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South West Indian Ocean islands tomato begomovirus populations represent a new major monopartite begomovirus group

Hélène Delatte,[1] Darren P. Martin,[2] Florence Naze,[1] Rob Goldbach,[3] Bernard Reynaud,[1] Michel Peterschmitt[4] and Jean-Michel Lett[1]

[1]CIRAD, UMR C53 PVBMT, CIRAD-Université de la Réunion, Pôle de Protection des Plantes, Ligne Paradis, 97410 Saint Pierre, Réunion, France

[2]Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa

[3]Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

[4]CIRAD, UMR BGPI, CIRAD-INRA, TA 41/K, 34398 Montpellier Cedex 5, France

Correspondence
Jean-Michel Lett
lett@cirad.fr

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Biological and molecular properties of Tomato leaf curl Madagascar virus isolates from Morondova and Toliary (ToLCMGV-[Tol], -[Mor]), Tomato leaf curl Mayotte virus isolates from Dembeni and Kahani (ToLCYTV-[Dem], -[Kah]) and a *Tomato yellow leaf curl virus* isolate from Réunion (TYLCV-Mld[RE]) were determined. Full-length DNA components of the five isolates from Madagascar, Mayotte and Réunion were cloned and sequenced and, with the exception of ToLCMGV-[Tol], were shown to be both infectious in tomato and transmissible by *Bemisia tabaci*. Sequence analysis revealed that these viruses had genome organizations of monopartite begomoviruses and that both ToLCMGV and ToLCYTV belong to the African begomoviruses but represent a distinct monophyletic group that we have tentatively named the South West islands of the Indian Ocean (SWIO). All of the SWIO isolates examined were apparently complex recombinants. None of the sequences within the recombinant regions closely resembled that of any known non-SWIO begomovirus, suggesting an isolation of these virus populations.

INTRODUCTION

The genus *Begomovirus* contains dicotyledonous infecting whitefly transmitted viruses in the family *Geminiviridae*. Most described begomoviruses have bipartite genomes encapsidated as circular single-stranded DNA (ssDNA) molecules within twin icosahedral (or geminate) particles. Whereas bipartite begomoviruses usually require both a DNA A and DNA B component to produce symptomatic infections, monopartite begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV), require only a DNA A-like component for infectivity (Navot *et al.*, 1991).

TYLCV is an important tomato pathogen that, following its emergence from the Mediterranean Basin in recent years (Moriones & Navas-Castillo, 2000), is progressively spreading throughout the world (Cohen & Antignus, 1994; Czosnek & Laterrot, 1997; Moriones & Navas-Castillo, 2000; Pico *et al.*, 1996; Polston *et al.*, 1999). In 1997, a severe outbreak of tomato yellow leaf curl disease occurred in Réunion, one of the South West islands of the Indian Ocean (SWIO). Yield losses reached 85 % the first year of the epidemic on the most susceptible cultivars (Reynaud *et al.*, 2003) and the disease has become the primary factor limiting both open field and protected greenhouse tomato production on the island. No begomoviruses had been detected in Réunion tomatoes prior to 1997 and it has now been determined that two strains of an exotic virus, the 'Israel' and the 'Mild' strains of TYLCV, are the causal agents of the disease (Peterschmitt *et al.*, 1999; Delatte *et al.*, 2005a). There is precedent for human spread of TYLCV into new habitats, i.e. the Carribean and Florida (Polston *et al.*, 1999), and the finding that whiteflies can acquire the virus from fruits demonstrates yet another route of potential dissemination (Delatte *et al.*, 2003). The influx of exotic viruses into SWIO is also not restricted to tomato begomoviruses. Other begomovirus of cassava such as the *African cassava mosaic virus* (ACMV) (Fauquet & Fargette, 1990), *East African cassava mosaic virus* (EACMV) (Swanson & Harrison, 1994) and *South African cassava mosaic virus* (SACMV) (Berrie *et al.*, 2001) have also been detected in Madagascar (Ranomenjanahary *et al.*, 2002).

In 2001, a tomato virus symptom survey on the islands of Madagascar and Mayotte identified both the association

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AJ865337–AJ865341.

of the begomovirus vector species, *Bemisia tabaci*, with tomato plants and the presence of plants displaying leaf curling and plant stunting symptoms characteristic of begomoviruses. Analysis of partial viral genome fragments isolated from leaf samples collected during this survey indicated the presence of two potentially new *Begomovirus* species (Delatte *et al.*, 2002; Lett *et al.*, 2004).

In this study, we report the construction of agro-infectious viral clones, symptom evaluation, whitefly transmission tests and analysis of the full-length DNA sequences of TYLCV-Mld[RE] and two isolates from two new monopartite begomovirus species. The new species, tentatively named Tomato leaf curl Madagascar virus (ToLCMGV) and Tomato leaf curl Mayotte virus (ToLCYTV), belong to the African begomoviruses but represent a distinctly unique monophyletic group that we refer to as the SWIO group. We report that the SWIO isolates appear to have been actively recombining amongst themselves.

METHODS

Plant material. Agro-inoculation and transmission experiments were carried out on the TYLCV susceptible tomato (*Lycopersicon esculentum*) genotype, Farmer (Known You Seed), in a growth chamber maintained at 25 °C with a 12/12 h photoperiod.

Sampling and DNA extraction. Tomato leaves presenting leaf-curling symptoms were collected from individual plants in Saint Pierre (Réunion), Morondova and Toliary (Madagascar), and Dembeni and Kahani (Mayotte). The leaves were preserved by dehydration with $CaCl_2$ (Bos, 1977). Total DNA was extracted from dried samples using the DNeasy Plant miniprep kit (Qiagen) according to the manufacturer's instructions.

PCR detection. PCR was used to amplify two fragments from the extracted DNA of all samples using two degenerate primer sets: MP16–MP82 (Umaharan *et al.*, 1998) and AV494–AC1048 (Wyatt & Brown, 1996). A less degenerate primer set was designed from previously obtained SWIO begomovirus sequences and used to amplify 904 nt of the core region of the coat protein (CP) gene (VD360–CD1266; Table 1). PCR reactions were carried out in 25 μl volumes with the following programme: a cycle of 5 min at 94 °C, then 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final cycle at 72 °C for 5 min. The presence/absence of a DNA B genome component was also determined for each of the isolates using the PCR primers: PBL1v2040 and PCRc1 (Rojas *et al.*, 1993; Table 1). The presence/absence of DNA β molecules was determined for each of the isolates using the primers Beta 1 and Beta 2 (Briddon *et al.*, 2002; Table 1).

Cloning strategies. Abutting primers with non-homologous tails were designed for each isolate in order to obtain a full-length DNA product (Patel *et al.*, 1993). Abutting primers were designed over a *Bam*HI restriction site for samples from Réunion (ReunionV160–ReunionC164; Table 1), and Madagascar (MadagascarV148Q–MadagascarC153Q; Table 1). For Mayotte samples, abutting primers were designed over the *Hin*dIII restriction site (Dembeni:

Table 1. PCR primers used in this study

Primer names	Primer sequences	Expected size (nt)
DNA-A (detection)		
VD360	5′-AGRCTGAACTTCGACAGC-3′	906
CD1266	5′-TCTCAACTTCARGGTCTG-3′	
V196	5′-CGGATTTCGTTGTATGTTAGC-3′	804
C1000	5′-AAGGGGTTTTCAGTATGGTT-3′	
AV494	5′-GCCYATRTAYAGRAAGCCMAG-3′	552
AC1048 (Wyatt & Brown, 1996)	5′-GGRTTDGARGCATGHGTACAT-3′	
MP16	5′-CCTCTAGATAATATTACCKRWKGRCC-3′	480
MP82 (Umaharan *et al.*, 1998)	5′-CGGAATTCYTGNACYTTRCANGGNCCYTCRCA-3′	
DNA-A (cloning)*		
ReunionV160	5′-GCACAGGATCCACTTCTAAATGAATTTCC-3′	Full sequence (2807)
ReunionC164	5′-TCGTAGGATCCCACATATTGCAAGACAAAC-3′	
MadagascarV148Q	5′-GGTGTGGATCCATTGTTAAATGAGTTCCC-3′	Full sequence (2793)
MadagascarC153Q	5′-GTGCGGGATCCCACATTGTGACAGGCCC-3′	
DembeniVQ	5′-TAGTGAAGCTTAGATAATCGTTTTTGTC-3	Full sequence (2791)
DembeniCQ	5′-CGCAGAAGCTTTGACGCGCGATTCTTATTG-3′	
KahaniVQ	5′-AAGAGAAGCTTAGATAATTGTTTTTGTC-3′	Full sequence (2781)
KahaniCQ	5′-CGCAGAAGCTTTGACGCGCGATTCTGATTG-3′	
DNA-B (detection)		
PBL1v2040	5′-GCCTCTGCAGCARTGRTCKATCTTCATACA-3′	600
PCRc1 (Rojas *et al.*, 1993)	5′-CTAGCTGCAGCATATTTACRARWATGCCA-3′	
DNA-β (detection)		
Beta 01	5′-GGTACCACTACGCTACGCAGCAGCC-3′	600–700 and 1350
Beta 02 (Briddon *et al.*, 2002)	5′-GGTACCTACCCTCCCAGGGGTACAC-3′	

*Cloning sites are underlined.

DembeniVQ–DembeniCQ; Kahani: KahaniVQ–KahaniCQ; Table 1). The PCR conditions used were: 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final elongation of 10 min at 72 °C. Amplicons of approximately full genome size ($\sim$2800 bp) were isolated using the Geneclean Turbo kit (Qbiogene) from 1 % agarose gels and cloned using the pGEM-T Easy vector system (Promega). The complete DNA components of five clones corresponding to the isolates from Dembeni, Kahani, Toliary, Morondova and Réunion were sequenced by gene walking using Sequentia (Clermont Ferrand).

Agro-inoculation. While the infectivity of the cloned DNA components of isolates from Réunion and Madagascar were tested using full head-to-tail DNA dimers (constructed at *Bam*HI restriction sites), partial DNA head-to-tail dimers were constructed (at *Hin*dIII restriction sites) for the viruses from Mayotte. Both full and partial dimers were inserted into the binary vector, pCAMBIA 2300 (Cambia). Recombinant plasmids were mobilized from *Escherichia coli* JM-109 cells (Promega) into *Agrobacterium tumefaciens* (strain C58) by triparental mating using *E. coli* HMB101 containing the plasmid helper pRK 2013 (Ditta *et al.*, 1980). The identity of all clones was verified by restriction endonuclease analysis. Ten day old susceptible tomato seedlings were agro-inoculated with the five constructs using a needle (Paximadis & Rey, 2001), and symptoms of infection were evaluated between 15 and 20 days post-inoculation. All the agro-inoculated plants showing symptoms were tested for the presence of viral DNA using either specific degenerate primers designed to amplify the isolates from the two new species (V360–CD1266; Table 1) or a specific non-degenerate primer designed to amplify TYLCV DNA (V196–C1000; Table 1).

Transmission tests. *B. tabaci* transmissibility of the viruses was evaluated by determining whether whiteflies could successfully transmit the viruses from symptomatic PCR-positive agro-inoculated plants to healthy tomato plants. *B. tabaci* adults used for the transmission tests were from a cabbage reared B biotype population initiated from whiteflies initially collected from cabbage in Réunion (Delatte *et al.*, 2005b). In each transmission test, 15 whiteflies were permitted a 3 day acquisition access period on PCR-positive symptomatic agro-inoculated tomato plants. These insects were then transferred onto healthy tomato plants and allowed an inoculation access period (IAP) of 3 days. Twenty-one days following the IAP, symptoms were evaluated and symptomatic plants tested for the presence of DNA by PCR, using the specific primers described above (V360–CD1266; V196–C1000; Table 1).

Sequence analysis. The full DNA sequences of the five isolates were arranged so that the first nucleotide in the sequence corresponds to the first base (A) of virion strand replication (Laufs *et al.*, 1995). Potential open reading frames in each of the isolate sequences were identified using DNAMAN (version 5.2.2, Lynnon Biosoft). Full DNA A-like and A sequences of related viruses used in phylogenetic analyses were obtained from public sequence databases using BLASTN. Two outgroup sequences were used during phylogenetic analyses, an isolate from Australia of the monopartite species *Tomato leaf curl virus* isolate (GenBank accession no. S53251; Stonor *et al.*, 2003) and an isolate from Florida of the bipartite species *Tomato mottle virus* isolate (NC_001938; Polston *et al.*, 1993). Multiple sequence alignments were performed using the optimal alignment method of DNAMAN. Phylogenetic trees were generated using the neighbour-joining method of PHYLIP (Felsenstein, 1989) or the Jukes–Cantor corrected distances, 2000 bootstrap replicates were performed.

Detection of potential recombinant sequences, identification of likely parental sequences and localization of possible recombination breakpoints in multiple sequence alignments were carried out using the RDP (Martin & Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), MAXIMUM χ^2 (Smith, 1992), CHIMAERA (Martin *et al.*, 2005a), RECSCAN (Martin *et al.*, 2005a) and SISTER SCAN (Gibbs *et al.*, 2000) methods as implemented in RDP2 (Martin *et al.*, 2005b). The analysis was performed with default settings for the different detection methods and a Bonferroni corrected *P*-value cut-off of 0·05.

RESULTS

Cloning and infectivity of isolates

While PCR amplification and cloning of apparently full-length DNA A-like components was possible from all symptomatic leaf samples, DNA B- and DNA β-specific PCR yielded no amplification products for any of the examined leaf samples. TYLCV susceptible tomato plants developed symptoms typical of those observed in the field, when agro-infected with cloned DNA of all isolates except for ToLCMGV-[Tol], which was non-infectious (Fig. 1).

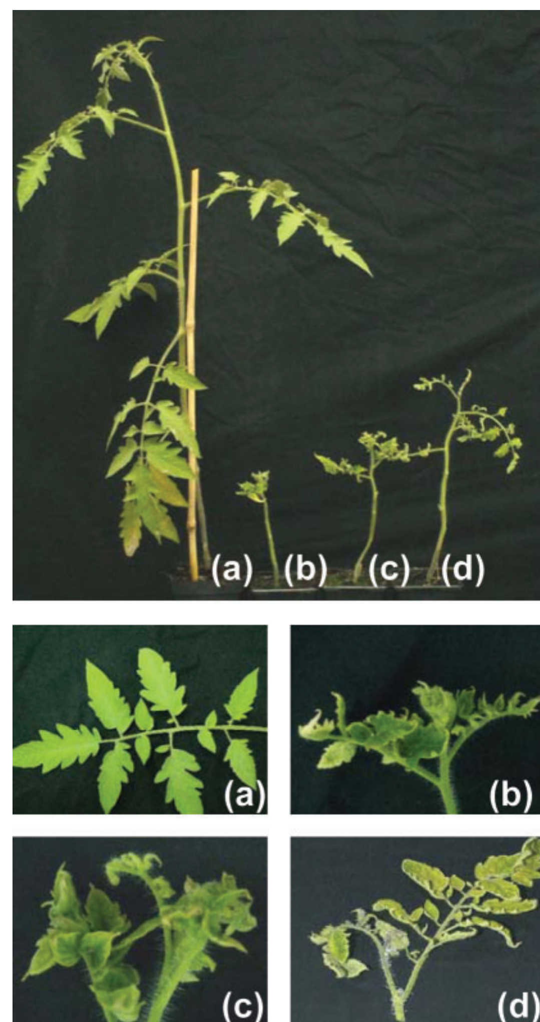

Fig. 1. Symptoms 20 days after agro-inoculation of the TYLCV susceptible tomato genotype, Farmer, with the various begomoviruses characterized in this study. (a) Corresponds to a non-inoculated control, (b) ToLCYTV-[Kah], (c) ToLCMGV-[Mor] and (d) TYLCV-Mld[RE].

Table 2. GenBank/EMBL/DDBJ accession numbers of complete begomovirus DNA A-like and A sequences used in this study

Begomovirus name	Acronym	Accession no.
Tomato leaf curl Mayotte virus-[Dembeni]*	ToLCYTV-[Dem]	AJ865341
Tomato leaf curl Mayotte virus-[Kahani]*	ToLCYTV-[Kah]	AJ865340
Tomato leaf curl Madagascar virus-[Morondova]*	ToLCMGV-[Mor]	AJ865338
Tomato leaf curl Madagascar virus-[Toliary]*	ToLCMGV-[Tol]	AJ865339
Tomato yellow leaf curl virus-Mild [Réunion]*	TYLCV-Mld[RE]	AJ865337
South African cassava mosaic virus	SACMV	AF155806
South African cassava mosaic virus-[M12]	SACMV-[M12]	AJ422132
Tomato yellow leaf curl Sardinia virus	TYLCSV	X61153
Tomato yellow leaf curl virus-Mild	TYLCV-Mld	X76319
Tomato yellow leaf curl virus	TYLCV	X15656
Tomato leaf curl virus	ToLCV	S53251
African cassava mosaic virus-[Kenya]	ACMV-[KE]	J02057
African cassava mosaic virus-Uganda Severe	ACMV-[UGSvr]	AF126802
East African cassava mosaic virus-[Tanzania]	EACMV-[TZ]	Z83256
Tobacco leaf curl Zimbabwe virus	TbLCZV	AF350330
Tomato yellow leaf curl Malaga virus	TYLCMalV	AF271234
Tomato leaf curl Sudan virus-[Gezira]	ToLCSDV-[Gez]	AY044137
Tomato mottle virus-[Florida]	ToMoV-[FL]	NC_001938
African cassava mosaic virus-[Cameroon-DO2]	ACMV-[CM/DO2]	AF366902
African cassava mosaic virus-[Ivory coast]	ACMV-[CI]	AF259894
African cassava mosaic virus-[Nigeria]	ACMV-[NG]	X17095
African cassava mosaic virus-[Nigeria-Ogo]	ACMV-[NG-Ogo]	AJ427910
African cassava mosaic virus-Uganda Mild	ACMV-UGMld	AF126800
African cassava mosaic virus-Uganda Severe	ACMV-UGSvr	AF126803
East African cassava mosaic virus-Uganda2 Mild	EACMV-UG2Mld	AF126804
East African cassava mosaic virus-Uganda2 Severe	EACMV-UG2Svr	AF126806
East African cassava mosaic Zanzibar virus	EACMZV	AF422174
East African cassava mosaic virus-[Kenya]	EACMV-[KE]	AJ516003
East African cassava mosaic virus-[Malawi]	EACMV-[MW]	AJ006461
East African cassava Malawi mosaic virus-[K]	EACMMV-[K]	AJ006460
Ageratum yellow vein virus	AYVV	X74516
Bhendi yellow vein mosaic virus-[301]	BYVMV-[301]	AJ002453
Bhendi yellow vein mosaic virus-[Madurai]	BYVMV-[Mad]	AF241479
Cotton leaf curl Gezira virus-[Sida]	CLCuGV-[Sida]	AY036007
Eupatorium yellow vein virus-[MNS2]	EpYVV-[MNS2]	AJ438936
Indian cassava mosaic virus	ICMV	Z24758
Okra yellow vein mosaic virus-[201]	OYVMV-[201]	AJ002451
Soybean crinkle leaf virus-[Japan]	SCLV-[JA]	AB050781
Tobacco curly shoot virus-[Y41]	TbCSV-[Y41]	AJ457986
Tomato leaf curl Gujarat virus-[Vadodara]	ToLCGV-[Vad]	AF413671
Tomato leaf curl Malaysia virus	ToLCMV	AF327436
Tomato leaf curl Laos virus	ToLCLV	AF195782
Tomato yellow leaf curl China virus	TYLCCNV	AF311734
Tomato yellow leaf curl virus-[Gezira]	TYLCV-[Gez]	AY044138
Tomato leaf curl Sudan virus-[Shambat]	ToLCSDV-[Sha]	AY044139
Tomato yellow leaf curl Sardinia virus-[Sicily]	TYLCSV-[Sic]	Z28390
Tomato yellow leaf curl Sardinia virus-[Spain1]	TYLCSV-[ES1]	Z25751
Tomato yellow leaf curl Sardinia virus-[Spain2]	TYLCSV-[ES2]	L27708
Tomato yellow leaf curl Thailand virus-[Y72]	TYLCTHV-[Y72]	AJ495812
Tomato yellow leaf curl virus-[Aichi]	TYLCV-[Aic]	AB014347
Tomato yellow leaf curl virus-[Almeria]	TYLCV-[Alm]	AJ489258
Tomato yellow leaf curl virus-[Cuba]	TYLCV-[CU]	AJ223505
Tomato yellow leaf curl virus-[Dominican Republic]	TYLCV-[DO]	AF024715

Table 2. cont.

Begomovirus name	Acronym	Accession no.
Tomato yellow leaf curl virus-[Iran]	TYLCV-[IR]	AJ132711
Tomato yellow leaf curl virus-[Portugal]	TYLCV-[PT]	AF105975
Tomato yellow leaf curl virus-[Puerto Rico]	TYLCV-[PR]	AY134494
Tomato yellow leaf curl virus-[Shizuokua]	TYLCV-[Shi]	AB014346
Tomato yellow leaf curl virus-[Spain7297]	TYLCV-[ES7297]	AF071228
Tomato yellow leaf curl Thailand virus-[1]	TYLCTHV-[1]	X63015
Watermelon chlorotic stunt virus	WmCSV	AJ012081
Watermelon chlorotic stunt virus-[IR]	WmCSV-[IR]	AJ245652

*Begomoviruses are named according to the ICTV guidelines (Fauquet *et al.*, 2003).

In all cases the presence of viral DNA could be confirmed in all symptomatic plants by PCR. Relative to healthy controls, symptomatic plants agro-inoculated with ToLCYTV-[Dem] and -[Kah], and ToLCMGV-[Mor] had a stunted bushy appearance with severely shortened rachis, curled petioles and curled leaves (Fig. 1). Plants agro-inoculated with TYLCV-Mld[RE] were stunted with yellow, curled leaves. All of the isolates that produced symptoms in agro-inoculated tomato could also be transmitted by whiteflies into TYLCV susceptible healthy tomato plants. Again, symptoms in the whitefly-inoculated plants resembled those observed in the field for the different isolates and could be confirmed by PCR detection of the viral genome. The ability of cloned DNA components to cause symptomatic infections of tomato resembling those observed in the field, coupled with our inability to confirm the presence of either DNA B or DNA β in field samples, indicated that TYLCV-Mld[RE], ToLCYTV-[Dem], ToLCYTV-[Kah] and ToLCMGV-[Mor] most likely have monopartite genomes.

Genome organization and molecular comparison with other begomoviruses

The lengths of the complete TYLCV-Mld[RE], ToLCMGV-[Mor], ToLCMGV-[Tol], ToLCYTV-[Dem], and ToLCYTV-[Kah] DNA sequences are 2791, 2777, 2775, 2765 and 2768 nt, respectively. The organization of inferred genes and intergenic regions for all five viruses is typical of that observed in begomoviruses, which characteristically have two virion senses and four complementary senses open reading frames (ORFs).

We detected three anomalies in the nucleotide sequence of ToLCMGV-[Tol] that might explain lack of infectivity of its clone. The first, and potentially most serious, is a single nucleotide frame-shift mutation near the beginning of the V2 ORF. The other anomalies were two unusual termination codons in the C4 ORF. For purposes of comparing the putative ToLCMGV-[Tol] V2 and C4 amino acid sequences with those of other viruses (Tables 2 and 3), we 'corrected' the sequence by inserting a T nucleotide at position 282, and changing an A at position 2163 and an A at position 2376 to a G and a C, respectively.

BLAST searches with the entire sequences of ToLCMGV-[Mor], -[Tol] and ToLCYTV-[Dem], -[Kah] indicated that these isolates were most closely related to TYLCV, SACMV and EACMV. As expected, a BLAST search with the entire sequence of TYLCV-Mld[RE] indicated that it was very closely related to TYLCV-Mld. Accordingly, seven African and five Mediterranean begomovirus sequences were chosen for detailed comparison with the five sequences described in this study (Table 3, Fig. 2).

ToLCMGV-[Mor] and ToLCMGV-[Tol], the two isolates from Madagascar, share 94 % genome sequence identity. ToLCYTV-[Kah] and ToLCYTV-[Dem], the two isolates from Mayotte, share 90 % identity which is close to the 89 % taxonomic threshold commonly used for begomovirus species distinction (Fauquet & Stanley, 2003). TYLCV-Mld[RE] is clearly a member of the Mediterranean and African tomato begomoviruses with its genome sequence sharing 98 % identity with that of TYLCV-Mld (Fig. 3).

Phylogenetic analysis of the sequences determined in this study and all other publicly available full-length African and Mediterranean isolate sequences indicated that ToLCMGV-[Mor], -[Tol] and ToLCYTV-[Dem], -[Kah] formed a distinct monophyletic subgroup within the African group that we have named the SWIO (Fig. 3).

The highest nucleotide identity of DNA detected between isolates of ToLCMGV and ToLCYTV, was 86 % when comparing ToLCMGV-[Mor] and ToLCYTV-[Dem]. The greatest degree of genome-wide sequence identity shared by ToLCMGV and ToLCYTV isolates with other currently described full-length sequences was 82 % for ToLCMGV-[Mor] with TYLCV-Mld and 83 % for ToLCYTV-[Dem] with SACMV (Fig. 2). We therefore propose that, according to the ICTV criteria for begomovirus species demarcation using DNA complete sequence (Fauquet *et al.*, 2003; Fauquet & Stanley, 2003), both ToLCMGV and ToLCYTV

Table 3. Percentage nucleotide and deduced amino acid sequence identities shared between full DNA-A, ORFs (CP/V1, MP/V2, C1, C2, C3 and C4) and intergenic region (IR) sequences of begomoviruses

The pairwise comparison concerned Tomato leaf curl Mayotte virus isolate Dembeni (ToLCYTV-[Dem]) (a) or Tomato leaf curl Madagascar virus isolate Morondova (ToLCMGV-[Mor]) (b), with those of selected begomoviruses originating from Africa or, in the case of Tomato leaf curl virus (ToLCV) and Tomato mottle virus (ToMoV-[FL]), from Australia and the USA, respectively. Abbreviations and accession numbers of viruses are provided in Table 2.

Virus isolate	DNA-A	CP/V1		MP/V2		C1		C2		C3		C4		IR
	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt
(a) ToLCYTV-[Dem]														
ToLCYTV-[Kah]	90	97	99	95	97	86	87	95	90	93	90	88	75	77
ToLCMGV-[Mor]	86	86	91	88	89	87	90	85	79	87	81	90	79	66
ToLCMGV-[Tol]	85	86	90	88	89	87	90	85	78	86	80	91	82	77
SACMV	82	84	90	86	85	84	88	83	73	83	75	86	75	75
SACMV-[M12]	83	86	91	86	83	83	88	83	73	83	77	87	75	75
TYLCSV	80	81	90	82	84	82	85	77	67	76	64	85	69	72
TYLCV-Mld	81	86	92	84	84	83	84	83	73	82	75	86	69	57
TYLCV-Mld[RE]	81	86	92	84	85	83	84	83	71	83	77	85	69	57
TYLCV	79	86	90	84	85	79	79	82	71	82	75	74	46	54
TYLCMalV	80	81	88	82	83	83	85	86	71	83	78	85	68	59
ToLCSDV-[Gez]	78	74	76	85	85	84	86	83	73	82	72	87	72	61
ToLCV	76	76	78	74	64	80	83	74	65	75	67	84	67	68
ACMV-[KE]	76	78	83	78	74	77	79	80	69	79	70	80	56	57
TbLCZV	76	76	83	85	81	78	78	78	67	77	70	81	60	58
EACMV-[TZ]	76	81	89	71	65	73	77	82	72	82	77	66	37	74
ACMV-[UGSvr]	75	80	85	71	64	74	76	83	75	82	75	67	42	70
(b) ToLCMGV-[Mor]														
ToLCMGV-[Tol]	94	99	100	99	87	92	95	99	99	98	96	90	77	79
ToLCYTV-[Dem]	86	86	91	88	89	87	90	85	79	87	81	90	79	66
ToLCMYV-[Kah]	83	87	91	87	89	85	86	85	79	85	81	91	82	62
SACMV	81	84	89	87	89	82	87	85	77	84	81	83	65	63
SACMV-[M12]	81	84	88	86	85	81	86	86	77	85	82	83	66	66
TYLCSV	79	82	88	84	87	81	84	77	68	77	64	84	66	62
TYLCV-Mld	82	84	88	88	89	84	86	85	80	83	79	87	70	61
TYLCV-Mld[RE]	82	84	88	87	88	84	86	85	79	84	81	87	71	61
TYLCV	81	84	87	88	89	81	82	85	79	83	78	78	52	60
TYLCMalV	81	81	87	85	86	84	86	85	79	85	82	87	70	64
ToLCSDV-[Gez]	80	76	77	88	87	84	88	83	79	84	78	88	72	63
ToLCV	75	74	75	71	62	80	83	73	65	75	68	87	72	64
ACMV-[KE]	76	78	83	79	74	76	79	81	71	81	73	77	54	58
TbLCZV	77	75	81	89	89	78	80	79	67	80	73	82	60	64
EACMV-[TZ]	74	80	89	71	64	72	76	85	75	84	82	70	41	64
ACMV-[UGSvr]	76	81	87	71	62	73	76	86	76	84	82	70	42	65

be considered new species as their nucleotide identities with other begomovirus are below 89 %.

Analysis of recombination

We analysed a 62-sequence alignment of full-length SWIO (Table 2), African and Mediterranean begomovirus DNA sequences for evidence that the SWIO isolates had undergone recombination. We initially screened the alignment looking for all evidence of recombination involving the SWIO isolates either as potential recombinants (i.e. as acceptors of sequence) or as parental donors of sequence in non-SWIO recombinants. Six different detection methods identified an enormous amount of evidence for recombination involving the SWIO sequences as either donors or acceptors of sequences (at least 130 unique events identified by RDP2). We analysed each of the identified events individually and used a phylogenetic approach to verify the parental/donor identifications made by RDP2. This involved construction and comparison of bootstrapped neighbour-joining trees from the two portions of the alignment corresponding to regions of potential recombinants originating from different parental sequences. Wherever there was good phylogenetic evidence that an

	ToLCMGV-[Tol]	ToLCMGV-[Mor]	ToLCYTV-[Dem]	ToLCYTV-[Kah]	TYLCV-Mld[RE]	TYLCV-Mld	TYLCV	TYLCSV	TYLCMalV	ToLCSDV-[Gez]	ToLCV	EACMV-[TZ]	EACMZV	SACMV-[M12]	SACMV	ACMV-[KE]	ACMV-UGSvr	TbLCZWV	ToMoV-[FL]
ToLCMGV-[Tol]	**																		
ToLCMGV-[Mor]	94	**																	
ToLCYTV-[Dem]	**86**	**85**	**																
ToLCYTV-[Kah]	**82**	**83**	90	**															
TYLCV-Mld[RE]	**81**	**82**	**81**	**80**	**														
TYLCV-Mld	**81**	**82**	**81**	**80**	98	**													
TYLCV	**80**	**81**	79	78	92	93	**												
TYLCSV	**80**	79	**80**	76	78	78	77	**											
TYLCMalV	**80**	**81**	**80**	79	91	90	**85**	**83**	**										
ToLCSDV-[Gez]	79	**80**	78	77	86	86	84	76	**84**	**									
ToLCV	75	75	76	75	74	74	74	73	74	74	**								
EACMV-[TZ]	76	74	76	74	72	72	72	73	72	70	69	**							
EACMZV	78	78	78	77	76	75	75	74	76	74	71	**86**	**						
SACMV-[M12]	**83**	**81**	**83**	79	79	78	77	78	78	76	72	**80**	**82**	**					
SACMV	**83**	**81**	**82**	79	79	79	78	78	78	76	73	**80**	**82**	93	**				
ACMV-[KE]	75	76	76	75	74	74	72	71	73	73	70	70	73	74	74	**			
ACMV-UGSvr	76	76	76	75	74	74	73	71	73	73	70	70	73	74	74	97	**		
TbLCZWV	76	77	76	75	77	77	75	75	77	75	73	71	74	74	75	71	71	**	
ToMoV-[FL]	65	66	64	64	64	64	64	64	64	65	64	62	65	63	63	61	61	64	**

Fig. 2. Matrix of pairwise identity percentages of A-component sequences of 19 begomoviruses. The matrix was generated using the full optimal alignment method and the observed divergency distance method options of DNAMAN software. Abbreviations and accession numbers of viruses are provided in Table 2. Percentages of identity above 90 % are underlined, percentages of identity between 80 and 90 % are in bold.

inferred recombination event involved an SWIO isolate as a donor sequence (i.e. there was little or no evidence that the SWIO isolate was the recombinant), we marked the 'recombinant region' in the non-SWIO recombinant sequence for later removal. Having examined all events with associated *P*-values $<1{\cdot}0\times10^{-6}$ (i.e. the most obvious events), we removed all the identified evidence of non-SWIO isolate recombination from the alignment. This was carried out by treating the identified 'recombinant region' in the recombinant sequence as missing data in subsequent analyses. We scanned the four SWIO isolates in pairs (i.e. six pairs in total) against the rest of the sequences in the alignment. Following identification of the more obvious recombination events (events identified with multiple comparison corrected *P*-values $<1{\cdot}0\times10^{-5}$) that involved SWIO isolates as acceptors of sequence (determined

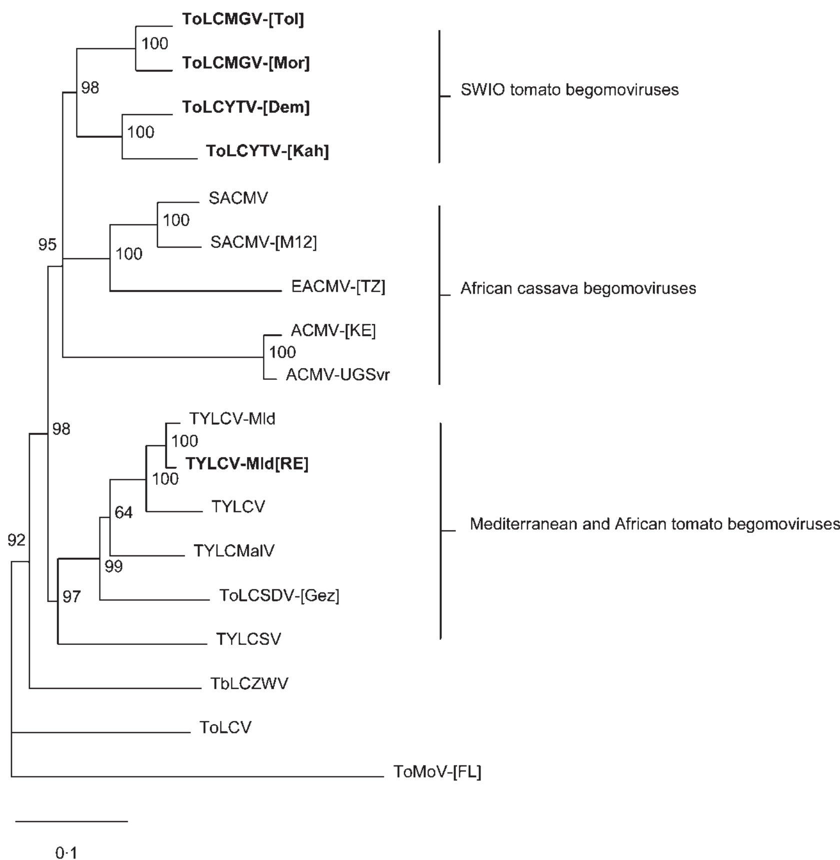

Fig. 3. Neighbour-joining tree indicating the phylogenetic relationships between the DNA sequences of ToLCMGV, ToLCYTV and TYLCV-Mld[RE] isolates and those of a representative sampling of publicly available African and Mediterranean begomovirus sequences. The tree was constructed using Jukes–Cantor corrected distances and rooted using ToMoV-[FL] as an outlier. Numbers associated with nodes indicate the percentage support for those nodes in 2000 bootstrap replicates. Whereas horizontal distances represent genetic distances as indicated by the scale bar, vertical distances are arbitrary. Abbreviations and accession numbers of viruses are provided in Table 2.

phylogenetically as described above) and removal of the identified recombinant regions from the alignment (also as described above), the six SWIO isolate pairs were screened one last time against the rest of the alignment for the least obvious detectable events.

It was apparent from this analysis that all of the SWIO isolates together bear detectable evidence of at least 15 past recombination events (Fig. 4). In all isolates other than ToLCMGV-[Tol] we detected a complex mosaic of sequences in an ~350 nt region spanning sequences encoding the N-terminal portion of Rep. Whereas there is statistically significant evidence that this region of the ToLCYTV-[Dem] sequence has three distinct origins, it has at least four distinct origins in both ToLCYTV-[Kah] and ToLCMGV-[Mor]. Importantly, in all cases the parental sequences identified were one of the SWIO isolates and a sequence only distantly related to previously characterized mainland African begomovirus isolates (either listed as 'unknown' or with a '~' prefix in Fig. 4).

DISCUSSION

We have isolated and characterized what appear to be isolates of two new begomovirus species that are the causal agents of tomato diseases in the SWIO of Madagascar and Mayotte. On the basis of the complete DNA sequences of two isolates from Toliary and Morondova, and in

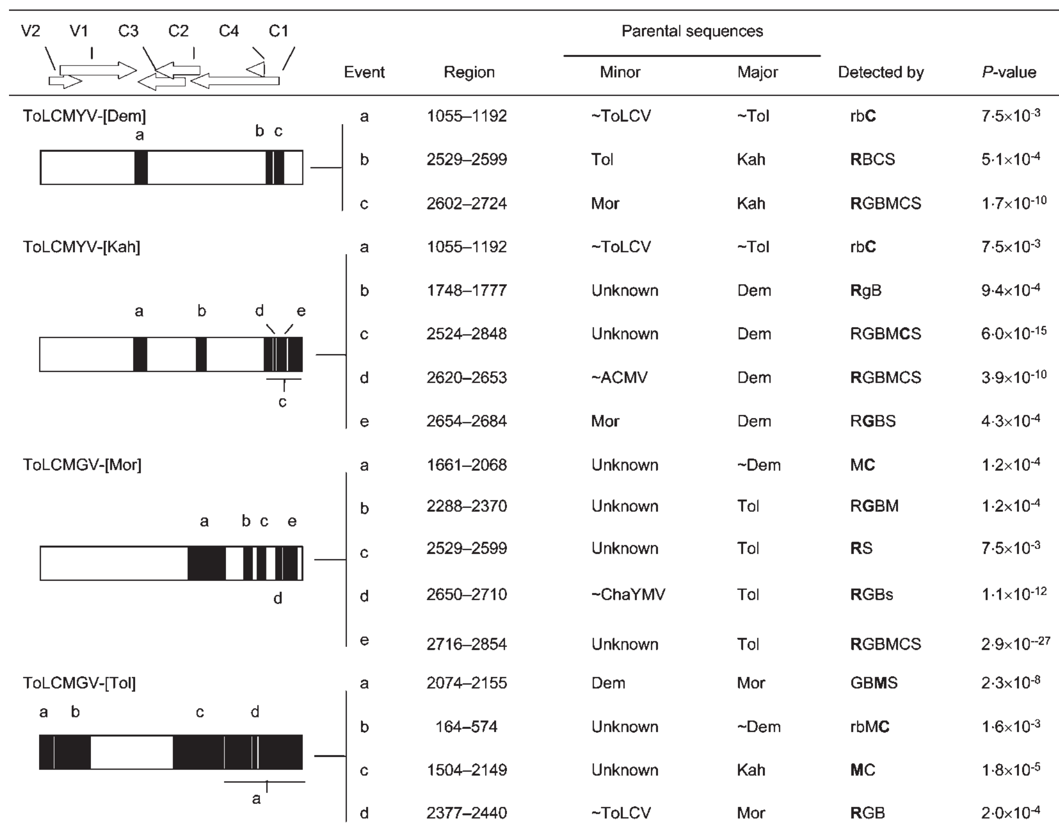

Fig. 4. Recombinant regions detected within SWIO virus sequences: Dem=ToLCYTV-[Dem], Kah=ToLCYTV-[Kah], Mor=ToLCMGV-[Mor] and Tol=ToLCMGV-[Tol]. The genome at the top of the figure corresponds with the schematic representation of sequences given below it. Region coordinates are nucleotide positions of detected recombination breakpoints in the multiple sequence alignment used to detect recombination. Wherever possible, parental sequences are identified. 'Major' and 'Minor' parents are sequences that were used, along with the indicated recombinant sequence, to identify recombination. Whereas for each identified event the minor parent is apparently the contributor of the sequence within the indicated region, the major parent is the apparent contributor of the rest of the sequence. Note that the identified 'parental sequences' are not the actual parents but are simply those sequences most similar to the actual parents in the analysed dataset. Whenever a '~' prefix is included before a parental sequence name, the isolate named is only a distant relative of the parental virus of that region. Recombinant regions and parental viruses were identified using the RDP (R), GENECONV (G), BOOTSCAN (B), and MAXIMUM χ^2 (M), CHIMAERA (C) and SISTER SCAN (S) methods. The reported *P*-value is for the method in bold type and is the best *P*-value calculated for the region in question. Whereas upper-case letters imply a method detected recombination with a multiple comparison corrected *P*-value $<0{\cdot}01$, lower-case letters imply the method detected recombination with a multiple comparison corrected *P*-value $<0{\cdot}05$ but larger than or equal to 0·01.

accordance with the ICTV *Geminiviridae* Study Group guidelines (Fauquet *et al.*, 2003; Fauquet & Stanley, 2003), the isolates should be considered members of the new species Tomato leaf curl Madagascar (ToLCMGV), with the two isolates designated names of ToLCMGV-[Tol] and -[Mor], respectively. Similarly we propose that the isolates from Dembeni and Kahani be considered members of a second new species, Tomato leaf curl Mayotte virus (ToLCYTV), with the two isolates designated names of ToLCYTV-[Dem] and -[Kah], respectively. We also propose that the monophyletic group to which these four isolates belong should be named the SWIO group.

We have demonstrated the infectivity and whitefly transmissibility of cloned DNA sequences for three of the four SWIO isolates. Our inability to detect either DNA B or DNA β in source leaf material, and the induction of leaf curling and stunting symptoms in agro-inoculated tomato plants similar to those observed in the field in the absence of these other genome components, indicates that the SWIO viruses are most likely monopartite.

Recently, a new biotype (Ms) of *B. tabaci* has been identified on Madagascar and other SWIO (Delatte *et al.*, 2005b). Although biotype Ms is genetically closely related to the *B. tabaci* B and Q biotypes, it has been estimated that biotype Ms diverged from biotype B and Q as long as 3 ($\pm 0{\cdot}3$) million years ago. It is possible that the SWIO viruses have evolved in relative isolation for a similar period and it will be interesting to determine whether the SWIO isolates have any transmission advantage relative to mainland African and Mediterranean isolates in biotype Ms.

The results of our recombination analysis support the fact that the SWIO viruses may have been evolving in relative isolation for a prolonged period. Had there been substantial influx of mainland begomovirus isolates onto the islands it would be expected that genetic exchange between mainland and island isolates would be detectable. Such exchanges are, for example, easily detectable both amongst and between divergent African and Mediterranean isolates (Padidam *et al.*, 1999). None of the sequences within the recombinant regions identified in the SWIO isolates closely resembled that of any known non-SWIO begomovirus, indicating that genetic exchange in these viruses has most likely been limited to that occurring between relatively unique island isolates. It is important to note, however, that the recombination analysis does not preclude the possibility of genetic exchange between viruses on different islands. In fact, there is highly significant evidence that, firstly, an 856 bp fragment of the Madagascar isolate, ToLCMGV-[Tol], originated from a virus closely resembling the Mayotte isolate, ToLCYTV-[Dem] (P-value$=2{\cdot}3\times10^{-8}$) (Fig. 4), and, secondly, that a 122 bp fragment of ToLCYTV-[Dem] originated from a virus closely resembling the Madagascar isolate, ToLCMGV-[Mor] (P-value$=1{\cdot}7\times10^{-10}$). When and where these potential recombination events occurred is an open question but it cannot be discounted that both ToLCYTV and ToLCMGV isolates might occur on both islands.

This study highlights the need for further sampling and monitoring of begomovirus diversity in both tomato and non-tomato hosts on the SWIO such as Madagascar, Mayotte and the Comoros archipelago. Such activities would almost certainly lead to the identification of more novel species and provide early warning of the presence of newly imported and potentially dangerous exotic begomoviruses. Many of the SWIO are small enough that repetitive and reasonably exhaustive begomovirus surveys on them are feasible. Isolated begomovirus populations on the smaller, remote SWIO such as Mayotte could provide one of the last and best remaining opportunities to non-destructively test begomovirus evolutionary hypotheses and population genetic models. Continuous maintenance of sampling projects on these islands might also provide opportunities for testing begomovirus epidemiological models whenever importation of exotic viruses to these islands does occur.

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