A novel capsid expression strategy for *Thosea asigna* virus (*Tetraviridae*)

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This paper presents evidence that *Thosea asigna* virus (TaV) has a unique capsid expression strategy and is a member of the *Nudaurelia* β-like genus of the *Tetraviridae*. Electron microscopy of TaV particles indicated a 38 nm, *T* = 4 icosahedral capsid similar in structure to that of *Nudaurelia* β virus (NβV). TaV particles have a buoyant density of 1.296 g/cm³ in CsCl and consist of two capsid proteins of 56 and 6 kDa. The virus genome contains a genomic RNA molecule of 6–5 kb and a subgenomic molecule of 2–5 kb. Northern blotting of TaV RNA indicated a genomic organization similar to that of NβV. The capsid gene of TaV is carried on both the genomic and subgenomic RNA molecules, while the RNA polymerase gene is present only on the genomic RNA. Cloning and sequencing of the TaV capsid gene identified an open reading frame that could potentially encode a capsid precursor protein of up to 82.5 kDa. The N-terminal sequences of the capsid proteins were compared with the nucleotide sequence of the capsid open reading frame. The sequences indicate that the pre-protein is cleaved at two positions to produce the 56 and 6 kDa capsid proteins as well as a predicted third protein that was not detected in the mature virion. Phylogenetic analysis of the capsid proteins indicated that TaV is more closely related to NβV than to the *Nudaurelia* ω-like viruses. The eight β-sheets that make up a jelly roll structure in the TaV capsid protein were identified by computer analysis.

Introduction

The family *Tetraviridae* consists of three well-characterized members: *Nudaurelia* β virus (NβV), *Nudaurelia* ω virus (NωV) and *Helicoverpa armigera* stunt virus (HaSV), plus a number of other recognized and potential members (Hendry *et al*., 1995). The family was divided into two genera (Hendry *et al*., 1995) on the bases of the appearance of the capsid and genomic organization. Viruses in the *Nudaurelia capensis* β-like (NβV-like) genus have a capsid with three distinct pits on each triangular face while the capsids of viruses in the *Nudaurelia capensis* ω-like (NωV-like) genus have more compact, closed triangular faces. Viruses in both genera have two RNA molecules. Members of the NωV-like genus carry the RNA polymerase gene and capsid gene on separate genomic RNA fragments and are therefore truly bipartite, while the NβV-like genus has two copies of the capsid gene. One copy of the capsid gene and the RNA polymerase gene are carried on the larger, genomic RNA fragment and the second copy of the capsid gene is located on the smaller, subgenomic RNA fragment (Hanzlik & Gordon, 1997). Although the recent review by Hanzlik & Gordon (1997) classified all poorly defined members of the *Tetraviridae* as part of the NβV-like genus, there is little evidence to support this assumption other than the historic grouping of these viruses as members of the *Nudaurelia* β virus family prior to the creation of the *Tetraviridae* and the NβV-like/NωV-like genera.

The larvae of *Setothosea asigna* (Lepidoptera: Limacodidae) are major defoliating pests of oil and coconut palms, with outbreaks recorded in north-eastern Sumatra, Borneo and western Malaysia (Entwistle, 1987). In 1978, Reinganum and co-workers reported a small RNA virus isolated from the larvae of *Setothosea asigna* (Reinganum *et al*., 1978), at that time...
Physical characteristics of virus particles. To visualize the capsid proteins of TaV, Tricine SDS–PAGE was used as described by Schagger & von Jagow (1987). Samples (0.9 µg) of TaV, NjV and NoV were electrophoresed through a 10% Tricine SDS–PAGE gel. A broad-range molecular mass protein marker (New England Biolabs) was included as a size standard. Proteins gels were stained with Coomassie blue for 30 min and then destained overnight. The density of TaV virus particles in CsCl was determined as described by Scotti (1985).

■ cDNA cloning of the TaV genome. RNA was extracted from 600 µl purified TaV by using TRIZol reagent (GIBCO-BRL) as specified by the manufacturer, except for the addition of 1 µl glycerol (20 mg/ml, Boehringer Mannheim) to enhance RNA precipitation. The RNA pellet was resuspended by the addition of 10 µl DEPC-treated water followed by heating for 10 min at 55 °C. The RNA was used to generate cDNA clones by using the random hexamers method from the SuperScript Choice system for cDNA synthesis (GIBCO-BRL). The resulting cDNA was blunt-ended by using Klenow DNA polymerase, ligated into EcoRV-digested pBluescript II SK(+) vector (Stratagene) and then transformed into E. coli XL-1 Blue cells by electroporation. Clones arising from TaV were identified by Southern blotting by using the product of first-strand cDNA synthesis of purified TaV RNA as a probe.

Two clones identified as arising from the RNA genome of TaV were sequenced by dye-termination sequencing (ABI Prism model 373A automated sequencer version 2.1.1; Centre for Gene Research, University of Otago). The clones were characterized further by using the Lasergene suite of DNA analysis programs (version 3.0; DNASTAR). Both DNA and protein sequences were used to search the GenBank database (BLAST algorithm) for similarity to other sequences. MegAlign (version 3.1; DNASTAR) was used to compare corresponding regions of proteins from several related insect viruses.

■ Analysis of the TaV genome. RNA was extracted from 250 µl purified virus by using TRIZol reagent (GIBCO-BRL) as described above. RNA was resuspended by the addition of 15 µl DEPC-treated water followed by heating to 55 °C for 10 min. The RNA was denatured by incubation at 50 °C for 15 min in 3 µl deionized 6 M glyoxal and 2 µl 0.1 M phosphate buffer, pH 7. Glyoxalated RNA was electrophoresed with a glyoxalated single-stranded RNA marker (GIBCO-BRL) in a 1% agarose gel at 50 V with 0.1 M phosphate buffer, pH 7 as the electrophoresis buffer and then transferred to a nylon membrane (Hybond-N, Amersham) by capillary transfer. After transfer, the RNA was fixed by exposure to UV for 2 min and then soaked in 10 mM Tris–HCl, pH 8.5 at 95 °C for 5 min to remove glyoxal. Membranes were probed with cDNA probes that were prepared by gel purification of insert DNA from TaV-containing clones by using the QIAEX DNA gel extraction kit according to the manufacturer’s instructions (Qiagen). Radioactively labelled probes were made by using the High Prime labelling kit (Boehringer Mannheim) and [α-32P]dCTP.

■ Complete sequencing of the capsid gene of TaV. The generation of clones to complete the sequence of the capsid gene of TaV was performed by using two different methods. The sequence of the clone described above, which contained part of the capsid gene of TaV, was used to design specific primers to extend the known sequence. These capsid-specific primers were utilized in conjunction with either universal forward (17-mer, 5′ GTAAAGACGCGGCAGT 3′) or universal reverse (19-mer, 5′ GGAAAGACGTATGACCATG 3′) primers to screen existing clones within the cDNA library by PCR. Products from the PCR were either sequenced directly by using the capsid-specific primers or, if there were convenient restriction enzyme sites close to the capsid primer-binding site, cloned and then sequenced as described above. The sequence of the 3′ end of the capsid gene was completed by RT–PCR by using a capsid-specific primer (capsid 5′ GCCGGTATGCTCCTGGTCTCGTTTTG 3′, bases 1873–1897) and an oligo(dt) primer (oligo(dT)20). RNA was extracted from purified virus RNA was extracted from purified virus as described above. A poly(A) tail was added to the 3′ end of the purified RNA by using poly(A) polymerase (GIBCO-BRL) as specified by the manufacturer. Following the addition of the poly(A) tail, RNA was used
A novel capsid expression strategy for TaV

Fig. 1. Comparison of capsid structures of TaV, NbV and NωV. (A) A single virus particle enlarged from a transmission electron micrograph of negatively stained, purified TaV particles. (B) Overlay highlighting the presence of three pits on the faces of the icosahedral virus capsid, indicating an NbV-like capsid arrangement. Two complete triangular faces of the TaV particle are outlined. This outline also highlights the 2-, 3- and 5-fold axes of symmetry that are characteristic of an icosahedral virus. The three pits on each triangular face are demonstrated by shaded circles. (C) Further enlargement of the virus particle in (A) and (B), to show detail of one triangular capsid face. Individual protein subunits are numbered. T = 4 symmetry is indicated by the presence of 12 protein subunits per face (240 proteins per virus particle). Note the arrangement of the subunits into Y-shaped trimers. (D) A single virus particle enlarged in Adobe Photoshop with the maximum filter applied. This filter heightens the lighter areas of an image, thus making the contours of the virus particle more visible. (E) Overlay demonstrating the arrangement of trimers on the capsid surface. Each small triangle represents one Y-shaped trimer. There are four trimers per triangular face. (F), (G) Image reconstruction of NbV (F) and NωV (G) capsids. The capsid structures of each genus within the Tetraviridae are markedly different. NbV-like viruses have three pits visible on each triangular face while NωV-like viruses have a smaller, more compact face with no pits visible. Images (F) and (G) generated by N. Olson and T. Baker, Purdue University, USA; reproduced from Hendry & Agrawal (1994) with permission of the publisher, Academic Press Limited, London.

in the mRNA capture kit (Boehringer Mannheim) according to the manufacturer’s instructions. The product from this reaction was cloned by using an NsiI site in the capsid gene with the NotI site in the oligodT17NotI primer, and sequenced as described previously.

- N-terminal sequencing of the capsid proteins of TaV. Purified TaV was electrophoresed through a 10% Tricine SDS–PAGE gel as described above. Pre-stained low-range molecular mass markers (Bio-Rad) were also included on the gel. Proteins were transferred to Immobilon P membrane (Millipore) by semi-dry blotting with the Pharmacia 2117 Multiphor II system. Proteins were visualized on the membrane by staining with 0.025% Coomassie blue in 40% methanol for 2 min. The membrane was destained in 50% methanol and then washed in deionized water. Bands corresponding to the large and small capsid proteins were excised and submitted to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, for N-terminal sequencing as described by Hubbard (1996). Ten cycles of sequencing were performed on the small capsid protein and five cycles were performed on the large capsid protein.

To confirm the result of N-terminal sequencing of the separate capsid proteins, intact virus was also sequenced. A drop of purified virus was applied to PVDF membrane and dried. The membrane was washed in methanol and then submitted to Jill McGovern at the Australian National University Biomolecular Resource Facility for five cycles of N-terminal sequencing.

- Confirming the novel capsid expression strategy of TaV. To verify the sequence of the 5′ end (bases 1–554) of the capsid contig, this region was re-isolated by two different methods and sequenced. Two primers, CP1 (5′ AATAGACCCGGGGCTCCAAGTGG, corresponding to bases 1–24) and CP2 (5′ TTAAGAATTCCGACCTCATTGTTG, corresponding to bases 2379–2355) were used to amplify the region covering bases 1–2379 of the capsid contig from viral RNA. The PCR product was cloned into pBluescript II SK(+) and the 5′ end was sequenced by using universal reverse primer and a capsid-specific primer, CP6 (5′ GAGCGGTTTTGGTTGCCTTG, bases 621–599). For further confirmation, two different primers, CP4 (5′ ATCCTCCCTTTCCATTTCGCT, bases 645–667) and RP2 (5′ ACTGCCCGGAGTGTTTG, corresponding to a region of the putative RNA
polymerase-containing clone), were designed for RT–PCR. This RT–PCR product was reamplified by using the capsid-specific primers CP1 and CP6 and then sequenced directly by using these primers (Fig. 5). All RT–PCRs were performed by using the Titan One-Step RT–PCR kit according to the manufacturer's instructions (Boehringer Mannheim).

Determining the relationship of TaV to other members of the Tetraviridae. The protein sequences of the capsid protein precursors of NβV (accession no. AF102884), HaSV (L37299), NooV (S43937) and black beetle virus (BBV; 75476), a nodavirus that was used as an outlier for alignments, were retrieved from GenBank. An alignment of the five capsid sequences was performed by using the Clustal W 1.7 algorithm available through the Human Genome Center, Baylor College of Medicine, Houston, USA (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). The resulting multiple sequence alignment was used in the MegAlign program (version 3.1; DNASTAR) and a phylogenetic tree was constructed by using the complete sequences of all five proteins.

The presence of β-sheets within the capsid protein of TaV was predicted by using the Protean program (Version 3.80; DNASTAR). The eight β-sheets that make up the jelly roll motif were additionally detected in TaV by comparison of its sequence, including the predicted β-sheets, to the sequences of the four other viruses used in the Clustal W alignment.

Results

Physical characteristics of TaV

Members of the Tetraviridae usually have buoyant densities of less than 1.3 g/cm³ in CsCl (Hendry & Agrawal, 1994). The buoyant density of TaV in CsCl was 1.296 g/cm³. Although this measurement differs from the value of 1.275 g/cm³ determined by Reinganum et al. (1978), this is to be expected due to the different method used to estimate the density (Scotti, 1985). The diameter of TaV particles was determined by comparison with tobacco mosaic virus as an internal standard. The icosahedral particles were estimated to be 38 nm in diameter, which is within the 35–41 nm range described for the Tetraviridae (Hendry & Agrawal, 1994).

The members of the Tetraviridae are the only non-enveloped viruses with T4 symmetry (Hendry et al., 1995). According to the quasi-equivalence theory proposed by Caspar & Klug (1962), isometric viruses accommodate 60T protein subunits. When TaV was visualized by TEM, 12 protein subunits were visible on the triangular faces of the icosahedral virus capsid (Fig. 1B). Each capsid contains 240 protein subunits, therefore the virus particles of TaV display T = 4 icosahedral symmetry. Within the Tetraviridae, the structure of
A novel capsid expression strategy for TaV

Fig. 4. Sequence of a cDNA clone derived from a 2484 base region of the TaV genome, including the entire capsid gene. The predicted amino acid sequence is shown below the nucleotide sequence from the first upstream methionine. All five methionine start codons are underlined. Residues from the first upstream methionine to the proposed N-terminal cleavage point are in italics. Bold residues indicate results of N-terminal sequencing of the large and small proteins. Thick underlining represents the eight β-sheets from β-B to β-I that make up the proposed jelly roll. The bold rectangle indicates the cleavage point between the large and small capsid proteins.

The virus capsid is one of the characteristics that separates the two genera. While the NβV-like viruses have three pits visible on each face of the virus capsid (Fig. 1F) (Finch et al., 1974; Olson et al., 1990), NωV-like particles lack these pits, having a smaller, closed face (Fig. 1G) (Hendry & Agrawal, 1994). Three pits were clearly visible on the TaV capsid, indicating that TaV is NβV-like in its capsid structure (Fig. 1A, B).

Two capsid proteins are present in TaV particles, a major protein of 56 kDa and a minor protein of 6 kDa. While the apparent molecular masses of the capsid proteins of TaV are lower than those of NβV (61 and 7–9 kDa) (Hendry & Agrawal, 1994; Hanzlik & Gordon, 1997) and NωV (62 and 7–8 kDa) (Agrawal & Johnson, 1992), the relative molarities of each protein are comparable (Fig. 2).
Fig. 5. The capsid expression strategy of TaV (not to scale). (A) The capsid precursor protein of TaV is produced from one of the five ATG codons (shaded arrows) at the start of the capsid contig. The proteins that are produced by cleavage of the precursor are shown. The 58–3 and 6–8 kDa proteins (shaded) are the large and small capsid proteins. A putative protein of up to 17 kDa is also produced by cleavage of the precursor. This protein has no known function and has not been detected in TaV virions. (B) Diagram showing the locations of primers used to verify the 5' sequence of the capsid contig. CP1 was used in conjunction with CP2 to amplify the entire capsid contig. This RT–PCR product was cloned into pBluescript and sequenced by using the universal reverse primer and CP6. CP4 was used with an RNA polymerase-specific primer, RP2, to generate an RT–PCR product that was then re-amplified with primers CP1 and CP6. The resulting PCR product was sequenced directly with CP6.

Analysis of the TaV genome

When a representative portion of the cDNA library was screened, two clones were identified as arising from TaV RNA. One clone contained an open reading frame encoding 310 amino acids, which revealed similarity to NoV and HaSV capsid proteins when used in a BLAST search of the GenBank database. The protein sequence from TaV was also compared to the capsid protein sequence of NoV, confirming that the clone contained a portion of the TaV capsid gene.

The other clone was positive when probed with the product of first-strand cDNA synthesis of TaV RNA. Although neither the DNA nor the protein sequence from this clone exhibited strong similarity to other sequences in the GenBank database, it is probable that the clone contains a portion of the RNA polymerase gene of TaV. Similarity between (+)-stranded virus RNA polymerases at the amino acid level is minimal, with only one strongly conserved motif in (+)-stranded virus RNA polymerases (Koonin & Dolja, 1993). The protein sequence from this clone does not contain this motif; however, it does hybridize to TaV genomic RNA. Therefore it is likely that this clone contains part of the RNA polymerase gene of TaV.

The TaV genome consists of two single-stranded RNA molecules, a 6.5 kb genomic RNA and a 2.5 kb subgenomic molecule (Fig. 3 C). Since the two genera within the Tetraviridae have distinct genome structures (Fig. 3 A, B), Northern blots of TaV genomic RNA were performed. When the TaV genome was probed with the putative RNA polymerase probe, only the genomic band showed cross-reactivity (Fig. 3 E). However, when the capsid gene probe was used, both RNA bands were visible, indicating that the capsid gene is repeated on the smaller subgenomic RNA molecule (Fig. 3 D).

Sequence of the TaV capsid protein

Sequence information has been determined for a 2484 base region of the TaV genome that includes the entire capsid gene (GenBank accession no. AF062037). An open reading frame of 2272 bp has the potential to encode a protein of up to 82.5 kDa (Fig. 4). Within the open reading frame there are five potential start codons, which would encode proteins of 82.5, 81.8, 81.3, 78.8 or 69.5 kDa. Stop codons located upstream of the five ATGs preclude the possibility of a larger polyprotein being produced, therefore it is likely that translation of the capsid precursor protein is initiated at one of the five start codons. The second of the five start codons has the best context (AAAatgA) for initiation of translation (Kozak, 1986). Furthermore, the 5' region of the capsid contig was re-isolated and sequenced by two different methods, as described above. The sequence obtained by each method was the same as the existing sequence, with the exception of two base changes in one clone. At base 84 of the capsid contig (Fig. 4), a C → T change introduced a stop codon upstream of the first of the five start codons. The other change was T → C at base 235 of the capsid contig. This resulted in a conservative Ile → Thr amino acid change within the region upstream of the predicted cleavage point that generates the N terminus of the large capsid protein.

Cleavage of the pre-protein results in the formation of the large and small capsid proteins. The cleavage point for generation of the small capsid protein was elucidated by N-terminal sequencing of this protein fragment. Ten sequencing
cycles performed on the small capsid protein resulted in the sequence GWGLMFSKVL. This protein sequence occurs within the predicted open reading frame (Fig. 4). The size of the small capsid protein as predicted from the sequence is 6–8 kDa.

From the sequence information available, it was observed that, after cleavage of the precursor protein into the large and small capsid subunits, regardless of which start codon initiated translation, the large capsid protein was predicted to be bigger than the protein visualized by SDS–PAGE. The N terminus of the large capsid proteins of tetraviruses studied to date have been shown to be blocked, those of NβV (Agrawal & Johnson, 1992), HaSV (Hanzlik et al., 1993) and NωV (Gordon et al., 1999). To determine whether the N terminus of the large capsid protein of TaV was blocked, sequencing of the large capsid protein was undertaken. This returned the amino acid sequence PTTVA, which occurs 39 amino acids downstream of the fifth putative methionine start codon (Fig. 4). The lack of a methionine residue at the N terminus of the sequence suggests that there is a second processing event of the capsid precursor. This hypothesis is supported by the predicted size of 58–3 kDa for the large capsid protein from PTTVA to the cleavage point from the small protein. This predicted size corresponds to the size of the large protein as obtained by SDS–PAGE analysis. It is also close to the size of 58–5 kDa determined by sequencing of the large capsid protein of NβV (Gordon et al., 1999).

To confirm that the N-terminal sequence obtained from the large capsid protein was not that of a breakdown product, the intact virus was also sequenced. The five cycles of sequencing performed on the intact capsid returned the residues PTTVA and GWGLM. The yields for the first three residues, PTT, of the large capsid protein were 9, 8–2 and 6–3 pmol, respectively. The yields for the first three residues, GWG, of the small capsid protein were 15, 6–2 and 8–6 pmol, respectively. This confirms that the N terminus of the large capsid protein is not blocked and also suggests that the sequence PTTVA results from the N-terminal cleavage of the large capsid protein, because it is present in approximately the same molar ratio as the small capsid protein.

Based on the sequence information determined for the capsid gene of TaV, we propose that the capsid protein precursor is cleaved twice, generating the unblocked N termini of the large and small capsid proteins (Fig. 5A). A predicted third protein, of up to 17 kDa, has not been detected in mature virions. The strategy, shown in Fig. 5B, used to confirm the sequence of the 5′ region of the capsid contig also confirms that the cDNA clone used to generate the RNA polymerase probe and primer does contain part of the TaV RNA polymerase gene.

**Determining the relationship of TaV to other members of the Tetraviridae**

The protein sequence of TaV, from PTTVA, the putative N terminus of the large capsid protein, to the end of the small capsid protein, was treated as equivalent to the capsid protein precursor sequences of the three tetraviruses and BBV and was aligned with these proteins by using the Clustal W algorithm. This comparison revealed that TaV was more closely related to NβV than to HaSV or NωV. However, it is not as closely related to NβV as the two NωV-like viruses are to each other. TaV exhibited 35–7% similarity to NβV, 23–3% to NωV and 22–5% to HaSV. In comparison, NβV has 21–6% similarity to NωV and 20–4% similarity to HaSV. The two viruses from the NωV-like genus are the most closely related according to this algorithm, with a similarity of 66%. The percentage of similarity of the tetraviruses to the outlier group, BBV, was below 19% in all cases. The alignment generated by this algorithm is shown in Fig. 6.
The folding of the large capsid proteins of other tetraviruses involves eight β-sheets that assemble into the jelly roll structure associated with icosahedral virus capsids (Chelvanayagam et al., 1992). Eight regions of β-sheets were identified in TaV that align (Fig. 6) with the β-sheets identified in another tetravirus, NοV, the crystal structure of which has been determined. The presence of these regions suggests that the capsid of TaV exhibits the jelly roll structure expected of an icosahedral virus particle.

The phylogenetic tree created in MegAlign by using the multiple sequence alignment generated with Clustal W had two main branches. The outlier group, BBV, was placed on one branch, while the other branch contained all the tetraviruses. This branch was further divided into two clades. TaV was grouped with NjV, and the two ω-like viruses were grouped together (Fig. 7).

**Discussion**

TaV is currently classified by the International Committee on the Taxonomy of Viruses (ICTV) as an unassigned virus that is considered to be a possible member of the Tetraviridae (Hendry et al., 1995). TaV has also been listed in a recent review by Hanzlik & Gordon (1997) as belonging to the NjV-like genus within the Tetraviridae. This virus, like many of the unassigned viruses listed in the Tetraviridae, has not been studied in depth, mainly because of the lack of suitable cell culture lines. In order to clarify the position of TaV within the Tetraviridae, it is necessary to expand upon previously published data (Reinganum et al., 1978) and to compare this data with the classification steps from the ICTV report and to other viruses in the family that have been studied in more detail.

The physical characteristics of TaV particles support the inclusion of this virus in the NjV-like genus of the Tetraviridae, as do the genome size and organization. The buoyant density of TaV virions in CsCl, the size of the icosahedral particle, the observation of $T = 4$ icosahedral symmetry and the presence of two single-stranded RNA molecules are all recognized features of the Tetraviridae (Hendry et al., 1995). The presence of three pits on each face of the TaV capsid and the organization of the genome, with the capsid gene present twice, once on the genomic RNA fragment and again on the subgenomic RNA molecule, are characteristics that have recently been described for NjV (Hanzlik & Gordon, 1997). A novel feature of TaV is the coding strategy of the capsid protein. The capsid protein precursor is cleaved into three proteins, the large and small capsid proteins and a third protein of unknown function that was not detected in the TaV virion. To confirm that the unblocked N terminus of the large capsid protein was not the result of the sequencing of a breakdown product, intact virus capsids were also N-terminally sequenced. The results that were obtained indicate that the capsid proteins are present in the same molar ratio, suggesting that the unblocked N terminus of the large capsid protein is the result of processing.

To verify the sequence of the 5′ region of the capsid contig, this region was re-isolated and sequenced by two different methods. Although there were some changes between clones, these were usually silent mutations. There were only two base changes that affected the sequence of this region. One resulted in the creation of a stop codon; however, this is upstream of the first start codon and will not alter the capsid precursor protein sequence. The other base change was a conservative amino acid substitution within the protein of unknown function. Apart from these two changes, the sequence obtained by three different methods, cDNA cloning, RT–PCR and PCR of an RT–PCR product, confirmed the existing sequence at the 5′ end of the capsid. This result also supports the hypothesis that the capsid protein precursor of TaV undergoes two cleavage events to generate the three proteins shown in Fig. 5.

The capsid proteins of TaV appear to be present in a similar arrangement to the major and minor capsid proteins of NjV and NοV. However, like the 55 kDa major capsid protein of the provisional tetravirus Pseudepistis includens virus (PIV; Chao et al., 1983), the size of the major capsid protein of TaV would currently exclude TaV from classification within the Tetraviridae. The most recent key from the ICTV includes a step requiring the major capsid protein to be 60 kDa or greater, thus excluding both TaV and PIV from the Tetraviridae, even though TaV exhibits many other characteristics in common with this virus family. This classification step would also exclude NjV, a type member of the Tetraviridae. Sequence data from NjV predicts a large capsid protein of 58-5 kDa (Gordon et al., 1999). It has now been demonstrated that both NοV-like and NjV-like members of the Tetraviridae have two capsid proteins (Agrawal & Johnson, 1992; Hanzlik & Gordon, 1997). Therefore, perhaps a more suitable classification step would be to require the presence of two capsid proteins, a major one of $\geq 55$ kDa and a minor one of 6–8 kDa. This would require confirmation as further studies of provisional members of the Tetraviridae provide more information.

The amino acid sequences of the capsid proteins of TaV demonstrated similarity to the type members of both genera, NjV and NοV, and also to HaSV, a member of the NοV-like genus. Hanzlik et al. (1995) demonstrated that there is an overall similarity of 76% between HaSV and NοV over the entire amino acid sequence. There are two regions within the

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**Fig. 7. Relationship of TaV to other members of the Tetraviridae.** A phylogenetic tree showing the relationship between TaV, HaSV, NjV, NοV, and BBV was created within the DNASTAR suite of programs by using an alignment generated by Clustal W. The alignment was performed on the capsid protein from each virus as described.
protein that have high similarity (approx. 80%) and two regions of low similarity (approx. 33%). However, comparison between the entire capsid proteins of NjV and NoV shows only 42% similarity overall, with no domains of strong similarity apparent (Hanzlik & Gordon, 1997). TaV does not exhibit domains of strong similarity with NjV or the NoV-like viruses, although there are conserved motifs present in both the large and small capsid proteins. The area of greatest similarity between NjV and TaV is directly upstream of the cleavage point between the small and large proteins. The slightly lower percentages of similarity determined for NoV/HaSV and NjV/NoV in this study probably result from the use of a different alignment algorithm.

This study supports the inclusion of TaV as a novel member of the Tetraviridae within the NjV-like genus. Although TaV has a unique capsid protein coding strategy and relatively low percentages of similarity to the other tetra-viruses, more viruses from the Tetraviridae must be studied to determine whether the expression strategy of TaV or NjV is typical of the NjV-like genus. It is also possible that the members of the Tetraviridae are more disparate than previously thought. Until further classification of different members of the Tetraviridae is completed, the taxonomy of this family of viruses should remain unchanged.

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References


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