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3′-Terminal sequences of the RNA genomes of narcissus latent and Maclura mosaic viruses suggest that they represent a new genus of the *Potyviridae*

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The nucleotide sequences of part of the nuclear inclusion body b (NIb) gene, the complete coat protein gene and the 3′ untranslated regions of narcissus latent virus (NLV) and Maclura mosaic virus (MacMV) were determined. Deduced amino acid sequences for the NIb and coat protein genes revealed that NLV and MacMV are closely related. Gel analysis and Western blotting of the coat proteins of NLV and MacMV from infected tissue or purified virus indicated that they have molecular masses of 39·5 kDa and 40 kDa (respectively), whereas estimates from deduced amino acid sequences suggested that they have molecular masses of 32·8 kDa and 34·1 kDa. Comparison of the NIb and coat protein sequences with other viruses showed that NLV and MacMV have close affinities with viruses of the *Potyviridae* and suggests that they should form a new genus of the family.

Narcissus latent virus (NLV) was first described in 1966 (Brunt & Atkey, 1967) as a member of the carlavirus genus. It has been reported to induce very mild leaf chlorosis in the tips of narcissus leaves and affects many commercially important cultivars (Brunt, 1977). NLV has flexuous filamentous particles ca. 657 nm long and ca. 13 nm wide, and a coat protein estimated to be 32·6 kDa (Brunt, 1977), properties suggesting that NLV is a carlavirus. In preliminary tests its coat protein cross-reacted with antiserum raised to only one carlavirus, lily symptomless virus (Brunt, 1977). However, it was later reported that NLV had a coat protein of 45 kDa and that in *Nicotiana clevelandii* it induces cylindrical cytoplasmic inclusions (CCIs) characteristic of potyviruses (Mowat *et al.*, 1991; Brunt *et al.*, 1994). In further serological tests NLV particles failed to react to antisera raised to 12 potyviruses and 9 carlaviruses (Mowat *et al.*, 1991; Shukla *et al.*, 1994).

Maclura mosaic virus (MacMV) was reported to cause mosaic symptoms on the leaves of the ornamental tree *Maclura pomifera* (Plese & Milicic, 1973) and to induce CCIs (Plese & Wrischer, 1978). Like NLV, MacMV has flexuous filamentous particles 650–710 nm long. Although some anomalies in the appearance of the isolated particles have been reported, measurements of NLV and MacMV particles have always shown a single modal length, suggesting that the viruses have monopartite genomes (Brunt & Atkey, 1967; Brunt, 1977; Mowat *et al.*, 1991; Plese & Wrischer, 1978). The coat protein size of MacMV was reported to be 45 kDa (Plese *et al.*, 1979). MacMV was tentatively classified as a member of the potyvirus genus due to the presence of CCIs in infected tissue and its weak cross-reaction with antisera to bean yellow mosaic potyvirus (Plese *et al.*, 1979).

Recently, it was demonstrated that NLV particles cross-react in ELISA and IEM with MacMV antiserum, results establishing a link between them. It was concluded that NLV was neither a potyvirus nor a carlavirus but could be a member of a new genus with MacMV (Mowat *et al.*, 1991). Moreover, a PCR primer designed to a sequence present in 80% of all carlaviruses sequenced to date failed to produce a product with NLV or MacMV cDNA (Badge *et al.*, 1996).

Due to the uncertain taxonomic status of NLV and MacMV, we have characterized these two viruses using molecular techniques. Analysis of the coat protein isolated from MacMV particles (isolate from Plese), by SDS–PAGE stained with Coomassie Blue, produced two or more bands ranging from 32 to 20 kDa (results not shown). N-terminal micro-sequencing of the 32 kDa protein showed the sequence 'SDPEE' to be present in the deduced amino acid sequence at position 487

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The nucleotide sequences reported in this paper have been submitted to GenBank and assigned the accession numbers U58770, U58771.

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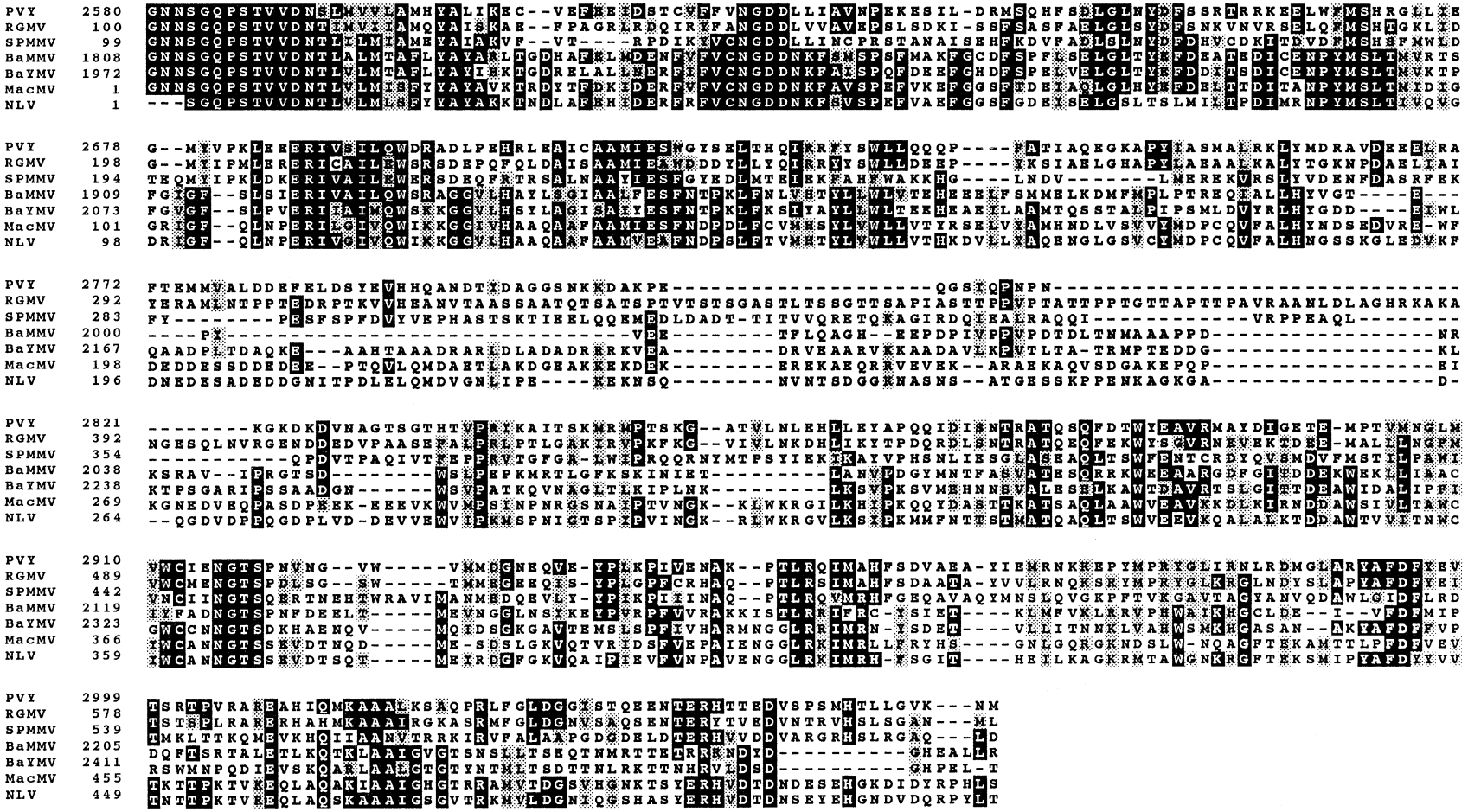

Fig. 1. CLUSTAL W alignment of the partial ORFs of NLV (GenBank U5870) and MacMV (GenBank U58771) with the C-terminal regions of two bymoviruses, barley mild mosaic virus (BaMMV, GenBank L49381) and barley yellow mosaic virus (BaYMV, GenBank D01092), one rymovirus, ryegrass mosaic virus (RGMV, GenBank U27383), one ipomovirus, sweetpotato mild mottle virus (SPMMV, GenBank Z48058) and one typical potyvirus, potato virus Y (PVY, GenBank A08776). Gaps (-) have been introduced for maximum alignment and the program PRETTYBOX was used to create boxing. Residues identical to MacMV are boxed with a black background, chemically similar residues are boxed in grey.

Table 1. Pairwise percent amino acid sequence identities between MacMV and NLV and other selected members of the *Potyviridae*

(*a*) Core coat protein*

	MacMV	BaYMV	BaMMV	SPMMV	RGMV	PVY
NLV	53·4	23·2	22·2	14·6	22·2	21·2
MacMV		22·2	21·2	15·1	18·0	19·4
BaYMV			33·7	11·0	11·5	13·4
BaMMV				11·1	12·9	14·8
SPMMV					18·0	18·9
RGMV						54·6

* The core coat protein is equivalent to D^{2748} to R^{3043} in PVY.

(*b*) Partial NIb proteins*

	MacMV	BaYMV	BaMMV	SPMMV	RGMV	PVY
NLV	76·3	57·2	60·3	23·0	21·3	26·7
MacMV		60·4	59·7	30·7	28·2	31·2
BaYMV			73·1	27·6	28·2	28·2
BaMMV				30·7	29·0	32·0
SPMMV					38·4	35·3
RGMV						51·1

* This region is equivalent to G^{2580} to A^{2711} in PVY. This region was used as cleavage sites between NIb and coat proteins have not been proposed for RGMV or SPMMV.

(Fig. 1). Further analysis using Western blotting of MacMV infected *N. clevelandii* tissue showed that coat protein obtained from purified particles was probably degraded, since a single protein of 40 kDa was observed. Similarly, NLV (isolate NLV-S) coat protein isolated from virus particles was estimated to be 32 kDa, but Western analysis of infected narcissus tissue indicated that the coat protein was 39·5 kDa. A reciprocal cross-reaction between NLV and MacMV from *N. clevelandii* was observed when using antiserum raised to either coat protein (results not presented).

In order to estimate more accurately the encoded molecular mass of the virus subunits, a number of overlapping (double-stranded) cDNA clones representing the 3′-terminal regions of both viruses were obtained and sequenced to completion on both strands. Agarose gel analysis of RNA extracted from virus particles and Northern analysis on infected material confirmed that for both viruses the full-length genomic RNA (8 kb) was of a single species (results not presented). A total of 1797 nucleotides (nt) was sequenced for NLV (GenBank U58770) and 2413 nt for MacMV (GenBank U58771). Analysis showed that each sequence contained a single continuous open reading frame (ORF). For MacMV this was 727 amino acids (aa), and for NLV this was 513 aa, leaving untranslated regions of 256 nt (NLV) and 231 nt (MacMV). Alignment of the coding regions indicated that NLV and MacMV RNAs are 52·4% identical at the nucleotide level.

A database search showed that the NLV and MacMV sequences had the highest amino acid similarity with those of barley yellow mosaic virus (BaYMV), the type member of the bipartite bymovirus genus within the *Potyviridae*. The presence of a single ORF within this 3′ region is analogous to a potyviral genome organization and is in contrast with the carlavirus genome, which contains two independent ORFs (Foster, 1992). We therefore conclude that the ORFs found in the deduced amino acid sequences of the 3′-terminal regions of NLV and MacMV RNAs may be C termini of large polyproteins. The NLV and MacMV sequences showed high similarity to a range of potyviruses in the nuclear inclusion body b (NIb) protein, which are present upstream of the coat protein (see Table 1*b*). To calculate the size of NLV and MacMV coat proteins, it was therefore necessary to estimate the position of the cleavage site between the NIb protein and the coat protein.

Alignment of the deduced amino acid sequence for NLV and MacMV partial polyprotein (Fig. 1) shows that in both sequences there is a possible cleavage site 'LQM'. Although

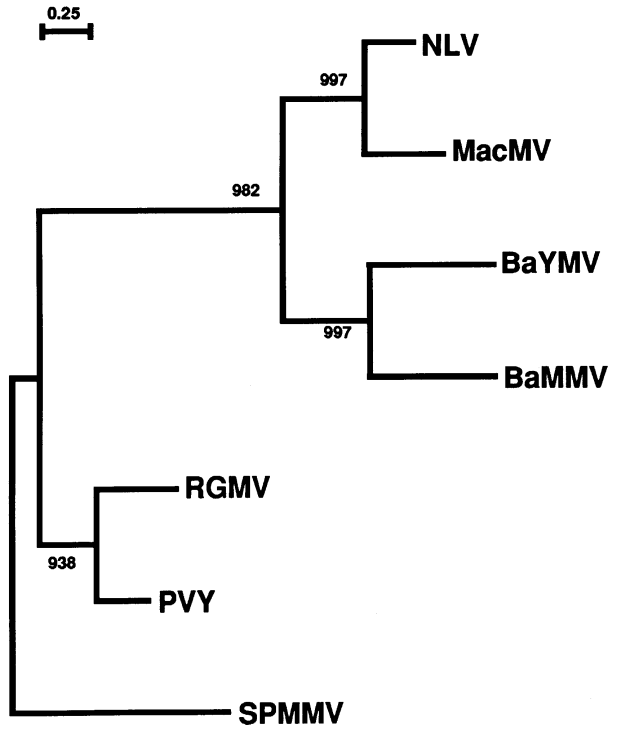

Fig. 2. Neighbour-joining tree produced using CLUSTAL W to demonstrate the taxonomic relationship between the core of the coat proteins of MacMV and NLV and those of selected members of the *Potyviridae* (as used in Table 1). Horizontal distances are proportional to percent divergence of tip species from nodes (scale given), vertical distances are arbitrary. Numbers shown on the branches are the results from bootstrapping using CLUSTAL W for 1000 replicates.

the consensus potyvirus cleavage site is a heptapeptide cleaving between glutamine (Q) and serine (S) or glycine (G), the bymoviruses are thought to have a consensus cleavage site 'LQA' (Dinant *et al.*, 1992). Cleavage after the glutamine residue would give alanine as the N-terminal residue of bymovirus coat proteins and methionine as that of NLV and MacMV coat proteins. It is noteworthy that both alanine and methionine are among N-terminal residues that are relatively resistant to degradation (Dinant *et al.*, 1992). The N termini of potyvirus coat proteins are highly diverse and the C terminus of the NIb protein is known to be well conserved (Shukla & Ward, 1988). With a cleavage site of 'LQM', NLV and MacMV deduced amino sequences follow this principle, with more similarity in the region up to the cleavage site than downstream of it (Fig. 1).

This putative cleavage site would produce coat proteins of a predicted size (32·8 kDa NLV, 34·1 kDa MacMV) smaller than those we have observed by Coomassie Blue stained SDS–PAGE or Western analysis. The difference in size of coat proteins observed by Western analysis, and that expected by deduced amino acid sequence has been noted previously (Shukla *et al.*, 1994), and an artefact of gel analysis could account for this discrepancy.

The sequence data from the C termini of the large polyproteins encoded by NLV and MacMV allows a re-examination of their classification. Alignments using all carlavirus coat proteins sequenced to date show only 9–12% sequence identity to NLV and MacMV coat proteins. Database searches over the whole region sequenced for NLV and MacMV revealed a much higher level of identity to the bymoviruses, which also utilize a polyprotein processing system, unlike the carlaviruses. Fig. 1 shows an alignment using CLUSTAL W of the deduced amino acid sequences of NLV and MacMV, two members of the bymovirus genus, BaYMV (Kashiwazaki *et al.*, 1989, 1991, 1992; Davidson *et al.*, 1991) and barley mild mosaic virus (BaMMV) (Foulds *et al.*, 1993; Kashiwazaki *et al.*, 1992), a typical potyvirus, potato virus Y (PVY) (Robaglia *et al.*, 1989), and type members of the two other genera recognized in the *Potyviridae* family, ryegrass mosaic virus (RGMV) (rymoviruses: Salm *et al.*, 1996) and sweetpotato mild mottle virus (SPMMV) (ipomoviruses: Colinet *et al.*, 1996). This region represents the C terminus of the NIb protein and the coat protein. In general, the potyviruses have a very good degree of identity in the coat protein ORFs, and this is particularly striking in a defined core region (Shukla *et al.*, 1994). Shukla & Ward (1988) have shown that distinct species of the potyvirus genus have coat protein core sequence similarities within the range 38–71% (average 54%). This suggests that NLV and MacMV form a distinct genus, as similarity in the core coat protein is low when compared with all the other accepted genera within the *Potyviridae* family (14–23%) (see Table 1*a*). This is further supported by the dendrogram of sequence relationships generated from the alignment of the coat protein core amino acid sequences (Fig. 2), which demonstrates that NLV and MacMV do not belong to any recognized genus in the *Potyviridae*.

The partial amino acid sequence available for the NIb protein includes several well conserved regions and further demonstrates that NLV and MacMV are more closely related to the bymoviruses than to the ipomo-, rymo- or potyviruses (see Table 1*b*). The consensus motifs (T/S)GXXXTXXXN(T/S) and GDD are highly conserved in positive-strand RNA viruses and are thought to be part of the active site for the RNA dependent RNA polymerase (Dougherty & Carrington, 1988). Other well conserved motifs within potyvirus coat proteins are found in NLV and MacMV; for example 'NGTS' (NLV, 363 aa; MacMV, 578 aa) is found in NLV, MacMV, and other members of the *Potyviridae* family. A tripeptide motif, 'DAG', is usually found within a heptapeptide block at or near the N terminus of the coat protein of aphid transmitted potyviruses (Atreya *et al.*, 1990, 1995). It was demonstrated by mutagenesis that this motif was essential for vector transmission (Atreya *et al.*, 1995). Two motifs similar to 'DAG' can be found directly after the cleavage site in NLV and MacMV. At position 218 aa NLV has the sequence 'DVG', and MacMV has the sequence 'DAE' at position 425 aa (Fig. 1). However, both tripeptides

are found directly after the proposed cleavage sites, and therefore probably do not form part of the suggested heptapeptide motif. It was also shown that in tobacco vein mottling potyvirus a mutation from 'DAG' to 'DAE', as observed for MacMV, gave a non-transmissible product, and that a mutation to 'DVG', which is found in NLV, left only minimal transmission function (Atreya *et al.*, 1990, 1995). The aphid transmissibility of these isolates of NLV and MacMV has not been determined experimentally.

In conclusion, sequence analysis has shown strong similarities of genome organization between MacMV, NLV and members of the *Potyviridae*. We have shown that NLV and MacMV have similar levels of amino acid sequence identity with the potyviruses to those shown by the bymoviruses, rymoviruses and ipomoviruses (Colinet *et al.*, 1996). This strongly supports the suggestion (Mowat *et al.*, 1991) that they should be classified as the fifth genus of the *Potyviridae*, tentatively designated the macluraviruses (Shukla *et al.*, 1994).

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