

## Partial Purification and Serology of Sugarcane Mild Mosaic Virus, A Mealybug-Transmitted Closterolike Virus

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### ABSTRACT

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A previously undescribed closterolike virus with particles measuring 1,500–1,600 nm × 12 nm was found in 11 cultivars of sugarcane (*Saccharum* sp.) from Florida, Mauritius, and Malawi. The virus, which was named sugarcane mild mosaic virus (SCMMV), occurred in all cases in mixed infections with sugarcane bacilliform virus (SCBV). SCMMV was transmitted by mechanical inoculation to sugarcane, rice, *Sorghum halepense*, and *S. bicolor* with partially purified, concentrated extracts but not with crude sap. SCMMV was not transmitted by *Melanaphis*

*sacchari* but was transmitted to both sugarcane and rice by the pink sugarcane mealybug, *Saccharicoccus sacchari*. SCMMV infection caused very mild mosaic or no foliar symptoms in sugarcane, no apparent symptoms in rice, and occasional chlorotic flecking in *S. halepense*. SCMMV could be detected by enzyme immunosorbent assay and immunosorbent electron microscopy and was unrelated serologically to six other closterovirus or closterolike viruses, including grapevine virus A, which is mealybug-transmitted, and to pineapple mealybug wilt associated virus.

A high percentage of clones of sugarcane, *Saccharum officinarum* L., the so-called noble canes, have recently been infected naturally by sugarcane bacilliform virus (SCBV) (4). SCBV (11) is a dsDNA plant virus (10) that is transmitted by the pink sugarcane mealybug *Saccharicoccus sacchari* (Cockerell) and by the gray sugarcane mealybug, *Dysmicoccus boninsis* (Kuwana) (B. E. L. Lockhart and J. C. Comstock, unpublished data). SCBV infection in sugarcane is not consistently associated with any distinctive foliar symptoms (Fig. 1) or readily observed changes in overall plant growth (11). However, several sugarcane clones (e.g., Selemi Bali, NG 51-39, M 27-16) infected by SCBV showed symptoms that included striate mosaic, abnormally narrowed leaves, and relatively slow growth. Examination of these clones by electron microscopy (EM), immunosorbent electron microscopy (ISEM), and enzyme immunosorbent assay (EIA) showed that they were not infected by sugarcane mosaic virus (SCMV) or any other virus reported to infect sugarcane. However, all of these sugarcane clones contained, in addition to SCBV, closterolike virus particles (Fig. 2A), which were absent from the symptomless sugarcane clones examined initially. This paper presents information on the occurrence, partial purification, morphology, transmission, and serology of sugarcane mild mosaic virus (SCMMV), the second closterovirus or closterolike virus demonstrated to be mealybug-transmitted.

### MATERIALS AND METHODS

**Virus source.** The SCMMV isolate initially used in these studies occurred in the naturally infected *S. officinarum* clone, Selemi Bali, in which it was maintained by vegetative propagation. Selemi Bali was also infected naturally by SCBV and had foliar symptoms consisting of a prominent chlorotic striate mosaic (Fig. 1A). SCMMV was subsequently separated from SCBV by mealybug

transmission to the *S. officinarum* clone IJ 76-319, which did not become infected with the SCBV isolate from Selemi Bali.

**Mechanical inoculation.** Crude sap inoculum was prepared by grinding young symptomatic Selemi Bali leaf tissue in 1% (w/v) K<sub>2</sub>HPO<sub>4</sub> containing 0.2% (w/v) Na<sub>2</sub>SO<sub>3</sub>. We added silicon carbide (600 mesh) as an abrasive to the crude extract, and we inoculated leaves of indicator plants by rubbing. Partially purified extracts used for mechanical inoculation were prepared as described below. Inoculated test plants were tested individually by ISEM with partially purified extracts after a single cycle of ultracentrifugation. Test plants were indexed by ISEM at periods varying from 6 wk to 6 mo after inoculation.

**Insect transmission.** Aphid transmission tests were done with *Melanaphis sacchari* (Zehntner), which occurred naturally on sugarcane in Florida and which readily colonized sugarcane in the greenhouse. *S. sacchari* was used in mealybug transmission tests. For both aphid and mealybug transmission tests, nonviruliferous insects were allowed to colonize plants of Selemi Bali infected naturally with both SCBV and SCMMV. Plants of IJ 76-319, infected with SCMMV only, were also used as virus source plants in subsequent mealybug transmission tests. After an acquisition feeding period of 2–6 days, 10–12 insects were transferred in each case to virus-free test plants of the sugarcane cultivar CL 61-620, the noble cane clones IJ 76-319 and IK 76-69, and to rice (*Oryza sativa* L. 'Taichung Native 1'). After an inoculation access period of 48 h, the insects were killed by insecticide. Inoculated test plants were observed in the greenhouse for up to 18 mo.

**Virus purification and EM.** SCMMV was purified from leaf tissue of naturally infected Selemi Bali or from inoculated IJ 76-319 and CL 61-620 by a modification of the method used previously for SCBV (11). Young and old leaf tissues were used. Fresh leaf tissue, minus midribs, was powdered in liquid nitrogen and extracted twice with 3 vol (tissue wt/vol) of 0.1 M Tris-HCl, pH 7.4, containing 0.5% (v/v) 2-mercaptoethanol and 0.5% (w/v) Na<sub>2</sub>SO<sub>3</sub>. The crude extract was centrifuged in a Sorvall SS-34 rotor at 10,000 g for 10 min, and the pellet was discarded.

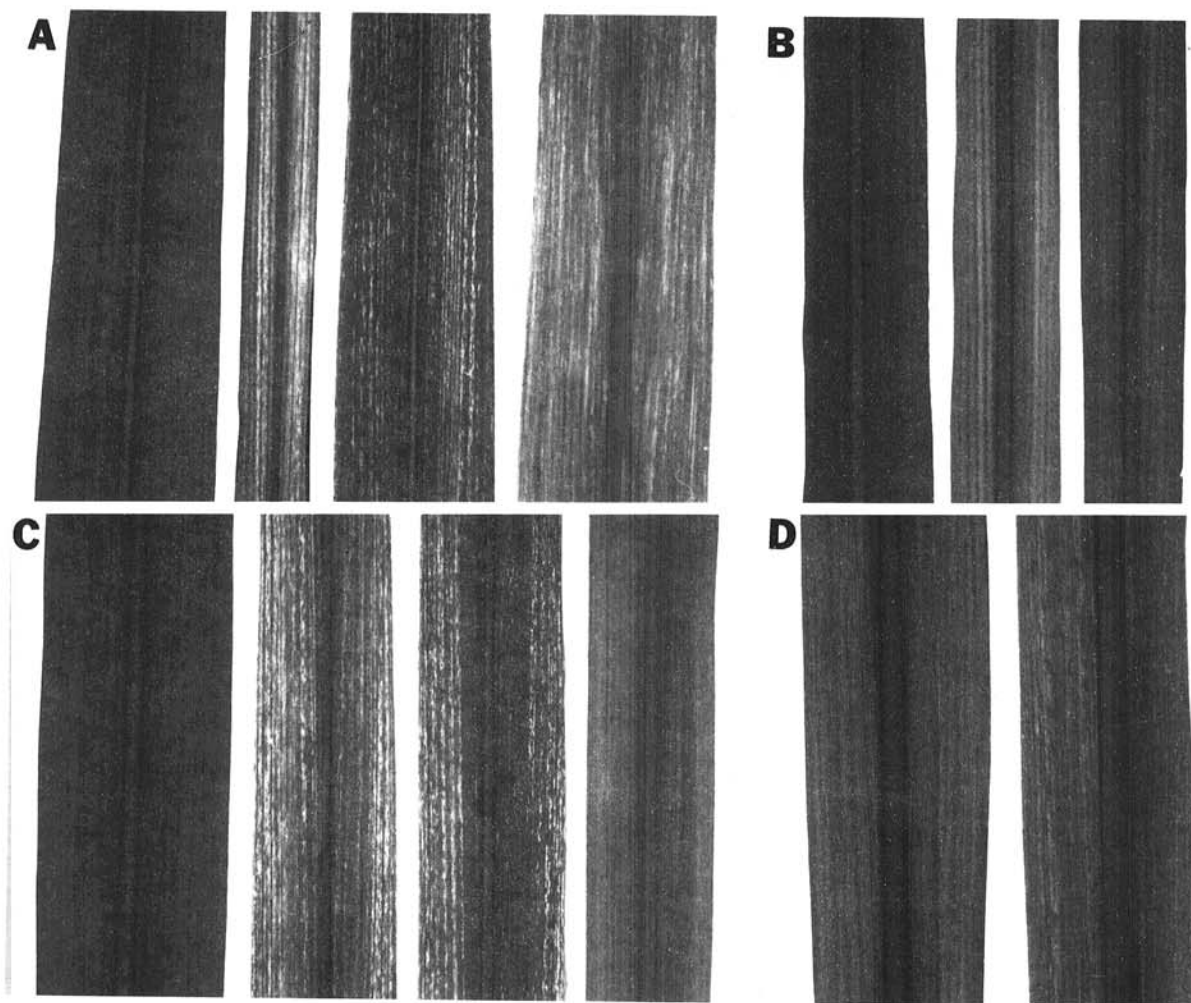
Triton X-100 was added to the supernatant to a final concentration of 2% (v/v), and the mixture was stirred for 5–10 min, then layered over 6 ml of 20% (w/v) sucrose in 0.01 M Tris-HCl, pH 7.4, and centrifuged for 1 h at 35,000 rpm (93,000 g max) in a Beckman 50.2Ti rotor (Beckman Instruments, Fullerton, CA). The resulting pellet was resuspended overnight in 0.01 M Tris-HCl, pH 7.4. The suspension was shaken briefly with 0.5 vol of chloroform and centrifuged in a Sorvall SS-34 rotor at 8,000 g for 10 min. The aqueous phase was re-extracted with chloroform as before and then layered on preformed sucrose-C<sub>2</sub>SO<sub>4</sub> gradients (11), which were centrifuged for 5 h at 140,000 g (max) in a Beckman SW 28.1 rotor. The gradients were fractionated into 0.25-ml aliquots, which were examined by EM. Gradients were fractionated from the top by using a gradient fractionator and Fluorinert FC-40 (ISCO Inc., Lincoln, NE) as chase solution. Fractions containing SCMMV were pooled, dialyzed against several changes of 0.01 M Tris-HCl, pH 7.4, and concentrated by ultracentrifugation in a Beckman 50.2Ti rotor at 193,000 g for 1 h; the pellets were resuspended in the same buffer to give what is subsequently referred to as a partially purified virus preparation.

For EM, partially purified virus preparations were stained with 2% neutral sodium phosphotungstate (PTA) or 4% aqueous uranyl acetate (UA), both containing 250 µg/ml of bacitracin (7). Particle length measurements of SCMMV were made from partially purified preparations obtained after a single cycle of ultracentrifuga-

tion. The preparations were negatively stained with PTA, and magnification calibration was based on catalase crystal lattice spacing (16). Pineapple wilt associated closterovirus was purified from infected pineapple leaf tissue as described (7).

**Serology.** An antiserum against SCMMV was prepared by intramuscular injection of a rabbit with partially purified virus emulsified with Freund's complete adjuvant. The quantity of viral antigen used for immunization in each case consisted of the partially purified preparation obtained from 300 g of fresh leaf tissue suspended in a final volume of 0.5 ml. Antigen concentration was not estimated spectrophotometrically. Four such immunizations were done at 2-wk intervals, and the animal was bled out 20 days after the final injection.

Immunodiffusion tests were done with 0.8% agarose, 0.5% sodium dodecyl sulfate (SDS), and 0.5% NaN<sub>3</sub> in distilled water. Partially purified virus, degraded with SDS (0.5% final concentration), was used as antigen. Double-antibody sandwich EIA was done by standard procedure (3) with a coating IgG concentration of 1 µg/ml, crude sap sample dilution of 1/5, and alkaline phosphatase-IgG conjugate dilution of 1/1,000. Results were expressed as absorbance values at A<sub>405nm</sub>. In indirect EIA tests, crude sap samples were prepared in EIA carbonate coating buffer, pH 9.6, at 1/5 and 1/10 dilutions and applied directly to plates. After a 4-h incubation, plates were rinsed, and whole serum, diluted 1/1,000 in EIA conjugate buffer, was added for 4 h. Cap-



**Fig. 1.** Association between foliar symptoms and infection by sugarcane bacilliform virus (SCBV) and sugarcane mild mosaic virus (SCMMV) in various clones of sugarcane (*Saccharum officinarum*). **A**, From left to right: virus-free CL 61-620; Selemi Bali and M 27-16, both doubly infected by SCBV and SCMMV and showing chlorotic striate mosaic symptoms; Raratonga 1 infected with SCBV plus sugarcane mosaic virus (SCMV). **B**, Left, healthy IK 76-319; center and right, B 41-227 and IJ 76-69, respectively, both doubly infected with SCBV plus SCMMV but showing no foliar symptoms. **C**, From left to right: virus-free CL 61-620; NG 77-042 and Jamaica Red, both infected by SCBV only and showing chlorotic striate mosaic symptoms; Akoki 22, infected with SCBV only and without foliar symptoms. **D**, Left, CL 61-620 infected with SCBV from NG 77-042 and showing no foliar symptoms; right, CL 61-620 infected with SCBV from Selemi Bali and showing slight chlorotic mosaic symptoms at 8 mo after inoculation. Symptoms became more pronounced at 16–18 mo after inoculation.

tured antibodies were detected by using goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad Laboratories, Richmond, CA) at a 1/3,000 dilution. All ISEM tests (12) were done with whole serum diluted 1/1,000 in 0.01 M Tris-HCl, pH 7.4. Carbon-coated Formvar grids were floated on diluted antiserum for 15 min, rinsed with 20 drops of 0.01 M Tris-HCl, pH 7.4, and then floated on a drop of partially purified virus suspension for 1 h. Grids were then rinsed with 15 drops of 0.01 M Tris-HCl, pH 7.4, followed by 15 drops of distilled water, and then 15 drops of either PTA or UA. For decoration, grids were coated with antiserum and floated on sample suspensions as described above. They were then rinsed with 20 drops of 0.01 M Tris-HCl, pH 7.4, and incubated for 10 min on a drop of antiserum diluted 1/500 in 0.01 M Tris-HCl, pH 7.4. Grids were then rinsed and stained as described above.

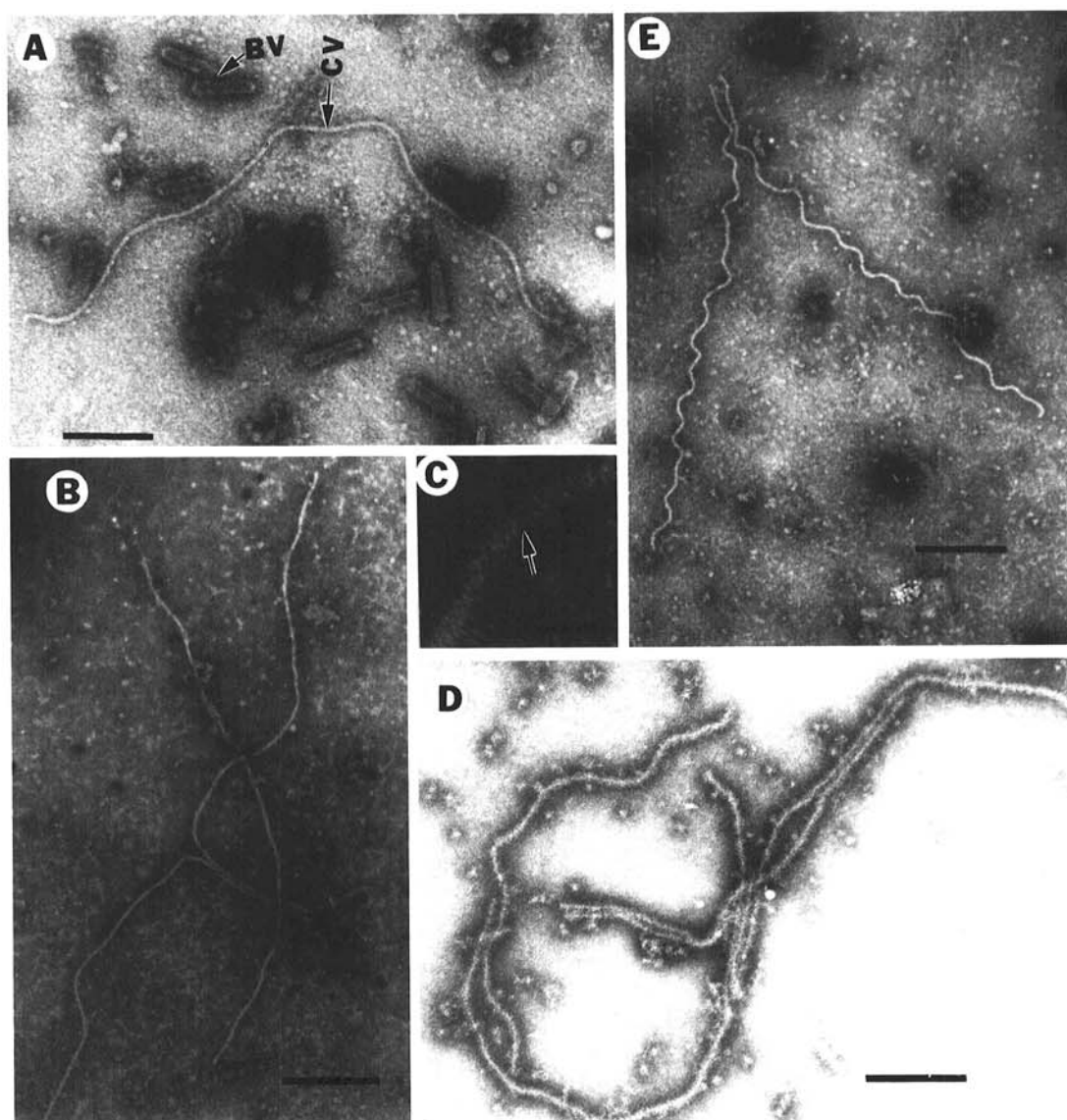
## RESULTS

**Natural occurrence of SCMMV and association with foliar symptoms in sugarcane.** SCMMV was detected by EM and ISEM

in the cultivars Selemi Bali, Seleri, NG 51-39, NG 77-109, and IK 76-94 from the USDA sugarcane germ plasm collections in Florida, in the cultivars Iscambine, Iscambine Striped, and M 27-16 from Mauritius, and in the commercial cultivars B 41-227, N 14, NCo 376, and Waya from Malawi. Sugarcane clones infected by SCMMV were also, in all cases, infected by SCBV. Several of the sugarcane clones infected by SCMMV and SCBV showed symptoms of pronounced chlorotic striate mosaic. These clones included Selemi Bali (Fig. 1A), Iscambine, and NG 51-39.

In other clones, including Seleri, B 41-227 (Fig. 1B), N 14, NCo 376, and Waya, no foliar striate mosaic symptoms were observed. All sugarcane cultivars infected by the combination of SCBV and SCMMV showed evidence of retarded growth, narrowing of leaves, and occasional dieback of ratoon shoots. However, because virus-free plants of these same cultivars were not available for direct comparison, these observations should be interpreted with caution; they were made relative to other sugarcane clones that were either virus-free or infected by SCBV only.

**Mechanical transmission.** Neither SCMMV nor SCBV was



**Fig. 2.** Particle morphology and immunosorbent electron microscopy of sugarcane mild mosaic virus (SCMMV). **A**, Particles of SCMMV (CV) and sugarcane bacilliform virus (SCBV) (BV) in an unfractionated partially purified preparation from sugarcane clone Selemi Bali. **B**, Particles of SCMMV from a  $\text{Cs}_2\text{SO}_4$  gradient fraction. **C**, Portion of a SCMMV particle photographed at high magnification to show characteristic closteroviruslike capsid appearance. **D**, Particles of SCMMV trapped and decorated by homologous antiserum from a partially purified preparation from sugarcane clone IJ 76-319. Particles were trapped for 1 h and decorated for 10 min. Antiserum dilution was 1/1,000 for trapping and 1/500 for decoration. **E**, Particles of SCMMV trapped from a partially purified preparation from rice cv. Taichung Native 1 infected by mealybug transmission from sugarcane clone IJ 76-319. Particles in A and B and E were stained with uranyl acetate, and in C and D with sodium phosphotungstate as described in text. Scale bar represents 200 nm in A, B, D, and E, and 25 nm in C.



transmitted mechanically in crude sap from Selemi Bali to sweet corn (*Zea mays* L. 'Early Sunglow'), barley (*Hordeum vulgare* L. 'Larker'), wheat (*Triticum aestivum* 'Era' and 'Mindum'), oats (*Avena sativa* L. 'Clintland 66'), Johnson grass (*Sorghum halepense* (L.) Pers.), sorghum (*S. bicolor* (L.) Moench) or *Nicotiana benthamiana* Domin. No symptoms developed on either inoculated leaves or new growth, and no virus was detected by EM or ISEM examination of partially purified leaf extracts. SCBV, but not SCMMV, was transmitted in crude extracts from Selemi Bali to the hybrid sugarcane clone CL 61-620. However, if partially purified extracts from IJ 76-319 and CL 61-620 were used, SCMMV was transmitted by mechanical inoculation to healthy CL 61-620 (two of two test plants), rice (four of six test plants), *S. bicolor* (seven of eight test plants), and *S. halepense* (14 of 16 test plants). A very mild vein-banding mosaic was observed in infected CL 61-620. Chlorotic flecking occasionally developed in *S. halepense*. Infected *S. bicolor* was slightly stunted and chlorotic relative to healthy controls. No symptoms were observed in rice. The presence of SCMMV in these hosts was verified by IEM. Other indicator plants, including corn, wheat, oats, barley, and *N. benthamiana*, were not infected by SCMMV in these inoculation tests with partially purified virus suspensions.

**Insect transmission.** Neither SCMMV nor SCBV was transmitted by *M. sacchari* from a mixed infection in Selemi Bali to any of four healthy test plants of CL 61-620. Both viruses were transmitted by the pink sugarcane mealybug, *S. sacchari*, from Selemi Bali to each of four test plants of CL 61-620 and IJ 76-69. Only SCMMV was transmitted to three of four test plants of IJ 76-319, a *S. officinarum* clone that was found to be free of SCBV in previous surveys (4). This sugarcane clone does not support replication of the SCBV isolate from Selemi Bali. Symptoms of leaf narrowing and stunting occurred in the mixed SCBV-SCMMV infection in CL 61-620 and IK 76-69, and chlorotic striate mosaic developed 8–10 mo later in these same plants of CL 61-620. No symptoms were observed in plants of IJ 76-319 infected by SCMMV only. SCMMV was detected in experimentally infected plants 4–6 wk after inoculation by viruliferous mealybugs. No symptoms developed on and no viruslike particles were detected in test plants of CL 61-620, on which nonviruliferous mealybugs were allowed to feed and multiply for 6 mo. Mealybugs, fed on IJ 76-319 infected with SCMMV alone, transmitted the virus to healthy CL 61-620 (four of four test plants) and rice (eight of eight test plants), demonstrating that transmission of SCMMV by *S. sacchari* is not dependent on the presence of SCBV. ISEM tests also demonstrated that infected rice contained particles of SCMMV (Fig. 2E) but not of SCBV.

**Purification and EM.** The purification method described above yielded preparations of SCMMV in which there was some breakage but little evidence of particle aggregation. Yields of virus were low as determined by EM examination. Stirring the initial

extracts overnight in the presence of higher concentrations of Triton X-100 did not improve virus yields, nor did the procedure described for purification of pineapple mealybug wilt associated virus (7). Virus yield and purity were not determined spectrophotometrically.

Particle length distribution of 53 PTA-stained particles of SCMMV is represented in Figure 3. Particles were trapped on antiserum-coated grids from the partially purified preparation obtained after the first cycle of ultracentrifugation. Particles ranged in length from 650 to 2,050 nm. Average particle length was 1,380 nm, but the length distribution profile indicates that the greatest number of particles (38%) were in the 1,500–1,600 nm range. Particles of SCMMV were 12-nm wide and displayed the undulating appearance and open helical structure (Fig. 2A–E) characteristic of plant closteroviruses (1,6).

**Serology.** The rabbit antiserum prepared against SCMMV had a specific homologous titre of only 1/2 in SDS-immunodiffusion tests and also reacted with healthy sugarcane leaf extracts. In EIA tests with IgG at 1 µg/ml for coating, leaf sample extracts at 1/5 dilution, and alkaline phosphatase-IgG conjugate at 1/1,000 dilution, SCMMV-infected sugarcane gave  $A_{405\text{nm}}$  readings of 0.30–0.40, whereas comparable healthy leaf extracts gave readings of 0.08–0.10. Readings for healthy and mealybug wilt infected pineapple tissue in the same experiments were 0.00–0.01. In ISEM tests, SCMMV antiserum trapped and decorated SCMMV particles (Fig. 2D) from extracts of all the SCMMV-infected sugarcane cultivars listed above, but did not trap particles of pineapple mealybug wilt associated closterovirus or unnamed closterolike viruses infecting *Piper* spp., *Rhamnus cathartica*, and *Polyscias* spp. (B. E. L. Lockhart et al, unpublished data). Particles of SCMMV were also neither trapped in ISEM tests nor detected in indirect EIA assays by whole antisera to citrus tristeza virus (CTV) (obtained from S. M. Garnsey, USDA, Orlando, FL), a Canadian isolate of grapevine virus A (GVA) (13), carnation necrotic fleck virus (CNFV) (obtained from A. A. Brunt, Glasshouse Crops Research Institute, Littlehampton, U.K.), or lilac chlorotic leafspot virus (LCLV) (obtained from A. A. Brunt). Additionally, SCMMV did not react with antiserum to sugarcane mosaic virus (SCMV) in either immunodiffusion, EIA, or ISEM tests. SCMMV also did not react in EIA tests with the monoclonal antibody that has detected a wide range of aphid-transmitted potyviruses (8) (supplied in kit form by Agdia Inc., Elkhart, IN) and gave positive results with an isolate of SCMV infecting the sugarcane clone Raratonga 1 (Fig. 1A).

## DISCUSSION

The results presented above show that SCMMV is mechanically transmissible, is transmitted by a mealybug vector in the absence of SCBV, and, in the sugarcane clones tested, causes very mild or no apparent foliar symptoms.

The majority of insect-transmitted clostero- or closterolike viruses have aphid vectors (1). Several other closterolike viruses, including lettuce infectious yellows virus (5) and Diodia vein chlorosis virus (9), are whitefly-transmitted. Grapevine virus A, 800 nm in length and a member of the proposed closterovirus subgroup A (1) or subgroup II (6), has been shown to be mealybug-transmitted (14), and the 1,200-nm closterolike virus associated with pineapple mealybug wilt (7,15) is also assumed to be mealybug-transmitted. In particle morphology, SCMMV resembles the definitive or tentative members of subgroup C (1) or subgroup I (6) of the closterovirus group. However, more information on the biochemical properties and histopathology of SCMMV is needed before this virus can be assigned to the closterovirus or any other plant virus group. The striate mosaic symptoms associated with SCMMV infection in some sugarcane clones appear to be due to accompanying infection by certain isolates of SCBV. Although it was reported previously that foliar symptoms in sugarcane could not be correlated with SCBV infection (11), striate mosaic symptoms occur in several clones, for example NG 77-042 and Jamaica Red (Fig. 1C), which are uninfected by SCMMV and in which SCBV virions are the only viruslike particles detect-

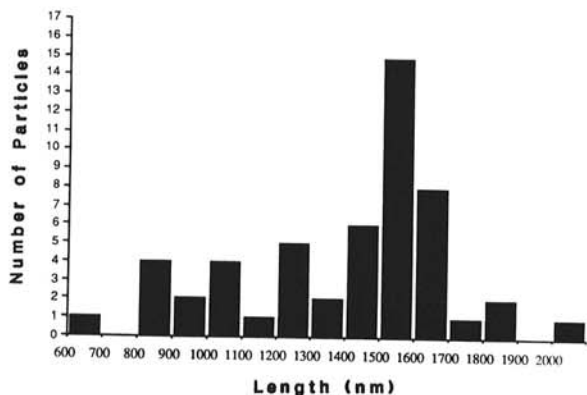


Fig. 3. Length distribution profile of 53 particles of SCMMV stained with 2% neutral sodium phosphotungstate. Particles were trapped on antiserum-coated grids from a partially purified preparation obtained after a single cycle of ultracentrifugation.

able. Mechanical transmission of purified SCBV from Selemi Bali to CL61-620, as described above, resulted in the development of mild striate mosaic symptoms in the latter variety (Fig. 1D). No SCMMV has been detected in these experimentally infected plants, suggesting that striate mosaic symptoms are associated with infection by SCBV not SCMMV. Maize streak virus (MSV) also causes striate mosaic symptoms in sugarcane (2), but gemini-viruslike particles were not isolated from any of the sugarcane clones mentioned above by using appropriate extraction procedures, and serological tests with MSV antiserum (provided by V. Damsteegt, USDA, Fort Detrick, MD) also gave negative results. It was recently demonstrated that SCBV is not, in fact, a single virus but is a mixed population encompassing a range of serological and genomic variabilities (B. E. L. Lockhart et al, *unpublished data*). On this basis, it may be suggested that, within the SCBV population, variants that produce striate mosaic symptoms in certain sugarcane genotypes exist.

Although the effect of single infection by SCMMV in sugarcane is not yet determined, the possibility of synergistic interaction between SCMMV and SCBV should be noted. Observations with mixed SCBV-SCMV infection in sugarcane indicate that the concentrations of both viruses increase markedly (B. E. L. Lockhart and J. C. Comstock, *unpublished data*), and similar indications of synergism have been noted for other mixed infections involving dsDNA bacilliform viruses (B. E. L. Lockhart, *unpublished data*).

The susceptibility of Johnson grass, sorghum, and rice to experimental infection by SCMMV implies that these plants are potential hosts of this virus, especially because these plants are grown in the same geographical zones. Whether SCMMV infection has any potential economic impact on sugarcane, rice, or sorghum has yet to be determined.

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