Occurrence of deformed wing virus variants in the stingless Melipona subnitida and honey Apis mellifera bee populations in North Eastern Brazil

de Souza, FS, Kevill, JL, Correia-Oliveira, ME, de Carvalho, CAL and Martin, SJ

http://dx.doi.org/10.1099/jgv.0.001206

Occurrence of Deformed wing virus variants in the stingless *Melipona subnitida* and honey *Apis mellifera* bee populations in North Eastern Brazil

Flaviane S. de Souza[1,2], Jessica L. Kevill[1], Carlos A. L. de Carvalho[2], Stephen J. Martin[1*]

[1]*School of Environment and Life Sciences, The University of Salford, Manchester M5 4WT, UK.*

[2]*Universidade Federal do Recôncavo da Bahia, Rua Rui Barbosa 710*, 44380-000, *Cruz das Almas, Bahia, Brazil*

*Corresponding author: s.j.martin@salford.ac.uk

Key Words: spill-over, Varroa, viral variants

Abbreviations DWV, Deformed wing virus

Abstract

Deformed wing virus (DWV) is now a global insect pathogen. Brazilian stingless bees are a diverse group often managed in close proximity to honey bees. We investigated the prevalence and load of DWV in 33 stingless bees (*Melipona subnitida*) and 12 honey bees (*Apis mellifera*) colonies from NE Brazil. DWV was detected in all colonies with the A and C-variants dominating *M. subnitida* and A-variant in *A. mellifera*. Viral loads were 8.83E+07 and 7.19E+07 in *M. subnitida* and *A. mellifera*, respectively. On Fernando de Noronha island DWV is low (<1E+03) in honey bees, but we detected high loads (1.6E+08) in nine island *M. subnitida* colonies, indicating no viral spill-over of DWV has occurred during the past 34 years. Furthermore, the ubiquitous presence of the DWV-C variant in *M. subnitida* colonies, and rarity in *A. mellifera*, may suggest limited viral exchange between these two species.

INTRODUCTION

The stingless bees (Apidae: *Meliponini*) are the most diverse group of eusocial bees, comprising of more than 400 species contained within 60 genera [1]. The majority of species occur in the Neo-tropics with colonies typically containing 200-700 adults and a perennial life-cycle [2]. Many species, particularly the large *Melipona* species have a long association with humans that harvest their highly prized honey [3], but they are also responsible for pollinating 40-90% of the native flora in some regions of Brazil [4]. Relative to the honey bees (*Apis* spp), very little is known about the pests and pathogens of stingless bees despite their importance.

Brazil has a long history of managing honey bees (*Apis mellifera*) originally imported from Europe, but in 1957, 26 colonies of imported African *A. m. scutellata* escaped quarantine and spread throughout Brazil, hybridising with existing honeybees to form the Africanised honey bee [5]. However, when in 1971 the parasitic Varroa (*Varroa destructor)* mite arrived in Brazil, the Africanised honey bees were naturally tolerant to the mite, whereas, the European honeybees suffered large scale losses. These losses are caused by a viral pathogen called Deformed Wing Virus (DWV) that is transmitted by the Varroa mite [6].

Although Varroa can only survive on honey bees, [7] showed that raised DWV levels in the honey bee population, initiated by the mite, has resulted in viral spill-over into other species of bees and wasps. This may explain why DWV has been detected in a wide range of non-*Apis* insects [8-11] and has even been detected in pollen [12]. The impact of DWV on these hosts remains unknown [13], although there is growing concern [11, 14-16].

In Brazil, the Africanised honey bee, Varroa mite and DWV have been present for decades so there have been ample opportunities for cross-species infections to occur, especially since both honey bees and stingless bees are often managed in close proximity, i.e. in nearby apiaries. Therefore, the aim of this study was to evaluate both the prevalence and viral load of the three described DWV master-variants (A, B and C) across a population of stingless bee (*Melipona subnitida*) and Africanised honey bees from North-Eastern Brazil. The stingless bee *M. subnitida* is a swarm founding species, brood development takes around 40 days, and workers survive for a few months. This species is endemic to the dryland-shrub forest ‘Caatinga biome’ found in NE Brazil and is the typical stingless bee maintained by beekeepers throughout the region. This Meliponiculture helps towards the conservation of local biodiversity, as well as provide extra income to the beekeepers [3].

RESULTS

Prevalence of DWV

We detected DWV in every *M. subnitida* and *A. mellifera* colony. Negative controls indicated no contamination had occurred in any of the runs. Furthermore, the housekeeping gene indicated all samples contain intact RNA (Fig. 1). The average Ct values indicated more β-actin in the *A. mellifera* samples (19.7Ct ± 1.91 S.D.) relative to the *M. subnitida* samples (23.5Ct ± 0.70 S.D.).

DWV viral loads, The A and C master-variants were detected in the *M. subnitida* population only (Fig. 2). The DWV-A variant was dominant in 78% of the colonies (Fig. 2) with the C-variant dominating the remaining 22%. Whereas, 92 % of honey bee colonies were dominated by the A-variant and only one colony (8%) was dominated by the C-variant. The DWV-B variant was quantifiable in a single *A. mellifera* colony (Table 1) whilst three others tested positive below the quantifiable limit of the qPCR assay but had visible bands when visualised on a gel (Mossoro, Garanhus and Cruz das Almas). The total DWV viral load detected in both species of bee averaged 8.8E+07 and 7.2E+07 in *M. subnitida* and *A. mellifera* respectively. On the remote Fernando de Noronha island, the *M. subnitida* colonies were dominated by the A-variant, and the C-variant was widespread. However, the viral load was an order of magnitude higher on the island (1.6E+08) relative to the mainland (3.6E+07).

DISCUSSION

This study provides the first report of DWV in *Meliponini* stingless bees, since DWV was not detected previously in *Melipona quadrifasciata* and *M. torrida* [17], although t the DWV-A variant was detected in Argentinian stingless bees (*Tetragonisca fiebrigi*). Furthermore, *M. scutellaris* tested negative for six bee-associated viruses including DWV, but did test positive for the honey bee associated acute bee paralysis virus [18]. The high prevalence of DWV in *A. mellifera* was expected since DWV is consistently the most prevalent viral pathogen of European and Africanised honey bees [19].

The dominance of the DWV-A variant found in this study reflects the situation found in honey bees in the USA in 2010 [20]. Although the B-variant is replacing the A-variant in the USA [20] and appears common in Europe [21], it was only detected in any quantity in a single Africanised colony (Fig. 2). This is despite the likely long-term infection of both stingless and honey bees in Brazil. The rarely detected C-variant [20, 22] was present in almost all the *M. subnitida* colonies.

Interestingly on the remote island of Fernando de Noronha where both *M. subnitida* and *A. mellifera* have been maintained in close proximity over the past 34 years, the DWV-A variant dominated all nine colonies with a mean viral load of 1.6E+08. Whereas in the European honey bees on this island have a low (~1E+03) viral load, and diverse range of DWV variants [2]. This provides further evidence that DWV may be a general hymenopteran or insect virus rather than a honey bee pathogen that has spilled over into the pollinator community. Again, the ubiquitous presence of the DWV-C variant in *M. subnitida* colonies, and rarity in *A. mellifera* colonies on the mainland again suggests limited viral exchange between these two species. The chance of spill-over may be reduced due to the low (8E+07) DWV viral loads present in both the stingless and honey bees of NE Brazil, relative to those found in asymptomatic (2.4E+09) and symptomatic (6.9E+11) European honey bees [23]. Whereas, when these high DWV loads are present in honey bees, DWV appears to spill-over into the neighbouring wasps and solitary bees [7]. These low DWV viral loads in Brazil may be attributed to the hygienic habit of stingless bees [24], and Varroa-tolerance in Africanised bees, both which will reduce the viral load in a colony.

METHODS

Samples

Pools of 30 *M. subnitida* workers were collected using a pooter directly at the entrance of 24 colonies from meliponiparies at ten mainland locations across NE Brazil. Samples from Fortaleza and Mossoro were collected in 2016 with all other samples collected in 2013. In addition, pools of ten *M. subnitida* workers from nine colonies located on the remote oceanic island of Fernando de Noronha were collected in 2013 using the same method. These samples are interesting since this population was originally established from 30 colonies brought to the island in 1983 from the mainland states of Ceara and Rio Grande do Norte [25]. In 1984 Kerr also established a small population of European honey bees on Fernando de Noronha that were accidentally infested by the Varroa mite, although the typically high levels of DWV were not present in either the honey bees or Varroa [26].

During the same period pools of 30 healthy adult worker Africanised honey bees where collected from the brood area of 12 colonies from six states across NE Brazil. All samples were collected in absolute ethanol and stored at -20° C before transportation to the UK under license to be analysed.

Detection and quantification of DWV variants

Total RNA was extracted from a pool of 10 heads per colony for both stingless and honey bees. Heads were used as this reduced sample processing and is based on sound scientific reasoning [27-30]. The heads were ground in liquid Nitrogen into a fine homogeneous powder, a 30mg sub-sample had its RNA extracted using a Qiagen RNeasy mini kit, which was enhanced by using a QIAshredder kit for the *M. subnitida* samples [31]. Nanodrop (8000 series) quantification was used to standardise the amounts of total RNA to 50 ng/µl using RNase free water, before been stored at -80° C.

In order to quantify the viral load of each DWV Master-variant we used a recently developed method [22]. Briefly, cDNA was synthesised using one-step SensiFAST SYBR No ROX One-step kit (Bioline, London, UK), the reactions contained 1µl RNA at a concentration of 50 ng/µl, 10µl Senifast mix, 0.2µl Reverse transcriptase, 0.4µl RNase inhibitor, 0.75 pmol of each primer (DWV F and R-Type A, B and C [Table 2]) and 7.5µl of $H_2$0. Reactions were run on a Rotor-Gene Q Thermocycler (Qiagen) with an initial reverse transcription stage at 45° C for 10 min and a denaturation step of 95° C for 10 min, followed by 35 cycles of denaturation for 15 s at 95° C, annealing for 15 s at 58° C for primers A and B, and 61.5° C for primer C and extension for 15 s at 72° C. A final dissociation melt curve was performed between 72° C and 90° C, at 0.5° C increments, each with a 90 s hold. The melt curve was used to ensure that a single targeted product was amplified, and that no contamination was present in the reverse transcription negative controls or in the no-template controls. The threshold cycle (Ct) value was determined for each sample using the QIAGEN Rotor—Gene Q Series Analysis software and viral quantification was done by using serial dilutions of the standard DWV RNA, ranging from 1E+02 to 1E+07 copies of DWV per reaction. All samples were run in triplicate and the average taken. Those samples which had a standard deviation of ≥3 Ct were repeated. Furthermore, PCR products were run on a 2% agarose gel stained with 0.001% GelRed to confirm the correct sized band had been amplified. A control housekeeping gene β-actin [23] was also run to ensure no degradation of the samples had occurred, due to large distances these samples were transported both within and between countries. Genome equivalents were calculated per sample using the following equation:

Genome equivalents = (average copy number) x (RNA dilution factor) x (elution volume of RNA) x (proportion of bee material)

Funding information

F. S. de Souza was funded by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) (Cod. SEG 02.11.01.029.00.00), CNPq (Proc. 400425/2014-9 and 305885/2017-0) and CAPES for the PDSE scholarship (Proc. 88881.132120/2016-01).S. J. Martin and C. A. L. de Carvalho were funded by a CNPq Special Visiting Researcher PVE (400425/2014-9).

Acknowledgement

The authors would like to acknowledge the Brazilian Institute of Environment and Renewable Natural Resources, Chico Mendes Institute for Biodiversity Conservation, DAI/DEFN and ADEFN, for the permission to conduct the survey in the island of Fernando de Noronha. We would like to thank Márcia de Fátima Ribeiro, Candida B. da Silva Lima, Maria Emilene Correia de Oliveira and Victor Hugo P. Dias for the collection of bee samples and the beekeepers, for allowing the sampling of bees from their colonies. Also we would thank Bárbara Lins Caldas de Moraes for support with the map.

Author Contributions

Conceptualization: All authors

Data curation: Flaviane S. de Souza, Jessica L. Kevill,

Formal analysis: Flaviane S. de Souza, Jessica L. Kevill, Stephen J. Martin.

Funding acquisition: Carlos A. L. de Carvalho, Stephen J. Martin.

Investigation: Flaviane S. de Souza, Jessica L. Kevill,

Methodology: Jessica L. Kevill,

Project administration: Carlos A. L. de Carvalho, Stephen J. Martin.

Resources: Carlos A. L. de Carvalho, Stephen J. Martin.

Supervision: Carlos A. L. de Carvalho, Stephen J. Martin.

Validation: Flaviane S. de Souza, Jessica L. Kevill,

Visualization: Flaviane S. de Souza

Writing - original draft: Flaviane S. de Souza

Writing - review & editing: Flaviane S. de Souza, Jessica L. Kevill, Stephen J. Martin.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

There are no ethical issues.

References

1. **Rasmussen C, Cameron SA.** Global stingless bee phylogeny supports ancient divergence, vicariance, and long-distance dispersal. *Biol J Linn Soc* 2010: 99:206–232

2. **Wille A**. Biology of the stingless bees. *Ann Rev Entomol* 1983: 28:41–64.

3. **Jaffé R, Pope N, Carvalho AT, Maia UM, Blochtein B *et al*.** Bees for Development: Brazilian Survey Reveals How to Optimize Stingless Beekeeping. *PLoS ONE*. 2015: 10(3): e0121157

4. **Nascimento VA, Matusita SH, Kerr WE.** Evidence of hybridization between two species of *Melipona* bees. *Genetic Mol Biol* 2000: 23:79-81.

5. **Winston ML.** *Killer Bees: The Africanized Honeybee in The Americas*. 1992.Cambridge, Mass.: Harvard University Press. ISBN 0-674-50353-8.

6. **Rosenkranz P, Aumeier P, Ziegelmann B.** Biology and control of *Varroa destructor*. *J Invert Path* 2010: 103 Suppl 1:S96-119. doi: 10.1016/j.jip.2009.07.016

7. **Santamaria J, Villalobos EM, Brettell LE, Nikaido S, Graham JR, Martin SJ.** Evidence of Varroa-mediated Deformed Wing virus spillover in Hawaii. *J Invert Path* 2018: 151:126-130

8. **Singh R, Levitt A, Rajotte E, Holmes E, Ostiguy N *et al.*** RNA viruses in Hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* Hymenopteran species. *PLoS ONE* 2010: 5(12):1-16.

9. **Evison SE, Roberts KE, Laurenson L, Pietravalle S, Hui J *et al.*** Pervasiveness of Parasites in Pollinators. *PLoS ONE* 2012: *7*(1) e30641.

10. **Levitt AL, Singh R, Cox-Foster DL, Rajotte E, Hoover K, Ostiguy N, Holmes EC.** Cross-species transmission of honey bee viruses in associated arthropods. *Virus Res* 2013: 176(1):232-240.

11. **Manley R, Boots M, Wilfert L.** Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. *J Appl Ecol* 2015: 52(2):331-340.

12. **Mazzei M, Carrozza ML, Luisi E, Forzan M, Giusti M *et al*.** Infectivity of DWV Associated to Flower Pollen: Experimental Evidence of a Horizontal Transmission Route. *PLoS ONE* 2014: 9(11): e113448.

13. **Tehel A, Brown MJ, Paxton RJ**. Impact of managed honey bee viruses on wild bees. *Curr Opinion Virol* 2016: 19:16-22.

14. Li J, Peng W, Wu J, Strange JP, Boncristiani H, Chen Y. Cross-species infection of Deformed wing virus poses a new threat to pollinator conservation. *J Eco Entomol* 2011: 104(3):732-739.

15. Fürst, M, McMahon D, Osborne J, Paxton R, Brown, M. Disease associations between honeybees and bumblebees as a threat to wild pollinator. *Nature* 2014: 506: 364-366.

16. Graystock P, Goulson, D, Hughes WO. Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proc Roy Soc B Biol Sci* 2015: 282:20151371

17. Alvarez LJ, Reynaldi FJ, Ramello PJ, Garcia MLG, Sguazza GH *et al.* Detection of honey bee viruses in Argentinian stingless bees (Hymenoptera: Apidae) *Insect. Soc* 2017: 65:191-197.

18. Ueira-Vieira C, Almeida LO, de Almeida FC, Amaral IMR, Brandeburgo AM, Bonetti AM. Scientific note on the first molecular detection of the acute bee paralysis virus in Brazilian stingless bees *Apidologie* 2015: 46: 628.

19. Wilfert L, Long G, Leggett HC, Schmid-Hempel P, Butlin R *et al*. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. *Science* 2016: 351: 594–597.

20. Ryabov EV, Childers AK, Chen Y, Madella S, Nessa A, van Engelsdorp JD. Recent spread of Varroa destructor virus-1, a honey bee pathogen, in the United States. *Sci Rep* 2017: 7: 17447.

21. McMahon DP, Natsopoulou ME, Doublet V, Furst M, Weging S *et al*. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proc Roy Soc B Biol Sci* 2016: 283:20160811.

22. Kevill JL, Highfield A, Mordecai GJ, Martin SJ, Schroeder DC. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. *Viruses* 2017: 9: 314.

23. Highfield AC, Nagar AE, Mackinder LC, Noel LM, Hall MJ *et al*. Deformed wing virus implicated in overwintering honeybee colony losses. *Appl Enviro Microbiol* 2009: 75: 7212-7220.

24. de Jesus JN, Chambó ED, da Silva-Sodré G, Oliveira NTE, Carvalho CA. Hygienic behavior in *Melipona quadrifasciata anthidioides* (Apidae, Meliponini). *Apidologie* 2017: 48:504-512.

25. Kerr WE, Cabeda M. Introdução de abelhas no território federal de Fernando de Noronha. *Rev of Ciencia e Cultura* 1985: 37: 467-471.

26. Brettell LE, Martin SJ. Oldest Varroa tolerant honey bee population provides insight into the origins of the global decline of honey bees. *Sci Rep* 2017: 7:45953.

27. Fujiyuki, T, Takeuchi H, Ono M, Ohka S, Sasaki TS *et al.* Novel Insect Picorna-Like Virus Identified in the Brains of Aggressive Worker Honeybees. *J. Virol* 2004: 78: 1093-1100.

28. Yue C. Genersch E. RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J Gen Virol* 2005: 86(12): 3419-3424.

29. Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C *et al.* The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie* 2010: 41(3):332-352.

30. Zioni N, Soroker V, Chejanovsky N. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. *Virology* 2011: 417(1):106-112.

31. Forsgren E, Locke B, Semberg E, Laugen AT, Miranda JR. Sample preservation, transport and processing strategies for honeybee RNA extraction: Influence on RNA yield, quality, target quantification and data normalization. *J Virol Meth*. 2017: 246:81-89.

Table. 1. The mean viral load of each DWV master variant detected in the 21 *Melipona subnitida* and 12 *Apis mellifera* samples collected from across NE Brazil.

	Melipona subnitida Average viral load	*Apis mellifera* Average viral load
DWV-A	8.10E+07	6.96E+07
DWV-B	n.d.	2.35E+05
DWV-C	7.31E+06	2.06E+06
All	8.83E+07	7.19E+07

Table 2. Primers used in this study were developed by [24].

Target	Primer Name	Sequence (5' - 3')	Size of product (bp)
DWV Forward	DWVnew-F1	TACTAGTGCTGGTTTTCCTTT	
DWV Type A	DWVA-R1	CTCATTAACTGTGTCGTTGAT	155
DWV Type B	DWVB-R1	CTCATTAACTGAGTTGTTGTC	155
DWV Type C	DWVC-R1	ATAAGTTGCGTGGTTGAC	152

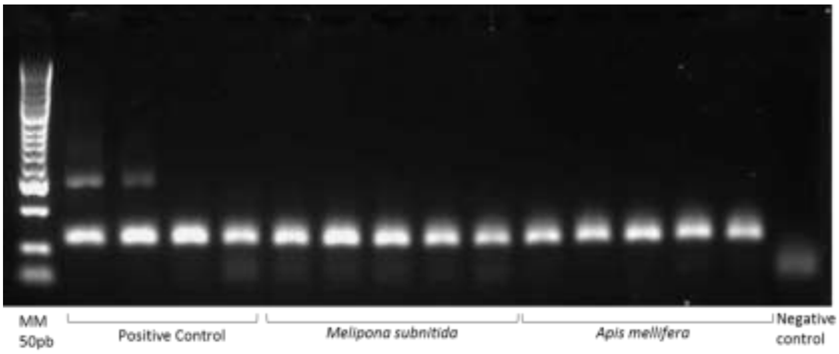

Fig. 1. Typical gel showing the presence of β-actin in all samples of *Melipona subnitida*, *Apis mellifera* and positive controls, confirming that the samples contained intact RNA.

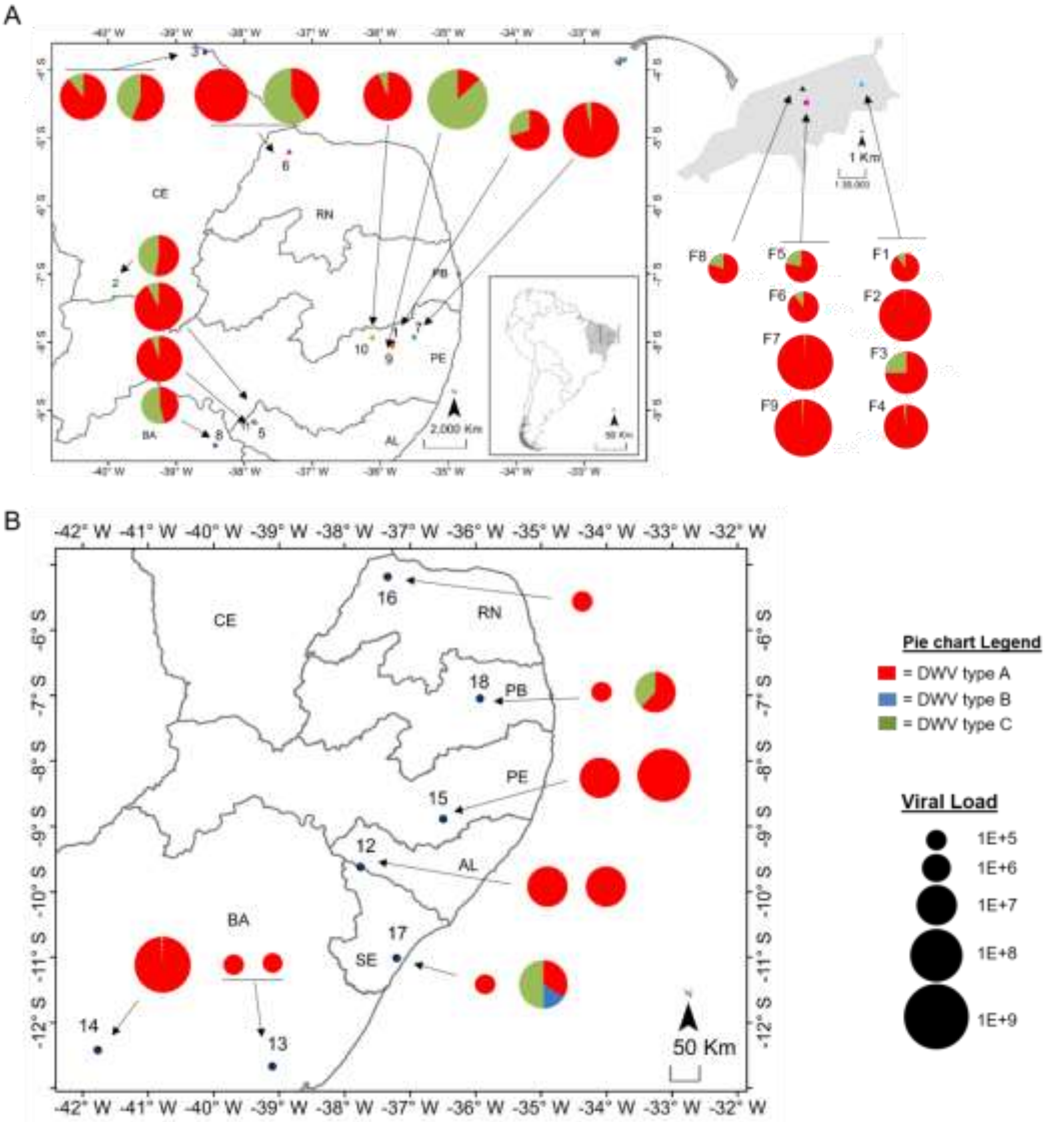

Fig. 2. Proportion and viral load of DWV-A (red), B (blue) and C (green) variants detected in A) *Melipona subnitida* stingless bees and B) *Apis mellifera* from across NE Brazil. The sample locations are 1. Cumaru, 2. Exu, 3. Fortaleza, 4. Fernando de Noronha, 5. Mata Grande, 6. Mossoró, 7. Passira, 8. Paulo Afosnso, 9. Riacho das Almas, 10. Taquaritinga do Norte, 11. Água Branca, 12. Piranhas, 13. Cruz das Almas, 14. Seabra, 15. Garanhuns 16. Mossoró, 17. São Cristóvão, 18. Areial. The states are CE= Ceara, RN= Rio Grande Do Norte, PB= Paraiba, PE= Pernambuco, AL= Alagoas, SE= Sergipe and BA= Bahia.