecotoxicological study on their Zn- and Pb-resistance.

MATERIALS AND METHODS

Organisms and culture conditions. Two strains (E120 and E5) of the unicellular, coccoid alga *Eustigmatos calaminaris* sp. nov. (Eustigmatophyceae, Stramenopiles) were isolated in 2007 from a surface layer (0–5 cm) of soil from two calamine mine spoils. These were over 120- and 5-year-old, respectively, located in the vicinity of the Zn/Pb ore mine in Bolesław (S. Poland). The soils differed significantly in their heavy metal contents. The total Zn and Pb contents in the ground of the older mine spoil (61.6 mg.g⁻¹ soil DW, 3.4 mg.g⁻¹ soil DW, respectively) were almost 2-times higher than in the younger one. However, nutrient contents (P-PO₄³⁻, N-NH₄⁺, N-NO₃⁻) in both mine spoils were low (TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2008).

Unialgal cultures of the isolated *Eustigmatos* strains (E120, E5) were obtained following TRZCIŃSKA & PAWLIK-SKOWROŃSKA (2008). They were cultured under laboratory conditions in the liquid Woods-Hole medium (pH 6.8 – 7.0; SIMONS et al. 1986) continuously aerated by sterile air, at 21±1°C. Illumination was provided by fluorescent lamps with an irradiance of 50 µmol.m⁻².s⁻¹ PPFD at the surface of cultures at light regime 16 h light:8 h dark. The exponentially growing cultures were kept in darkness for 18 h at 10 °C to induce zoospore formation.

Both strains were deposited in the Culture Collection of Autotrophic Organisms (CCALA) of the Institute of Botany, Academy of Sciences of the Czech Republic in Třeboň and have been kept alive on agar slants.

Effect of Zn and Pb on algal growth. Algal cells from the exponential growth phase of cultures were separated by centrifugation (2 700×g, 17 °C, 10 min) and suspended in the sterile modified Woods-Hole medium (pH 6.8) according to KALINOWSKA & PAWLIK-SKOWROŃSKA (2008). The biomass of *Eustigmatos* strains was estimated spectrophotometrically by measuring the optical density OD₆₅₀ (Helios Unicam, UK) of alive cells and comparing OD₆₅₀ with standard calibration curve (OD versus biomass dry weight). The dry weight (DW) of algal biomass was obtained after filtering algae from aliquots of culture of a known density and drying to stable mass at 90 °C. For assessment of chlorophyll-*a*, the algal biomass was collected on GF/C filters (Whatman, UK), extracted (1 h, 65 °C) in dimethyl sulfoxide (DMSO) in the dark and centrifuged (15 500×g, 17 °C, 10 min). The absorbance at 649, 665 and 730 nm of the pigment extracts was measured spectrophotometrically. Concentrations of chl-*a* were calculated using Wellburn's equation (1994). The influence of high Zn (400 µM) and Pb (25 µM) concentrations on algal biomass and chlorophyll-*a* contents was observed after 96 h exposure. Heavy metals were supplied to algal cultures as Zn(NO₃)₂·6H₂O and Pb(NO₃)₂. The initial biomass density was 35 mg DW.l⁻¹ and chl-*a* content in cultures was 0.9 mg.l⁻¹. All determinations were made in triplicate and data expressed as means ± the standard deviation (SD). Statistical tests were carried out using the software packed STATISTICA ver. 5.0. The data were

Table 1. List of algal taxa used in this study with their GenBank accession numbers for 18S rDNA (*) and ITS1–5.8S rDNA–ITS2 (**) sequences.

Species/strain	GenBank accession number	Reference
E120 strain CCALA 1014 (<i>Eustigmatos</i> sp. MT–2012 isolate E120)	JX188078(*) JX202554(**)	This study
E5 strain CCALA 1013 (<i>Eustigmatos</i> sp. MT–2012 isolate E5)	JX188079(*) JX202555(**)	This study
<i>Eustigmatos magnus</i> (J.B. PETERSEN) HIBBERD SAG 36.89	JX202557(**)	This study
<i>Eustigmatos polyphem</i> (PITSCHMANN) HIBBERD SAG 38.84	JX188077(*) JX202558(**)	This study
<i>Eustigmatos vischeri</i> HIBBERD SAG 860–1	JX274590(*) JX202556(**)	This study
<i>Vischeria helvetica</i> (VISCHER et PASCHER) HIBBERD SAG 876–1	JX188080(*) JX202559(**)	This study
<i>Eustigmatos magnus</i> (J.B. PETERSEN) HIBBERD CCMP387	U41051.1(*)	ANDERSEN et al. 1998
<i>Nannochloropsis gaditana</i> L.M. LUBIÁN CCMP536	AF045040.1(*)	ANDERSEN et al. 1998
<i>Nannochloropsis granulata</i> B. KARLSON et D. POTTER CCMP535	AF045043.1(*)	ANDERSEN et al. 1998
<i>Nannochloropsis</i> sp. JL2/1–4	DQ977727.1(*)	FAWLEY & FAWLEY 2007
<i>Nannochloropsis limnetica</i> L. KRIENITZ, D. HEPPERLE, H.–B. STICH et WEILER	DQ977726.1(*)	FAWLEY & FAWLEY 2007
<i>Nannochloropsis oceanica</i> S. SUDA et H. MIYASHITA	HQ201712.1(**)	Li (unpublished)
<i>Nannochloropsis oculata</i> (DROOP) HIBBERD CCMP533	AF045045.1(*)	ANDERSEN et al. 1998
<i>Nannochloropsis salina</i> HIBBERD CCMP537	AF045049.1(*)	ANDERSEN et al. 1998
<i>Monodopsis subterranea</i> (J.B. PETERSEN) HIBBERD UTEX151	U41054.1(*)	ANDERSEN et al. 1998
<i>Pseudocharaciopsis minuta</i> (BRAUN) HIBBERD UTEX2113	U41052.1(*)	ANDERSEN et al. 1998
<i>Mischococcus sphaerocephalus</i> VISCHER SAG847–1	AF083400.1(*)	BAILEY & ANDERSEN (unpublished)

tested for homogeneity of variance and normality and then subjected to one-factor analysis of variance (ANOVA). The difference between individual means was determined by Tukey's post hoc multiple range test $P < 0.05$.

Taxonomy. Identification and classification of the isolates was based on microscope observations of cell morphology, ultrastructure, life cycle with reference to descriptions given by HIBBERD & LEADALE (1972), Ettl & GÄRTNER (1995) and VAN DEN HOEK et al. (1995). Images of vegetative cells and non-fixed zoospores were taken using a Zeiss light microscope with a Canon (PowerShot G9) digital camera. Morphological characteristics of isolates were compared with other soil-dwelling *Eustigmatos* species i.e. *E. vischeri*, *E. magnus*, *E. polyphem* and the closely related *Vischeria helvetica* (VISCHER et PASCHER) HIBBERD (*Eustigmatophyceae*) obtained from SAG (strain numbers in Table 1).

Transmission electron microscopy (TEM). For ultrastructure examination, cells from the logarithmic phase of growth were harvested by centrifugation (2 700×g, 17 °C, 10 min). Cell pellets were suspended in phosphate buffer (pH 7.3) containing 2% paraformaldehyde (PFA) and 2%

glutaraldehyde (GA) for 4.5 h at 4 °C. Samples were then rinsed three times in phosphate buffered saline (PBS; 1.5 h, 4 °C), preserved by 1% osmium tetroxide (12 h, 4 °C), dehydrated in a series of alcohol and acetone and embedded in LR White resin. Polymerization of resin was conducted for 48 h at 50–55 °C. Ultrathin sections were obtained with an ultramicrotome (RMC MT – XL Tucson, USA), collected on copper grids, contrasted with uranyl acetate and lead citrate and examined using a Carl Zeiss LEO 912 AB electron microscope (Oberkochen, Germany).

Molecular analyses. Nucleotide sequences of the small subunit ribosomal DNA (18S rDNA) and the first and second internal transcribed spacer including 5.8S rDNA (ITS1–5.8S rDNA–ITS2) were analyzed in both isolates of *Eustigmatos* (E120, E5). Similar analyses were carried out for *Eustigmatos vischeri*, *E. polyphem* and *Vischeria helvetica* (from SAG). For *E. magnus* (SAG) only ITS1–5.8S rDNA–ITS2 sequence was determined. All new sequences were deposited in the GenBank. The list of algal taxa used in this study with their GenBank accession numbers is given in Table 1.

For molecular analyses, algal cells were harvested by centrifugation (2 700×g, 17 °C, 10 min), washed in ice cold

PBS and resuspended in 0.7 ml buffer containing 50 mM TRIS-HCl (pH 8), 2.5 mM EDTA, 100 mM NaCl, 1% SDS (sodium dodecyl sulphate). 0.2 ml of acid washed glass beads (Sigma) were then added and shaken vigorously 10 times at intervals of 30 s with 1 min cooling on ice between each burst. Samples were subsequently incubated (65 °C, 30 min), cooled on ice, 0.2 ml of 5 M potassium acetate added and kept on ice for a further 30 min. Cell debris and protein precipitate were pelleted by centrifugation (13 000×g, 4 °C, 15 min). Nucleic acids were precipitated from the supernatant by the addition of 1/3 (v/v) of isopropyl alcohol. The obtained pellets were washed with ice cold 70% ethanol, dried and resuspended in 0.1 ml of TE buffer (1 mM TRIS-HCl, pH 8.0, containing 0.1 mM EDTA) and used for PCR reaction. For 18S rDNA amplification, Taq polymerase (Fermentas) and the primers SR1F – SR12R were used (NAKAYAMA et al. 1996). Its sequencing was performed using additional primers set: SR1 – SR7 and SR4 – SR12. ITS1–5.8S rDNA–ITS2 sequences of the isolates and other Eustigmatophyceae species were obtained by using primers (18S-PCR–5'F and ITS-PCR–3'R) described by FIETZ et al. (2005). PCR-amplified DNA fragments (obtained from two independent amplifications) were isolated after agarose gel electrophoresis using the Qiagen gel extraction kit according to manufacturer's instructions, then sequenced (IBB, Warsaw, Poland) using the dye-terminator method. Partial DNA sequences of the E120 and E5 strains and other Eustigmatophyceae species were combined manually. Alignment of the sequences was performed using CLUSTAL W. As an outgroup *Mischococcus sphaerocephalus* (Xanthophyceae) and *Nannochloropsis oceanica* (Eustigmatophyceae) were used for 18S rDNA and ITS1–5.8S rDNA–ITS2 alignments, respectively. The evolutionary history was inferred using the Neighbor-Joining method (NJ; SAITOU & NEI 1987). Consensus from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches (FELSENSTEIN 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method (TAMURA 1992) and are presented in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the alignment. For studied E120 and E5 strains, there were a total of 1796 positions in the final dataset for 18S rDNA and 749 positions for ITS1–5.8S rDNA–ITS2. Phylogenetic analyses were conducted in MEGA4 (TAMURA et al. 2007).

RESULTS

Eustigmatos calaminaris M. TRZCIŃSKA et B. PAWLIK–SKOWROŃSKA sp. nov. (Figs 1 – 5)

Description: Algae unicellular, yellow–green. Adult cells globose to oval (7–9(12) µm in diameter), daughter cells more or less angular. Large cells (18–30 µm) occasional. Cells uninucleate. Cell wall thin (50–

60 nm), smooth, multilayered, without ornamentation and mucilage. Chloroplast single, parietal, massive, cup-shaped and lobed. Polyhedral pyrenoid surrounded by lamellate vesicle projects on a stalk from the inner side of the chloroplast. Conspicuous, single, globular, reddish body present in cells. In older, larger cell the protoplast pushed towards the periphery by a huge vacuole surrounded by refractile granules. Reproduction occurs through autospore formation (two – hemispherical or four – tetrahedral) and zoospores. Zoospores (7–12 µm long), uniflagellate, spindle-shaped, with conspicuous extraplastidial orange eyespot in the flagellar swelling region at the anterior extremity and a single, bowl-shaped chloroplast in the posterior two-thirds of the body.

The strain E120 (CCALA 1014; Figs 1a, c, 2, 3, 5a) and the strain E5 (CCALA 1013; Figs 1b, d, 4, 5b) of the *Eustigmatos calaminaris* sp. nov. were isolated in March 2007 and have been deposited in the CCALA (Třeboň, Czech Republic).

GenBank accession numbers: the E120 strain (*Eustigmatos* sp. MT–2012 isolate E120): JX188078 (18S rDNA), JX202554 (ITS1–5.8S rDNA –ITS2); the E5 strain (*Eustigmatos* sp. MT–2012 isolate E5): JX188079 (18S rDNA), JX202555 (ITS1–5.8S rDNA –ITS2).

Type locality of the authentic strain E120: soil of the over 120-year-old (50°17'50" N, 19°28'35" E) calamine mine spoil in Bolesław (Southern Poland). The strain E5 was isolated from 5-year-old mine spoil in Bolesław (50°17'49" N, 19°28'23" E). Both mine spoils are highly polluted with heavy metals (mainly Zn and Pb).

Etymology: the epithet *calaminaris* was derived from the English term „calamine” (a historic name for an ore of zinc) and refers to the specific locality where the alga was found.

Microscopical observation of two strains (E120 and E5) of the unicellular alga *Eustigmatos calaminaris* sp. nov. from two different Zn- and Pb-polluted sites did not reveal any morphological differences (Figs 1, 3–5). Vegetative cells appearing yellow–green were spherical to oval, 7–9(12) µm in diameter (Fig. 1). However, there was always a proportion of younger cells with an angular, slightly polyhedral shape. Large, so-called “giant cells”, 18–30 µm in diameter were also observed in the life cycle (Figs 1, 2). The protoplast of the “giant cells” was pushed towards the periphery by a huge vacuole surrounded by refractile granules (Fig. 2). The cell wall of both young and mature cells was smooth, thin (50–60 nm), multilayered, without any ornamentation and mucilage (Figs 3, 4). Within the mature cells a conspicuous, single, globular, reddish body was present, more visible in mature than in younger or “giant cells” (Fig. 1). TEM observation revealed that this red vesicle was enclosed by a single cell membrane and contained numerous dark droplets

(Figs 3b, 4). One nucleus and a single, parietal, massive, cup-shaped, slightly lobate chloroplast were present. The chloroplast lamellae consisted of three thylakoids, but there was no girdle lamella. At the inner side of the chloroplast, a huge, stalked polyhedral pyrenoid surrounded by a sheath of lamellate vesicles was present (Figs 3a, b; Figs 4a, c). Reproduction of both E120 and E5 strains occurred through the formation of two hemispherical “bean-shaped” or four angular autospores that later expanded to produce a more spherical shape (Fig. 1). Higher number of autospores was not observed. Production of uniflagellate, clearly spindle-shaped zoospores (7–12 µm long) with an elongated posterior end, was also noticed (Fig. 5). The zoospores of E120 and E5 strains were identical but differed in shape from the other *Eustigmatos* species as described by Ettl & Gärtner (1995). They were motile for 3–4 min and contained a single, elongate, bowl-shaped chloroplast located in the posterior two-thirds of their body (Fig. 5). Outside the chloroplast, at the anterior end of a zoospore in the flagellar swelling region, a conspicuous, orange eyespot was present.

Various Zn- and Pb-resistance of E120 and E5 strains

Experimental exposure of the morphologically identical E120 and E5 strains to high Zn (400 µM) or Pb (25 µM) concentrations revealed differences in their Zn- and Pb-resistance (Fig. 6). Generally, toxic effects of Zn or Pb on biomass and chl *a* content in algal cultures were stronger in the E5 than in the E120 strain. For example, 96 h-exposure of E5 to 400 µM Zn or 25 µM Pb caused a 55% and 34% decrease in biomass, respectively, whereas in E120 those metal concentrations caused only 36% and 20% decrease, respectively (Fig. 6). Similarly, during 96 h-exposure to 400 µM Zn chl-*a* concentration in E5 culture decreased by 75%, but in E120 by 48% compared to controls (Fig. 6a). The toxic effect of 25 µM Pb on chl-*a* content in the culture of E5 was ca. 2-fold stronger than in E120 (Fig. 6b).

Molecular analyses

Due to the morphological identity of the algal strains (E120, E5; Figs 1, 3–5) isolated from two calamine mine spoils and their considerable differences in Zn- and Pb-resistance (Fig. 6), molecular analyses of 18S rDNA sequences and the more rapidly evolving ITS1–5.8S rDNA–ITS2 region were carried out. An analysis of the phylogenetic relationship based on the 18S rDNA sequences (NJ; Fig. 7) revealed that the strains (E120 and E5) belong to the genus *Eustigmatos* (100% bootstrap), a paraphyletic group within the Eustigmatophyceae class. Using an alignment of the complete 18S rDNA sequences (1 796 base pairs), both strains (E120, E5) were found to be identical but different from all other known *Eustigmatos* species, for which 18S rDNA sequences were also determined

in this work for the first time. In total, differences in up to 3 nucleotide positions were found between E120 and E5 strains and all the other examined *Eustigmatos* species (Table 2). For example, the isolated strains differed from *E. magnus* in two nucleotide substitution in positions: 254 (A→G), 401 (G→A) and from *E. vischeri* and *E. polyphem* in 3 nucleotide substitutions in the following positions: 254 (A→G), 258 (A→T), 456 (C→G) or 254 (A→G), 258 (A→T), 456 (C→T), respectively. One to three substitutions in 18S rDNA were also found among the other examined *Eustigmatos* species: *E. magnus*, *E. polyphem* and *E. vischeri* (Table 2). Slightly higher numbers (3–6) of differences in 18S rDNA sequences were found between all the examined *Eustigmatos* species and the closely related eustigmatophycean alga *V. helvetica* (Table 2). A phylogeny (NJ) based on the sequence of ITS1–5.8S rDNA–ITS2 also confirmed that both E120 and E5 strains differed considerably from the other *Eustigmatos* species (Fig. 8); overall 15–18 differences (substitutions, deletions or insertions) were found (Table 3). 16–22 differences in ITS1–5.8S rDNA–ITS2 sequences were detected among *E. magnus*, *E. polyphem* and *E. vischeri* (Table 3; GenBank accession numbers in Table 1), and 19–23 differences between them and *V. helvetica* (Table 3). Interestingly, the alignment of the ITS1–5.8S rDNA–ITS2 sequence of E5 strain of the lower Zn- and Pb-resistance revealed one substitution (G→T) in the 5.8S rDNA region in comparison with the more resistant E120 strain and all the other *Eustigmatos* species.

DISCUSSION

Microalgae belonging to the genus *Eustigmatos*, reported mainly from soil, seem to be ubiquitous (LUKEŠOVÁ 2001; FLECHTNER et al. 2008; M. TRZCIŃSKA & B. PAWLIK-SKOWROŃSKA 2008; CZERWIK-MARCINKOWSKA & MROZIŃSKA 2009; ŠKALOUD 2009; KHAYBULLINA et al. 2010; NEUSTUPA & ŠKALOUD 2010). The two strains (E120, E5) of the unicellular alga isolated from two different Zn- and Pb-polluted calamine mine spoils in southern Poland shared identical morphological characteristics and were described here as members of the new species *Eustigmatos calaminaris*. Several morphological and ultrastructural features of vegetative cells and zoospores of the strains E120 and E5 are unique to members of the class Eustigmatophyceae (e.g., chloroplast organization, polyhedral stalked pyrenoid surrounded by lamellate vesicles, enlarged “giant cells” and extraplasmidial eyespot in zoospores; HIBBERD & LEEDALE 1971; Ettl & Gärtner 1995; SANTOS et al. 1996). Moreover, HIBBERD & LEEDALE (1972) described a red-pigmented body with globular contents present in older vegetative cells of Eustigmatophyceae. In mature cells of E120 and E5 strains, a conspicuous, single, globular, reddish body

Table 2. The number of differences in 18S rDNA sequences among different *Eustigmatos* species and *Vischeria helvetica*.

Species/strain	1	2	3	4	5	6
1 E120 strain (CCALA 1014)						
2 E5 strain (CCALA 1013)	0					
3 <i>Eustigmatos magnus</i>	2	2				
4 <i>Eustigmatos vischeri</i>	3	3	3			
5 <i>Eustigmatos polyphem</i>	3	3	3	1		
6 <i>Vischeria helvetica</i>	3	3	5	6	6	

Table 3. The number of differences in ITS1–5.8S rDNA–ITS2 sequences among different *Eustigmatos* species and *Vischeria helvetica*.

Species/strain	1	2	3	4	5	6
1 E120 strain (CCALA 1014)						
2 E5 strain (CCALA 1013)	1					
3 <i>Eustigmatos magnus</i>	15	16				
4 <i>Eustigmatos vischeri</i>	17	18	22			
5 <i>Eustigmatos polyphem</i>	15	16	20	16		
6 <i>Vischeria helvetica</i>	16	17	19	23	20	

(vesicle) was also observed. TEM analysis revealed that this vesicle was enclosed by a single membrane layer and contained numerous dark globules. In older cells of E120 and E5 strains, a large, central vacuole surrounded by refractile granules was observed. It was previously recorded in Heterokontophyta (PASCHER 1939) and in eustigmatophycean species (HIBBERD & LEEDALE 1972; GÄRTNER et al. 2012). The E120 and E5 strains resemble morphologically algae from the genus *Eustigmatos*. However, some differences found between them and the other *Eustigmatos*, as well as the closely related *Vischeria*, precluded naming them as any previously described species (ETTL & GÄRTNER 1995; GÄRTNER et al. 2012). The strains never the less share morphological features with other Eustigmatophyceae, i.e., *Eustigmatos* and *Vischeria* species. For example, vegetative cells of E120 and E5 strains from the exponential growth phase were similar in shape and size (7–9(12) µm) to *Eustigmatos vischeri* and the closely related *Vischeria helvetica*, but smaller than cells of *Eustigmatos magnus* (14–34 µm; ETTL & GÄRTNER 1995) and *Eustigmatos polyphem* (14–25 µm; PITSCHMANN 1969; ETTL & GÄRTNER 1995). Angular daughter cells released from parental cell wall observed in the life cycle of the strains were characteristic also for *E. vischeri*, *E. magnus* as well as *Vischeria helvetica* and *V. punctata* VISCHER (ETTL & GÄRTNER 1995). However, contrary to all reported *Eustigmatos* species, vegetative cells of *Vischeria* spp. had a true

polyhedral shape with a variable number of faces (ETTL & GÄRTNER 1995) and warty cell wall (BROADY 1978), or according to GÄRTNER et al. (2012) with folded cell wall with protrusions. The isolated strains (E120, E5) in all growth phases had a completely smooth cell wall and never developed folded cell walls with hump-like protrusions. These are the most striking differences between the isolated *Eustigmatos* strains and *Vischeria* species. Differences between E120, E5 strains and the other *Eustigmatos* species in the reproduction process were also found. Asexual propagation of E120 and E5 strains, similar to *E. vischeri*, occurred by division of parental cell into 2 or 4 angular autospores, whereas in *E. polyphem* and *E. magnus* into 2, 4 or 8 autospores (PITSCHMANN 1969; ETTL & GÄRTNER 1995). The phylogenetic importance of zoospore features, such as shape, dimension and ultrastructure were ascertained for eustigmatophycean taxonomy (HIBBERD & LEEDALE 1972). For example, within the Eustigmatophyceae the genera *Eustigmatos*, *Vischeria* and *Trachydiscus* were clearly defined by having uniflagellate, lageniform zoospores (SANTOS et al. 1996; PŘIBYL et al. 2012). The zoospores of the E120 and E5 strains possessed an extraplastidic orange eyespot in the flagellar swelling region, a typical photoreceptor for some Eustigmatophyceae (HIBBERD & LEEDALE 1972; SANTOS et al. 1996). They were similar in size (7–12 µm) to those reported for *E. vischeri*, *E. magnus* (STARMACH 1968; ETTL & GÄRTNER 1995; NEUSTUPA & NĚMCOVÁ

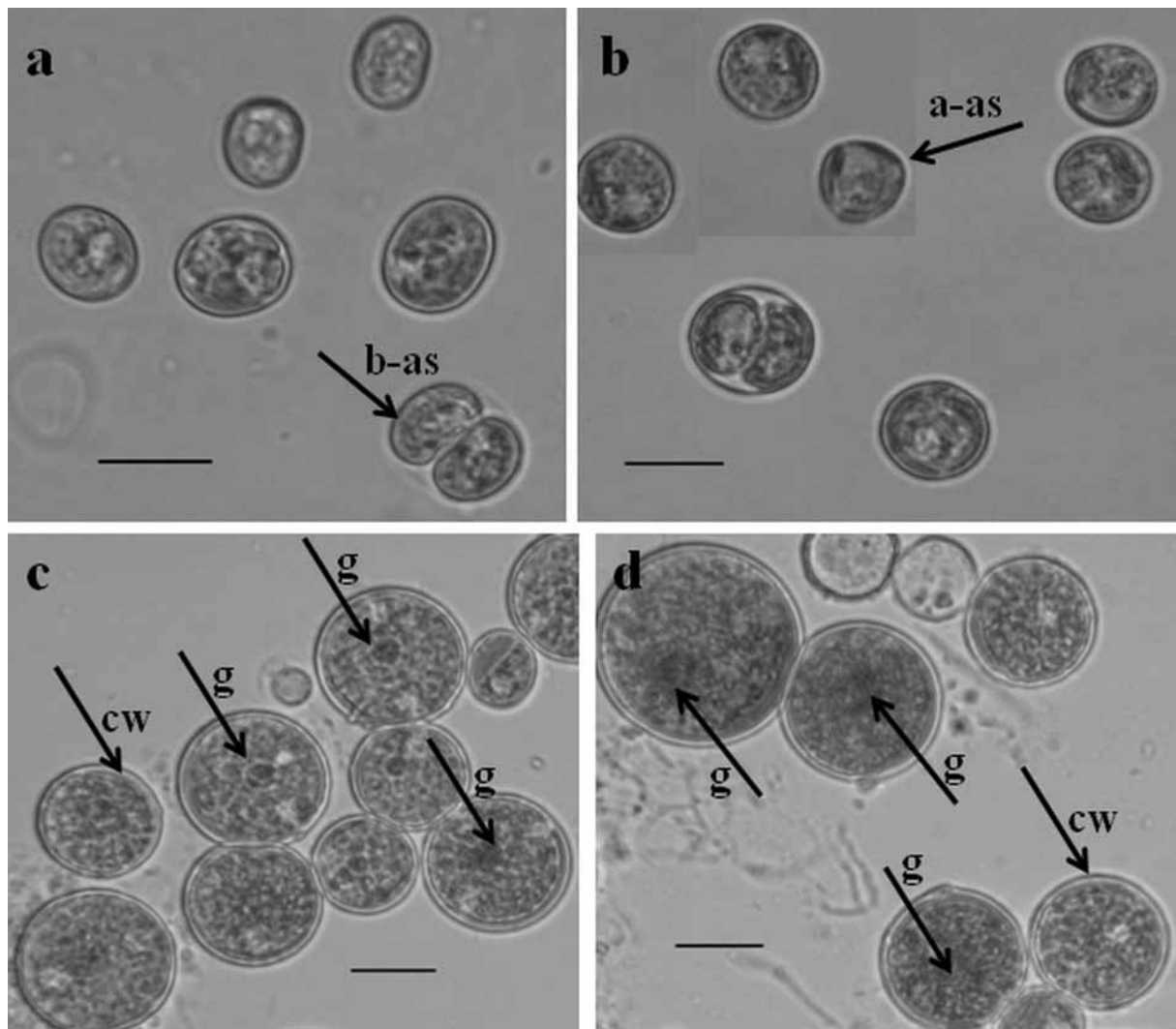


Fig. 1. Morphology of vegetative cells of *Eustigmatos* E120 and E5 strains; (cw) cell wall: (a) bean-shaped autospores (b-as) of E120 strain; (b) angular autospore (a-as) of E5 strain; (c) mature cells of E120 strain with a conspicuous reddish globule (g); (d) mature cells of E5 strain with conspicuous reddish globule (g). Scale bars 10 µm.

2001) and *Vischeria* sp. (NEUSTUPA & NĚMCOVÁ 2001), but almost 2 – 3-times shorter than those reported for *E. polyphem* (ETTL & GÄRTNER 1995). What is noteworthy, the zoospores of E120 and E5 strains were clearly spindle-shaped with an elongated posterior end containing a bowl-shaped chloroplast in the posterior two-thirds of the zoospore body. In *E. vischeri* and *E. magnus*, more lageniform zoospores with a plate-shaped chloroplast were reported (HIBBERD & LEEDALE 1971; ETTL & GÄRTNER 1995). This contrasts with the ribbon-shaped chloroplast located in the centre of a zoospore described in *E. polyphem* (PITSCHMANN 1969; ETTL & GÄRTNER 1995). The zooids of E120 and E5 were motile for 3–4 min., unlike the stigma-lacking zoospores of the eustigmatophycean *Trachydiscus minutus* motile for 8–10 min (PŘIBYL et al. 2012). Traditional identification and classification methods based on cell morphology and morphometry are inappropriate due to the overall morphological similarity of the documented *Eustigmatos* species.



Fig. 2. “Giant cell” (*Eustigmatos* E120 strain) with huge vacuole (v). Scale bar 10 µm.

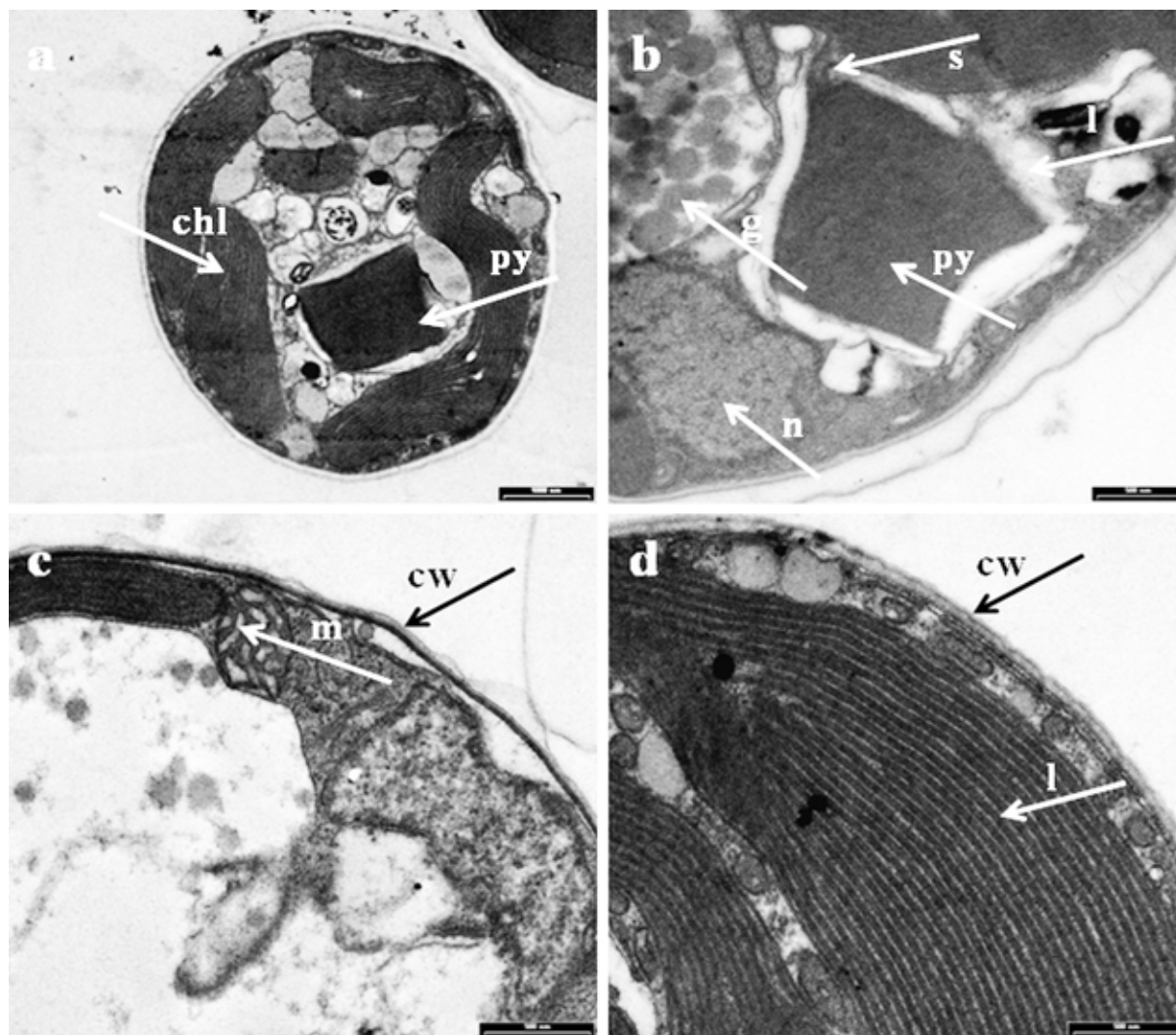


Fig. 3. Ultrastructure of *Eustigmatos* E120 strain; (c) chloroplast; (py) pyrenoid; (lv) lamellate vesicle surrounding the pyrenoid; (s) stalk; (g) reddish globule with droplets; (n) nucleus; (m) mitochondrion; (l) lamella composed of stack of three thylakoids; (cw) multilayered cell wall. Scale bars 1000 nm (a), 500 nm (c–d).

Sequences of 18S rDNA, a very conserved region, as well as of the ITS1–5.8S rDNA–ITS2 regions were therefore analysed in the isolated strains (E120, E5). Sequences were compared to each other and to the sequences of three *Eustigmatos* species obtained from the SAG collection. Nucleotide sequencing of 18S rDNA is an effective and generally accepted genetic marker for algal identification, classification and species differentiation (ANDERSEN et al. 1998). For example, SUDA et al. (2002) on the basis of differences in 18S rDNA sequences of ten strains of *Nannochloropsis* classified them into four different species. FIETZ et al. (2005) examined the 18S rDNA sequences of three other freshwater picoplanktonic *Nannochloropsis* strains and found them to be identical to each other and to the type strain of *Nannochloropsis limnetica* L. KRIENITZ, D. HEPPERLE, H.–B. STICH et W. WEILER. In contrast to *Nannochloropsis*, the molecular phylogeny of the genus *Eustigmatos* has not yet been described. Only the 18S rDNA sequences of *E. magnus*

(CCMP387) and *E. vischeri* (UTEX 310) are currently available in the GenBank for similarity analysis. The phylogenetic tree, constructed on the basis of the complete 18S rDNA sequences of fourteen different eustigmatophycean species, confirmed that E120, E5 strains belong to the genus *Eustigmatos*. The identical 18S rDNA sequences of E120 and E5 strains support the finding (based on the morphology of vegetative cells and zoospores) that they belong to the same, and not to any previously described *Eustigmatos* species. The number of divergences (2–3) in 18S rDNA between the isolates and other *Eustigmatos* species were similar to those (1–3) found among all the other nominal *Eustigmatos* examined. For example, the nucleotide sequence of 18S rDNA of the isolated strains differed in 2 or 3 positions from *E. magnus* and *E. vischeri*, respectively, while 3 divergences were found between *E. magnus* and *E. vischeri* and only 1 substitution between *E. vischeri* and *E. polyphem*. Genetic divergences and morphological differences between the isolated strains

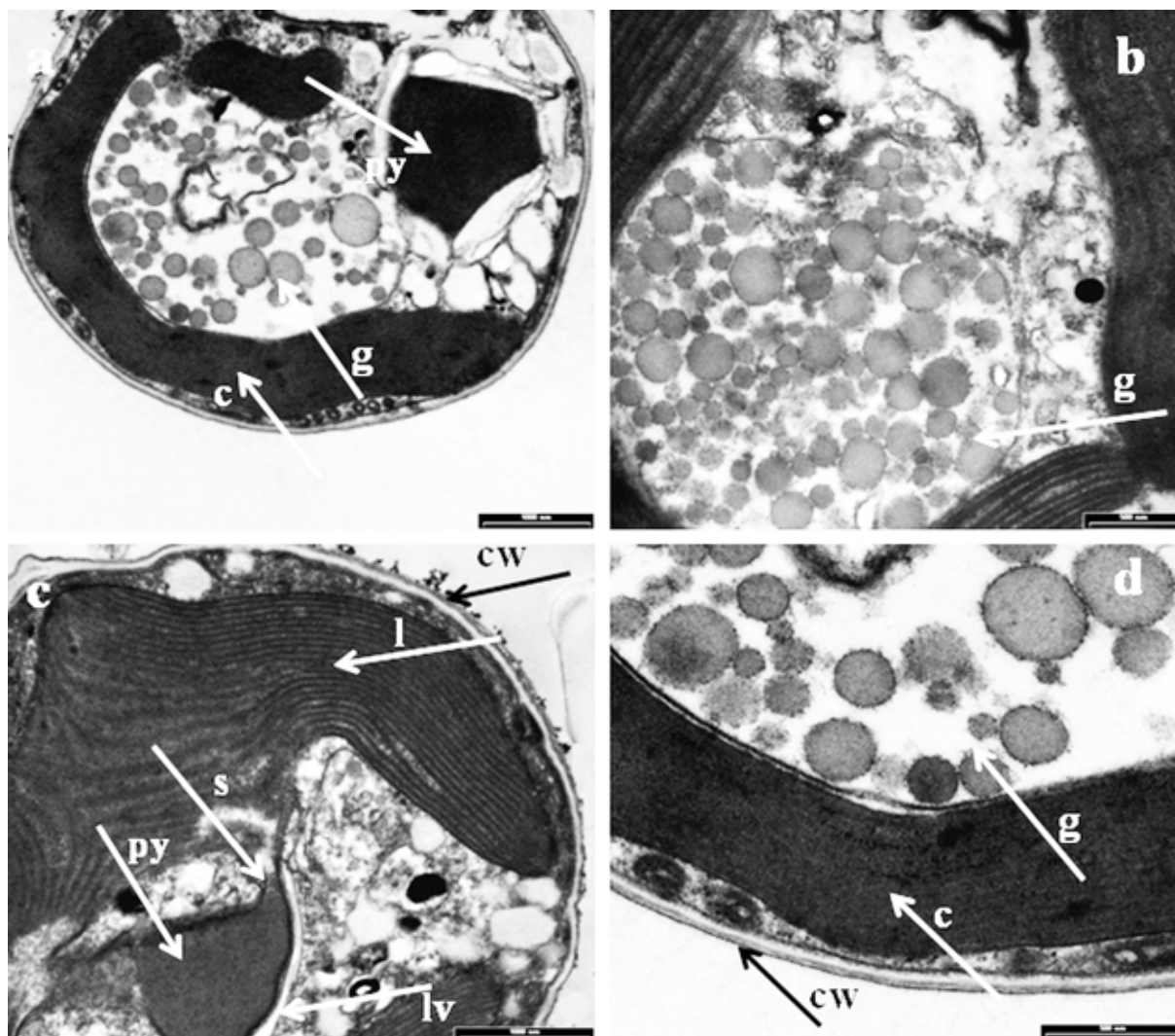


Fig. 4. Ultrastructure of *Eustigmatos* E5 strain; (c) chloroplast; (py) pyrenoid; (lv) lamellate vesicle surrounding the pyrenoid; (s) stalk; (g) reddish globule with droplets; (l) lamella composed of stack of three thylakoids; (cw) multilayered cell wall. Scale bars 1000 nm (a, c), 500 nm (b, d).

(E120, E5) and other *Eustigmatos* species indicate that they are a new species. Interestingly, the low number of divergences in 18S rDNA (3–6) were also found between the examined *Eustigmatos* species and *Vischeria helvetica*. This is in agreement with the report of ANDERSEN et al. (1998) concerning three differences between *E. magna* and *V. helvetica*. Although the 18S rDNA data (NJ tree) suggests placing the E120 and E5 strains into the genus *Vischeria*, morphological differences in cell shape and cell wall structure suggest they belong to *Eustigmatos*. In contrary to Eustigmataceae, the number of divergences in 18S rDNA sequences of the closely related *Nannochloropsis* spp. (Monodopsidaceae) was much higher. For example, 18S rDNA of *Nannochloropsis granulata* B. KARLSON et D. POTTER, *N. oculata* (DROOP) HIBBERD and *N. salina* HIBBERD differed from each other in 9 to 32 nucleotide substitutions and 1–4 insertions or deletions (KARLSON et al. 1996), while 2 or 4 substitutions in 18S rDNA (similar number to those stated among

particular *Eustigmatos* species) were reported for three subspecies of the *Nannochloropsis limnetica* (FAWLEY & FAWLEY 2007). The degree of intra-specific variation may be as extensive as that occurring between species or even orders. For example, LOWE et al. (2005) reported that differences in 18S rDNA of the *Oxyrrhis marina* DUJARDIN (dinoflagellates) inhabiting waters of different salinity were, in some cases, even higher (10.5%) than those found between two orders within dinoflagellates: *Prorocentrum micans* C.G. EHRENBURG and *Gonyaulax spinifera* (CLAPARÈDE et LACHMANN) DIESING (7.3%). This indicated that *O. marina* consists of many distantly related genotypes. According to FENCHEL (2005), genetic variation in 18S rDNA reflects the accumulation of neutral mutations and may or may not correlate with phenotypic differentiation. SCHECKENBACH et al. (2006) suggested that the phylogenetic markers, like slowly evolving 18S rDNA, might not be sufficient to detect ecophysiological differences between populations of

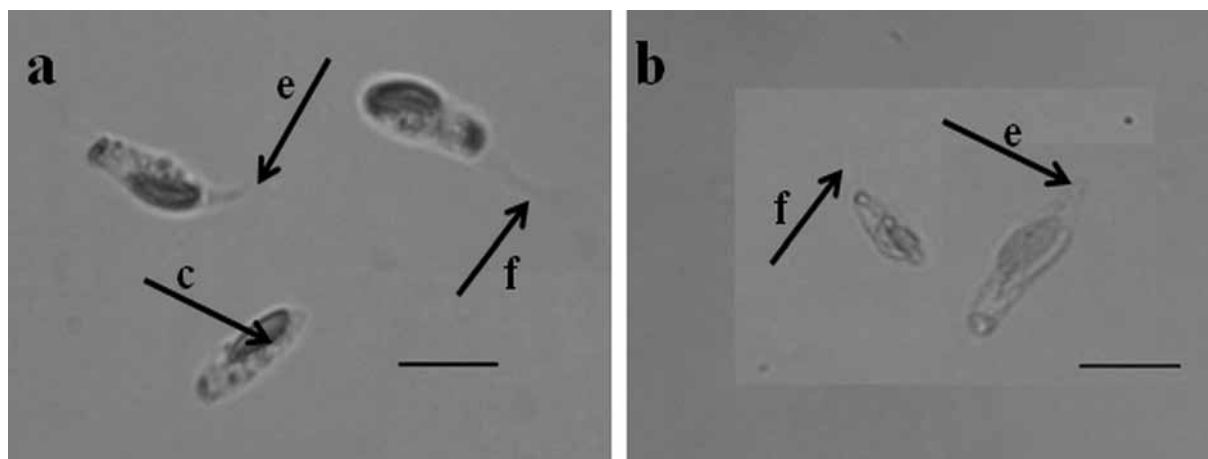


Fig. 5. Spindle-shaped zoospores of *Eustigmatos*; (c) chloroplast; (f) flagellum; (e) eyespot; (p) elongated posterior end of zoospore: (a) E120 strain; (b) E5 strain. Scale bars 10 μ m.

the same morphospecies. Comparison of 18S rDNA may therefore be applied only for identification at the genus level, while for species and subspecies, sequence analyses of the more variable regions such as ITS, are more appropriate (COLEMAN 2003). Thus far, no ITS sequences of any *Eustigmatos* species are available in GenBank. Our study revealed that ITS1–5.8S rDNA–ITS2 region of all nominal *Eustigmatos* species was more diverse (15–22 divergences) than 18S rDNA (1–3 substitutions). The number of differences (15–18 positions) between the isolated strains (E120, E5) and other *Eustigmatos* species was similar to those found among all examined *Eustigmatos* species. The morphological and genetic differences stated between them and the other *Eustigmatos* species were comparable to those observed among all the nominal *Eustigmatos* species (*E. vischeri*, *E. magnus*, *E. polyphem*). This suggests that the isolated strains represent a new species and the name *Eustigmatos calaminaris* sp. nov. (referred to the locality where they were found) has been proposed. Interestingly, the E5 and E120 strains only differed in one nucleotide substitution in the 5.8S rDNA region, whereas ITS1 and ITS2 sequences were identical. The small genetic difference between the E120 and E5 strains, together with the observed different response to Zn and Pb stress, suggests that they represent two ecotypes of the new species. Significant differences in Zn and Pb accumulation, as well as in the metal-induced oxidative stress (membrane lipid peroxidation) has recently been documented between the two strains (TRZCIŃSKA & PAWLIK-SKOWROŃSKA 2013). Both strains revealed also higher Zn and Pb resistance than *E. vischeri* (from SAG). This suggests that heavy metal stress experienced in natural habitats has induced the evolution of intrinsic defence mechanisms, such as metal avoidance or intracellular detoxification, in some cells. Selection of the more resistant species or ecotypes in heavy metal polluted sites has then occurred. FENCHEL (2005) suggested that genetic variation caused by selection in response to different

habitats (e.g., climates, salinities, temperatures) may evolve rapidly and/or independently on different occasions. Studies on microalgal ecotypes living in various habitats have revealed their diversity at ecophysiological, morphological and/or genetic levels (FIETZ et al. 2005; LOWE et al. 2005; RODRIGUEZ et al. 2005). LÓPEZ-RODAS et al. (2008) suggested that metal resistant microalgae inhabiting Cu-rich mine waters could be the descendants of chance mutants that arrived in the past or are even arriving at present. TAKAMURA et al. (1990) previously demonstrated genetically stable Cu-tolerance of eukaryotic benthic algae (occurring in metal polluted sites) which did not change even after 2-year cultivation in a Cu-free medium. It is therefore highly probable that the ecotypes E120 and E5 of the *E. calaminaris* sp. nov. exhibit different degrees of Zn- and Pb-resistance. These may have evolved through spontaneous mutations occurring at random which were preserved during their existence in diverse habitats. The E120 and E5 ecotypes displayed evident physiological differences in terms of metal-resistance in spite of there being very little genetic difference (regarding 5.8S rDNA). Mechanisms highlighting this phenomenon require further elucidation.

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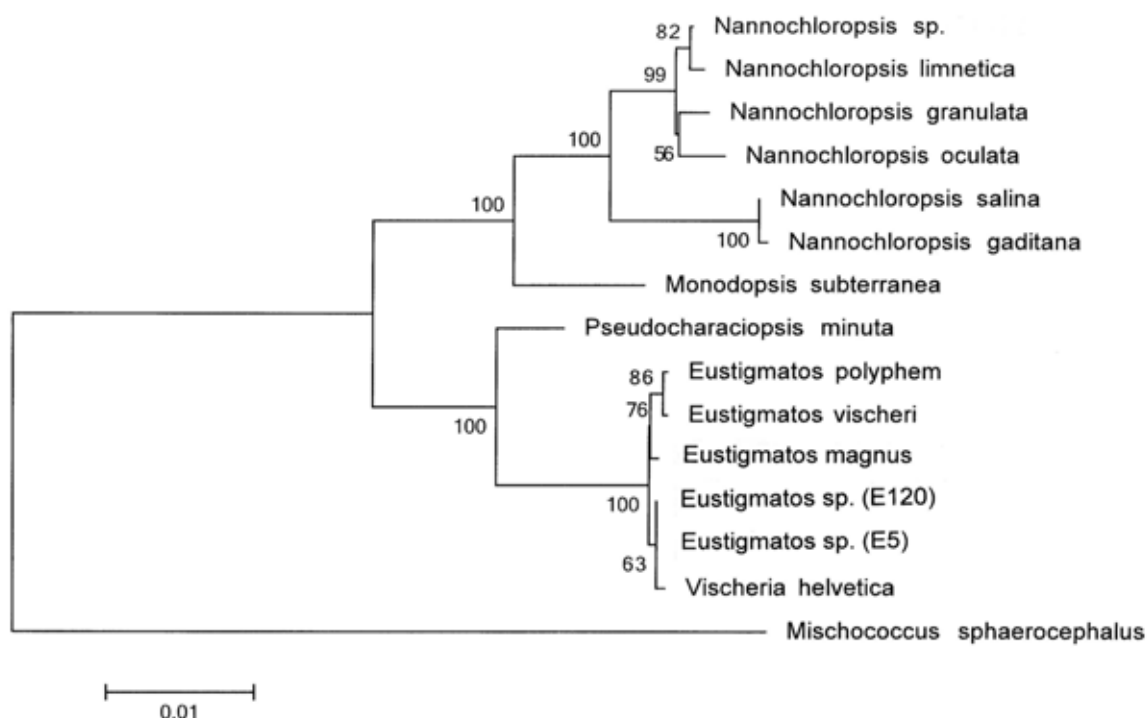


Fig. 6. A neighbor-joining phylogenetic tree based on 18S rDNA sequences of Eustigmatophyceae with *Mischococcus sphaerocephalus* (Xanthophyceae) as an outgroup taxon. Bootstrap value greater than 50% are shown. Scale bar 0.01 substitution per site.

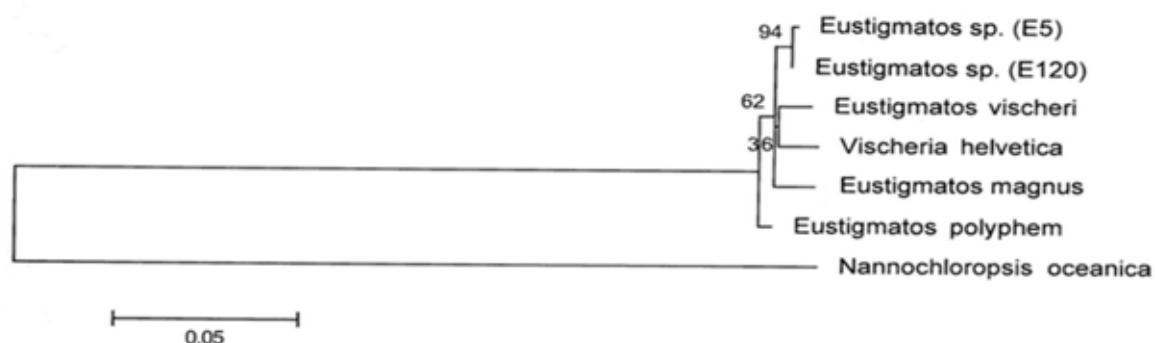


Fig. 7. A neighbor-joining phylogenetic tree of ITS1–5.8S rDNA–ITS2 for *Eustigmatos* species with *Nannochloropsis oceanica* (Eustigmatophyceae) as an outgroup taxon. Bootstrap values are shown above the branches. Scale bar 0.05 substitution per site.

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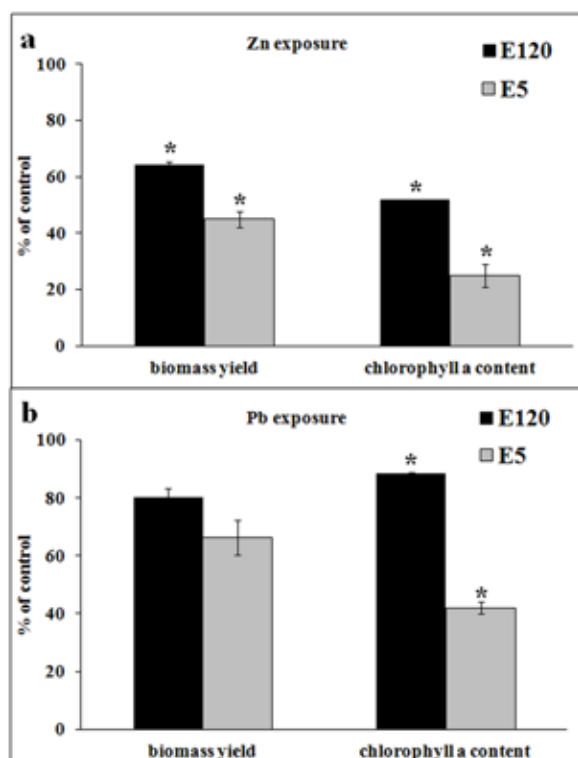


Fig. 8. Effect of high Zn (400 µM) and Pb (25 µM) concentrations on biomass and chlorophyll-*a* content in cultures of E120 and E5: (a) effect of 400 µM Zn; (b) effect of 25 µM Pb. Data are expressed as mean \pm SD ($n = 3$). Biomass and chl *a* in control cultures is set as 100%; Asterisk (*) indicate significant differences between strains in their biomass and chl-*a* content ($P < 0.05$).

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