The Journal of Experimental Biology 208, 2433-2445
Published by The Company of Biologists 2005
doi:10.1242/jeb.01629

The anhydrobiotic potential and molecular phylogenetics of species and strains of *Panagrolaimus* (Nematoda, Panagrolaimidae)

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Accepted 31 March 2005

Summary

Members of the genus *Panagrolaimus* are bacterial-feeding nematodes that occupy a diversity of niches ranging from Antarctic and temperate soils to terrestrial mosses. Some members of this genus are able to survive extreme desiccation by entering into a state of suspended animation known as anhydrobiosis. We have assembled a collection of *Panagrolaimus* species and strains and have investigated their anhydrobiotic phenotypes. Our data show that within the genus *Panagrolaimus* there is a continuum of strains ranging from those unable to survive exposure to low relative humidity (RH) without prior preconditioning at high RH (slow desiccation strategists), through strains that have limited ability to survive rapid desiccation but whose anhydrobiotic ability improves upon preconditioning, to strains such as *P. superbus* that can readily survive immediate exposure to severe desiccation (fast desiccation strategists). Using this panel of nematodes we investigated the effect of preincubation at high RH on the accumulation of trehalose and on the nematodes' anhydrobiotic potential. We found that there is a strong correlation between trehalose induction and anhydrobiotic survival in *Panagrolaimus*. Furthermore, the high trehalose levels observed in fully hydrated *P. superbus* (10% dry mass) suggest that constitutive expression of trehalose pre-adapts this fast dehydration strategist to combat desiccation. All the strains observed, regardless of survival rates, undertook both coiling and clumping, which has the effect of reducing surface area and slowing the rate of water loss during desiccation. Phylogenetic analyses were carried out to investigate whether the observed anhydrobiotic phenotypes were the result of convergent evolution or represented a single phylogenetic lineage. These analyses, derived from alignments of the rDNA ITS and D3 sequences, indicate that the strongly anhydrobiotic strains of *Panagrolaimus* form a single phylogenetic lineage, which is separate from the weakly anhydrobiotic strains. The weakly anhydrobiotic strains are also phylogenetically divergent from each other. Our data indicate that *Panagrolaimus* has the potential to be an excellent model system for the investigation of molecular aspects of nematode anhydrobiosis.

Key words: *Panagrolaimus*, nematode, anhydrobiosis, desiccation, trehalose, phylogeny, rDNA ITS, rDNA D3.

Introduction

Many organisms from diverse taxonomic groups possess the ability to survive extreme desiccation by entering into a state of suspended animation known as anhydrobiosis (reviewed by Crowe et al., 1992; Tunnacliffe and Lapinski, 2003). This state of suspended animation in which metabolism is not detectable is also known as cryptobiosis (Clegg, 2001; Keilin, 1959). Anhydrobiotic individuals can survive in the dry state for long periods of time, after which, upon rehydration, they are able to resume normal metabolic activity. For example, the nematode *Panagrolaimus* sp. PS443 was isolated from dried soil that had been stored for 8 years (Aroian et al., 1993). Members of some taxa can undergo anhydrobiosis at all stages of their life cycle, e.g. some bacteria, algae, lichens, mosses, ferns, certain angiosperm genera (known as 'resurrection plants'), as well as representatives of rotifer, tardigrade and nematode taxa. Other taxa have stage-specific anhydrobiotic forms, e.g. the spores of some bacteria and fungi (Aguilera and Karel, 1997; Potts, 1994), the embryonic cysts of brine shrimps and other microcrustaceans (Clegg, 1965), larvae of the chironomid *Polypedilum vanderplanki* (Watanabe et al., 2003), and some plant seeds and pollen.

Anhydrobiotic organisms have the ability to tolerate conditions ranging from those in which there is no continuous aqueous phase within the cell cytoplasm to those (at 1–5% of normal hydration levels) in which the hydration shell of molecules is gradually lost (Barrett, 1991; Clegg, 1979). Womersley (1987) recognised two broad categories of anhydrobiotic nematodes: slow-dehydration and fast-

dehydration strategists. Fast-dehydration strategists are able to withstand rapid dehydration, whereas slow-dehydration strategists are unable to survive exposure to extreme desiccation unless they have first experienced a period of preconditioning to moderate reductions in relative humidity. This categorisation has general applicability to all anhydrobiotic organisms, with most anhydrobiotic animals belonging to the slow-dehydration group.

Nematodes are aquatic animals and a moisture film is necessary for normal nematode activity. However, nematodes occupy a great diversity of terrestrial habitats, ranging from the relative protection of the lower soil profile to highly exposed surfaces such as plant foliage. These habitats are often the subject of either partial or severe drought and thus many nematodes have become adapted to withstand desiccation. A large number of plant and animal parasitic nematodes have anhydrobiotic eggs or infective juvenile stages (Antoniou, 1989; Perry, 1999). Some nematodes can survive immediate and prolonged exposure to rapid dehydration (Nicholas, 1984). These include *Panagrolaimus rigidus* (Ricci and Pagani, 1997), which inhabits extremely exposed environments on the surface of moss cushions. Such fast dehydration strategist nematodes are probably pre-adapted at a cellular level to survive desiccation. Nematodes that experience slow rates of water loss have the time needed to induce the biochemical changes necessary to survive in an anhydrobiotic state. The majority of anhydrobiotic nematodes are believed to fall into this latter group of slow dehydration strategists (Womersley, 1987). Many nematode species that are unable to enter into cryptobiotic anhydrobiosis can nevertheless survive more modest water losses by entering a quiescent state in which metabolic activity is reduced but not suspended (Womersley, 1990).

One of the best characterised metabolic changes that occurs during the induction of anhydrobiosis in slow-dehydration strategists is the accumulation of high concentrations of disaccharides; sucrose is the predominant sugar accumulated in plants, while animals and yeast accumulate trehalose. Trehalose accumulation has been associated with the successful induction of anhydrobiosis in invertebrates such as brine shrimps (Clegg, 1965), tardigrades (Westh and Ramlov, 1991) larvae of the chironomid *Polypedilum vanderplanki* (Watanabe et al., 2003) and nematodes such as *Aphelenchus avenae* (Madin and Crowe, 1975), *Anguina tritici* and *Ditylenchus dipsaci* (Womersley and Smith, 1981). Trehalose accumulation is believed to protect membranes and proteins from desiccation damage by replacing structural water (Carpenter et al., 1987; Crowe et al., 1984). This sugar may also contribute to the formation of an intracellular glass (Crowe et al., 1998), which is believed to stabilise the cell contents and prevent damage associated with water loss. While the accumulation of trehalose in *A. avenae* is believed to be necessary for anhydrobiotic survival, it is not sufficient. A further period of preconditioning following maximum trehalose accumulation is needed before maximum survival is seen, suggesting that other changes must also occur in *A. avenae* before it can successfully enter the anhydrobiotic state (Higa and Womersley, 1993; Browne et al., 2004). An extensive series of studies has revealed that desiccation tolerance in plants requires the co-ordinated expression of a large array of genes at the onset of desiccation. This leads to the accumulation of various osmolytes and to the synthesis of a variety of proteins, particularly hydrophilic proteins, as well as proteins involved in cellular protection and repair and in the detoxification of reactive oxygen species (Bartels and Sunkar, 2005; Collett et al., 2004; Ingram and Bartels, 1996). Similarly to anhydrobiotic plants, there is also a dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *A. avenae* (Browne et al., 2002, 2004). Data from microorganisms (Potts, 1994, 1996) and brine shrimps (Liang et al., 1997) also demonstrate that successful entry into anhydrobiosis requires a coordinated set of biochemical and cellular adaptations in these organisms.

We have initiated a research programme aimed at gaining a deeper understanding of the molecular biology of anhydrobiosis in nematodes. Our initial studies on the molecular biology of anhydrobiosis in nematodes were carried out with *A. avenae*, one of the best characterised anhydrobiotic nematodes. *A. avenae*, a soil dwelling fungivore, was originally selected by J. H. Crowe as an ideal model in which to study the biochemical changes associated with anhydrobiosis because it can be cultured in large quantities on autoclaved wheat seed inoculated with the fungus *Rhizoctonia solani* (Evans, 1970). Many anhydrobiotic soil nematodes are bacterial feeders and such nematodes can be cultured in the laboratory using methods developed for the model nematode *Caenorhabditis elegans* (Brenner, 1974). Members of the genus *Panagrolaimus* are bacterial feeding nematodes that occupy a diversity of niches ranging from Antarctic, temperate and semi-arid soils to terrestrial mosses, and includes both fast dehydration and slow dehydration anhydrobiote strategists (Aroian et al., 1993; Ricci and Pagani, 1997; Wharton and Barclay, 1993). We have assembled a collection of *Panagrolaimus* species and strains and have investigated their anhydrobiotic phenotypes. Our data show that these strains fall into three broad categories: fast and slow dehydration strategists and desiccation sensitive strains; the majority being slow desiccation strategists. Using this panel of nematodes we investigated the effect of preincubation at high relative humidity (RH), on the accumulation of trehalose and on the nematodes' anhydrobiotic potential. Our data indicate that there is a strong correlation between trehalose induction and anhydrobiotic survival in *Panagrolaimus*. Indeed the high trehalose levels observed in fully hydrated *P. superbus* (10% dry mass) suggest that constitutive expression of high levels of trehalose pre-adapt this fast dehydration strategist to combat desiccation. Phylogenetic analyses were carried out to investigate whether the observed anhydrobiotic phenotypes were the result of convergent evolution or represent a single phylogenetic lineage. Our analyses show that the strongly

anhydrobiotic strains of *Panagrolaimus* form a single phylogenetic lineage which is separate from the phylogenetically divergent weakly anhydrobiotic strains.

Materials and methods

Source and culturing of Panagrolaimus

The source and origin of the *Panagrolaimus* isolates used in this study are listed in Table 1. The nematodes were cultured at 20°C in the dark on nematode growth medium (NGM) plates containing a lawn of *E. coli* strain OP50 (Brenner, 1974). Liquid cultures were set up by adding axenised eggs to liquid culture S Medium (Sulston and Hodgkin, 1987) containing concentrated *E. coli* OP50. Liquid cultures (80 ml) were incubated in 250 ml culture flasks and were shaken at 90 rpm in the dark at 20°C. Liquid cultures were harvested after approximately 14 d, before the appearance of any dauer larvae. The nematodes were cleaned by allowing them to pass through two layers of Kleenex® tissue paper mounted over a coarse plastic sieve into S Basal buffer (Sulston and Hodgkin, 1987) overnight. Clean nematodes moved into the S Basal buffer leaving dead ones and debris from the substrate behind. The clean nematodes were rinsed several times in S Basal buffer.

Axenization of nematodes from plate cultures

Gravid worms were rinsed from the agar surface with S Basal buffer. The worms were centrifuged (2000 ***g*** for 5 min) at room temperature and washed three times with S Basal buffer in a final volume of 30 ml. Worms were allowed to settle and then treated for 4 min at room temperature with 8 ml of 1:3 NaOCl solution and 2 ml freshly prepared 10 mol l^{-1} NaOH, during which time the samples were vigorously shaken to assist the disintegration of the worms and release of eggs. Released eggs and embryos were washed three times with S Basal buffer, collected by centrifugation (2000 ***g***), and added directly to liquid culture (Lewis and Fleming, 1995). A sample of the embryos was transferred to a Petri dish containing S Basal buffer and incubated at room temperature overnight. The following day, the embryos were examined under the microscope to ensure that they developed into L1 larvae and that the axenization procedure had been successful.

Control of relative humidity

Relative humidity (RH) was controlled by the use of saturated salt solutions as described by Solomon (1951) and Winston and Bates (1960). Saturated salt solutions (200 ml) were maintained at 20°C in the dark in desiccation chambers for 3 d prior to the addition of nematodes in order to allow them to equilibrate. A hygrometer was used to confirm that the correct relative humidity had been established. The required relative humidities were maintained as follows: 100% RH, distilled water vapour; 98% RH, potassium dichromate ($K_2Cr_2O_7$); 95% RH, sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$); 90% RH, magnesium sulphate ($MgSO_4.7H_2O$) and ~10% RH, freshly activated silica gel. All experiments were performed at 20°C using mixed stage populations.

Desiccation tolerance

A 1 ml suspension of nematodes in S Basal buffer (concentration 2000 nematodes ml^{-1}) was vacuum filtered onto a 2.5 cm Supor®-450 filter (0.45 μm, Gelman Science East Hills, New York, USA) using a Sartorius funnel (25 mm glass vacuum filter holder with a 30 ml funnel (cat. no. 5m16315, Sartorius AG, Goettingen, Germany) and a vacuum flask attached to a pump. The filters were then transferred to 3 cm Petri dishes without lids and placed in a 2.5 l desiccation chamber containing approximately 200 ml of the appropriate saturated salt solution required to maintain the desired relative humidity. The nematode dishes were then transferred to sealed plastic boxes containing freshly activated silica gel for 48 h. Following this drying period the nematodes were prehydrated at 100% RH over distilled water for 24 h before immersion in S Basal buffer. Percentage survival was assessed by microscopic observation of motility and response to probing 24 h after rehydration. Five replicates were prepared for each experiment. In a preliminary experiment, *Panagrolaimus* sp.

Table 1. *Source of the* Panagrolaimus *isolates used in this study*

Strain	Geographical location	Obtained from	Reproduction
Panagrolaimus superbus (DF5050)	Surtsey Island, Iceland	Bjorn Sohlenius	Male/female (Gonochoristic)
Panagrolaimus detritophagus (BSS8)	Surtsey Island, Iceland	Bjorn Sohlenius	Gonochoristic
Panagrolaimus rigidus (AF36)	Pennsylvania, USA	CGC* (isolated by Andras Fodor)	Gonochoristic
Panagrolaimus rigidus (AF40)	Pennsylvania, USA	CGC (isolated by Andras Fodor)	Gonochoristic
Panagrolaimus sp. BW287	Beijing, China	CGC (isolated by Bill Wood)	Parthenogenetic (possibly hermaphroditic)
Panagrolaimus sp. PS443	Armenia	CGC (isolated by Paul Sternberg)	Gonochoristic
Panagrolaimus sp. PS1159	North Carolina, USA	CGC (isolated by Paul Sternberg)	Parthenogenetic
Panagrolaimus sp. PS1579	California, USA	CGC (isolated by Paul Sternberg	Parthenogenetic
Panagrolaimus sp. PS1732	California, USA	CGC (isolated by Paul Sternberg)	Parthenogenetic
Panagrolaimus sp. WS94	Berlin, Germany	Walter Sudhaus	Parthenogenetic
Panagrolaimus davidi (DNA only)	McMurdo Sound, Antartica	David Wharton	Parthenogenetic

*CGC, Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN 55455, USA.

WS94 nematodes were exposed to various relative humidities (100%, 98%, 95% and 90% RH) for 48 h before exposure for 48 h to freshly activated silica gel (<10% RH). Nematodes pre-treated at 98% RH had the highest survival (Kruskal–Wallis test, $P<0.001$; data not shown). Thus 98% RH was used as standard in all subsequent preconditioning experiments.

Measurement of length

Nematodes were vacuum filtered onto Supor®-450 membrane filters as described above. The control group was maintained at 98% RH for 96 h, rehydrated for 24 h in S Basal buffer and then allowed to migrate through two layers of Kleenex® tissue paper in a wide meshed sieve in order to exclude dead nematodes. The experimental group was treated as follows: 98% RH for 48 h, <10% RH (silica gel) for 24 h, 100% RH for 24 h, followed by rehydration for 24 h in S Basal buffer, after which the animals were allowed to migrate though tissue to exclude dead nematodes. The recovered nematodes were heat killed and straightened by plunging a 0.5 ml suspension of nematodes in an Eppendorf tube into a water bath at 60°C. Animals were then fixed in TAF (triethanolamine, formalin, distilled water, 2:7:91), and measured using a micrometer eyepiece, previously calibrated using a micrometer slide (Perry, 1976).

Carbohydrate extraction

For biochemical analyses, a 1 ml suspension containing 10 000 nematodes per ml was filtered onto a 2.5 cm filter and placed in a 3 cm Petri dish. The Petri dishes containing the nematodes were exposed to 98% RH for the appropriate time period (0–96 h) and then transferred to silica gel for 24 h. The filter papers containing the nematode samples were placed into individual 1.5 ml Eppendorf tubes and stored at –80°C until carbohydrates were extracted. Three replicates were prepared for each experiment and fresh untreated nematodes were used as controls.

The samples (nematodes and filter) were suspended in 1 ml of 30% ethanol for 30 min. Then 0.75 ml of 95% ethanol was added along with 0.75 g of glass beads (<106 μm; cat. no. G 8893; Sigma-Aldrich, St Louis, MO, USA). The samples were homogenised (Mini-beadbeater, Biospec Products, Bartlesville, OK, USA) on ice for six 30 s pulses at high speed. 83% ethanol (1 ml) was then added and the sample was centrifuged at 10 000 ***g*** for 30 min at 4°C. The supernatant was decanted into 2× 1.5 ml Eppendorf tubes, which were placed in a Speedivac and dried for approximately 12 h at room temperature. The samples were then resuspended by the addition of 50 μl of deionised water to each tube. The samples were then recombined and filtered using 0.22 μm centrifuge filter tubes (Spin-X, Costar, Baar, Germany). These samples were then used for low molecular mass carbohydrate analysis. Dry mass was determined by weighing identical control samples of nematodes (minus the mass of the dry filter paper), which had been incubated over silica gel in a small airtight plastic box at 70°C for 3 days.

Analysis of carbohydrate content

Carbohydrates were analysed using high performance liquid chromatography (HPLC). The HPLC instrument (Spectra Physics; model SP 8800, Mountain View, CA, USA) was attached to a Shodex Rl SE-61 refractive index detector. Samples were run through a hypersil hyperREZ carbohydrate H^+ column (300 mm × 7.8 mm; Shandon, Cheshire, UK) which separated the carbohydrates (range 1, attenuation 64, running time 15 min) using 2.5 mmol l^{-1} H_2SO_4 as the mobile phase (flow rate 1 ml min^{-1}). Carbohydrates were identified and quantified by running serial dilutions of known carbohydrate standards through the column.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the morphology of desiccated *Panagrolaimus* sp. The nematodes were washed repeatedly using S Basal buffer and filtered on a Supor®-450 (Pall Corporation, Portsmouth, Hampshire, UK) membrane filter (100 nematodes per filter) using vacuum filtration. The nematodes were then preconditioned at 98% RH for 48 h, followed by exposure for 48 h to freshly activated silica gel. Specimens treated in this way did not require fixation and were mounted on aluminium stubs using double-sided Sellotape®, coated with gold using a Polaron SC500 sputter coater (Polaron plc, Watford, UK) for 3 min at 20 mV and viewed at 20 kV using a Hitachi S4300 field emission scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

Phylogenetic analyses

Gemomic DNA was prepared using standard phenol chloroform extraction described by Maniatis et al. (1982). PCR amplification of the D3 expansion region of the 28S rDNA subunit was achieved using the D3A and D3B primers (Nunn et al., 1996) and each strain of *Panagrolaimus* yielded a single PCR product of approximately 320 bp. PCR cycling conditions were: 94°C, 3 min; 94°C, 1 min; 52°C, 1 min; 72°C, 1 min; 35 cycles followed by 72°C, 10 min. Each PCR amplification was performed in 25 μl volumes containing 0.25 U of Hi-Fidelity Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA; 2.5 μl 10× High Fidelity PCR Buffer (Invitrogen); 1.5 μl 50 mmol l^{-1} $MgSO_4$; 1 μl 10 mmol l^{-1} dNTP solution; 10 pmol of each primer; 100–1500 ng of genomic DNA (which was optimised for each DNA sample) and sterile distilled H_2O to 25 μl. All reactions were analysed by agarose gel electrophoresis.

A fragment of approximately 1.1 kb comprising the rDNA ITS1, 5.8S rDNA and rDNA ITS2 was amplified from *Panagrolaimus* PS1159 using the rDNA1 and rDNA2 primers (Vrain et al., 1992). This DNA fragment was cloned and sequenced as described below. The resulting DNA sequence was aligned with that of *Caenorhabditis elegans* rDNA (accession number X03680.1) to identify conserved regions within the 5.8S rDNA. This information was used to design the PCR primers Pana5.8R (5′-GGTAAGTAACGCAGCAAGC-3′) and Pana5.8F (5′-GCTTGCTGCGTTACTTACC-3′).

Pana5.8R was used with the rDNA1 primer to amplify the rDNA ITS1 region (≈690 bp) and Pana5.8F rDNA was used with the ITS2 to amplify the rDNA ITS2 region (≈590 bp) from all *Panagrolaimus* strains. The PCR reagents and their concentrations were as described above. The PCR cycling conditions were: 94°C, 3 min; 94°C, 1 min; 50°C, 2 min; 72°C, 2 min; 35 cycles followed by 72°C, 10 min.

PCR products were excised from the gel and gel purified using the Montage DNA Gel extraction Kit (Millipore, Billerica, MD, USA). The purified PCR products were cloned into the pCR2.1 TOPO vector using the TOPO-TA Cloning kit (Invitrogen). The insert sizes of the recombinant clones were confirmed by colony PCR using M13 forward and reverse primers. Selected clones were sequenced (AGOWA, Berlin, Germany). These sequences have been deposited in GenBank with the following accession numbers: rDNA D3 sequences, AY878376 to AY878386; rDNA ITS1 sequences, AY878387 to AY878397 and rDNA ITS2 sequences, AY878398 to AY878408.

Alignments of the rDNA D3, ITS1 and ITS2 fragments from the *Panagrolaimus* strains were made using ClustalW 1.8 at the University of Aberdeen (http://www.abdn.ac.uk/~mbi094/SEQANAL.htm#DNA). The rDNA D3, ITS1 and ITS2 sequences from *C. elegans* were included in the alignment and used as an outgroup when constructing phylogenetic trees. For pairwise alignments the gap opening penalty was set to 10.0 and the gap extension penalty was set to 6. For multiple alignments the gap opening penalty was set to 12.0 and the gap extension penalty was set to 5. The alignments were then edited manually and the three sequences were concatenated into one sequence of approximately 1.4 kb for phylogenetic analysis.

To ensure that there was phylogenetic signal within the alignment a permutation tail probability (PTP) test (Archie, 1989) as implemented in PAUP 4.0b10 (Swofford, 2003) was performed. Phylogenetic analyses were performed using the maximum parsimony (MP), maximum likelihood (ML), Bayesian and distance based criteria. Distance based trees were constructed using the neighbor-joining (NJ) program (Saitou and Nei, 1987), with distances corrected by Kimura's two parameter model (Kimura, 1980). MP, ML and distance based analyses were performed in PAUP 4.0b10. MP trees were created using the random stepwise addition option of the heuristic search for 1000 replicates with tree bisection-reconnection branch swapping. Characters were treated as unordered and unweighted. For the ML analysis the optimal model of sequence substitution was found by comparing substitution model likelihood scores using Modeltest 3.04 (Posada and Crandall, 1998). The optimal model of substitution was found to be HKY (Hasegawa et al., 1985). The statistical robustness of the MP, NJ and ML tree topologies were assessed using the bootstrap resampling technique (Felsenstein, 1985), with 1000 replicates for the MP and NJ trees and 100 replicates for ML tree.

A phylogeny was also reconstructed using the Bayesian framework as implemented in MR BAYES 3.0B4 (Huelsenbeck and Ronquist, 2001). Model parameters were estimated during the run and four Markov chains were used. The Markov chain Monte Carlo was set to ten million generations and the chain was sampled every 100th generation. The trees generated after the initial burn in period of two hundred thousand generations were summarised using the majority rule consensus method implemented in PAUP 4.0b10. The first 500 trees were discarded and the remaining trees were combined into a single file which was imported into PAUP* 4.0b10 to compute the 50% majority rule consensus tree. The statistical robustness of branch support was determined by calculating Bayesian posterior probabilities (BPPs) using the *sumt* command of MR BAYES 3.0B4. Two tests: the Shimodaira Hasegawa test (Shimodaira and Hasegawa, 1999) and the approximately unbiased test (Shimodaira, 2002) were used to compare the robustness of phylogenetic trees inferred by the four phylogenetic reconstruction methods.

Statistical analyses

All statistical analyses were carried out using Minitab™ version 13.1 (Minitab Inc., State College, PA, USA). Simple linear regression was carried out alongside fitted line plots and correlation was considered significant at $P<0.05$ in all cases. Analysis of variance (ANOVA) was carried out with confidence intervals at the 95% level to test for differences between means. Means were considered to be significantly different at $P<0.05$. The 2-tailed Mann–Whitney *U*-test was used as a non-parametric method to test for difference between population means. The null hypothesis that population means are all equal was tested at the $P=0.05$ level of significance. The Kruskal–Wallis method was used as a non-parametric test of the equality of medians. The null hypothesis that medians are all equal was tested at the $P=0.05$ level of significance.

Results

Effect of preconditioning at high RH on the anhydrobiotic survival of Panagrolaimus

Ten strains of *Panagrolaimus* representing three species and six unassigned strains were preconditioned at 98% RH for various time periods (0–96 h) followed by exposure to dried silica gel for 48 h. Nematode survival following rehydration was then estimated (Fig. 1). Analysis of the ability of these *Panagrolaimus* strains to survive desiccation without preconditioning at 98% RH shows that these strains fall into three broad categories: the 'fast dessication strategist' *Panagrolaimus superbus* (Fuchs 1930), which demonstrates 93% survival when transferred directly to silica gel; the 'slow dehydration strategists' *P. rigidus* AF36 (Schneider 1866, Thorne 1937), *Panagrolaimus* spp. PS443, PS1159, PS1579 and WS94 whose survival upon direct exposure to silica gel ranged from 17% to 31%; and the 'poor desiccators' *P. detritophagus* (Fuchs 1930), *P. rigidus* AF40 and *Panagrolaimus* spp. BW287 and PS1732, which exhibited very limited ability to survive rapid desiccation, with less than 5% survival upon direct exposure to <10% RH over silica gel.

The anhydrobiotic survival of *Panagrolaimus* increases on

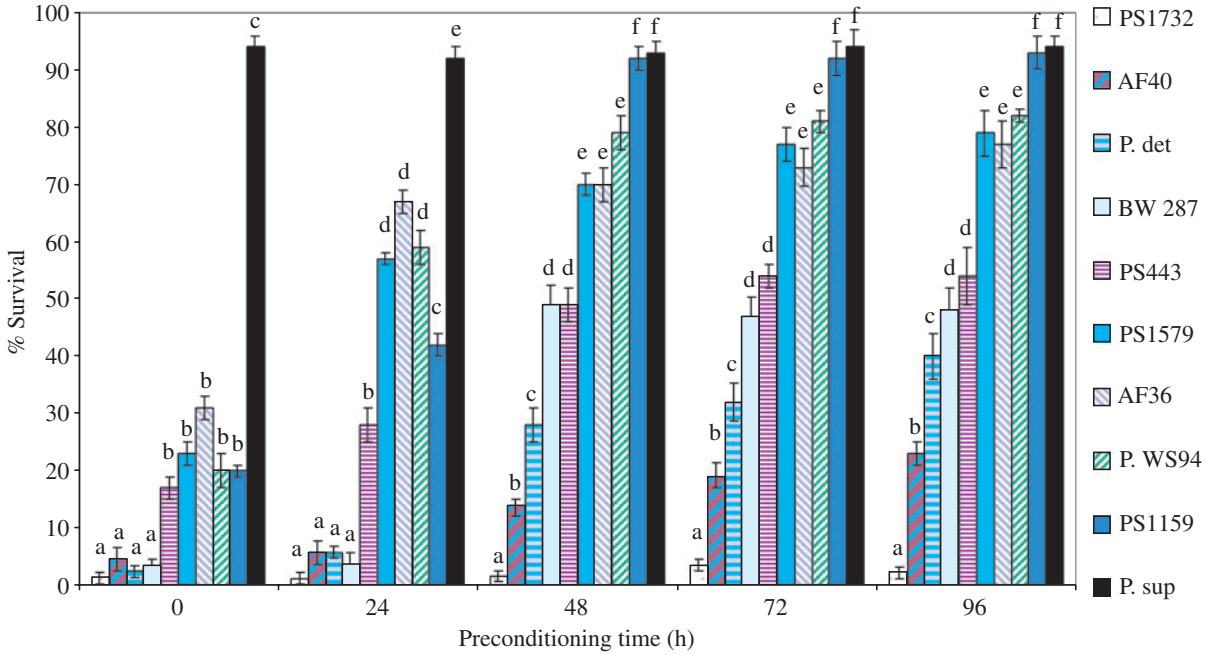

Fig. 1. The effect of preconditioning time at 98% RH (relative humidity) on the survival of 10 strains of *Panagrolaimus*. Treated nematodes were preconditioned at 98% RH at 20°C for 0–96 h, desiccated for 48 h over activated silica gel, 'prehydrated' at 100% RH for 24 h and rehydrated in S Basal buffer for 24 h before survival was determined. Survival values are the mean of five replicates ± S.E.M. Mixed stage populations were used for all strains. For each preconditioning time means designated with the same letter are not significantly different (ANOVA, $P<0.05$). The full names of the *Panagrolaimus* species and strains used in this experiment are as follows: *Panagrolaimus* sp. (PS1732); *Panagrolaimus rigidus* (AF40); *Panagrolaimus detritophagus* (P. det; BSS8); *Panagrolaimus* sp. (BW287); *Panagrolaimus* sp. (PS443); *Panagrolaimus* sp. (PS1579); *Panagrolaimus rigidus* (AF36); *Panagrolaimus* sp. (P. WS94); *Panagrolaimus* sp. (PS1159) and *Panagrolaimus superbus* (P. sup; DF5050).

average with the length of the preconditioning time at 98% RH (regression analysis over the entire data set: r^2=15.4%, $P<0.05$). When regression analysis was carried out for the slow dehydration strategists alone this correlation was much stronger (r^2=73.4%, $P<0.01$). This suggests that for the slow dehydration strategists survival is strongly correlated with preconditioning time, whereas this relationship is weaker for the other two groups.

Following 48 h preincubation the survival capacity of three of the four poor desiccators (*P. detritophagus*, *P. rigidus* AF40 and *Panagrolaimus* sp. BW287) had improved significantly and this trend was maintained for these strains when preconditioned at 98% RH for 72 h and 96 h. Nevertheless, the maximal survival for these strains was less than 50%, ranging from 23% *P. rigidus* AF40 to 48% *Panagrolaimus* sp. BW287. The survival capacity of the poor desiccator *Panagrolaimus* sp. PS1732 did not improve significantly upon preincubation at 98% RH at any of the time points tested over a 96 h period, its maximal survival value never exceeding 4%.

Effect of anhydrobiosis on size distribution of survivors

The length of the surviving nematodes of the slow desiccation strategist strain *Panagrolaimus* sp. WS94 was measured following recovery from anhydrobiosis and compared with the length of control samples (Fig. 2). Nematodes were harvested prior to swarming and a mixed stage population was used for this experiment. The nematodes have been grouped into classes of 50 µm increments. From observation, it was ascertained that adult worms had a length of 800 µm or greater. A 2-tailed Mann–Whitney test (Z=–3.094, $P<0.01$) shows that there is a significant difference between the length distribution of the treated population and

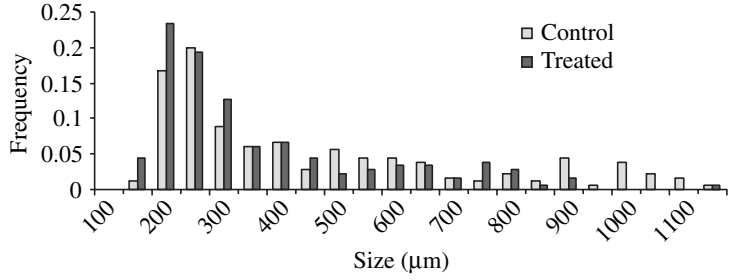

Fig. 2. The length–frequency distribution of nematodes that survived anhydrobiosis compared with a matched control. Treated nematodes were preconditioned at 98% RH (relative humidity) for 48 h, desiccated for 24 h over activated silica gel, 'prehydrated' at 100% RH for 24 h and rehydrated in M9 buffer for 24 h before survivors were isolated and measured. The control samples were maintained at 98% RH for 96 h and rehydrated in S Basal buffer for 24 h before survivors were isolated and their lengths determined.

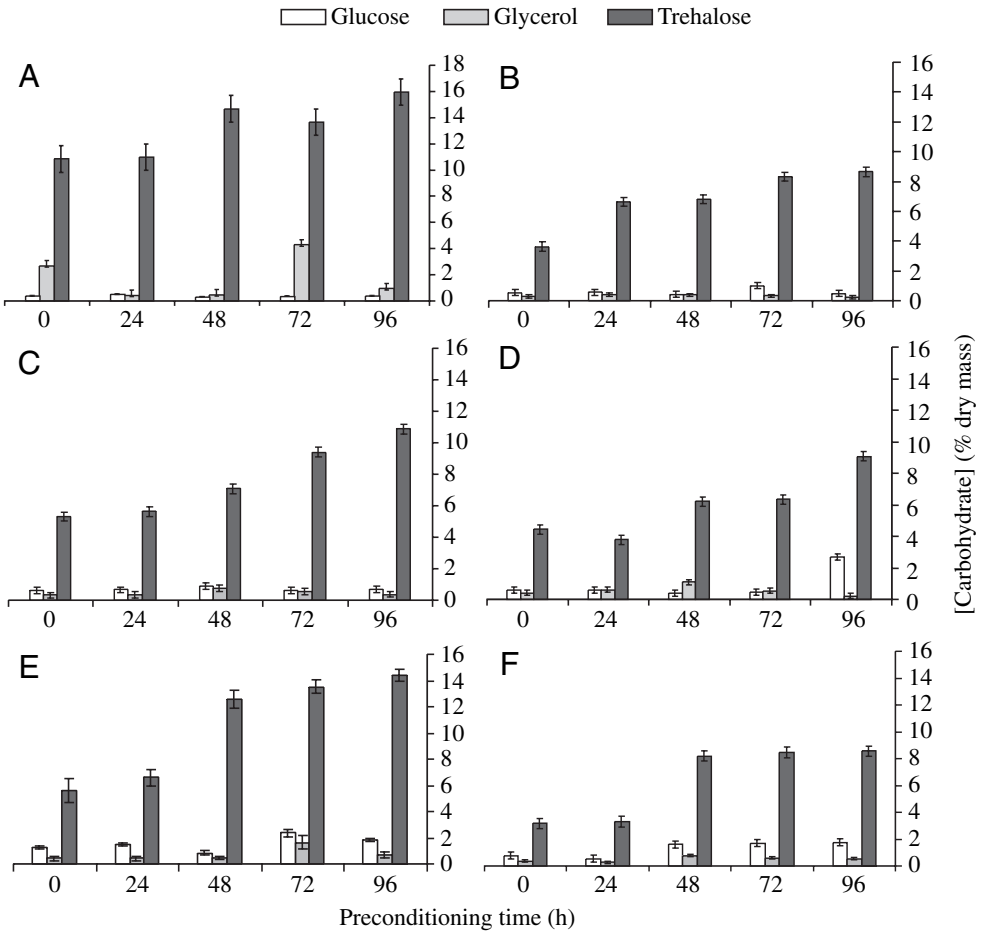

Fig. 3. The effect of preconditioning at 98% RH (relative humidity) on the levels of trehalose, glucose and glycerol in *P. superbus*, a 'fast dehydration strategist' and five 'slow dehydration strategist' strains of *Panagrolaimus*. Nematodes were exposed to 98% RH for various time periods (0–96 h) before carbohydrates were extracted. Carbohydrate levels are expressed as percentage dry mass. (A) *P. superbus* DF5050; (B) *P. rigidus* AF36; (C) *Panagrolaimus* sp. PS1159; (D) *Panagrolaimus* sp. PS1579; (E) *Panagrolaimus* sp. WS94; (F) *Panagrolaimus* sp. PS443.

the untreated controls. Very few nematodes longer than 800 μm survived the desiccation treatment. This suggests that within the slow dehydration strategist strains of *Panagrolaimus* it is the shorter, and therefore the younger, nematodes that preferentially survive anhydrobiosis.

Effects of preconditioning on the carbohydrate content of Panagrolaimus *sp.*

The mean initial concentration of trehalose from the slow dehydration strategist group was 4.5% dry mass (Fig. 3) and this value had risen to 10.3% dry mass after 96 h preincubation (ranging from 8.5% in *Panagrolaimus* sp. PS443 to 14.2% dry mass for *Panagrolaimus* sp. WS94). The levels of trehalose among this slow dehydration strategist group correlate with preconditioning time (regression analysis r^2=76.9%, P<0.01). Fig. 4 shows that a correlation between length of preincubation at 98% RH and trehalose induction was also observed for the poor desiccator strains *Panagrolaimus* sp. BW287 and *P. detritophagus* (r^2=64.4%, P<0.01). In the case of the two remaining poor desiccator strains, *P. rigidus* AF40 and *Panagrolaimus* sp. PS1732, there is no correlation between length of preincubation at 98% RH and trehalose induction (r^2=0.7%, NS). These two strains had the least ability to survive desiccation and the weakest response to preconditioning for improved desiccation tolerance. The fast desiccation strategist *P. superbus,* which demonstrates 93% survival when transferred directly to silica gel, had an initial concentration of 10.8% trehalose and this value increased to 15.9% after 96 h of preconditioning at 98% RH, although no further increase in survival was detected for this strain upon preincubation at 98% RH.

The level of glycerol was low initially; the mean initial value for all ten strains was 0.4% dry mass. Glycerol levels did fluctuate moderately during the preconditioning period (ANOVA, F=3.01, P<0.03) however, the fluctuation did not correlate with preconditioning time (r^2=0.1, P>0.8). The levels of glucose remained low throughout the experiment, and no significant fluctuations (ANOVA, F=0.22, P>0.9) occurred during the preconditioning period. Glucose levels did not correlate with preconditioning time (r^2=0.3%, P>0.5).

Scanning electron microscopy of desiccated Panagrolaimus

When exposed directly to silica gel, individuals of *P. superbus,* a fast dehydration strategist nematode, displayed the typical coiled morphology of anhydrobiotic nematodes (Fig. 5A). This nematode does not appear shrunken or distorted and seems structurally intact after exposure to activated silica gel for 48 h without preconditioning. The slow dehydration strategist nematode *Panagrolaimus* sp. PS1159 (Fig. 5B) was exposed to 72 h preconditioning at 98% RH prior to exposure

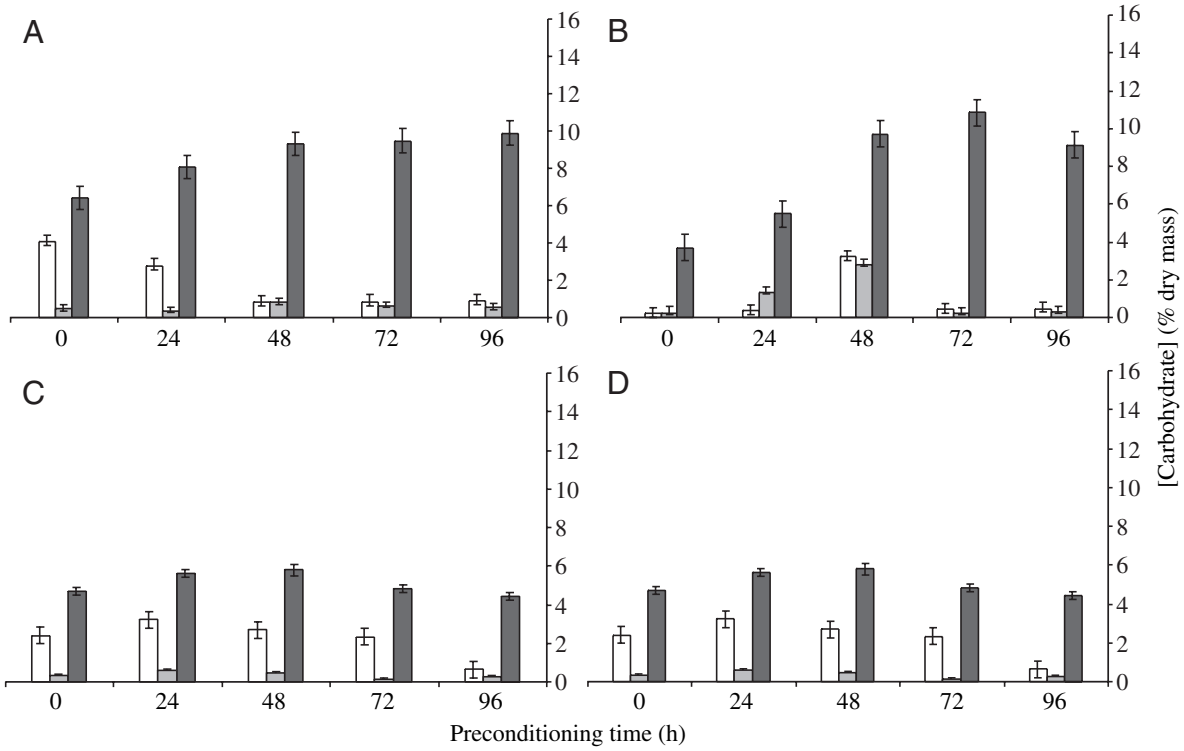

Fig. 4. The effect of preconditioning at 98% RH (relative humidity) on the levels of trehalose, glucose and glycerol in four strains of *Panagrolaimus* sp. classified as 'poor desiccators'. Nematodes were exposed to 98% RH for various time periods (0–96 h) before carbohydrates were extracted. Carbohydrate levels are expressed as percentage dry mass. (A) *Panagrolaimus* sp. BW287; (B) *P. detritophagus* BSS8; (C) *Panagrolaimus rigidus* AF40; (D) *Panagrolaimus* sp. PS1732.

to silica gel for 48 h. This individual also appears to be structurally intact. There are, however, small areas where the cuticle has fused to the filter surface. *Panagrolaimus* sp. BW287 (whose maximal survival following 72 h preconditioning at 98% RH is 49%) shows moderate shrinkage and, in addition, large areas of its cuticle have fused to the filter surface (Fig. 5C). *Panagrolaimus* sp. PS1732 (Fig. 5D) was also exposed to 72 h preconditioning at 98% RH prior to exposure to silica gel for 48 h. This nematode shows extreme structural collapse and severe cuticle and membrane damage and leakage of body fluids, despite being coiled. This poor desiccator has a very limited ability to synthesise trehalose upon preincubation at 98% RH (Fig. 3) and does not show an increase in desiccation survival following preincubation at 98% RH (Fig. 1).

Phylogenetic analyses

Two competing topologies were consistently inferred by the four phylogenetic reconstruction methods applied to the data (Fig. 6). Both topologies were in agreement regarding the branching order of *C. elegans*, *P. detritophagus*, *P. rigidus* AF40 and *Panagrolaimus* spp. BW287 and PS1732. The MP and NJ analysis grouped clade 1 (*Panagrolaimus* spp. WS94 and PS443) and clade 2 (*P. davidi*, *Panagrolaimus* spp. PS1159 and PS1579) as sister taxa to the exclusion of *P. superbus* and *P. rigidus* AF36 (clade 3) with relatively high bootstrap support (Fig. 6, Tree A). However, the ML and Bayesian methods inferred clades 1 and 3 as sister taxa (Fig. 6, Tree B). Branch supports for this tree derived from Bayesian posterior probabilities (BPP) were found to be highly significant with all internal nodes having support values equal to 1. BPPs have been shown to be excessively liberal (Suzuki et al., 2002). Because of this, only the bootstrap supports derived from the ML analysis were considered. ML bootstrap support (56%) is weak for the grouping of clades 1 and 3 as sister taxa in Tree B.

To assess the likelihood that any differences in topology between the two inferred trees is no more significant that that expected by chance, we performed the Shimodaira Hasegawa (Shimodaira and Hasegawa, 1999) test and the approximately unbiased test (Shimodaira, 2002). Both tests indicated that tree A receives a better likelihood score than tree B. The difference in likelihood scores are not significant however (ln –11456.16 *vs* ln –11457.41; χ^2 NS) and it is not possible to select one tree over the other based on the available data.

Discussion

The ability of nematodes to enter anhydrobiosis in response to desiccation has enabled them to exploit diverse ecological niches in unfavourable or variable environments ranging from desert to Antarctic soils to exposed plant foliage. The importance of anhydrobiosis as a survival mechanism in economically important plant parasitic nematodes has been

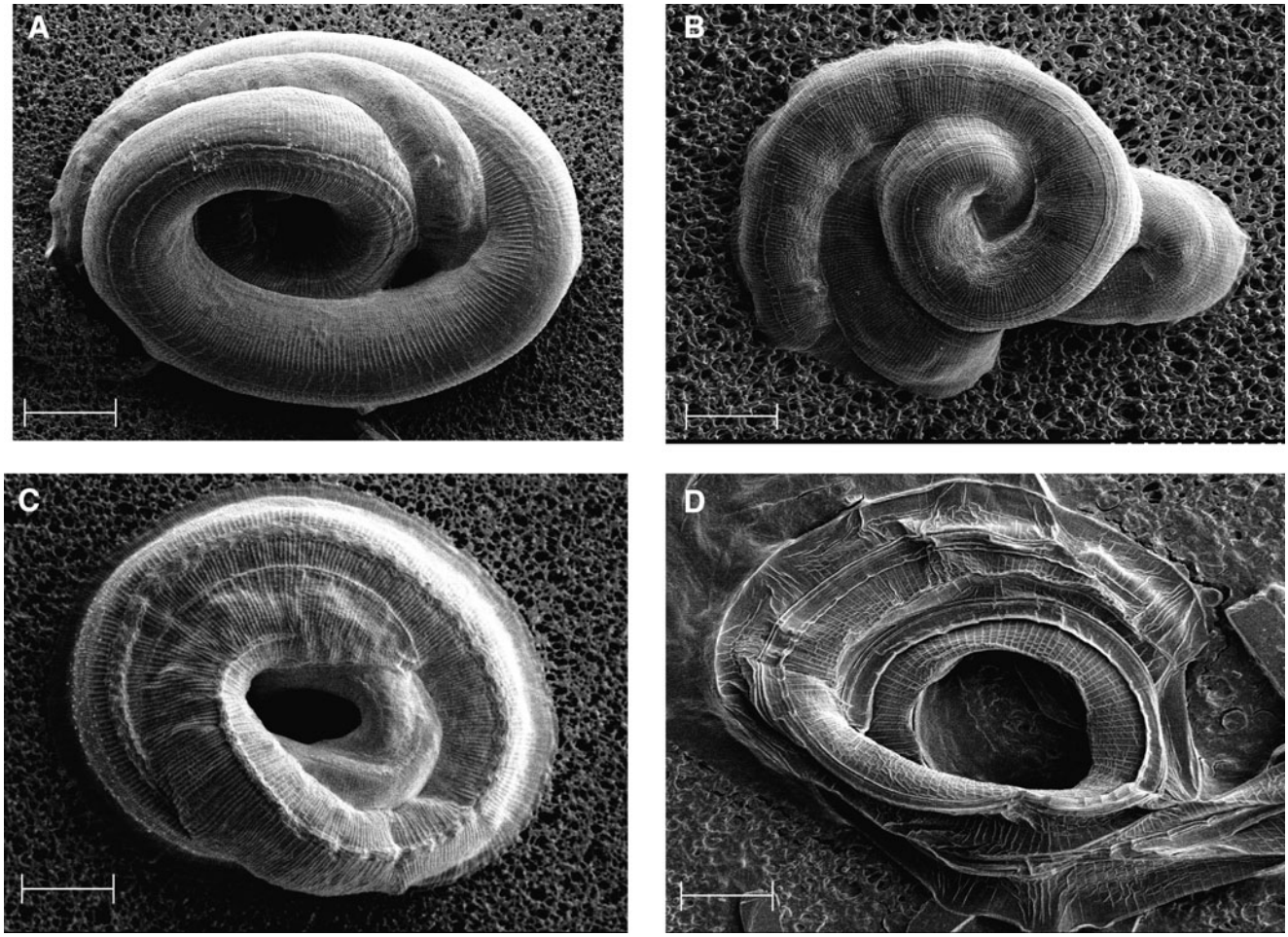

Fig. 5. Scanning electron micrographs of desiccated *Panagrolaimus* following exposure to dried silica gel (<10% RH; relative humidity) for 48 h. (A) *Panagrolaimus superbus* DF5050 exposed directly to silica gel without preconditioning; (B) *Panagrolaimus* sp. PS1159 exposed to silica gel following preconditioning at 98% RH for 72 h; (C) *Panagrolaimus* sp. BW287 exposed to silica gel following preconditioning at 98% RH for 72 h; (D) *Panagrolaimus* sp. PS1732 exposed to silica gel following preconditioning at 98% RH for 72 h. Scale bars, 15 μm (A–C), 10 μm (D).

documented in several studies (reviewed by Antoniou, 1989; Perry, 1999). While in the anhydrobiotic state, nematodes show increased resistance to many other environmental stresses including high and low temperatures and ionising radiation, and they are also resistant to damage caused by metabolic poisons (Demeure and Freckman, 1981). The majority of studies on anhydrobiosis in free living nematodes have utilised a restricted range of nematode species and have been concentrated mainly on *A. avenae*. However, it is clear that many nematode species from the upper soil profile are slow-dehydration strategists, which are unable to survive exposure to extreme desiccation, but can undergo anhydrobiosis if subjected to slow and controlled levels of water loss (Demeure et al., 1979; Womersley, 1987; Womersley and Ching, 1989). Slow rates of water loss enable the nematodes to undergo coiling and aggregation, behavioural adaptations that help to reduce the rate of water loss. The synthesis of trehalose (Madin and Crowe, 1975) and of hydrophilic proteins as well as proteins involved in cellular protection (Browne et al., 2002, 2004) has also been shown to occur in *A. avenae* during the preincubation period.

The ten strains of *Panagrolaimus* investigated in this study display great variability in their anhydrobiotic ability. The 93% survival observed for *P. superbus* following direct exposure to activated silica gel for 48 h is remarkable and provides and excellent example of a fast dehydration strategist. This nematode was isolated from the mosses making up a gull's nest in a small lava cavity in a nunatak on Surtsey island, Iceland (Bostrom, 1988; Sohlenius, 1988). Surprisingly, we have found that the *P. detritophagus* strain, which was also isolated at the same site from the same nest material has a very limited ability to survive rapid desiccation and is a slow desiccation strategist. Womersley (1987) distinguished between slow-dehydration and fast-dehydration strategist nematodes on the basis that slow-dehydration strategists could not survive direct exposure to harsh desiccation. Examples of such slow dehydration strategists are *A. avenae* (Crowe and Madin, 1975), *Rotylenchus reniformis* (Womersley and Ching, 1989)

and *P. davidi* (Wharton and Barclay, 1993). All the *Panagrolaimus* strains investigated in this study show some survival upon direct exposure to silica gel, so they could all be described as fast dehydration strategists *sensu* Womersley. However, with the exception of the fast dehydration strategist *P. superbus* and the poor desiccator *Panagrolaimus* sp. PS1732, all of the other strains show substantial improvements in their desiccation tolerance to low RH following preincubation at 98% RH. Thus it appears that within the genus *Panagrolaimus* there is a continuum of strains from those that are unable to survive exposure to low RH without prior preconditioning at high RH such as *P. davidi* (Wharton and Barclay, 1993), through strains that have limited ability to survive rapid desiccation but whose anhydrobiotic ability improves upon preconditioning (e.g. *P. detritophagus*, *P.* sp. PS1159), to strains such as *P. superbus* that can survive immediate exposure to severe desiccation.

Trehalose accumulation has been associated with the successful induction of anhydrobiosis in invertebrates such as brine shrimps, tardigrades, larvae of the chironomid *Polypedilum vanderplanki* and the nematodes *A. avenae* (Madin and Crowe, 1975), *Anguina tritici* and *Ditylenchus dipsaci* (Womersley and Smith, 1981). However, data on trehalose accumulation in response to desiccation in other anhydrobiotic nematodes are lacking (reviewed by Behm, 1977). While the accumulation of trehalose in *A. avenae* is believed to be necessary for anhydrobiotic survival, it is not sufficient. A further period of preconditioning following maximum trehalose accumulation is needed before maximum survival is seen, suggesting that other changes must also occur in *A. avenae* before it can successfully enter the anhydrobiotic state (Higa and Womersley, 1993; Browne et al., 2004). Data, especially from plants (reviewed by Ingram and Bartels, 1996; Hoekstra et al., 2001; Ramanjulu and Bartels, 2002) and microorganisms (Potts, 1994, 1996), but also from brine shrimps (Liang et al., 1997) and nematodes (Browne et al., 2004) demonstrate that successful entry into anhydrobiosis requires a coordinated set of biochemical and cellular adaptations. Such studies show that the accumulation of trehalose or other disaccharides represents one possible anhydrobiotic mechanism, but alternative biochemical adaptations may also be utilised by organisms to achieve desiccation tolerance (reviewed by Crowe et al., 2001; Oliver et al., 2001).

In fully hydrated *A. avenae* trehalose levels are 1.5–2.0% dry mass, but these levels rise to a maximum of 11–12% dry mass following 72 h preconditioning at high RH (Madin and Crowe, 1975; Browne et al., 2004). *A. avenae* is

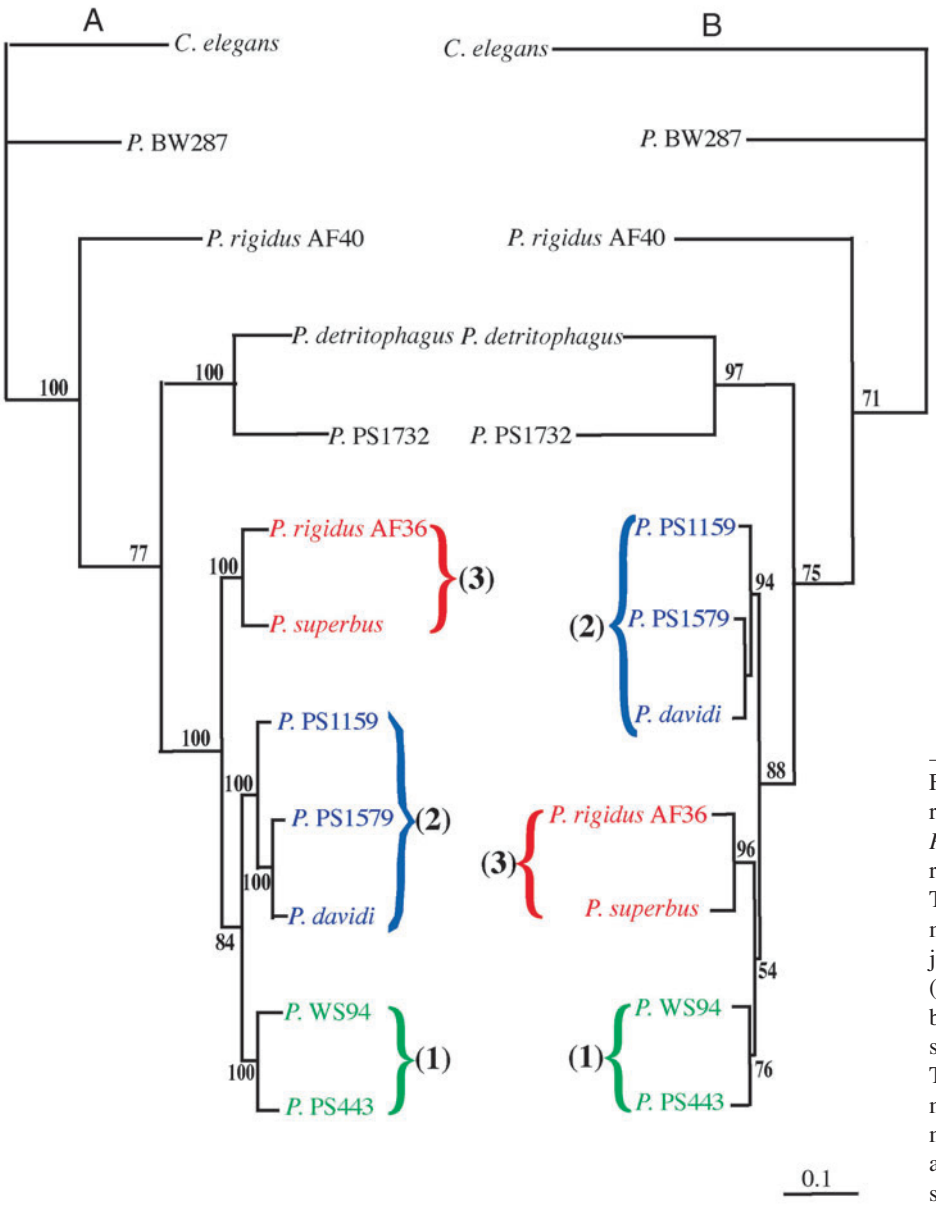

Fig. 6. Hypotheses of phylogenetic relationships among isolates and species of *Panagrolaimus* derived from alignments of the rDNA ITS1, ITS2 and D3 DNA sequences. (A) The phylogenetic tree obtained using maximum parsimony (MP) and neighbor-joining (NJ) algorithms. NJ bootstrap values (1000 replicates) are indicated on the internal branches. MP bootstrap analysis retrieves scores nearly identical to those displayed. (B). The phylogenetic tree obtained using maximum likelihood (ML) and Bayesian methods. ML bootstrap values (100 replicates) are indicated. The scale bar represents 0.1 substitutions per site.

unable to survive immediate exposure to activated silica gel, however, all of the *Panagrolaimus* strains tested in this study (even the poor desiccator *Panagrolaimus*. sp. PS1732) showed some ability to survive direct exposure to silica gel. The mean concentration of trehalose in the 10 *Panagrolaimus* strains when fully hydrated was 4.5% dry mass – more than twice the levels recorded in undesiccated *A. avenae*. We found that the correlation between preconditioning at high RH and trehalose accumulation was strongest in the slow dehydration strategist strains of *Panagrolaimus*, but trehalose accumulation also occurred in the fast dehydration strategist *P. superbus* following preincubation although no further improvement on the initial survival (93%) of this strain was detected. The two poorest desiccator strains show only moderate levels of trehalose accumulation (which increased by ≈1.5%). The high trehalose levels observed in fully hydrated *P. superbus* (10% dry mass) suggest that constitutive expression of high levels of trehalose pre-adapt this fast dehydration strategist to combat desiccation.

Overall, these data indicate that trehalose accumulation is required for successful induction of anhydrobiosis in *Panagrolaimus*. However it is also apparent that *Panagrolaimus* strains that accumulate similar levels of trehalose during preincubation (e.g. *P. detritophagus*, *Panagrolaimus* sp. BW287 and *Panagrolaimus* sp. PS1732) show different levels of anhydrobiotic survival. This suggests that the anhydrobiotic capacity resulting from a given level of trehalose accumulation in *Panagrolaimus* may be strain specific and that other factors in addition to trehalose accumulation are also involved in anhydrobiotic survival in this genus. The behavioural responses of the strains of *Panagrolaimus* in response to desiccation appear to be more conserved than the production of trehalose. All the strains observed, regardless of survival rates, undertook both coiling and clumping, which have the effect of reducing surface area and slowing the rate of water loss during desiccation. It can be seen, however, that the ability to coil is not sufficient to enable nematodes to survive extreme desiccation especially in the absence of sufficient levels of trehalose, as in the case of *Panagrolaimus* sp. PS1732.

Our phylogenetic analyses indicate that the strongly anhdyrobiotic strains of *Panagrolaimus* form a single phylogenetic lineage, which is separate from the four poor desiccation strains. It is also clear that these poor desiccators are phylogenetically divergent from each other. Clade 3 contains *P. superbus* a fast desiccation strategist, and *P. rigidus* AF36 whose desiccation tolerance improves upon preconditioning, but whose capacity for fast desiccation tolerance results in over 30% survival without preconditioning. Whether this fast desiccation strategist clade evolved from within the slow desiccation clades, as indicated by Tree B, or whether it represents a distinct sister taxon to the slow desiccation strategists in clades 1 and 2 (Tree A) cannot be elucidated from our data. The phylogenetic trees presented here are derived from alignments of the rDNA ITS and D3 sequences. These fast evolving regions have been found to provide phylogenetically informative data for closely related genera and species in a range of invertebrates, including nematodes (Adams et al., 1998; Al-Banna et al., 1997; Carta et al., 2001; Livaitis et al., 1994; Nguyen et al., 2001). However, it will be necessary to find other more polymorphic molecular markers to fully resolve the phylogenetic relationships between *Panagrolaimus* clades 1-3. Few species within the genus *Panagrolaimus* possess diagnostic morphological characters (Andrássy, 1984; Bostrom, 1995; Williams, 1987) and species diagnosis of *Panagrolaimus* is difficult, being based predominantly on morphometric data. Thus many *Panagrolaimus* strains remain unassigned to species level. The positioning of the two strains of *P. rigidus* in our phylogram suggests that the taxonomic designation of these strains needs to be reevaluated.

Oliver et al. (2000) have analysed the evidence for differing mechanisms of vegetative desiccation tolerance (i.e. anhydrobiosis) in land plants. They hypothesise that vegetative desiccation tolerance was primitively present in land plants, but was lost in the evolution of the tracheophytes and subsequently re-evolved at least eight times in the angiosperms. They postulate that this re-evolution of desiccation tolerance in angiosperm 'resurrection plants' was achieved by the expression in vegetative tissues of genes involved in seed development (and seed desiccation tolerance). Thus each time the desiccation tolerant phenotype re-evolved in an angiosperm lineage, the response patterns and biochemical adaptations differed. Some free-living nematodes can undergo anhydrobiosis at all stages of their life cycle, and in addition, a large number of plant and animal parasitic nematodes have anhydrobiotic egg, cyst or infective juvenile stages. In a phylogenetic analysis based on rDNA small subunit sequences Blaxter et al. (1998, 2000) recognise six clades within the phylum Nematoda, referred to as clades I to V, the sixth clade, the Chromadorida, is considered by these authors as basal to clades III, IV and V. Clade IV contains the best described anhydrobiotic nematodes, including *Panagrolaimus* and *Aphelencus* and the plant parasitic order Tylenchida (which frequently has anhydrobiotic encysted infective stages, e.g. *Globodera*, *Meliodogyne*). Clade III contains four animal parasitic orders, one of which, the Ascaridida, contains members with anhydrobiotic encysted infective stages. There has been no systematic investigation of anhydrobiotic phenotypes across the phylum Nematoda, but all the remaining clades in the phylogeny of Blaxter et al. (2000) contain some terrestrial and parasitic forms (either plant or animal parasites), thus it is possible that nematodes with anhydrobiotic capabilities may also occur in these taxa. The nematode families contained in clade IV are phylogenetically divergent (Blaxter et al., 2000) and in addition to containing many anhydrobiotic taxa, this clade also has many desiccation sensitive nematode species. A more detailed phylogenetic investigation will be required to determine whether the anhydrobiotic members of clade IV are derived from an anhydrobiotic ancestor, or whether they independently acquired or re-evolved an anhydrobiotic capability. In the plant

parasitic order Tylinchida of clade IV, taxa with the best developed anhydrobiotic capabilities are not closest relatives, suggesting independent evolutionary modifications (Baldwin et al., 2004). However, it is noteworthy that all the anhydrobiotic taxa that have been investigated to date in clade IV accumulate trehalose in response to desiccation.

In comparison with the large research effort expended in investigating anhydrobiosis and desiccation tolerance in land plants, research in nematodes has been limited. Our data indicate that *Panagrolaimus* has the potential to be an excellent model system for the investigation of molecular aspects of nematode anhydrobiosis. This genus contains closely related nematodes that possess a diverse range of anhydrobiotic abilities. This should facilitate researchers in correlating gene expression data with anhydrobiotic phenotypes and thereby help in the elucidation of the key molecular strategies employed by anhydrobiotic nematodes.

This work was funded by a Science Foundation Ireland Investigator Programme Grant to A.M.B. We thank the scientists who kindly provided us with cultures of *Panagrolaimus*. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

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