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七株眼虫基于微卫星 DNA 指纹图谱的区分和关系分析*

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摘　要:尽管以前对眼虫进行过大量的形态发育研究和基于核糖体 RNA 基因的系统发育分析,但对于株系之间的关系仍然知之甚少. 因其形态特征有限并且易变,很难鉴定眼虫的相似种和同种内不同的株. 作者利用微卫星 DNA 指纹图谱,在七株眼虫中扩增了七个微卫星 DNA 位点,成功扩增的六个微卫星引物都得到了四到八个条带. 从微卫星 DNA 指纹图谱计算得到的相似性系数范围从 0.000 到 0.957. 根据相似性系数得到的树状结构,七株眼虫在距离为 0.9346 处分为三支:*E. mutabilis*, *E. intermedia* 和 *E. gracilis*. 其中,五株 *E. gracilis* 分为两组:来自日本的和美国的. 不同地区的株得到不同的基因型,并初步分析了它们之间的关系. 研究表明七株眼虫根据微卫星 DNA 指纹图谱被明显区分开. 微卫星 DNA 指纹图谱具有很高的分辨率,是鉴定和区分原生动物相似种和同种内不同株的一种有用的新方法.
关键词:眼虫;微卫星 DNA;原生动物;相似性关系;株的区分

Separation and relationships of seven *Euglena* strains based on microsatellite DNA fingerprinting

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Abstract: The genetic relationships among *Euglena* strains remain poorly understood, despite a rich history of morphological examination and an extensive phylogenetic analysis of the ribosomal RNA genes. It is difficult to identify related species of the genus *Euglena* and to separate different strains of the same species because the morphological characters are variable and limited. We address this question using microsatellite DNA fingerprinting. Seven microsatellite loci were amplified from 7 *Euglena* strains. A total of 4 – 8 bands were successfully amplified for each of 6 microsatellite primer pairs. Microsatellite DNA fingerprinting and similarity values ranging from 0.000 to 0.957 among the seven strains were obtained, which were used to construct a similarity tree among them. According to the tree, at the linkage distance of 0.9346 seven strains were separated into three clusters: *E. mutabilis*, *E. intermedia* and *E. gracilis* consisting of Japanese and USA groups. Further, in the cluster *E. gracilis*, different genotypes were recognized for the strains from different geographical origins. The relationships among species and strains were analyzed preliminarily. We demonstrates that the seven strains of *Euglena* can be clearly separated by their microsatellite DNA fingerprinting patterns. The microsatellite DNA fingerprinting has a high resolution and is a new useful method to identify and separate similar species and intraspecific strains in free-living protozoa.
Keywords: *Euglena*; microsatellite DNA; protozoa; similarity relationships; strains separation

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Traditionally, for a long time, the taxonomy of the genus *Euglena* has been mainly based on morphological characters such as cell size and shape, pellicle, striations, transverse section, chloroplasts and paramylons. Unfortunately, the morphological characteristics of *Euglena* are not distinguishable when the ambiguous form is found under certain cultured conditions and among strains collected from nature. Many phylogenetic analyses of *Euglena* strains at the species level or above based on the ribosomal RNA genes (rDNA) were taken recently[1-5]. But the deep-level taxonomic relationships among *Euglena* strains remain poorly understood. Furthermore, identification and separation of different strains of the same *Euglena* species based on microsatellite DNA has not been reported.

Microsatellite DNA regions are abundant in eukaryotic genomes, which have a co-dominant inheritance, are highly polymorphic, easy to use and score, and are selectively neutral with a high mutation rate[6-8]. In the past decade, microsatellite DNA has been developed into one of the most popular genetic markers because of its ability to detect and characterize multiple alleles at a given locus; and it has been widely used in studies on polymorphism of gene, evolution, linkage analysis as well as population genetics etc. in a large range of eukaryotic organisms including a few parasite protozoa [9-12]. However, studies on microsatellite DNA in free-living unicellular eukaryotic organisms are scarce[13,14].

In this study, seven microsatellite primer pairs designed in our previous research[15], were used to separate seven *Euglena* strains. Similarity coefficients between particular strains were calculated on the basis of the results of microsatellite fingerprinting. These values were analyzed and used in dendrogram construction in order to elucidate relationships in *Euglena* strains.

1 Materials and methods

1.1 Origin of strains

Seven *Euglena* strains from the Freshwater Algae Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences, Wuhan, China, were selected for the present study. Each strain was cultured clonally. Strains´names and geographical origins are listed in Tab. 1 Two mutant achlorophyllous strains *E. gracilis* Ofland *E. gracilis* Sm were formed, after *E. gracilis* FACHB848 was treated with Ofloxacin (Ofl) and Streptomycin (Sm) respectively. The morphological features of each strain were investigated by light microscopy and compared to those reported in previous papers[16,17].

Tab. 1 Species names and geographical origins of seven *Euglena* strains

FACHB[a] number.	Specie names	Geographical origins
FACHB745	*E. gracilis*	USA
FACHB412	*E. intermedia*	China
FACHB Ofl	*E. gracilis*	Japan
FACHB Sm	*E. gracilis*	Japan
FACHB850	*E. gracilis* var. *bacillaris*	Japan
FACHB848	*E. gracilis*	Japan
FACHB851	*E. mutabilis*	Japan

FACHB[a] means the Freshwater Algae Collection of the Institute of Hydrobiology, CAS.

1.2 DNA extraction

After cells were starved overnight in distilled sterile water, they were washed several times with distilled sterile water. The genomic DNA was extracted from about 500 mg of freshly centrifuged cells using a standard SDS-proteinase K procedure, as described by Sambrook et al[18].

1.3 PCR amplification

PCR reaction mixtures of 25 μl volume contained the following components/concentrations: 1 unit Taq DNA polymerase (MBI, Fementers), 1 × reaction buffer with $(NH_4)_2SO_4$ (supplied with the enzyme), 2 mM $MgCl_2$, 0.1 mM of each dNTP (MBI, Fementers), 0.6 μM of each primer, and 20 ng DNA. PCR amplification was performed on a Perkin Elmer™ 9600 thermal cycler under the following conditions: 5 min at 94 ℃, 30 cycles of 1 min at 94 ℃, 55 s at *T* ℃ (Tab. 2) and 55 s at 72 ℃, final extension at 72 ℃ for 10 min. Seven microsatellite primer pairs were used. Their sequences, amplified microsatellite loci names and annealing temperature

are listed in Tab. 2. Negative controls and replicates were included to verify repeatability of the results.

Tab. 2 Microsatellites loci names, primers sequences and annealing temperature (T) in the present work

Microsatellites loci names	Accession number in GenBank	PCR primers 5′-3′	T(℃)
EGMS1	AY513252	1 CCAACCGAAGAGGAAGG 2 CGCCCACATTAACACGTTC	54
EGMS2	AY513253	1 CGTTGGACATTTCTCAGCCC 2 GCAGTAAGCCGATGGTATG	50
EGMS3	AY513254	1 GTGACCTTGTGCGAGCAAG 2 CTTGGATGGTTTGCTGGCA	50
EGMS4	AY513255	1 GTCTCTGTTTGCCACCAC 2 CACCTGAGTCACATTGGAG	50
EGMS5	AY513256	1 GAGGAAACAGCTTACATCAC 2 CTTTGTCTCAGGGTGCCTG	50
EGMS6	AY513257	1 CATCCAGCAACACTGGCA 2 ACATCTAGGGAGAGCTCC	49
EGMS7	AY513258	1 GTCGGAGGGTGTGTTG 2 GTCCGGCTACTACCAATGG	51

1.4 Polyacrylamide gel electrophoresis (PAGE)

PCR products were mixed 1∶5 with loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), then 12μl mixtures were electrophoresed on 10% non-denaturing polyacrylamide gels (PAG) in 1×TBE buffer at 1V/cm for 5 hours or so at 4 ℃. After gels were dyed with ethidium bromide, exact identification of the bands was done through comparison with a PBR322 DNA/*Msp* Ladder (Huamei Co. Ltd., Luoyang, Henan, China).

1.5 Data analysis

Microsatellite fingerprinting patterns were visually analyzed and scored from the photographs. The distinct and well-separated bands were selected for analysis and comparison. Polymorphic bands were scored qualitatively as present (1) or absent (0). It should be mentioned that some other weak bands were amplified because the simple repeat sequence (microsatellite DNA) was easily chain-slipped in the process of PCR amplification. These relatively weak bands in a lane were scored as absent (0) because they were not true microsatellite DNA bands. Genetic distances (D) and similarity values (S) among the samples were calculated based on the formula: $D = 1 - S = 1 - 2N_{AB}/(N_A + N_B)$ where N_A and N_B are the number of bands scored in species A and B respectively, and N_{AB} is the number shared by both[19]. The resulting genetic distance matrixes were then used to build a tree using the unweighted pair-group method for arithmetic averages analysis (UPGMA) method as implemented by the software package PHYLIP version 3.5c[20]. Finally, TREE-VIEW 1.5[21] was used to illustrate the tree.

2 Results

2.1 Morphological comparison

Euglena gracilis, *E. intermedia* and *E. mutabilis* can be separated with some morphological characters (Tab. 3). *E. gracilis* differs from *E. intermedia* and *E. mutabilis* in its discoid chloroplasts and sheath around pyrenoid. *E. intermedia* is the largest in body length and has discoid chloroplasts without pyrenoid or sheath. *E. mutabilis* is distinguished from both *E. gracilis* and *E. intermedia* by its half-ring or flat chloroplasts, pyrenoid without sheath and the loss of the emergent flagellum (it resides inside the reservoir). *E. mutabilis* can not swim. Among five strains of *E. gracilis*, *E. gracilis* var. *bacillaris* FACHB850, *E. gracilis* FACHB745 and *E. gracilis* FACHB848 have chloroplasts and can photosynthesize; on the contrary, neither *E. gracilis* FACHB Ofl nor *E. gracilis* FACHB Sm have chloroplasts[22].

Tab. 3 Morphological comparison among *Euglena mutabilis*, *E. gracilis* and *E. intermedia* [16,17]

Species	Body length (μm)	Posterior	Chloroplast shape	Pyrenoid	Sheath around pyrenoid	Flagella length/Body length
E. gracilis	31 – 40	rotund end with a aboral protruberance or tapering gently to a sharp point	discoid	present	present	1/2 – 1
E. intermedia	60 – 174	rotund end tapering to a point	discoid	absent	absent	1/7 – 1/4
E. mutabilis	60 – 95	taper	curved	present	absent	absent

2.2 Microsatellite DNA fingerprinting

Among a total of seven sets of microsatellite DNA primers, six microsatellite DNA fingerprinting successfully revealed different polymorphic band patterns with the exception of EGMS2. Fig. 1 shows two amplification fingerprinting pattern examples of the microsatellite loci. The number and size range of bands are shown in Tab. 4. Fingerprinting experiments revealed four to eight bands depending on the strains and the primers used. In the amplified profile of primer EGMS4, *E. gracilis* FACHB Ofl showed one band less than *E. gracilis* FACHB Sm. *E. mutabilis* FACHB851 was distinctly different from the other six strains because it did not share any band with others for each of the six primers.

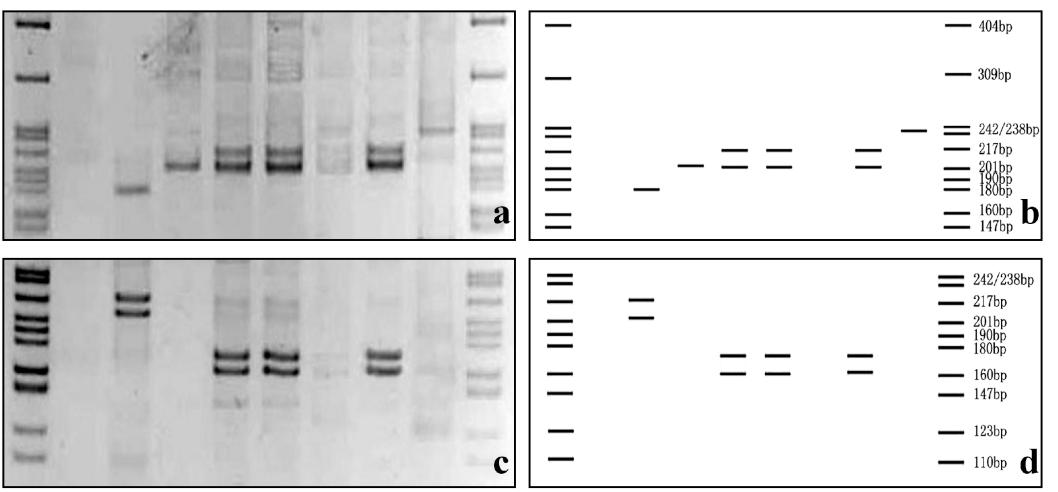

Fig. 1a-d. Microsatellite DNA fingerprinting patterns of seven *Euglena* strains for two microsatellite loci. The sized ladder PBR322 DNA/*Msp* I Ladder (Promega) was run on both sides of the gel. Respectively, figs a and b: EGMS1; figs c and d: EGMS5. Lanes: 0, blank control; 1, *E. gracilis* FACHB745; 2, *E. intermedia* FACHB412; 3, *E. gracilis* FACHB Ofl; 4, *E. gracilis* FACHB Sm; 5, *E. gracilis* var. *bacillaris* FACHB850; 6, *E. gracilis* FACHB848; 7, *E. mutabilis* FACHB851.

Tab. 4 Size range and number of bands of microsatellites loci

Microsatellites loci names	EGMS1	EGMS3	EGMS4	EGMS5	EGMS6	EGMS7
Size range (bp)	180 – 240	180 – 410	201 – 404	160 – 217	150 – 264	217 – 410
Number of amplified bands	4	8	5	4	6	6

2.3 Genetic variation

The genetic variation was estimated by the genetic distance and the similarity value among seven *Euglena*

strains (Tab. 5).

Genetic distances ranged from 0.889 to 1.000 showed apparent differences between different species. For example, the maximum genetic distance was 1 between the strain *E. mutabilis* FACHB851 and six other *Euglena* strains, and between the strain *E. intermedia* FACHB412 and two*E. gracilis* strains (*E. gracilis* FACHB745 and *E. gracilis* var. *bacillaris* FACHB850). The intraspecific genetic distances of *E. gracilis* varied from 0.043 to 0.826 in a wide range. The minimum was 0.043 between *E. gracilis* FACHB Ofl and *E. gracilis* FACHB Sm, and between *E. gracilis* FACHB Ofl and *E. gracilis* FACHB848.

Tab. 5 Similarity values (lower half) and genetic distances (upper half) for seven *Euglena* strains based on microsatellite DNA fingerprinting

	FACHB745	FACHB412	FACHB Ofl	FACHB Sm	FACHB850	FACHB848	FACHB851
FACHB745	—	1.000	0.826	0.818	0.684	0.818	1.000
FACHB412	0.000	—	0.895	0.889	1.000	0.889	1.000
FACHB Ofl	0.174	0.105	—	0.043	0.400	0.043	1.000
FACHB Sm	0.182	0.111	0.957	—	0.474	0.091	1.000
FACHB850	0.316	0.000	0.600	0.526	—	0.474	1.000
FACHB848	0.182	0.111	0.957	0.909	0.526	—	1.000
FACHB851	0.000	0.000	0.000	0.000	0.000	0.000	—

Accordingly, all interspecific similarity values (0.000 – 0.111) were very low. The similarity value between *E. gracilis* and *E. intermedia* FACHB412 was higher than that between *E. gracilis* and *E. mutabilis* FACHB851. So, *E. mutabilis* FACHB851 showed the greatest difference from the others.

2.4 Similarity Tree

A similarity tree was constructed based on the data obtained in the present study (Fig. 2). At the linkage distance of 0.9346, the dendrogram divided the seven strains into three clusters: *E. mutabilis*, *E. intermedia* and *E. gracilis* consisting of Japanese and USA groups. In the group of *E. gracilis*, the USA strain was separated from the Japanese strains. Interestingly, *E. gracilis* var. *bacillaris* from Japan was clustered with three Japanese *E. gracilis* at first. Further, in four Japanese strains, *E. gracilis* FACHB Ofl had the highest similarity to *E. gracilis* FACHB Sm strain.

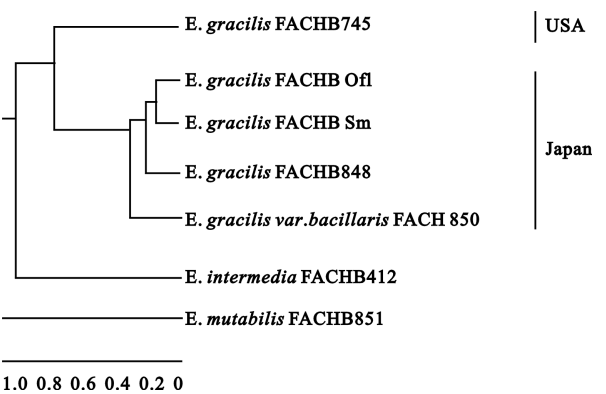

Fig. 2 Similarity tree inferred from the microsatellite DNA fingerprinting of seven *Euglena* strains based on UPGMA method. The numerical scale indicates increasing genetic distance.

3 Discussion

This is the first report on the use of microsatellite DNA to identify and display relationships of free-living proto-

zoa. Many microsallite loci were applied to avoid the artificial pollution in the PCR proceeding, and this makes the results more reliable. It clearly demonstrates microsatellite DNA as a useful molecular marker in discriminating different protozoan strains within a species.

3.1 Strains identification and the value of microsatellite DNA fingerprinting

The different species can be identified by their different morphological characters, except that different strains of *E. gracilis* can not. Moreover, the morphological differences of seven *Euglena* strains are not obvious, especially for the person who is not an expert in the classification of *Euglena*. The five strains of *E. gracilis* are so morphologically similar that it is impossible to separate them only by morphological characters. This shows that it is necessary to find a method to identify these strains. Our data indicate that the seven strains of *Euglena* can be clearly identified by their microsatellite DNA fingerprinting patterns.

In this study, microsatellite DNA fingerprinting patterns revealed the existence of many differences among the seven *Euglena* strains, specifically in different species. It was easy to identify the species *E. mutabilis* FACHB851 from other *Euglena* strains based on microsatellite fingerprinting. The genetic distances among *E. gracilis* strains were so high (0.043 – 0.826), which make intraspecific identification easy. The distance between *E. gracilis* FACHB Ofl and *E. gracilis* FACHB Sm from the same strain was 0.043. This indicated microsatellite DNA fingerprinting is adaptable to study intraspecific identification and genetic variance. The microsatellite DNA fingerprinting method could provide alternatives to the traditional morphological and molecular approaches for identifying the strains within a species of protozoa.

Although many gene sequence markers, for example, SSU rDNA[23], large subunit rDNA[24], Hsp70[25], histone[26], DNA polymerase α subunit sequences[27] etc. were successfully used to reconstruct phylogenetic trees at the species level or above, they were not suitable for identification of different strains from the same species. Zakryś et al.[28] showed a broad inter- and intraspecific variation of genotypes in the common species *Euglena pisciformis* using randomly amplified polymorphic DNA (RAPD) markers. But results of RAPD often cannot be reproduced in different laboratories[29]. In addition, the restricting fragment length polymorphism (RFLP, riboprinting) does not discover intraspecific differences[30]. Allozyme diversity may have always been lower than PCR-based variation[31]. Comparing the above methods, as a new source of genetic markers, microsatellite fingerprinting overcomes the limitations in resolution of organelle gene sequence, RAPD, RFLP and allozyme.

3.2 Relationships among species

The similarity tree generated from the microsatellite DNA fingerprinting clearly separated the strains of *Euglena* into three distinct subgroups. As shown in Fig. 2, *E. mutabilis* acted as a separate clade, which indicates much more differences from the other six *Euglena* strains. The relation between *E. intermedia* and *E. gracilis* is much nearer to *E. mutabilis* and *E. gracilis*. This is supported by previous report[4], in which the Euglenophyceae were analyzed based on all available SSU rDNA sequences (9 Eutreptiales, 101 Euglenales). They constructed a phylogeny of Euglenophyceae based on 1588 aligned positions with *Petalomonas cantuscygni* and *Peranema trichophorum* as outgroup taxa. As a result, the Euglenales showed a basal dichotomy separating the mutabilis-clade from the remaining Euglenales. The mutabilis- and non-mutabilis-clades displayed significant rate differences. In another study, Sittenfeld et al.[3] also showed the *E. mutabilis* is obviously different from other *Euglena* in the phylogenetic tree. *E. mutabilis* plays a special role in *Euglena* strains which may be correlated with adaptation to extreme habits. *E. mutabilis* has been described as one of the dominant phytobenthic species in acid mining lakes. It is well known for its high metal and acid tolerance, and is able to grow at a pH of 1.3[3]. Moreover *E. mutabilis* has an extremely reduced emergent flagellum, and consequently its locomotion is restricted to gliding movements[16].

But in Wang's cladistic analysis, *E. gracilis* had the closer relation with *E. mutabilis* than *E. intermedia* according to the characters of pyrenoid and its sheath[32]. This discrepancy may be due to differences in study methods. Wang's analysis was based on morphological characters. In Marin et al.[4], Sittenfeld et al.[3] and our stud-

ies, all results are at the molecular level and the relations are concluded from the information of DNA.

3.3 Relationships among *Euglena gracilis* strains

E. gracilis var. *bacillaris* FACHB 850 did not separate from *E. gracilis* strains, showing it has little difference with *E. gracilis* strains at the molecular level. However, *E. gracilis* var. *bacillaris* FACHB 850 from Japan shares closer relationship with *E. gracilis* strains from Japan than *E. gracilis* from the USA. That is, *E. gracilis* var. *bacillaris* has genetic coherence with *E. gracilis* strains from Japan.

Our tree indicates *E. gracilis* FACHB Ofl and *E. gracilis* FACHB Sm have the closest relation. Oda et al. [33] and Wang et al. [22] proved that Streptomycin is able to eliminate chloroplasts from *E. gracilis*, to form a mutant achlorophyllous strain without interfering with cell division. Both *E. gracilis* FACHB Ofl and *E. gracilis* FACHB Sm were originated from *E. gracilis* FACHB848, so they have very similar genetic structures. But *E. gracilis* FACHB Sm lacked a band in the amplification. It is presumed that different genetic mutations took place in their genome and the mutation possibly lied in or near to the microsatellite DNA locus. Thus this affected the pair-band location of the DNA template with microsatellite primers.

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