Acta Protozool. (2000) 39: 209 - 216

ACTA
PROTOZOOLOGICA

Studies on Six *Euplotes* spp. (Ciliophora: Hypotrichida) Using RAPD Fingerprinting, Including a Comparison with Morphometric Analyses

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Summary. Species separation among six morphologically similar *Euplotes* spp. was investigated using random amplified polymorphic DNA fingerprinting (RAPD fingerprinting). Distinctly different banding patterns were obtained for each taxon (the band sharing index (D) among the six was 0.36-0.59), which indicates that all those concerned are well-defined species. Phylogenetic relationships among the six species were also analyzed using both RAPD fingerprinting and morphological analysis. Based on the molecular data, the ciliates were split into two clusters: *E. vannus-minuta-woodruffi-charon*, and *E. eurystomus-octocarinatus*. By contrast, a phenetic dendogram for these six species derived from morphometric characters indicated that they are divided into two rather different clusters, namely *E. vannus-minuta-charon* and *E. eurystomus-octocarinatus-woodruffi.* The most significant difference between the two analyses is that *E. woodruffi* and *E. charon* belong to two different clusters according to the dendogram based on morphology, but are closely related in the dendogram based on RAPD fingerprinting. The present investigation shows that RAPD fingerprinting is a useful method for species separation, but it seems to be of limited use for constructing phylogenetic relationships among the six species investigated. The results of the morphometric analysis support the view that the pattern of the frontoventral cirri, type of silverline system, arrangement of dorsal kineties and the structure of adoral zone of membranelles are important characters for determining phylogenetic relationships among species of *Euplotes*, whereas body shape and size are less significant.

Key words: *Euplotes*, morphometry, phylogenetic relationships, RAPD fingerprinting, species separation.

INTRODUCTION

Reconstruction of phylogeny and species identification remain two of the main tasks for systematists working on ciliated protozoa. Conventional research techniques, such as morphological investigations based on light and electron microscopic analyses, behavioural studies, and mating tests with living strains *etc.*, have been the bedrock on which ciliate systematics is based. These techniques, however, have their limitations as evidenced by the numerous examples of taxonomic confusion cited in the literature (Tuffrau 1960, Curds 1975, Fleury *et al*. 1992, Prescott 1994, Kołaczyk and Wiąckowski 1997, Song and Bradbury 1997).

The introduction of biochemical and molecular techniques, such as enzyme electrophoresis, PCR/RFLP, DNA diversity, and small subunit ribosomal RNA

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(SSUrRNA) sequence comparisons, have helped to resolve some of the systematic problems within the phylum Ciliophora, particularly with respect to species separation and the reconstruction of phylogenetic relationships (Schlegel *et al*. 1988, 1991; Orias *et al*. 1991; Jerome and Lynn 1996; Kusch and Heckmann 1996; Lynn *et al*. 1999). Random amplified polymorphic DNA (RAPD) fingerprinting, which detects polymorphic fragments of DNA throughout the genome by the polymerase chain reaction, offers new possibilities for analyzing genetic diversity at genus and species levels and is particularly useful in this respect because previous sequence information is not necessary (Weish and McClellane 1990, Williams *et al*. 1990).

Traditionally, species separation and phylogenetic reconstruction within the genus *Euplotes* have mainly relied on morphological and morphometric studies (Tuffrau 1960, Carter 1972, Curds 1975, Dragesco and Dragesco-Kernéis 1986, Song and Bradbury 1997, Song and Wilbert 1997). However, it has long been known that some characters of presumed taxonomic value are quite variable, even among the descendents of a single individual (Gates 1978, Schlegel *et al*. 1988). Furthermore, many features overlap among species and this often yields great confusion for species separation. RAPD fingerprinting can provide useful guidance for species separation, and possibly for indicating interrelationships, among morphologically similar and dissimilar taxa within the genus *Euplotes* (Kusch and Heckmannn 1996).

The aims of the current work were; (i) to determine whether RAPD fingerprinting can be used for a clear separation of some closely related species of *Euplotes*, and; (ii) to compare RAPD fingerprinting with morphometric analyses as methods for examining the phylogenetic relationships among these species.

MATERIALS AND METHODS

Ciliate strains and culture. Of the six *Euplotes* species used in the present study, five were isolated from environmental samples collected in or around Qingdao, P. R. China. The sources of the isolates were as follows: *E. woodruffi* and *E. eurystomus*, both from a small freshwater pond in the Zhongshan Park, Qingdao; *E. charon*, from a marine shellfish-farm pond in Xunshan, Qingdao; *E. minuta*, from a marine prawn-farm pond in Hongdao, near Qingdao; *E. vannus*, from the coastal waters off Taipingjiao, Qingdao. Clonal cultures of each were established in the laboratory. Cultures were uniprotistan, the only other organisms present being the bacterial food sources. In the case of *E. octocarinatus*, genomic DNA was kindly provided by Dr. Liang Aihua (University of Shanxi, Taiyuan, China) and it was on this material that RAPD fingerprinting was carried out.

Table 1. Oligonucleotide sequences of primers for RAPD reaction

Primer	5' to 3' sequence
E1 (22bp)	ATG TAA GCT CCT GGG GAT TCA C
No.832 (17bp)	GGA AGA ATA CAG CAG CA
S101 (10bp)	GGT CGG AGA A

DNA extraction and PCR reaction. Amplifications were performed using either 30 ng of genomic DNA prepared from *Euplotes octocarinatus* cells as described previously (Liang *et al*. 1994) or 25 µl of cell lysates. For preparations of *Euplotes* lysates as DNA-templates for RAPD polymerase chain reactions, test ciliates were first transferred from culture medium to sterile double distilled water or sterile artificial marine water. A further transfer to 5 ml of sterile water was performed in order to remove any contamination. Individual ciliates were isolated in a volume of 10 µl with the help of a dissecting microscope and transferred into PCR-tubes. Samples of 10 µl volume without ciliates were processed as controls. To each tube was added 90 µl of lysis buffer and the mixtures were incubated at 56°C for 60 min followed by 95°C for 15 min. Afterwards the ciliate lysates and controls were stored at-20°C (Kusch and Heckmann 1996, Brünen-Nieweler *et al*. 1998).

Amplifications by PCR were carried out in a total volume of 50 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of one oligonucleotide primer (Table 1) and 2.5 U of Taq DNA polymerase (Promega). A total of 45 oligonucleotide primers were tested initially, the catalogue numbers of which were as follows: E1, E2, No. 9 and No. 832 (TaKaRa Bio. Co., Japan); S21-S40, S101-S120 and S2001 (Sangon Bio. Co., Canada). The amplification mixtures were covered with 25 µl of mineral oil and placed in a PCR thermal cycler. For amplification the reaction mixtures were denatured at 94°C for 5 min, followed by the first 5 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 30 s at 35°C, and extension for 1 min at 72°C. The subsequent 35 cycles comprised denaturation for 30 s at 94°C, primer annealing for 30 s at 40°C, and extension for 1 min at 72°C. Cycling was followed by a final extension step for 5 min at 72°C. PCR products (10 µl), along with a DNA molecular weight marker, were run on a 1.5% agarose gel. Three repetitions of the PCR reaction were performed in order to assess the reproducibility of the data.

The requisite concentration of DNA template for RAPD fingerprinting was determined by carrying out the PCR reaction with different numbers of *E. vannus* cells. To PCR-tubes containing 25 µl of lysis buffer was added 1, 3, 5, 10, or 20 cells of *E. vannus* respectively, which provided the source DNA template for the PCR reaction. The resulting PCR product was run on a 1.5% agarose gel.

Morphometric data. Data for the morphometric analysis was obtained from the literature as indicated in Tables 2 and 3. In addition

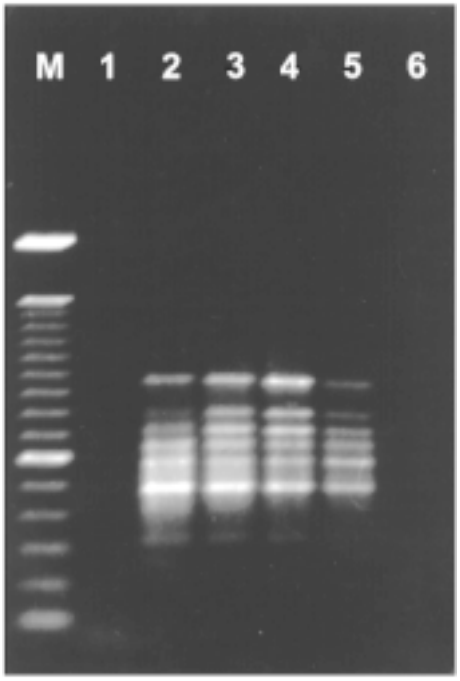

Fig. 1. Gradient of DNA template of *Euplotes vannus* RAPD fingerprinting with primer E1. Lanes: M - 100 bp marker, 1 - control without cells, 2 - 1 cell, 3 - 3 cells, 4 - 5 cells, 5 - 10 cells, 6 - 20 cells

to the 11 morphometric characters listed, two others were also included: the dorsal ridges (present *vs*. absent) and macronucleus shape. No character weighting was included.

Data analysis. The Cluster program in Statistica software (ver 5.1, StatSoft, Inc. 1984-1996) was used both for calculating the phylogenetic relationships among the six taxa and for tree construction. The band-sharing index (D) was calculated for all possible pairwise comparisons. The band-sharing index for two individuals is given by the formula: $D = 2N_{AB} / (N_A+N_B)$ where N_A and N_B are the number of bands scored in ciliates A and B respectively, and N_{AB} is the number shared by both (Wetton *et al.* 1987).

RESULTS AND DISCUSSION

RAPD fingerprinting using different numbers of cells of *E. vannus*

The results of RAPD fingerprinting using different numbers of *E. vannus* cells are shown in Fig. 1. The control solution (without DNA template, lane 1) did not produce bands. Lanes 2-5, representing original inocula of 1, 3, 5, and 10 cells respectively, each had 8 bands. There were no bands in lane 6 (20 cells), possible reasons for which include; (i) the concentration of DNA template was too high; (ii) other cellular constituents (i.e. proteins, lipids, etc.) were present in excessive amounts; (iii) a combination of these two factors.

The most intense bands were found in lanes 3 and 4 (3-5 cells). Although the intensities of several amplified segments varied with the concentration of template DNA, the positions of bands did not change relative to one another indicating that our results had satisfied the criterion of reproducibility. The fact that a single cell was

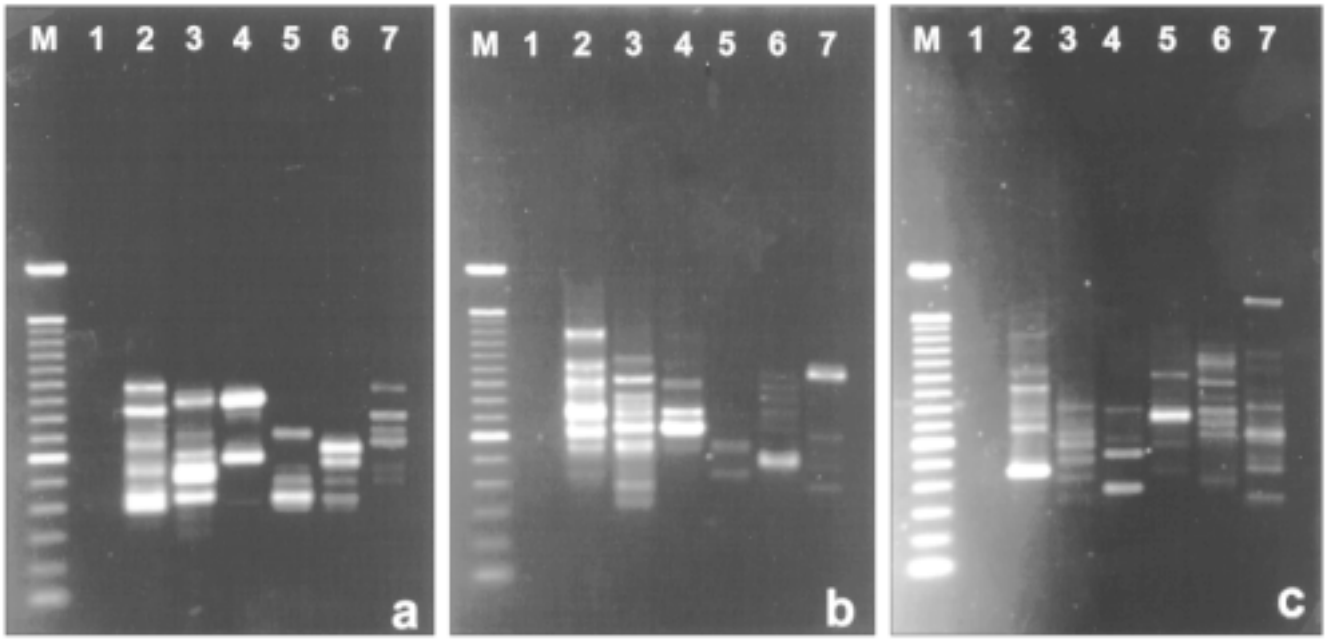

Fig. 2. RAPD fingerprinting of six *Euplotes* species with primers E1 (**a**), No.832 (**b**) and S101 (**c**). Lanes: M -100 bp marker, 1 - control without cells, 2 - *E. woodruffi*, 3 - *E. eurystomus*, 4 - *E. octocarinatus*, 5 - *E. charon*, 6 - *E. minuta*, 7 - *E. vannus*

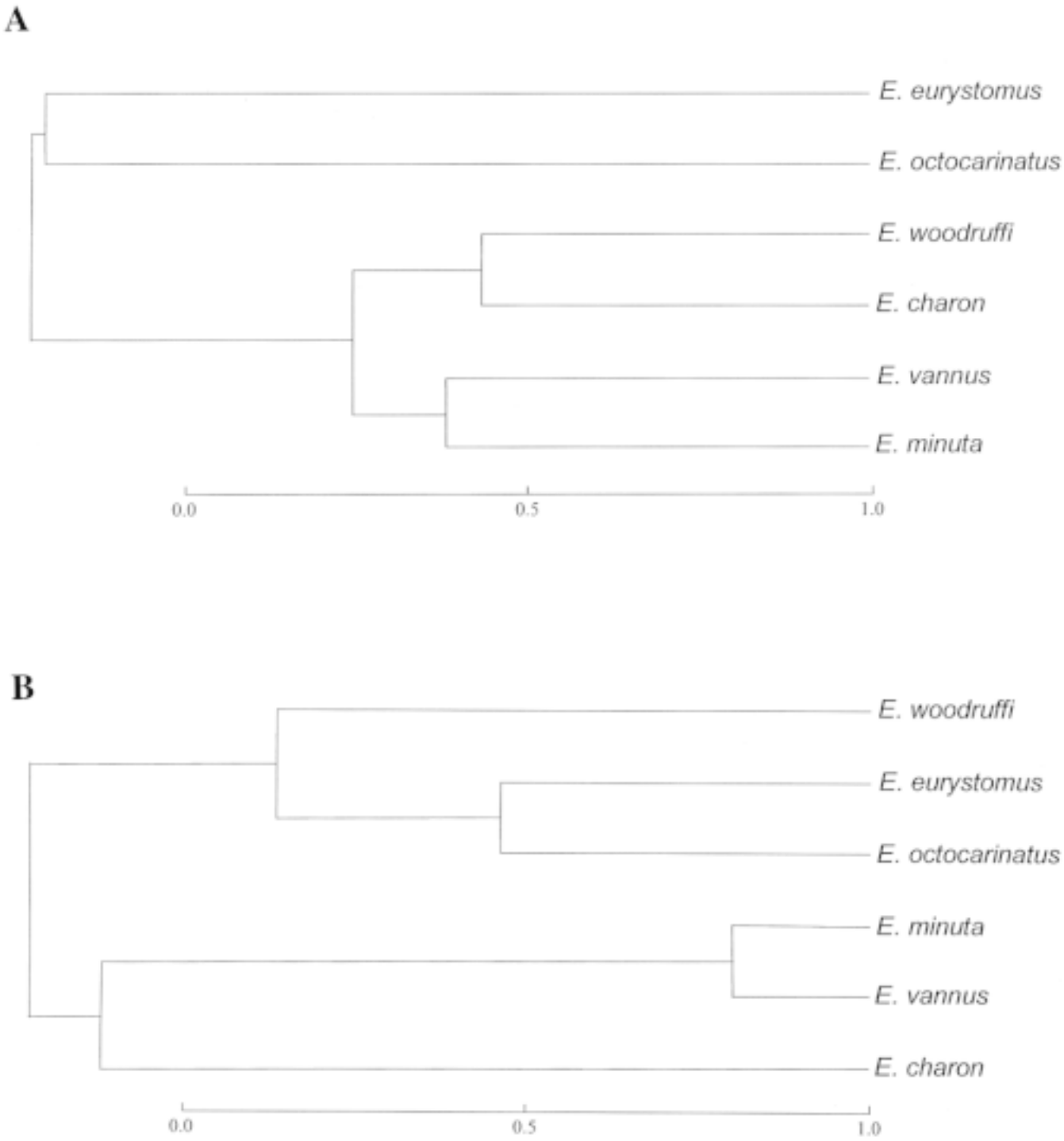

Fig. 3. A - phylogenetic dendogram of six *Euplotes* species, based on RAPD fingerprinting. **B** - phylogenetic dendogram of the same six species, based on morphometric characters. Both dendograms prepared using UPGMA in PHYLIP (ver 3.57c, Felsenstein 1995). The calculated mutation distances are indicated for each branch

Table 2. Morphometric characterization of *Euplotes woodruffi* (1st line), *E. eurystomus* (2nd line), *E. octocarinatus* (3rd line), *E. charon* (4th line), *E. minuta* (5th line) and *E. vannus* (6th line). Data are based on protargol impregnated specimens. Measurements in µm. AZM - adoral zone of membranelles, Max - maximum value, Min - minimum value, n - sample size, SD - standard deviation, SE - standard error of arithmetic mean, CV - coefficient of variation in %, (-) - data not available. Data sources: *E. woodruffi* (after Song and Bradbury 1997); *E. eurystomus*, present paper; *E. octocarinatus* (after Carter 1972); *E. charon* and *E. vannus* (after Song and Packroff 1996/97); *E. minuta* (after Song and Wilbert 1997)

Character	Min	Max	Mean	SD	SE	CV	n
Length of body	105	134	117.0	5.69	1.58	4.9	13
	108	136	117.9	9.34	2.33	7.9	16
	61.5	99	80.0	-	-	-	-
	75	98	85.1	7.33	1.83	8.6	16
	45	78	57.2	8.37	2.09	14.6	16
	96	135	124.2	8.69	2.17	7.0	16
Body width	84	93	87.7	2.59	0.72	3.0	13
	64	100	81.6	8.89	2.22	10.9	16
	33	66	50.0	-	-	-	-
	67	90	74.6	8.12	2.03	10.9	16
	31	54	40.1	5.76	1.44	14.3	16
	61	78	69.2	5.18	1.30	7.5	16
Length of buccal field	84	90	86.7	1.97	0.55	2.3	13
	68	88	79	5.93	1.48	7.5	16
	33	55.5	50.1	-	-	-	-
	61	77	66.3	4.60	1.15	6.9	16
	35	43.0	38.4	2.13	0.53	5.5	16
	84	108	98.1	6.99	1.75	7.1	16
No. of frontoventral cirri	9	9	9	0	0	0	18
	9	9	9	0	0	0	16
	9	9	9	-	-	-	-
	10	10	10	0	0	0	16
	10	10	10	0	0	0	25
	10	10	10	0	0	0	16
No. of transverse cirri	5	5	5	0	0	0	18
	5	5	5	0	0	0	16
	5	5	5	-	-	-	-
	4	6	5.1	0.44	0.11	8.7	16
	5	5	5	0	0	0	25
	5	5	5	0	0	0	16
No. of caudal cirri (MC and CC)	4	4	4	0	0	0	20
	4	5	4.1	0.25	0.06	6.2	16
	4	4	4	-	-	-	-
	4	7	4.4	0.63	0.16	14.2	16
	4	4	4	0	0	0	25
	4	6	4.5	0.51	0.13	11.6	16
No. of dorsal kineties	10	10	10	0	0	0	13
	8	9	8.6	0.50	0.13	5.8	16
	8	8	8	-	-	-	-
	9	10	9.5	0.52	0.13	5.4	16
	7	9	8.6	0.63	0.16	7.4	16
	9	10	9.8	0.40	0.10	4.1	16
No. of basal bodies in mid-dorsal kineties	23	28	24.5	1.45	0.40	5.9	13
	17	25	20.9	2.43	0.54	11.6	20
	ca 18-21						
	10	25	22.3	1.92	0.48	8.6	16
	9	11	10.1	0.81	0.20	7.9	16
	16	21	18.3	1.34	0.27	7.3	24
No. of membranelles in AZM	57	64	60.1	1.94	0.65	3.2	9
	42	54	46.2	3.36	0.87	7.3	15
	36	42	38	-	-	-	-
	51	60	54.5	3.88	1.17	7.1	11
	33	41	36.6	2.15	0.54	5.8	18
	53	66	57.1	4.46	1.11	7.8	16

Table 3. Pattern of silverline system on dorsal side, and the biotope, of six *Euplotes* species (after Curds 1975)

	Single-*vannus*	Double-*eurystomus*	Double-*patella*	Habitat
E. woodruffi	+			Freshwater
E. eurystomus	+			Freshwater
E. octocarinatus			+	Freshwater
E. charon		+		Marine
E. minuta	+			Marine
E. vannus	+			Marine

found to produce sufficient DNA template for the RAPD reaction to work successfully shows that this method can be used without the need to cultivate the organism.

Evolutionary relationships inferred from RAPD fingerprinting

As a result of the findings reported above, 3-5 cells from each clonal culture were used to provide the DNA template for the investigations of DNA polymorphism and genetic diversity. Three out of 45 oligonucleotide primers tested generated informative bands; altogether 10 bands were scorable with primer E1, 13 bands with primer No.832, and 19 bands with primer S101 (Fig. 2). The same band patterns were produced for each taxon on each of three separate occasions thereby verifying the reproducibility of these results. Other primers gave less discernible or no band patterns. The control without template DNA amplified nothing

An examination of Fig. 2 shows that, for each of the three primers used, there are significant differences in the banding patterns among all six taxa. This indicates that even morphologically very similar species, e.g. *E. vannus* and *E. minuta*, can be clearly separated using RAPD fingerprinting. From Table 4 it can be seen that genetically (i.e. as measured by the band sharing index, D), the most closely related taxa were *E. vannus* and *E. minuta* (D = 0.5854) followed by *E. vannus* and *E. woodruffi* (D = 0.5455); *E. charon* was relatively distantly related to the other species, particularly *E. octocarinatus* (D = 0.3571). Thus, the range of the band sharing indices among the six taxa was *ca* 0.36-0.59. This compares with the range of 0.38-0.46 reported by Kusch and Heckmann (1996) in their investigation of three species of *Euplotes*.

Based on the results of the RAPD fingerprinting a phylogenetic dendogram was constructed (Fig. 3A). According to this dendogram, the six taxa grouped into two identifiable clusters; *E. vannus-minuta-charon-woodruffi*, and *E. eurystomus-octocarinatus*. Within the first cluster there were two clades, one formed by *E. minuta* and *E. vannus*, the other by *E. woodruffi* and *E. charon*. By contrast, *E. eurystomus* and *E. octocarinatus* were only distantly related, both to each other and to *E. minuta* and *E. vannus* (Fig. 3A). This was somewhat unexpected and probably erroneous because these four species have several important morphological characters in common (viz. patterns of silverline system and infraciliature) and could reasonably be expected to group together. A possible explanation for this is that the molecular data derived from RAPD fingerprinting are largely dependent on the choice of primer used. In this case, entirely different banding patterns were obtained for each taxon according to which of the three primers was used (see Fig. 2a,b,c). Furthermore, there were very few similarities between the banding patterns for any of the taxa studied, regardless of the primer used, suggesting that there is little justification for supposing that their phylogenetic relationships could be inferred from the RAPD fingerprinting data.

Morphometric analysis

The phylogenetic dendogram based on morphometric characters is shown in Figure 3B and reveals two main clusters: *E. vannus-minuta-charon*, and *E. eurystomus-octocarinatus-woodruffi*. Among these, *E. minuta* and

Table 4. Band sharing index (D) of RAPD fingerprinting for interspecies genetic diversity of six *Euplotes* species

	E. woodruffi	*E. eurystomus*	*E. octocarinatus*	*E. charon*	*E. minuta*	*E. vannus*
E. woodruffi	-	0.4390	0.5143	0.5143	0.5128	0.5455
E. eurystomus	0.4390	-	0.5294	0.4706	0.5263	0.5116
E. octocarinatus	0.5143	0.5294	-	0.3571	0.5000	0.4324
E. charon	0.5143	0.4706	0.3571	-	0.3750	0.4324
E. minuta	0.5128	0.5263	0.5000	0.3750	-	0.5854
E. vannus	0.5455	0.5116	0.4324	0.4324	0.5854	-

E. vannus were the most closely related, followed by *E. octocarinatus* and *E. eurystomus*. Although *E. charon* was revealed as an isolated branch and sister taxon to the *E. minuta-vannus* group, the support for this relationship did not appear to be very high. Thus, the dendogram based on morphological data differs significantly from that based on RAPD analysis, particularly with respect to the relative positions on the trees of *E. woodruffi* and *E. charon* (Fig. 3A, B).

Morphological differences among *Euplotes* spp. have previously been analyzed by Borror and Hill (1995) who recognized four main groups and, as a result, proposed the quadripartition of the genus. Unsurprisingly, the phylogenetic tree based on morphological characters presented here reflects Borror and Hill's (1995) groupings for the six taxa in question. However, because of the small number of taxa included in the present study, it was not possible to reach any conclusion as to the taxonomic level these groupings represent, i.e. generic or subgeneric level.

Conclusions

As this study has demonstrated, RAPD fingerprinting is potentially a very useful technique for the separation of morphologically similar taxa. For example, *E. minuta* and *E. vannus*, or *E. eurystomus* and *E. octocarinatus*, have relatively minor morphologically differences but could be clearly separated by RAPD fingerprinting (Fig. 2). This study also confirms a previous report that RAPD fingerprinting can be successfully applied even when only single cells are available (Kusch and Heckmann 1996). However, this technique seems to be less useful for determining phylogenetic relationships at species level since the banding patterns vary considerably according to the primer used (Fig. 2). Furthermore, in the present study the high degree is dissimilarity among the banding patterns rendered it difficult to make any inference about the phylogenetic interrelationships among the six taxa investigated. Nevertheless, the application of molecular biological techniques such as enzyme electrophoresis, DNA/DNA-hybridizations, and SSUrRNA sequence comparisons (Schlegel *et al*. 1988, 1991; Orias *et al*. 1991; Jerome and Lynn 1996; Kusch and Heckmann 1996; Lynn *et al*. 1999), in parallel with the continued use of morphological characters, should facilitate a better understanding of phylogenetic relationships among the ciliated protozoa in general, and euplotids in particular.

Acknowledgements. We are grateful to Dr. Liang Aihua, Shanxi University of China, for supplying DNA material of *E. octocarinatus* and for providing technical support, and to Dr. William Liang, San Francisco State University, USA, and Dr Robert Hirt, Natural History Museum, London, for providing constructive suggestions. This work was supported by the National Natural Science Foundation of China (Project no. 39770093) the Cheng Kong Scholars Programme and a Royal Society Exquota grant to WS.

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Received on 6th March, 2000; accepted on 25th May, 2000