

Genetic characterization of Spondweni and Zika viruses and susceptibility of geographically distinct strains of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* (Diptera: Culicidae) to Spondweni virus

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1 **Abstract**

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3 **Background:** Zika virus (ZIKV) has extended its known geographic distribution to the
4 Western Hemisphere and is now responsible for severe clinical complications in a sub-set
5 of patients. While substantial genetic and vector susceptibility data exist for ZIKV, less is
6 known for its sister flavivirus, Spondweni virus (SPONV). Both ZIKV and SPONV have
7 been known to circulate in Africa since the mid-1900s, but neither has been genetically
8 characterized by gene and compared in parallel. Furthermore, the susceptibility of
9 cosmopolitan mosquito species incriminated or suspected in the transmission of ZIKV to
10 SPONV was unknown.

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12 **Methodology/Principal Findings:** In this study, two geographically distinct strains of
13 SPONV were genetically characterized and compared to nine genetically and
14 geographically distinct ZIKV strains. Additionally, the susceptibility of both SPONV
15 strains was determined in three mosquito species. The open reading frame (ORF) of the
16 SPONV 1952 Nigerian Chuku strain, exhibited a nucleotide and amino acid identity of
17 97.8% and 99.2%, respectively, when compared to the SPONV 1954 prototype South
18 African AR 94 strain. The ORF of the SPONV Chuku strain exhibited a nucleotide and
19 amino acid identity that ranged from 68.3%-69.0% and 74.6%-75.0%, respectively, when
20 compared to nine geographically and genetically distinct strains of ZIKV. The ORF of
21 the nine African and Asian lineage ZIKV strains exhibited limited nucleotide divergence.

22 *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* susceptibility and
23 dissemination was low or non-existent following artificial blood feeding of moderate
24 doses of both SPONV strains.

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26 **Conclusions/Significance:** SPONV and ZIKV nucleotide and amino acid divergence
27 coupled with differences in geographic distribution, ecology and vector species support
28 previous reports that these viruses are separate species. Furthermore, the low degree of
29 SPONV dissemination in *Ae. albopictus*, *Ae. aegypti*, and *Cx. quinquefasciatus* following
30 exposure to two geographically and genetically distinct virus strains suggest a low
31 potential for these species to serve as vectors.

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33 **Keywords**

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35 Spondweni virus, Zika virus, mosquito, arbovirus, sylvatic, febrile, host range

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45 **Author Summary**

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47 Spondweni virus (SPONV) is a mosquito-transmitted flavivirus reported in Africa.
48 Human infection with SPONV may result in a febrile illness similar to symptomatic Zika
49 virus (ZIKV) infection, as well as many other tropical infections. Previously, little was
50 known about the genetic relationships between SPONV and ZIKV. Additionally, the
51 ability of SPONV to infect cosmopolitan mosquito species associated or incriminated in
52 ZIKV transmission was unknown. Both SPONV strains exhibited a high degree of
53 nucleotide and amino acid identity to each other, but considerable nucleotide and amino
54 acid divergence to ZIKV. The open reading frame (ORF) of the nine African and Asian
55 lineage ZIKV strains originally isolated in West Africa, Central Africa, East Africa,
56 Southeast Asia, the Pacific Islands, and the Western Hemisphere all exhibited limited
57 nucleotide divergence. Both strains of SPONV exhibited a low degree of infection and
58 dissemination in *Aedes albopictus*, *Ae. aegypti*, and *Culex quinquefasciatus* mosquitoes
59 suggesting that these species have a low potential to serve as vectors. These results
60 coupled with differences in geographic distribution, ecology and vector species indicate
61 that SPONV and ZIKV are similar but separate species.

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68 **Introduction**

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70 The Spondweni serogroup, genus *Flavivirus* (*Flaviviridae*), includes two species – Zika
71 virus (ZIKV) and Spondweni virus (SPONV) [1]. Both ZIKV and SPONV are associated
72 with human illness [2]. SPONV can cause a self-limiting febrile illness characterized by
73 headache, myalgia, nausea, and arthralgia [3-6], signs and symptoms similar to most
74 reported symptomatic ZIKV infections [7-12], making diagnosis challenging in those
75 regions of Africa with virus co-circulation. Although SPONV is not typically associated
76 with serious disease, a sub-set of patients report: conjunctivitis, macropapular and pruritic
77 rash suggestive of vascular leakage; while reports of headache, vertigo, photophobia,
78 disorientation, bilateral transient ocular paresis, and meningismus point to neurological
79 involvement [3-6,13]. The close genetic relationship and the similarity in those signs and
80 symptoms observed in typical ZIKV infections suggest the possibility of a low incidence
81 of more severe neurologic disease.

82

83 In 1952, the Chuku strain of SPONV was isolated from the blood of a febrile patient in
84 Nigeria [5]. This strain was initially misclassified as ZIKV [14], leading to the 1955
85 South African AR 94 *Mansonia uniformis* mosquito isolate being classified as the
86 prototype SPONV strain [15]. Since its initial isolation, SPONV activity has been
87 reported throughout sub-Saharan Africa (Table 1). In nature the virus is likely maintained
88 in a zoonotic primate/mosquito cycle like that of ZIKV [14], and has been isolated from
89 several mosquito genera (Table 1).

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91 Like other flaviviruses SPONV has a positive-sense single stranded RNA genome of
92 approximately 11 kilobases in length [16]. The genome contains 5' and 3' untranslated
93 regions flanking a single open reading frame (ORF) that encodes a polyprotein that is
94 cleaved into three structural proteins: the capsid (C), premembrane/membrane (prM), and
95 envelope (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K,
96 NS4B, and NS5) [16].

97

98 We further characterize SPONV strains and investigate their potential for urban
99 emergence as seen with ZIKV as well as with other flaviviruses including yellow fever
100 and dengue viruses [17,18]. We determined the genetic relationship between the
101 prototype South African AR 94 and the Nigerian Chuku sequences of SPONV and
102 compared those sequence data to nine geographically and genetically distinct strains of
103 ZIKV. We also determined the susceptibility of both SPONV strains to three mosquito
104 species that have been incriminated or suspected in the transmission of ZIKV: *Aedes*
105 *aegypti* [19-21], *Ae. albopictus* [19,22], and *Culex quinquefasciatus* (C. F. Junqueira
106 Ayres pers. comm.).

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109 **Methods**

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111 Virus strains and virus propagation

112 Virus strains were obtained from the World Reference Center of Emerging Viruses and
113 Arboviruses Collection at the University of Texas Medical Branch in Galveston, Texas.

114 Both the South African AR 94 and Nigerian Chuku strains prior passage histories were
115 unknown and therefore could exhibit passage-associated mutations. For this study, each
116 virus was passaged once in *Ae. albopictus* cells (C6/36; ATCC #CCL-1660) for
117 sequencing, and subsequently passaged once in African green monkey kidney cells
118 (Vero; ATCC #CCL-81) for vector susceptibility experiments (virus stocks frozen at -
119 80°C).

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121 RNA preparation, genomic amplification, and sequencing

122 Viral RNA was extracted from cell culture supernatant using the QIAamp Viral RNA Kit
123 (Qiagen, Valencia, CA, USA). Overlapping primer pairs were used to amplify the entire
124 open reading frame (ORF) using the Titan OneStep RT-PCR kit (Roche, Mannheim,
125 Germany) and purified amplicons were directly sequenced using the Applied Biosystems
126 BigDye Terminator version 3.1 Cycle Sequencing Kit (Foster City, CA, USA) and the
127 Applied Biosystems 3100 Genetic Analyzer (Foster City, CA, USA). Nucleotide
128 sequences derived from both SPONV strains were assembled in Vector NTI Suite
129 (Invitrogen, Carlsbad, CA, USA), aligned in SeaView [23] using MUSCLE [24], and
130 edited in MacVector (Apex, NC, USA). These consensus sequences were deposited in
131 GenBank, SPONV Chuku accession no. KX227369 and SPONV AR 94 accession no.
132 KX227370.

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134 Genetic analyses

135 ZIKV strains currently fall into either the African or Asian lineages [11,25]; as such nine
136 geographically and genetically distinct sequences (i.e. strains) were used as representative

137 members of these lineages for nucleotide and amino acid comparisons with both SPONV
138 strains. The selected strains were isolated in West Africa (n = 1), Central Africa (n = 1),
139 East Africa (n = 1), Southeast Asia (n= 2), the Pacific Islands (n = 2), and the Western
140 Hemisphere (n = 2). These strains include the prototype strain MR-766 (Uganda 1947)
141 GenBank accession no. AY632535 [16]; ArB 13565 (Central African Republic 1976)
142 GenBank accession no. KF268948.1 [26]; ArD 41519 (Senegal 1984) GenBank
143 accession no. HQ234501.1 [11]; P6-740 (Malaysia 1968) GenBank accession no.
144 HQ234499 [11]; CPC-0740 (Philippines 2010) GenBank accession no. KM851038.1; EC
145 Yap (Yap Island 2007) GenBank accession no. EU545988.1 [27]; H/FP/2013 (French
146 Polynesia 2013) GenBank accession no. KJ776791.1 [28]; Z1106033 (Suriname 2015)
147 GenBank accession no. KU312312.1 [29]; and PRVABC59 (Puerto Rico 2015) GenBank
148 accession no. KU501215.1 [30]. The MR-766 sequence used in these analyses exhibited
149 a deletion the potential glycosylation site that has been noted previously [11,16].

150

151 Mosquito rearing, maintenance, and artificial infectious blood feeds

152 Three geographically distinct strains of both *Ae. albopictus* and *Ae. aegypti*, and one
153 strain of *Cx. quinquefasciatus* were used to determine susceptibility (Table 3).
154 Mosquitoes were reared and maintained during experiments using a 12:12 hour light/dark
155 photoperiod in approximately 80% relative humidity, and adult mosquitoes were
156 provided a 10% sucrose solution via a cotton ball. Four- to seven-day-old female
157 mosquitoes were sugar starved for 24 hours prior to infectious blood meal feeding, with
158 *Ae. albopictus* and *Cx. quinquefasciatus* having access to deionized water up to 12 hours
159 prior to feeding to reduce physiological stress.

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161 Mosquito infections were performed in an Arthropod Containment Level-3 (ACL3)
162 laboratory following the guidelines set forth under the Biosafety in Microbiological and
163 Biomedical Laboratories (BMBL) 5th Edition Appendix E (Arthropod Containment
164 Guidelines). Groups of 100 mosquitoes were allowed to feed from artificial membrane
165 feeders (Discovery Workshops, Lancashire, UK) covered by rat skins and containing a
166 suspension of one part defibrinated sheep blood (Colorado Serum Company, Denver, CO,
167 USA) and virus. Blood meal titers were 5.1 (Chuku) and 5.3 (AR 94) \log_{10} PFU/mL. Post
168 feeding, mosquitoes were sorted on ice and fully engorged individuals meeting the
169 criteria for stages 4 to 5 were retained [31].

170

171 Mosquito processing and virus assay

172 On day 14 post-feeding, mosquitoes were chilled for immobilization, then dissected,
173 pooled and homogenized (legs/wings and body separately) in a tubes containing a steel
174 BB and 500 μ l of media [Dulbecco's Modified Eagle Medium supplemented with 20%
175 (vol/vol) fetal bovine serum, 100 U/ml of penicillin, 100 μ g/ml of streptomycin, and
176 0.5 mg/ml amphotericin B (Sigma Aldrich, St. Louis, MO, USA)], and frozen at -80°C.
177 Pools were assayed on C6/36 cells for the presence of SPONV antigen by an indirect
178 fluorescent antibody (IFA) test using hyperimmune mouse ascitic fluid (HMAF) directed
179 against the SPONV Chuku strain and a commercial fluorescein isothiocyanate-
180 conjugated goat antimouse immunoglobulin G (Sigma Aldrich, St. Louis, MO, USA)
181 [32,33].

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183

184 **Results**

185

186 Genetic analysis

187 The complete sequence of both SPONV strains was translated and aligned with selected
188 ZIKV strains. The ORF of SPONV AR 94 and Chuku strains displayed >98% nucleotide
189 and amino acid identity to each other, whereas they displayed ~68% and ~75% percent
190 nucleotide and amino acid identity to ZIKV. Next we compared nucleotide and amino
191 acid identity in the individual genes of SPONV and ZIKV. The lengths of individual
192 genes were determined by utilizing putative cleavage sites of ZIKV genes. The individual
193 SPONV gene sizes were similar to ZIKV genes: Capsid, prM, NS1, NS4A, and NS5 were
194 identical, whereas the E (505 vs. 504 amino acid), NS2A (226 vs. 217 amino acid), NS2B
195 (130 vs. 122 amino acid), NS3 (619 vs. 617 amino acid) and NS4B (255 vs. 251 amino
196 acid) were larger than ZIKV. The individual structural gene comparison of SPONV and
197 ZIKV showed nucleotide and amino acid identity ranging from 61% to 68% and 64% to
198 72%, respectively, with the E gene displaying greater sequence identity (68% nucleotide
199 and 72% amino acid). The nonstructural gene comparison displayed nucleotide and
200 amino acid identity ranging from 59% to 73% and 58% to 82%, respectively. The NS4B
201 and NS3 genes displayed the greater identity, 70% to 72% nucleotide and 81-82% amino
202 acid. The NS2A gene was the most divergent gene with 59% to 60% nucleotide and 58%
203 to 59% amino acid identity between SPONV and ZIKV.

204

205 Mosquito infection and dissemination

206 Exposure to the SPONV Chuku strain by artificial blood meal did not result in any
207 infection or dissemination in any of the three mosquito species (Table 2). Exposure to the
208 SPONV AR 94 strain by artificial blood meal resulted infection in 8.3% of *Ae. aegypti*
209 (Galveston) and 12.5% *Ae. aegypti* (Thailand), while only *Ae. aegypti* (Galveston)
210 developed disseminated infection (8.3%).

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212

213 **Discussion**

214

215 Prior to this study, one SPONV strain had been sequenced, but its geographic origin and
216 passage history was not reported [34]. Our analyses demonstrated that both SPONV
217 strains sequenced in this study (Chuku and AR 94) are genetically similar, but exhibit a
218 high degree of nucleotide and amino acid divergence when compared to ZIKV strains
219 from West Africa, East Africa, Southeast Asia, the Pacific Islands, and the Western
220 Hemisphere (Fig. 1). The similarity between the two SPONV strains isolated in different
221 geographic regions approximately 2.5 years apart may indicate the possibility of
222 continuous enzootic transmission and maintenance between Nigeria and South Africa,
223 although interpretation is limited due to the lack of spatial and temporally spaced
224 sequences (i.e. multiple isolates). ZIKV strains within each lineage, African and Asian,
225 also exhibited a low degree of nucleotide divergence when compared to one another, as
226 seen in previous work [11]. With the exception of the MR-766 ZIKV strain, neither
227 SPONV strain nor any of the other eight ZIKV strains used in this study exhibited a

228 deletion in the potential N-linked glycosylation site as reported in some ZIKV strains that
229 had prior passage histories in mouse brains [11,16,26,35].

230

231 The susceptibility and dissemination to moderate doses of both SPONV strains in all
232 three species was low or non-existent (Table 3). The Chuku strain did not cause any
233 infection/dissemination in any of the species, while the AR 94 strain was only observed
234 to cause disseminated infection in *Ae. albopictus* Galveston (8.3%). Work by Bearcroft
235 also failed to show transmission of the Chuku strain by *Ae. aegypti* [3]. Unlike SPONV,
236 *Ae. aegypti* and *Ae. albopictus* have been incriminated as vectors of ZIKV [19-22] and
237 recently *Cx. quinquefasciatus* has been discussed as a potential vector in Brazil (C. F.
238 Junqueira Ayres pers. comm.). Early work demonstrated that *Ae. aegypti* was a
239 competent vector of ZIKV following feeding on an artificial blood meal containing the
240 MR-766 prototype strain, with three mosquitoes transmitting ZIKV to a single rhesus
241 monkey 72 days post-exposure [21]. Since that time, several studies have shown that
242 various geographically distinct strains of *Ae. aegypti* or *Ae. albopictus* mosquitoes
243 exposed to ZIKV strains from either the African and Asian lineages exhibit a wide range
244 of susceptibility and/or vector competence in these two mosquito species [19,20,22,36].
245 Caution should be exercised regarding the over interpretation of the results of vector
246 susceptibility/competence studies, as variation in vector competence between
247 geographically distinct mosquito populations has been reported in other arboviruses [37].
248 Also, many of these studies used very high passage ZIKV strains.

249

250 Unlike its sister ZIKV, which has a broad geographic distribution, SPONV isolations and
251 seroprevalence have thus far been confined to Africa (Table 1) [11,25,38]. While it is
252 possible that the difference in the geographic distribution between ZIKV and SPONV is a
253 result of prior infection with ZIKV or SPONV resulting in a refractory status among
254 amplification hosts, another explanation is there are differences in the vector species
255 between these two viruses. Intensive mosquito collections and subsequent virus isolation
256 attempts over a number of years by laboratories in sub-Saharan Africa yielded isolations
257 of SPONV from eight species of mosquitoes in the genera *Aedes*, *Culex*, *Eretmapodites*,
258 and *Mansonia* (Table 1), while ZIKV has been isolated in 20 species in the genera *Aedes*,
259 *Anopheles*, *Eretmapodites*, and *Mansonia* [11]. Although many of these species are
260 found in the same regions where both SPONV and ZIKV have been isolated, both viruses
261 have only been isolated in 2 species, *Ae. fowleri* and *Ma. uniformis*. Further studies are
262 needed to determine the potential for sylvatic mosquito species to transmit both ZIKV
263 and SPONV.

264

265 Previous to the Ninth Report of the International Committee on the Taxonomy of Viruses
266 (ICTV) [39], SPONV was considered a species of the Genus *Flavivirus*: Family
267 *Flaviviridae*, and both SPONV and ZIKV were considered members of the Spondweni
268 Serogroup [2]. According to the current report, SPONV has now been categorized as a
269 member of the genus *Flavivirus* that has not been approved as a species. SPONV clearly
270 exhibits a greater nucleotide (~ 32%) and amino acid (~25%) divergence from its sister
271 virus – ZIKV as has been previously reported (Fig. 1) [26]. This is particularly evident
272 when comparing individual proteins rather than the entire ORF (Figs 2, 3, 4).

273 Comprehensive historic work using neutralization, hemagglutination-inhibition,
274 complement fixation, and antibody absorption tests also differentiate SPONV and ZIKV
275 as distinct viruses based on limited cross-reactivity [2,14,40,41]. Furthermore, both
276 viruses exhibit differences in vector associations, ecology, and geographic distribution.
277 These data suggest that although both SPONV and ZIKV are related, they are separate
278 species.

279

280 In conclusion, this study determined the genetic relationship between two sequences of
281 SPONV, as well as to nine representative African and Asian lineage ZIKV strains. The
282 SPONV Chuku and AR 94 strains exhibited poor infection and dissemination in *Ae.*
283 *aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes, indicating a low potential
284 for these species to serve as vectors and probably limited emergence potential into urban
285 cycles characteristic of ZIKV, yellow fever virus, and dengue viruses. Nucleotide and
286 amino acid divergence coupled with differences in geographic distribution, ecology and
287 vector species support previous reports that SPONV and ZIKV are separate species.

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300 **Disclosure Statement**

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302 policy or position of the U.S. Department of Defense, the Department of the Army,
303 Centers for Disease Control and Prevention, or the Kingdom of Thailand.

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502 Table 1. Reported geographic distribution of Spondweni virus*.
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Country	Seroprevalence [†] (Humans)	Virus isolation (Human)	Virus isolation (Mosquito)	Reference(s)
Angola	X			[42]
Botswana	X			[43]
Burkina Faso	X			[13]
Cameroon	X	X	<i>Eretmapodites spp.</i>	[13,44]
Ethiopia	X			[45]
Gabon	X			[13]
Mozambique	X		<i>Aedes fryeri/fowleri</i>	[46]
Namibia	X			[43]
Nigeria	X	X		[5]
South Africa	X		<i>Ae. circumluteolus, Ae. cummingsi, Culex neavi, Cx. univittatus, Er. silvestris, Mansonia africana, Ma. uniformis</i>	[6,15,47,48]

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*Does not include laboratory acquired infections.
[†] Seroprevalence was determined by one or more of the following methods: Haemagglutination inhibition, neutralization, and/or complement-fixation.
 Of note, it is possible due to antigenic cross-reactivity among flaviviruses that seropositive individuals may have been previously exposed to one or more flaviviruses and not to Spondweni virus.

543 Table 2. Mosquito susceptibility to Spondweni virus.

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545 A. Susceptibility of selected mosquito species to Spondweni Chuku strain, dose 5.1

546 \log_{10} PFU/mL.

Mosquito (origin)	No.	Infection, no. (%)	Dissemination, no. (%)
<i>Aedes aegypti</i> (Galveston, USA)	19	0 (0.0)	0 (0.0)
<i>Aedes aegypti</i> (Iquitos, Peru)	20	0 (0.0)	0 (0.0)
<i>Aedes aegypti</i> (Thailand)	4	0 (0.0)	0 (0.0)
<i>Aedes albopictus</i> (Galveston, USA)	24	0 (0.0)	0 (0.0)
<i>Aedes albopictus</i> (Thailand)	12	0 (0.0)	0 (0.0)
<i>Aedes albopictus</i> (Venezuela)	3	0 (0.0)	0 (0.0)
<i>Culex quinquefasciatus</i> (Galveston, USA)	24	0 (0.0)	0 (0.0)

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548 B. Susceptibility of selected mosquito species to Spondweni AR 94 strain, dose 5.3

549 \log_{10} PFU/mL.

Mosquito	No.	Infection, no. (%)	Dissemination, no. (%)
<i>Aedes aegypti</i> (Galveston, USA)	24	0 (0.0)	0 (0.0)
<i>Aedes aegypti</i> (Iquitos, Peru)	24	0 (0.0)	0 (0.0)
<i>Aedes aegypti</i> (Thailand)	24	1 (4.2)	0 (0.0)
<i>Aedes albopictus</i> (Galveston, USA)	24	2 (8.3)	2 (8.3)
<i>Aedes albopictus</i> (Thailand)	24	3 (12.5)	0 (0.0)
<i>Aedes albopictus</i> (Venezuela)	24	0 (0.0)	0 (0.0)
<i>Culex quinquefasciatus</i> (Galveston, USA)	24	0 (0.0)	0 (0.0)

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567 **Figure 1. Genome structure and pairwise comparison of the open reading frame**
 568 **(ORF) of Spondweni (SPONV) and Zika (ZIKV) viruses.***

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 570 A) SPONV genome organization: capsid (C), premembrane/membrane (prM), envelope
 571 (E), NS1, NS2A, NS2B, NS3, NS4A, 2K (not shown), NS4B, and NS5. Numbers
 572 indicate amino acids in each protein.

573
 574 B) Pairwise comparison of the ORF of SPONV and ZIKV strains. SPONV AR 94;
 575 SPONV Chuku; ZIKV MR-766; ZIKV ArB 13565; ZIKV ArD 41519; ZIKV P6-740;
 576 ZIKV CPC-0740; ZIKV EC Yap; ZIKV H/PF/2013; ZIKV Z1106033; ZIKV
 577 PRVABC59. *Boldface type (upper diagonal) = Percent amino acid identity; Lightface
 578 type (lower diagonal) = Percent nucleotide identity.

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 580 **Figure 2. Pairwise comparison of the structural proteins of Spondweni (SPONV)**
 581 **and Zika (ZIKV) viruses.***

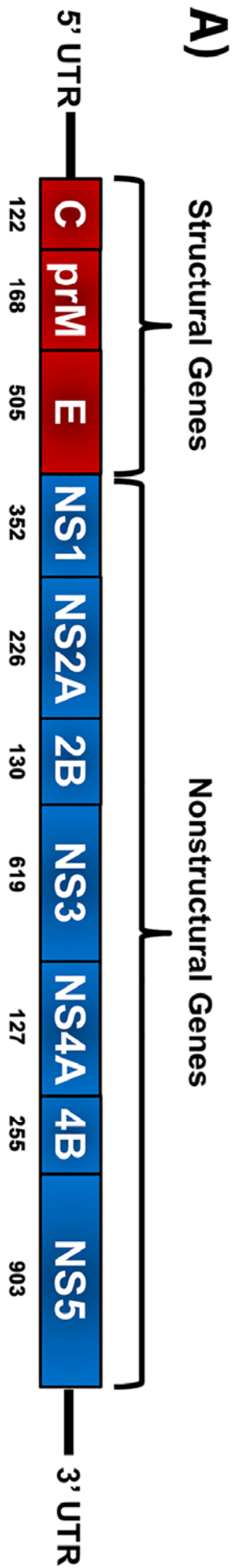
582
 583 Capsid (C), premembrane/membrane (prM), and envelope (E). SPONV AR 94; SPONV
 584 Chuku; ZIKV MR-766; ZIKV ArB 13565; ZIKV ArD 41519; ZIKV P6-740; ZIKV CPC-
 585 0740; ZIKV EC Yap; ZIKV H/PF/2013; ZIKV Z1106033; ZIKV PRVABC59.
 586 *Boldface type (upper diagonal) = Percent amino acid identity; Lightface type (lower
 587 diagonal) = Percent nucleotide identity.

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 590 **Figure 3. Pairwise comparison of the non-structural proteins NS2b, NS3, and NS5 of**
 591 **Spondweni (SPONV) and Zika (ZIKV) viruses.***

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 593 SPONV AR 94; SPONV Chuku; ZIKV MR-766; ZIKV ArB 13565; ZIKV ArD 41519;
 594 ZIKV P6-740; ZIKV CPC-0740; ZIKV EC Yap; ZIKV H/PF/2013; ZIKV Z1106033;
 595 ZIKV PRVABC59. *Boldface type (upper diagonal) = Percent amino acid identity;
 596 Lightface type (lower diagonal) = Percent nucleotide identity.

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 599 **Figure 4. Pairwise comparison of the non-structural proteins NS1, NS2a, NS4a, and**
 600 **NS4b of Spondweni (SPONV) and Zika (ZIKV) viruses.***

601
 602 SPONV AR 94; SPONV Chuku; ZIKV MR-766; ZIKV ArB 13565; ZIKV ArD 41519;
 603 ZIKV P6-740; ZIKV CPC-0740; ZIKV EC Yap; ZIKV H/PF/2013; ZIKV Z1106033;
 604 ZIKV PRVABC59. *Boldface type (upper diagonal) = Percent amino acid identity;
 605 Lightface type (lower diagonal) = Percent nucleotide identity.



B)

ORF	AR 94	Chuku	MR-766	A/B 13565	A/D 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	99.2	74.6	75.0	74.9	74.8	74.9	74.6	74.8	74.8	74.7
<i>Chuku</i>	97.8	-	74.6	75.0	74.9	74.8	75.0	74.7	74.9	74.8	74.8
MR-766	68.5	68.5	-	98.4	98.4	97.0	96.4	96.4	96.5	96.4	96.4
A/B 13565	68.9	68.8	94.8	-	98.9	97.6	97.2	96.8	97.2	97.1	97.1
A/D 41519	69.0	69.0	93.1	93.2	-	97.4	97.0	96.7	97.1	97.0	97.0
P6-740	68.4	68.5	89.8	90.0	90.0	-	98.9	98.6	98.9	98.9	98.9
CPC-0740	68.2	68.3	88.4	88.6	88.5	95.6	-	99.2	99.4	99.4	99.4
EC YAP	68.2	68.3	88.7	88.6	88.6	95.8	98.2	-	99.2	99.1	99.1
H/PF/2013	68.3	68.4	88.5	88.7	88.6	95.6	97.9	98.1	-	99.9	99.9
Z1106033	68.2	68.3	88.4	88.7	88.5	95.4	97.6	97.9	99.7	-	99.9
PRVABC59	68.2	68.3	88.5	88.8	88.6	95.5	97.6	97.9	99.7	99.7	-

	AR 94	Chuku	MR-766	ARB 13565	A/D 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	97.5	64.5	66.1	66.1	66.9	66.9	65.3	66.1	66.1	65.3
Chuku	96.4	-	65.3	66.9	66.9	67.7	67.7	66.1	66.9	66.9	66.1
MR-766	63.4	64.2	-	95.1	94.3	90.2	90.2	94.3	91.1	91.1	90.2
ARB 13565	65.1	65.3	94.3	-	98.4	95.1	95.1	89.4	95.9	95.9	95.1
A/D 41519	64.5	65.3	91.3	94.0	-	95.1	95.1	90.2	95.9	95.9	95.1
P6-740	64.8	65.1	89.7	92.1	92.1	-	100.0	94.3	99.2	99.2	98.4
CPC-0740	64.8	65.6	89.2	91.3	91.3	96.7	-	94.3	99.2	99.2	98.4
EC YAP	64.2	65.1	91.9	88.6	87.8	94.6	95.7	-	93.5	93.5	92.7
H/PF/2013	65.3	65.6	88.9	91.6	90.8	97.0	98.1	95.9	-	100.0	99.2
Z1106033	65.3	65.6	88.9	91.6	90.8	97.0	98.1	95.9	100.0	-	99.2
PRVABC59	65.1	65.3	88.6	91.3	90.5	96.7	97.8	95.7	99.7	99.7	-

DRM

	AR 94	Chuku	MR-766	ARB 13565	A/D 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	99.4	64.3	63.7	64.3	64.3	64.9	64.3	64.3	63.7	64.3
Chuku	98.0	-	64.9	64.3	64.9	64.9	65.5	64.9	64.9	64.3	64.9
MR-766	62.7	62.3	-	98.8	100.0	95.2	94.0	94.6	94.0	93.4	94.0
ARB 13565	61.9	61.5	94.8	-	98.8	95.2	94.0	94.6	94.0	93.4	94.0
A/D 41519	62.3	63.1	91.8	91.2	-	95.2	94.0	94.6	94.0	93.4	94.0
P6-740	61.7	62.5	88.8	88.0	86.0	-	98.8	99.4	98.8	98.2	98.8
CPC-0740	61.9	63.1	87.2	87.0	85.0	95.6	-	99.4	98.8	98.2	98.8
EC YAP	61.3	62.9	88.0	87.8	85.8	96.0	98.0	-	99.4	98.8	99.4
H/PF/2013	61.3	62.5	88.0	87.4	85.8	95.6	96.4	97.2	-	99.4	100.0
Z1106033	60.9	62.1	88.0	87.4	85.8	95.6	96.4	97.2	99.6	-	99.4
PRVABC59	61.1	62.3	88.2	87.6	86.0	95.6	96.4	97.2	99.6	99.6	-

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	AR 94	Chuku	MR-766	ARB 13565	A/D 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	99.2	71.5	72.7	72.5	72.5	72.5	72.3	72.5	72.7	72.5
Chuku	97.8	-	71.5	72.9	72.5	72.1	72.3	72.1	72.3	72.5	72.3
MR-766	69.1	68.4	-	98.2	98.4	96.8	96.8	96.6	96.4	96.2	96.4
ARB 13565	69.6	69.0	94.0	-	99.6	98.0	98.0	97.8	97.6	97.4	97.6
A/D 41519	68.6	68.4	92.3	93.0	-	98.2	98.4	98.2	98.0	97.8	98.0
P6-740	67.9	67.7	89.0	90.3	89.5	-	99.4	99.2	99.0	98.8	99.0
CPC-0740	68.3	68.0	87.3	88.2	88.1	95.5	-	99.8	99.6	99.4	99.6
EC YAP	68.0	67.5	87.7	88.9	88.5	96.1	98.5	-	99.4	99.2	99.4
H/PF/2013	68.4	67.9	87.7	89.0	88.4	95.9	98.3	98.7	-	99.8	100.0
Z1106033	68.5	68.0	87.4	88.8	88.0	95.4	97.9	98.3	99.4	-	99.8
PRVABC59	68.3	67.8	87.6	88.9	88.1	95.6	98.1	98.5	99.6	99.5	-

NS2b

	AR 94	Chuku	MR-766	ARB 13565	ARD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRV/ABC59
AR 94	-	100.0	76.2	76.2	76.2	76.9	77.7	76.9	76.9	76.9	76.9
Chuku	99.2	-	76.2	76.2	76.2	76.9	77.7	76.9	76.9	76.9	76.9
MR-766	72.1	72.3	-	100.0	100.0	98.5	97.7	98.5	98.5	98.5	98.5
ARB 13565	72.8	73.1	95.1	-	100.0	98.5	97.7	98.5	98.5	98.5	98.5
ARD 41519	71.8	72.1	94.9	94.6	-	98.5	97.7	98.5	98.5	98.5	98.5
P6-740	71.5	72.3	91.5	93.1	92.6	-	99.2	100.0	100.0	100.0	100.0
CPC-0740	72.3	73.1	90.0	91.0	91.0	95.1	-	99.2	99.2	99.2	99.2
EC YAP	71.8	72.6	90.3	90.8	91.3	95.4	98.2	-	100.0	100.0	100.0
H/PF/2013	72.3	73.1	89.7	90.8	90.8	95.4	97.2	96.9	-	100.0	100.0
Z1106033	72.3	73.1	89.7	90.8	90.8	95.4	97.2	96.9	100.0	-	100.0
PRV/ABC59	72.3	73.1	89.7	90.8	90.8	95.4	97.2	96.9	100.0	100.0	-

NS3

	AR 94	Chuku	MR-766	ARB 13565	ARD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRV/ABC59
AR 94	-	99.7	81.1	81.4	81.3	81.4	81.3	81.1	81.3	81.3	81.3
Chuku	98.2	-	80.9	81.3	81.1	81.3	81.1	80.9	81.1	81.1	81.1
MR-766	71.9	71.9	-	99.7	99.0	98.7	98.1	98.1	98.2	98.2	98.2
ARB 13565	72.3	72.1	95.4	-	99.4	99.0	98.4	98.4	98.5	98.5	98.5
ARD 41519	72.3	72.3	93.7	93.5	-	98.4	97.7	97.7	97.9	97.9	97.9
P6-740	71.5	71.9	90.9	90.7	90.7	-	99.4	99.4	99.5	99.5	99.5
CPC-0740	70.6	70.9	88.8	88.6	89.0	95.8	-	99.0	99.2	99.2	99.2
EC YAP	70.8	71.2	88.8	88.7	89.1	96.3	98.2	-	99.2	99.2	99.2
H/PF/2013	70.8	71.1	88.9	88.9	89.3	96.3	97.5	98.1	-	100.0	100.0
Z1106033	70.8	71.0	88.8	88.8	89.0	96.1	97.2	97.9	99.7	-	100.0
PRV/ABC59	70.8	71.0	89.0	88.9	89.2	96.1	97.3	97.9	99.8	99.6	-

NS5

	AR 94	Chuku	MR-766	ARB 13565	ARD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRV/ABC59
AR 94	-	98.6	77.6	77.6	77.6	77.1	77.3	77.2	77.3	77.2	77.1
Chuku	97.7	-	77.5	77.5	77.5	77.2	77.6	77.5	77.6	77.5	77.4
MR-766	69.8	69.7	-	97.9	98.7	96.5	96.0	95.9	96.0	95.9	95.8
ARB 13565	70.0	69.8	94.9	-	98.6	96.7	96.6	96.5	96.6	96.5	96.3
ARD 41519	70.6	70.4	93.5	93.1	-	96.7	96.5	96.3	96.5	96.3	96.2
P6-740	70.0	70.0	89.7	89.2	90.0	-	98.3	98.2	98.3	98.3	98.2
CPC-0740	69.9	69.9	88.3	88.1	88.2	95.2	-	99.9	99.8	99.7	99.6
EC YAP	69.8	69.7	88.6	88.1	88.5	95.7	98.6	-	99.9	99.8	99.7
H/PF/2013	70.0	70.1	88.2	87.9	88.3	95.2	98.0	98.6	-	99.9	99.8
Z1106033	69.8	69.9	88.2	87.9	88.3	95.0	97.7	98.4	99.8	-	99.9
PRV/ABC59	69.9	70.0	88.3	88.0	88.4	95.1	97.7	98.4	99.7	99.9	-

NS1

	AR 94	Chuku	MR-766	ArB 13565	ArD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	99.4	74.1	75.0	74.4	74.1	74.4	74.1	74.4	74.4	74.4
Chuku	97.3	-	73.9	74.7	74.1	73.9	74.1	73.9	74.1	74.1	74.1
MR-766	67.9	68.8	-	98.3	98.0	97.4	97.4	96.6	97.4	97.4	97.4
ArB 13565	68.6	69.5	95.5	-	98.9	98.0	98.3	97.4	98.3	98.3	98.3
ArD 41519	69.1	69.6	93.3	93.7	-	97.7	98.0	97.2	98.0	98.0	98.0
P6-740	69.1	69.4	90.8	90.1	90.1	-	99.4	99.1	99.4	99.4	99.4
CPC-0740	68.7	68.8	90.1	89.5	89.3	95.3	-	99.1	99.4	99.4	99.4
EC YAP	68.7	68.9	90.0	89.4	89.4	95.4	98.8	-	99.1	99.1	99.1
H/PF/2013	68.8	69.0	89.8	89.3	89.4	94.6	98.2	98.7	-	100.0	100.0
Z1106033	68.7	68.9	89.8	89.3	89.4	94.5	97.9	98.4	99.7	-	100.0
PRVABC59	68.6	68.8	89.9	89.4	89.3	94.3	97.9	98.4	99.7	99.8	-

NS2a

	AR 94	Chuku	MR-766	ArB 13565	ArD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	99.1	59.3	58.4	58.0	57.5	57.5	57.1	57.5	57.5	57.5
Chuku	97.5	-	59.3	58.4	58.0	57.5	57.5	57.1	57.5	57.5	57.5
MR-766	59.3	59.3	-	97.3	96.9	96.0	96.0	95.6	96.0	96.0	96.0
ArB 13565	59.6	59.0	94.2	-	98.7	96.5	96.5	96.0	96.5	96.5	96.5
ArD 41519	59.7	59.4	93.1	92.9	-	96.0	96.0	95.6	96.0	96.0	96.0
P6-740	58.8	58.4	88.2	89.2	87.6	-	99.1	98.7	99.1	99.1	99.1
CPC-0740	58.3	58.1	88.1	89.2	87.2	97.1	-	99.6	100.0	100.0	100.0
EC YAP	58.8	58.7	87.3	88.1	86.3	96.3	97.8	-	99.6	99.6	99.6
H/PF/2013	58.6	58.4	87.0	88.3	86.0	96.6	97.9	97.2	-	100.0	100.0
Z1106033	58.6	58.4	87.5	88.5	86.1	96.5	97.8	97.1	99.6	-	100.0
PRVABC59	58.7	58.6	87.2	88.5	85.8	96.5	97.8	97.1	99.6	99.7	-

NS4a

	AR 94	Chuku	MR-766	ArB 13565	ArD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	100.0	75.6	75.6	74.8	75.6	76.4	75.6	75.6	75.6	75.6
Chuku	99.0	-	75.6	75.6	74.8	75.6	76.4	75.6	75.6	75.6	75.6
MR-766	65.1	65.1	-	99.2	99.2	99.2	97.6	97.6	99.2	99.2	99.2
ArB 13565	65.6	65.4	95.0	-	98.4	98.4	96.9	97.6	98.4	98.4	98.4
ArD 41519	65.1	65.1	92.4	93.2	-	98.4	96.9	96.9	98.4	98.4	98.4
P6-740	64.8	64.8	91.3	89.8	91.3	-	98.4	98.4	100.0	100.0	100.0
CPC-0740	63.5	63.5	90.8	89.5	89.5	96.1	-	96.9	98.4	98.4	98.4
EC YAP	63.3	63.3	90.3	89.8	89.5	95.3	97.4	-	98.4	98.4	98.4
H/PF/2013	64.0	63.5	91.6	90.3	89.8	95.5	98.4	97.1	-	100.0	100.0
Z1106033	63.8	63.3	91.9	90.6	90.0	95.8	98.2	96.9	99.7	-	100.0
PRVABC59	63.8	63.3	91.6	90.6	90.0	95.8	97.9	96.9	99.5	99.7	-

NS4b

	AR 94	Chuku	MR-766	ArB 13565	ArD 41519	P6-740	CPC-0740	EC YAP	H/PF/2013	Z1106033	PRVABC59
AR 94	-	100.0	80.0	81.2	81.6	82.0	81.2	80.8	81.2	81.2	81.2
Chuku	97.1	-	80.0	81.2	81.6	82.0	81.2	80.8	81.2	81.2	81.2
MR-766	69.5	69.7	-	98.0	96.8	97.2	95.2	94.4	94.8	94.8	94.8
ArB 13565	70.3	70.5	93.9	-	98.4	98.4	96.4	95.6	96.0	96.0	96.0
ArD 41519	71.4	71.6	92.6	93.2	-	98.0	96.8	96.0	96.4	96.4	96.4
P6-740	70.1	70.1	89.1	90.3	90.6	-	97.6	96.8	97.2	97.2	97.2
CPC-0740	71.4	70.8	87.4	87.8	89.0	95.0	-	98.8	99.2	99.2	99.2
EC YAP	71.1	70.8	87.8	88.2	89.1	95.0	98.3	-	98.8	98.8	98.8
H/PF/2013	70.8	70.8	87.6	87.8	89.0	94.7	97.9	98.3	-	100.0	100.0
Z1106033	70.8	70.8	87.5	87.6	89.1	94.7	97.7	98.1	99.7	-	100.0
PRVABC59	70.8	70.8	87.8	87.9	89.4	94.7	97.5	97.9	99.5	99.7	-