GENETIC VARIABILITY OF *EUGLENA AGILIS* (EUGLENOPHYCEAE)

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ABSTRACT

The results of the internal transcribed spacer (ITS2) of extrachromosomal rDNA and the chloroplast SSU rDNA sequence analysis presented here confirmed elevated genetic polymorphism revealed earlier by RFLP and RAPD for seven clones of the cosmopolitan species – *Euglena agilis* Carter. High diversity among these clonal strains was not reflected by morphological criteria, with the exception of the only one character – the ability of the cell in its non-motile dividing states (palmella) to produce mucus and form a slimy envelope. Evolutionary adaptation as formation of slimy envelope may be attributed to different survival strategy of the species by which it adapts to life in a highly variable environment.

KEY WORDS: *Euglena*, *Euglena agilis*, Euglenida, 16S rDNA, ITS2, phylogeny, molecular systematics, taxonomy.

INTRODUCTION

Until very recently, all practice of distinguishing and identifying euglenoid species was based on morphological criteria, usually revealed by light microscope. Given large morphological plasticity of euglenoids, there is a need of verification of diagnostic morphological characters, because phenotypic diversity, reflecting perhaps adaptations to different environmental conditions or/and particular physiological state of the organism, may not correspond well to genetic variability.

In spite of intensive molecular research into euglenoid diversity, almost all effort is concentrated on the phylogeny of the entire group, its green or colorless forms, or the whole genera, rather than particular species (Thompson et al. 1995; Mountegut-Felkner and Triemer 1997; Linton et al. 1999, 2000; Preisfeld et al. 2000, 2001; Leander and Farmer 2000, 2001a, 2001b; Leander et al. 2001; Milanowski et al. 2001; Moreira et al. 2001; Müllner et al. 2001; Busse and Preisfeld et al. 2002a, b, 2003; Nudelman et. al. 2003; Morin et al. 2003). Few works using combined molecular and morphological approach may be found in the literature dealing with taxonomic subjects on the intra- or inter-specific level, even though, this may yield a practical results helping identifying particular taxa. We have used such an approach with respect to three *Euglena* species: *E. agilis* Carter (Zakryś and Kucharski 1996; Zakryś et al. 1996; Zakryś 1997, 1997a), *E. gracilis* Ehr. (Zakryś et al. 1997) and *E. geniculata* Duj. (Zakryś et al. 2002). Only in the last case the sequence analysis was used (16S, 18S, ITS2), while the two first dealt with analysis of fragment lengths (RAPD and RFLP).

The most unexpected results were obtained for *E. agilis* (Zakryś and Kucharski 1996; Zakryś et al. 1996). Genetic polymorphism sampled by RFLP and RAPD analysis revealed high diversity among 12 clonal strains, which was not reflected by morphological criteria, with the exception of the only one character – the ability of the cell in its non-motile dividing states (palmella) to produce mucus. On the basis of these findings a taxonomic revision of the entire group consisting of fourteen variations and four species, all with similar morphology to *Euglena agilis*, was proposed. We thus concluded that all diversity should be contained within the single taxon – *E. agilis* Carter (Zakryś 1997). The difference in the ability to produce a mucilage layer by palmelloid stages was not taken into account then, because

we felt that this may not be done without further insight into genetic diversity by sequence analysis approach. This approach is used herein. We believe that this approach should help defining the concept of euglenoid species, as well as, at the practical level, be helpful in devising clear criteria to identify taxa in the genus *Euglena*. So far, the existence of numerous so called critical groups of species (similar morphologically), as well as the lack of consensus on which morphological characters should be considered diagnostically important are causing a considerable difficulty in identifying euglenoid species.

MATERIAL AND METHODS

Euglenoid strains and culture conditions

All strains were cultivated in liquid soil-water medium, enriched with a small piece of garden pea (medium 3c, SAG Göttingen; Schlösser 1994). Seven clones of *E. agilis*, isolated in 1992-1993 from different localities in Poland (Zakryś et al. 1996), the same that were used for RAPD and RFLP analyses (Zakryś and Kucharski 1996; Zakryś et al. 1996) were used in this study. With respect to the mucus production three types of strains were identified by localities from which they came: Wisła k-1 (Wi),Wąwocko k-1 (Wa) (substantial mucus); Kołczewo k-1 (Ko), Pruszków k-1 (Pr) (minute mucus); Górki k-3 (Go), Piaski k-1 (Pi), Nowy Targ k-1 (NT) (no mucus).

DNA Isolation, amplification and sequencing

Isolation of DNA, amplification, purification of PCR products and sequencing of chloroplast SSU and ITS2 rDNA was performed as previously described (Milanowski et al. 2001; Zakryś et al. 2002). The GenBank accession numbers for SSU and ITS2 rDNA sequences reported in this paper are shown in Table 1.

Sequence alignment and phylogenetic analysis

The sequences used for phylogenetic analyses are shown in Table 1. Alignment of sequences was obtained using the Clustal X program (Thompson et al. 1994; Thompson et al. 1997) with default options, manually checked and edited according to the secondary structure of *Euglena gracilis* 16S rRNA as suggested by Van de Peer et al. (1999). Regions, which could not be unambiguously aligned, were omitted from analyses. The alignments used for analyses are available in EMBL [alignment 16S: ALIGN_000698; alignment ITS2 agilis + gracilis: ALIGN_000699; alignment ITS2 same agilis: ALIGN_000700]. The address of EMBL is: ftp: //ftp.ebi.ac.uk/pub/databases/embl/align/. The 16S as well as ITS rDNA trees were built and their stability assessed by posterior probabilities using Bayesian inference (Huelsenbeck et al. 2001) and general time reversible model (GTR + I + G) (Tavaré 1986; Yang 1993), with the rates of nucleotide substitutions, the base frequencies, the shape of the gamma distribution and the proportion of invariant sites estimated, as implemented in MrBayes 2.01 program (Huelsenbeck and Ronquist 2001). The sequences of *Euglena* subgenus were used for comparison and as an outgroup for 16S rDNA, while *E. gracilis* sequence was an outgroup for the ITS2 trees. The trees were drawn by Tree View, Version 1.6.1 for Microsoft Windows (Page 1996).

RESULTS

Phylogenetic analysis of 16S rDNA from several euglenoid species show that the sequences of all three *E. agilis* clones form a well defined clade with *E. gracilis* as a sister group (Fig. 1). The branching order between the three clones, representing different levels of mucilage forming, could not however be established. The distances between the three groups are in the range of the distances between two of the most divergent clones of *E. geniculata*.

In order to further assess the genetic similarity of the *E. agilis* clones, we have surveyed the fragment of extrachromosomal DNA for large subunit (LSU) rRNA species 1 and 2, encompassing the part corresponding to 5.8 S rRNA, ITS2 and the beginning of 28S rRNA (Schnare et al. 1990). We have sequenced this region in seven clones of *E. agilis*, isolated in 1992-1993 from different localities in Poland, the fraction of the same clones that were used for RAPD and RFLP analyses (Zakryś and Kucharski 1996; Zakryś et al. 1996). Unequivocal alignment of all the sequence fragments used for analysis could only be done within the re-

TABLE 1. GenBank accession numbers, culture, and collection information for members of the genus *Euglena* used in this study. Accession numbers of sequences obtained in this study are printed in bold.

Taxon	Strain	GenBank accession number	
		16S rDNA	ITS2
Euglena agilis Carter	UW Górki k-3		**AY158149**
Euglena agilis Carter	UW Kołczewo k-1		**AY158145**
Euglena agilis Carter	UW Piaski k-1		**AY158147**
Euglena agilis Carter	UW Nowy Targ	**AY158150**	**AY158148**
Euglena agilis Carter	UW Wąwocko k-1	**AY158151**	**AY158144**
Euglena agilis Carter	UW Pruszków k-1	AF289239	**AY158146**
Euglena agilis Carter	UW Wisła k-1		**AY158143**
Euglena gracilis Klebs	unknown	X12890	
Euglena geniculata Dujardin	SAG 1224-4b	AF289241	
Euglena geniculata Dujardin	SAG 1224-4f	AY070252	
Euglena stellata Mainx	SAG 1224-14	AF289244	
Euglena tristella Chu	SAG 1224-35	AF289246	
Euglena viridis Ehrenberg	SAG 1224-17d	AF289248	

SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany; UW, Department of Plant Systematics and Geography of Warsaw University, Poland; CCAP, Culture Collection of Algae and Protozoa, UK.

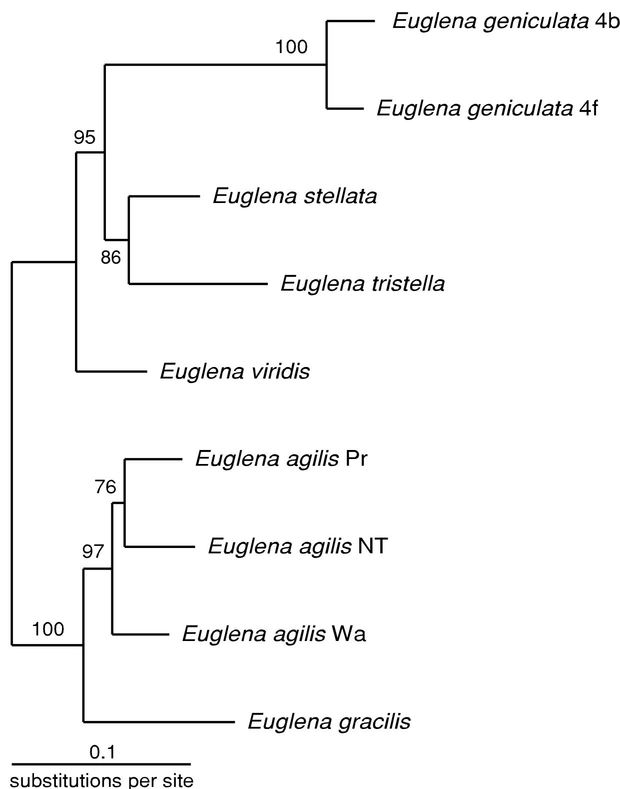

Fig. 1. Phylogenetic tree of 16S rDNA of several euglenoid species, including three representative clones of *E. agilis*, obtained by Bayesian inference. The GTR+I+G model of sequence evolution (Tavaré 1986; Yang 1993) was used with likelihood parameters estimated by Markov Chains MonteCarlo method. Numbers at nodes show posterior probabilities of the tree bipartitions.

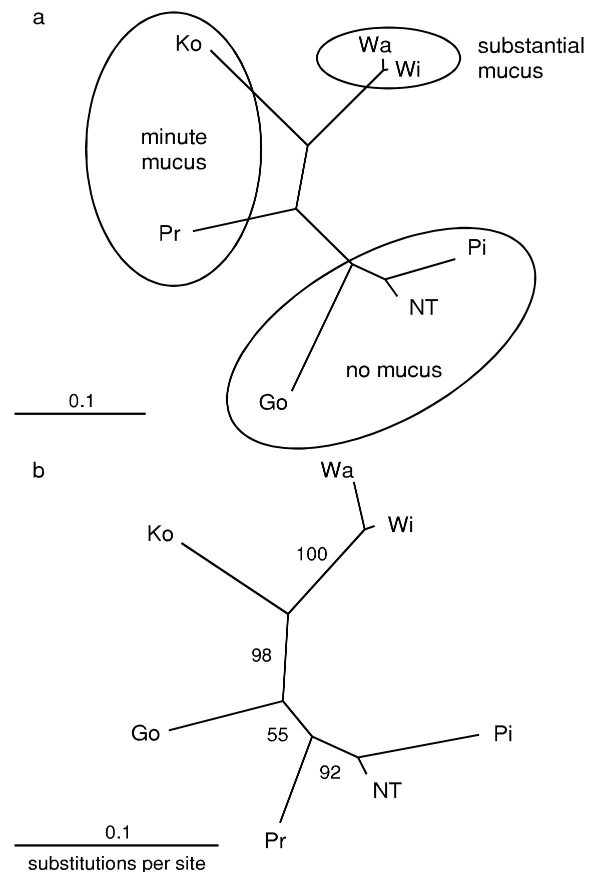

Fig. 2. Unrooted phenogram using 462 (a) and the phylogenetic tree of seven *E. agilis* clones, obtained by Bayesian inference (GTR+I+G model of sequence evolution) using 277 sites (b). Numbers at nodes show posterior probabilities of the tree bipartitions.

gion corresponding to LSU rDNA and some parts of ITS2. The parts corresponding to large regions of ITS2 could be aligned only within the groups of very similar strains. Figure 2a shows the ClustalX guide tree generated with the default parameter values for gap penalties. Since there are many regions of uncertain homology in the alignment used for building this tree, it could not be considered as a true phylogenetic tree, rather a phenogram representing pair wise sequence similarity. However, it was shown that in case of ITS2, such a tree could reflect quite accurately the partitions between groups of divergent sequences (Hershkovitz and Lewis 1996).

The seven sequences form two groups consisting of sequences represented by clones with or without the ability to form mucus (Fig. 2a), when the whole length of the aligned sequences was used (462 nt). The first group, represented on Figure 1 by the NT clone, also contains the sequences of Pi and Go clones, all without the ability to form mucus. The group consists of sequences from Wa and Pr localities, represented on Figure 1, and additionally the sequences from Wi (substantial mucus) and Ko (minute mucus). Within the clones which are able to form mucilage, those with substantial mucus form a separate group, while clones forming a minute mucus branch first. When sites which could not be unambiguously aligned were eliminated in order to perform Bayesian maximum likelihood analyses, the strains did not form two well-defined groupings corresponding to the clones with and without ability to form mucus (Fig. 2b). The situation is somewhat similar when the sequence of *E. gracilis* was used as an outgroup (Fig. 3). The clones form two sister groups, one with the ability to form mucus and the other without that ability, and within the latter group the clones with minute mucilage branch of first, only on a phenogram when all sites were used for analysis (Fig. 3a). However, the portion of unambiguously aligned sites used for maximum likelihood analysis decreased further to 211, resulting in lower posterior probabilities precluding inferring of real relationships between clones. Only two clones with substantial mucus form a well-defined clade.

DISCUSSION

Analysis of 16S rDNA (Fig. 1) shows that the three clones of *E. agilis* form a robust clade. The differences between them are similar to those between *E. geniculata* strains showing high morphological variability. Results of ITS2 sequence analysis are generally consistent with the indirect molecular probing (RAPD, RFLP) (Zakryś and Kucharski 1996; Zakryś et al. 1996) (Figs 2, 3). Taken together with earlier findings concerning morphology (microscopic observations and biometry) they indicate that different genetic clones of *E. agilis* coexist in natural populations, but genetic differences are not reflected in morphological characteristic, with the one possible exception of the ability to form a slimy envelope during cellular division in the state of non-motile palmella. Judging by differences in ITS2 sequences (Figs 2a, 3a) *E. agilis* clones seem to form two gro-

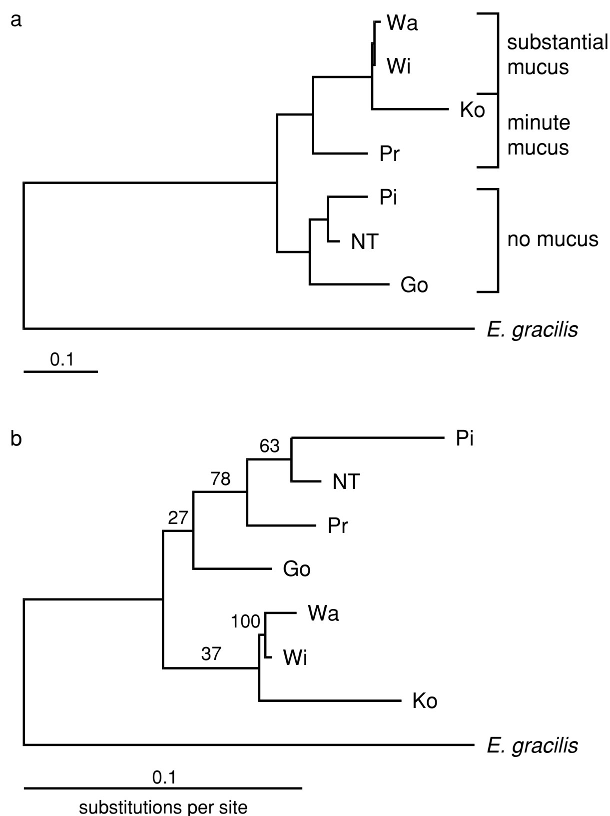

Fig. 3. Rooted phenogram using 475 (a) and the phylogenetic tree of seven *E. agilis* clones, obtained by Bayesian inference (GTR+I+G model of sequence evolution) using 221 sites (b). Numbers at nodes show posterior probabilities of the tree bipartitions.

ups – non-slimy clones (Go, Pi, NT) and slimy clones (Wi, Wa, Ko and Pr). However, due to a weak phylogenetic signal in ITS2 sequences such grouping could not be confirmed with significant probabilities.

Evolutionary adaptation as formation of slimy envelope may be attributed to different survival strategy (Zakryś and Kucharski 1996). Euglenoids live in a highly variable environment such as small astatic bodies of water like puddles, ponds, ditches, drainage canals in which sudden changes of environmental conditions often take place. Some clones of *E. agilis* multiply very fast and expansively, giving up protection of their dividing stages (palmellas). Within a short time the population becomes too dense and survival stages are formed. Those which do not manage to finish that process before a reservoir they inhabit dries out, must die. Others clones invest more energy to protect their division stages at the expense of the rate of reproduction. However, surrounded by the thick layer of mucus palmellas are able to survive temporary periods of drought (Zakryś and Kucharski 1996).

The question arises whether it is desirable to erect a taxon based on the level of variance or form, which would take into account the differences at the morphological level (ability to produce a slimy envelope). In our view this is not warranted for three reasons:

1) the difference in mucus formation is manifested only at palmella stage, whereas *E. agilis* is identified in environmental samples in the flagellate stage (motile stage) hence it is not particularly useful in identifying the species,

2) the lack or the presence of this feature may be related merely to different life strategies, therefore this should be regarded as related to different ecological forms,

3) in spite of the relative high polymorphism between clones of *E. agilis* all of them form a well defined clade.

For these reasons we believe that a new reclassification of taxa in *E. agilis* group is not necessary and the proposition of Zakryś (1997) in the wake of the additional findings presented here should stay. The former study proposes to take as synonyms of *Euglena agilis* Carter four taxa in the rank of species (*E. bichloris* Schiller, *E. bipyrenoida* Prošk.-Lavr., *E. nana* Johnson and *E. van-goori* Deflandre), fourteen intraspecific taxa of the species *E. agilis* (var. *apyrenoida* Schiller, var. *circumsulcata* Schiller, f. *coeruleoviridis* Schiller, var. *praeexcisa* Schiller, var. *varians* Schiller) and *E. pisciformis* Klebs (var. *minor* Hansgirg, var. *granulosa* Playfair, var. *piriformis* Szabados, var. *fallax* Pringsheim, var. *lata* Pringsheim, var. *mucronata* Pringsheim, var. *obtusa* Pringsheim, var. *procera* Pringsheim, var. *striata* Pringsheim).

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