Zucchini yellow mosaic virus Populations from East Timorese and Northern Australian Cucurbit Crops: Molecular Properties, Genetic Connectivity, and Biosecurity Implications

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Abstract

Zucchini yellow mosaic virus (ZYMV) isolates from cucurbit crops growing in northern Australia and East Timor were investigated to establish possible genetic connectivity between crop viruses in Australia and Southeast Asia. Leaves from symptomatic plants of pumpkin (*Cucurbita moschata* and *C. maxima*), melon (*Cucumis melo*), and zucchini (*C. pepo*) were sampled near Broome, Darwin, and Kununurra in northern Australia. Leaves from symptomatic plants of cucumber (*C. sativus*) and pumpkin sampled in East Timor were sent to Australia on FTA cards. These samples were subjected to high-throughput sequencing and 15 complete new ZYMV genomic sequences obtained. When their nucleotide sequences were compared with those of 48 others from GenBank, the East Timorese and Kununurra sequences (three per location) and single earlier sequences from Singapore and Reunion Island were all in major phylogroup B. The seven Broome and two Darwin sequences were in minor phylogroups I and II, respectively, within larger major phylogroup A. When coat protein (CP) nucleotide sequences from the 15 new genomes and 47 Australian isolates

sequenced previously were compared with 331 other CP sequences, the closest genetic match for a sequence from Kununurra was with an East Timorese sequence (95.5% nucleotide identity). Analysis of the 63 complete genomes found firm recombination events in 12 (75%) and 2 (4%) sequences from northern Australia or Southeast Asia versus the rest of the world, respectively; therefore, the formers' high recombination frequency might reflect adaptation to tropical conditions. Both parents of the recombinant Kununurra sequence were East Timorese. Phylogenetic analysis, nucleotide sequence identities, and recombination analysis provided clear evidence of genetic connectivity between sequences from Kununurra and East Timor. Inoculation of a Broome isolate to zucchini and watermelon plants reproduced field symptoms observed in northern Australia. This research has important biosecurity implications over entry of damaging viral crop pathogens not only into northern Australia but also moving between Australia's different agricultural regions.

Zucchini yellow mosaic virus (ZYMV; family *Potyviridae*; genus *Potyvirus*) causes severe diseases of tropical and subtropical cucurbit crops globally (Coutts et al. 2011a; Desbiez and Lecoq 1997; Lecoq and Katis 2014; Zitter et al. 1996). It is transmitted nonpersistently by more than 10 aphid species (Castle et al. 1992; Desbiez and Lecoq 1997; Katis et al. 2006; Yuan and Ullman 1996) and a low rate of seed transmission occurs (Coutts et al. 2011b; Robinson et al. 1993; Schrijnwerkers et al. 1991). It can also be transmitted by plant-to-plant contact (Coutts et al. 2013). Its virions consist of flexuous filaments 680 to 730 nm in length (Desbiez and Lecoq 1997; Lisa et al. 1981). Volunteer cucurbitaceous crop and weed plants act as natural reservoirs of infection for spread to cucurbit crops (Coutts and Jones 2005; Coutts et al. 2011b; Lecoq and Desbiez 2008). When ZYMV infects cucurbit plants, foliage symptoms include mosaic, chlorosis, leaf deformation,

and plant stunting. Infected fruit develop malformation and surface discoloration, rendering them unmarketable (Blua and Perring 1989; Coutts et al. 2011a; Desbiez and Lecoq 1997; Gal-On 2007).

ZYMV causes major losses to Australian cucurbit crops in its tropical north (Northern Territory [NT]) and northwest (Kimberley region of Western Australia [WA]), and the subtropical west (Gascoyne region of WA) (Coutts and Jones 2005; Jones 1996). It also infects cucurbit crops in its tropical and subtropical northeast (Queensland [QLD]) and subtropical east (northern New South Wales [NSW]) (Büchen-Osmond et al. 1988; Greber et al. 1987, 1988). In Australia, the main crops in which ZYMV causes disease are melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima* and *C. moschata*), zucchini (*C. pepo*), and cucumber (*Cucumis sativus*) (Büchen-Osmond et al. 1988; Coutts and Jones 2005; Greber et al. 1987, 1988). In neighboring countries to Australia's north, ZYMV infects cucurbit crops in Indonesia and Papua New Guinea (PNG) but has not been reported from East Timor (CABI 2016; Daryono and Natsuaki 2016; Davis et al. 2002; Somowiyarjo 1993). Elsewhere in Southeast Asia, ZYMV occurs in Malaysia, Singapore, and Vietnam (AVA 2001; CABI 2016; EPPO 2014; Fujisawa et al. 1986, 1990; Ha et al. 2008; Wong et al. 1992, 1994).

In Southeast Asia, agriculture has been practiced for more than 8,000 years (Hutterer 1983) but, in Australia, it only started following European colonization in 1788 and, in remote regions, as recently as the 1960s. Therefore, many viruses and virulent virus strains that cause damaging crop diseases elsewhere in the world are still absent from Australia, and several others only arrived recently. Human activities involving trade in plants and plant products involving unknowingly infected seed or vegetative propagules are responsible for most introductions (Gibbs et al. 2008; Jones 2009). Between

Ethics statement: Approval for sampling in East Timor was obtained from the Plant Quarantine Department of the National Directorate of Quarantine and Biosecurity, Ministry of Agriculture and Fisheries, Dili. Permission to import plant material blotted on the FTA cards was obtained from the Australian Federal Department of Agriculture who granted permit No. IP15004160.

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November and April every year, northwesterly monsoonal winds blow from East Timor and Indonesia across the sea toward northern Australia. These monsoonal winds could bring viruliferous insect vectors or migrating birds with infected seed in their guts, thus introducing viruses (Eagles et al. 2013). Evidence that this could have occurred already was obtained by analysis of coat protein (CP) sequences from Australian ZYMV isolates. Isolates from near Kununurra in the northeast Kimberley region of WA were the only ones showing genetic connectivity (genetic connectivity is a biosecurity term used to describe matching nucleic acid sequence identities among samples sourced from different countries) with isolates from two countries to Australia's north, Singapore and Vietnam (Coutts et al. 2011b). Because ZYMV is aphid borne, it could have been carried to northern Australia by a viruliferous aphid vector blown from a nearby country.

The hypothesis that "important plant viral pathogens of economically important crops are arriving in tropical northern Australia by crossing the sea separating it from its northern neighbours" (Maina et al. 2016a–f, 2017a,b) is tested in this article. No complete genomic ZYMV nucleotide sequences are available from Australia, East Timor, Indonesia, or PNG, and only one from Southeast Asia (Singapore). Coutts and Jones (2005) obtained 47 complete ZYMV CP sequences from cucurbit crops or cucurbitaceous weeds from five locations in mainland Australia (four locations in the continent's north). None are available from Indonesia, East Timor, or PNG. Here, we compared complete coding regions (polyproteins) of 15 ZYMV isolates from cucurbit crops growing in northern Australia or East Timor. These were used to investigate (i) whether genetic connectivity occurs between these ZYMV populations or between them and ZYMV populations from other world regions and (ii) whether any are recombinants. We also investigated whether any genetic connectivity occurs between 393 other CP sequences from GenBank and CP sequences from the 15 new genomes. Important biosecurity implications arising from this work are discussed.

Materials and Methods

Isolate collection. In May 2015, 15 leaf samples were collected from cucumber or pumpkin plants with virus-like symptoms growing on small farms in the Aileu, Dili, Loes, and Maubisse districts of East Timor (Fig. 1A; Table 1); no details of their foliage symptoms are available. Sap from each sample was blotted onto FTA Classic Cards (GE Healthcare), as described by Ndunguru et al. (2005). The cards were sent to Perth, WA. In August 1996, five leaf samples collected from symptomatic plants of pumpkin or melon were taken from crops

in the Ord River Irrigation Area near Kununurra, East Kimberley region, and transported to Perth, WA (Fig. 1B; Table 1). When leaf sap from each sample was examined by electron microscopy, all five samples contained 750-nm-long flexuous filamentous particles typical of potyviruses (Jones 1996). The five potyvirus-infected samples were sent to Dennis Persley, QLD Department of Agriculture and Fisheries, Brisbane, who tested them for ZYMV presence by enzyme-linked immunosorbent assay (ELISA) and detected ZYMV in four of them. Leaf tissue was preserved over silica gel for 20 years in Brisbane before being returned to Perth in 2016. In 2011, leaves infected with ZYMV isolates 38NT and 75NT from symptomatic melon crops growing near Darwin, NT were sent to Perth and freeze dried in glass vials, and the vials were lodged in the Department of Agriculture and Food Western Australia virus isolate collection, Perth. In October 2015, leaf samples from 22 potentially symptomatic plants were collected from pumpkin, melon, and zucchini crops growing at Broome, West Kimberley region (Maina et al. 2017a). Leaf tissue from seven samples was ZYMV positive but *Papaya ringspot virus* (PRSV; family *Potyviridae*; genus *Potyvirus*) negative by ELISA (Table 1).

Glasshouse grown plants, virus culture, and inoculations. Seed were sown in plastic pots filled with potting mix and plants grown in insect-proofed, air-conditioned glasshouses maintained at 19 to 21 °C. Plants of 'Black Jack' zucchini, 'Sugar Baby' watermelon, 'Borlotti' common bean (*Phaseolus vulgaris*), *Nicotiana benthamiana*, *Chenopodium amaranticolor*, and *C. quinoa* were grown. To inoculate plants, virus-infected zucchini leaves were ground in extraction buffer containing monosodium phosphate (NaH_2PO_4) at 3 g liter⁻¹ and disodium hydrogen phosphate (Na_2HPO_4) at 11.5 g liter⁻¹, and the infective sap was mixed with "celite" before being rubbed onto leaves of healthy plants. To establish a ZYMV culture, infective leaf sap containing Broome isolate 13Br was inoculated to zucchini plants. A culture of PRSV isolate B9 (Maina et al. 2017a) was maintained similarly.

Host responses to inoculation. In experiment 1, infective sap containing isolate 13Br was inoculated to two plants or species of watermelon, *N. benthamiana* and *C. quinoa*. In experiment 2, infective sap was inoculated to two plants or species of zucchini, watermelon, common bean, *N. benthamiana*, *C. amaranticolor*, and *C. quinoa*. One control plant of each species was mock inoculated with healthy leaf sap. Experiment 3 was a duplicate of experiment 2. Plants were observed for virus symptoms at weekly intervals for 6 weeks after inoculation. Samples from inoculated and uninoculated upper leaves

Fig. 1. Maps showing locations in East Timor and the north of the Australian mainland where leaf samples infected with *Zucchini yellow mosaic virus* were collected from cucurbit crops. Another cucurbit-growing location (Carnarvon) is also indicated because this is referred to in the text. **A**, Sampling locations in East Timor. **B**, Sampling locations in northern Australia and the position of East Timor in relation to them. Arrows show the prevailing monsoonal wind direction from November to April every year.

were tested for ZYMV by ELISA 2 or 4 weeks after inoculation, respectively. Uninoculated upper leaves recorded as uninfected were retested after 6 weeks.

ELISA. Leaf samples were tested for ZYMV and PRSV by double-antibody sandwich ELISA (Clark and Adams 1977), as described by Coutts and Jones (2005). Sap from ZYMV isolate 13Br-infected, PRSV isolate B9-infected, and healthy zucchini leaves was included in paired wells to provide positive or negative controls. Polyclonal antisera to ZYMV and PRSV from DSMZ GmbH were used.

RNA extraction and reverse-transcription polymerase chain reaction. The 15 East Timorese samples blotted onto FTA cards were sampled by punching up to 20 discs (approximately 2 mm in diameter) for each sample. Total RNA was extracted from these discs using a ZR Plant RNA MiniPrep™ kit (Zymo Research) according to manufacturer's instructions. The seven leaf samples positive from Broome for ZYMV by ELISA, five desiccated leaf

samples from Kununurra, and two frozen dried leaf samples from Darwin were sampled, and their RNA was extracted as described above. Crude RNA was treated with RNase-free DNase (Invitrogen) and quality control done as described previously (Guerret et al. 2017; Maina et al. 2016a–f). Polymerase chain reaction was done using GoTaq Green master mix (Promega Corp.) and generic *Potyvirus* LegPotyF and LegPotyR primers (Webster 2008).

High-throughput sequencing. Libraries were prepared from 0.1 to 1 µg of total RNA using the TruSeq stranded Total RNA Sample Preparation kit with RiboZero Plant (Illumina), as described previously (Guerret et al. 2017; Maina et al. 2016a–f). The final size and concentration of each library was verified using Qubit and D1000 ScreenTape (TapeStation 2200; Agilent Technologies). The first run involved 10 nanomolar library pools prepared by mixing the libraries to achieve an equal molar concentration of each. A pool of denatured libraries was sequenced using a 2-by-251 v2 kit to generate

Table 1. Details of the 15 *Zucchini yellow mosaic virus* isolates sequenced in this investigation

Isolate	Host	Symptoms	Geographical origin ^a	Year of isolation	Isolate origin or supplier
TM16	Pumpkin	Virus-like	Loes, East Timor	2015	This study
TM39	Pumpkin	Virus-like	Aileu, East Timor	2015	This study
TM40	Cucumber	Virus-like	Aileu, East Timor	2015	This study
13Br	Zucchini	Mosaic, leaf deformation	Broome, WA, Australia	2015	This study
14Br	Zucchini	Mosaic, leaf deformation	Broome, WA, Australia	2015	This study
17Br	Zucchini	Mosaic, chlorosis, reduction in leaf size, plant stunting	Broome, WA, Australia	2015	This study
19Br	Zucchini	Mosaic, leaf deformation	Broome, WA, Australia	2015	This study
20Br	Zucchini	Mosaic, leaf deformation	Broome, WA, Australia	2015	This study
21Br	Zucchini	Mosaic, leaf deformation	Broome, WA, Australia	2015	This study
56Br	Halloween pumpkin	Mosaic	Broome, WA, Australia	2015	This study
694K	Jarrahdale pumpkin	Yellow mosaic	Kununurra, WA, Australia	1996	Jones (1996)
695K	Jarrahdale Pumpkin	Mosaic	Kununurra, WA, Australia	1996	Jones (1996)
697K	Honeydew melon	Mosaic	Kununurra, WA, Australia	1996	Jones (1996)
38NT	Honeydew melon	Mosaic, leaf deformation	Darwin, NT, Australia	2011	Barry Conde
75NT	Rockmelon	Mosaic, leaf deformation	Darwin, NT, Australia	2011	Barry Conde

^a WA = Western Australia and NT = Northern Territory.

Fig. 2. Symptoms caused by **A**, natural infection with *Zucchini yellow mosaic virus* (ZYMV) in a zucchini (*Cucurbita pepo*) plant growing at Broome, Western Australia in 2015 or **B** to **D**, infection induced by mechanical inoculation of cucurbit plants with ZYMV isolate 13Br. **A**, Foliage of zucchini showing obvious mosaic and leaf deformation; **B**, leaf of Black Jack zucchini showing obvious vein clearing; **C**, fruit of Black Jack zucchini showing obvious yellow mottling, severe malformation, and diminished size; and **D**, leaf of Sugar Baby watermelon (*Citrullus lanatus*) with mosaic and leaf deformation.

paired-end reads (Illumina) and a 1% PhiX v3 spike control was included. The MiSeq platform was used to sequence sample TM39 in one lane multiplexed with 12 other samples that formed part of another study. The other samples were sequenced by sending ready stranded libraries to Macrogen Inc., where the libraries were subjected into HiSeq 2500 using a Truseq SBS KIT v4 (Illumina) with 151 cycles to generate paired-end reads in a multiplex of 24 samples in one lane.

Sequence analysis. After sequencing, RNA-seq reads were subjected to quality control using CLC Genomics Workbench 6.5 (CLCGW) (CLC bio; Qiagen) followed by *Denovo* assembly, as described previously (Guerret et al. 2017; Kehoe et al. 2014a; Maina et al. 2016a–f). All the contigs were subjected to BLAST in the National Center for Biotechnology Information (NCBI) using CLCGW and, later, to pairwise sequence comparisons (PASC) (Altschul et al. 1990; Bao et al. 2014). All contigs with plant virus matches were used for further analysis. In addition, trimmed reads from CLCGW were imported into Geneious 8.1.7 (Biomatters Ltd.) (Kearse et al. 2012) for reference mapping, as described by Kehoe et al. (2014a). Consensus sequence integrity was observed and its full length used with the contig of interest from CLCGW in Geneious by alignment with MAFFT (Katoh et al. 2002) to create a final sequence. ZYMV coding regions were improved by aligning nucleotide sequences to the aligned deduced amino acid sequences using MUSCLE available in Geneious. Open reading frames were predicted and annotations made using Geneious. Fifteen ZYMV sequences were obtained: these were from Loes (one) and Aileu (two) in East Timor and Broome (seven), Kununurra (three), and Darwin (two) in northern Australia (Table 1; Fig. 1). Final sequences were deposited in GenBank under the accession numbers KY225542 to KY225556. Apart from PRSV with isolate 38NT from Darwin (Maina et al. 2017a) and *Squash mosaic virus* (genus *Comovirus*, family *Secoviridae*) with 697K from Kununurra (S. Maina, O. R. Edwards, and R. A. C. Jones RAC, unpublished), no other viruses were detected by high-throughput sequencing in these samples.

Phylogenetics. Using Clustal W in MEGA 7.0.14 (Kumar et al. 2016), the coding regions of the new ZYMV sequences were aligned with those of other complete ZYMV genome sequences from other parts of the world retrieved from GenBank (Supplementary Table S1). Further alignments were obtained using MAFFT in Geneious (Katoh et al. 2002). Several additional ZYMV sequences available on GenBank were excluded from the analysis due to poor editing, redundancy, or unequal lengths. Phylogenetic analysis compared (i) the coding regions of the 2 nonrecombinant complete genomes each from Australia and East Timor, respectively, with those of 27 other nonrecombinant and nonredundant complete genomes; (ii) the coding regions of the 12 and 3 complete genomes from Australia and East Timor, respectively, with 33 other complete ZYMV genomes (recombinants and highly similar sequences included but 18 almost identical U.S. sequences obtained by Simmons et al. [2012] during plant-to-plant transmission studies excluded); and (iii) the extracted CP genes from these 15 new genome sequences and 20 Australian isolates sequenced previously (27 highly similar Australian CP sequences excluded) with 162 representative complete CP sequences from GenBank. Maximum-likelihood trees were prepared using the Tamura-Nei model, all with bootstrap values of 1,000. All ambiguous positions were removed for each sequence pair, and the trees were rooted with a *Bean common mosaic virus* (family *Potyviridae*; genus *Potyvirus*) sequence (KU896809). The within-virus species phylogroup classification system used was the CP sequence nomenclature of Coutts et al. (2011b), which uses Latin numerals for minor phylogroups (Jones and Kehoe 2016).

Sequence identities. When the coding regions of the 15 new ZYMV genomes were analyzed along with the 48 others retrieved from GenBank (including all recombinant and highly similar sequences), their nucleotide identities were determined as described previously (Guerret et al. 2017; Maina et al. 2016a–f). Their nucleotide sequences were translated to proteins in Geneious and their protein sequences exported to Clustal Omega (EMBL-EBI database) for alignment of amino acid sequences. To determine nt sequences

identities based on CPs, the CP sequences from the 15 new ZYMV genomes were compared with the 47 previously reported Australian CP sequences and 331 other CP sequences from GenBank. Their nt sequence identities were determined as described above.

Recombination detection. The complete coding sequences of the 15 new genomic nucleotide sequences from northern Australia and East Timor, and 48 others retrieved from GenBank, were assessed for evidence of recombination. The Recombination Detection Program No. 4 (RDP4) package, version 4.72 (Martin et al. 2010), was used to detect putative recombination break points between these 63 sequences and excluded any events that carried the warning “this event may not have been caused by recombination”. Default parameters were implemented for the seven programs within RDP4:RDP (Martin et al. 2005), GENECONV (Padidam et al. 1999), Bootscan (Martin et al. 2005), MaxChi (Smith 1992), Chimaera (Posada and Crandall 2001), 3Seq (Boni et al. 2007), and SiScan (Gibbs et al. 2000). The analysis also included using a Bonferroni-corrected *P* value cut-off of 0.05. All sequences for which recombination patterns were detected by four or more programs within RDP4 were considered to contain firm events providing strong evidence of actual recombination but recombination patterns supported by fewer than four programs were not considered (Ohshima et al. 2002). Using the RDP4 package, the recombination sites identified were examined individually, and a phylogenetic approach confirmed the parental assignments made (Martin et al. 2010, 2015).

Results

Natural infection. The plants from which three ZYMV isolates were obtained in 1996 came from cucurbit crops growing on two Kununurra farms. One plant each of the two ZYMV-infected Jarrahdale pumpkin plants had mosaic symptoms (695K) or displayed a distinct yellow mosaic (694K), whereas the infected honeydew melon plant showed mosaic (697K) (Table 1). The plants from which two ZYMV isolates were obtained near Darwin in 2011 both had symptoms of leaf mosaic and deformation, and were honeydew melon (38NT) or rockmelon (75NT). Of the 22 samples collected at Broome in 2015, the seven ZYMV-infected samples detected by ELISA came from a commercial farm and an educational institution, and were from plants of zucchini (six samples) and Halloween pumpkin (one sample). The infected Halloween pumpkin plant displayed mosaic symptoms whereas, among the infected zucchini plants, five had leaf mosaic and deformation (Fig. 2A) and one had mosaic, chlorosis, reduction in leaf size, and plant stunting (Table 1).

Host responses to inoculation. In experiment 1, ZYMV induced vein clearing in young leaves followed by severe leaf mosaic and deformation in watermelon plants, and the virus was detected by ELISA in samples from leaves with symptoms. No ZYMV was detected in inoculated or tip leaf samples of *C. quinoa* or *N. benthamiana*. In experiments 2 and 3, the foliar symptoms induced in zucchini started with vein clearing in young leaves (Fig. 2B) followed by severe leaf mosaic and deformation, and fruit showed yellow mottle, malformation, and size reduction (Fig. 2C). In watermelon, systemic infection induced initial vein clearing followed by leaf mosaic and deformation (Fig. 2D). No symptoms ever developed in inoculated plants of *C. amaranticolor*, *C. quinoa*, *N. benthamiana*, or common bean. ZYMV was detected by ELISA in leaf samples from inoculated and tip leaves of zucchini and watermelon but not in similar leaf samples from the inoculated plants of the other four species. No symptoms ever developed in mock-inoculated plants of any species, and no virus was detected by ELISA in their leaf samples.

High-throughput sequence data. RNA-seq reads analysis of the ZYMV samples revealed that the numbers of raw paired-end reads obtained from both MiSeq and HiSeq 2500 platforms were 7,450,998 to 19,458,216; however, these numbers were reduced to 7,450,535 to 18,461,142 after quality control (Supplementary Table S2). Following *Denovo* assembly using CLCGW, the numbers of contigs produced were 4 to 2,284 and the number of reads mapping to the contig of interest were 24,569 to 2,738,665. The contig of interest lengths were 2,000 to 9,688 nucleotides (nt), with average coverage of 278 to 42,227 times. After mapping to reference genomes in Geneious, the lengths of the consensus sequences were 9,113 to 9,604 nt, with average coverage of 164 to 36,508 times.

The numbers of reads mapped to the reference sequence were 10,513 to 2,524,041. Final sequence lengths consisted of the consensus of the contig from CLCGW and the consensus from Geneious mapping, and were 9,309 to 9,656 nt. All of the sequenced samples had a Q30 of >98%. All the contigs of interest were closely related to ZYMV when subjected to BLAST using the PASC tool (Bao et al. 2014). All three Kununurra sequences were identical (694K, 695K, and 697K), as were four of the Broome (13Br, 17Br, 20Br, and 21Br) and the two East Timorese sequences from Aileu (TM39 and TM40).

Phylogenetic relationships. Of the new genomic sequences, all seven from Broome and all three from Kununurra were recombinants, as was one from East Timor (see below). When the coding regions of the 4 new nonrecombinant ZYMV sequences were compared with the 27 nonrecombinant, nonredundant sequences from other countries retrieved from GenBank, phylogenetic analysis

revealed clustering into two major phylogroups (A and B) (Fig. 3A). Major phylogroup A contained minor phylogroups I to V, which included the Darwin sequences and sequences from Asia, Europe, and North America. Minor phylogroups 1 to IV were consistent with the CP phylogroup classification of Coutts et al. (2011b) but minor phylogroup V was an additional one. Major phylogroup B consisted of only the two East Timorese and single sequence RU (L29569) from Reunion island in the Indian Ocean, close to Africa. When the coding regions of all 15 new complete genomes from Australia and East Timor were compared with those of the 33 genomes from other countries, the analysis again revealed clustering into major phylogroups A and B (Fig. 3B). Major phylogroup A contained minor phylogroups I to V and tentative additional minor phylogroup VI, which contained a single recombinant genome. Among the minor phylogroups in group A, the Australian sequences grouped as follows: phylogroup I consisted of the seven new Broome sequences and nine sequences from

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Fig. 3. Maximum-likelihood phylograms obtained from alignment of *Zucchini yellow mosaic virus* (ZYMV) genomic nucleotide sequences. **A**, Comparison between the coding regions of the two nonrecombinant complete genomes each from Australia and East Timor, and the 27 other nonrecombinant and nonredundant complete genomes available on GenBank. **B**, Comparison between the coding regions of the 12 and 3 complete new genomes from Australia and East Timor, respectively, and 33 other complete ZYMV genomes obtained from GenBank (recombinants and highly similar sequences included, apart from 18 almost identical U.S. sequences obtained by Simmons et al. [2012] during plant-to-plant transmission studies). Trees were created in MEGA 7.0.14 using Clustal W with 1,000 replicates. Bootstrap values are percentages, with only values over 60% shown at the nodes. They were rooted with *Bean common mosaic virus* nucleotide sequence KU896809. Symbols show the 12 and 3 newly sequenced isolates from East Timor (●) and Australia (■), respectively.

Europe, the Middle East, and South Asia, whereas phylogroup II contained the two new Darwin sequences and nine from the United States. Phylogroup III contained sequences from East Asia and phylogroup IV contained sequences from Europe and East Asia. Phylogroup V consisted of one sequence each from China and South America, and tentative new phylogroup VI contained a single sequence from East Asia. Major phylogroup B consisted of the three new sequences each from Kununurra and East Timor, the only other available Southeast Asian

sequence (SIN from Singapore), and sequence RU from Reunion Island.

When the 15 new ZYMV CP nucleotide sequences from East Timor and 20 other Australian sequences from GenBank were compared with 162 others from the rest of the world, the new Darwin CP sequences grouped together with the previous sequences from Darwin (Supplementary Fig. S1). The new Broome sequences all grouped together with the previous sequences from Carnarvon,

Fig. 3. Continued from previous page.

which is located 1,177 km further south in WA (Fig. 1). The three new CP sequences from Kununurra grouped together with the Kununurra sequences obtained previously. They also grouped together with the three new East Timorese CP sequences, CP sequence RU from Reunion Island, and two other CP sequences from Southeast Asia (SIN from Singapore and Cm3 from Vietnam).

Sequence identities. When the 63 complete ZYMV coding regions were analyzed, their nucleotide identities were $\geq 78.4\%$ across all sequences. Within major phylogroup A, the closest genetic matches to the Broome sequences were identical sequences B* and NAT from Israel, with 94.9% nucleotide identity to the four identical Broome sequences. The closest genetic match to the Darwin sequences was CAL from the United States, with 98.8% nucleotide identity to sequence 75NT. Within major phylogroup B, the paired sequence comparison nucleotide identities were as follows: SIN with RU, 82.7%; SIN with East Timor, $\geq 83.6\%$; SIN with Kununurra, 82.4%; RU with East Timor, $\geq 85.6\%$; and RU with Kununurra, 85.5%; East Timor with Kununurra, $\geq 87.1\%$. The closest genetic match with the three identical Kununurra sequences was with isolate TM40 from Aileu in East Timor, with a nucleotide identity of 88.9%. The Kununurra and East Timorese sequences had an amino acid identity of $>86.8\%$. Within the amino acid alignment, there was a substitution from aspartic acid to glutamic acid at one position in the CP gene.

The 12 new Australian CP sequences from Broome, Darwin, and Kununurra had an overall $\geq 85.9\%$ nucleotide identity. The closest genetic match between the two new Darwin sequences was 99.9% with the three previous identical Darwin sequences Nt-1, Nt-2, and Nt-3. The seven new Broome sequences had an overall $\geq 99.6\%$ nucleotide CP identity. The closest genetic match was 98.4% between sequence Cvn-18 from Carnarvon, WA, and both CP sequence 56Br and the four identical Broome CP sequences. The East Timorese CP sequences had $\geq 86.0\%$ overall nucleotide identity. The closest genetic match to the East Timorese CP sequences was the 95.5% nucleotide identity between sequence TM39 and previously sequenced virus isolate Knx-1 from Kununurra.

Fig. 4. Evidence for recombination between the coding regions of the 15 new isolates of *Zucchini yellow mosaic virus* (ZYMV) sequenced in this study and the 48 complete ZYMV genomes obtained from GenBank. Locations of genes in the ZYMV genome are indicated by the diagram at the top of the figure. Black shading shows the beginnings and ends of putative recombination breakpoints. Lack of shading designates the nonrecombined portions of each genome. Lettering on the right-hand side shows the names of each sequence with evidence of recombination. Six combinations of breakpoints were suggested by the RDP4 package.

Recombination detection. The RDP4 package detected six firm recombination events, each supported by five or more programs among 14 ZYMV isolates (Fig. 4; Table 2). All seven Broome sequences and three from Kununurra were recombinants. Among the three East Timorese sequences, only TM16 from Loes was recombinant. By contrast, only 3 of the 48 sequences from GenBank were recombinants, each with a separate event: WM from China, Z5-1 from Japan, and SIN from Singapore. All seven Broome sequences had one event involving the P1, HC-Pro, and P3 regions. Their major parent was 38NT and their minor parent was KR-PA from South Korea. The three Kununurra sequences had an entirely different event involving their Nib and CP regions. This was the only event detected by all seven RDP4 programs. Its major parent was TM16 and its minor parent TM39. East Timorese sequence TM16 had an event involving its P1 and HC-Pro regions. Its major parent was 694K from Kununurra and its minor parent TM39 from East Timor. Southeast Asian sequence SIN had an event in its CP region. It was the only sequence with parents in different major clades. These were major parent TM39 from the B clade and minor parent 1st (JN192428) from the A clade.

Discussion

This article describes the first complete ZYMV genomes obtained from cucurbit crops growing in tropical northern Australia and East Timor. Evidence of genetic connectivity between these countries' ZYMV populations was obtained by analyzing their sequence identities, phylogeny, and recombination patterns. The closest genetic match to the three identical 20-year old sequences from Kununurra in north-west Australia was a sequence from East Timor. Moreover, the Kununurra sequences grouped together with the three East Timorese sequences within major phylogroup B, previously called the Southeast Asian/Reunion Island phylogroup, which seems adapted to tropical conditions. Analysis of CP genes also gave similar results. This evidence of genetic connectivity is consistent with those of the CP study of Coutts et al. (2011b) mentioned above. Moreover, recombination analysis revealed that the three identical genomic sequences from Kununurra contained a single recombination event supported by all seven RDP4 programs that had East Timorese sequences as both its major and minor parents. Therefore, this body of evidence all supports the hypothesis that "important plant viral pathogens of economically important crops are arriving in tropical northern Australia by crossing the sea separating it from its northern neighbours". Unlike the new Kununurra genomic sequences, those from Broome and Darwin in northern Australia were both in major phylogroup A, within which the seven Broome and two Darwin sequences were in minor phylogroups I and II, respectively. There were no Southeast Asian genomic sequences in phylogroup A. These findings agree with the previous CP sequence study of Coutts et al. (2011b), suggesting three distinct ZYMV introductions to tropical northern Australia, where agriculture only commenced relatively recently. However, because they were all in phylogroup I, our new Broome sequences and previous CP sequences from other widely separated locations around Australia (Coutts et al. 2011b) might represent multiple introductions of very similar isolates. Zucchini and watermelon plants inoculated with an Australian isolate developed systemic foliage and fruit symptoms characteristic of ZYMV in plants growing under field conditions in

Table 2. Recombination between the coding regions of the 15 new *Zucchini yellow mosaic virus* isolates and 48 others from GenBank

Events	Sequence	Break points ^a	Genes affected	Parental sequences		Methods ^b	P value
				Major	Minor		
1	13Br, 14Br, 17Br, 19Br, 20Br, 21Br, 56Br	105–2,874	P1, HC-Pro, P3	38NT	AY278998	3,S,C,M,R,B	6.185×10^{-8R}
2	TM16	58–2,351	P1, HC-Pro	694K	TM39	3,S,C,M,R,B	7.172×10^{-08B}
3	Z5-1	4,072–5,943	CI, 6K2, VpG, N1a	AY278998	EF062582	S,C,M,G,R,B	8.359×10^{-06B}
4	SIN	8,563–9,225	CP	TM39	JN192428	3,S,C,M,R,B	8.898×10^{-06C}
5	694K, 695K, 697K	7,247–9,238	Nib, CP	TM16	TM39	3,S,C,M,G,R,B	6.364×10^{-31R}
6	WM	8,543–9,032	CP	AY278998	AY278999	S,C,M,G,R,B	8.553×10^{-09C}

^a Detected break points.

^b Programs within the RDP4 package: 3 = Seq, B = Bootscan, C = Chimera, G = Gencov, M = Maxchi, R = RDP, and S = Siscan.

northern Australia. This research has critical implications regarding entry of damaging viral pathogens into northern Australia and within Australia.

Within major phylogroup A, phylogenetic analysis of nonrecombinant, nonredundant sequences found the four minor phylogroups (I to IV) reported previously (Coutts et al. 2011b) but also revealed a new one (V). Although Coutts et al. (2011b) previously subdivided major phylogroup B into two based on CP sequence analysis, there were too few genomic sequences and insufficient sequence identity differences to subdivide it in our study. Australian sequences from Kununurra were closer to the three East Timorese sequences than to the other two sequences in phylogroup B. Analysis of CP sequences found the new Kununurra sequences grouped closely with the previous Kununurra sequences of Coutts et al. (2011b). The closest CP genetic match to a Kununurra sequence was to an East Timorese sequence (nucleotide identity of 95.5%). The new Darwin CP sequences grouped with the two previous NT CP sequences. The Broome CP sequences grouped closely with the previous CP sequences from Carnarvon. This closeness suggests the likelihood of a recent introduction from Carnarvon to Broome because, in large-scale surveys of cucurbit crops in 2003 to 2004, ZYMV was widespread at Carnarvon but absent from Broome (Coutts and Jones 2005). Discarded ZYMV-infected cucurbit fruit can introduce this virus to new locations (Lecoq et al. 2003); therefore, this introduction might have occurred by visitors traveling from Carnarvon to Broome, a major tourist resort. Of the four northern Australian cucurbit-growing regions (including Carnarvon), their remoteness from each other (Fig. 1) was reflected by the three genetically distinct populations found.

The close relationship between the complete genomic and CP sequences from Kununurra and East Timor suggest recent ZYMV introduction across the sea from Southeast Asia to Kununurra, where agriculture only commenced in the 1960s. Plausible routes for its introduction include via (i) viruliferous aphids blown by monsoonal winds, (ii) infected cucurbit seed carried in the intestines of migrating birds, or, (iii) as suggested previously for other potyviruses (Gibbs et al. 2010), possibly inadvertently when fishermen from nearby countries land for supplies and might discard virus-infected cucurbit fruit (Lecoq et al. 2003). Such virus introductions would not be intercepted by the stringent quarantine and surveillance procedures that Australian biosecurity authorities have in place currently that are designed to detect infections entering via imported seed or other plant propagules. Also, they might be expected to occur at an increasing frequency as the intensity of the monsoonal winds increases in future years due to climate change (Jones 2009, 2016; Jones and Barbeti 2012). Although evidence of other plant viruses arriving via windborne insect vectors is lacking, this is not the case for animal viruses; for example, incursions of Japanese encephalitis virus into northeast Australia (QLD) have occurred when windborne viruliferous mosquitos were blown from PNG (Eagles et al. 2013; Johansen et al. 2001, 2003). Based on more sequence information on dated isolates, in the future, it would be interesting to develop a phylogeographic approach to obtain more information.

Recombination provides an important method by which potyviruses evolve and more damaging strains arise (Gibbs et al. 2008; James et al. 2016; Jones 2009). The RDP program we used to detect recombination provides evidence for recombination and identifies putative recombination breakpoints. The frequency of recombination it identified among the 16 ZYMV genomes from northern Australia and Southeast Asia (75%) seems unexpectedly high when compared with the frequency among the 48 genomes from other parts of the world (4%). Because recombination constitutes an important means by which RNA viruses such as potyviruses adapt to changing environmental conditions and new hosts (Gibbs and Ohshima 2010; Kehoe et al. 2014b; Revers et al. 1996; Valli et al. 2007), the high recombination frequency suggested by the RPP4 program might reflect an evolutionary pathway enabling ZYMV to adapt to the tropical conditions encountered in these two regions. However, proof that it is adapted to tropical conditions would require a fitness comparison with isolates from other groups under both tropical and temperate conditions. The high recombination frequency might also allow them to infect alternative hosts not encountered previously (e.g., species

of Australia's unique native flora), enabling them to survive better outside the annual cucurbit-growing season. All seven genomic sequences from Broome were recombinants, the virus only arrived there recently (<12 years ago), and the major parent of the Broome sequence recombination event is from the same phylogroup as the CP sequences from Carnarvon reported previously (Coutts et al. 2011b). Also, based on CP sequences, Carnarvon sequences have the closest percent nucleotide identities to Broome sequences, and Carnarvon is also where ZYMV was first found in Australia in 1983 (Büchen-Osmond et al. 1988; Greber et al. 1987; McLean and Price 1984). However, complete genomes are lacking from Carnarvon so whether the recombination event found in Broome sequences occurred first in Carnarvon or before its introduction to Australia is unknown. All seven Broome sequences and the one from East Timor had firm recombination events that included their P1 regions but the Kununurra sequence included a firm event in the CP region at the opposite end of the genome. Potyviruses often have recombination events occurring in their P1, CI, and Vpg genomic regions but less often in their CP region (Desbiez and Lecoq 2008; Ohshima et al. 2007; Tan et al. 2004). These regions are associated with virus amplification, pathogenicity, movement, and symptom expression (Choi et al. 2013; James et al. 2016; Urcuqui-Inchima et al. 2001; Wen and Hajimorad 2010). Although Gal-On et al. (1998), who studied ZYMV recombination using cobombardment of truncated ZYMV clones and in silico analyses, suggested the presence of recombinants (Hasiów-Jaroszewska et al. 2013; Revers et al. 1996), ours appears to be the first recombination study using complete coding regions of isolates obtained directly from naturally infected cucurbit crops.

This study describes how occurrence of the unique ZYMV CP sequences found previously in samples from Kununurra (Coutts et al. 2011a) can now be explained by the presence of similar sequences in a nearby country, East Timor. Moreover, in the future, this might be found to be the case for other Australian virus sequences for which no similar sequences have been found locally. This highlights the need to sample cucurbit-growing areas in northern Australia to obtain more genomic ZYMV sequences, especially in tropical and subtropical QLD, where the cucurbit industry is largest. It also highlights the need for sampling and genomic analysis of ZYMV isolates from Indonesia and PNG. Such sequences could then be used to determine (i) whether any ZYMV introductions undetected thus far that involve its other phylogroups have occurred and (ii) whether there has been any recombination between them and members of ZYMV variants already present that might form more damaging new ZYMV strains. Stringent quarantine regulations directed at imported plants and plant products are playing a critical role in reducing the risk of further damaging viral pathogen incursions into Australia via airports, major seaports, and international mail centers. However, our findings with ZYMV suggest that the risk of entry via insect vectors or migrating birds blown by wind currents from the continents' northern neighbors is also cause for concern. Such concern would justify enhanced surveillance and monitoring to identify potentially damaging viral pathogens and their insect vectors entering cultivated areas of northern Australia from outside the country and moving between the isolated agricultural regions within Australia's north. More broadly, there is a need to follow up the hypothesis we tested here using a wind dispersion model to evaluate the likely distribution of viruliferous insect vectors blown over long distances by prevailing winds. Its findings would likely facilitate rapid action to eliminate or contain any new virus incursions detected. This would help sustain the current situation regarding absence from Australia of many viral pathogens, or their virulent strains, that cause damaging crop diseases in tropical and subtropical regions elsewhere in the world.

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