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A Comparative Study of *Willaertia magna* (Free-Living *Amoeba*) from Different Geographic Areas Using Whole-cell and Small-subunit rDNA Restriction Fragment Length Polymorphisms

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ABSTRACT

Nine strains of the free-living amoeba *Willaertia magna* and one of *Naegleria fowlen* were compared by detection of whole-cell and small subunit ribosomal DNA (ssurDNA) restriction fragment length polymorphisms (RFLPs). Ail strains of *W. magna* showed identical Pst I whole-cell RFLPs, although variation in Bg1 II, Hae III and Kpn I RFLPs were found. These RFLPs did not correlate with the geographic origin of the strains. Phylogenetic analysis of the whole-cell RFLPs separated the *W. magna* strains into three distinct clusters. However, these were not sufficiently diverse to indicate interspecies variation. Homologous ssurDNA RFLPs were found for ail strains of *W. magna* regardless of the restriction endonuclease used. Both the whole-cell and ssurDNA RFLPs were different for *W. magna* and *N. fowleri* and can be used to identify the species. On the basis of homologous whole-cell Pst I and ssurDNA RFLPS, the findings of this study confirm that *W. magna* is a single species genus.

INTRODUCTION

Willaertia magna is a small free-living amoeba (FLA) characterized by a trophozoite, cyst and flagellate stage (De Jonckheere et al. 1984; Page 1988,

Robinson et al. 1989). Although the original isolate of the species was made from a bovine enteric specimen, *W. magna* is commonly found in warm freshwater habitats world-wide (De Jonckheere et al. 1984; Dobson et al. 1993). Such sites can also contain the thermophilic amoebo-flagellate *Naegleria fowlen*, the causative organism of fatal primary amoebic meningoencephalitis (PAM) in man (Carter 1972; John 1993; Kilvington and Beeching 1995). Whilst the morphological forms are larger in *W. magna*, the trophozoites of both species show limax movement, have pores in the cyst wail, form temporary flagellates that in *W. magna* can also divide (John 1993, Page 1988; Robinson et al. 1989). In addition, both species can tolerate growth up to 45°C (De Jonckheere et al. 1984). It is therefore important to reliably differentiate the two species when monitoring environments for the presence of *N. fowlen.*

As in all members of the schizopyrenid amoebae, the ribosomal DNA (rDNA) genes of *W. magna* are present on extrachromosomal plasmids (Clark and Cross 1987). Endonuclease digestion of whole-cell DNA preparations enables the direct detection on agarose gel electrophoresis of restriction fragment length polymorphisms (RFLPs) derived from mitochondrial and rDNA. These RFLPs enable the demonstration of both inter and intraspecific differences within genera (De Jonckheere 1986; De Jonckheere 1987; Kilvington and Beeching 1995). Most recently, amplification of the small-subunit rDNA gene (ssurDNA) by the polymerase chain reaction (PCR) and digestion with restriction endonucleases (riboprinting) has also been used to detect species variation in both parasitic and free-living amoebae (Clark and Diamond 1991a; Clark and Diamond 1991b; De Jonckheere 1994a; Brown and De Jonckheere 1994).

Minor differences in RFLPs and isoenzyme profiles have been demonstrated in strains of *W magna* (De Jonckheere et al. 1984; De Jonckheere 1986; Dobson et al 1993) but these are not sufficiently diverse to indicate that the genus is comprised of more than a single species (De Jonckheere et al. 1984; De Jonckheere 1986). Descriptions of *Protonaegleria westphali* (Michel and Raether 1985) and *W. minor* (Dobson et al. 1993) are now considered to be synonyms of *W. magna* (Robinson et al. 1989; De Jonckheere and Brown 1995). In this present study we have used whole-cell and ssurDNA RFLP typing to compare 9 strains of *W. magna* from various geographical locations and a clinical isolate of *N. fowleri.* The objectives were to determine the level of genetic variation in *W. magna* and to establish whether these techniques can reliably differentiate the species from *N. fowleri.*

MATERIALS AND METHODS

Origin and Culture of Strains

Strains of *W. magna*, *N. fowleri* and *N. Iovaniensis* examined in this study are

listed in Table 1. Trophozoites were maintained by culture on non-nutrient agar plates seeded with live *Escherichia coli* (NNA-*E. coli*) at 37°C (Page 1988) and also adapted to axenic growth at 32°C in serum-casein-glucose-yeast extract medium (Aufy et al. 1986). Strains were tested for growth tolerance in air at 42°C and 44°C on NNA-*E. coli*. Ability of the trophozoites to transform into flagellates was tested by resuspending NNA-*E. coli* cultures in distilled water and incubating at 30°C in air for 5.5-22 hrs.

Isolation and Restriction Endonuclease Analysis of Whole-Cell DNA

Approximately $1x10^8$ late log phase trophozoites were used for the isolation of whole-cell DNA. Total nucleic acids isolated by standard methodology of detergent lysis, proteinase K digestion, phenol extraction and iso-propanol precipitation, as described previously (De Jonckheere 1987; Kilvington et al. 1991). The final pellet was dissolved in 200 µl of TE buffer (10 mM tris.HCl-1 mM EDTA, pH 8.0) containing 5 µg/ml Rnase A (Sigma Chemical Company, Poole, England). DNA samples (2-3 mg) were digested at 37°C for 2 hrs with 5-10 U of the restriction endonucleases Bgl II, Hae III, Kpn I or Pst I using appropriate reaction buffers provided by the supplier (Northumbria Biologicals Limited, Northumberland, England). Samples were loaded on to horizontal 0.8-1.2% agarose gels prepared in trisborate-EDTA (TBE) buffer for electrophoresis in TBE buffer at 1.5 V/cm overnight (Sambrook et al. 1989). DNA standards of γ-Hind III/ΦX-174 RF-Hae III digests (Pharmacia LKB Ltd, Milton Keynes, England) were included as size markers. Gels were stained with 1 mg/ml ethidium bromide in distilled H_20 and photographed under shortwave W trans illumination using Polaroid 665 film and a Kodak Wratten #23A orange filter (Sambrook et al. 1989).

Table 1. Species, strain, source and year of isolation.

Lab code	Species	Strain	Source	Tem tolerance (°C)	Flagellate formation
A-1	*N. fowleri*	MCM	PAM[a], Bath, England, 1978	44	+
A-2	*W. magna*	Z503[b]	Bovine faeces, France, 1979	44	+
A-3	*W. magna*	F 3228	Thermal spa water, Bath, England, 1987	42	+
A-4	*W. magna*	O1/B	Thermal polluted water, Scotland, 1987	42	+
A-5	*W. magna*	VU5/1	Thermal polluted water, Germany, 1984	44	+
A-6	*W. magna*	PAOB Cl_4	Pond, Spain, 1986	42	+
A-7	*W. magna*	A_1PW_1Cl	River Nile, Egypt, 1987	42	+
A-8	*W. magna*	$A_1PW_1Fl_1$	River Nile, Egypt, 1987	42	+
A-9	*W. magna*	NI13	Pond, Mulund, India, 1983	44	-
A-10	*W. magna*	NI_4Cl_1(CCAP 1517/1)[c]	Pond, Mulund, India, 1983	44	+

[a]primary amoebic meningoencephalitis, [b]denotes type strain of the species, [c]previously *Protonaegleria westphali*

PCR Riboprinting

The ssurDNA for each strain was amplified using the universal eukaryotic primers for the gene: 5' AYCTGGTTGATYYTGCCAG; 3' TGATCCATCTGCAG GTTCACC (Embley et al. 1992a). The letter Y denotes either a cytosine or thymine pyrimidine nucleotide. Each amplification reaction comprised: 300 ng of whole-cell DNA template; 0.2 mM each dNTP, 1 mM of each primer, 2 mM $MgCl_2$, 1x Taq polymerase buffer and 2.5 U of Taq DNA polymerase (Advanced Biotechnologies, Surrey, England), in a final volume of 100 ml. After an initial denaturation step of 95°C for 5 min, the amplification comprised: 10 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; followed by 30 cycles of 92°C for 1 min, 55°C for 1 min, 72°C for 2 min. After a further 10 min at 72°C the tubes were chilled to 4°C. The amplified ssurDNA was purified by spin column chromatography with Sephacryl S400 (Pharmacia Biotech Ltd, Hertfordshire, England), digested separately with the restriction enzymes Alu I, Cfo I, Hae III, Msp I and Sau 3A, and separated on 1.2% agarose TBE gels at 90 volts for 2 hrs (Sambrook et al. 1989).

Phylogenetic analysis

The whole-cell RFLPs obtained for the *W. magna* strains were compared for the presence or absence of common restriction endonuclease sites. Jaccard (1901) similarity indexes were generated and the matrices analysed by the Neighbor-Joining programme within PHYLIP (Phylogeny Inference Package) Version 3.4 (Felsenstein 1989).

RESULTS

Restriction endonuclease digestion of whole-cell DNA with Bgl II, Hae III, Kpn I or Pst I enabled the detection of RFLPs directly on agarose gel electrophoresis (Figs. 1-4). All *W. magna* strains showed identical Pst I RFLPs (Fig. 4). With Bgl II, variation in RFLPs was found (Fig. 1). Strains A-2, A-3, A-8 and A-10 formed one RFLP profile, and A-4, A-5, A-6 and A-9 another. Strain A-7, whilst similar to the latter profile, had a unique band of 1.6 kilobase pairs (kbp). For Hae III, the strains shared common restriction fragment length bands although A-2, A-3 and A-10 were characterized by a unique band of 2.6 kbp (Fig. 2). A-8 showed an additional 2.2 kbp band not present in any other strains. Most variation in RFLPs was seen with Kpn I (Fig. 3). Identical RFLP profiles were found with A-2 and A-8; A-4 and A-9; and A-3 and A-7. All other strains were unique. The RFLP profiles shown by *N. fowlen* MCM were completely distinct from *W. magna* and there was no co-migration of any individual band between the two species regardless of the restriction enzyme used (Figs. 1-4).

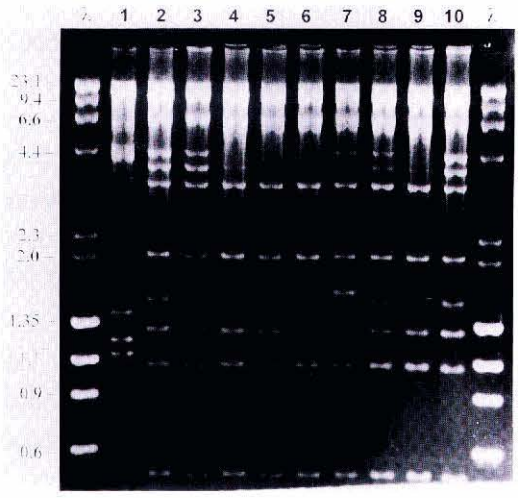

Figure 1. Bgl II whole-cell DNA RFLPs. DNA standards of λ-Hind III/ΦX-174 RF-Hae III digests in kbp (λ). *N. fowleri* (1). *W. magna* strains A-2 to A-10 (2-10).

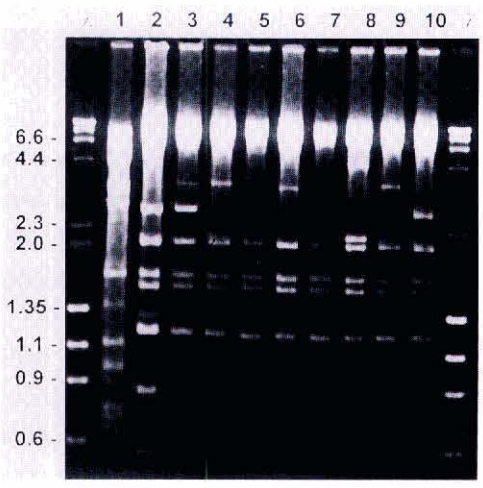

Figure 2. Hae III whole-cell DNA RFLPs. DNA standards of λ-Hind III/ΦX-174 RF-Hae III digests in kbp (λ). *N. fowlen* (1). *W. magna* strains A-2 to A-10 (2-10).

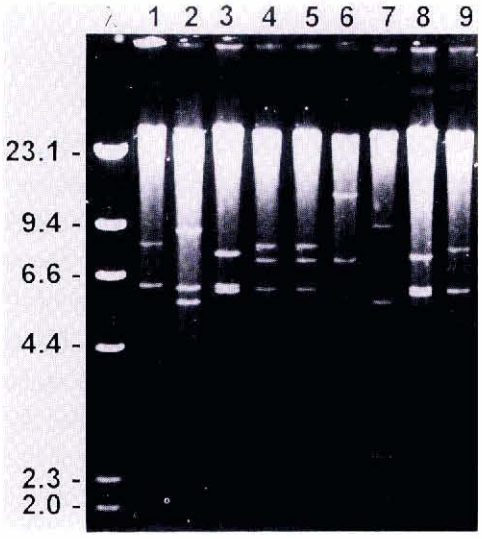

Figure 3 . Kpn I whole-cell DNA RFLPs. DNA standards of λ-Hind III/ΦX-174 RF-Hae III digests in kbp (λ). *W. magna* strains A-2 to A-10 (1-9).

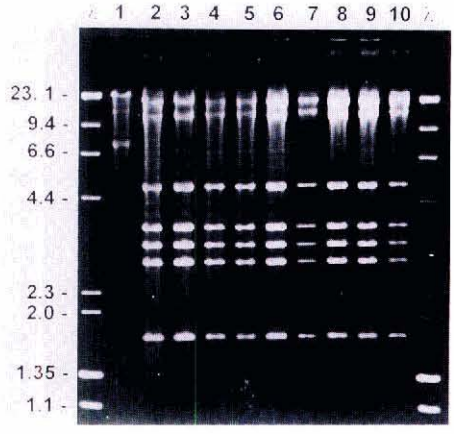

Figure 4. Pst I whole-cell DNA RFLPs. DNA standards of 1,.λ-Hind III/ΦX- 174 RF-Hae III digests in kbp (λ). *N. fowlen* (1). *W. magna* strains A-2 to A-10 (2-10).

Amplification of the ssurDNA gene detected a product of approximately 2.0 kbp in all *W. magna* strains and *N. fowlen* MCM (results not shown). Restriction endonuclease digestion of the product with Alu I, Cfo I, Hae III, Msp I and Sau 3A enabled RFLPs to be detected. All strains of *W. magna* showed homologous RFLPs for the respective endonucleases, however these were distinct from those obtained with *N. fowleri* MCM. The riboprints obtained with Cfo I, Hae III and Msp I are shown in Fig. 5. The photographs of the Alu I and Sau 3A riboprints are not shown. The sizes of the restriction fragments in kbp with Alu I were: 0.57, 0.53, 0.41, 0.35, 0.30 for *N. fowleri;* and 0.60, 0.55, 0.41, 0.37, 0.25 for *W. magna.* With Sau 3A the sizes were: 0.77, 0.49, 0.40, 0.34 for *N. fowlen;* and 0.70, 0.45, 0.33, 0.27, 0.21 for *W. magna.*

The phylogenetic tree obtained for the whole-cell RFLPs is shown in Fig. 6. The riboprinting results were not included in the data analysis as these were homologous for all the strains regardless of the restriction endonuclease tested. Two discrete and one divided clusters were formed. Cluster I comprised strains A-4, A-9, and cluster 2 A-5, A-6. The third cluster contained three subgroups of strains A-2, A-8; A-10; and A-3, A-7.

Of the *W. magna* strains studied here, only A-2, A- 10, A-9 and A-5 showed a maximum growth temperature of 44°C on NNA-*E. coli* (Table 1). No correlation between maximum temperature tolerance and RFLP profile or phylogenetic clustering was found (see Fig. 6). For example, Strains A-4 and A-9 of cluster I showed maximum temperature tolerances of 42°C and 44°C respectively. Flagellate transformation was observed in all strains except A-9 when trophozoites were incubated at 30°C in distilled water (Table 1). The number of flagellates produced was sparse and incubation times of up to 22 hrs were required before they were detected. *N. fowleri* MCM grew at 44°C and readily formed flagellates.

DISCUSSION

In this study the comparison of 9 strains of *W. magna* from various geographic localities was undertaken using the detection of whole-cell and ssurDNA RFLPs. All strains gave homologous ssurDNA RFLPs for all restriction endonucleases tested. Variation in whole-cell DNA RFLPs was, however, detected with Bgl II, Hae III and Kpn I. Identical Pst I RFLPs were obtained in all strains indicating this particular profile may be used to definitively identify *W. magna.* Phylogenetic analysis produced three distinct clusters for the strains based on the whole-cell RFLPs. Cluster 1 comprised strains isolated from Scotland and India, and cluster 2 contained strains from Germany and Spain. Cluster 3 which was subdivided into three branches included strains from France and Egypt, India, and England and Egypt respectively. Therefore, strains of *W. magna* from the same locality exist

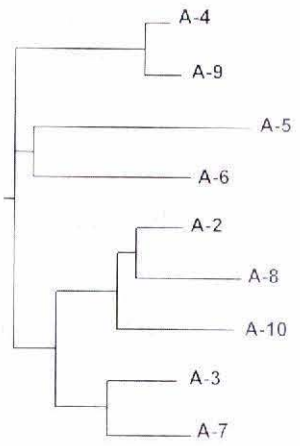

Figure 5. Cfo I, Hae III, Msp I ssurDNA riboprints. DNA standards of λ-Hind III/ΦX-174 RF-Hae III digests in kbp (λ). *N. fowleri* (I). *W. magna* strains A-2 to A- 10 (2-10).

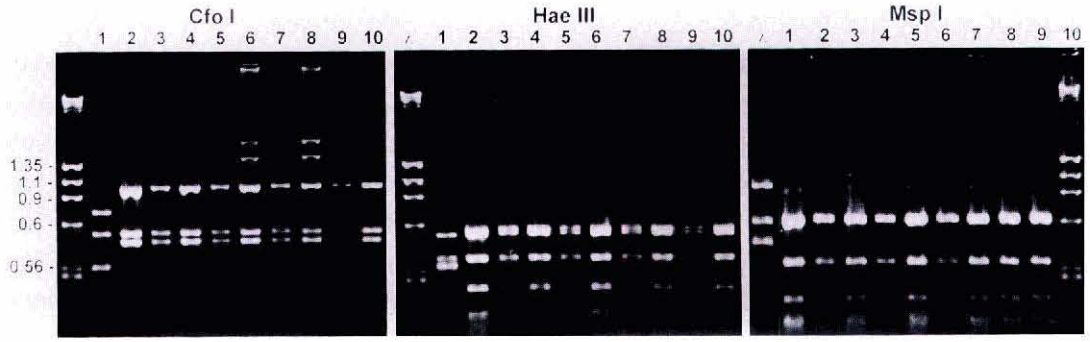

Figure 6. Phylogenetic tree derived from *W. magna* whole-cell DNA RFLPs.

with different RFLP types which do not correspond with geographic locality. This is in contrast to the findings for *N. fowleri* in which strain RFLP type appears to correlate with the geographic continent of origin (De Jonckheere 1988; Kilvington et al. 1995).

The ssurDNA is a highly conserved region in eukaryotes (Hillis et al. 1993). The homology found within *W. magna* strains by riboprinting further indicates that the genus is comprised of a single species. The approximate 2.0 kbp ssurDNA PCR product obtained for all strains of *W. magna* and *N. fowlen* corresponds to the expected size of the gene in FLA (Embley et al. 1992b; De Jonckheere 1993) and indicates the absence of intron elements as has been discovered in *N. andersoni*, some *N. gruberi* strains and *Acanthamoeba* (FLA) species (Embley et al. 1992b; De Johnckheere 1993; Gast et al. 1994). Although riboprinting is a rapid approach for

inferring phylogenetic relationships, it only analyses a small proportion of the total gene. Use of a wider range of restriction endonucleases or, definitively, sequencing of the entire ssurDNA gene may identify intraspecies variation as has been found in the *Naegleria* (De Jonckheere 1993; De Jonckheere 1994a). The riboprints obtained for *W. magna* and *N. fowleri* were distinct and enabled the differentiation of the species. Riboprinting with Alu I of the ssurDNA has previously been shown to identify all *Naegleria* spp. and *W. magna* (De Jonckheere 1994). Thus, the sensitivity and specificity of PCR riboprinting is a valuable technique for the identification of these FLA. Importantly, strains do not need to be adapted to axenic culture for testing and would enable the rapid identification of species soon after primary isolation from the environment.

The flagellate formation test is that used to identify the genus *Naegleria*, members of which readily produce transformants regardless of age or type of culture (Page 1988). However, flagellate formation in *W. magna* is difficult to induce (Page 1988; Robinson et al. 1989) and in this study could not be demonstrated with strain A-9 (NI 13). The use of the test for the identification of *W. magna* is therefore unreliable and further studies are required to establish the optimal conditions for inducing transformation in the trophozoites of this organism. The failure to recognize a flagellate stage in the original description of *W. magna* inadvertently led to the description of the amoeba-flagellate *P. westphali* (Michel and Raether 1985). On the basis of whole-cell and ssurDNA RFLPs, this study has confirmed that *P. westphali* NI_4 Cl_1, (CCAP 1517/1) is a strain of *W. magna*. Recently, Dobson et al. (1993) examined 12 strains of *W. magna* by morphological, physical and isoenzyme analysis. No allelic differences were found between the strains for 18 out of 23 enzymes studied indicating low genetic diversity within the species. However, one strain isolated from the Northern Territory, Australia shared alleles with *W. magna* at only 3 loci and had a maximum growth temperature of 38°C. The strain was designated as a new species, *W. minor*. Sequence analysis of the ssurDNA of the strain has, however, shown it to belong to the genus *Naegleria* (De Jonckheere and Brown 1995).

In conclusion, the homology seen in the Pst I whole-cell DNA and ssurDNA riboprinting RFLPs confirms that *W. magna* is a single species genus. These RFLPs are distinct from those of *N. fowleri* and the methods can be used to differentiate the two species that could by morphological criteria and maximum growth temperature be confused.

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