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Species Separation and Identification of *Uronychia* spp. (Hypotrichia: Ciliophora) using RAPD Fingerprinting and ARDRA Riboprinting

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Summary. The 3 most common morphospecies of *Uronychia*, i.e. *U. setigera*, *U. transfuga* and *U. binucleata*, were examined *in vivo* and following protargol impregnation. Among these, *U. transfuga* is morphologically different to the others (large cell size, more macronuclear segments etc.). By contrast, *U. setigera* and *U. binucleata* are very similar and difficult to separate based only on their morphologies. Random amplified polymorphic DNA (RAPD) fingerprinting and amplified ribosomal DNA restriction analyses (ARDRA riboprinting) were therefore performed in order to confirm the division between them and to aid species identification. Using 4 different random primers the RAPD fingerprinting revealed 3 distinct patterns. Thus, 7 strains could be separated into 3 species with a similarity index of over 82% between different strains of the same species and only 30% to 40% between strains of different species. The unique restriction pattern of highly-conserved rDNA fragments (ARDRA) of different strains of *U. setigera* and *U. binucleata* using the enzyme *Msp* I was found to be species-specific and could be applicable for both species identification and species separation. According to our molecular analyses, the *Uronychia*-populations comprised at least 3 taxa (morphospecies). Moreover, the morphologically similar *U. setigera* and *U. binucleata* could be reliably separated and identified at the molecular level.

Key words: ARDRA riboprinting, identification, RAPD fingerprinting, species separation, *Uronychia.*

INTRODUCTION

The genus *Uronychia* is one of the most commonly-reported hypotrichous ciliates and is found worldwide in a range of marine and other saline biotopes (Müller 1786; Stein 1859; Quennerstedt 1867; Wallengren 1900; Buddenbrock 1920; Young 1922; Mansfeld 1923; Wang and Nie 1932; Kirby 1934; Wang 1934; Ozaki and Yagiu 1941; Fenchel 1965; Reiff 1968; Kattar 1970; Agamaliev 1971; Borror 1972a, b; Wilbert and Kahan 1981; Song 1997; Shi and Song 1999). In terms of species separation and taxonomy, however, *Uronychia* is possibly one of the most confused of all ciliate genera despite the fact that several studies concerning its taxonomy, morphology and morphogenesis have been carried out in recent years (Curds and Wu 1983, Dragesco and Dragesco-Kernéis 1986, Hill 1990,Valbonesi and Luporini 1990, Petz *et al*. 1995, Wilbert 1995, Song 1996).

Up to 1983,10 species of *Uronychia* had been described in the literature (Müller 1786, Dujardin 1841,

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Claparède and Lachmann 1858, Wallengren 1900, Calkins 1902, Pierantoni 1909, Buddenbrock 1920, Young 1922, Taylor 1928, Kahl 1932, Bullington 1940, Fenchel 1965). In their taxonomic revision of the Euplotidae, Curds and Wu (1983) recognised only 4 species of *Uronychia*: *U. transfuga* (Müller, 1786) Stein, 1859, *U. setigera* Calkins, 1902, *U. binucleata* Young, 1922 and *U. magna* Pierantoni, 1909. Since then two additional species, *U. antarctica* Valbonesi and Luporini, 1990 and *U. multicirrus* Song, 1997, have been described.

Following their detailed investigation of over twenty *Uronychia*-populations collected over a 6-year period, Song and Wilbert (1997) concluded that *U. magna* and *U. antarctica* are conspecific with *U. transfuga* and *U. binucleata* respectively. Consequently, all known putative *Uronychia* spp. were allocated to one of 4 revised morphospecies: *U. transfuga*, *U. setigera*, *U. binucleata* (all of which are common) and *U. multicirrus* (which is rare).

Although Song and Wilbert (1997) significantly enhanced our knowledge and understanding of *Uronychia*-populations based on morphometric analyses, some morphological features at the species level are weak and easily overlooked. For example, the separation of *U. setigera* and *U. binucleata* is based almost exclusively on cell size and on the difference in the number of basal body pairs in the leftmost dorsal kinety, the latter character only being discernible with careful observation of high quality protargol preparations.

It has previously been reported that PCR-based methods such as RAPD (random amplified polymorphic DNA) fingerprinting and ARDRA (amplified ribosomal DNA restriction analyses, also called 'riboprinting') are suitable for the study of microbial diversity (Persing *et al*. 1993). In the field of protozoology, RAPD fingerprinting has been used to identify different parasite species (Neto *et al*. 1993), to separate different scuticociliate species (Song *et al*. 2002), to study genetic diversity within *Trypanosoma* spp. (Dirie *et al*. 1993, Mathieu-Daudé *et al*. 1995, Stothard *et al*. 2000) and in the genus *Tetrahymena* (Lynch *et al*. 1995), and to investigate the population structure of *Euplotes* spp. (Kusch and Heckmann 1996). Jerome and Lynn (1996) even suggested that PCR-based RFLP riboprinting (i.e. ARDRA) provides an alternative to traditional techniques for identifying and distinguishing *Tetrahymena* spp., while Stoeck *et al*. (2000) successfully combined two molecular techniques (RAPD and ARDRA) to reject the hypothesis of sibling species in *Paramecium caudatum*. By contrast, Foissner *et al*. (2001) failed to separate two morphospecies of the hypotrich ciliate *Gonostomum* using RAPD-fingerprinting.

During the present study, RAPD and ARDRA were used in order to investigate the relatedness between 3 species of *Uronychia*: *U. setigera* (three strains), *U. binucleata* (three strains) and *U. transfuga* (one strain). The main objective was to determine whether the separation of these 3 morphospecies is supported at the molecular level.

MATERIALS AND METHODS

Origin of isolates

Seven strains of *Uronychia* were isolated from diverse marine biotopes between 1998 and 2002. Three strains of *U. setigera* were from: (i) a crab-farming pond at Xunshan (strain 99062701); (ii) off the coast of Qingdao (strain 99102305); (iii) a shrimp-farming pond at Laizhou (strain 01041607). Three strains of *U. binucleata* were from: (i) a shellfish-farming pond at Hongdao (strain 01042001); (ii) a shrimp-farming pond at Huangdao (strain 01042106); (iii) a shrimp-farming pond at Weihai (strain 01051801). One strain of *U. transfuga* (strain 00042803) was from the shellfish-farming pond at Hongdao. Clonal cultures of each strain were established and maintained in sterile seawater at room temperature with rice grains to enrich natural bacteria as food for the ciliates. The protargol method according to Wilbert (1995) was used to reveal the infraciliature. The infraciliature and other morphological features of each strain were compared to those reported in previous papers (Song 1996, Song and Wilbert 1997).

DNA extraction and RAPD reaction

The nucleotide extraction protocols used in this study have been described in detail elsewhere (Chen and Song 2001) and involved the following steps: cells were rinsed 3 times with sterile artificial marine water after being starved overnight and were then pelleted by centrifugation (about 1000 g). 0.5 ml lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.6 % Tween 20, 0.6 % Nonidet P40, 60 µg/ml Proteinase K) was added and the mixture was incubated at 56°C for 1-2 h. After incubation, the DNA was extracted using an equal volume of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 70% ethanol. DNA samples were stored at -20°C.

Amplifications by PCR were carried out in a total volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of one oligonucleotide primer, 15 ng of genomic DNA and 1.5 units of Taq DNA polymerase (TaKaRa, Japan). 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. Two or 3 repetitions of the PCR reaction were performed in order to assess the reproducibility of the data

(Chen *et al*. 2000). Four random primers were used, the sequences of which were as follows: S040 - 5' GTT GCG ATC C - 3'; S103 - 5' AGA CGT CCA C - 3'; S104 - 5' GGA AGT CGC C - 3'; S113 - 5' GAC GCC ACA C - 3' (Sangon Bio Co., Canada).

Ribosomal DNA amplification and digestion (ARDRA)

PCR amplifications were performed in a PCR thermal cycler. Genome DNA (50 ng) was mixed with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 mM of each oligonucleotide primer (16S-like F: 5' - AAC CTG GTT GAT CCT GCC AGT - 3'; 23S-like R: 5' - TTG GTC CGT GTT TCA AGA CG - 3') and 5 units of Taq Ex polymerase (TaKaRa, Japan). Distilled water was added to the mixture to a total volume of 100 µl. The reaction mixtures were denatured at 94°C for 5 min before the polymerase was added, followed by the first 5 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 2 min at 56°C and extension for 2 min at 72°C. In the subsequent 35 cycles, the annealing temperature was raised to 62°C. The circulation was followed by a final extension step for 5 min at 72°C (Elwood *et al*. 1985, Medlin *et al*. 1988, Jerome and Lynn 1996).

The amplified ribosomal DNA products were purified using the UNIQ-5 DNA Gel Extraction Kit (Sangon Bio. Co., Canada) and dissolved in 50 µl Tris-EDTA solution (pH 8.0). Restriction of amplified ribosomal DNA was carried out by separate incubations at 37°C for 1.5 h with each of 10 restriction enzymes: *Kpn* I, *Taq* I (65°C), *Xba* I, *Bam*H I (30°C), *Eco*R I, *Hin*d III, *Eco*R V, *Hae* III, *Msp* I, *Pst* I (TaKaRa Bio. Co., Japan). The total volume of restriction mixtures was 10 µl containing 0.5 µl of each restriction enzyme solution, 1 µl 10x buffer for each enzyme, 1 µl BSA (0.1% w/v) and 7.5 µl PCR product (Shang *et al*. 2002).

Data analysis

The band-sharing index of RAPD fingerprinting for two individuals is based on that used by Wetton *et al*. (1987) for comparing DNA fingerprints and is given by the formula: $S = 2\ N_{AB} / (N_A + N_B)$, 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.

RESULTS AND DISCUSSION

Morphological comparison

The strain of *U. transfuga* could be clearly separated from the 6 strains belonging to the other two species by the following characters: more elongated, rectangular body shape; body size *in vivo* 110-250 x 80-180 µm; macronucleus moniliform comprising several to many (6-13) segments which form a horseshoe shape; base of buccal cirrus conspicuously long and narrow (Fig. 2, Table 1). The number of macronuclear segments is considered a particularly reliable character for *U. transfuga* thereby confirming its identity unequivocally (Song and Wilbert 1997).

Uronychia setigera and *U. binucleata* are morphologically similar and are characterized as follows: two macronuclear segments often connected by short funiculus, but sometimes only one segment in globular forms of *U. setigera* (Song and Wilbert 1997); invariably with one small micronucleus between the macronuclear segments; rapidly crawling or very fast swimming movement; 4 frontal and 2 ventral cirri; 4 large and one small transverse cirri; 3 strong left marginal cirri; constantly 6 dorsal kineties. *Uronychia setigera* differs from *U. binucleata* by having fewer basal body pairs in the leftmost dorsal kinety (Fig. 1, arrowed), smaller body size, a conspicuous (*vs* inconspicuous) spur on the left cell margin and its characteristic buccal apparatus (Figs 1, 2; Table 1).

Thus, our findings confirm those of Song and Wilbert (1997) who also reported that *U. transfuga* is morphologically distinct from *U. binucleata* and *U. setigera*, and that the latter two are so morphologically similar that they can only be separated following meticulous observations of cells *in vivo* and of the infraciliature following protargol impregnation. It has been suggested that in cases such as this, molecular methods, which have the potential to provide informative data independent of the morphological organisation, might be usefully employed (Clark 1997).

RAPD fingerprinting

The RAPD fingerprinting patterns obtained with the 4 random primers revealed different polymorphic band patterns (Fig. 3). The control with no DNA template amplified no bands. Lane 7 (*U. transfuga*) was distinctly different from the other 6 samples for each of the

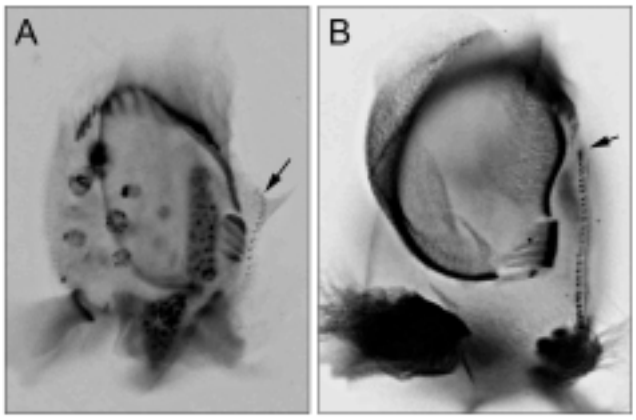

Fig. 1. Photomicrographs of *Uronychia setigera* (A) and *U. binucleata* (B) (protargol impregnations). Arrows mark leftmost dorsal kinety; note the different number of basal body pairs.

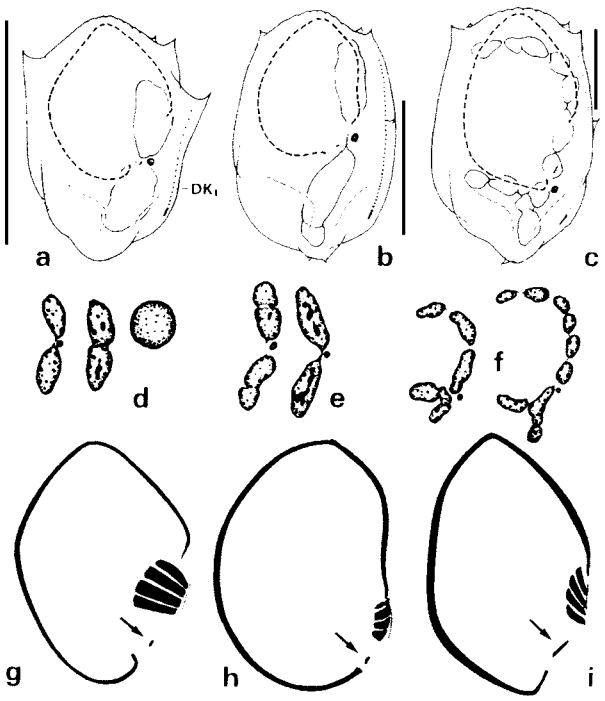

Fig. 2. Comparative diagrams of 3 *Uronychia* species: *U. setigera* (a, d, g); *U. binucleata* (b, e, h) and *U. transfuga* (c, f, i). **a-c** - showing the cell size and general body shape; **d-f** - different forms of macronuclear segments; **g-i** - showing the buccal apparatus, arrows indicate the buccal cirrus. Scale bars 50 μm (a-c) (from Song and Wilbert 1997).

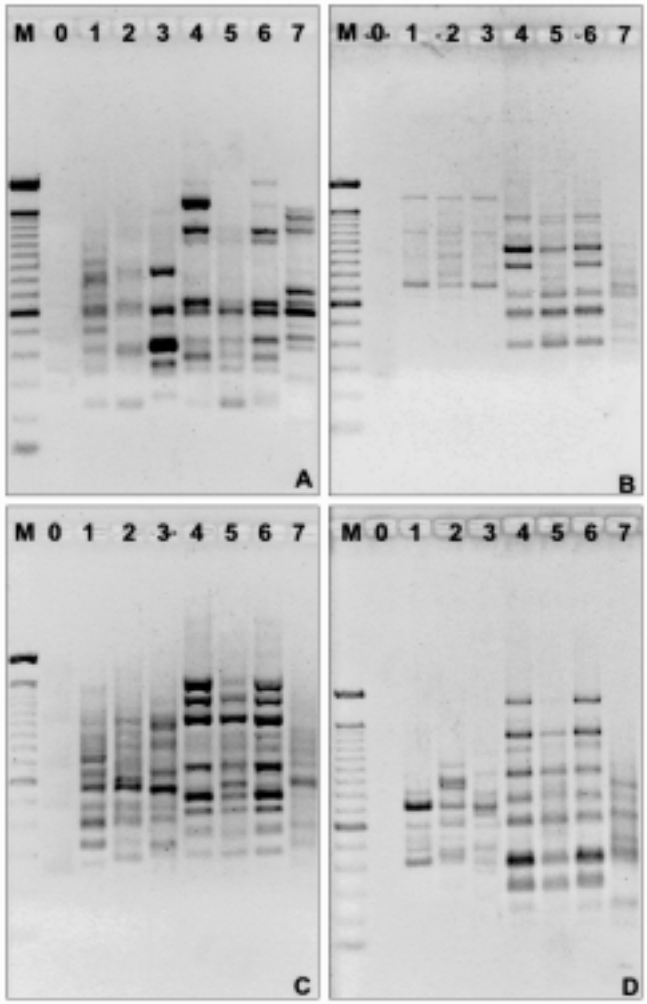

Fig. 3. RAPD banding patterns of 7 *Uronychia* strains using oligonucleotide random primers S040 (A), S103 (B), S104 (C) and S113 (D). Lane M - 100 bp molecular markers. Lane 0 - control without DNA. Lanes 1-3 - three strains of *U. setigera* (strains 99062701, 99102305, 01041607). Lanes 4-6 - three strains of *U. binucleata* (strains 01042001, 01042106, 01051801). Lane 7 - one strain of *U. transfuga* (strain 00042803).

4 primers. Lanes 4 to 6 (*U. binucleata*) shared similar band patterns though the intensity of some amplified bands varied due to the different concentration of template DNA. The same is true for lanes 1 to 3 (*U. setigera*).

Calculating the mean similarity index of the DNA banding patterns for each pair of strains, we found a genetical relatedness of 82-100% between different strains of the same species (Table 2, in bold) and only 30-40% between strains of different species (Table 2, in italics). Specifically, there was an 82-89% similarity among the 3 strains of *U. setigera*, and over 92% among the 3 strains of *U. binucleata*. The 11-18% dissimilarity among *U. setigera* strains and <8% dissimilarity among *U. binucleata* strains in the RAPD patterns can be explained by genotypical variance and genetic diversity within these species (Mathieu-Daudé *et al.* 1995, Kusch and Heckmann 1996, Kusch 1998, Stoeck and Schmidt 1998, Stoeck *et al.* 2000). On other hand, there was a mean similarity of only 35% between *U. setigera* and *U. transfuga*, 39% between *U. binucleata* and *U. transfuga* and 34% between *U. setigera* and *U. binucleata* (Table 2).

Kusch and Heckmann (1996) revealed that the similarity index was 0.38-0.46 (i.e. a 38-46% similarity) when different species of *Euplotes* were compared, and 0.60-0.78 (60-78% similarity) among strains of the same species. Our findings are consistent with these results. Thus, based on the polymorphic RAPD patterns we can confidently separate the 3 *Uronychia* species. Notably, our results confirm the separation of uronychias with two macronuclear segments into two distinct species, *U. setigera* and *U. binucleata*. This is in contrast to the findings of Foissner *et al.* (2001) who were unable to

Table 1. Morphological comparison of three *Uronychia* species. Data based on Song and Wilbert (1997).

Species	Cell length *in vivo* (µm)	Number and appearance of macronuclear segments	Base of buccal cirrus	Body shape	Number of basal body pairs in leftmost dorsal kinety
Uronychia setigera	40-70 (mostly 45-55)	1 or 2 (usually 2) sausage-like (occasionally as a single spherical mass)	short, *ca* 2-3 µm long	oval, lateral spur conspicuous	*ca* 15 (12-19)
Uronychia binucleata	70-120 (mostly 90-110)	constantly 2, sausage-like sometimes in two-doublets form	short, *ca* 3 µm long	long oval, lateral spur inconspicuous	*ca* 30 (23-40)
Uronychia transfuga	110-250 (mostly 150-210)	6-13, moniliform, forming a C-shape	conspicuously long, 10-14 µm in length	elongated rectangular, lateral spur inconspicuous	*ca* 50 (46-56)

Table 2. Similarity indices (S) of pairwise comparisons of RAPD patterns of 7 *Uronychia* strains. S was calculated according to Nei and Li (1979) from RAPD fingerprinting with 4 different primers with a total 56 polymorphic bands (*U. setigera* - strains 99062701, 99102305, 01041607; *U. binucleata* - strains 01042001, 01042106, 01051801; *U. transfuga* - strain 00042803).

	99062701	99102305	01041607	01042001	01042106	01051801	00042803
99062701	1.00						
99102305	**0.82**	1.00					
01041607	**0.89**	**0.86**	1.00				
01042001	*0.30*	*0.31*	*0.35*	1.00			
01042106	*0.36*	*0.37*	*0.42*	**0.92**	1.00		
01051801	*0.30*	*0.31*	*0.35*	**1.00**	**0.92**	1.00	
00042803	*0.36*	*0.37*	*0.33*	*0.39*	*0.40*	*0.39*	1.00

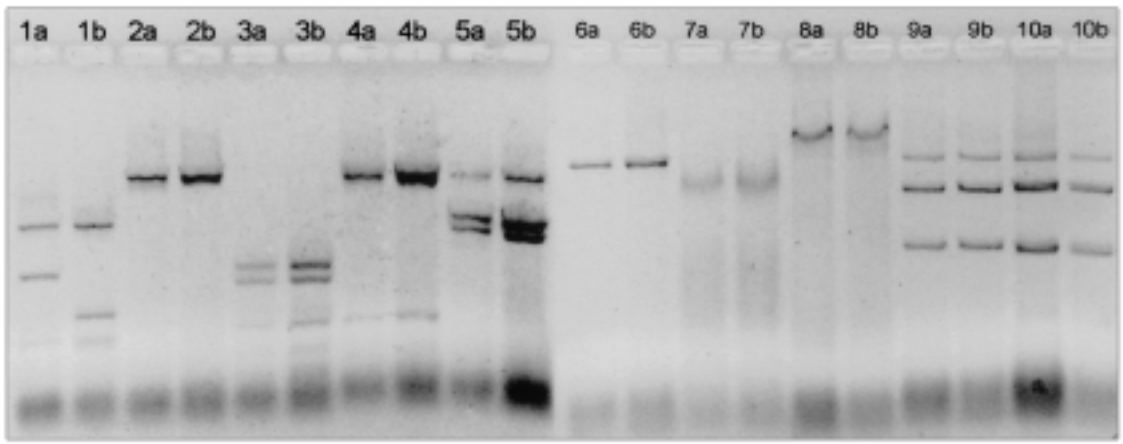

Fig. 4. ARDRA riboprints of; *U. setigera* (strain 99102305) (a**)** and *U. binucleata* (strain 01042106) (b)**,** with *Msp* I, *Xba* I, *Hae* III, *Pst* I, *Eco*R I, *Bam*H I, *Taq* I, *Kpn* I, *Hin*d III and *Eco*R V (lanes 1-10 respectively).

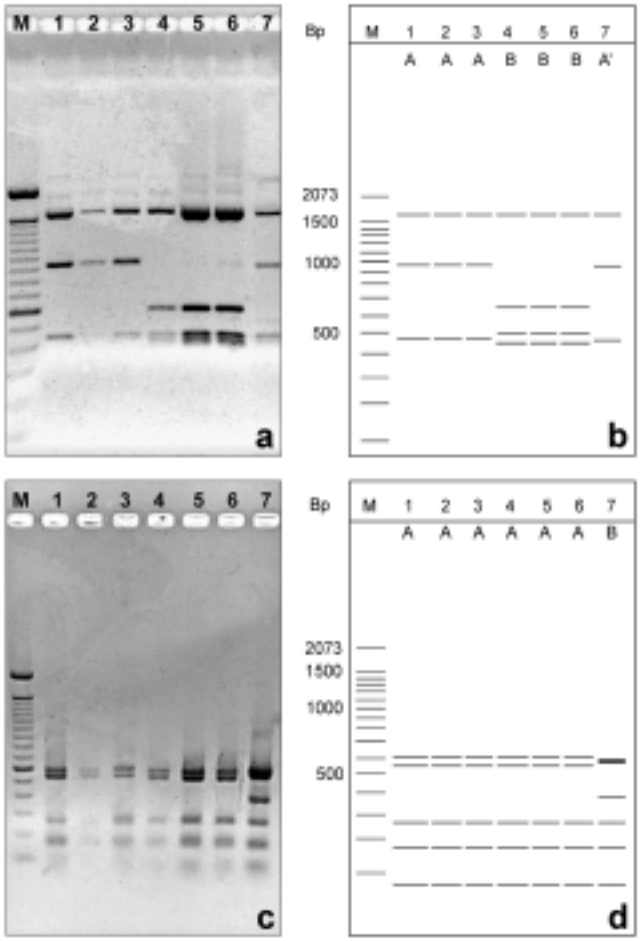

Fig. 5. ARDRA riboprint patterns and schematic representation of 7 *Uronychia* strains by *Msp* I enzymes (a, b) and by *Alu* I enzyme (c, d). Lane M - 100 bp molecular markers. Lanes 1-3 - three strains of *U. setigera* (strains 99062701, 99102305, 01041607). Lanes 4-6 - three strains of *U. binucleata* (strains 01042001, 01042106, 01051801). Lane 7 - *U. transfuga* (strain 00042803).

separate two morphospecies of the hypotrich ciliate *Gonostomum* using RAPD. Nothing is known about the DNA sequences amplified in RAPD fingerprinting, however, and specific bands cannot be associated with particular loci in the genome (Lynch 1991). Consequently RAPD data on their own are not sufficiently reliable to unambiguously identify *Uronychia setigera* or *U. binucleata*.

ARDRA

Two primers were used in order to amplify the complete small subunit (16S), and part of the large subunit (23S), ribosomal RNA gene (including internal transcribed spacers). The lengths of these amplified products were similar (*ca* 3000 bp) for each of the 7 strains. Ten restriction enzymes were used to select informative bands between two species (strain 99102305 *vs* strain 01042106) beforehand (Fig. 4). All enzymes with the exception of *Kpn* I could digest the DNA product while only *Msp* I revealed different restriction patterns between the two strains. Subsequently the amplified products were restricted with *Msp* I enzyme which revealed two kinds of patterns among the 7 strains; the 3 strains of *U. setigera* and the *U. transfuga* strain had one pattern, whereas the 3 strains of *U. binucleata* had another (Figs 5a,b). By using a second restriction enzyme (*Alu* I) it was possible to separate *U. transfuga* from *U. setigera* and *U. binucleata* since the former had a species-specific restriction band of about 400 bp which was absent in the other two (Figs 5c,d). None of the other restriction enzymes tested produced riboprints with identifiable patterns.

ARDRA riboprinting, has previously been successfully applied for the separation and identification of various protozoan taxa including species of *Entamoeba* (Clark and Diamond 1997), trypanosomes (Clark *et al*. 1995) and sibling species belonging to the *Tetrahymena pyriformis* species complex (Jerome and Lynn 1996). The results of the present study suggest that the morphologically similar *U. setigera* and *U. binucleata* can be identified among *Uronychia*-populations by their species-specific ARDRA patterns using restriction enzyme *Msp* I. Interestingly, the riboprint pattern for *U. transfuga* is similar to that of *U. setigera* (compare lanes 1-3 and lane 7, Figs 5a,b), although the two are morphologically distinct.

In conclusion, although we have shown that different strains of *Uronychia* can be characterized by the unique digestion patterns of their rDNA fragments, it cannot be stated unequivocally that these riboprints are absolutely species-specific since currently no published riboprint data are available for *Uronychia multicirrus*. Furthermore, although we were unable to separate the 3 *Uronychia* spp. using a single enzyme, *U. transfuga* could be separated from *U. setigera* and *U. binucleata* using *Alu* I, while the restriction patterns produced with *Msp* I enzyme provide a reliable basis for the identification of these latter two.

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