Frequent occurrence of *Mungbean yellow mosaic India virus* in tomato leaf curl disease affected tomato in Oman

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Next generation sequencing (NGS) of DNAs amplified by rolling circle amplification from 6 tomato (*Solanum lycopersicum*) plants with leaf curl symptoms identified a number of monopartite begomoviruses, including *Tomato yellow leaf curl virus* (TYLCV), and a betasatellite (*Tomato leaf curl betasatellite* [ToLCB]). Both TYLCV and ToLCB have previously been identified infecting tomato in Oman. Surprisingly the NGS results also suggested the presence of the bipartite, legume-adapted begomovirus *Mungbean yellow mosaic Indian virus* (MYMIV). The presence of MYMIV was confirmed by cloning and Sanger sequencing from four of the six plants. A wider analysis by PCR showed MYMIV infection of tomato in Oman to be widespread. Inoculation of plants with full-length clones showed the host range of MYMIV not to extend to *Nicotiana benthamiana* or tomato. Inoculation to *N. benthamiana* showed TYLCV to be capable of maintaining MYMIV in both the presence and absence of the betasatellite. In tomato MYMIV was only maintained by TYLCV in the presence of the betasatellite and then only at low titre and efficiency. This is the first identification of TYLCV with ToLCB and the legume adapted bipartite begomovirus MYMIV co-infecting tomato. This finding has far reaching implications. TYLCV has spread around the World from its origins in the Mediterranean/Middle east, in some instances, in live tomato planting material. The results here may suggest that begomoviruses which do not commonly infect tomato, such as MYMIV, could be spread as a passenger of TYLCV in tomato.

Cultivation of tomato in Oman has in recent years suffered serious losses due to tomato leaf curl disease (ToLCD). ToLCD is caused by begomoviruses; viruses of the genus *Begomovirus* (family *Geminiviridae*). To date five distinct begomovirus species have been identified in tomato with ToLCD symptoms in the country, including *Tomato yellow leaf curl virus* (TYLCV). TYLCV was initially identified in the Middle East in the 1960s and has since spread to most tropical and sub-tropical regions of the world. Viruses with circular single-stranded (ss) DNA genomes encapsidated in paired quasi-icosahedral capsids are classified in the family *Geminiviridae*. Geminiviruses are transmitted plant-to-plant by specific arthropod vectors. The family comprises of nine genera, of which the most widespread and most destructive are viruses of the genus *Begomovirus* that are transmitted by the whitel fly *Bemisia tabaci*. The New World (NW) native begomoviruses typically have two-component genomes (components DNA A and DNA B), whereas most native to the Old World (OW) have monopartite genomes (a homolog of the DNA A of bipartite viruses). Few bipartite begomoviruses have been identified in the OW but only three monopartite begomoviruses have so far been identified in the NW.

The genomes of monopartite begomoviruses, and their bipartite begomovirus homolog DNA A, originating from the OW encode six genes. Virion-sense strand transcribed genes encode the coat protein (CP) and V2 protein. The complementary-sense strand codes for four genes. These encode the replication-associated protein (Rep), the transcriptional-activator protein (TrAP), the replication enhancer protein (REn) and the C4 protein. Bipartite begomovirus DNA B components encode one gene on the complementary-sense, the movement protein

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virus movement in plants and has suppressor of RNA silencing activity17. LYMVs cause distinctive yellow mosaic symptoms in, and extensive losses to, grain legume production24. In planta are reliant on helper viruses for movement not essential for the infectivity of the helper virus, alphasatellites may enhance or attenuate virus symptoms in plants and rely upon helper viruses for movement in planta as well as plant-to-plant transmission. The Rep encoded by alphasatellites has been shown to suppress RNA silencing14,15. In contrast betasatellites (previously known as DNA β) rely on helper viruses for replication, movement in plants and transmission. Betasatellites may enhance helper virus induced symptoms as well as raising viral DNA levels in plants. The structure of betasatellite is highly conserved, encompassing a sequence that is highly conserved among betasatellites (the satellite conserved region [SCR]), a sequence rich in adenine residues (A-rich) and a single, complementary-sense strand encoded, gene known as βC116. The βC1 protein is a dominant pathogenicity determinant, may have a role in virus movement in plants and has suppressor of RNA silencing activity27.

Five distinct begomovirus species have been identified that affecting tomato cultivation in Oman (reviewed by Khan et al.18). These include viruses which are believed to have been introduced into Oman, TYLCV20, Chilli leaf curl virus (ChilCV)20 and Tomato leaf curl Sudan virus (ToLCSDV)21. as well as begomoviruses so far identified only in Oman, Tomato leaf curl Liwa virus (ToLCLiwaV, previously known as Tomato leaf curl Al-Batinah virus)22 and Tomato leaf curl Barka virus (ToLCBv)23. Only two betasatellites have been identified in Oman, the most important of which is Tomato leaf curl betasatellite (ToLCB), which has its origin on the Indian sub-continent18. ToLCB in Oman has been shown to occur in tomato with TYLCV, ChilCV and ToLCBv19,21.

The begomoviruses that infect legumes in the OW, known collectively as “legume yellow mosaic viruses” (LYMVs), are amongst the most unusual of the begomoviruses (reviewed by Qazi et al.24). In phylogenetic analyses LYMVs segregate basal to all the OW begomoviruses and are distinct from the many legume-infecting begomoviruses identified in the NW25,26. The distinction between LYMV and other OW begomoviruses has been proposed to be due to genetic isolation; there possibly being either a virus or vector host range barrier that prevents genetic exchange between viruses that infect legumes and non-leguminous plants24. LYMVs are bipartite begomoviruses and the group includes Mungbean yellow mosaic India virus (MYMIV)27, Mungbean yellow mosaic virus (MYMIV)24, and Dolichos yellow mosaic virus24. They occur widely across the Indian subcontinent and, in the case of MYMIV, Thailand. Recently MYMIV has been identified in Vietnam and MYMIV in Indonesia20. The LYMVs cause distinctive yellow mosaic symptoms in, and extensive losses to, grain legume production24.

The study detailed here has analysed, as part of the routine screening of crops for viruses, the diversity of begomoviruses associated with ToLCD affected tomato in Oman. In contrast to earlier studies, which used PCR with either universal or specific primers, the study here has used the more sensitive next generation sequencing (NGS) of circular DNAs amplified by rolling circle amplification (RCA) to identify DNA viruses in tomato. The results obtained are, broadly, consistent with earlier studies showing that the major viruses infecting tomato in the Al-Batinah region of Oman are TYLCV and ChilCV in association with the betasatellite ToLCB. Surprisingly the analysis also identified a significant incidence of MYMIV in ToLCD affected tomato across all tomato growing regions of the country. The possible effect of the presence of MYMIV on TYLCV-ToLCB infected plants was investigated by inoculating cloned virus components to plants. The wide ranging implications of these findings are discussed.

Results

Preliminary screening of tomato plants for virus infection. During a survey in December 2016, fields of tomato were observed with plants showing yellowing, curling and stunting symptoms (Fig. 1) in the Barka Governorate, Al-Batinah region of Oman. Leaves from six symptomatic plants (Tom26, Tom30, Tom31, Tom32, Tom34 and Tom35), originating from four separate farms, were collected as well as leaves from a non-symptomatic plant from each farm. On the sampled farms 65–70% of tomato plants were showing typical ToLCD symptoms. Initially DNA samples extracted from leaves were screened by PCR with primer pair TYLCD-356/ TYLCD-1044, designed to conserved regions of the genomes of begomoviruses prevalent in Oman. Amplification products of the expected size, ~700 nt, were obtained for PCR reactions with DNA extracted from all symptomatic
### Table 1. NGS sequence reads mapped to TYLCV, ChiLCV, ToLCBrV, ToLCB and MYMIV.

| Sample | Total Sequence Reads | TYLCV | ChiLCV | ToLCBrV | ToLCB | MYMIV DNA A | MYMIV DNA B |
|--------|----------------------|-------|--------|---------|-------|--------------|-------------|
| Tom26  | 46,559,532           | 32,337,132 (69.4%) | 1,669,278 (3.6%) | 9,913,951 (21.2%) | 259,115 (0.55%) | 1,062 (0.002%) | 2,152 (0.005%) |
| Tom30  | 86,458,498           | 52,571,104 (60.57%) | 15,529,268 (17.96%) | 18,696,674 (21.62%) | 268,415 (0.31%) | 108 (0.000%) | 1,120 (0.000%) |
| Tom31  | 83,950,036           | 15,854,183 (18.9%) | 993,391 (1.18%) | 6,370,118 (7.58%) | 47,816,644 (56.9%) | 4 (0.000%) | 148 (0.000%) |
| Tom32  | 46,010,628           | 28,234,471 (61.3%) | 2,680,269 (5.8%) | 8,458,790 (18.4%) | 218,163 (0.47%) | 9 (0.000%) | 19 (0.000%) |
| Tom34  | 120,459,810          | 88,318,623 (73.3%) | 4,822,183 (4.0%) | 26,595,641 (22.07%) | 254,998 (0.21%) | 373 (0.000%) | 14 (0.000%) |
| Tom35  | 68,869,822           | 48,399,000 (70.27%) | 5,340,667 (7.75%) | 14,229,098 (22.07%) | 254,998 (0.21%) | 13 (0.000%) | 19 (0.000%) |

samples but not from the non-symptomatic plants. This finding confirmed the association of a begomovirus with the symptoms in tomato.

**NGS of virus-positive tomato plants.** DNA extracted from the six tomato samples were subjected to RCA to amplify all circular DNA molecules. This yielded high-molecular weight DNA products (concatamers) for all six samples. RCA products were sequenced by DNA-seq on an Illumina HighSeq. 4000PE101 platform. Raw sequencing data was processed to remove adapter sequences, low quality reads (below Phred score 20) and host-related sequences. The remaining good quality sequences were assembled de novo. Assembled sequences were identified using Blast comparison to sequences available in the GenBank database. The results of the NGS (accession PRJNA531683) suggested that all the tomato plants were infected with TYLCV, ChiLCV, ToLCBrV and ToLCB (Table 1). For each sample the vast majority of the reads were mapped to TYLCV. Additionally, for at least two of the samples (Tom 26 and Tom 30), the NGS data suggested the presence of the DNA A and DNA B genomic components of MYMIV. However, in comparison to the numbers of reads which could be mapped to TYLCV, ChiLCV, ToLCBrV and ToLCB, the reads that could be mapped to MYMIV were very low (Table 1).

**Confirmation of NGS results.** RCA products from the six tomato samples were digested with various restriction endonucleases. Restriction yielded ~2.7 kb DNA fragments from all samples, which were cloned in pUC19 and Sanger end sequenced. After partial sequencing and blast analysis the sequences formed three groups, representative of three distinct begomovirus species (results not shown). For each species a single clone from each tomato sample was fully sequenced using the primer-walking strategy. Analysis of these sequences is detailed in the next section.

Specific primers for the PCR-mediated amplification of MYMIV DNA A (MYMIV-AF/MYMIV-AR) and DNA B (MYMIV-BA/MYMIV-BR) were designed based on the Illumina HighSeq contigs. PCR reactions with primer pairs MYMIV-AF/MYMIV-AR and MYMIV-BA/MYMIV-BR resulted in the amplification of ~2.7 and ~2.6 kb DNA fragments, respectively, for Tom26, Tom30, Tom31 and Tom35. No amplification products were obtained for PCR reactions with DNA extracted from samples Tom32, Tom34 using primers MYMIV specific primers. This result confirmed the presence of MYMIV for the four samples and the absence of MYMIV in samples Tom32, Tom34.

**Characterization of clones of monopartite begomoviruses and betasatellite obtained from tomato with ToLCD.** The sequences of six clones (Tom 26–10, Tom 30–3, Tom 31–5, Tom 32–29, Tom 34–34 and Tom 35–26), obtained from RCA products digested with XbaI, showed high levels of sequence identity to isolates of TYLCV available in the databases. Four sequences (Tom 30–3, Tom 31–5, Tom 32–29 and Tom 34–34) obtained from the tomato plants in which MYMIV was not identified showed between 99.1 and 100% sequence identity to the two clones (Tom 32–29 and Tom 34–33) obtained from the tomato plants in which MYMIV was not identified. The four sequences showed the highest levels of sequence identity (99.3 to 100%) to an isolate of TYLCV strain "Iran" (TYLCV-IR) recently obtained from common bean in Oman (MG970362) whereas the two other isolates showed the highest levels of sequence identity (99.9%) to two isolates of TYLCV-IR obtained from tomato originating from Oman (DQ644565, FJ956702). A closer analysis of the six TYLCV-IR sequences obtained here showed the two sequences from tomato in which MYMIV was not identified to differ from the four sequences in which MYMIV was identified in the intergenic region which contain numerous sequence changes as well as several insertions and deletions (results not shown). In a phylogenetic analysis the two sequences from tomato in which MYMIV was not identified segregate apart from the other four sequences (Fig. 2A). An analysis of the six TYLCV sequences produced here from clones obtained by PCR amplification for possible recombination using the Recombination Detection Program (RDP) showed the two clones (Tom 32–29 and Tom 34–33) obtained from the tomato plants in which MYMIV was not identified to differ from the four clones from the tomato plants in which MYMIV was identified. All sequences have potential recombination events in the intergenic region and the sequences obtained from tomato plants in which MYMIV was identified additionally having a recombination event at the N-terminal end of the C4 gene (Supplementary Fig. 1). These recombination events possibly explain the differences seen between the two groups of TYLCV sequences in the phylogenetic analysis.

The sequences of six clones (Tom 26–12, Tom 30–19, Tom 31–23, Tom 32–30, Tom 34–34 and Tom 35–26) obtained from RCA product digested with HindIII showed the highest levels (98.8 to 100%) of sequence identity to two ChiLCV isolates available in the database; HG969264 isolated from radish originating from Oman and...
KX787939 isolated from watermelon originating from Oman. In a phylogenetic analysis these sequence segregated with the sequences of ChiLCV isolates previously obtained from Oman (Fig. 2A). Six clones (Tom 26–16, Tom 30–21, Tom 31–24, Tom 32–31, Tom 34–35 and Tom 35–27), obtained from RCA product digested with XbaI, showed the highest levels of sequence identity (99.7 to 100%) to an isolate of ToLCBrV from tomato in Oman. In a phylogenetic analysis these sequence segregated with previously published sequences of ToLCBrV; a begomovirus species so far only identified in Oman (Fig. 2A). Six potentially full-length clones, Tom26–11, Tom30–9, Tom31–14, Tom32–7, Tom 34–38 and Tom35–2, obtained from PCR amplifications with universal betasatellite primers were sequenced. These sequences are between 1,375 and 1,386 nt in length and are available in the GenBank database under the accession numbers given in Table 2. The six sequences show the features typical of betasatellites consisting of a single conserved open reading frame, in the complementary-sense, with the capacity to encode a product of 118 amino acids (Table 2), a region of sequence rich in adenine (coordinates 721–967) and a sequence conserved between all betasatellites, the satellite conserved region (coordinates 1255–16). All the betasatellite sequences obtained showed high nucleotide sequence identity (> 90%) to ToLCBrV species previously reported from Oman. An analysis using the Species Demarcation Tool (SDT) showed the highest levels of identity to be to an isolate of ToLCBrV associated with Chili leaf curl virus from watermelon (KX787940) for Tom31–14 and Tom35–2 (99.3 and 99.6%, respectively), to an isolate associated with Tomato leaf curl Barka virus from tomato (KF293292) for Tom26–11 (96.6%) and to an isolate from tomato (KF229727) for Tom30–9 (94.5%). A phylogenetic analysis based upon an alignment with selected other betasatellite sequences from the databases showed the four betasatellite sequences obtained here to be most closely related to other isolates obtained from Oman, with which they form a separate clade, followed by ToLCBrV isolates from Iran (Fig. 2B).

Efforts to identify the presence of possible alphasatellites, either by PCR with universal primers for alphasatellites or by cloning from RCA products, were uniformly negative.

Figure 2. Phylogenetic dendrograms based upon alignments of the complete nucleotide sequences of the sequences of Tomato yellow leaf curl virus (TYLCV), Chili leaf curl virus (ChiLCV) and Tomato leaf curl Barka virus (ToLCBrV) isolated from tomato with the genome sequences of selected monopartite begomoviruses from the databases (A) and the sequences of Tomato leaf curl betasatellite (ToLCB) isolated from tomato with selected betasatellite sequences available in the databases (B). Begomovirus acronyms used are Cotton leaf curl Gezira virus (CLCuGeV), Papaya leaf curl virus (PaLCuV), Tomato leaf curl Bangalore virus (ToLCBaV), Tomato leaf curl Liwa virus (ToLCLvV), Tomato leaf curl Sudan virus (ToLCSDV) and Tomato leaf curl virus (ToLCV). Betasatellite acronyms used are Chili leaf curl betasatellite (ChilLCB), Cotton leaf curl Multan betasatellite (CLCuMuB), Okra leaf curl Oman betasatellite (OLCOMB), Pea leaf distortion betasatellite (PLDB), Tomato leaf curl Bangladesh betasatellite (ToLCBDDB) and Tomato leaf curl Karnataka betasatellite (ToLCKB). The trees were arbitrarily rooted on the sequence of Tomato pseudo-curly top virus (TPCTV, X84735) for the virus tree and the sequence of Ageratum yellow vein Singapore alphasatellite (AYVSGA, AJ416153) for the betasatellite tree as outgroup. The database accession numbers are indicated in each case. The sequences originating from tomato are indicated by bold text in each case.
| Clone | Virus | Acc. no.* | Size (nt) | CP | (A)/V2 | Rep | TrAP | REn | (A)/C4 | DNA B/Betasatellite | Segment/acc. no.* | Size (nt) | Position of BY1/BCI or 3C gene (coordinates)/no. of amino acids [predicted coding capacity in kDa] |
|-------|-------|-----------|-----------|----|--------|-----|------|-----|-------|----------------------|------------------|-----------|-------------------------------|
| Tom 26–4 | MYMIV DNA A | MK757218 | 2,746 | 316–1089 | 257 | 29.7 | 141–497 | 118 | 13.53 | 1538–2626 | 362 | 41.30 | 1228–1680 | 1529 | 17.24 | 1086–1490 | 134 | 15.7 | 2176–2459 | 99 | 11.36 | Tom 26–7 | MYMIV DNA B/ MK757222 | 2,652 | 419–1189 | 267 (30.47)/1220–2116 298 (33.76) |
| Tom 26–10 | TYLCV | MK757238 | 2,767 | 304–1080 | 258 | 30 | 141–494 | 116 | 13.43 | 1531–2616 | 343 | 40 | 1242–1628 | 120 | 13.2 | 1079–1483 | 134 | 14.7 | 2166–2459 | 91 | 10.4 |
| Tom 26–12 | ChiLCV | MK757212 | 2,761 | 309–1082 | 257 | 29.7 | 149–514 | 121 | 14 | 1526–2616 | 361 | 39.2 | 1219–1623 | 134 | 15.7 | 1074–1478 | 134 | 15.7 | 2197–2454 | 85 | 11.36 |
| Tom 26–16 | ToLCBrV | MK757232 | 2,753 | 301–1077 | 258 | 30 | 141–491 | 116 | 13.43 | 1538–2626 | 362 | 41.30 | 1228–1680 | 150 | 17.24 | 1086–1490 | 134 | 15.7 | 2176–2459 | 99 | 11.36 |
| Tom 30–6 | MYMIV DNA A | MK757219 | 2,746 | 316–1089 | 257 | 29.7 | 141–497 | 118 | 13.53 | 1538–2626 | 362 | 41.30 | 1228–1680 | 150 | 17.24 | 1086–1490 | 134 | 15.7 | 2176–2459 | 99 | 11.36 |
| Tom 30–3 | TYLCV | MK757239 | 2,768 | 305–1081 | 258 | 30 | 145–495 | 116 | 13.43 | 1554–2618 | 354 | 39.2 | 1223–1630 | 135 | 16 | 1078–1482 | 134 | 15.7 | 2168–2467 | 99 | 11.36 |
| Tom 30–19 | ChiLCV | MK757213 | 2,761 | 309–1082 | 257 | 29.7 | 149–514 | 121 | 14 | 1531–2616 | 343 | 40 | 1242–1628 | 120 | 13.2 | 1079–1483 | 134 | 14.7 | 2166–2459 | 91 | 10.4 |
| Tom 30–21 | ToLCBrV | MK757233 | 2,753 | 301–1077 | 258 | 30 | 141–491 | 116 | 13.43 | 1526–2616 | 361 | 39.2 | 1219–1623 | 134 | 15.7 | 1074–1478 | 134 | 15.7 | 2197–2454 | 85 | 11.36 |
| Tom 31–1 | MYMIV DNA A | MK757220 | 2,746 | 316–1089 | 257 | 29.7 | 141–497 | 118 | 13.53 | 1538–2626 | 362 | 41.30 | 1228–1680 | 150 | 17.24 | 1086–1490 | 134 | 15.7 | 2176–2459 | 99 | 11.36 |
| Tom 31–5 | TYLCV | MK757240 | 2,768 | 305–1081 | 258 | 30 | 145–495 | 116 | 13.43 | 1554–2618 | 354 | 39.2 | 1223–1630 | 135 | 16 | 1078–1482 | 134 | 15.7 | 2168–2467 | 99 | 11.36 |
| Tom 31–23 | ChiLCV | MK757214 | 2,761 | 309–1082 | 257 | 29.7 | 149–514 | 121 | 14 | 1531–2616 | 343 | 40 | 1242–1628 | 120 | 13.2 | 1079–1483 | 134 | 14.7 | 2166–2459 | 91 | 10.4 |
| Tom 31–24 | ToLCBrV | MK757234 | 2,753 | 301–1077 | 258 | 30 | 141–491 | 116 | 13.43 | 1526–2616 | 361 | 39.2 | 1219–1623 | 134 | 15.7 | 1074–1478 | 134 | 15.7 | 2197–2454 | 85 | 11.36 |
| Tom 32–29 | TYLCV | MK757241 | 2,765 | 291–1067 | 258 | 30 | 131–481 | 116 | 13.43 | 1540–2604 | 354 | 39.2 | 1209–1616 | 135 | 16 | 1064–1468 | 134 | 15.7 | 2154–2447 | 97 | 11.36 |
| Tom 32–30 | ChiLCV | MK757215 | 2,761 | 309–1082 | 257 | 29.7 | 149–514 | 121 | 14 | 1531–2616 | 343 | 40 | 1242–1628 | 120 | 13.2 | 1079–1483 | 134 | 14.7 | 2166–2459 | 91 (10.4) |
| Tom 32–31 | ToLCBrV | MK757235 | 2,753 | 301–1077 | 258 | 30 | 141–491 | 116 | 13.43 | 1526–2616 | 361 | 39.2 | 1219–1623 | 134 | 15.7 | 1074–1478 | 134 | 15.7 | 2197–2454 | 85 (11.36) |
| Tom 34–33 | TYLCV | MK757242 | 2,765 | 291–1067 | 258 | 30 | 131–481 | 116 | 13.43 | 1540–2604 | 354 | 39.2 | 1209–1616 | 135 | 16 | 1064–1468 | 134 | 15.7 | 2154–2447 | 97 | 11.36 |
| Tom 34–34 | ChiLCV | MK757216 | 2,761 | 309–1082 | 257 | 29.7 | 149–514 | 121 | 14 | 1531–2616 | 343 | 40 | 1242–1628 | 120 | 13.2 | 1079–1483 | 134 | 15.7 | 2166–2459 | 91 (10.4) |

Continued
Characterization of MYMIV clones obtained from tomato with ToLCD. The PCR amplicons obtained with primers specific for MYMIV DNA A and DNA B were cloned into pTZ57R/T. Eight potentially full-length clones, four (Tom26–4, Tom30–6, Tom31–1 and Tom35–8) obtained with the DNA A primers and four (Tom26–7, Tom30–13, Tom31–15 and Tom35–18) obtained with the DNA B primers, were selected for further analysis. The eight clones were sequenced in their entirety and the sequences are available in the GenBank sequence database under the accession numbers given in Table 2.

The sequences of clones Tom26–4, Tom30–6, Tom31–1 are 2,746 nt in length and that of Tom35–8 is 2,748 nt. The sequences have all characteristics typical of the genomes/DNA A components of begomoviruses originating from the Old World, encoding four genes in the complementary-sense and two in the virion-sense orientation (Table 2). The four sequences showed greater than 99.5% nucleotide identity with each other while the highest nucleotide sequence identities (99 to 99.3%) with the DNA A components of MYMIV isolates previously identified in Oman38,39, followed by 99 to 99.3% identity to the DNA A sequence of a MYMIV isolate from cowpea in India (AY937195)40. Based on the presently applicable criteria for species demarcation of begomoviruses Tom26–4, Tom30–6, Tom31–1 and Tom35–8 are the DNA A component of isolates of MYMIV44. In a phylogenetic analysis the four sequences from tomato segregated with the DNA A components of other MYMIV isolates (Table 2) and to share greater than 99.2–99.8% nucleotide sequence identity. An SDT analysis of selected MYMIV isolates previously reported in Oman38,39 followed by 98–98.5% to a MYMIV DNA B sequence isolated from cowpea in India (AY937196)40. A phylogenetic analysis of the DNA B sequences showed the isolates from tomato to be most closely related to isolates previously identified in Oman. All the isolates from Oman were most closely related to AY937196 and formed a distinct clade with the DNA B sequences of MYMIV isolates from Indonesia, some isolates from India as well as some isolates of MYMIV (Fig. 3B).

The MYMIV sequences obtained by NGS agreed well with the sequences obtained from Sanger sequencing of clones. For example, for plant Tom 26 the DNA A determined by NGS showed 99.7% identity to Tom26–4 whereas the DNA B sequence showed 99.2% identity to Tom26–7. The sequences of the MYMIV DNA A and DNA B determined by NGS are given in Supplementary Fig. 2. The sequence differences likely are due to natural sequence variation in the populations of viral molecules in the plant.
To determine the geographical incidence of infection of tomato with MYMIV a total of 82 tomato plants (collected between 2015 and 2016), originating from across Oman, were screened by PCR with primers to detect MYMIV. A total of 21 tomato plants screened showed the presence of either MYMIV DNA A and/or MYMIV DNA B, giving an incidence of 25% (Supplementary Table 1). The samples were collected from across the country, including the main agricultural area in Al Batinah Governorate and in the south around Salalah (Dhofar province), showing the presence of MYMIV in tomato to be widespread.

Analysis of the infectivity of the MYMIV, TYLCV and ToLCB clones isolated from tomato. Agrobacterium-mediated inoculation of just the DNA A component of the MYMIV isolate from tomato to N. benthamiana plants resulted no apparent symptoms of infection at 24 days post inoculation (dpi) (Fig. 4; Table 3). However, for a single plant, out of seventeen inoculated plants, the presence of the DNA A component was shown by PCR diagnostics. Similarly there were no apparent symptoms following inoculation with both the DNA A and DNA B components of MYMIV, although more plants (n = 3) were found to harbour the DNA A at 24 dpi (Fig. 5; Table 3). Of these only two plants were also found to harbour the DNA B component.

Agroinoculation of MYMIV DNA A and ToLCB to N. benthamiana led to the majority of plants (10 plants out of 18 inoculated) showing symptoms of infection at 16 dpi, consisting of mild foliar crumpling, downward leaf curling and vein thickening (Fig. 5). Diagnostic PCR further confirmed the association of MYMIV DNA A component and ToLCB with the symptomatic N. benthamiana plants. Co-inoculation of N. benthamiana with MYMIV DNA A, DNA B and ToLCB induced symptoms that were qualitatively similar to those induced without the DNA B in 16 out of 18 inoculated plants (Fig. 5). However, the symptoms were slightly milder, with less pronounced upward leaf curling, and appeared one day earlier (Table 3). Although diagnostic PCR showed the DNA A component to be present in all (n = 16) symptomatic plants the DNA B was only detected in four plants and the betasatellite in 12 plants.

Figure 3. Phylogenetic dendrograms based upon alignments of the complete nucleotide sequences of the DNA A sequences of *Mungbean yellow mosaic India virus* (MYMIV) isolated from tomato with the genome or DNA A sequences of selected begomoviruses from the databases (A) and the DNA B sequences of MYMIV isolated from tomato with selected DNA B components of begomoviruses available in the databases (B). Vertical branches are arbitrary; horizontal branches are proportional to calculated mutation distance. Values at nodes indicate percentage bootstrap values (1000 replicates). Begomovirus acronyms used are *Blhendi yellow vein mosaic virus* (BYVMV), *Catharanthus yellow mosaic virus* (CaYMV), *Dolichos yellow mosaic virus* (DoYMV), *Horsemgram yellow mosaic virus* (HgYMV), *Mungbean yellow mosaic virus* (MYMV), *Papaya leaf curl virus* (PaLCuV), *Rhynchosia yellow mosaic India virus* (RhYMIV), *Rhynchosia yellow mosaic virus* (RhYMV), *Tomato leaf curl Taiwan virus* (ToLCTV), *Velvet bean severe mosaic virus* (VBSMV) and *Vigna yellow mosaic virus* (ViYMV). The trees were arbitrarily rooted on the DNA A sequence of *Tomato leaf curl New Delhi virus* (ToLCNDV-[PK:MS4:09]FN435310) for the DNA A tree and DNA B of ToLCNDV-[PK:MS2:09]FN435311 for the DNA B tree as outgroup. The database accession numbers are indicated in each case. The sequences originating from tomato are indicated by bold text in each case.

Geographical incidence of MYMIV in tomato in Oman. To determine the geographical incidence of infection of tomato with MYMIV a total of 82 tomato plants (collected between 2015 and 2016), originating from across Oman, were screened by PCR with primers to detect MYMIV. A total of 21 tomato plants screened showed the presence of either MYMIV DNA A and/or MYMIV DNA B, giving an incidence of 25% (Supplementary Table 1). The samples were collected from across the country, including the main agricultural area in Al Batinah Governorate and in the south around Salalah (Dhofar province), showing the presence of MYMIV in tomato to be widespread.
All *N. benthamiana* plants (n = 15) inoculated with a TYLCV isolate from tomato obtained in this study developed symptoms of infection, consisting of a reduction in leaf size, severe upward leaf curling of leaf margins and yellowing, at 9 dpi. Inoculation of *N. benthamiana* plants with TYLCV and ToLCB induced severe downward leaf curling, leaf crumpling and yellowing in all inoculated plants (n = 16) at 8 dpi. Diagnostic PCR showed the presence of TYLCV DNA in all plants but ToLCB was only detected in 15 plants (Fig. 5; Table 3). For plants inoculated with TYLCV and either the DNA A or DNA B of MYMIV, the symptoms of infection were indistinguishable from plants inoculated with only TYLCV and in none of the plants were the MYMIV components detected by diagnostic PCR. However, the latent period was possibly one day longer than for plants inoculated with only TYLCV (10 days in comparison to 9 days, respectively). Co-inoculation of TYLCV with both the DNA A and DNA B components of MYMIV induced symptoms that were qualitatively similar to *N. benthamiana* plants inoculated with only TYLCV. However, the symptoms were a little less severe and were delayed in comparison to plant inoculated with only TYLCV (12 days compared to 9 days, respectively; Fig. 5; Table 3). When ToLCB was co-inoculated with TYLCV and MYMIV DNA A and DNA B, *N. benthamiana* plants developed symptoms indistinguishable from the symptoms induced by a TYLCV with ToLCB infection, although the symptoms were delayed by approx. 2 days (Fig. 5; Table 3).

Agroinoculation of tomato plants with either DNA A alone or with MYMIV DNA A and DNA B did not lead to symptoms and neither DNA A nor DNA B were detected by diagnostic PCR or by PCR reactions using RCA products, obtained from amplifications with DNA extracted from inoculated plants, as template (RCA-PCR; Fig. 5; Table 3). In contrast, agroinoculation of tomato with either MYMIV DNA A and ToLCB or with MYMIV DNA A, DNA B and ToLCB led to very late developing symptoms (approx. 28 dpi) consisting of mild downward leaf curling and mild yellowing for a few plants (Fig. 5; Table 3). Neither of the virus components nor the beta-satellite were detected in diagnostic PCR. However RCA-PCR showed the presence of the virus components in the plants with symptoms. The TYLCV isolate obtained here was highly infectious to tomato and induced severe downward leaf curling and yellowing symptoms by 12 dpi in all inoculated plants. Co-inoculation of TYLCV with ToLCB reduced the latent period (10 days) and altered the symptoms to mild downward leaf curling with slightly more yellowing. Maintenance of the betasatellite by TYLCV was shown in 14 out of 16 tomato plants inoculated by diagnostic PCR (Fig. 5; Table 3). Co-inoculation of tomato plants with TYLCV and either MYMIV DNA A or MYMIV DNA B resulted in mild downward leaf curling and yellowing symptoms and extended the latent period over plants inoculated with only TYLCV, with the greatest effect seen for MYMIV DNA B (18 days). However, only TYLCV was detected in inoculated plants by diagnostic PCR and RCA-PCR. Similarly, inoculation of tomato plants with TYLCV, MYMIV DNA A and DNA B resulted in mild downward leaf curling and yellowing symptoms and extended the latent period (14 days) but both MYMIV DNA A and DNA B were detected in only two plants by RCA-PCR. Inoculation of tomato plants with all four components (TYLCV, MYMIV DNA A, MYMIV DNA B and ToLCB) led to very late developing symptoms (approx. 28 dpi) consisting of mild downward leaf curling and mild yellowing for a few plants (Fig. 5; Table 3).
Inoculum | N. benthamiana | Tomato
---|---|---
MYMIV A | 0/(1)/17 | 0/(0’)/18
MYMIV A + MYMIV B | 0/(3, 2)/18 | 0/(0’), 0’)/15
MYMIV A + ToLCB | 10/(10, 10)/18 | 2/(2’, 2’)/18
MYMIV A + MYMIV B + ToLCB | 16/(16, 4, 16)/18 | 4/(4’, 2’, 4’)/16
TYLCV | 15/(15)/15 | 8/(18)/18
TYLCV + MYMIV A | 18/(18, 0)/18 | 16/(16, 0’)/16
TYLCV + MYMIV B | 18/(18, 0)/18 | 18/(18, 0’)/18
TYLCV + ToLCB | 16/(16, 15)/16 | 15/(15, 14)/15
TYLCV + MYMIV A + MYMIV B | 17/(17, 3, 1)/18 | 16/(16, 2’, 2’)/16
TYLCV + MYMIV A + MYMIV B + ToLCB | 18/(18, 5, 2, 17)/18 | 17/(17, 4’, 2’, 16)/18

Table 3. Infectivity of MYMIV, TYLCV and ToLCB in agroinoculated N. benthamiana and tomato plants.

| Inoculum | N. benthamiana | Tomato |
|---|---|---|
| | Plants symptomatic/(plants infected)/plants inoculated | Symptoms | Latent period (days) | Plants symptomatic/(plants infected)/plants inoculated | Symptoms | Latent period (days) |
| MYMIV A | 0/(1)/17 | Nr | — | 0/(0’)/18 | Nr | — |
| MYMIV A + MYMIV B | 0/(3, 2)/18 | Nr | — | 0/(0’), 0’)/15 | Nr | — |
| MYMIV A + ToLCB | 10/(10, 10)/18 | mCr, mDlc, Vt | 16 | 2/(2’, 2’)/18 | mDlc, Y | 28 |
| MYMIV A + MYMIV B + ToLCB | 16/(16, 4, 16)/18 | mCr, mDlc, Y | 15 | 4/(4’, 2’, 4’)/16 | mDlc, Y | 28 |
| TYLCV | 15/(15)/15 | sUlr, Y | 9 | 18/(18)/18 | sDlc, Y | 12 |
| TYLCV + MYMIV A | 18/(18, 0)/18 | sUlr, Y | 10 | 16/(16, 0’)/16 | mUlr, Y | 16 |
| TYLCV + MYMIV B | 18/(18, 0)/18 | sUlr, Y | 10 | 18/(18, 0’)/18 | mUlr, Y | 18 |
| TYLCV + ToLCB | 16/(16, 15)/16 | sCr, sDlc, sVt, Y | 8 | 15/(15, 14)/15 | Dlc, Y | 10 |
| TYLCV + MYMIV A + MYMIV B | 17/(17, 3, 1)/18 | sUlr, Y | 12 | 16/(16, 2’, 2’)/16 | mUlr, Y | 14 |
| TYLCV + MYMIV A + MYMIV B + ToLCB | 18/(18, 5, 2, 17)/18 | sCr, sDlc, sVt, Y | 10 | 17/(17, 4’, 2’, 16)/18 | Dlc, Y | 12 |

Inoculum | N. benthamiana | Tomato
---|---|---
| MYMIV A | 0/(1)/17 | 0/(0’)/18 |
| MYMIV A + MYMIV B | 0/(3, 2)/18 | 0/(0’), 0’)/15 |
| MYMIV A + ToLCB | 10/(10, 10)/18 | 2/(2’, 2’)/18 |
| MYMIV A + MYMIV B + ToLCB | 16/(16, 4, 16)/18 | 4/(4’, 2’, 4’)/16 |
| TYLCV | 15/(15)/15 | 8/(18)/18 |
| TYLCV + MYMIV A | 18/(18, 0)/18 | 16/(16, 0’)/16 |
| TYLCV + MYMIV B | 18/(18, 0)/18 | 18/(18, 0’)/18 |
| TYLCV + ToLCB | 16/(16, 15)/16 | 15/(15, 14)/15 |
| TYLCV + MYMIV A + MYMIV B | 17/(17, 3, 1)/18 | 16/(16, 2’, 2’)/16 |
| TYLCV + MYMIV A + MYMIV B + ToLCB | 18/(18, 5, 2, 17)/18 | 17/(17, 4’, 2’, 16)/18 |

Table 3. Infectivity of MYMIV, TYLCV and ToLCB in agroinoculated N. benthamiana and tomato plants.

Discussion

During a small survey of tomato crops conducted during 2015 in the Al-Batinah region of Oman using NGS, the presence in some plants of multipartite begomoviruses (TYLCV, ChiLCV, ToLCBrV), a betasatellite (ToLCB) and a bipartite begomovirus (MYMIV) was shown. The NGS results were confirmed by cloning the viruses/virus components and betasatellite, either from NGS product or by cloning and sequencing of the viral DNA. The viruses were detected using diagnostic PCR and confirmed by cloning the viruses/virus components and betasatellite.

In the phylogenetic analyses the MYMIV DNA A and DNA B components originating from Oman, including those identified earlier, form a distinct clade with MYMIV isolates from Indonesia and some isolates from India and Bangladesh. All these isolates are unusual in that, as first noted by John et al., they are MYMIV but with a DNA B component derived from the closely related MYMV, likely by a process known as pseudo-recombination (component exchange). This was confirmed for the Oman isolates by alignment methods, which show the majority of the DNA B sequence to have the highest levels of identity to the sequences of MYMIV DNA B components but the intergenic region to have higher levels of sequence identity to MYMIV isolates (results not shown).

Based on their results, Tsai et al. concluded that MYMIV was introduced into Indonesia only very recently. The collective results from Oman similarly suggest that MYMIV has only been introduced quite recently. The very close relationship of the isolates in Oman and Indonesia also suggests that they have a common origin, likely having been introduced from South Asia. The likely mechanism of introduction is unclear, although the demonstration for an increasing number of geminiviruses that seed transmission can occur, including for the LYMVs DoYMV and possibly MYMV, which are closely related to MYMIV, may suggest that the mechanism of geographic dispersal was via seed.

In Oman both TYLCV-IR and MYMIV have previously been identified separately in legumes: common bean and kidney bean, respectively. Although generally considered a tomato virus, TYLCV can infect and cause problems in legumes; for example the type strain of TYLCV is one of two viruses shown to cause bean leaf crumple disease in Spain. The unusual aspect of the findings of Shahid et al. was the identification of MYMIV with a betasatellite (ToLCB) in kidney bean. This virus is rarely encountered in the presence of a betasatellite.
Rouhibakhsh and Malathi showed MYMIV in cowpea with Cotton leaf curl Multan betasatellite (CLCuMuB) and Ilyas et al. showed the virus in soybean with Tobacco leaf curl betasatellite (TobLCB). In all three cases the symptoms of the infections suggested that the betasatellite was enhancing symptoms—the symptoms in each case having features not usually associated with MYMIV infection. MYMIV has on one occasion been identified in tomato without, apparently, the presence of a betasatellite. The MYMIV identified in tomato in that case was also of the pseudo-recombinant type, with a DNA B originating from MYMV.

The infectivity analysis with cloned virus/virus components/betasatellite shows that both N. benthamiana and tomato are non-hosts of MYMIV. For N. benthamiana, both components could be detected in a small number of plants after inoculation with the DNA A and DNA B components of MYMIV, although no symptoms were induced. The inability of MYMIV, and other LYMVs, to infect N. benthamiana has been demonstrated previously. However, in the presence of ToLCB MYMIV DNA A spread efficiently in N. benthamiana plants and induced symptoms. When MYMIV DNA B was included in the inoculum with DNA A and ToLCB, the DNA B was maintained in a few plants. This suggests that the inability of MYMIV to infect N. benthamiana is due to a deficiency in DNA B. Since the DNA B component of bipartite begomoviruses encodes proteins involved in virus movement in plants, the deficiency of MYMIV may be in the ability to spread. Certainly the efficient spread (infectivity) of the DNA A component in the presence of the betasatellite supports this. Betasatellites have previously been shown to complement DNA B functions suggesting that the only protein encoded by betasatellites, βC1, has movement functions. However, the demonstration that βC1 has suppressor of both post-transcriptional and transcriptional gene silencing may indicate that rather than providing movement functions, betasatellites instead overcome a host resistance to movement based on gene silencing. This is supported by the finding here that inoculation with the betasatellite reduces the latent period (faster spread of the virus following inoculation) and alters the phenotype of TYLCV infection (indicating differing plant tissues affected and/or timing of the infection of those tissues), as noted previously for a number of monopartite begomovirus-betasatellite interactions including TYLCV/ToLCB.

In co-inoculations with TYLCV, MYMIV was only maintained in N. benthamiana in the presence of ToLCB or in inoculations that also included MYMIV DNA B. However, in inoculations with MYMIV DNA B, the DNA B was not maintained in most plants. This suggests that, although not well adapted to N. benthamiana, this component does contribute to the infectivity of the DNA A (more plants that ultimately contain the DNA A).

For inoculations of tomato MYMIV DNA A and/or DNA B were only maintained in the presence of ToLCB. In the few plants that MYMIV DNA A and DNA B were detected the genomic components were below the detection threshold of PCR, indicating that the titres were very low. Nevertheless, in the inoculations with MYMIV DNA A and/or DNA B there were changes in the latent periods and symptoms exhibited by infections of TYLCV.
Total nucleic acid was isolated from tomato leaf samples using a CTAB-based method. DNA extracts were resuspended in sterile distilled water and stored at −20°C. Plants showing typical ToLCD infections were collected from the Barka Governorate, Al-Batinah region of Oman. 

Chain reaction (PCR) in initial screening with primer pair TYLCD-356/TYLCD-1044, designed to conserved sequence motifs of tomato-infecting begomoviruses in Oman. 

Materials and Methods

Detection of viruses and betasatellite. During a survey for begomoviruses in January 2015, tomato plants showing typical ToLCD infections were collected from the Barka Governorate, Al-Batinah region of Oman. Total nucleic acid was isolated from tomato leaf samples using a CTAB-based method. DNA extracts were resuspended in sterile distilled water and stored at −20°C. The nucleic extracts were used as a template in polymerase chain reaction (PCR) in initial screening with primer pair TYLCD-356/TYLCD-1044, designed to conserved sequences of tomato-infecting begomoviruses in Oman.

Next-generation sequencing. Circular DNA molecules in nucleic acid samples were amplified by rolling circle amplification (RCA) using a TempliPhi 100 Amplification System kit (GE Healthcare) as described by the manufacturer. Resulting high molecular weight products were sent to the Beijing Genomics Institute (Hong Kong) for sample preparation and paired-end 101 sequencing on an Illumina HighSeq. 4000 platform. NGS data received in fastq format was processed by using Trimgalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) on a High Performance Computing cluster and adapter sequences and low quality (Phred score < 20) sequence reads were removed. Sequence reads were mapped to the tomato genome sequence (Solanum lycopersicum iTag2.4) using Bowtie2 to remove host related sequences and enrich viral sequences. Sequence reads not mapping to the tomato genome sequence were isolated and de novo assembled using SPAdes version 3.12.0. Contigs obtained from this assembly were identified by using stand-alone Blast search tool.

Amplification by PCR, RCA and cloning of viruses and betasatellite. MYMIV infection of tomato plants was detected by PCR with primers for the detection of MYMIV DNA A (MYMIV-CPF [5'-TCCCC CCGGATGCACCAAGCGACCTAC-3']/MYMIV-CPR [5'-CAAGTGCAGACTAATTCAATATCGAATCTA-3']) and
MYMIV DNA B (MYMIV-NSPF [5'-AACATCGATAGGCGGCATGAAGTGA-3']/MYMIV-NSPR [5'-TTAGTCCATTCCTTTGAGTCTTTCA-3']). The full length components of MYMIV were PCR-amplified from DNA extracts using specific primers MYMIV-AF (5'-GTAAAGCTTACCTCTCCACCGTG-3')/MYMIV-AR (5'-TGTAACGTTAGCTTACGATAATCTCTGTTCAATAC-3') for the DNA A component and MYMIV-BA (5'-CAGGTACGTATGCTCCTGGCA-3')/MYMIV-BR (5'-TTGGATCTGGAGATCTATCT-3') for the DNA B component. These primers for the amplification of full-length components were designed to the sequences obtained from the NGS analysis. Betasatellites were amplified with primer pair beta01/beta02. The amplification products were cloned in pTZ257/R/T (Fermentas). Concatameric RCA products were digested with restriction endonucleases Xhol or PstI. Resulting ~2.7 kb fragments were purified from agarose gels using a GeneJet Gel Extraction Kit (Thermo Fisher Scientific) and cloned in pUC19.

Sanger sequencing, sequence assembly and sequence analysis. Selected clones harbouring potentially full-length begomovirus or satellite inserts were sequenced commercially (Macrogen Inc., South Korea). Sequence reads were assembled using SeqMan, part of the Lasergene package of sequence analysis software (DNA Star Inc., Madison, WI, USA). The resultant sequences were initially analyzed using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related sequences in the nucleotide sequence databases. Percentage sequence identity values quoted were determined using SITD with the MUSCLE alignment option. Pairwise multiple sequence alignments using the MUSCLE algorithm were produced using MEGA6. Evolutionary relationships were determined by constructing phylogenetic trees using Clustal X (neighbor-joining method) and displayed using Treeview. Possible recombination events in sequences were determined using RDP4.

Production of constructs for Agrobacterium-mediated inoculation. A full-length clone of MYMIV DNA A (Tom26–4) was digested with HindIII and XhoI to release a fragment of ~1,600 bp that was ligated into the binary vector pGreen0029 to yield pGMYA-0.4. Then the full-length insert of Tom26–4 was released using HindIII and ligated into pGMYA-0.4, linearised with XhoI, to yield the partial direct repeat construct pGMYA-1.4. Similarly, MYMIV DNA B (Tom26–7) was digested with BamHI and Clai to release a fragment of ~1,700 bp and ligated to into pGreen0029 to yield pGMYB-0.4 into which the full-length insert of Tom26–7, released with BamHI, was ligated to yield pGMYB-1.4. A partial direct repeat construct of TYLCV (Tom26–7) in pGreen0029 was produced using an ~1,249 bp BamHI-XhoI fragment and the full-length insert of Tom26–10, released using Xbal, to yield pGTB-1.4. A partial direct repeat construct of ToLCB (Tom26–10) in pGreen0029 was produced using an ~600 bp BamHI-XbaI fragment and the full-length insert released using KpnI to yield pGTYB-1.4. The pGreen0029 constructs were finally electroporated into Agrobacterium tumefaciens strain LBA4444.

Agrobacterium-mediated inoculation and maintenance of plants. Agrobacterium cultures harbouring the pGreen0029 constructs were grown for 48 h in 50 ml LB liquid medium supplemented with which antibiotics (kanamycin 25 µg/µl, tetracycline 10 µg/µl, rifampicin 50 µg/µl). The bacteria were pelleted by centrifugation at 4800 g for 10 min at 4°C and resuspended in infiltration solution (10 mM MgCl2, 200 µM acetosyringone) and adjusted to a final optical density at 600 nm of ~1.0. Agroinoculation was performed on 4–5 week old tomato (Solanum lycopersicum L. cv. Pusa Ruby) plants as described previously. For each combination of begomovirus and/or betasatellite, 5–6 plants were used for inoculation. Inoculated plants were maintained in a secure, insect-free growth room with a day length of 12 h, a day/night temperature of 25°C/25°C and a relative humidity of 65%. The plants were observed daily for the appearance of symptoms of virus infection.

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Author contributions
M.S.S. and A.M.A. conceived the study and supervised the work. M.S., A.R. and M.N.A. performed the experimental work. M.I. performed N.G.S. analysis. M.S.S. and R.W.B. interpreted the data. M.S.S. and M.I. wrote the first draft of the manuscript, which was edited by R.W.B.

Competing interests
The authors declare no competing interests.

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