Virus Genes
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Distribution and genetic variability of alfalfa dwarf virus, a cytorhabdovirus associated with alfalfa dwarf disease in Argentina

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Abstract

In 2010, a novel cytorhabdovirus named alfalfa dwarf virus (ADV) was detected for the first time in lucerne crops in Argentina showing dwarfism, in mixed infections with several other viruses. ADV appears to be endemic to Argentina and has not been reported elsewhere. In this study, we have investigated the genetic variability of ADV based on the complete nucleoprotein (N) gene of 13 isolates from different lucerne-growing regions in Argentina. Phylogenetic and sequence identity analyses showed that all ADV isolates are closely related and have not diverged more than 1% in the N gene despite geographical separation. These data provide further evidence that ADV is new to science and emerged and spread very recently. A total of 43 single-nucleotide polymorphisms were identified between the ADV isolates studied. Analysis of N gene ORF sequence revealed a mutational bias, with more transitions than transversions. In all cases, the ratio of non-synonymous/synonymous nucleotide changes was < 1, indicating that ADV N gene is under predominantly purifying selection.

Keywords Genetic diversity · Cytorhabdovirus · Phylogenetic analysis · Single-nucleotide polymorphism · Nucleoprotein gene · Alfalfa dwarf disease

Lucerne or Alfalfa (*Medicago sativa* L., Fabaceae) is a widely cultivated perennial pasture legume in more than 80 countries due to its high biomass production and nutritional value for livestock [1–3]. In 2010, a new severe disease named alfalfa dwarf disease (ADD) was observed in Argentina that caused up to 30% annual hay yield reduction [4, 5]. Next generation sequencing of the diseased plants identified a novel cytorhabdovirus named alfalfa dwarf virus (ADV), the bromovirus alfalfa mosaic virus (AMV), the luteoviruses bean leafroll virus (BLRV) and alfalfa enamovirus 1 (AEV-1), and the geminivirus alfalfa leaf curl virus (ALCV) [5–10]. The disease is distinguished by dwarfed, bushy appearance of infected plants, and leaf puckering and vein enations on abaxial leaf surface (Fig. S1) [5]. Different insect pests including a number of aphid species, in particular black legume aphid (*Aphis craccivora*) and leafhoppers, have been observed in ADD-affected paddocks. AMV, AEV-1, and ALCV appear to be transmitted by *A. craccivora*; however, the vector of ADV remains to be determined (V. Trucco, personal communication). ADV, AEV-1, and ALCV are newly discovered viruses from lucerne plants [6, 9, 10]. ADV has so far only been detected in Argentina [5, 6], whereas ALCV was detected in France and Spain [11, 12], and a strain of AEV-1 named alfalfa enamovirus 2 (AEV-2) was detected in Sudan [13]. ADV is taxonomically classified in the species *Alfalfa dwarf cytorhabdovirus*, genus *Cytorhabdovirus*, family *Rhabdoviridae*, order *Mononegavirales* [14]. ADV has a negative-sense, single-strand RNA genome of 14,494 nucleotides and encodes 7 proteins in gene order from 3′ to 5′: nucleoprotein (N), phosphoprotein (P), movement protein (P3), matrix protein (M), glycoprotein (G), accessory protein (P6), and large RNA-dependent RNA polymerase (L) [6]. ADV P protein is a local and systemic RNA silencing suppressor and P3 is a

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30K-like cell-to-cell movement protein [15, 16]. Only the 'Manfredi' isolate of ADV has been studied thus far, and the extent of genetic diversity between ADV isolates from different geographic lucerne-growing areas of Argentina is unknown. This knowledge will be essential to refine diagnostic tools to take into account potential sequence diversity.

Here, we report the genetic variability among 13 ADV isolates based on sequence of the complete N gene. Leaf samples from ADD-affected lucerne were collected from paddocks located in 12 provinces of Argentina between 2010 and 2015 (Table S1). Total nucleic acids were extracted using cetyltrimethylammonium bromide method [17]. ADV N gene including open reading frame (ORF) and non-coding 3′ and 5′ regions was amplified using Superscript III One Step RT-PCR kit with Platinum *Taq* DNA polymerase (Life Technologies) and primers ADV-77F (5′-CCTTTTCCTAATATCTACCG-3′) and ADV-2007R (5′-CGACACTGATATCACTAAG-3′). The amplicon of approximately 1.93 kb was directly purified from the PCR reaction using Wizard® DNA clean up system (Promega), cloned into pGEM-T Easy vector (Promega) and sequenced at the Australian Genome Research Facility (AGRF, Brisbane). Internal primers ADV-463F (5′-TTGAATGATGACGACTGTGT-3′) and ADV-1524R (5′-CCTAGCTTTCTGATCTTCCC-3′) as well as M13F and M13R primers were used for sequencing of at least three independent clones per virus isolate. Individual consensus N gene sequences for all ADV isolates were aligned to the published 'Manfredi' isolate sequence (GenBank KP205452.2). Multiple sequence alignment (ClustalW) of ADV N gene sequences was used for distance analysis in Geneious R9.1 (Biomatters, New Zealand). Maximum-likelihood (ML) phylogenetic trees were constructed using MEGA 7 [18]. Four representative cytorhabdoviruses were included, and potato yellow dwarf virus (PYDV; genus *Nucleorhabdovirus*) N gene sequence was used to root the tree, and bootstrapping used 1000 replicates. Recombination analysis was done using Recombination Detection Program v4 [19]. The extent and distribution of genetic variation among ADV N gene ORF sequences was estimated by the average number of nucleotide differences per site (π) using DnaSP v5 using a sliding window of 100 nts with a 25-nt step size [20]. Transitions and transversions ratio was estimated by the maximum composite likelihood method via MEGA version 7 [18]. Confidence estimation for the non-synonymous and synonymous nucleotide substitution rates ($d_N/d_S = \omega$) and degree of selective constraints imposed on ADV N ORF was computed using the bootstrap method (500 replicates) by Tamura–Nei model using MEGA version 7 [18]. A P value < 0.05 was considered to be significant. Single-nucleotide polymorphisms (SNPs) and insertion or deletion of nucleotides were analyzed within multiple sequence alignment of all sequences using Geneious.

The ORF nucleotide sequence identity between the geographically diverse isolates including the corrected 'Manfredi' N gene nucleotide sequence was 99.0–99.9% (Table S2) indicating that ADV had very little time to diverge since its emergence in Argentina in 2010. ADV N genes from Neuquen and Rio Negro were highly similar to the reference sequence, showing the same nucleotide sequence identity (99.7%). On the other hand, ADV isolate from La Pampa showed the lowest N gene nucleotide sequence identity (99.2%) with the corrected reference sequence. There were 43 isolate-specific SNPs within N gene ORF (3% diversity) that can be used to differentiate the known ADV isolates. There were also eight isolate-specific SNPs within 3′untranslated regions (UTRs). A single SNP in the 5′UTRs differentiated isolate 'Jujuy' from all other ADV isolates. ADV diagnostic primers that were previously designed from isolate 'Manfredi' N gene sequence [21] were mapped to the nucleotide sequences of all 13 isolates to determine if any SNPs were positioned in the primers binding sites. Except 'La Pampa' which had a single SNP coincident with the 3′ end of the forward primer, all other isolate N gene sequences were 100% complementary to the primers. Because ADV N gene is highly conserved among all studied isolates, no optimization will be required to the current diagnostic RT-PCR assay that we developed in our previous study to detect all known ADV variants. Some SNPs with significance at the 5% level and strand bias $> 65\%$ P value were identified between positions 170–210, and 1281 nt downstream from the N gene ORF start codon (Table S3); they can be used to differentiate some isolates from the 'Manfredi' reference. No nucleotide insertion or deletion was observed in our dataset.

Nucleotide substitution analysis of the N gene ORF sequences revealed a mutational bias, with 11.5 times more transitions than transversions (Fig. S2). There was no evidence of recombination in the complete N gene (data not shown). When the extent and distribution of genetic diversity in the N gene ORF were analyzed, the variation rates ranged from 0.5 to 0.8% and the distribution was uneven, with a peak at the beginning of the ORF (100–300 bp downstream of the start codon) (Fig. 1b). For both, N gene and its ORF, the d_{NS}/d_S ratio was significantly < 1, and P values determined in the models $d_N \neq d_S$ and $d_N < d_S$ [22] were below 0.05, implying that ADV N gene is under predominantly purifying selection. A whole genome study of isolates of the nucleorhabdovirus eggplant mottled dwarf virus similarly indicated purifying selection [23].

Construction of a ML phylogenetic tree based on the 1449 nucleotide sequences of the N gene ORF indicated that ADV isolates are closely related (Fig. 1a). ADV isolates were $< 1\%$ divergent in the N gene ORF, thus

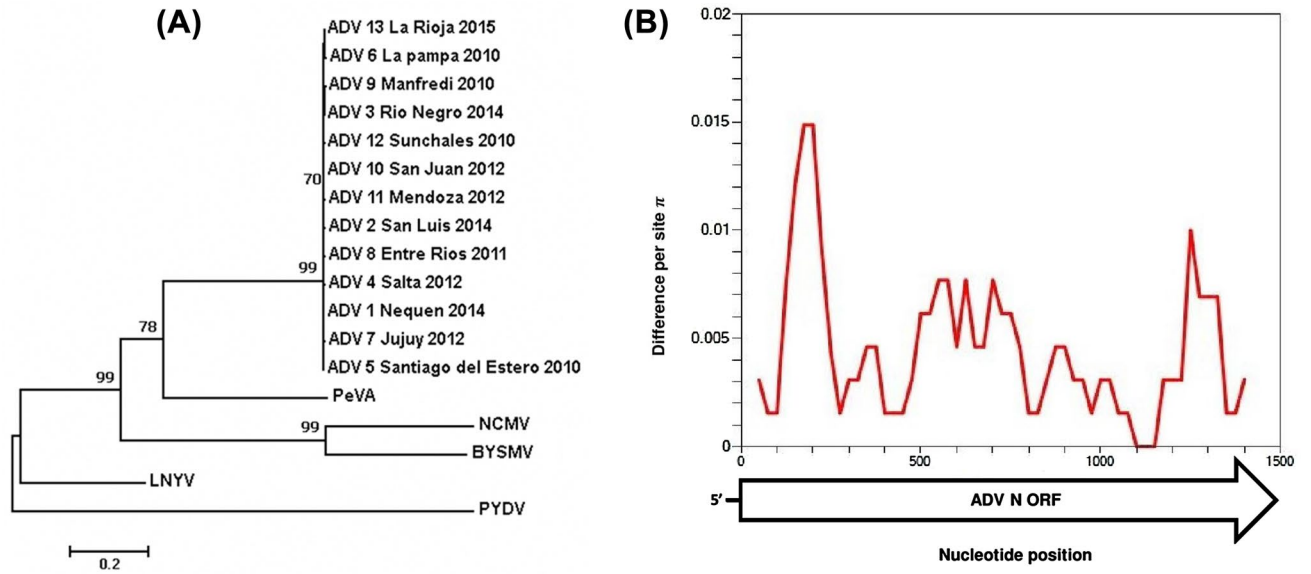

Fig. 1 ADV genetic diversity. **a** ML phylogenetic tree of ADV isolates based on nucleoprotein gene ORF nucleotide sequences. Bootstrap values above 50% are shown as percentage of 1000 replications. The scale bar indicates the number of substitutions per site. Geographic origin, year of sample collection, and isolate reference number are shown for each isolate. Representative cytorhabdoviruses persimmon virus A (PeVA; GenBank AB735628), lettuce necrotic yellows virus (LNYV; KP109940), northern cereal mosaic virus (GU985153) and barley yellow striate mosaic virus (KM213865), and the nucleorhabdovirus PYDV (EU183122) are also shown. **b** Distribution of genetic variation along ADV N gene ORF (arrow) estimated by nucleotide diversity (π). A 100-nt wide sliding window was used with a 25-nt step size

supporting the phylogenetic results. By comparison, N gene ORF sequences of the well-established cytorhabdovirus LNYV fell into two subgroups and differed significantly between them, about 20% at the nucleotide and 4% at the amino acid levels [24]. This provides some evidence that ADV is a "new" pathogen that emerged and spread very recently. Multiple sequence alignments of deduced amino acid sequences of the ADV N protein revealed nine amino acid changes distributed across the N protein, showing an amino acid sequence diversity of 1.87%.

ADV appears to be less diverse than the other plant rhabdoviruses like taro vein chlorosis virus (TaVCV), isolates of which differed by 19.3 and 6.3% in the N gene at the nucleotide and amino acid sequence levels, respectively [25]. Recently, a d_N/d_S ratio of 0.03 or less was estimated for coffee ringspot virus (CoRSV; genus *Dichorhavirus*) N gene indicating purifying selection like we found for ADV. However, unlike ADV, phylogenetic study of the CoRSV N gene identified a strong geo-spatial relationship among isolates [26].

A recent study of orchid fleck virus (OFV; genus *Dichorhavirus*) genetic diversity revealed that N protein sequence was less genetically diverse than other RNA1-encoded proteins, and similar to findings for ADV, LNYV, and CoRSV, OFV N gene appears to be under purifying selection [27].

The current and previous studies show lower variability of plant rhabdoviruses' N gene at amino acid than at nucleotide level [25, 28]. This may indicate evolutionary restrictions to preserve essential N protein functions [28, 29]. The comparatively low genetic diversity within ADV N gene provides evidence that ADV is a pathogen that is new to science, and which has not had time to evolve in different locations since its emergency. More detailed studies are required to find if variants of ADV can be differentiated by symptom severity, alternative hosts or insect vectors. Answering these questions will require further studies of ADV and ADD epidemiology.

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Author contributions SS, NEB, and RGD designed the study; SS performed the experiments and analyzed the data. SS drafted the manuscript and all authors edited and approved the final version.

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