Characterization of an alpha tubulin gene sequence from *Neospora caninum* and *Hammondia heydorni*, and their comparison to homologous genes from Apicomplexa

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SUMMARY

The gene coding for α tubulin has been isolated by the polymerase chain reaction and sequenced from 2 isolates of *Neospora caninum* (Nc-Liverpool and Nc-SweB1)‡. The data show that the gene, as in *Toxoplasma gondii*, is single copy and contains 3 exons and 2 introns and is identical in sequence in the 2 isolates studied. Comparison of the predicted protein sequence shows it to be identical to the α tubulin protein encoded by the *T. gondii* gene. The majority of the nucleotide substitutions that have occurred during the evolution of the *T. gondii* and *N. caninum* genes from their common ancestor have occurred in the third codon position. A partial coding sequence for α tubulin was also obtained from *Hammondia heydorni* and compared to other α tubulin sequences from Apicomplexa. The results show the sequences of the *T. gondii*, *N. caninum* and *H. heydorni* α tubulin genes to be similar but not identical in sequence, thereby providing new evidence that *N. caninum* and *H. heydorni* are genetically distinct species.

Key words: *Neospora caninum, Hammondia heydorni*, tubulin, phylogeny.

INTRODUCTION

Microtubules are found in all eukaryotes, from yeasts to multicellular organisms where they are principally involved in maintenance of cell structure and division. In concert with actin and intermediate filaments, microtubules form part of a cell's cytoskeleton which is responsible for establishing and maintaining cell shape. Another important role for microtubules is in cell division, where they make up the spindle fibres. Microtubules contain dimers of 2 essential tubulin proteins: α and β tubulin.

Alpha tubulin is considered a relatively abundant protein in eukaryotic organisms with a typical mass of 50 kDa and it contains approximately 450 amino acids. Within the cell, tubulin is normally post-translationally modified by acetylation at the C-terminus and factors such as GTP and microtubule associated proteins (MAPs) bind to it and influence the polymerization of tubulin into microtubules. This is a dynamic process, and is reasonably well understood (MacRae & Langdon, 1989; Mandelkow & Mandelkow, 1990, 1995; MacRae, 1992, 1997; Drewes, Ebneth & Mandelkow, 1998). A number of chemical reagents are known to inhibit cell function and division, including herbicides, by inhibiting microtubule formation and function (Bell, 1998).

The gene encoding alpha tubulin exists as either a single copy or low copy number gene in lower eukaryotes and are found as large multigene families in higher eukaryotes. *Toxoplasma gondii*, for example, contains single copy genes for both α and β tubulin (Nagel & Boothroyd, 1988). Tubulin isotypes, where one molecule differs from the next by one or more amino acid substitutions, can also exist. In *Plasmodium falciparum*, for example, 2 isotypes of α tubulin exist (Holloway *et al.* 1989, 1990; Delves *et al.* 1990; Rawlings *et al.* 1992), and expression of these is stage dependent: one form (Tub II) is expressed only in the axoneme of male gametes (Rawlings *et al.* 1992). The amino acid sequence similarity between the 2 *P. falciparum* isotypes and that of *T. gondii* is 94%. There are also many similarities in the structure of the α tubulin gene from these 2 taxa. They all have 3 exons, which are separated by 2 introns, and the exon/intron boundaries seem to be conserved at amino acid positions 21/22 and 62/63. Tubulin therefore exhibits a high degree of conservation at both the gene and protein sequence level, and for this reason alone, tubulin gene sequence data have also been exploited for use in evolutionary studies (Tourancheau *et al.* 1998; Edgcomb *et al.* 2001).

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‡ The nucleotide sequence data reported in this paper are available from the GenBank database under the accession numbers AF508031 (*N. caninum*) and AY169962 (*H. heydorni*).

Neospora caninum was first identified in 1988 (Dubey *et al.* 1988) and so relatively little is known about the molecular biology of this species, which is now recognized as an important cause of abortion in cattle (Reichel, 2000). The structure of the infective zoite stages of *N. caninum* show the presence of subpellicular microtubules and a conoid which are common in Apicomplexa (Speer *et al.* 1999) and presumably contain tubulin (Nagel & Boothroyd, 1988). In this study, we report the results of investigations into the structure and organization of the α tubulin gene from *N. caninum* and *Hammondia heydorni*, both of which are cyst-forming coccidians closely related to *T. gondii*. The results show that *N. caninum* contains a single copy gene encoding α tubulin, which contains 3 exons and 2 introns. Furthermore, comparison of the α tubulin gene sequences amongst these taxa show that *T. gondii*, *N. caninum* and *H. heydorni* are all genetically different at this locus. Hence evidence is provided which shows that *N. caninum* and *H. heydorni* are clearly different species.

MATERIALS AND METHODS

Parasites

Tachyzoites of *N. caninum* (Nc-Liverpool or Nc-SweB1 strains) were grown on a monolayer of Vero cells as described previously (Barber *et al.* 1995) except that horse serum (5–10%) was used in the media. Tachyzoites were purified by filtration through a 3 μm nitrocellulose filter and genomic DNA was purified from them as described (Ellis *et al.* 2000).

Oocysts of *H. heydorni* were kindly supplied by Dr B. Pomroy (Massey). They were purified from the faeces of a greyhound, and identified as *H. heydorni* by the polymerase chain reaction (PCR) and sequencing of the ITS1 and partial large subunit ribosomal DNA sequences (GenBank accession numbers AF508020 and AF508029 respectively) (Ellis & Pomroy, 2003).

PCR of genomic alpha tubulin sequences

Primer pairs NCATF (5'ACATGGTATCCAGC-CGGAT3') and NCATR (5'GAGAGCAGCCAA-ATCCTCAC3') (giving a PCR product of 1354 bp) and cDNAFAT (5'GTTTTCAGCCGCATGGA-C3') and cDNARAT (5'CTCCCCGTTCAGAAC-ATATCG3') (454 bp) were used to amplify genomic DNA from Nc-Liverpool. PCR products were purified by the QiaQuick purification kit (Qiagen) and sequenced with both primers. As a negative control for PCR, DNA from the Vero host cells were included in the experimental design. Primers FTGAT (5'AGCATCCACGTCGGCCAG3') and RTGAT

(5'TGCTCCGGGTGGAACAGG3') gave a product from *N. caninum* DNA containing multiple bands, 1 of which was approximately 900 bp. This band was subsequently gel purified (Qiagen gel extraction kit) and sequenced with both primers. The 5' end of the gene was PCR amplified by primers Alpha-F1 (5'ACGCGGGGAGAAACA-TTTTGT3') and Alpha-R1 (5'GCACCACAT-GTGGCAGTACGTA3'). The PCR product produced from genomic DNA was gel purified and sequenced with both primers.

Nc-SweB1 DNA was amplified by primers BVFAT (5'GGAGCTCTTCTGCCTGGAG3') and BVRAT (5'CGAGCCTCAGAGAATTCAC-C3') (1349 bp), 5FATBV (5'CCAGGCCGGTAT-CCAAATC3') and 5RATBV (5'AGAAGGTGT-TGAAGGCGTCG3') (640 bp) and cDNAFAT/cDNARAT (454 bp).

All PCR products (except those derived from cDNAFAT/cDNARAT and Alpha-F1/Alpha-R1 which were sequenced directly by cycle sequencing) were cloned into pGEM-T using the pGEM cloning kit. Recombinants were identified by blue/white screening on agar plates containing ampicillin, Xgal and IPTG, and by PCR using M13 forward and reverse primers. Plasmids containing the correct inserts were then grown in 500 ml bacterial cultures of *Escherichia coli* strain DH5α, and further purified by the standard alkaline SDS lysis method, followed by caesium chloride density gradient centrifugation.

Isolation of cDNA coding for alpha tubulin from Nc-Liverpool

A cDNA library from Nc-Liverpool (Ellis *et al.* 2000) was screened using the method of Benton & Davis (1977) with a ^{32}P-labelled probe made from a PCR product amplified by primers FTGAT and RTGAT. This probe covers much of the 3' coding sequence present in the largest exon of the α tubulin gene. Plaques were rescreened repeatedly until they were considered plaque pure. They were then subject to PCR using primers RpB2 (5'GCCG-CTCTAGAACTA3') and FpB (5'GTAAAACG-ACGGCCAGT3') which gave PCR products, approximately 1050 bp in size, which were purified by the QiaQuick purification kit (Qiagen) and sequenced.

Rapid Amplification of cDNA Ends (RACE)

Total cellular RNA was purified from Nc-Liverpool tachyzoites as described (Ellis *et al.* 2000). SMART RACE (Clontech) was used for making RACE ready cDNA from Nc-Liverpool which was amplified by 5' RACE with primer 5'at-4 (5'GTCCTCTT-ACCAGCAGGCGTTACCG3'). A band approximately 400 bp in size was purified from other

products by preparative gel electrophoresis and a Qiagen gel extraction kit. The purified product was cloned into pGEM-T and transformants screened by PCR for products of the correct size. Three clones were subsequently identified and sequenced by standard cycle sequencing methods.

Copy number

Nc-Liverpool and Vero genomic DNA were independently restricted with *Hind*III, *Hinf*I, *Pst*I, *Hae*III, and *Eco*RI, and the digests were separated by agarose gel electrophoresis. The gel was Southern blotted by standard methods onto nitrocellulose. The filter was then hybridized to a ^{32}P-labelled cloned probe derived by PCR from primers NCATF and NCATR which covers the middle of the *N. caninum* α tubulin gene.

Computer analyses of sequence data

The non-redundant nucleotide sequence database maintained by the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm. nih.gov/) and the Apicomplexa nucleotide sequence database at the Parasite Genome Blast Server (PGBS; http://www.ebi.ac.uk/parasite/parasite_ blast_server.html) were searched with the sequences obtained using the program BlastN and TBlastX in order to detect homologies with nucleotide sequences currently in the nucleotide sequence databases. Further searches were also made of the *Toxoplasma* and *Neospora* Databases of Clustered ESTS (http://ParaDB.cis.upenn.edu/).

Alpha tubulin DNA or protein sequences were aligned from *N. caninum, T. gondii* (GenBank accession number M20024), *Eimeria acervulina* (X88776), *P. falciparum* (I and II, X15979 and M34390 respectively) and *Cryptosporidium parvum* (AF082877) using PILEUP run on the Australian National Genomic Information Service. They were analysed phylogenetically by maximum likelihood using the Jones-Taylor-Thornton model of amino acid change (proteins) or the Felsenstein-Churchill model of nucleotide (DNA) using PHYLIP. The robustness of the branches was assessed using 200 bootstrap replicates; and the robustness of the phylogenetic reconstructions was assessed by comparing the trees to those from both neighbour-joining analyses using the Kimura 2-parameter model and parsimony analyses.

Distance analysis was performed using Ednadist (on ANGIS) and Kimura's model of nucleotide substitution. Coding sequence was also analysed using the method of Li *et al.* run on ANGIS as NewDiverge (described by Hemphill & Gottstein, 2000). This program estimates the number of synonymous and non-synonymous substitutions per site between 2 nucleic acid sequences that code for proteins.

Table 1. Summary of nucleotide substitutions found between the coding regions of the α tubulin genes of *Neospora caninum* and *Toxoplasma gondii*

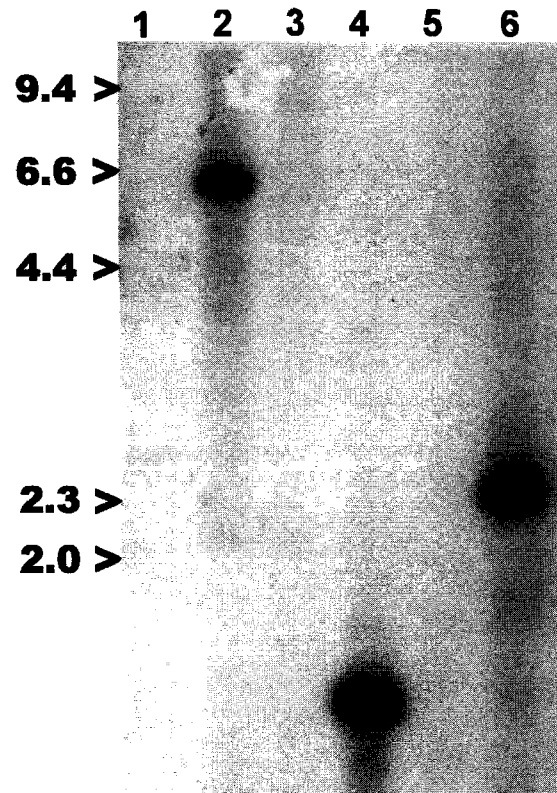| | | N. caninum | | | |
		T	C	A	G
T. gondii	T	—	25	2	4
	C	27	—	1	9
	A	3	2	—	7
	G	7	11	6	—

Fig. 1. Results from Southern hybridization of restriction digests of *Neospora caninum* (lanes 2, 4, 6) and Vero DNA (lanes 3, 5) with a radioactive probe made from an *N. caninum* α tubulin PCR product (using primers NCATF and NCATR). Genomic DNA was digested with either *Hind*III (lanes 2 and 3); *Hinf*I (lanes 4 and 5) or Pst1 (lane 6). Lane 1 contained a set of molecular weight markers (in kb).

Isolation and sequence analysis of α tubulin from Hammondia heydorni

Genomic DNA from *H. heydorni* was extracted and amplified by PCR using primers CRATF (5'GG-TATCCAAATCGGT) and CRATR (5'GCCT-TCTCTGCGCTG). The PCR product obtained was gel purified using a Qiagen PCR purification kit and then cloned into the pGEM-T vector. Recombinant colonies were PCR screened with M13F/ M13R primers to check for insert size and 2 clones were randomly selected for sequencing on both strands. Since the sequences obtained from each

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              1        11        21        31        41        51
consensus  MREVISIHVGQAGIQIGNACWELFCLEHGIQPDGQMP-SD   GG DDAFNTFFSETGAG

Nc         .....................................KTI..G..............

Tg         .....................................KTI..G..............

Ea         ...............................AE...P.QTI..I.............

Pf(II)     .................................QVVA.G..............

Pf(I)      ...............V.................KASRAN..............

Cp         ...........................N...T..M.EQNM.IS.............
```

```
            61        71        81        91        101       111
           KHVPRCVF DLEPTVVDEVRTGTYR LFHPEQLISGKEDAANNFARGHYTIGKEIVD   L

           ..........L...................H.........................LS.

           ..........L...................H.........................LS.

           ..........L...................H.........................LS.

           ..........V...................Q.........................VC.

           ..........V...................Q......................VI.VC.

           ....A...V.........I.S....Q.......N.............V....LEVC.
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           121       131       141       151       161       171
           DRIRKLADNCTGLQGFLMFNAVGGGTGSGLGCLLLERLSVDYGKKSKLNFCSWPSPQVST

           .....................................................

           .....................................................

           ........Q............................................

           ..V..................................AI..............

           ...................S........F...M.................C........

           .....................A..T..................T.....L..
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           181       191       201       211       221       231
           AVVEPYNSVLSTHSLLEHTDVAVMLDNEAIYDICRRNLDIERPTYTNLNRLIAQVISSLT

           ....|.....................................................

           ....|.....................................................

           ....|.....................................................

           ....|.......................I..........KK..................

           ....|.......................I.............................

           ....|...A.........A.................N..Q.A..............
```
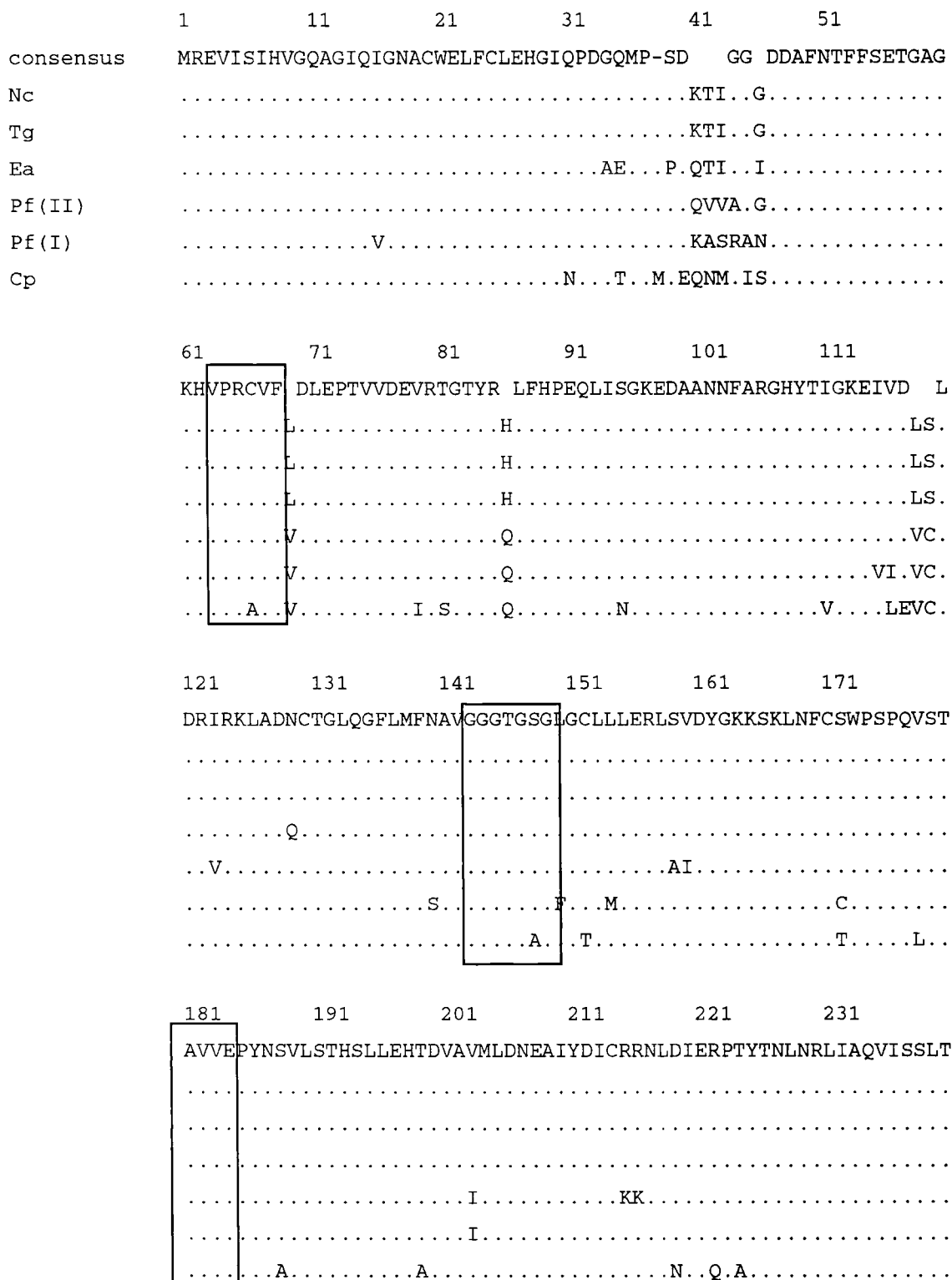
Fig. 2. Alignment of the predicted amino acids for the α tubulin proteins from *Neospora caninum* (Nc), *Toxoplasma gondii* (Tg), *Eimeria acervulina* (Ea), *Plasmodium falciparum* (PfI), *P. falciparum* (PfII) and *Cryptosporidium parvum* (Cp). A consensus amino acid sequence for α tubulin derived from members of the data set is shown above the alignment. The numbers refer to the residue position in the alignment. Gaps are represented by dashes. Identical residues are represented by dots. The boxed sequences represent the GTP and MAP binding motifs referred to in the text.

clone only just overlapped in the middle, a sequencing primer (HhatubseqF; (5'TCTTCTGCCTG-GAGCATG3') was designed 550 bp from the 5' end to clear up ambiguities in the centre region of each

sequence. Alignment of the partial *H. heydorni* α tubulin coding region and its analysis with others from Apicomplexa was performed as described above.

```
 241       251       261       271       281       291
ASL RFDGAL NVDVTEFQTNLVPYPRIHFMLSSYAPIISAEKAYHEQLSVAEITNSAFEPA
... ...... .................................................
... ...... .................................................
... ...... .................................................
... ...... ........................................S........
... ...... ...........................VV.............S........
... ...... ..I.............................F..........AV...Q
```

```
 301       311       321       331       341       351
SMMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQP
...........................................................
...........................................................
...........................................................
.....................................S.....................
N..........................................................
NQ.....................T...................................
```

```
 361       371       381       391       401       411
PTVVPGGDLAKVMRAVCMISNSTAIAEVFSRMDHKFDLMYAKRAFVHWYVGEGMEEGEFS
...........................................................
...........................................................
...........................R...............................
.............................Q.............................
.............................Q.............................
..............C.............N.........S....................
```

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 421       431       441       451
EARE DLAALEKDYEEVGIE AE GEGEEEGY      *
.... ...............T.. ........GDEY.
.... ...............T.. ........GDEY.
.... ...............T.. ..A.....GDEF.
.... ...............SND ....D...E*---
.... ...............SN. A...D...EADY.
.... ...............I.D ..D..VH.EGDF.
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Fig. 2. (Cont.)

RESULTS

Isolation and characterization of the α *tubulin gene from* Neospora caninum

Genomic sequence from Nc-Liverpool was determined by sequencing over-lapping PCR products derived from genomic DNA extending across the gene (5′ to 3′) from primer pairs Alpha-F1/Alpha-R1, FTGAT/RTGAT, NCATF/NCATR and cDNAFAT/cDNARAT. Blast searches of the sequence databases showed the consensus sequence obtained from genomic DNA was related to the α tubulin gene from many other taxa, and so this gene was called NcTUBA.

A similar PCR-based strategy was used to sequence most (but not all) of the same gene from NcSweB1. The very 5' and 3' ends of the gene (approximately 300 bp at either end) were not determined for Nc-SweB1. The gene sequence data from Nc-Liverpool and Nc-SweB1 were compared and found to be identical.

Characterization of the α tubulin transcript from Nc-Liverpool was limited to 5' RACE and isolation of cloned cDNA molecules. Screening of 4400 clones from a cDNA library of Nc-Liverpool detected 5 positive clones whose inserts were amplified by PCR and sequenced giving approximately 1050 bp of cloned cDNA sequence representing the 3' end of the mRNA. 5' RACE was used to isolate and sequence 350 bp at the 5' end of the mRNA.

Comparative analyses of cDNA and genomic sequences from Nc-Liverpool, along with database comparisons, showed the presence of three exons (397, 121 and 1450 bp; 57·6% GC) and 2 introns (509 and 150 bp; 49·9 and 44% GC respectively) in NcTUBA. The 5' and 3' splice site signals correspond well to the universal consensus sequences for these sites. Comparison of the 2 introns with those found in T. gondii showed them to be of similar size but with little sequence similarity (intron 1, 55·2% sequence similarity and intron 2, 54·7% similar). After comparison, the 5' and 3' untranslated regions of the N. caninum mRNA are smaller than those found in T. gondii (335 vs 386; 271 vs 395 bp).

The coding sequence of NcTUBA was compared to that from T. gondii and found to be 92·4% similar at the DNA level. Comparison of the NcTUBA open reading frame (ORF) with that from T. gondii showed that from a total of 454 codons, the useage of 352 codons was identical (Ks = 0·386; Ka = 0·003). Approximately one half of the nucleotide substitutions which exist between the coding regions of N. caninum and T. gondii are changes in pyrimidines (T-C or C-T) at the third codon position (Table 1).

Southern blotting of Nc-Liverpool DNA with an α tubulin gene probe revealed specific hybridization to single bands in the genomic DNA restricted by PstI, HindIII and EcoR1 (Fig. 1). These observations are consistent with the α tubulin gene being single copy in N. caninum. No hybridization was detected to Vero host cell DNA.

The N. caninum gene encodes a polypeptide predicted to be 50113 in molecular weight with a pI of 4·84. Comparison of the predicted α tubulin polypeptides between N. caninum and T. gondii (P10873) shows them to be identical in composition. Both start with Met-Arg-Glu, which is typical for this protein, and finish with Glu-Tyr. Both proteins are highly similar to that derived for E. acervulina, and more distantly related to those from P. falciparum and C. parvum (Table 1 and Fig. 2). The 5 motifs involved in GTP binding (residues 63–68, 143–149, 181–184, 244–249) is highly conserved across all

the 6 sequences analysed, except for 2 amino acid changes in the C. parvum sequence. Similarly the MAP binding (425–442) region of the tubulin protein is also well conserved. The C-terminus of all the predicted protein sequences are rich in Glu (E).

The coding and predicted amino acid sequences of the apicomplexan α tubulin were analysed phylogenetically. The overall structure of the trees obtained was identical in all analyses performed with either DNA or aa sequences, and all branches had 100% bootstrap support. The distance data of Table 2A and the tree in Fig. 3A shows the N. caninum and T. gondii sequences to be more closely related to that from E. acervulina.

Isolation and characterization of partial α tubulin gene sequences from H. heydorni

Sequencing of the PCR product from H. heydorni showed that it covered the majority of the 3 exons and 2 introns predicted to be present in the α tubulin gene, following its comparison to those from N. caninum and T. gondii. The 2 introns (495 and 198 bp respectively) possessed no sequence similarity to the introns found in the α tubulin genes from N. caninum and T. gondii. The DNA sequence compiled from the 3 exons was approximately 90% similar to homologous regions from the N. caninum and T. gondii genes. The genetic distance calculated between the N. caninum and H. heydorni sequences was greater than that found between N. caninum and T. gondii (Table 2B), and the phylogenetic analyses show that H. heydorni is the sister to the N. caninum + T. gondii clade (Fig. 3B).

The partial gene sequence of H. heydorni encoded 269 amino acids, and the predicted protein sequence was identical to that predicted for N. caninum, except for 2 amino acid changes (E:G; A:T). Since the available protein sequence from H. heydorni was not complete, it was not included in any phylogenetic analysis. However, the 2 observed amino acid changes are consistent with the placing of H. heydorni as ancestral to the N. caninum + T. gondii clade.

DISCUSSION

Alpha tubulin is a highly conserved protein and this property has been utilized previously to isolate α tubulin genes from other apicomplexan parasites such as P. falciparum (Holloway et al. 1989; Rawlings et al. 1992) and T. gondii (Nagel & Boothroyd, 1988). In the case of P. falciparum, an oligonucleotide probe based on a highly conserved region of the tubulin protein of other organisms was used to probe a genomic library (Holloway et al. 1989, 1990; Sen & Godson, 1990). The α- and β tubulin genes of T. gondii were isolated using α1- and β2 tubulin gene probes from Chlamydomonas (Nagel & Boothroyd,

Table 2. Distance analysis of (A) the complete coding sequence of α tubulin from Apicomplexa (DNA, above diagonal and protein below diagonal) sequence alignments; (B) partial α tubulin coding DNA sequences from Apicomplexa homologous to *Hammondia heydorni*

(A)						
N. caninum	0·0000	0·0812	0·2561	0·3861	0·3897	0·3904
T. gondii	0·0000	0·0000	0·2649	0·3690	0·3759	0·4002
E. acervulina	0·02174	0·02174	0·0000	0·3120	0·3099	0·3644
P. falciparum (II)	0·04841	0·04841	0·6455	0·0000	0·1778	0·3491
P. falciparum (I)	0·06449	0·06449	0·08331	0·05287	0·0000	0·3462
C. parvum	0·11191	0·11191	0·11823	0·117931	0·12783	0·0000
(B)						
N. caninum	0·0000					
T. gondii	0·0806	0·0000				
H. heydorni	0·0982	0·1071	0·0000			
E. acervulina	0·2558	0·2563	0·2558	0·0000		
P. falciparum (II)	0·4366	0·4149	0·4025	0·3499	0·0000	
P. falciparum (I)	0·4228	0·3965	0·4033	0·3387	0·2042	0·0000
C. parvum	0·3907	0·4154	0·3944	0·3874	0·3679	0·3599
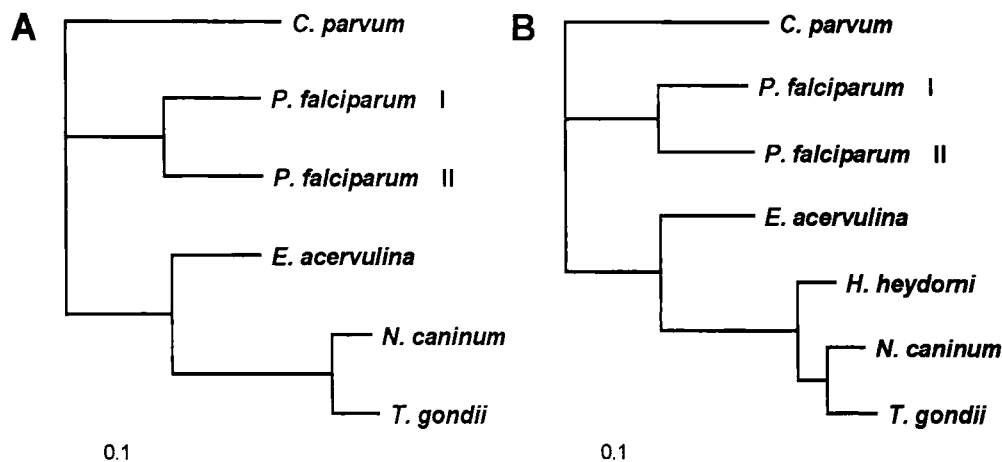
Fig. 3. Unrooted trees from the maximum likelihood analysis of the DNA sequences of α tubulin from the complete sequences (A) or the partial sequences (B) of some members of the Apicomplexa. The length of the branches indicates the amount of inferred evolutionary change (as indicated by the scale), and all of the branches have 100% bootstrap support. The evolutionary trees from the parsimony and neighbour-joining analyses have identical topography to these trees, as do the maximum likelihood analyses of the predicted aa sequences.

1988). In our study, the close relationship between *N. caninum* and *T. gondii* was utilized to design primers which were used to amplify parts of the α tubulin gene from both the Nc-Liverpool and Nc-SweB1 isolates. The PCR products were then cloned into pGEM-T vectors for subsequent sequence analysis or alternatively they were sequenced directly. A cDNA library was also screened using a PCR produced probe in order to identify cloned cDNA sequences.

The limitation of this PCR approach was in designing suitable primers to amplify regions like the 5' flanking region including the first few bases of exon 1 and the 3' flanking region, although after many attempts 5' RACE was eventually used to isolate the 5' end by PCR. The carboxy terminus is said to be the most variable region of the entire α tubulin polypeptide (Holloway *et al.* 1989; Mandelkow & Mandelkow, 1990), making it difficult to design a

universal primer for this region. Consequently, the 3' end of the gene was isolated by screening a cDNA library and 5 positive clones were identified. Unfortunately, the positive clones isolated from the cDNA library, all carried partial cDNA of α tubulin, with truncated 5' ends.

The α tubulin gene of *N. caninum* consists of 3 exons, which are interrupted by 2 intron sequences, much like its counterpart in *T. gondii* and *P. falciparum*. In comparison, the *C. parvum* gene is intron-less (Bonafonte, Garmon & Mead, 1999) and intron-less α tubulin genes are also known to exist in flagellates such as *Leishmania* (MacRae & Langdon, 1989). The position of intron/exon boundaries is also conserved between the α tubulin gene of the 3 apicomplexan parasites, *N. caninum*, *T. gondii* (Nagel & Boothroyd, 1988) and *P. falciparum* (Holloway *et al.* 1989). The introns are found at position 21/22 and 62/63 in the respective sequences

and they are conserved in both Nc-Liverpool and Nc-SweB1.

Alpha tubulin is widely described in the literature as a highly expressed gene (Tittawella & Normak, 1987; Parker & Detrich, 1998). The number of clones obtained by screening the N. caninum cDNA library with the α tubulin probe does not support the idea that this gene is highly expressed in this species. Furthermore, α tubulin mRNA does not appear as an abundant EST in the N. caninum database (http://ParaDB.cis.upenn.edu/). In P. falciparum α tubulin is not an abundant protein (Delves et al. 1990).

A comparison of the nucleotide sequences of T. gondii and N. caninum reveal that the majority of the base substitutions have taken place in the third exon, which comprises 86·5% of the coding region. The α tubulin genes of Nc-Liverpool and T. gondii differ by 6 nucleotide substitutions in the second exon and 96 substitutions in the third exon. Of the 104 substitutions that have taken place in the α tubulin gene during the evolutionary divergence of N. caninum and T. gondii, 94 have taken place in the 'silent' third codon position. A comparison of the α tubulin gene sequences derived by PCR from the two isolates of N. caninum showed them to be identical, which is in keeping with the observations that limited genetic variation occurs amongst different isolates of N. caninum (Atkinson et al. 1999; Schock et al. 2001).

Most of the essentially conserved regions of the α tubulin protein that have been described previously, are also conserved in N. caninum. Thus if one considers the predicted amino acid sequences for T. gondii, N. caninum, E. acervulina and P. falciparum the 5 motifs that are involved in GTP binding and MAP binding (Mandelkow et al. 1985; Sanchez et al. 1994) are conserved across all 5 sequences. The N-terminus of the N. caninum protein commences with Met-Arg-Glu as do all other α Tub (Holloway et al. 1989). Similarly, the C-terminus of the N. caninum α Tub ends with tyrosine as it does in the majority of the α tubulins sequenced to date. The end terminus Tyr is said to contribute towards the stability of microtubules and it is reported that only an α Tub molecule carrying a terminal Tyr can be assembled into microtubules. The terminal Tyr is removed from α tubulin of assembled microtubules by the enzyme tyrosine carboxypeptidase (TTC), exposing the Glu residue that is commonly found before the terminal Tyr (MacRae, 1997). The Tyr-depleted α tubulin molecules are normally recycled in the cell by the addition of tyrosine by the enzyme tubulinyl tyrosine carboxypeptidase (TTC), to the Glu residue. Given the importance of the end-terminal Tyr, it is therefore surprising to note that only half of the apicomplexan tubulin sequences shown in Fig. 2 are terminated with Tyr.

The motif TIGGG, which is conserved in most α tubulins sequenced to date, is also found in the

N. caninum sequence (residues 42–45) (Holloway et al. 1989). This sequence is usually preceded by a lysine, which is also conserved in the majority of α tubulins, and also is found in the N. caninum sequence. The lysine residue is thought to play an important role in a highly specific post-translational acetylation (LeDizet & Piperno, 1987).

It has been widely reported that the C-terminal region of most α tubulin proteins studied to date are highly acidic due to the presence of 6–8 Glu (E) or Asp (D) residues in the terminal sequence (Mandelkow, Herrman & Ruhl, 1985). Similarly, there are 6 Glu residues in the amino acid sequence from N. caninum.

Finally, considerable discussion has occurred recently over the relationship and identity of N. caninum and H. heydorni (Mehlhorn & Heydorn, 2000; Dubey et al. 2002b). The comparison presented here of the α tubulin gene sequences from T. gondii, N. caninum and H. heydorni shows these genes to possess a similar level of DNA sequence similarity amongst each other. Nevertheless, they are all sufficiently different from each other to recognize that they originate from genetically, quite distinct species. The trees obtained are in complete agreement with that hypothesized for the phylogeny of these Apicomplexa by rDNA comparisons (Morrison & Ellis, 1997). Thus this study of tubulin sequences, along with data from ITS1 and rDNA (Ellis et al. 1999), is consistent with N. caninum and H. heydorni being different species as suggested by others (Dubey et al. 2002a).

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