Evolutionary Origin of *Euglena*

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Abstract

Euglenids (Excavata, Discoba, Euglenozoa, Euglenida) is a group of free-living, single-celled flagellates living in the aquatic environments. The uniting and unique morphological feature of euglenids is the presence of a cell covering called the pellicle. The morphology and organization of the pellicle correlate well with the mode of nutrition and cell movement. Euglenids exhibit diverse modes of nutrition, including phagotrophy and photosynthesis. Photosynthetic species (Euglenophyceae) constitute a single subclade within euglenids. Their plastids embedded by three membranes arose as the result of a secondary endosymbiosis between phagotrophic eukaryovorous euglenid and the *Pyramimonas*-related green alga. Within photosynthetic euglenids three evolutionary lineages can be distinguished. The most basal lineage is formed by one mixotrophic species, *Rapaza viridis*. Other photosynthetic euglenids are split into two groups: predominantly marine Eutreptiales and freshwater Euglenales. Euglenales are divided into two families: Phacaceae, comprising three monophyletic genera (*Discoplastis*, *Lepocinclis*, *Phacus*) and Euglenaceae with seven monophyletic genera (*Euglenaformis*, *Euglenaria, Colacium*, *Cryptoglena*, *Strombomonas*, *Trachelomonas*, *Monomorphina*) and polyphyletic genus *Euglena*. For 150 years researchers have been studying *Euglena* based solely on morphological features what resulted in hundreds of descriptions of new taxa and many artificial intra-generic classification systems. In spite of the progress towards defining *Euglena,* it still remains polyphyletic and morphologically almost undistinguishable from members of the recently described genus *Euglenaria*; members of both genera

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have cells undergoing metaboly (dynamic changes in cell shape), large chloroplasts with pyrenoids and monomorphic paramylon grains. Model organisms *Euglena gracilis* Klebs, the species of choice for addressing fundamental questions in eukaryotic biochemistry, cell and molecular biology, is a representative of the genus *Euglena*.

Keywords

Euglena • euglenids • Euglenales • Euglenophyceae • evolution • Excavata • phylogeny • taxonomy

Abbreviations

cpSSU	Cytoplasmic small subunit
EGT	Endosymbiotic gene transfer
hsp90	Heat shock protein 90
ITS	Internal transcribed spacer
LGT	Laterar gene transfer
nLSU	Nuclear large subunit
nSSU	Nuclear small subunit
psbO	Photosystem II manganese-stabilizing polypeptide
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase

1.1 The Phylogeny of Euglenids

Most of the knowledge about the group of protists called euglenids (Euglenida) comes from extensive studies of the photosynthetic model species *Euglena gracilis*. However, there are thousands of other euglenid species, which differ substantially from *E. gracilis* in morphology or mode of nutrition. Euglenids are a monophyletic group of free-living, single-celled flagellates (in most cases with two flagella) living predominantly in aquatic environments. Their common feature is a unique cell covering called the pellicle, a complex structure consisting of proteinaceous strips which are covered by a cell membrane and underlain by the microtubule system and the cisternae of the endoplasmic reticulum. The pellicle strips extend along the entire length of the cell. The adjacent strips are connected to each other and a sliding movement of one strip relative to another is possible. Cells with helically arranged strips and without fusions of adjacent strips can dynamically change their shape, a process called metaboly, euglenoid motion or euglenoid movement (Leander et al. 2007; Farmer 2011).

Euglenids are closely related to heterotrophic flagellates Symbiontida (free-living flagellates living in low-oxygen marine sediments), Diplonemea (free-living marine flagellates) and Kinetoplastea (free-living and parasitic flagellates, e.g. *Trypanosoma*) (Fig. 1.1). Euglenids, symbiontids, diplonemids and kinetoplastids form a monophyletic group Euglenozoa (Cavalier-Smith 1981; Breglia et al. 2010; Burki 2014). There are three main synapomorphies shared by all members of Euglenozoa: (a) a unique pattern of flagellar root with two basal bodies and three asymmetrically arranged microtubular roots, (b) the presence of a paraxial rod in one or both flagella, (c) the presence of tubular extrusomes (e.g. trichocysts, mucocysts) (Cavalier-Smith 1981; Simpson 1997; Farmer 2011). Molecular and morphological analyses also indicate that Euglenozoans are members of a larger group known as Excavata – one of the five supegroups of Eukayotes – where they form a sister clade to Hetrolobosea (e.g. parasitic species *Naegleria fowleri*) (Hampl et al. 2009; Yabuki et al. 2011; Adl et al. 2012; Burki 2014). However, there are also some reports indicating that the root of Eukayotes should be placed within excavates and that Euglenozoa is the first group which branched off from the main evolutionary lineage of eukaryotes (Cavalier-Smith 2010; Burki 2014). Whereas the monophyly of the Euglenozoa is indisputable, the internal relationships among kinetoplastids, diplonemids, symbiontids

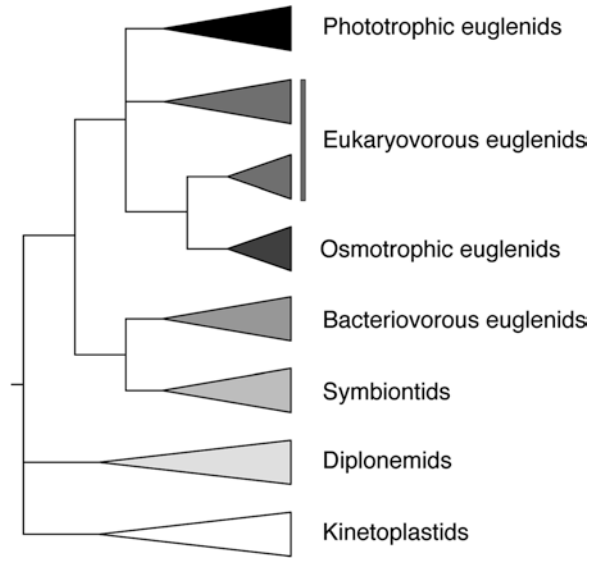

Fig. 1.1. The schematic phylogenetic tree of Euglenozoa according to Breglia et al. 2013

and euglenids are less clear. The most controversial is the phylogenetic position of symbiontids, which group together with bacteriovorus euglenids – in effect the Euglenida seems to be paraphyletic (Yamaguchi et al. 2012; Breglia et al. 2013). Symbiontids are a small group of microaerophilic or anaerobic euglenozoans enveloped by episymbiotic bacteria. In contrast to euglenids, their cells are not covered by a pellicle. So far only three species were classified as symbiontids and further analysis is obviously needed to resolve their relationships with euglenids (Yubuki et al. 2009, 2013; Breglia et al. 2010).

Several different modes of nutrition are observed within euglenids. Most species are heterotrophs (bacteriotrophs, eukaryotrophs or osmotrophs), but there is also one lineage of plastid-bearing photoautotrophs and mixotrophs (Fig. 1.1). The changes of nutritional mode can be tracked on phylogenetic trees (Leander et al. 2007). The basal lineages of euglenids contain phagotrophic species with rigid cell covering; they feed on small prey cells like bacteria. Later more flexible and larger species evolved which feed on larger cells such as other protists. A more elastic cell covering of eukaryovores is the effect of a higher number of helically arranged pellicle strips. Two monophyletic groups of euglenids evolved independently from eukaryovorous ancestors: osmotrophs and photoautotrophs. This indicates that changes in the mode of nutrition, from phagotrophy to osmotrophy as well as from phagotrophy to phototrophy, took place once in the evolution of the group (Linton et al. 1999; Preisfeld et al. 2000, 2001; Müllner et al. 2001; Marin et al. 2003). The rise of phototrophic euglenids (ophyceae) was the result of an unprecedented evolutionary event: a secondary endosymbiosis between a phagotrophic eukaryovorous euglenid and a green alga closely related to extant members of the *Pyramimonas* genus (Gibbs 1978; Turmel et al. 2009; Hrdá et al. 2012). In the most likely scenario, the alga was engulfed and enslaved by the host cell, resulting in chloroplasts surrounded initially by four membranes, two membranes of the primary plastid, the plasma membrane of the green alga and the food vacuole membrane of the euglenid. Subsequently, one of the membranes has been lost, since plastids in all known phototrophic euglenids are surrounded by three membranes. The alternative scenario assumes that chloroplasts of euglenids have never been surrounded by four membranes because the endosymbiont was not acquired by phagocytosis but rather by myzocytosis so that only the cellular contents of the green alga including the chloroplasts were sucked into the food vacuole of a heterotrophic species (Delwiche 1999; Farmer 2011). Apart from the number of membranes, euglenid chloroplasts are

very similar to chloroplasts found in green algae. They contain chlorophyll *a* and *b* and their tylakoids are arranged in groups of three. However, their storage carbohydrate is different. While green algae synthesize starch in the plastids, phototrophic as well as heterotrophic euglenids synthesize a β-1,3-glucan, paramylon, in the cytoplasm. (Kiss et al. 1987). This suggests that the ability to synthesize paramylon must have been present before acquisition of the chloroplasts. Chloroplasts have so far been shown to be present among all genera of Euglenophyceae. There are however known secondary heterotrophic species, which have lost photosynthetic ability. The best known example of such an euglenid is *Euglena longa*, a close relative of *E. gracilis*. This species contains photosynthetically inactive plastids with a reduced plastid genome (Gockel and Hachtel 2000). It is worth mentioning that phototrophic euglenids are not the only eukaryotic group with secondary plastids acquired from green algae. Chlorarachniophytes and dinoflagellates from the genus *Lepidodinium* have chloroplasts acquired from green algae but this does not reflect a close evolutionary relationship. The chloroplasts of euglenids (Excavata), chlorarachniophytes (Rhizaria) and *Lepidodinium* dinoflagellates (Alveolata) were acquired in independent endosymbiotic events from different groups of green algae: prasinophytes, core chlorophytes (Rogers et al. 2007; Turmel et al. 2009) and peridinophytes (Kamikawa et al. 2015), respectively.

The acquisition of chloroplasts by the ancestor of green euglenids was accompanied by endosymbiotic gene transfer (EGT) from the plastid and nuclear genomes of the endosymbiont to the nuclear genome of the host. This process not only integrated the new organelle into the euglenid cell but it also allowed new metabolic pathways to emerge. Surprisingly, it was revealed that the *E. gracilis* nuclear genome contains not only genes of green algal origin but also genes of red algal origin. Genes of red algal origin have also been found in the primary heterotrophic euglenid *Peranema trichophorum* (Maruyama et al. 2011). It is uncertain whether red algal genes were acquired by lateral gene transfer (LGT) or whether they are evidence of a past endosymbiosis with a red alga. Nonetheless, the observed mosaicism of euglenid genome is a result of acquisition of genes derived from not only a distant ancestor and a green algal endosymbiont but also from other evolutionary lineages (Maruyama et al. 2011). For example, several genes encoding enzymes of the Calvin-Benson cycle share an ancestry with red algae and/or chromophytes (organisms with secondary plastids derived from red-algae) (Markunas and Triemer 2016). It seems that some of these genes were already present in the cell of the phagotrophic euglenid which enslaved the green algal endosymbiont and their presence would have facilitated the transition from an endosymbiont to a chloroplast (Maruyama et al. 2011; Markunas and Triemer 2016). Such examples show the impact of lateral/endosymbiotic gene transfers (LGT/EGT) on the evolutionary history of euglenids, the structure of their genomes and the position they hold in ecological niches.

Within the group of Euglenophyceae, three evolutionary lineages can be distinguished (Adl et al. 2012) (Fig. 1.2). The most basal lineage is formed by the genus *Rapaza* with one known marine mixotrophic species, *Rapaza viridis*. This species contains functional chloroplasts but its photosynthetic capacity is probably not sufficient to support growth. The crucial component of the *R. viridis* diet is a green alga prey *Tetraselmis* sp., which is engulfed by phago- or myzocytosis (Yamaguchi et al. 2012). Other green euglenids are split into two groups: Eutreptiales, which comprises species living in marine and brackish environment and Euglenales (=Euglenea) containing species living mostly in small, astatic freshwater reservoirs. The cells of Eutreptiales (two genera: *Eutreptia* and *Eutreptiella*) have 2–4 emergent flagella of equal or unequal length. Because of the presence of helically arranged pellicle strips without fusions they undergo dynamic metaboly. The members of Euglenales have a single emergent flagellum, whereas the second one is hidden within a reservoir – an invagination at the anterior end of the cell (Adl et al. 2012). The Euglenales group is divided into two families: (a) Phacaceae, comprising three monophyletic genera (*Discoplastis*, *Lepocinclis*,

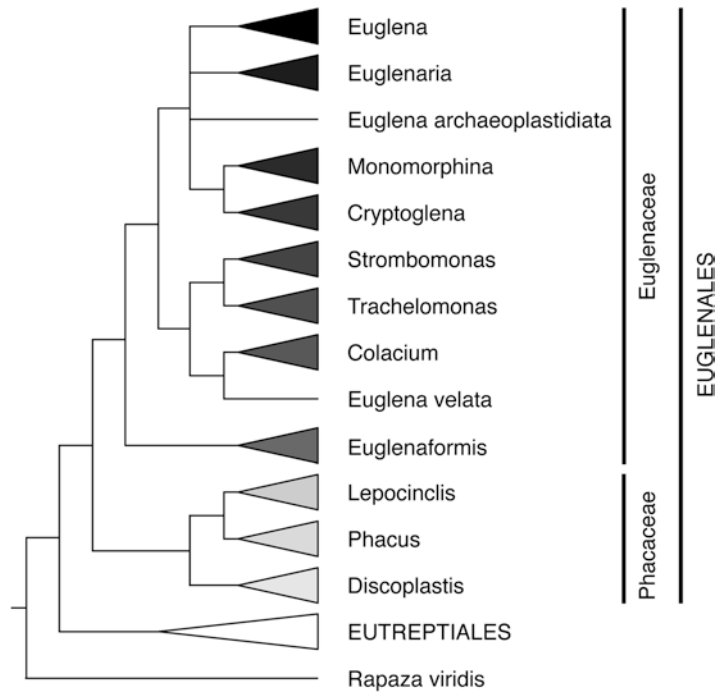

Fig. 1.2. The schematic phylogenetic tree of phototrophic euglenids (Euglenea) according to Karnkowska et al. 2015 and Kim et al. 2015

Phacus) and (b) Euglenaceae with seven monophyletic genera (*Euglenaformis*, *Colacium*, *Strombomonas*, *Trachelomonas*, *Monomorphina*, *Cryptoglena*, *Euglenaria*) (Kim et al. 2010). *Euglena*, the eighth genus of the Euglenaceae family, seems to be polyphyletic and needs reclassification (Karnkowska et al. 2015; Kim et al. 2015). Two species, *Euglena archaeoplastidiata* and *Euglena velata* always remain outside of the main *Euglena* clade and their phylogenetic position is uncertain (Fig. 1.2).

1.2 The Taxonomy of *Euglena*: Nineteenth and Twentieth century

Taxonomic research on phototrophic euglenids dates back to 1830 when Ehrenberg established the genus *Euglena* by grouping together four already described taxa: *Euglena viridis* (= *Cercaria viridis* Müller), *E. sanguinea* (= *Enchelys sanguinea* Ness and Golgfufs), *E. acus* (= *Vibrio acus* Müller), *E. pleuronectes* (= *Cercaria pleuronectes* Müller) with two new species he discovered (*E. pyrum* and *E. longicauda*) (Ehrenberg 1830). During his lifetime Ehrenberg described many new taxa in the genus *Euglena* and established three new genera: *Cryptoglena* Ehrenberg (1831), *Colacium* Ehrenberg (1833) and *Trachelomonas* Ehrenberg (1833); Ehrenberg's original drawings are now available online at the Museum in Berlin (http://download.naturkunde-museum-berlin.de/Ehrenberg/Ec%20Drawings/).

For the next 150 years many researchers have been studying the diversity of euglenids and many new species have been described based solely on their morphological features. This resulted in descriptions of thousands of new taxa (3200 validly published names listed in AlgaeBase: http://www.algaebase.org) and new genera (*Phacus* Dujardin 1841, *Lepocinclis* Perty 1849, *Eutreptia* Perty 1852, *Monomorphina* Mereschkowsky 1877, *Eutreptiella* Da Cunha 1913, *Strombomonas* Deflandre 1930). Many species from the genus *Euglena* were successively transferred to other genera but at the same time, the genus *Euglena* served as kind of a "bag" into which all newly described species that "do not fit" morphologically to other genera were "thrown". That resulted in a morphologically heterogeneous group and correct identification of its

representatives was very difficult. To facilitate the recognition of *Euglena* members, subsequent investigators proposed various intra-generic classification systems. Unfortunately, they were based only on morphological features that different researchers considered as diagnostic in an arbitrary manner. For example, Gojdics (1953) distinguished eight "groups" (A-E) based on chloroplast organization. Pringsheim (1956) distinguished five "tentative Groups (Taxa) or subgenera" (Rigidae, Lentiferae, Catilliferae, Radiatae, Serpentes) based on cell shape and chloroplast morphology. Zakryś (1986) distinguished three subgenera (Euglena, Calliglena and Discoglena) taking into account the number, morphology and position of the chloroplasts in the cells, the number and morphology of paramylon grains and presence or absence of pyrenoids. As mentioned before, our understanding of morphological features was changing substantially over the time and more recently it started to be interpreted in the context of the evolution of autotrophic euglenids.

1.3 The Main Morphological Diagnostic Features

Detailed studies on morphological plasticity and tracing morphological characters on the reliable phylogenetic tree were unable to decipher the evolution of the characters and define diagnostic features for individual clades. Two characters, the presence of lorica and mucilaginous stalks, are synapomorphic (lorica for the clade grouping *Strombomonas* and *Trachelomonas* and mucilaginous stalks for *Colacium*). Evolution of other characters, cell metaboly, morphology of chloroplasts, presence of pyrenoids, morphology of paramylon grains and the presence and shape of mucocysts, was more complicated but they also can serve as useful diagnostic features.

Metaboly: The morphology and organization of the pellicular strips correlates well with the cell plasticity. Cells with fewer strips tend to be rigid while those with many strips often exhibit euglenoid movement called also metaboly (Leander et al. 2007; Farmer 2011). The ancestral state reconstruction suggested that the ancestor of the freshwater photosynthetic euglenids was characterized by cell undergoing dynamic metaboly and that is the predominant character observed among extant photosynthetic euglenids, including *Euglena*. Rigid cells among Euglenophyceae arose twice. Once in the common ancestor of *Phacus* and *Lepocinclis* and once in the common ancestor of *Monomorphina* and *Cryptoglena* (Karnkowska et al. 2015).

Chloroplasts: Chloroplasts of euglenids exhibit great morphological diversity. The ancestor of freshwater autotrophic euglenids probably had numerous small discoid plastids (a few micrometers in diameter) without a pyrenoid. This type of chloroplast is characteristic for representatives of the family Phacaceae and the genus *Euglenaformis,* the most basal clade of the family Euglenaceae (Karnkowska et al. 2015). Other Euglenaceae are characterized by larger chloroplasts but they vary in shape (stellate, lobed or spherical), size, number per cell (from one to numerous) and location (parietal, partly parietal or axial). Typically, they possess pyrenoids.

Pyrenoid: One of the structural features associated with many euglenoid chloroplasts is the pyrenoid, an area in which ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) tends to be concentrated (Osafune et al. 1990). Pyrenoids are not visible under the light microscope but often the pyrenoids are accompanied by a bilateral paramylon cap, "diplopyrenoid", a single paramylon cap "haplopyrenoid" or a cluster of small paramylon grains, "paramylon center"; in some species pyrenoid is "naked". Often the presence of the pramylon cap indicates the presence of the pyrenoid. The common ancestor of the freshwater photosynthetic euglenids most probably had pyrenoids as supported by the presence of pyrenoids in the green algae related to the possible donor of the *Euglena* chloroplast. However, not all autotrophic euglenids possess pyrenoids and it was proposed that they were lost independently twice, in the common ancestor of

the family Phacaceae, and in the genus *Euglenaformis* (Karnkowska et al. 2015).

Paramylon: The storage material paramylon, a β-1, 3 glucan, can be found free in the cytoplasm or may be associated with the chloroplasts forming "caps" or "paramylon centers" over the pyrenoids. Small paramylon grains are observed in all euglenoid cells. In addition to these ubiquitous small grains, many phototrophic species produce a larger type of paramylon grains (Monfils et al. 2011). The presence of small and large grains is referred to as "dimorphic" in contrast to "monomorphic" when only small grains are present in the cell. An ancestral state reconstruction suggested that dimorphic paramylon grains evolved twice, once in the common ancestor of the family Phacaceae, and once in the common ancestor of *Monomorphina* and *Cryptoglena*, members of the family Euglenaceae (Karnkowska et al. 2015).

Mucocysts: Mucocysts are membrane bound small bodies with openings, positioned under the pellicle, which contain mucilaginous threads. Mucocysts are always uniform in size and shape, either spherical or spindle-shaped, arranged in parallel rows which follow the spiral alignment of the pellicle strips. In some taxa, the mucocysts are readily visible but in most cases they are visible only after staining with neutral red. Mucocysts are only characteristic for some representatives of *Euglena*. Ancestral state reconstruction indicated that the common ancestor of *Euglena* lacked mucocysts so they probably appeared independently at least two times in the genus *Euglena* (Karnkowska et al. 2015).

1.4 The Current View on the Taxonomy and Phylogeny of *Euglena*

The advent of molecular phylogenetics and combination of molecular and morphological data has had a huge impact on our understanding of the relationships within the genus *Euglena* and in the whole group of euglenids. The first phylogenetic tree was based on nuclear small subunit (nSSU) rDNA sequences including only four species of euglenids (Montegut-Felkner and Triemer 1997). Later, the number of species used for phylogenetic analyses increased (Linton et al. 1999, 2010; Moreira et al. 2001; Müllner et al. 2001; Marin et al. 2003; Nudelman et al. 2003; Shin and Triemer 2004; Kosmala et al. 2007a, b; Karnkowska-Ishikawa et al. 2010, 2011, 2012, 2013; Łukomska-Kowalczyk et al. 2015) and other molecular markers were employed: internal transcribed spacer (ITS) (Zakryś et al. 2004), nuclear large subunit (nLSU) rDNA (Brosnan et al. 2003; Ciugulea et al. 2008; Linton et al. 2010; Kim et al. 2012, 2013; Kim and Shin 2014), cytoplasmic small subunit (cpSSU rDNA) (Zakryś et al. 2002; Milanowski et al. 2001, 2006; Linton et al. 2010), chloroplast LSU rDNA (Kim and Shin 2008; Kim et al. 2015) and nuclear encoded protein coding sequences of *hsp*90 and *psb*O genes (Karnkowska et al. 2015).

The molecular phylogenetic trees clearly demonstrated that the "bag all-catch" genus *Euglena* was indeed paraphyletic and/or polyphyletic (Linton et al. 1999, 2010) and required the transfer of several of the more rigid "*Euglena*" species with numerous small discoid plastids without pyrenoids (classified into subgenus *Discoglena sensu* Zakryś 1986, e.g. *Euglena acus* (O.F. Müller) Ehrenberg, *E. oxyuris* Schmarda, *E. tripteris* (Dujardin) Klebs, *E. spirogyra* Ehrenberg, *E. fusca* (Klebs) Lemmermann) to the genus *Lepocinclis* (Marin et al. 2003; Kosmala et al. 2005). That was the first step in a major reorganization of the genus *Euglena*. The new genus *Discoplastis* was created for two taxa, *E. spathirhyncha* Skuja and *E. adunca* Schiller, both having highly metabolic cells and numerous small discoid plastids without pyrenoids, and formed a well suported clade near the base of the euglenoid lineage independent of the *Euglena* clade (Triemer et al. 2006). Similar plastids were also observed in *E. proxima*, but the species formed an independent lineage from *Discoplastis* and *Euglena* (Milanowski et al. 2006), and was finally described as a new genus *Euglenaformis* based on phylogenomic analyses (Bennett et al. 2014). After this change,

all taxa with small numerous discoid chloroplasts without pyrenoids were moved from the genus *Euglena*, which now contains only species with large stellate or lobed chloroplasts with pyrenoids. Surprisingly, phylogenetic analyses revealed that three more species, *Euglena anabaena* Mainx, *E. caudata* Hübner and *E. clavata* Skuja, formed well-supported clade outside of the *Euglena* despite having morphology that fit well into the genus *Euglena*. This group of cryptic species was transferred into a new genus *Euglenaria* (Linton et al. 2010). Currently the genus *Euglenaria* contains four species after including *Eu. clepsydroides* Zakryś, a newly discovered species recently found in Poland (Zakryś et al. 2013). Nevertheless, the genus *Euglena* still remains paraphyletic/polyphyletic with two species branching out of the main *Euglena* trunk, *Euglena archaeoplastidiata* and *Euglena velata*. On phylogenetic trees, *E. archaeoplastidiata* diverges prior to the *Monomorphina/Cryptoglena* or the *Euglenaria* clade (Karnkowska et al. 2015; Kim et al. 2015) while the *E. velata* strain complex is sister to the genus *Colacium* (Kim et al. 2015) (Fig. 1.2). *Euglena archaeoplastidiata* has a single, parietal chloroplast similar to those observed in *Monomorphina* and *Cryptoglena* but some characters like the presence of only small paramylon grains (monomorphic), diplopyrenoids (pyrenoid with two paramylon caps) and metaboly are more similar to the characters of *Euglena* or *Euglenaria*. *Euglena velata* shares morphological features with representatives of the genus *Euglena* (or *Euglenaria*).

Comparative morphological and molecular studies have revealed that many taxa from the genus *Euglena* described in the literature are taxonomically not justified. For many species, diagnostic descriptions were emended, epitypes established, keys to correct identification were created and complicated nomenclature issues were sorted out (Zakryś et al. 2002; Shin and Triemer 2004; Kosmala et al. 2009; Karnkowska-Ishikawa et al. 2012, 2013), and two new species were described (*E. pseudostellata* Zakryś and Kosmala and *E. pseudochadefaudii* Zakryś and Kosmala) (Kosmala et al. 2009). In summary, to-date about 150 taxa have been verified taxonomically representing almost one third of all the taxa described in the genus *Euglena* (AlgaeBase: http://www.algaebase.org provides 560 validly published names of species, varieties and forms).

1.5 The Taxonomic Future of the Genus *Euglena*

Recent advances in the taxonomy of the genus *Euglena* are the result of the increased taxon sampling in phylogenetic analyses. New lineages, not related with *Euglena*, but previously classified in this genus based on their morphology (Marin et al. 2003; Triemer et al. 2006; Linton et al. 2010) have been revealed. In spite of the progress in the taxonomy of *Euglena*, it still remains polyphyletic. Moreover, many more taxa resembling *Euglena* have been described but not studied at the molecular level so far.

The most commonly used molecular marker for autotrophic euglenids is nuclear SSU rDNA, with available sequences representing 148 taxonomically verified taxa. That is less than 5% of all described taxa and as a consequence, most genera are poorly represented on the phylogenetic trees (Fig. 1.2). Those sequences mainly represent most common species deposited in culture collections and do not reflect the large morphological diversity of phototrophic euglenids. Only 20% of the sequences are from taxa recently isolated from the environment.

Almost all of the strains from the culture collections have been already sequenced, therefore more strains need to be isolated from the environment. However, establishing new cultures is a very labour-intensive and time-consuming process with a very low efficiency because many of species do not survive in the laboratory conditions (Bennett and Triemer 2012; Lax and Simpson 2013). New approaches such as multiple displacement amplification using just a few cells (Bennett and Triemer 2012) or the single-cell approach (Lax and Simpson 2013) have recently been shown to be a valuable tool to overcome those limitations and have been employed to study the biodiversity and phylogeny of both

phototrophic (Bennett and Triemer 2012; Łukomska-Kowalczyk et al. 2015) and phagotrophic euglenids (Lax and Simpson 2013).

1.6 The Molecular Identification of Photosynthetic Euglenids

Knowledge about the biodiversity of euglenids is limited mainly due to difficulties with species identification based on morphological characters and species definition in an asexual group. Nowadays, molecular techniques of identification are an increasingly valuable tool for studying the diversity of this group. One of the most widely used methods for molecular identification is DNA barcoding. This technique is based on the assumption that the diversity of short DNA fragments reflects the diversity of the organisms themselves (Hebert et al. 2003). This is true for many groups of organisms and valuable barcodes have been assigned and successfully used in animals, fungi and plants. The species assignment is based on a comparison of the sequence of interest with the reference database. Once a good barcode and reference database are established, the method is fast and accurate (Blaxter 2004). It is a valuable tool for biodiversity research (Hajibabaei et al. 2007), detection of a particular species in the environment (Hajibabaei et al. 2011), detection of new species (Hajibabaei et al. 2007) and ecological studies (Joly et al. 2014). A database of nSSU rDNA sequences of taxonomically validated species (reference sequences) was recently established for autoptrophic euglenids and the variable regions, V2-V3 and V4, of nuclear rDNA were recommend as DNA barcodes (Łukomska-Kowalczyk et al. 2016). Using DNA barcoding will facilitate research on biodiversity of autotrophic euglenids and will enable research on species other than *Euglena gracilis.* The majority of applied and basic research utilize *E. gracilis* Klebs 1883 mainly because it is easy to isolate, maintain in the laboratory, and there are many strains of *E. gracilis* in the culture collections of algae including the best-known strain "Z". It is not a species commonly found in a typical for euglenids eutrophic habitats that have a high content of organic compounds and it does not create blooms as seen for many other representatives of the autotrophic euglenids e.g. *Euglena stellata*, *Euglena sanguinea*, *Euglenaria anabaena*, *Lepocinclis oxyuris* or *Phacus salina* (long-term observations of the authors). For these reasons, *E. gracilis* is not a typical representative inhabitant of the eutrophic and hyper eutrophic environment where euglenids are very common. Molecular identification methods (e.g. barcodes) can change the status quo by allowing isolation of new species (characteristic for some environments, toxic or rare) from natural populations for many kinds of basic and applied research such as industrial wastewater purification, the production of biofuels or the development of new monitoring methods which could predict the mass appearance of toxic species (like *Euglena sanguinea*) in fish breeding ponds, recreational places or drinking water reservoirs.

1.7 The Current Classification of Euglenids

Taking into account the recent changes in the taxonomy of euglenids, the current classification is as follows:

EXCAVATA Cavalier-Smith 2002, *emended* by Simpson 2003

Euglenozoa Cavalier-Smith 1981, *emended* by Simpson 1997

- Euglenida Bütschli 1884, *emended* by Simpson 1997
- Euglenophyceae Schoenichen 1925 (in Eyferth and Schoenichen 1925), *emended* by Marin and Melkonian 2003 (in Marin et al. 2003)
- Euglenales Leedale 1967, emended by Marin and Melkonian 2003 (in Marin et al. 2003) (= Euglenea Bütschli 1884, emended by Busse and Preisfeld 2003)
- Euglenaceae Dujardin 1841, emended by Kim, Triemer and W. Shin 2010 (in Kim et al. 2010)
- *Euglena* Ehrenberg 1830, emended by Marin and Melkonian 2003 (in Marin et al. 2003)
- *Cryptoglena* Ehrenberg 1831, emended by Kosmala and Zakryś 2007 (in Kosmala et al. 2007a)
- *Colacium* Ehrenberg 1833

- *Trachelomonas* Ehrenberg 1833
- *Monomorphina* Mereschkowsky 1877, *emended* by Kosmala and Zakryś 2007 (in Kosmala et al. 2007a)
- *Strombomonas* Deflandre 1930
- *Euglenaria* Karnkowska, Linton and Kwiatowski 2010 (in Linton et al. 2010)
- *Euglenaformis* M. S. Bennett and Triemer 2014 (in Bennett et al. 2014)
- Phacaceae Kim, Triemer and W. Shin 2010 (in Kim et al. 2010)
- *Phacus* Dujardin 1841, *emended* by Linton and Karnkowska 2010 (in Linton et al. 2010)
- *Lepocinclis* Perty 1849, *emended* by Marin and Melkonian 2003 (in Marin et al. 2003)
- *Discoplastis* Triemer 2006 (in Triemer et al. 2006)
- Eutreptiales Leedale 1967, *emended* by Marin and Melkonian 2003 (in Marin et al. 2003)
- Eutreptiaceae Hollande 1942
- *Eutreptia* Perty 1852
- *Eutreptiella* Da Cunha 1913, *emended* by Marin and Melkonian 2003 (in Marin et al. 2003)
- *Rapaza* Yamaguchi, Yubuki and Leander 2012 (in Yamaguchi et al. 2012)

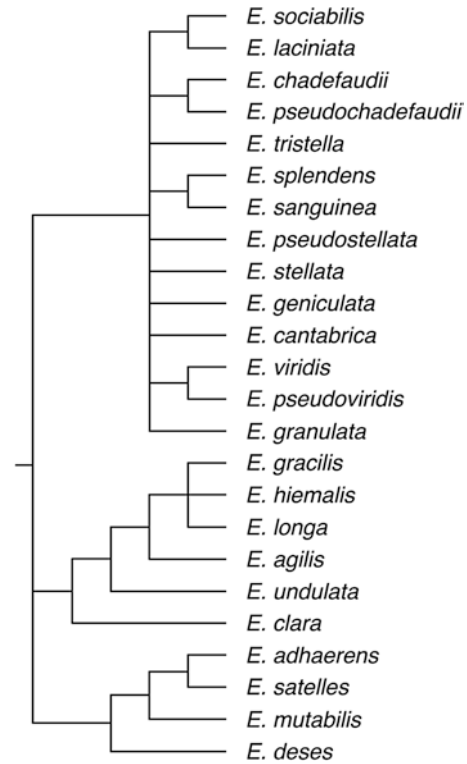

Fig. 1.3. The schematic phylogenetic tree of the genus *Euglena* according to Karnkowska et al. 2015 and Kim et al. 2015

1.8 Short Descriptions of the Families and Genera Classified in the Euglenales

The family Euglenaceae Dujardin 1841, *emended* by Kim et al. 2010

Comprise eight genera whose members have one emergent flagella and large chloroplasts with pyrenoids. The exception to this is the genus *Euglenaformis* with chloroplasts without pyrenoids.

Euglena Ehrenberg 1830, *emended* by Marin and Melkonian 2003

After all the recent reclassifications the genus *Euglena* includes only species with not flattened cells undergoing dynamic metaboly, characterized also by monomorphic paramylon grains (except some physiological forms of *E. deses*) and large chloroplasts with pyrenoids. Based on the number and shape of the chloroplasts, their location in the cell, parietal, partly parietal, axial, and the form of paramylon accompanying the pyrenoids, diplopyrenoids, haplopyrenoids, paramylon centers, naked pyrenoids, all species can be divided into four morphological groups (Triemer and Zakryś 2015).

1. Species with a single, parietal, spherical chloroplast and two diplopyrenoids. Only one species (*E. archaeoplastidiata*) represents this morphogroup but its position on the phylogenetic tree is unstable (Fig. 1.2).
2. Species with axial, stellate chloroplasts (1, 2 or 3) with each chloroplast having a paramylon center. There are nine species in this group which are accepted after the latest taxonomic revision: five taxa with one chloroplast: *E. cantabrica, E. stellata, E. pseudostellata, E. viridis E. pseudoviridis*; three taxa with two chloroplasts: *E. geniculata, E. chadefaudii, E. pseudochadefaudii,* and one species with three chloroplasts; *E. tristella* (Kosmala et al. 2009). Most species are common and cosmopolitan

in bodies of water rich in organic compounds. Fusiform or spherical mucocysts are present in most species which is a very useful diagnostic feature. Members of this group do not form a monophyletic clade on the phylogenetic tree (Fig. 1.3).

3. Species with parietal, lobed chloroplasts. Each chloroplast has a single pyrenoid (naked, diplo- or haplopyrenoid). *Euglena gracilis* belongs to this morphogroup and is closely related to *E. hiemalis*, a species hardly distinguishable morphologically from *E. gracilis,* and *E. longa*, a colourless species. Although *Euglena gracilis* is a model species for studies on phototrophic euglenids, its taxonomy and taxonomy of closely related species has not been emended so far based on molecular phylogenetic analyses. Some species are very common and cosmopolitan, *E. agilis, E. deses* or *E. granulata.* They can be identified based only on morphological diagnostic features but in the case of most species it is much easier to distinguish them at the molecular level and sometimes this is the only way to separate them (Karnkowska et al. 2012) (Figs. 1.2 and 1.3).
4. Species with plate-like, partially parietal chloroplasts where the centers of the chloroplasts are located deep within the cytoplasm while the rest of the chloroplast surface, which is radially cut into long bands, reaches the pellicle. Each chloroplast has a diplopyrenoid. Fusiform mucocysts are present in cells of all species. There are four currently accepted species in this group, *E. laciniata*; *E. sanguinea, E. sociabilis* and *E. splendens* (Karnkowska-Ishikawa et al. 2013). All of them are common and cosmopolitan. Toxic blooms of *E. sanguinea* were recently observed (Zimba et al. 2004). Ichthyotoxins produced by this species cause important economic losses in commercial agriculture ponds in the United States (Zimba et al. 2004, 2010). Members of this group do not form a monophyletic clade on the phylogenetic tree (Fig. 1.3).

- ***Euglenaria*** Karnkowska, Linton and Kwiatowski 2010

Species of the genus *Euglenaria* are morphologically indistinguishable from the members of the genus *Euglena* that have plate-like chloroplasts with diplopyrenoids. Instead, the molecular nSSU characters are the diagnostic characters that separate *Euglenaria* from *Euglena* (Linton et al. 2010). There are four species in this genus: *E. anabaena, E. clavata, E. caudata* and *E. clepsydroides*. Most of them are common and cosmopolitan in eutrophic environments rich in organic material, where they very often form blooms.

- ***Euglenaformis*** M.S. Bennett and Triemer 2014

This genus contains only one species *Euf. proxima* which differs from the members of *Euglena* by having chloroplasts without pyrenoids.

- ***Colacium*** Ehrenberg 1833

Members of this genus have *Euglena*-like cells that attach to a substratum (mostly planktonic animals - copepods, rotifers) by production of mucilaginous stalks.

- ***Monomorphina*** Mereschkowsky 1877, *emended* by Kosmala and Zakryś 2007

The presence of a single spherical chloroplast with haplopyrenoids in rigid pear-shaped cells and large parietal paramylon plates, located between the pellicle and the chloroplast are diagnostic features of eight species classified in this genus after recent taxonomical verification. Only one, *M. aenigmatica*, is morphologically identifiable. The remaining seven species can only be identified at the molecular level (Kim et al. 2012).

- ***Cryptoglena*** Ehrenberg 1831, *emended* by Kosmala and Zakryś 2007

This genus contains five species taxonomically verified and all of them can be identified only at

the molecular level. Cells of the members of this genus resemble a coffee bean in shape. The single, parietal, large chloroplast forms an open cylinder in the shape of the letter "C" and contains two haplopyrenoids whose presence is not sufficiently described. Two large paramylon plates are located laterally between the chloroplast and the pellicle.

- ***Trachelomonas*** Ehrenberg 1833, ***Strombomonas*** Deflandre 1913

In both genera, cells undergoing metaboly (*Euglena*-like) are enclosed in a mineralized envelope, the lorica, with an apical pore through which the locomotory flagellum emerges. Shape and ornamentation of the lorica are used as classical diagnostic features for distinguishing both genera.

The family Phacaceae Kim, Triemer and Shin 2010

Contains three genera whose members have one emergent flagella and numerous, parietal, small, disc-shaped chloroplasts that lack pyrenoids.

- ***Phacus*** Dujardin 1841, *emended* by Linton and Karnkowska 2010

All members of this genus have dimorphic paramylon grains and rigid, flattened, leaf-shaped, sometimes twisted cells. There are only two exceptions from this characteristic; *P. limnophila* and *P. salina* recently moved into *Phacus*. Cells of both species are not flattened and *P. salina* has monomorphic paramylon grains. Colorless forms are known [e.g. *Phacus ocellatus* (Pringsheim) Marin and Melkonian].

- ***Lepocinclis*** Perty1849, *emended* by Marin and Melkonian 2003

Cells are not flattened, only rarely compressed or triangular in apical view, rigid or semi rigid and sometimes twisted. Paramylon grains are dimorphic in size. Colorless forms are known (*L. cyclidiopsis* M. S. Bennett and Triemer).

- ***Discoplastis*** Triemer 2006

This genus currently contains two species, *D. adunca* and *D. spathirhyncha,* reclassified from the genus *Euglena*. Morphologically these species are very similar to *Lepocinclis* and *Phacus* because of the presence of small, parietal, discoid chloroplasts lacking pyrenoids but they are distinguishable from the other two genera by having cells undergoing dynamic metaboly.

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