Generating viral metagenomes from the coral holobiont

Karen D. Weynberg, Elisha M. Wood-Charlson, Curtis A. Suttle, and Madeleine J. H. van Oppen

Abstract

Reef-building corals comprise multipartite symbioses where the cnidarian animal is host to an array of eukaryotic and prokaryotic organisms, and the viruses that infect them. These viruses are critical elements of the coral holobiont, serving not only as agents of mortality, but also as potential vectors for lateral gene flow, and as elements encoding a variety of auxiliary metabolic functions. Consequently, understanding the functioning and health of the coral holobiont requires detailed knowledge of the associated viral assemblage and its function. Currently, the most tractable way of uncovering viral diversity and function is through metagenomic approaches, which is inherently difficult in corals because of the complex holobiont community, an extracellular mucus layer that all corals secrete, and the variety of sizes and structures of nucleic acids found in viruses. Here we present the first protocol for isolating, purifying and amplifying viral nucleic acids from corals based on mechanical disruption of cells. This method produces at least 50% higher yields of viral nucleic acids, has very low levels of cellular sequence contamination and captures wider viral diversity than previously used chemical-based extraction methods. We demonstrate that our mechanical-based method profiles a greater diversity of DNA and RNA genomes, including virus groups such as Retro-transcribing and ssRNA viruses, which are absent from metagenomes generated via chemical-based methods. In addition, we briefly present (and make publically available) the first paired DNA and RNA viral metagenomes from the coral *Acropora tenuis*.

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Guidelines

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Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

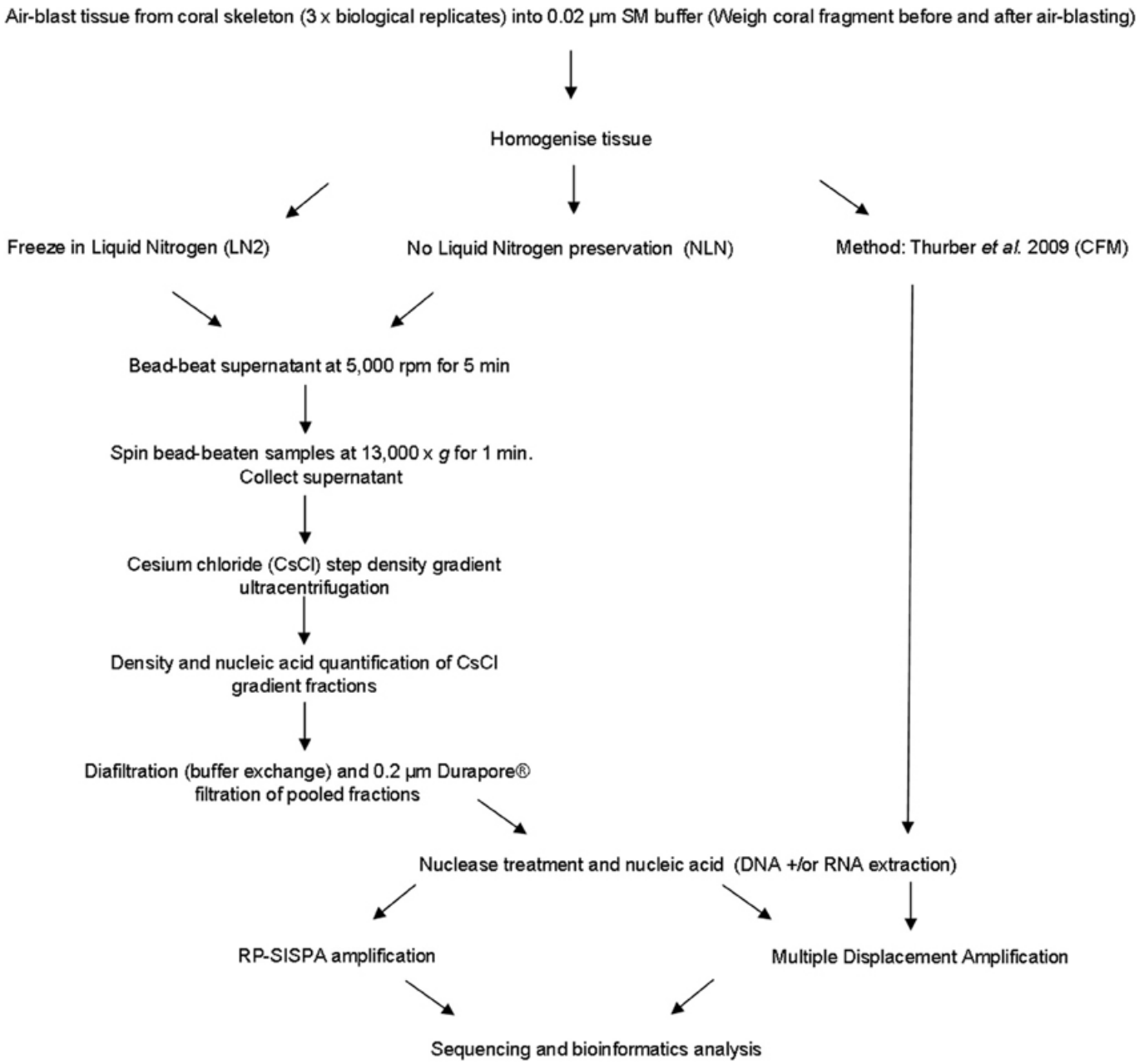
For the *P. damicornis* samples, isolation of the viral metagenomes associated with the coral tissue was undertaken in a 3-way comparison of methodologies. The first approach was to replicate

previously published protocols for isolating viruses from coral tissue (Marhaver et al., 2008; Thurber et al., 2008,2009) using a chloroform disruption step, which we term the chloroform (CFM) method.

New Mechanical-Based Method Omitting the Use of Chloroform

A number of viruses are sensitive to chloroform as it acts to remove the lipid envelope surrounding the exterior of the viral capsid (Feldman and Wang, 1961; Ackermann, 2006). We developed an alternative approach to avoid the use of chloroform, instead using mechanical disruption to break open host cells, which we term the mechanical (MECH) method. In addition, the MECH protocol was used to test the effects of storage in liquid nitrogen on samples post-tissue homogenization. Sampling of coral colonies in the field frequently means working in remote locations with limited access to resources specific to virus purification, such as an ultracentrifuge. Therefore, preservation of fresh coral tissue homogenate using liquid nitrogen is often necessary until further processing in a laboratory can occur. We tested the effect of storage in liquid nitrogen (LN2) prior to nucleic-acid isolation vs. the immediate processing of fresh coral tissue homogenate (no liquid nitrogen, NLN). A simplified outline of the optimized method using MECH for generating viral metagenomes from coral tissue is shown in Figure 1. To standardize the sample for method testing, we pooled tissue homogenate from three *P. damicornis* colonies and subdivided the pooled sample for processing by MECH (NLN and LN2) and the published CFM method for viral metagenome isolation, purification and amplification.

Figure 1



Nucleic Acid Extraction and Amplification for Sequencing

The amplification methods used were Phi 29 polymerase-based Multiple Displacement Amplification (MDA) RepliG® (QIAGEN) technology and a modified Random Priming-mediated Sequence-Independent Single-Primer Amplification (RP-SISPA) approach. Samples processed via the CFM method were only amplified using RepliG®, as this is similar to other Phi 29 polymerase-based amplification techniques used in this method (e.g., GenomiPhi®) (Thurber et al., 2008; Hewson et al., 2012). The NLN and LN2 samples were amplified using both RepliG® and a RP-SISPA method for DNA viral metagenomes modified from a published protocol for amplifying RNA viruses extracted from seawater samples (Culley et al., 2010). The modified method converts viral DNA to dsDNA through a two-step Klenow reaction, which also adds the primer sites to both DNA strands prior to amplification by PCR.

Table 1

Virus type	CFM	MECH			
		NLN	LN2	NLN*	LN2*
dsDNA viruses, no RNA stage	11.3	10.9	6.6	78.9	71.9
Bacteriophage	(98.6%)	(93.5%)	(93.5%)	(99%)	(99%)
Eukaryotic	(1.4%)	(6.5%)	(6.5%)	(1%)	(1%)
Reverse transcribing viruses	0	1.8	4.9	0.4	1.0
Satellites	3.3	4.7	6.1	0.9	0.1
ssDNA viruses	84.9	82.1	82.3	17.5	24.6
ssRNA viruses	0	<0.1	<0.1	<0.1	<0.1
Unclassified archaeal viruses	0	<0.1	0	0	<0.1
Unclassified phages	0.4	0.4	0.1	2.2	2.3
Unclassified viruses	<0.1	0.1	<0.1	<0.1	0.1
Virophage	<0.1	0	0	<0.1	<0.1

The viral metagenomic data sets generated by the previously published chloroform treatment method (CFM) and our novel mechanical disruption method (MECH). The latter method was tested with (LN2) and without (NLN) liquid nitrogen tissue preservation. Data sets with an asterisk (*) were amplified using RP-SISPA prior to sequencing and were analyzed after removal of contaminating ssDNA phiX sequences (spiked during Illumina sequencing). All other data sets were amplified using RepliG® technology. Percent of dsDNA viruses as phage or eukaryotic viruses are highlighted in brackets.

Protocol

Sampling Locations and Collection of Coral Tissue

Step 1.

At Trunk Reef, approximately 45 g of coral tissue was sampled from three healthy, freshly collected coral colonies of *Pocillopora damicornis*.

NOTES

Karen Weynberg 16 Feb 2016

Field sampling occurred at Trunk Reef (18°20′49″S, 146°49′46″E) in November 2012 and in Pioneer Bay off Orpheus Island (18°38′3″S, 146°29′57″E) in March 2013, in the central Great Barrier Reef.

Sampling Locations and Collection of Coral Tissue

Step 2.

Approximately 20 g of *Acropora tenuis* tissue was sampled from three healthy, freshly collected coral colonies collected in Pioneer Bay.

Sampling Locations and Collection of Coral Tissue

Step 3.

Fragments were washed in autoclaved, 0.02 µm filtered virus-free seawater.

Sampling Locations and Collection of Coral Tissue

Step 4.

Subsequently, tissue was blasted from the coral skeleton, using an air-gun, into 15 mL 0.02 µm filtered (Anotop, Whatman) SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris pH 7.5) in a zip-lock bag.

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 5.

Briefly, 5 mL of chloroform per 40 mL of coral blastate was added and samples were agitated gently for 1 h at room temperature.

DURATION

01:00:00

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 6.

Coral blastates were homogenized at 5000 rpm for 1 min (Heidolph SilentCrusher™).

DURATION

00:01:00

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 7.

Samples were immediately centrifuged at 1000 g for 15 min.

DURATION

00:15:00

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 8.

The supernatant was transferred to sterile glass corex tubes and spun at 12,000 g for 15 min to pellet the majority of microbial cells (Beckman Coulter JA 25.50 rotor).

DURATION

00:15:00

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 9.

A cesium chloride (CsCl) density gradient was then formed by layering 1 mL of 1.7, 1.5, and 1.35 g mL^{-1} CsCl into 13.2 mL UltraClear™ ultracentrifuge tubes (Beckman Coulter) with 9 mL sample layered on the top of the gradient.

Chloroform Extraction and Cesium Chloride Density Gradient Centrifugation

Step 10.

Gradients were then centrifuged for 2 h at 60,000 g at 4°C in a swinging bucket rotor.

DURATION

02:00:00

New Mechanical-Based Method Omitting the Use of Chloroform

Step 11.

In the MECH method, the coral tissue blastate was homogenized at 10,000 rpm for 1 min.

DURATION

00:01:00

New Mechanical-Based Method Omitting the Use of Chloroform

Step 12.

The coral tissue blastate was then spun at 400 g for 5 min.

DURATION

00:05:00

New Mechanical-Based Method Omitting the Use of Chloroform

Step 13.

The supernatant was then aliquoted into 1.5 mL aliquots in 2 mL eppendorf tubes containing 0.3 mL acid-washed glass beads (425–600 µm diameter) (Sigma-Aldrich).

New Mechanical-Based Method Omitting the Use of Chloroform

Step 14.

The tubes were placed in a bead beater and cells were disrupted at 5000 rpm for 5 min.

DURATION

00:05:00

New Mechanical-Based Method Omitting the Use of Chloroform

Step 15.

Tubes were centrifuged at top speed in a bench-top Eppendorf centrifuge for 1 min.

DURATION

00:01:00

New Mechanical-Based Method Omitting the Use of Chloroform

Step 16.

The supernatant was collected for viral fractionation using step CsCl density gradients.

PROTOCOL

. MECH step CsCl density gradients

CONTACT: Karen Weynberg

Step 16.1.

CsCl solutions were made with solid molecular-grade CsCl (Sigma-Aldrich) dissolved in 0.02 µm filtered (Anotop, Whatman) SM buffer.

Step 16.2.

A 3 mL cushion of 1.6 g mL^{-1} CsCl was added to the bottom of a 13.2 mL UltraClear™ ultracentrifuge tube (Beckman Coulter).

Step 16.3.

Add 2.5 mL of 1.45 g mL^{-1} density on top of the 1.6 g mL^{-1} layer.

Step 16.4.

The density of sample homogenate supernatant was adjusted to 1.12 g mL^{-1} with CsCl.

Step 16.5.

Add 2.5 mL of 1.3 g mL^{-1} density.

Step 16.6.

Add 2 mL of 1.2 g mL^{-1} density.

Step 16.7.

2 mL of sample was placed on top of the layered gradient.

Step 16.8.

Gradients were then centrifuged in an Optima XL-80K ultracentrifuge (Beckman Coulter) in a swinging bucket rotor (SW 41 Ti, Beckman Coulter) for 2.5 h at 40,000 rpm and 4°C.

DURATION

02:30:00

Step 16.9.

Fractions (0.5 mL) from the gradients were collected in 1.5 mL tubes using an 18 bore gauge needle and luer-lok syringe, puncturing the tube 1 mL from the bottom.

Step 16.10.

The density of fractions was determined gravimetrically and DNA concentration of each fraction was measured using a Quant-It Picogreen dsDNA High Sensitivity assay kit (Invitrogen, Life Technologies).

Step 16.11.
Diafiltration and buffer exchange were performed to remove CsCl salts.

NOTES

Karen Weynberg 23 Feb 2016
Their presence may interfere with downstream processing, such as DNA extraction.

Step 16.12.
Fractions containing the nucleic acid peaks were pooled and buffer exchange was performed with Amicon® centrifugal spin columns (30 kDa, Millipore) against 0.02 µm filtered SM buffer.

Step 16.13.
The diafiltrated sample was then filtered using a 0.2 µm pore size Durapore® syringe filter to remove remaining contaminating bacteria.

New Mechanical-Based Method Omitting the Use of Chloroform

Step 17.
To confirm that the MECH method was not disrupting virus particles, two dsDNA viruses, OtV-2 (Weynberg et al., 2011) and EhV-86 (Wilson et al., 2005b), were subjected to the same mechanical disruption protocol.

New Mechanical-Based Method Omitting the Use of Chloroform

Step 18.
Flow cytometry was used to enumerate viruses before and after disruption.

NOTES

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We could find no discernible differences in virus populations following MECH (data not shown).

Nucleic Acid Extraction and Amplification for Sequencing

Step 19.
All samples were treated with DNase and RNase (Ambion) prior to nucleic acid extraction.

Nucleic Acid Extraction and Amplification for Sequencing

Step 20.
DNA was extracted and RNase treated using a MasterPure kit (Epicentre, Illumina) following manufacturer's instructions.

Nucleic Acid Extraction and Amplification for Sequencing

Step 21.
RNA was extracted using a Qiagen QIAamp viral RNA kit (Qiagen) following manufacturer's instructions, including the final DNase step (Ambion).

Nucleic Acid Extraction and Amplification for Sequencing

Step 22.
Two different amplification methods were used.

NOTES

Karen Weynberg 23 Feb 2016
See guidelines for more information.

Amplification of Viral DNA Genomes With Replig®

Step 23.
In order to reduce some of the inherent biases in multi-displacement amplification (MDA), such as a

preference for ssDNA viral genomes, DNA extractions were converted to dsDNA prior to amplification.

Amplification of Viral DNA Genomes With Replig®

Step 24.

Triplicate 10 µ L aliquots of the DNA extractions, containing ds and ssDNA viral genomes, underwent a single round of Klenow reaction (3′–5′ exo-, 5U/µ L) by mixing 1.5 µ L of 10× reaction buffer (New England Biolabs Buffer 2), 1.5 µ L of dNTPs (2.5 mM stock), 1 µ L of random hexamer primers (50 ng/µ L, Invitrogen).

Amplification of Viral DNA Genomes With Replig®

Step 25.

The reaction was incubated at 94°C for 3 min.

DURATION

00:03:00

Amplification of Viral DNA Genomes With Replig®

Step 26.

The reaction was then placed on ice for 3 min to allow for primer annealing.

DURATION

00:03:00

Amplification of Viral DNA Genomes With Replig®

Step 27.

1 µ L of Klenow (3′–5′ exo-) was added and incubated at 25°C for 10 min.

DURATION

00:10:00

Amplification of Viral DNA Genomes With Replig®

Step 28.

It was then incubated at 37°C for 60 min.

DURATION

01:00:00

Amplification of Viral DNA Genomes With Replig®

Step 29.

It was then incubated with a termination step of 75°C for 20 min.

DURATION

00:20:00

Amplification of Viral DNA Genomes With Replig®

Step 30.

After termination, reactions were pooled and cleaned using a Qiagen QIAamp DNA mini kit and eluted in 50 µL of Buffer AE.

Amplification of Viral DNA Genomes With Replig®

Step 31.

Replicate MDA reactions (n = 3 for each sample) were amplified using 2.5 µ L dsDNA template and the Qiagen RepliG® kit using the standard protocol.

Amplification of Viral DNA Genomes With Replig®

Step 32.

All reactions were run on a 0.8% agarose gel in 1× TAE at 100 V for 30 min to confirm amplification, pooled and cleaned with QIAampl DNA minikit and eluted in 200 µ L of Buffer AE.

DURATION

00:30:00

Amplification of Viral DNA Genomes With Replig®

Step 33.
Negative controls were treated the same and also sent for sequencing to confirm that no viral contamination was present.

Amplification of Viral DNA Genomes with RP-SISPA

Step 34.
As with the RepliG® protocol, Klenow Fragment (3′–5′ exo-) was used to convert all DNA genomes to dsDNA using RP-SISPA primers with a 3′ random hexamer sequence that is used for downstream PCR amplification.

Amplification of Viral DNA Genomes with RP-SISPA

Step 35.
To label the first strand with the RP-SISPA primer, 5 µL of nucleic acid was added to 9 µL reaction mix containing:

PROTOCOL

. 9 µL reaction mix

CONTACT: Karen Weynberg

Step 35.1.
1.5 µ L of 10× PCR buffer (New England Biolabs Buffer 2)

Step 35.2.
1.0 µL of 2.5 mM dNTPs

Step 35.3.
1.5 µL of primer FR26RV-N (GCCGGAGCTCTGCAGATATCNNNNNN, 10 µ M stock)

Step 35.4.
5 µL of DNase-free distilled water

Amplification of Viral DNA Genomes with RP-SISPA

Step 36.
The reaction was incubated at 94°C for 3 min.

DURATION
00:03:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 37.
The reaction was then placed on ice for 3 min to allow for primer annealing.

DURATION
00:03:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 38.
1 µL of Klenow Fragment (3′–5′ exo-, 5U/µL, NEB #) was added and incubated at 37°C for 60 min.

DURATION
01:00:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 39.
A second round of Klenow Fragment reaction (3′–5′ exo) labeled the second strand with the SISPA primer, by adding an additional 1 µL of primer and 1 µL dNTP.

Amplification of Viral DNA Genomes with RP-SISPA

Step 40.
The reaction then underwent a 94°C for 3 min heating step.

DURATION
00:03:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 41.
The reaction was then put on ice for 3 min.

DURATION
00:03:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 42.
The reaction then underwent a final addition of 1 µL of Klenow Fragment (3′–5′ exo-).

Amplification of Viral DNA Genomes with RP-SISPA

Step 43.
The reaction was incubated at 37°C for 60 min.

DURATION
01:00:00

Amplification of Viral DNA Genomes with RP-SISPA

Step 44.
The reaction was then terminated at 75°C for 20 min.

DURATION
00:20:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 45.
Briefly, in preparation for cDNA synthesis, 10 µ L purified RNA viral template was mixed with 1 µ L of 2.5 mM dNTPs and 1.3 µ L of FR26RV-N (GCCGGAGCTCTGCAGATATCNNNNNN, 10 µ M stock) and FR40RV-T primer (GCCGGAGCTCTGCAGATATC(T)20, 50 nM stock).

NOTES
Karen Weynberg 25 Feb 2016
Amplification of viral RNA genomes with RP-SISPA was performed as described by Culley et al. (2010) in Manual of Aquatic Viral Ecology (MAVE).

Amplification of Viral RNA Genomes with RP-SISPA

Step 46.
The reaction was heated to 65°C for 5 min.

DURATION
00:05:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 47.
The reaction was then cooled on ice for 3 min to allow the primers to anneal.

DURATION
00:03:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 48.
While still on ice, 1 µL DTT (Invitrogen) was added to the reaction as an enzyme stabilization reagent with 1 µL RNase OUT (Invitrogen) to protect the sample from RNAse activity.

Amplification of Viral RNA Genomes with RP-SISPA

Step 49.
The complementary DNA strand was synthesized with 200 U of Superscript III reverse transcriptase.

Amplification of Viral RNA Genomes with RP-SISPA

Step 50.
The reaction was incubated initially at 25°C for 10 min to allow annealing of the hexamer 3′ end of primer FR26RV-N and the poly(T)20 3′ end of primer FR40RV-T to the template while cDNA synthesis commenced.

DURATION
00:10:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 51.
The temperature was then increased to 50°C for 60 min.

DURATION
01:00:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 52.
The first strand synthesis reaction was heated immediately to 94°C for 3 min and then rapidly cooled on ice.

DURATION
00:03:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 53.
A complementary second strand was subsequently synthesized at 37°C for 60 min with the addition of 1 µL Klenow Fragment (3′–5′ exo-, 5U/µL).

DURATION
01:00:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 54.
The Klenow reaction was terminated with a final incubation at 75°C for 20 min.

DURATION
00:20:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 55.
PCR amplification of the SISPA primer labeled template (DNA and RNA) was done in triplicate 25 µL reactions containing:

PROTOCOL

. PCR amplification of the SISPA primer reaction mixture

CONTACT: Karen Weynberg

Step 55.1.
2.5 µ L 10× reaction buffer

Step 55.2.
4 µ L dNTPs (2.5 mM stock)

Step 55.3.
2 µ L FR20RV primer (GCCGGAGCTCTGCAGATATC, 10 µ M stock)

Step 55.4.
1 µ L of template

Step 55.5.
0.25 µ L of TaKaRa LA HS Taq polymerase (5 U/µ L, Scientifix)

Amplification of Viral RNA Genomes with RP-SISPA

Step 56.
The reaction was incubated at 95°C for 10 min.

DURATION
00:10:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 57.
The reaction then underwent 30 cycles of denaturation at:

PROTOCOL

. PCR amplification of the SISPA primer denaturing cycles

CONTACT: Karen Weynberg

Step 57.1.
95°C for 30 s.

DURATION
00:00:30

Step 57.2.
60°C for 60 s.

DURATION
00:01:00

Step 57.3.
72°C for 90 s.

DURATION
00:01:30

Step 57.4.
A final extension step at 72°C for 13 min to allow the completion of complementary strand synthesis.

DURATION
00:13:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 58.
The PCR reactions were loaded on to a 0.8% agarose gel in 1×TAE at 100 V for 30 min.

DURATION
00:30:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 59.
If amplification resulted in visible PCR products (typically a smear; products should be longer than 250 bp), a reconditioning PCR was performed on pooled reactions as follows.

Amplification of Viral RNA Genomes with RP-SISPA

Step 60.
One reconditioning PCR contained 10 µL of pooled SISPA reaction template, 10 µL 10× buffer, 16 µL

dNTP (2.5 mM stock), 8 µL FR20RV primer (10 µ M stock) and 0.75 µL TaKaRa LA HS Taq.

Amplification of Viral RNA Genomes with RP-SISPA

Step 61.
The reaction was incubated at 95°C for 10 min.

DURATION
00:10:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 62.
The reaction then underwent 5 cycles of denaturation at:

PROTOCOL

. Reconditioning PCR amplification of the SISPA primer denaturing cycles

CONTACT: Karen Weynberg
Step 62.1.
95°C for 30 s.

DURATION
00:00:30
Step 62.2.
60°C for 60 s.

DURATION
00:01:00
Step 62.3.
72°C for 90 s.

DURATION
00:01:30
Step 62.4.
An extension at 72°C for 13 min.

DURATION
00:13:00

Amplification of Viral RNA Genomes with RP-SISPA

Step 63.
Reactions were cleaned and QC was assessed.

Sequencing and Bioinformatics Analysis

Step 64.
After amplification, samples were cleaned with a QIAamp® DNA Mini kit (RepliG® amplification) or a MinElute® PCR purification kit (RP-SISPA).

Sequencing and Bioinformatics Analysis

Step 65.
Samples were checked for quantification using a Quant-iT PicoGreen® kit on a NanoDrop 3300 fluorospecrometer, for quality (260:280 ratios) on a NanoDrop 2000, and were run on a 0.8% agarose gel in 1× TAE at 100 V for 30 min to confirm a size range appropriate for sequencing (250–500 bp) was present without contamination of smaller fragments.

DURATION
00:30:00

Sequencing and Bioinformatics Analysis

Step 66.
All metagenomes were sequenced using Nextera XT MiSeq 250 bp paired-end sequencing (Illumina) at the Ramaciotti Centre, University of New South Wales, Sydney, Australia.

Sequencing and Bioinformatics Analysis

Step 67.
Raw sequence reads were processed in CLC Genomics Workbench 5.5.

Sequencing and Bioinformatics Analysis

Step 68.
Sequences were imported as Illumina paired-end reads, adaptor sequences were trimmed, and reads were checked for quality using a PHRED score of 20 and a minimum length of 100 bp.

Sequencing and Bioinformatics Analysis

Step 69.
Paired reads were merged and a final data set containing merged reads and ORFans was checked again for QC with a minimum length of 200 bp.

Sequencing and Bioinformatics Analysis

Step 70.
To carry out the taxonomic assignment, these non-assembled read data sets were uploaded to the Metavir web server, which is dedicated to the analysis of viral metagenomes (http://metavir-meb.univ-bpclermont.fr) (Roux et al., 2011).

LINK:
http://metavir-meb.univ-bpclermont.fr/

NOTES
Karen Weynberg 25 Feb 2016
This server computes the taxonomic composition using tBLASTx against the NCBI viral Refseq genomes (release 2013-09-12, bit-score = 50) and normalizes the results to viral genome length using the GAAS tool (Angly et al., 2009).

Sequencing and Bioinformatics Analysis

Step 71.
All virus sequences were further classified into families using the taxonomic information from the top BLAST hit.

Sequencing and Bioinformatics Analysis

Step 72.
Tetranucleotide clustering and rarefaction curves were generated using tools available through Metavir.

NOTES
Karen Weynberg 25 Feb 2016
The *P. damicornis* metagenomes arose from the same biological samples and, therefore, tetranucleotide clustering was used to detect relative changes in sequence diversity caused by different methodologies. For the rarefaction curves, sequences were clustered at 75% identity because these data sets originated from the same pooled tissue homogenate sample from *P. damicornis*.

Nucleotide Sequence Accession Numbers

Step 73.
The five datasets generated from the *P. damicornis* samples were submitted to Genbank Sequence Read Archive (SRA) and are available under the accession numbers SRR1207981, SRR1207983, SRR1207980, SRR1207984, and SRR1246941 (Table 1 in guidelines).

Nucleotide Sequence Accession Numbers

Step 74.

The two datasets generated from the *A. tenuis* samples have also been deposited in the SRA under the accession numbers SRR1207979 and SRR1210582.