# Metagenomic approach to study viruses and virus-like pathogens in native potatoes from Chiloé Archipelago

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*To Juan and Juan Pablo*

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# Contents

# Chapter 1

## General Introduction

Potato (*Solanum tuberosum* L.) belongs to the *Solanaceae* family, which includes economically important species such as tomato, eggplant, petunia, tobacco and chili. It is listed together with wheat, rice and corn as main food source, with a world production of around 388 million tons in 2017 (FAOSTAT, 2019). By 2020 it is estimated that more than two million people, especially in the regions of Asia, Africa and Latin America will depend on this crop for both food and fodder (Kuang *et al*., 2005). In this way, optimization of production levels and resistance to biotic and abiotic stress are the fundamental objectives of the global potato breeding programs.

Potato cultivation is widely distributed throughout the world. However, it is affected by a series of diseases caused mainly by fungi, bacteria and viruses, resulting in losses of approximately USD 3.8 billion per year (Haverkort *et al*., 2008). In Chile, potatoes are grown in several regions of the country (http://www.odepa.cl). During the last years a great interest has arisen for native varieties originated in the Chiloé and Los Chonos Archipelagos through the domestication of wild species, which have as main characteristic the adaptation to long days to initiate the tuberization process. These native varieties show great diversity, in terms of color, shape, texture, size and flavor, making them of great interest to initiate breeding programs that seek characteristics of agronomic interest, such as organoleptic quality, source of anthocyanins and resistance to different diseases. However, studies that include

both the characterization of the phytopathogenic agents responsible for the diseases that affect them, and the genetic characteristics of the germplasm of these species are scarce or focused on only a few accessions.

Until now, it is known that during the process of domestication of wild species until the production of monocultures, the phytopathogens capable of infecting them have co-evolved together in conglomerates of communities (Cooper and Jones, 2006). This co-evolutionary process has given specific characteristics to both the wild host and its pathogens, including viruses (Jones, 2009). In this way, the study of viruses and/or related pathogens in native species has the potential to provide critical information not only in the face of threats to the biodiversity of these species, but also to the pathogen host interactions that could be involved (Jeger *et al*., 2006), including the movement of phytopathogens between traditional and native species.

Plant viruses are obligate intracellular pathogens that need the cellular machinery for their multiplication (Agrios, 2005, Hull, 2009). Even though viruses must overcome a series of obstacles to complete their proliferative cycle, for some time now, they have become one of the main concerns of developing countries, because the diseases they cause have a negative impact on the welfare of people through economic losses associated with agriculture, also may affect the conservation of biodiversity of different species (Anderson *et al*., 2004, Jones, 2009). In this regard, viral diseases seem to proliferate exponentially over the years, in part, due to their attribution as responsible for diseases previously described or thanks to the use of more sensitive and specific technologies for the identification and detection of pathogens, mainly based on the polymerase chain reaction (PCR) (Hull, 2009).

Research carried out in this area have been mainly restricted to viruses that cause diseases in economically important crops, not worrying about other plant-virus interactions in other natural hosts such as weeds and native plants, generating information that can not necessarily be extrapolate to the real interactions that are established in native hosts (Roossinck, 2011; Wren *et al*., 2006).

Special interest has arisen in the study of plant-virus interactions that occur with wild or native species, in order to form a broader and more realistic map of the evolution and origin of plant viruses (Jones, 2009; Roossinck, 2008). One of the main proposals is that wild and native species would be reservoirs of new pathogens, or otherwise, of pathogens whose host range is greater than previously observed, which could be the key in the study of virology evolutionary (Wren *et al*., 2006). However, suspecting new phytopathogenic agents present in native or wild species is more difficult to identify since there is no knowledge regarding their type of genetic material (DNA, RNA), form of dissemination, host range, and forms to facilitate their control (Hull, 2009; Kreuze *et al*., 2009).

Accurate disease diagnosis and precise identification of any pathogens involved is an essential prerequisite for understanding plant diseases and controlling them effectively (Ward *et al*., 2004). While the identification and diversity of bacteria or fungi can be studied using rRNA genes or barcodes (Cataldo *et al*., 2011; Lee *et al*., 1998; Seifert, 2009), viruses lack of universal genes that have enough similarity to be useful in their identification or detection (Roossinck, 2011) limiting access to diversity studies and viral composition in native environments (Wommack *et al*., 2012).

In the past, at the time of identifying and / or detecting plant viruses, nonspecific diagnostic tools were used, which include the use of indicator plants or electronic microscopy that allowed, in the best of cases, the successful identification of the virus or an approximation of the family or genus to which it belonged (Adams *et al*., 2009). Then, antibody-based techniques, such as ELISA, were a significant step in adding sensitivity, accuracy and precision to virus and other pathogen detection, along with simplicity and low cost (Makkouk and Kumari, 2006). Subsequently, the traditional techniques were complemented by molecular techniques, which can be classified into two types based on prior knowledge or not of the nucleotide sequences of the virus. Sequence-dependent methods include polymerase chain reaction (PCR, RT-PCR) or those based on hybridizations as in the case of microarrays (Boonham *et al*., 2007; Hull, 2009). On the other hand, sequence-independent techniques do not need to have prior knowledge of the viruses present in the samples. Subtractive subtraction hybridization (SSH) technique and Representational difference analysis (RDA) are examples of methods used for the discovery of new viruses (Mokili *et al*., 2012). SSH was originally used for studies of gene expression, but was later successfully used in the study of the etiology of viral diseases that affect the respiratory tract in humans (Ambrose and Clewley, 2006).

Although these techniques often generate satisfactory results, they may be insufficient when new viruses are suspected, a virus that extends their host range or one that is not fully characterized (Adams *et al*., 2009). One way to perform an evaluation for a previously unidentified virus is, in principle, to perform specific assays; which require a prediction of the behavior of the virus. However, these data

are generally scarce or completely absent, for example, in the case of wild and native species.

Usually, the identification of a particular virus may require the use of multiple tests, and its corresponding molecular characterization can take weeks or even months, although it corresponds to a previously described virus. While the identification of a new viral population can take an even longer time, for example, the identification of the *Black currant reversion virus* (BRV) took several years (Susi, 2004) using traditional techniques described above. For this reason, research has continued on tools to improve and decrease the time invested for the identification of new viruses, one of these approaches corresponds to the use of metagenomics.

The term metagenomics, described for the first time by Handelsman *et al*. (1998), refers to the isolation, sequencing and analysis of the genetic material recovered from a sample from a specific environment, contrary to the traditional genomics in which individual samples are worked. Subsequently, metagenomics has been defined as "the application of modern genomic techniques for the direct study of communities of microorganisms in their natural environment, avoiding the need to isolate and cultivate each of the species of the community" (Chen and Pachter, 2005).

In parallel, deep sequencing platforms became to be available since 2005. These are capable of producing small sequences of DNA, between 25-500 bases and generate thousands of billions of readings per run without the need to know a priori the genomic sequences from the samples analyzed (Ansorge, 2009, Mardis, 2008, Metzker, 2010, Lister *et al*., 2009, Sogin *et al., 2006*). This last characteristic is what

makes them very attractive for the identification of new pathogens or those not previously described for a given host (Roossinck *et al*., 2010).

Thus, by joining metagenomics and deep sequencing technologies, great possibilities are generated to increase scientific knowledge thanks to obtaining a large amount of information through nucleotide sequences, enabling the detection, identification and discovery of viruses and other plant pathogens of impartial way, without requiring prior knowledge of the pathogen (Dinsdale *et al*., 2008).

## Hypothesis

The use of deep sequencing technologies linked to metagenomics will allow the identification of viruses or related pathogens, not previously described in native potato (*Solanum tuberosum* ssp. *tuberosum* L.) from Chiloé Archipelago.

## Objectives

### General Objectives

Develop and evaluate a metagenomic approach for detection and identification of viruses and / or related pathogens infecting native potatoes from the Chiloé Archipelago, using next generation sequencing platform.

### Specific Objectives

(i) Implement a strategy of deep sequencing of small RNAs derived from native potatoes.

(ii) Identify the virome present in samples of native potatoes and to determine the prevalence of these.

(iii) Characterize biologically and molecularly viruses and/or related pathogens not previously described in Chile.

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# Chapter 2

# Deep sequencing of siRNAs reveals the presence of a quarantine virus affecting native potatoes in Chile

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**Research paper**

# Deep sequencing of siRNAs reveals the presence of a quarantine virus affecting native potatoes in Chile

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**Abstract**

In Chile, the potato crop is present in several regions along the country, including native potato varieties from the Chiloé Archipelago, which are very diverse in terms of colour, shape, size and flavour. On the other hand, viral diseases are an important factor limiting crop production. Through a metagenomic approach, using deep sequencing followed by assembly of viral small RNAs (vsRNAs) to a reference virus genome, several potato virus such as *Potato virus A* (PVA)*, Potato virus M* (PVM)*, Potato virus S* (PVS)*, Potato virus Y* (PVY) and *Potato mop-top virus* (PMTV), were found infecting native potatoes in Chiloé Archipelago, in the south of Chile. PMTV is an important pathogen of potato crop, vectored by *Spongospora subterranea* f.sp. *subterranea* through zoopores. Potato mop top disease can cause serious economic losses in susceptible cultivars and the virus is a quarantine organism for the country. Thereby, the identity of the virus was confirmed through bait bioassay and reverse transcription-polymerase chain reaction (RT-PCR) for partial regions of the capsid gene (CP) and the second gen of the Triple Gene Block (TGB2). Amplicons sequencing revealed a 99-100% sequence identify with most PMTV coat protein or TGB2 sequences of isolates from Europe and USA, which was confirmed by further phylogenetic analysis.

**Key words:** Viral diagnostics, potato virus, *Pomovirus,* quarentine, metagenomic

## Introduction

An adequate detection of pathogens is key to maintaining the phytosanitary status of a crop, either because of its agronomic interest or as a contribution to biodiversity. A diagnostic tool must be sensitive enough to detect low quantities of nucleic acids or proteins of the pathogen, in turn, it must be able to identify between races or variants present.

While the identification and diversity of other microbes or organisms can be studied using 16S ribosomal RNA genes, or other universal genes called barcode sequences (Cataldo *et al*., 2011; Lee *et al*., 1998; Seifert, 2009), viruses lack of universal genes that are sufficiently similar to be useful in their identification or detection (Roossinck, 2011), the traditional techniques used enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), southern hybridization assay are species or genus specific, decreasing the detection capacity of unknown, emerging, or highly variable pathogens (Candresse *et al*., 2014) limiting access to diversity studies and viral composition in native environments (Wommack *et al*., 2012).

One way to face the challenge of detection and identification of plant viruses present without a priori knowledge is the small-RNA deep sequencing. This is a generic method to identify both viruses that have RNA or DNA genomes. Most plant viruses possess RNA genomes and produce a double-stranded RNA intermediate during their replication. Thus, these double-stranded structures are recognized and the plant uses as a defense mechanism the degradation of these RNAs called gene silencing or RNA interference (RNAi) (Waterhouse *et al*., 2001). The silencing

process consists of three main stages: initiation, amplification and dissemination of the signal (Llave, 2010). In general terms, plants are susceptible to viruses and these are revealed as well through the formation of double-stranded RNA intermediates, either during their replication or through the formation of hairpin structures. Then, Dicer proteins are able to recognize these intermediaries and process them into small interfering RNAs (21-24 nt), which interfere with viral replication (Llave, 2010). In addition, the silencing signal is capable of being amplified by RNA-dependent RNA polymerases that generate new double-stranded RNA, to be processed by Dicer proteins, and thus, new small RNAs can move from cell to cell through of plasmodesmata or systemically through the phloem (Kalanditis *et al*., 2008). Although viruses can adopt a series of strategies to overcome this type of response, the most common is the production of silencing suppressors (Li and Ding, 2006).

Deep sequencing of small RNAs is based in a natural anti-viral defense, which is present in all eukaryotic organisms and generates and leads to the accumulation of 21–24 nt virus derived siRNAs (Mlotshwa *et al*., 2008), which can be sequenced and assembled to posteriorly identify viruses by homology to known virus sequences. Successful examples of this tool has been employed to identify new viruses and to detect know plant viruses that have RNA o DNA genomes (Kreuze *et al*., 2009, 2013; Bi *et al*., 2012; Wu *et al*., 2012; Zhang *et al*., 2011; Li *et al*., 2012; Fuentes *et al*., 2012; Untiveros *et al*., 2010).

Today, about 50 viruses are known to infect potato crop globally (Jones, 2014). In Chile, potato is grown in several regions along the country, including varieties of native potatoes (*Solanum tuberosum* L. ssp. *tuberosum*) from Chiloé archipelago,

one of the subcenters of origin and diversity of cultivated potatoes. These native potatoes are preserved in the fields of small farmers, and they are very diverse in terms of color, shape, texture, size and flavour (Contreras and Castro Urrutia, 2008; Solano *et al*., 2007). However, viral diseases limit production. It has been described that viruses such as *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS) and *Potato virus M* (PVM) can to cause severe symptoms and yield losses (Li *et al.,* 2013). Crop yield reductions attributed to specific virus in specific crops may vary from less than 10% to more than 80–90% (Waterworth and Hadidi, 1998; Hadidi *et al*., 2011; Barba and Hadidi, 2015).

In this study, we applied a metagenomic approach using siRNA deep sequencing to detect viruses in a pool of samples of native potatoes from the Chiloé Archipelago.

## Materials and methods

***Plant and virus.*** 98 potato field samples were collected from 12 places of Chiloe Archipelago in the south of Chile during summer of 2011. Also, native potatoes showing PMTV symptoms and cystosori of *Spongospora subterranea* f.sp. *subterranea* were collected from several points within the Archipelago in later samplings.

***Deep sequencing and bioinformatics analysis of siRNAs.*** Total RNAs were extracted from foliar samples with CTAB, RNA samples were sent to Macrogen Inc.

(Seoul, South Korea) for library preparation and was sequenced using Genome Analyser platform. The sequence quality was checked by use of fastQC-0.10.1 (Andrews, 2010), and Novoalign (V2.07.18) was used for discarding of adaptors. Contigs were screened for homology to known viruses by BLASTN and BLASTX against the nr database (http://www.ncbi.nlm.nih.gov/) with an e-value threshold of 10−6 in both. Contigs mapping to the *Solanum tuberosum* L. genome, as well as those mapping to bacterial, fungal and protozoa genomic fragments were omitted from further work. A list of the potential viruses present in the analyzed samples was created using the virus Genbank database and reference sequences (RefSeq database) were selected for mapping of reads. Mapped reads were checked by Novoalign allowing 2-8 nucleotide and mismatches allowed (penalty: 40 open gap, 6 extended gap). The parameters were used for Global alignment, and the reads were matched randomly. Alignments and genome coverage, respectively, were visualized and estimated by Tablet 1.14. (Milne *et al*., 2013) and Geneious v8 (Biomatters Limited) was also used for mapping of reads on reference genomes, with a mismatches tolerance of two.

***Virus detection and prevalence determination by RT-PCR.*** Virus detected by deep sequencing was confirmed further using reverse transcription PCR (RT-PCR). cDNA was synthesized using random hexamer primers (IDT Technologies) and MMLV (Invitrogen) following the manufacture instructions, then cDNA was stored at -20 °C until use.

The 98 samples used to form the pool of total RNA used in deep sequencing were screened for *Potato leaf-roll virus, Potato mop-top virus, Potato virus A, Potato virus M, Potato virus S, Potato virus X* and *Potato virus Y*. PCR amplification was carried out with 2,5 μl of cDNA in final volume of 25 μl, containing 0,2 μM of each primer (forward and reverse) described in Table 1, 1,5 mM $MgCl_2$, 200 μM each dNTP with 1 unit of Taq DNA Polymerase (Invitrogen). After 5 min at 94°C, the cycling scheme used (35 cycles) was 30 s at 94°C, 30 s at 55 to 64°C (according to each virus), and 30 s at 72°C, followed by a final extension of 5 min at 72 °C. The final PCR products were visualized under UV light after electrophoresis on agarose gels. Randomly chosen PCR products were directly sequenced (Macrogen, Seoul).

***Detection PMTV in Spongospora subterranea cystosori.*** Cystosori (resting spores) samples of *Spongospora subterranea* f.sp. *subterranea* were provided by the Unidad de Fitopatología of the Laboratorio Regional-Servicio Agrícola y Ganadero (SAG, Osorno, Los Lagos Region). The samples were used to prepare total RNA using RNA-Solv Reagent (Omega) following the manufacturer's instructions. Then, cDNA was synthesized using random hexamer primers (IDT Technologies) and MMLV (Invitrogen) following the manufacture instructions and RT-PCR for partial fragments of CP and TBG genes were perform in the same way described above.

***Phylogenetic analysis PMTV*.** Nucleotide sequence of partial fragments of capside protein (CP) and second gene of triple gen block (TGB) were translated into amino acid sequences and phylogenetic trees were constructed using the neighbor-joining

algorithm implemented in MEGA 6 (Tamura *et al*., 2013) or Geneious v8 (Biomatters Limited) using a randomized bootstrapping (1000 replicates) for the evaluation of branching validity. All nodes supported by >50% confidence values are shown.

## Results and discussion

### Deep Sequencing

Chile is considered to be a sub-center of origin for the cultivated potato (Spooner *et al*., 2005), with introduced and native genetic material coexisting. Native potatoes varieties are mostly located in the Chiloé Archipelago preserved in the fields of small farmers, but the deterioration of their phytosanitary status can negatively affect its conservation (Solano *et al*., 2007). Through a metagenomic approach for a siRNA deep sequencing, a total of 2.197.732 reads in the 21 to 24 nt size range of siRNAs (Figure 1) were generated after filtered process.

Following of deep sequencing, assembly of viral small RNAs (vsRNAs) to a reference virus genome evidenced eleven different viruses infected the sample pool included in the analysis (Table 2). PVX and PVS had the highest number of reads with 116.331 and 57.184 respectively, followed by PVY (14397 reads), PLRV (8135 reads) and PVM (1339 reads). All previously described infecting traditional potato crops in the country. Among the viruses identified, the presence of *Potato mop top virus* (PMTV, 6.831 reads), a quarantine virus absent in Chile, prior to this investigation, standed out. Assembled contigs contained sequences corresponding to PMTV RNA1 showed coverage of bases of 77.3% of reference sequence (NC

003723.1) with 5.2X of average coverage depth, contigs assembled for PMTV RNA2 showed 76.8 % of coverage (Ref.seq: NC 003723.1) and 5.4X of coverage depth, while for PMTV RNA3 the contigs assembled showed 68 % of coverage (Ref.seq: NC 003724.1) with 3.2X of coverage depth. The distribution of the contigs with respect to the reference sequences for the three PMTV RNAs was only seen in the coding regions (Figure 2), which agrees with previous experiments (Kreuze *et al*., 2009). According to Kreuze (2014), a poor coverage of the UTRs regions would be due to an evolutionary strategy to avoid degradation/targeting by RNA silencing.

**Characterization of PMTV isolates and phylogenetic relationships**

Further sample collecting during summer of 2013 and 2014, found potatoes with PMTV symptoms (See supplemental figure) at two farms growing native potatoes in Puqueldón (Chiloé). In these samples, PMTV was identified by RT-PCR and amplicon sequencing of the TGB2 and CP fragments, revealing a 99-100% sequence identify with most PMTV coat protein or TGB2 sequences of isolates from Europe and USA, which was confirmed by further phylogenetic analysis (Figures 3 and 4).

PMTV was also detected on resting spores from its only known fungal vector, *Spongospora subterranea* f.sp. *subterranea (Sss),* from different potatoes growing areas of the country, such as Castro, La Serena, Los Muermos, Puqueldón and Puerto Montt. Under wet conditions, zoospores of *Sss* can germinate from the cysts and infect potato roots and tubers, while transmitting PMTV. The virus can survive for long periods of years in resting spores of Sss (Campbell, 1996) and this was

corroborated by a soil bait test with *Nicotiana tabacum* as bait. Soil to which resting spores positive to the presence of the virus were added y later were observed to efficiently transmit the virus to tobacco bait plants in controlled greenhouse conditions by immune tissue printing (Data not shown).

**Validation and determination of prevalence of potato virus by RT-PCR**

Finally, viral prevalence was determined for all 98 samples collected from the Chiloé Archipelago, revealing that PLRV has the highest prevalence (63%), followed by PMTV (59%), PVS (44%), PVX (42%), PVA (33%), PVY (16%) and PVM (13%). The detail of the positive samples can be seen in Table S1 of the supplementary material.

The high number of native potatoes infected with viruses is of great concern, because their cultivation is predominantly linked to small agriculture they are not under regulations of programs for the production of certified tuber potato seeds. Thus, the natives potatoes infected can act as reservoirs of emerging viruses such as PMTV, a virus considered quarantine in Chile, that can cause severe damage at the level of tubers (necrosis). Furthermore, there are no known sources of resistance to PMTV.

This study provides sufficient evidence of the ability to detect viruses using siRNAs derived from viral silencing. In addition, relevant information is presented regarding the phytosanitary status of native potatoes of Chilean origin.

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## Tables

**Table 1.** Primers used in the present study.

| Virus | Primers | Sequence 5'-> 3' | Tm (°C) | Fragment size (bp) | Reference |
|---|---|---|---|---|---|
| PLRV | PLRV-F | CCACTCCAACTCCCCAGAAG | 57 | 208 | Du *et al.,* 2006 |
| | PLRV-R | TACATAGGGACGGCTTGCAT | 56 | | |
| PVY | PVY-F | ATACTCGRGCAACTCAATTCACA | 55 | 166 | Du *et al.,* 2006 |
| | PVY-R | CCATCCATCATAACCCAAACTC | 53 | | |
| PVX | PVX-F-337 | TCCTTATTCCAACGGCATC | 52 | 337 | Maoka *et al.,* 2010 |
| | PVX-R-337 | ATCTAGGCTGGCAAAGTCG | 55 | | |
| PVS | PVS-F102 | GGGGCTCATACGCAGRCCCACAC | 65 | 485 | Salari *et al.,* 2011 |
| | PVS-R102 | TCTGACTTTGCACCATGGGTGG | 60 | | |
| PVA | PVAF | TTTCTATGAGATCACTGCACCCACT | 56 | 116 | Du *et al.,* 2006 |
| | PVAR | TGACATTTCCGTCCAGTCCAA | 56 | | |
| PVM | PVM3 | TGAGCTCGGGACCATTCATAC | 59 | 520 | Hu *et al.,* 2010 |
| | PVM4 | ACATCTGAGGACATGATGCGC | 60 | | |
| PMTV | H360 | CATGAAGGCTGCCGTGAGGAAGT | 62 | 460 | MacKenzie *et al.,* 1996 |
| | C819 | CTATGCACCAGCCCAGCGTAACC | 62 | | |
| PMTV | Pmt4F | CAGCAACCACAAACAGACAGG | 57 | 417 | Xu *et al*., 2004 |
| | Pmt4R | AAGCCACTAACAAAACATACTGC | 54 | | |

Acronymus used are as follows: PLRV (*Potato leaf roll virus*), PVY (*Potato virus* Y), PVX (*Potato virus* X), PVS (*Potato virus S*), PVA (*Potato virus* A), PVM (*Potato virus* M)

**Table 2. Number of reads mapped to reference virus genomes by Novoalign**

| Virus genus | Virus ID | Number of reads | Reference sequence |
|---|---|---|---|
| *Carlavirus* | *Potato virus S* | 57184 | NC_007289.1 |
| | *Potato virus M* | 1339 | NC_001361.2 |
| | *Potato rough dwarf virus* | 179 | NC_009759.1 |
| | *Potato latent virus* | 118 | NC_011525.1 |
| *Polerovirus* | *Potato leafroll virus* | 8135 | NC_001747.1 |
| *Pomovirus* | *Potato mop-top virus RNA 1* | 1509 | NC_003723.1 |
| | *Potato mop-top virus RNA 2* | 3506 | NC_003725.1 |
| | *Potato mop-top virus RNA 3* | 1816 | NC_003724.1 |
| *Potexvirus* | *Potato virus X* | 111631 | NC_011620.1 |
| *Potyvirus* | *Potato virus Y* | 14397 | NC_001616.1 |
| | *Potato virus A* | 3321 | NC_004039.1 |

## Figures

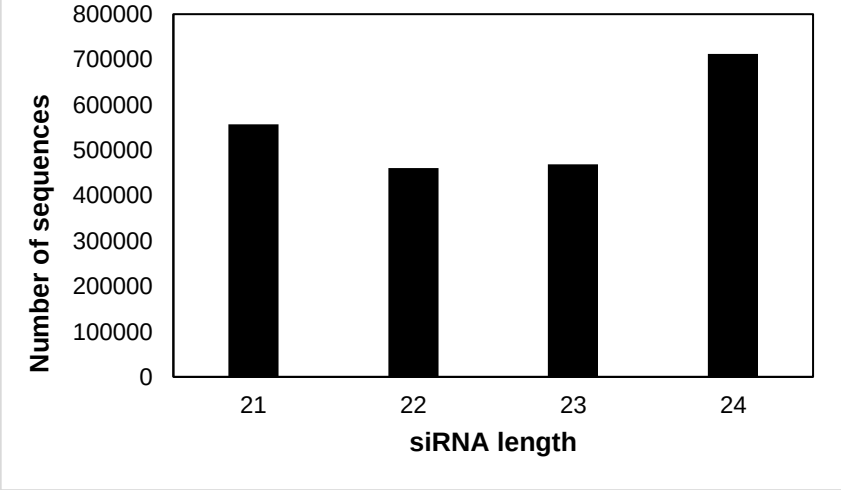


**Figure 1.** Numbers of siRNA obtained from Ilumina plataform (Genome Analyzer) from native potatoes pool samples.

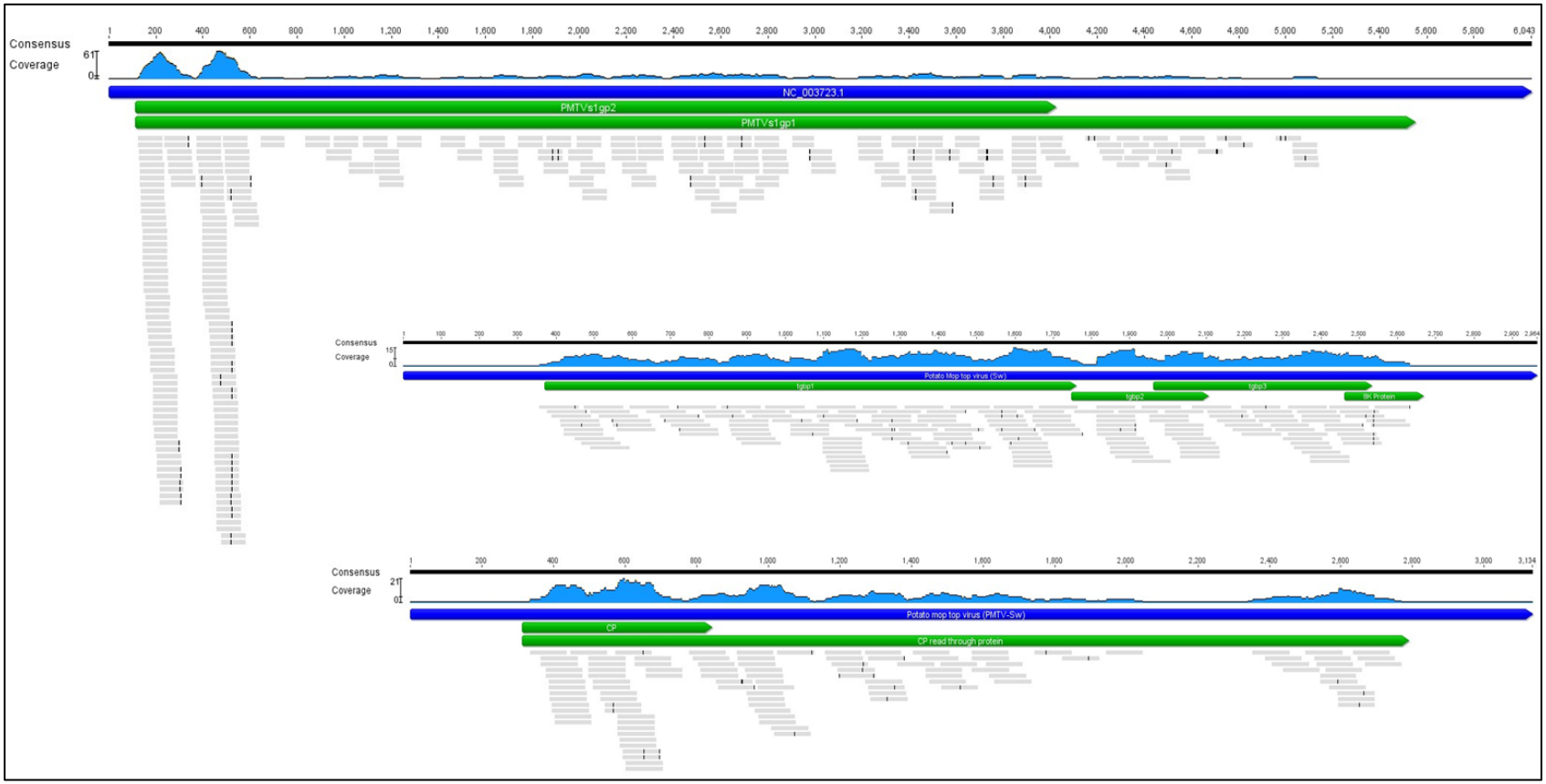


**Figure 2.** PMTV genome coverage following deep sequencing. The genomic organization of PMTV is schematically shown in blue and green, the coverage achieved after a mapping of reads produced by Illumina-based siRNA sequencing against the PMTV genome is indicated in gray.

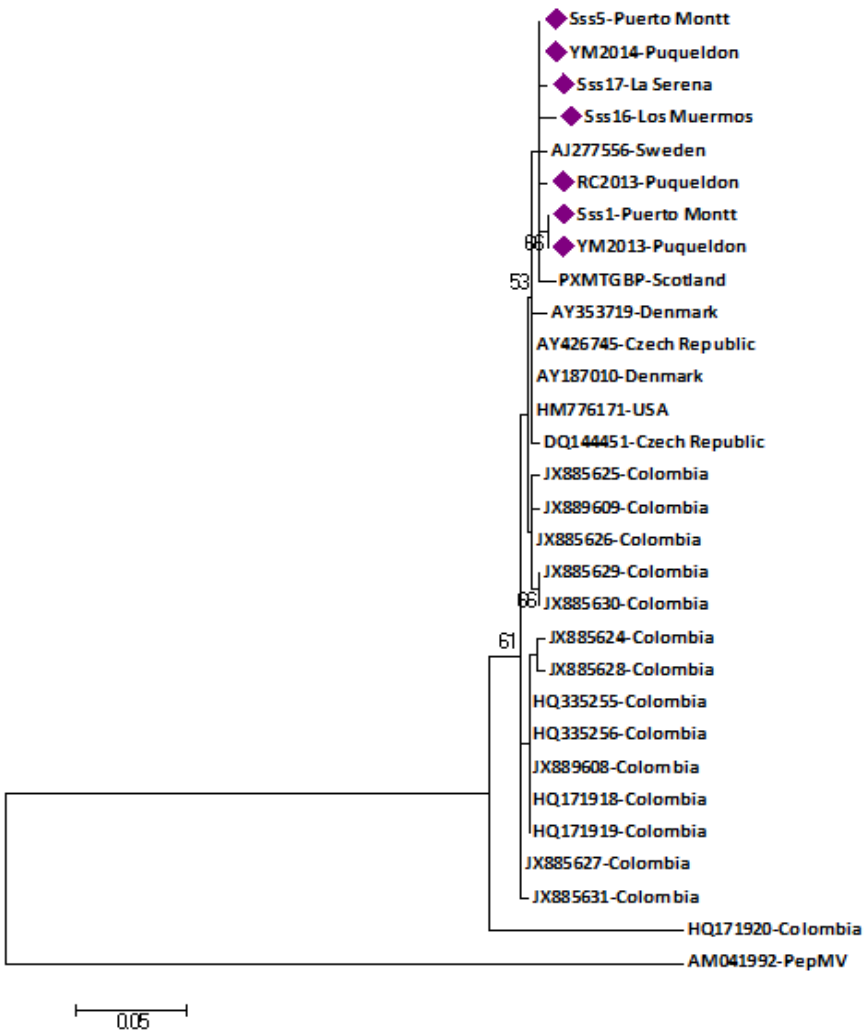


**Figure 3.** Phylogenetic tree constructed for PMTV isolates from the multiple alignments of amino acid sequences from RNA 3 (encoding gene 2 of TGB). The tree was constructed by the neighbor-joining method in MEGA 6 and the statistical significance of branches was evaluated by bootstrap analysis with 1,000 replicates, only bootstrap values >50% are shown. The scale bar represents 5% amino acid divergence. The accession number of the sequences retrieved from GenBank is indicated, together with the origin country. The Chilean isolates are indicated by a purple diamond and isolate of *Pepino mosaic virus* was used as outgroup.

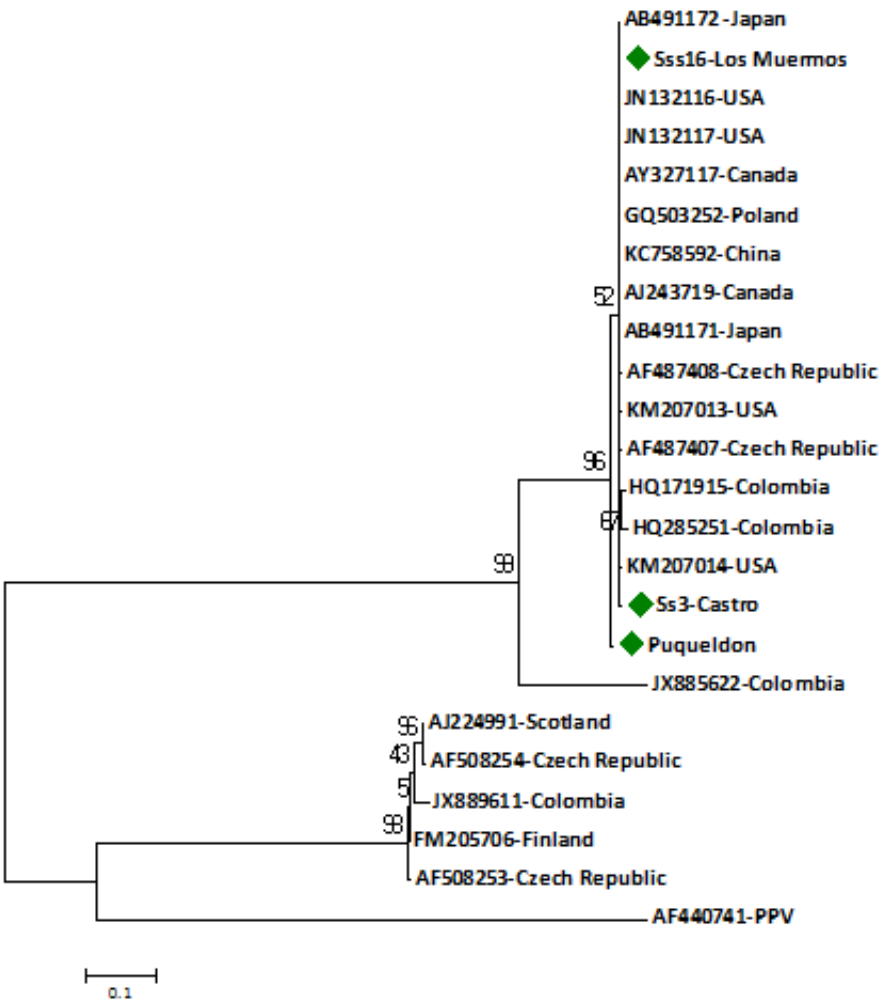


**Figure 4.** Phylogenetic tree constructed for PMTV isolates from the multiple alignments of amino acid sequences from RNA 2 (CP). The tree was constructed by the neighbor-joining method in MEGA 6 and the statistical significance of branches was evaluated by bootstrap analysis with 1,000 replicates. The scale bar represents 10% amino acid divergence. The accession number of the sequences retrieved from GenBank is indicated, together with the origin country. The Chilean isolates are indicated by a green diamond and isolate of *Plum pox virus* was used as outgroup

.

**Suplemental material**

**Table S1.** Multiple infections

| Sample | PMTV | PVS-O | PVS-A | PVX | PVY | PVA | PVM | PLRV | TRV |
|---|---|---|---|---|---|---|---|---|---|
| 1.1 | - | - | - | - | - | - | - | - | + |
| 1.2 | - | + | - | - | - | - | + | - | - |
| 1.3 | - | + | - | - | - | - | + | - | - |
| 1.4 | + | + | - | + | - | - | + | - | - |
| 1.5 | - | + | - | - | - | - | + | - | + |
| 1.6 | + | - | + | - | - | - | - | - | + |
| 1.7 | - | + | + | - | + | - | + | - | + |
| 1.8 | - | - | + | - | - | - | - | - | + |
| 1.9 | + | + | + | + | - | - | - | - | + |
| 1.10 | - | - | - | + | + | - | - | + | + |
| 1.11 | - | + | - | - | - | - | - | + | + |
| 1.12 | + | - | - | + | - | - | - | - | - |
| 1.13 | + | - | + | + | - | - | - | + | - |
| 2.1 | + | - | - | + | - | - | - | + | - |
| 2.3 | - | - | - | - | + | - | - | - | - |
| 2.4 | + | - | - | - | - | - | - | - | + |
| 2.5 | - | - | - | - | - | - | - | - | - |
| 2.6 | - | - | - | - | - | - | - | - | + |
| 2.7 | - | - | - | - | - | - | - | + | - |
| 2.8 | - | - | - | + | - | - | - | - | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 2.9 | + | - | - | - | - | + | - | + | + |
| 2.10 | - | - | - | + | - | + | + | + | - |
| 2.11 | - | - | - | - | - | + | - | + | + |
| 2.12 | - | - | - | - | - | + | - | + | + |
| 3.1 | - | - | - | - | - | - | - | + | + |
| 3.2 | - | - | + | - | - | - | - | + | - |
| 3.3 | + | - | - | - | - | - | - | + | - |
| 3.4 | - | - | - | - | - | - | - | - | - |
| 3.5 | - | - | - | - | - | - | + | + | + |
| 4.1 | + | + | - | - | - | + | - | + | + |
| 4.2 | + | - | - | - | - | - | - | - | - |
| 4.3 | + | + | - | - | + | + | - | + | + |
| 4.4 | + | - | + | + | - | - | - | + | + |
| 4.5 | + | - | - | - | - | + | - | + | - |
| 4.6 | + | - | - | - | + | + | - | + | + |
| 4.7 | + | - | - | + | + | - | - | - | - |
| 4.8 | + | - | - | + | + | + | - | + | - |
| 4.9 | + | - | - | - | + | + | - | + | - |
| 4.10 | + | + | - | - | - | - | - | - | - |
| 4.11 | + | - | - | + | - | + | - | + | - |
| 4.12 | + | + | - | - | - | - | + | + | - |
| 4.13 | + | + | - | + | + | + | - | + | - |
| 4.14 | + | + | - | + | + | + | - | + | - |
| 4.15 | + | + | + | + | + | - | - | + | - |
| 4.16 | + | - | - | - | - | - | - | - | + |
| 4.17 | + | - | - | - | - | + | - | + | + |
| 4.18 | + | - | - | + | - | - | - | + | - |
| 4.19 | + | - | - | - | - | - | - | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 4.20 | + | - | - | - | - | - | - | - | - |
| 4.21 | + | + | - | - | - | - | - | - | + |
| 5.1 | - | + | - | - | - | - | + | + | - |
| 5.2 | - | - | + | - | - | + | - | + | - |
| 5.3 | - | - | - | - | - | - | - | + | - |
| 5.4 | - | - | - | - | - | - | - | - | - |
| 6.1 | - | - | - | - | - | + | - | + | - |
| 6.2 | + | - | - | - | - | + | + | + | + |
| 6.3 | - | - | - | - | - | + | - | + | - |
| 6.4 | - | - | - | - | + | - | + | + | + |
| 6.5 | - | - | - | - | - | + | - | - | + |
| 6.7 | + | - | + | - | - | - | - | + | + |
| 6.8 | - | - | - | - | - | - | - | - | + |
| 6.10 | + | - | - | + | - | - | - | + | + |
| 6.11 | + | - | - | + | - | - | - | + | - |
| 7.2 | + | + | - | + | - | - | - | - | + |
| 7.3 | + | + | - | - | - | + | - | + | - |
| 7.6 | - | - | + | - | - | + | - | + | + |
| 7.7 | + | + | - | + | - | - | - | + | + |
| 7.8 | + | - | - | - | - | - | - | + | - |
| 7.9 | + | - | - | + | - | + | - | - | - |
| 7.10 | + | - | - | + | + | + | - | + | + |
| 7.11 | - | + | - | + | - | + | - | + | + |
| 7.12 | - | - | - | - | - | - | - | - | - |
| 7.13 | - | + | - | + | + | + | - | + | + |
| 7.14 | - | + | - | + | - | - | - | + | - |
| 7.15 | - | - | - | + | - | - | - | + | + |
| 7.16 | + | + | - | + | - | - | - | - | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 7.17 | - | - | - | + | - | + | - | + | + |
| 7.18 | - | + | - | + | - | - | - | - | - |
| 7.19 | + | - | - | + | - | - | - | - | + |
| 7.20 | - | - | - | - | + | - | - | - | - |
| 8.2 | - | + | - | + | - | - | + | + | + |
| 8.3 | - | - | - | - | - | - | - | + | + |
| 8.4 | + | - | - | - | - | - | - | - | + |
| 9.1 | - | - | - | - | - | - | - | - | + |
| 9.2 | + | + | - | - | - | - | - | + | + |
| 9.3 | - | - | - | + | - | - | - | + | + |
| 9.4 | + | + | - | + | - | - | - | + | - |
| 9.5 | + | + | - | + | - | - | - | + | - |
| 10.1 | + | - | + | + | - | + | - | + | - |
| 11.1 | - | - | - | + | - | + | - | + | - |
| 11.2 | - | + | - | - | - | + | - | + | - |
| 11.3 | + | - | - | - | - | - | - | + | + |
| 11.4 | + | + | - | + | + | + | - | + | + |
| 11.5 | - | + | - | + | - | + | - | - | + |
| 11.6 | + | + | - | - | - | - | - | + | - |
| 11.7 | + | + | - | + | - | + | - | + | - |
| 11.8 | + | + | + | + | - | - | - | + | + |
| 12.1 | + | - | - | + | - | - | + | + | + |

# Chapter 3

## First Report of *Potato mop-top virus* in Chile

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**Disease notes**

# First Report of *Potato mop-top virus* in Chile

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*Potato mop-top virus* (PMTV) is an important pathogen of potato (*Solanum tuberosum* L.), occurring in Northern Europe, North and South America, and Asia, and significantly reducing potato production (Santala *et al*. 2010). PMTV is transmitted by spores of *Spongospora subterranea* f. sp. *subterranea*, the causal agent of powdery scab of potato (Jones and Harrison 1969). During the summer season of 2012, native potato (*S. tuberosum* L. ssp. *tuberosum)* leaves showing virus-like symptoms were collected on several points within the Chiloé Archipelago (Chiloé Province, southern Chile), one of the centers of origin and diversity of

cultivated potatoes (Montaldo 1984). The presence and identity of viruses in the samples were determined through next-generation high-throughput sequencing (Solexa-Illumina) followed by assembly of viral small RNAs (vsRNAs) to reference plant virus genomes. The plant material used for the construction of the sRNA library corresponded to pools of symptomatic leaves sampled in the Archipelago. This analysis revealed that PMTV was infecting native potatoes in Chiloé, since the assembled contigs covered 77.3, 76.8, and 68.0% of PMTV RNA1 (reference sequence, GenBank Accession No. NC_003723.1), RNA2 (reference sequence, NC_003725.1) and RNA3 (reference sequence, NC_003724.1), respectively. Subsequently, three farms growing native potatoes (local varieties Michuñe negra, Michuñe roja, and Clavela lisa) with symptoms associated with PMTV infections were identified in Puqueldón (Lemuy Island, Chiloé Province) during the summers of 2013 and 2014. Disease symptoms observed included stunting of stems, shortening of internodes, and mosaic patterns (V-shaped) on leaves, but necrotic symptoms on the surface or the flesh of tubers were not observed. To detect PMTV, total nucleic acid was extracted from three symptomatic plants each year and tested by reverse transcription (RT)-PCR using two sets of primers. In all samples analyzed, primer set C819/H360, targeting the coat protein (CP) gene in RNA3 yielded a 460-bp amplicon (Xu *et al*. 2004). Primer set pmtF4/pmtR4 amplified a 417-bp fragment from the second gene of the triple gene block (TGB2) in RNA2 (Xu *et al*. 2004). PCR products were directly sequenced (Accession Nos. KT267163 and KT267164). BLAST analysis showed that the amplicons were 99 to 100% identical to most of the CP or TGB2 sequences from several PMTV isolates from Europe and

the United States. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA, Bioreba AG) on the same symptomatic potato leaf samples confirmed the presence of the virus in all cases. To our knowledge, this is the first report of PMTV in Chile. However, the powdery scab pathogen, *S. subterranea* f. sp. *subterranea*, is widespread in the potato-production areas near Chiloé Province. These results provide important and cautionary information to be considered by programs managing seed tuber production and plant quarantine for potato pathogens in Chile.

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**Supplemental Material**

Supplementary Fig. 1

Symptoms of *Potato mop top virus* infection observed in the field. V-shaped, chlorotic chevrons were occasionally observed on potato leaves collected within the Chiloé Archipelago, in the south of Chile.

# Chapter 4

## Molecular characterization of new *Carlavirus* infecting potato in Chile

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## Virology

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## ABSTRACT

Potato is one of the most important foods crops in the world. In Chile, potato crops are present in several regions along the country, including native potato varieties originating in the Chiloé archipelago, which are very diverse in terms of color, shape, texture, size and flavor. However, viral diseases are an important factor limiting potato production. Many viruses, such as *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS), *Potato virus M* (PVM) cause from mild to severe symptoms, and yield losses. Preliminary studies of potato viruses led to the discovery of a new virus. The determination and analysis of its partial nucleotide sequence provided evidence that this agent represents a new species of

the genus *Carlavirus*. Complete RNA genomic sequences of one isolate of a new virus was determined using reverse transcription-long distance PCR and the 5′ rapid amplification of cDNA ends (5' RACE) method. Sequence analysis revealed that the new virus had the typical genomic organization of members of the genus *Carlavirus*, with a positive-sense single-stranded genome of 8422 nt. The amino acid sequences of the coat protein and RNA dependent RNA polymerase showed a sequence identity between 28.3 to 68.4% and 34.9 to 56.7 %, respectively, compared with other Carlaviruses.

## INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important foods crops in the world. In Chile, potatoes are grown in several regions along the country, including more of 200 accessions of native potatoes from Chiloe archipelago (Contreras and Castro Urrutia, 2008), one of the sub centers of origin and diversity of cultivated potatoes (Jones, 2005; Spooner, 2005). These native potatoes are very diverse in terms of color, shape, texture, size and flavour (Contreras and Castro Urrutia, 2008) and have been maintained over the time by small-scale farmers practicing subsistence agriculture (Solano *et al*., 2013). However, some of the native potatoes from Chiloe are being lost because to the introduction of potato cultivars from Europe and national breeding programs (Solis *et al*., 2007; Solano *et al*., 2013). Furthermore, phytopathological deterioration, such as that produced for fungi and viruses, has also contributed to the loss of native potatoes.

Like other vegetative propagated crops, potato is infected with many virus diseases. Nowadays, about 50 viruses are known to infect crop potato globally (Valkonen, 2007; Jones, 2014). In Chile, the presence of 11 viruses infecting potato has been described, including the well-studied virus it was found *Potato virus Y*, *Potato virus X*, *Potato virus A*, *Potato leaf-roll virus*, *Potato virus S*, *Potato virus M* and *Potato mop-top virus* (Acuña, 2008; Peña *et al*., 2016). Of these, PVS and PVM belong to the genus *Carlavirus* in the family *Betaflexiviridae* (Adams *et al*., 2004; Martelli *et al*., 2007; Ryu and Lee, 2008).

The genus *Carlavirus* comprise *47* species (ICTV, release 2017). PVS, PVM, *Potato latent virus* (PotLV), *Potato virus P* (PVP, also known as *Potato rough dwarf virus*,

PRDV) and *Potato virus H* have been reported infecting potatoes cultivars worldwide (Brattey *et al*., 2002; Massa *et al*., 2006; Nisbet *et al*., 2006; Li *et al*., 2013; Jones, 2014).

Our studies in the Chiloé Archipelago, south of Chile, led to the discovery of a new virus infecting potato plants. The results showed that genome analysis of this new virus have enough differences to distinguish from *Potato virus P* (PVP, *Carlavirus, Betaflexiviridae),* the most closely related virus. We propose its classification as a separate species in the genus *Carlavirus*, and the name *Chiloe potato virus* (ChPV) is propose.

## MATERIALS AND METHODS

### Plant material and virus isolates

Ten native potato leaves samples from Curaco de Vélez, Chonchi and Puqueldón municipalities in the Chiloé archipelago were collected during summer of 2011 and selected for *Carlavirus* detection by polyvalent degenerated oligonucleotides nested PCR. For prevalence analysis of ChPV, 98 leaves of native potato samples were collected in 12 places distributed in Chiloé archipelago. Potato leaf samples of commercial potatoes from La Araucanía and Los Lagos regions, were provided by M. Gutiérrez of Servicio Agrícola y Ganadero, Laboratorio Regional-Osorno, Chile.

**Total nucleic acids extractions**

Total acid nucleic (TNA) extraction for all samples was performed according to the procedure two, described by Foissac *et al*. (2005).

**Universal *Carlavirus* detection by polyvalent degenerated olinucleotides (PDO) nested RT-PCR**

Carlaviruses detection were realized by PDO nested RT-PCR for conserved motifs of viral replicase (RdRp). First, TNA samples (5µL) were submitted to a reverse transcription with random hexamers primers and using the Superscript II Reverse Transcriptase in 20 µl volume reaction following the manufacturer's recommendations (Invitrogen/Fischer Scientific). Each PCR assay was used with combined primers, for the first: MC 1 (5'-TITTYWYIAARWSICARHTITGYAC-3'), RMC 6 (5'-GMRCACATRTCRTCICCIGCIAARCA-3'), and RMC 7 (5'-AVIYKCCAICCRCARAAISTIGG-3'), from which an aliquot was used in a second nested PCR assay using primers MC 2 (5'-GCIAARSBIGYICARWSIATYGTITG-3') and RMC 5 (5'-TCICCIGARAAICGCATRATIGC-3'). The first RT-PCR was carried out with 4 µl of cDNA in a final volume of 40 µl (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 0.09% Triton X-100, 4 mM $MgCl_2$, 200 µM each dNTP, and 1 µM each of primers MC 1, RMC 6, and RMC 7) and 1 unit of DyNAzyme II DNA Polymerase (Termofisher, France). Reactions were incubated at 95°C for 3 min, 35 amplification cycles (30 s at 95°C, 30 s at 42°C, and 30 s at 72°C) were performed. The nested PCRs were performed using 1 µL of the first amplification reaction in a final volume

of 40 µL (10 mM Tris-HCl [pH 8.8], 50 mM KCl, 0.09% Triton X-100, 4 mM $MgCl_2$, 200 µM each dNTP, and 1 µM each of primers MC 2 and RMC 5) with 1 unit of DyNAzyme II DNA Polymerase (Termofisher, France). After 3 min at 95°C, the cycling scheme used (40 cycles) was 30 s at 95°C, 30 s at 42°C, and 30 s at 72°C. The final PCR product of 358 bp was visualized under UV light after electrophoresis on ethidium bromide-stained 2% agarose gels. PCR products were directly sequenced (GATC Biotech AG, Germany).

**Determination of complete sequence of ChPV isolate by LD-PCR and 5' RACE**

The 5' sequence was determined using 5' rapid amplifications of cDNA ends (RACE) strategy and internal specific primer following the manufacture instructions (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). The 3' genomic region was amplified in long distance PCR amplifications reactions using forward internal primers designed from conserved domains of RdRp sequence between Carlaviruses in long-distance PCR amplification reactions following the supplier instructions. All amplification products were sequenced on both strands (GATC Biotech AG, Mulhouse, France) directly and the sequences obtained were assembled with to generate the complete genomic viral sequence.

**Sequence and phylogenetic analyses**

Multiple alignments of nucleotides or deduced amino acid sequences were performed using Clustal W program (Larkin *et al*., 2007) implemented in Geneious v8.1.8 (Biomatters) or MEGA6 (Tamura *et al*., 2013). Phylogenetic trees were constructed using the neighbour-joining method and randomized bootstrapping (1000 replicates) for the evaluation of branching validity. All nodes supported by >50% confidence values are shown.

**Determination of prevalence of ChPV**

Based on the sequences obtained, a diagnostic assay was developed for detection of NEW CARLA infecting native or commercial potatoes. Total acid nucleic extraction was performed following the procedure two described by Foissac *et al*. (Foissac *et al*., 2005). Specific primers for the new virus were designed, CPi-F (5'-CCTACCACACACGTCAATGC-3') and CPi-R (5'- CCTAAGCCCCACATTTTTCA-3'). PCR was performed with 2.5 µl of cDNA in a final volume of 25 µl (10mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 50 ng each of primers CPiF and CPiR) and 1 unit of Platinum Taq (Invitrogen/Thermofisher, Foster City, CA). After 5 min at 95°C, the cycling scheme used (35 cycles) was 95°C for 30 s, 61°C for 30 s, and 30 s at 72°C followed by a final extension step of 72°C for 5 min. The final PCR product of 489 bp was visualized under UV light after electrophoresis on ethidium bromide-stained 2% agarose gels.

# RESULTS

## Universal Carlavirus detection

Ten randomly selected native potato samples were used to detect Carlavirus by PDO RT-PCR. Eight samples were positive for Carlavirus, obtaining fragments of about 356 bp, which were subsequently sequenced. The result of sequencing and subsequent BLAST analysis revealed that most fragments corresponded to RdRp from PVM and PVS, with the exception of a sample from Puqueldón municipality in the Chiloé archipelago that showed similarity of 75%, to PVP (data not show).

## Complete sequence

The complete genome of this new potato Carlavirus was determined and consists of 8422 nt plus a poly-A tract at the 3'-terminus (GenBank accession n°). Sequence analysis showed the same organization as other Carlaviruses with six putative open reading frames (ORFs) (Adams *et al*., 2004; Martelli *et al*., 2007) (figure 1). The ORF1 spans from 64 to 5886 nt and encode the viral replicase protein (RdRp) with a predicted molecular mass of 218.98 kDa. Using the Conserved Domain Database (CDD) search at the NCBI website (Marchler-Bauer *et al*., 2015), 4 viral domains were found for ORF 1 (RdRp): metyltransferase (MTR), Carla endopeptidase, Superfamily 1 viral RNA helicase and RNA dependent RNA polymerase.

Then, there are three overlapping ORFs downstream of ORF1. ORF2 from 5917 nt to 6606, ORF3 from 6584 nt to 6904, and ORF4 from 6886 nt to 7080, that encode proteins for the Triple gene block (TGB), involved in viral movement (Morozov, 2003; Verchot-Lubicz *et al*., 2010) with a predicted molecular weight of 25.26 kDa, 11.37 kDa, and 7.06 kDa proteins, respectively. ORF5 (nt 7122-8030) encodes the coat protein (CP) with a predicted molecular weight of 32.92 kDa and contains three highly conserved domains typical of carlaviruses and potexviruses. The 3' proximal ORF encodes a putative nucleic acid binding protein of 12.19 kDa containing a domain that includes a zing finger cysteine motif (C-X2-C-X9-C-X4-C), a conserved feature of carlaviruses (Gramstat *et al*., 1990; Martelli *et al*., 2007). Non-coding sequences correspond to 69 nt at the 5 'end and 76 nt at the 3' end, not including the Poly-A tail.

**Phylogenetic and sequence analysis**

The complete nucleotide sequence of ChPV was compared with others Carlaviruses. The highest identity found was 60.1 % with *Potato virus P* (or PRDV) and the lowest identity was to *Sweet potato yellow mottle* virus with 40.4 %. The ORF 1, which encode the replicase viral showed nucleotide and amino acid identities of 40.9-56.2 and 34.9-56.7%, respectively, with other carlaviruses. The highest identity was with PVP, whereas the lowest identity level was with *Nerine latent virus* and *Narcissus symptomless virus.* The OR5, which encodes CP, showed a nucleotide identity between 40.4-65.2% with other carlaviruses. The highest identity was with PVP and

lowest identity was with *Sweet potato chlorotic fleck virus*. The predicted amino acid sequence of CP shared 68.4% identity with PVP and 28.3% of identity with *Poplar mosaic virus*, the lowest value of identity (table 1)

Multiple sequence alignments were made using the ClustalW included in the Geneious package (Biomatters Ltda.), which was also used for the construction of phylogenetic trees. Apple stem pitting virus, ASPV, (genus *Foveavirus*, family *Betaflexiviridae*) was used as outgroup. Phylogenetic relationship of ChPV and members of the genus Carlavirus based on viral polymerase and coat protein indicate that ChPV formed a distinct branch and was most closely related to Potato virus P (figure 2).

**Prevalence and distribution of ChPV**

The virus was detected in potato leaves using RT-PCR strategy with specific primers designed to amplify a fragment of the CP. The virus was present in 11 of the 12 sampling sites at Chiloé archipelago (figure 3), where 57/98 native potato leaves samples were infected. All positives samples were infected with the ChPV and at least one of the following potato virus such as PLRV, PMTV, PVA, PVM, PVS, PVX, PVY and/or TRV. Additionally, potato commercial varieties from three regions in the south of Chile (La Araucanía, Los Ríos and Los Lagos) were analyzed, and 62 out of 63 samples were infected with the virus.

## DISCUSSION

The particular characteristics of Chiloé and Chonos archipelagos have allowed the proliferation of a great number of native potatoes varieties (Spooner *et al*., 1991; Contreras *et al*., 1993; Contreras and Castro Urrutia, 2008). These native potatoes have high allelic richness representing wide genetic diversity in comparison with commercial varieties (Solis *et al*., 2007; Solano *et al*., 2013). However, studies on the phytosanitary status of these native varieties are limited, probably because they are preserved in the fields of small farmers.

RT-PCR PDO assays have been successfully reported for the detection of members of three viral genera in the family *Flexiviridae*, the *Trichovirus*, *Capillovirus*, and *Foveavirus*. (Foissac *et al*., 2005). For *Carlavirus,* an amplicon of 356 bp of the targeted polymerase motifs was generated and have the potential for to detect previously unknown agents. Here, an amplicon showed a lowest nucleotide identity with PVP (75%), suggesting the presence of a new species of the genus. To corroborate this, we used a LD-PCR strategy and subsequent sequencing to obtain the complete virus sequence.

This is the first report of the complete genomic sequence of ChPV (*Chiloe potato virus)*. Analysis of the virus genome structure and phylogenetic indicates that ChPV belongs to a new species of the genus *Carlavirus* according to species demarcation criteria where distinct species have less than about 72% nucleotides or 80% amino acids identical between their coat protein (CP) or replicase genes (Martelli *et al*.,

2007; Ryu and Lee, 2008), demonstrating that ChPV should be considered a member of a new species within this genus.

The molecular detection of >150 samples analyzed between native and traditional potatoes showed a prevalence of 73.9% of the virus in the main seed production area of the country. Biological information of ChPV is difficult, because the virus was found in mixed infections in all cases which makes complicate to associate a specific symptomatology to the virus infection.

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**Tables**

TABLE 1. Nucleotide and amino acid identity between *Chiloe potato virus and* other Carlaviruses.

| Region | nt (%) | | aa (%) | |
|---|---|---|---|---|
| | min | max | min | max |
| 5’ UTR | 20.6 | 58.7 | - | - |
| ORF 1 (RdRp) | 40.9 | 56.2 | 34.9 | 56.7 |
| ORF 2 (TGB1) | 36.4 | 53.6 | 27.5 | 58.1 |
| ORF 3 (TGB2) | 40.2 | 60.7 | 33.0 | 65.1 |
| ORF 4 (TGB3) | 28.8 | 56.9 | 13.6 | 40.6 |
| ORF 5 (CP) | 40.4 | 65.2 | 28.3 | 68.4 |
| ORF 6 (NABP) | 22.4 | 66.4 | 10.5 | 68.2 |
| 3’ UTR | 30.8 | 88.9 | - | - |
| FULL LENGHT | 40.4 | 60.1 | - | - |

**Figures**

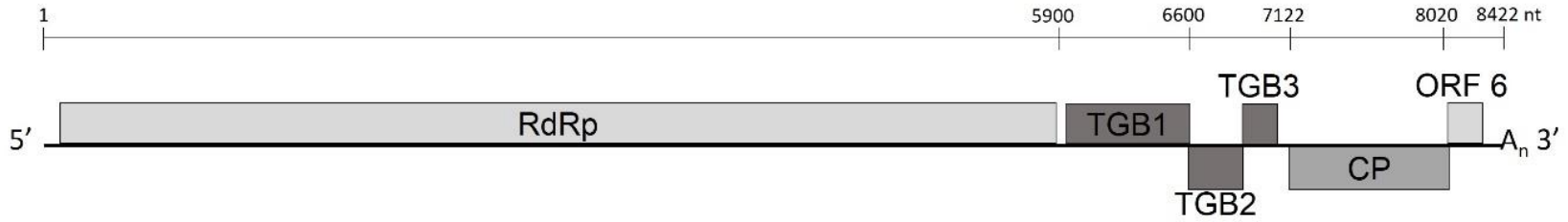
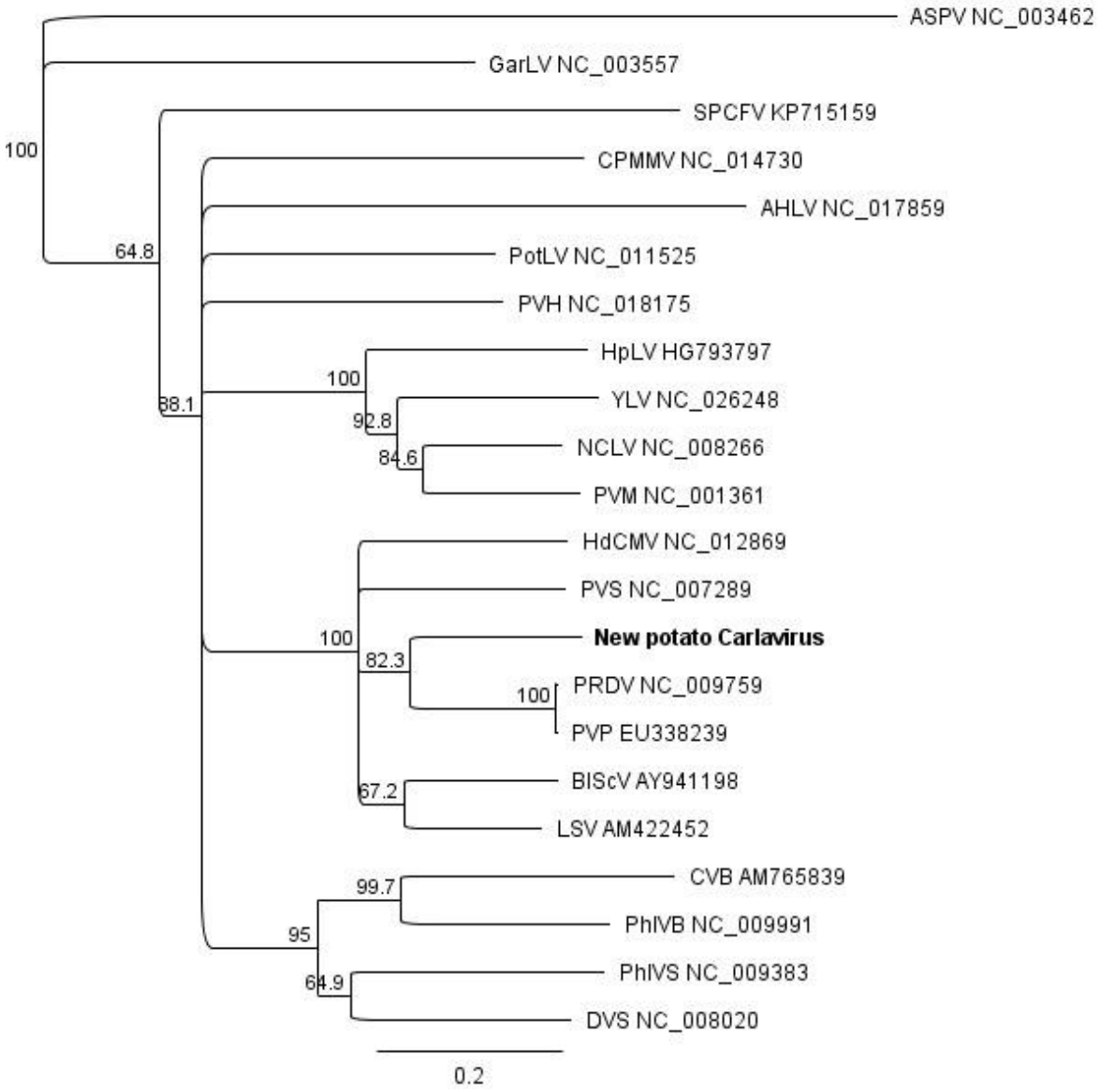



Figure 1. Schematic representation of genome organization of ChPV RNA encoding the viral RNA-dependent RNA polymerase (RdRp), triple block proteins (TGBs), coat protein (CP) and 12kDa protein (ORF6).

A)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GarLV NC_003557
SPCFV KP715159
CPMMV NC_014730
AHLV NC_017859
PotLV NC_011525
PVH NC_018175
HpLV HG793797
YLV NC_026248
NCLV NC_008266
PVM NC_001361
HdCMV NC_012869
PVS NC_007289
New potato Carlavirus
PRDV NC_009759
PVP EU338239
BIScV AY941198
LSV AM422452
CVB AM765839
PhlVB NC_009991
PhlVS NC_009383
DVS NC_008020
100
64.8
100
88.1
100
92.8
84.6
100
82.3
100
67.2
99.7
95
64.9
0.2

B)

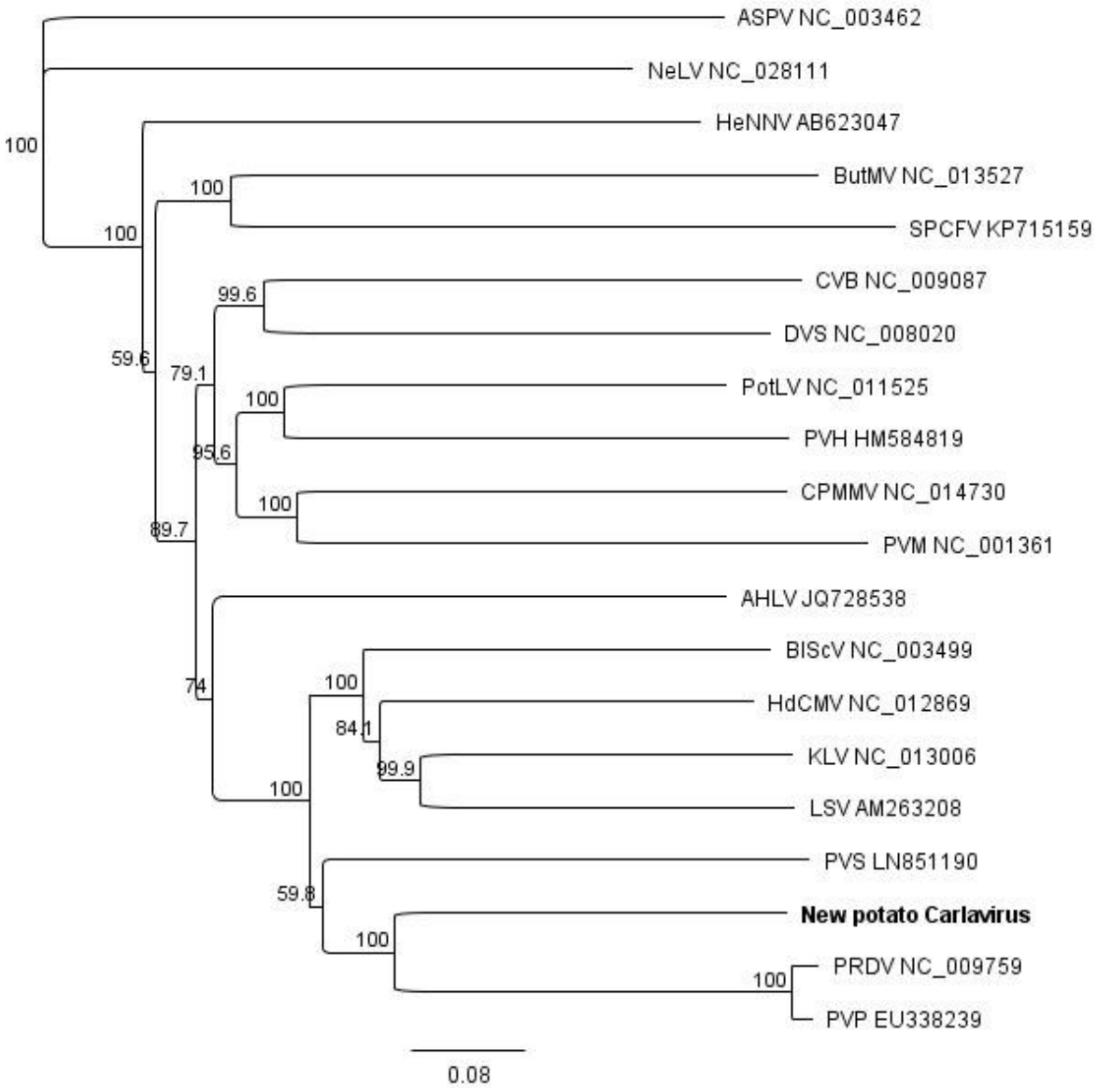


Figure 2. Phylogenetic analysis of the coat protein (A) and replicase (B) amino acid sequences of ChPV and selected carlaviruses. The neighbour-joining tree was generated using Geneious v8.1.8 software, horizontal scale indicates sequence divergence. The data set was subjected to 1,000 bootstrap replicates. All nodes supported by >50% confidence values are shown. *American hop latent virus,* AHLV;

*Blueberry scorh virus,* BlScV; *Apple stem pitting* virus, ASPV; *Butterbur mosaic virus,* ButMV; *Chrysanthemum Virus B,* CVB; *Cowpea mild mottle virus,* CPMMV; *Daphne virus S*, DVS; *Gaillardia latent virus*, GLV; *Garlic latent virus*, GarLV; *Helleborus net necrosis virus*, HeNNV; *Hop latent virus*, HpLV; *Hydrangea chlorotic mottle virus*, HdCMV; *Kalanchoe latent virus,* KLV; *Lily symptomless virus*, LSV; *Narcissus common latent virus*, NCLV; *Nerine latent virus*, NeLV; *Phloxvirus virus B*, PhlVB; *Phloxvirus virus S*, PhlVS; *Potato latent virus*, PotLV; *Potato rough dwarf virus*, PRDV; *Potato virus H*, PVH; *Potato virus M*, PVM; *Potato virus P*, PVP; *Potato virus S*, PVS; *Sweet potato chlorotic fleck virus*, SPCFV.

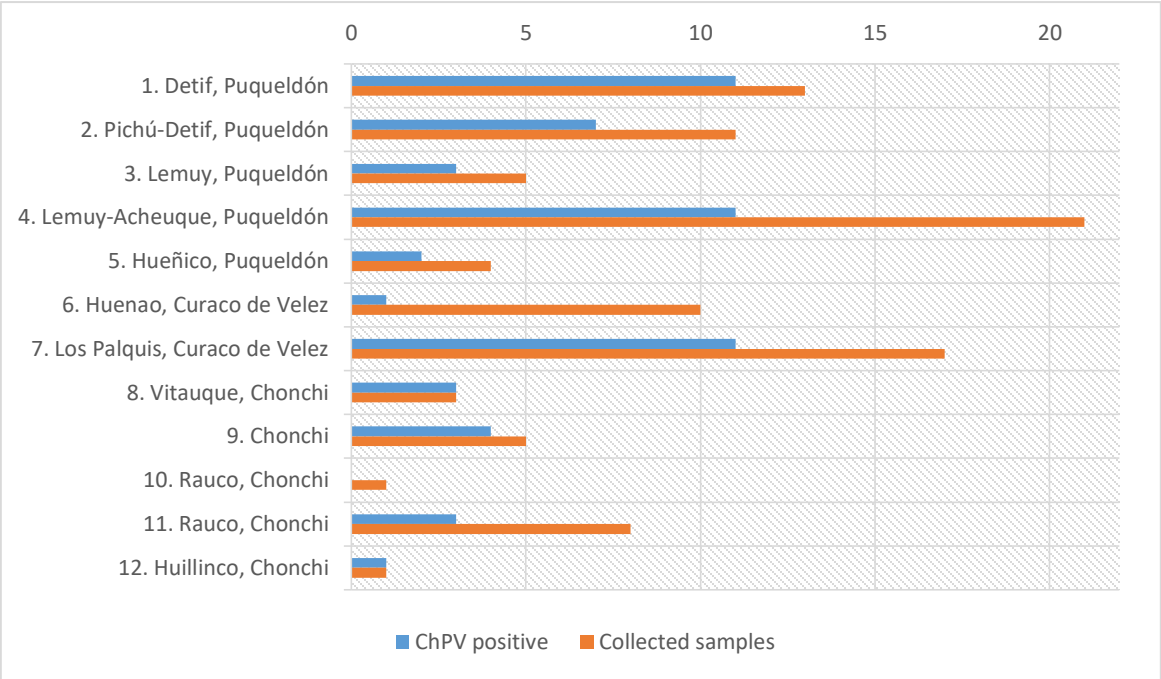


Figure 3. Prevalence of *Chiloe potato virus* (ChPV) infection in samples of potato leaves in relation to Chiloé Archipelago municipalities.

# Chapter 5

## General Conclusions

The approach used in this study, through the use of a massive sequencing platform, allowed to obtain a viral profile of a pool of samples of native potatoes collected in the Chiloé Archipelago.

For the first time in the country, the PMTV presence was identified in samples of native potatoes. In addition, the detection of the virus in resistant spores of vector by means of specific RT-PCR was successfully implemented.

There were evidence of the presence of a new species of virus belonging to the genus *Carlavirus* in samples of native potatoes collected in the Chiloé Archipelago. This virus presents the characteristic organization of the members of the genus including a zinc finger motif rich in cysteine at the 3 'end, motif conserved among the species

Phylogenetic analysis based on the coding sequences of the CP and RpRd showed that this *Carlavirus* does not group with any virus specie previously described, confirming the existence of a new specie infecting potatoes in Chile.