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MICROCALORIMETRY AS A POSSIBLE TOOL FOR PHYLOGENETIC STUDIES OF *TETRAHYMENA*

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Using isothermal microcalorimetry, the growth power-time curves of three strains of *Tetrahymena* were determined at 28°C. Their Euclidean distances and cluster analysis diagram were obtained by using two thermokinetic parameters (r and Q_{log}), which showed that *T. thermophila* BF_1 and *T. thermophila* BF_5 had a closer relationship. Compared with the single molecular biomarker (ITS1) method, microcalorimetry was maybe a simpler, more sensitive and more economic technique in the phylogenetic studies of *Tetrahymena* species.

Keywords: *Euclidean distances, ITS1, microcalorimetry, phylogeny, Tetrahymena, thermokinetic*

Introduction

Tetrahymena is a ciliated protozoa belonging to a free-living, fresh-water genus that is highly successful ecologically. Its ultrastructure, cell physiology, development, biochemistry, genetics, and molecular biology have been extensively investigated. The richness of *Tetrahymena*'s biology makes it a unicellular animal model organism [1]. And the *Tetrahymena thermophila* macronulear genome sequencing project has been finished. However, *Tetrahymena* species provide many interesting challenges, which require an understanding of their evolutionary history for a full explanation [2, 3]. So far, the application of the molecular techniques to phylogenetic studies provides us with the opportunity to acquire gene sequences. Then the genes are used to reconstruct phylogenetic trees. Different molecular sequences including small subunit rRNA (SSRNA) [4], large unit rRNA (LSRNA) [5], internal transcribed spacer1 (ITS1) [6] have been used to study the phylogeny of *Tetrahymena*. However, the molecular techniques are usually complicated, expensive and dangerous to operators. It's highly urgent to explore a simple, economical and safe method for studying the phylogeny of *Tetrahymena*.

Microcalorimetry as a useful technique has been widely used in microbiology [7], pharmacological analysis [8], biotechnology [9], ecology [10], genetics [11] and environmental sciences [12] because of its high sensitivity, high accuracy, non-destructivity and automaticity [13]. Furthermore, microcalorimetric studies of *Tetrahymena* have also been applied in many fields [14, 15]. However, phylogenetic studies of *Tetrahymena* with this method have not been reported. As to the complex living systems, microcalorimetry can easily record their specific growth power-time curves that reveal both thermal and kinetic data. The thermokinetic characteristics are helpful for studying differences in overall energy metabolism, which maybe contribute to phylogenetic relationship studies of organisms. In this study, microcalorimetry and ITS1 gene were used to investigate phylogenetic relationships of the three strains of *Tetrahymena*, respectively. And microcalorimetry as a tool for phylogenetic studies of *Tetrahymena* compared with ITS1 gene method was discussed.

Experimental

Species and culture medium

Tetrahymena thermophila BF_1, *Tetrahymena thermophila* BF_5 and *Tetrahymena pyriformis* GL were provided by East China Normal University and Peking University, respectively. The culture medium contained tryptone 15 g (Oxoid), yeast extract 5 g (Oxoid) and glucose 1 g per 1000 mL, and then was sterilized by autoclaving for 20 min at 0.1 MPa.

Calorimeter

The calorimetric measurements of *Tetrahymena* growth were performed with TAM Air Isothermal Microcalorimeter (Thermometric AB, Sweden). The detection limit of calorimeter was 2 μW and its baseline stability over 24 h is 5 μW.

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Measurements of growth power-time curves of Tetrahymena

After microcalorimeter was thermostated at 28°C, the power-time curves of *Tetrahymena* were determined by using the ampoule method. Firstly, stationary-stage cells were counted using a Leitz microscope. Then appropriate cell suspensions and sterilized culture medium were added into the 20 mL glass ampoules. Cell suspensions in ampoules were finally adjusted to 1500 cells mL^{-1} with a volume of 5 mL. The ampoules were then sealed and put into microcalorimeter to determine the thermal power of *Tetrahymena* growth.

DNA extractions and sequencing

Cells were incubated in the lysis buffer (100 mM NaCl, 10 mM Tris pH 7.8, 10 mM EDTA, 0.2% SDS, 0.2 mg L^{-1} proteinase K) at 55°C for 12–20 h and then extracted using a standard phenol/chloroform method [16]. DNA content was determined by spectrophotometry using a DU Series 500 Spectrophotometer (Beckman Instruments Inc., Fullerton, USA).

PCR amplification of the ITS1 genes was performed in a Perkin-Elmer GeneAmp PCR System 9600 (PE Applied Biosystems, Mississauga, ON, Canada). Forward primer was 5'-GTT CCC CTT GAA CGA GGA ATT C-3', reverse primer was 5'-CGC ATT TCG CTG CGT TCT TC -3' [17]. The fragments were amplified in every species tested using the following PCR procedure: an initial denaturation step of 94°C for 10 min; followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, and a final extension step at 72°C for 10 min.

PCR products were purified using the Biostar Glassmilk DNA Purification Kit (BioStar International Toronto, Canada). Ligation reactions were performed by overnight incubation at 4°C of 10 μL mixtures containing approximately 50 ng pure DNA, 1 μL p GEM-T Easy Vector (Promega Biotech, Madison, WI), and 1 μL T_4 DNA ligase, 5 μL ligation buffer. 5 μL ligation reactions were used to transform 50 μL HB101 High Efficiency Competent Cells following the method of [16]. The clones containing the amplified fragment were selected and then sequenced by Shanghai United Gene Holdings, Ltd.

Data analysis

Thermokinetic equations and correlation coefficients (R) of *Tetrahymena* metabolism were calculated by making use of the data P_t and t taken from the log phase of growth to simulate logistic equation.

Euclidean distances, which can reveal the relationship of two samples, are calculated by the following formula:

$$\mathrm{EUCLID} = \sqrt{\sum_{i=1}^{n} (x_i - y_i)^2}$$

EUCLID is Euclidean distances of two samples; n is number of variables, x_i is value of variable i of one sample, y_i is value of variable i of another sample.

Then cluster analysis diagram can be obtained by Euclidean distances [18].

In this study, thermokinetic equations and correlation coefficients (R) were gained by non-linear estimation of Statistica 6.0, and Euclidean distances and clustering analysis diagram were gained by cluster analysis of Statistica 6.0 [19]. Base on ITS1 gene sequences, the genetic distances among *Tetrahymena* were calculated by Neighbor-joining method [20] of PAUP* 4.0 [21].

Results

Growth power-time curves and thermokinetic parameters of Tetrahymena

The growth power-time curves of three strains of *Tetrahymena* at 28°C were shown in Fig. 1. The thermokinetic process in the log phase of growth was simulated by the classical logistic model in order to quantify the metabolic characteristics of *Tetrahymena* species [22]:

$$\ln(K/P_t - 1) = \alpha - rt$$

P_t is power output at time t, r is intrinsic rate of metabolic increase, K is power output when cell number got to environmental carrying capacity (maximum number in specific environment). α is a constant which stands for the orientation of logistic curves relative to origin.

The thermokinetic equations and correlation coefficients (R) of *Tetrahymena* metabolism were shown in Table 1. The values of r and other thermokinetic parameters were revealed in Table 2. All of the experimental results had a very good reproducibility and consistency. From their power-time curves and thermokinetic characteristics, we clearly found that there were significant differences in their metabolic processes even between *T. thermophila* BF_1 and *T. thermophila* BF_5. However, intrinsic rate of metabolic increase, r and thermal effect in log phase, Q_{log} indicated the same well-regulated change. Using them as two variables of three strains of *Tetrahymena*, we could obtain their Euclidean distances (Table 3) and cluster analysis diagram (Fig. 2). From Table 3 and Fig. 2, it was found that the relationship between *T. thermophila* BF_1 and *T. thermophila* BF_5 was closer than that between them and *T. pyriformis*.

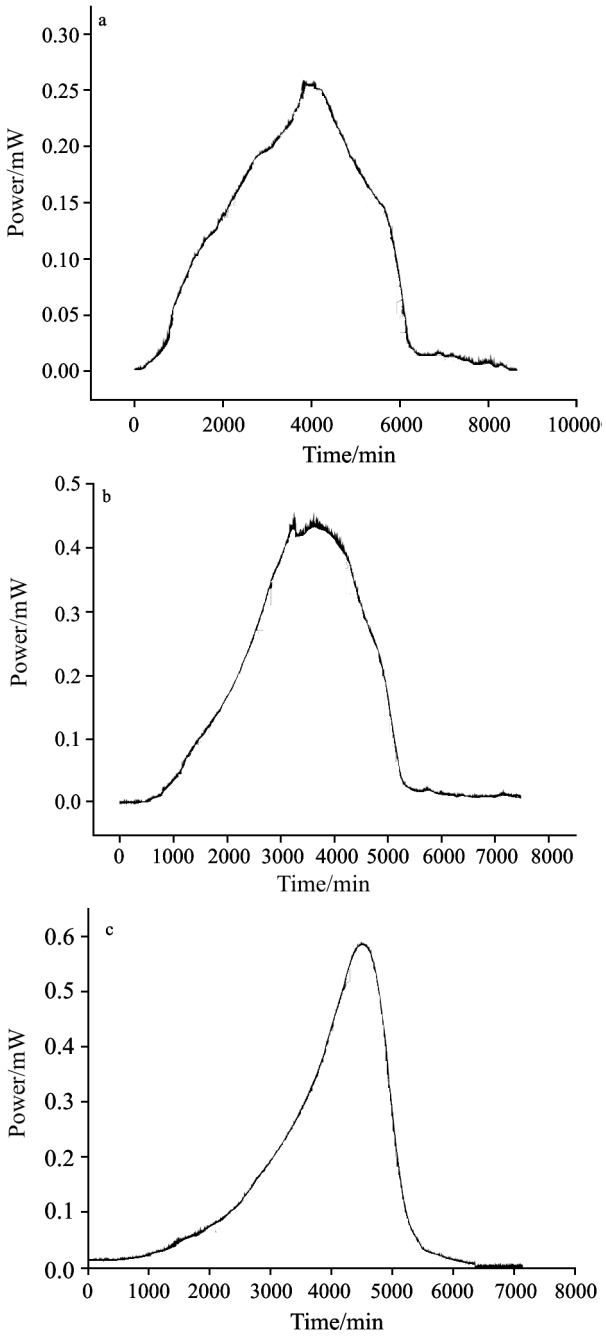

Fig. 1 Growth power-time curves of three stains of *Tetrahymena* at 28°C; a – *T. thermophila* BF_1, b – *T. thermophila* BF_5 and c – *T. pyriformis* GL

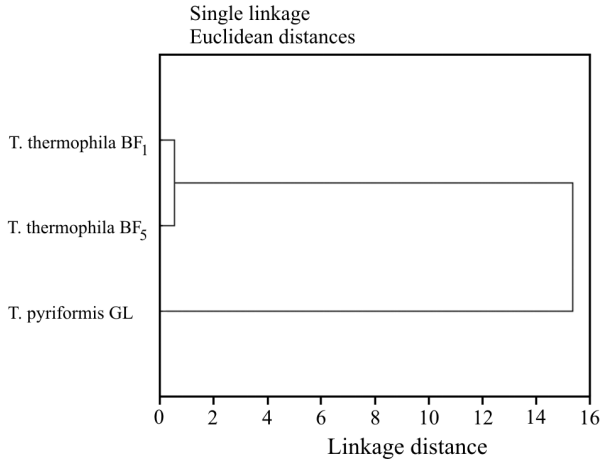

Fig. 2 Cluster analysis diagram of *Tetrahymena* by thermokinetic parameters (r, Q_{log})

Gene sequences and genetic distances

The obtained ITS1 gene sequences (Fig. 3) contained ITS1 region, the partial sequences of 3' end of 18S rDNA and 5' end of 5.8S rDNA. The 18S-ITS1-5.8S rDNA sequences of *T. thermophila* BF_1 and *T. thermophila* BF_5 were 414bp. They were exactly the same. But the sequence of *T. pyriformis* was 416bp and had variations in 12 base positions with those of *T. thermophila*. According to the sequences, genetic distances among them were shown in Table 3. The genetic distance (0) between *T. thermophila* BF_1 and *T. thermophila* BF_5 was less than those (0.024) between them and *T. pyriformis*. Obviously, *T. thermophila* BF_1 and *T. thermophila* BF_5 showed a closer relationship.

Discussion

T. pyriformis and *T. thermophila* were two different species. *T. thermophila* BF_1 and *T. thermophila* BF_5 belonged to two strains with different mating type of *T. thermophila* species. Due to the similarities in morphology, they belonged to the *T. pyriformis* complex in the past. Therefore, it was difficult to choose a morphological characteristic as a phylogenetic marker. Since ITS1 gene was regarded as an effective bio-

Table 1 Thermokinetic equations of metabolism of *Tetrahymena* at 28°C

Species	Thermokinetic equation	R
T. thermophila BF_1	$\ln(0.227157/P_t-1)=2.73322-0.001628t$	0.9897
T. thermophila BF_5	$\ln(0.576684/P_t-1)=4.12912-0.001605t$	0.9971
T. pyriformis GL	$\ln(1.13713/P_t-1)=4.8379-0.001083t$	0.9987

Table 2 The values of intrinsic rate of metabolic increase (r), total thermal effect (Q_T), thermal effect in log phase (Q_{log}), maximum power output (P_{max}) of *Tetrahymena* at 28°C

Species	r/mW min^{-1}	Q_T/J	Q_{log}/J	P_{max}/mW
T. thermophila BF_1	0.001628	55.8024	28.7743	0.2584
T. thermophila BF_5	0.001605	70.3276	29.3037	0.4475
T. pyriformis GL	0.001083	63.5522	44.6618	0.5859

Table 3 Euclidean distances (marked with *) and genetic distances (without mark) of three strains of *Tetrahymena*

	T. thermophila BF_1	*T. thermophila* BF_5	*T. pyriformis* GL
T. thermophila BF_1	–	0	0.024
T. thermophila BF_5	0.5*	–	0.024
T. pyriformis GL	15.9*	15.4*	–

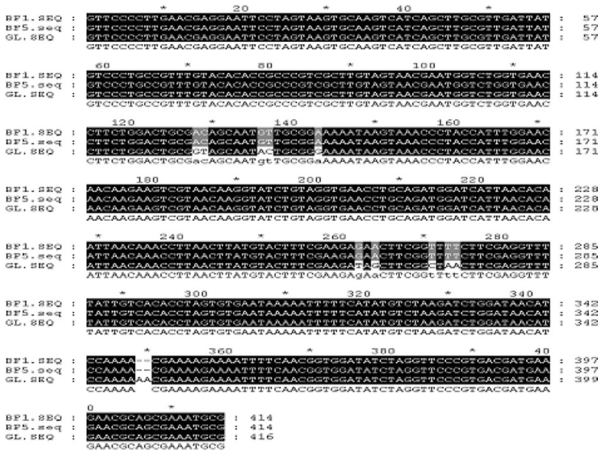

Fig. 3 ITS1 sequences of three strains of *Tetrahymena*; BF_1 is *T. thermophila* BF_1, BF5 is *T. thermophila* BF_5 and GL is *T. pyriformis* GL

marker for phylogenetic studies among species, it was used to investigate the phylogeny in our study. Our analyses based on ITS1 gene sequences suggested the relationships among them, which were consistent with the classification of three strains of *Tetrahymena.* However, two strains of *T. thermophila* showed no differences in ITS1 sequences. It may be explained by that only using ITS1 gene as biomarker was inadequate within the lower taxonomic rank due to its conservative inheritance. In the phylogenetic studies, single gene could never provide complete molecular evolutionary information. It is necessary to use multigene, various sequences and even the genomes to analyze the phylogenetic relationships in order to obtain the real distances among species [23]. However, the work must be very complicated. Microcalorimetry provided us an alternative method. All living systems produce heat and the heat evolution can be measured calorimetrically, without any interference with the processes. The non-specific calorimetric signal from a complex reaction system is usually difficult to interpret on a molecular level. However, the calorimetric signal does give an overall account of the complex process, which a specific analytical signal rarely will give. Further, it should be noted that calorimetric results, although non-specific, are expressed in terms of well-defined thermokinetic quantities, which significantly strengthen the obtained analytical information above that from most other non-specific measurements [24]. In present study, thermokinetic characteristics of three strains of *Tetrahymena* were obtained by microcalorimetry. Their Euclidean distances and cluster analysis diagram were then obtained by r and Q_{log}, which showed similar phylogenetic results to those by molecular data. We could also find out that there was a good linear relationship between Euclidean distances and genetic distances. Especially, the distance between *T. thermophila* BF_1 and *T. thermophila* BF_5, which could not be shown by molecular data, had been obtained by them. Therefore, r and Q_{log} were maybe two proper thermokinetic parameters as phylogenetic markers.

To conclude, comparing with the single molecular biomarker (ITS1) method, microcalorimetry was maybe a simpler, more sensitive and more economic technique in the phylogenetic studies of three strains of *Tetrahymena*. Certainly, it was safe to operators too. The appropriate thermokinetic parameters (r, Q_{log}) could also quantitatively study the distances among *Tetrahymena* species. However, why r and Q_{log} could reveal their distances? Could they be applied to phylogenetic studies of other organisms? A further study is necessary.

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