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ORIGINAL ARTICLE

Sequence analyses of RT-PCR products obtained from seven infected leaf samples revealed existence of three potyvirus species in Indian narcissus (*Narcissus tazetta* L.)

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Abstract
Potyvirus species associated with yellow leaf stripe disease of Indian narcissus (*Narcissus tazetta* L.) var. Paperwhite has been studied by sequence analyses of ~ 1.5 kb genomic fragments obtained from seven RT-PCR amplifications of infected samples. Sequence analysis revealed the occurrence of three potyvirus species: *cyrtanthus elatus virus*-A (CEVA: KF430815, KF430816, KM066973, KM066974); *narcissus yellow stripe virus* (NYSV: KM066972, JQ686724) and *narcissus degeneration virus* (NDV: MK572806). The existence of three potyvirus species: CEVA, NYSV and NDV are being reported in Indian narcissus.

Keywords *N. tazetta* var. paperwhite · Leaf yellow stripe · RT-PCR amplification · Sequence identity · Phylogenetic relationships · CEVA · NYSV · NDV

Introduction

Indian narcissus (*Narcissus tazetta* L.) of the family Amaryllidaceae is a bulbous ornamental plant. It is popular for its beautiful flowers in the garden beds and used as a cut-flower for bouquets, vases and also for the production of fragrant oil and perfumes in India. *N. tazetta* is common ornamental species in the Mediterranean region (from Portugal to Turkey), considered as its native place, though, its dissemination extended throughout the Asian countries with demand-based international trading (Harvey and Selby 1997; Hanks 2002; Kamenetsky and Okubo 2012). Now, *N. tazetta* are widely grown in China, Israel, India, and Japan, and the large volumes of field-grown cut-flowers are traded, along with other commercially important flowers and pot-grown plants as well as the bulbs (Hank and Chastagner 2018).

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Narcissus are reported worldwide to be infected by *narcissus mosaic virus* (NMV), *narcissus yellow stripe virus* (NYSV), *narcissus late season yellow virus* (NLSYV), *narcissus degeneration virus* (NDV), *narcissus latent virus* (NLV), *narcissus tip necrosis virus* (NTNV), *cyrtanthus elatus virus*-A (CEVA) and *ornithogalum mosaic virus* (OrMV) causing streaks, yellow stripe and tip necrosis symptoms on the leaf (Brunt 1977, 2008; Wylie and Jones 2012). Other viruses such as *raspberry ring spot virus* (RRSV), *nerine latent virus* (NeLV), *narcissus symptomless virus* (NSV), *arabis mosaic virus* (ArMV), *cucumber mosaic virus* (CMV), *tobacco rattle virus* (TRV) and *tomato black ring virus* (TBRV) are also reported to infect narcissus though their frequency of infection is reported considerably less as compared to above said viruses (Brunt 1995). Literature survey revealed that infection of said viruses reduced the quality and productivity of narcissus bloom and bulbs (Hanks and Chastagner 2018). Among them, potyviruses are the most prevalent viruses of narcissus including NYSV, NLSYV, NDV, CEVA and OrMV (Chen et al. 2006; Yadav and Khan 2008; Kumar et al. 2015; Ohshima et al. 2016; Raj et al. 2018).

Narcissus propagates vegetatively through its bulbs and, therefore, growing plants, if infected by virus, continuously pass infection from generation to generation through propagations of infected mother stocks (Milosevic et al.

2012). Consequently, whole population of narcissus may be infected by the virus if not protected timely and hence reliable early diagnosis and identification of virus is essential for designing their efficient disease management. In the Indian scenario, previous works revealed that narcissus are infected by potyviruses based on study of symptoms, electron microscopy, molecular weight of protein subunits and virus detection by serological methods, and reverse transcription-polymerase chain reaction (RT-PCR) followed by sequence analyses of PCR amplicons (Aminuddin et al. 1999; Yadav and Khan 2008, 2015; Chandel et al. 2010; Kumar et al. 2015). In the present study, we report the existence of three potyvirus species: CEVA, NYSV and NDV in *N. tazetta* var. Paperwhite based on the analyses of 3′partial genome sequences of viral genome amplified by RT-PCR using potyvirus degenerate primers.

Materials and methods

For detection and molecular characterization of virus, leaf samples from eight (*N. tazetta* L.) var. Paperwhite plants showing yellow stripe symptoms were collected from three locations: cultivated fields, garden beds, and experimental plots of CSIR- National Botanical Research Institute (NBRI), Lucknow. The total genomic RNA was isolated from 100 mg leaf samples of infected, healthy narcissus plants and a positive control (Kumar et al. 2015) using Sigma kit Spectrum™ Plant total RNA kit (Sigma-Aldrich, Missouri, USA) and used as template for RT-PCR.

For potyvirus detection, RT-PCRs were performed in Thermal cycler (PTC 200 DNA engine of MJ Research, USA) following the protocol described elsewhere (Raj et al. 2019) using total RNA and Pot-I/Pot-II degenerate primers capable of amplifying 3′-partial genome of all potyvirus (Gibbs and Mackenzie 1997). The obtained PCR products were separated through electrophoreses in 1% agarose gel to check the presence of amplicon using standard DNA marker.

For cloning and sequencing, the amplicons were eluted using Wizard SV Gel and PCR Clean-Up System (Promega, CA, USA). The purified products were ligated into pGEM-T Easy Vector System and transformed into competent *E. coli* (DH5α) cells. The transformants were screened by restriction digestion with *Eco*R1 enzyme and three positive clones of each sample were sequenced. The obtained sequence data were analysed and assembled using BIOEDIT tool (https://www.mbio.ncsu.edu/bioedit/bioedit.html) to eliminate any sequence ambiguity, and consensus sequences were determined and submitted to the GenBank database.

The sequences were analyzed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for potyvirus identification. Also, the *DiAlign* tool (https://www.genomatix.de/cgi-bin/dialign/dialign.pl) was used to obtain nucleotide and amino acid identities with the selected potyviruses listed in Table 1. The phylogenetic analysis of sequences was performed by Molecular Evolutionary Genetics Analysis (MEGA) v7.0 tool using the Maximum Likelihood algorithm at 1000 bootstrap value (Kumar et al. 2016).

Results

During our surveys, leaf yellow stripe symptoms on *N. tazetta* var. Paperwhite plants were observed in cultivated fields, garden beds and experimental plots of CSIR-NBRI, Lucknow. The RT-PCRs using potyvirus degenerate primers (Pot-I/Pot-II) revealed the presence of expected size ~ 1.5 kb amplicons in all eight leaf samples similar to as the positive control, while no such amplicon could be obtained in a healthy narcissus sample, indicated the presence of potyvirus (Fig. 1).

The obtained RT-PCR amplicons of ~ 1.5 kb size were cloned and transformed into *E. coli* cells as described in MM. The plasmids from positive clones when digested with *Eco*R1 showed presence of about 1.5 kb of DNA insert. The three positive clones of each sample were sequenced and consensus sequences of each sample were submitted in GenBank under the accession numbers: KF430816 (NBRI-1), KM066974 (NBRI-2), KM066973 (NBRI-3), KF430815 (NBRI-4), JQ686724 (NAR-1), KM066972 (NAR-2) and MK572806 (NBRI-NDV1R).

The submitted NAR-1 and NAR-2 sequences shared 100% nucleotide sequence identity with each other and 75–97% with other NYSV sequences reported all over the word during the BLASTn analysis. They also revealed highest 97% nucleotide identity with NYSV-Zhangzhou (Acc. No. AJ311372) reported from China and 72–77% identities with NLSYV (the other potyvirus species) reported on the same host, *N. tazetta*. BLASTn analysis of NBRI-1, NBRI-2, NBRI-3 and NBRI-4 sequences revealed 88–100% nucleotide sequence identities to each other and 80–98% with CEVA. The highest 98% nucleotide sequence identity was obtained with CEVA reported from Australia (GU812283) and New Zealand (DQ417604), *w*hile 67–68% identities were with NDV reported all over the world on *N. tazetta.* Whereas, NBRI-NDV1R sequence showed highest 97% nucleotide sequence identity with partial polyprotein gene of *Chinese narcissus potyvirus* (ChNP)-Chongming Island (AJ311374), ChNP-Zhangzhou (AJ311373); *narcissus degeneration virus* (NDV)-Zhangzhou (AM182028) reported from Republic of China. NBRI-NDV1R also showed 96% identity with NDV-NV-3 (China, EU200456); NDV-NaN14-CL8 (Japan, LC158506); NDV-NaN9-CL12 (Japan, LC158504); NDV-Marijiniup 2 (Australia, JQ395041); NDV-NaF1-CL16 (Japan, LC158495); NDV-NaN9-CL14 (Japan,

Table 1 Details of sequence data of samples under study and other potyvirus isolates taken for this study

Virus	Isolate	Host	Location	Genome length (nt)	GenBank Accession	Source
NYSV	NAR-1	*Narcissus tazetta*	India	1559	JQ686724	Under study
NYSV	NAR-2	*Narcissus tazetta*	India	1559	KM066972	Under study
NYSV	Zhangzhou	*Narcissus tazetta*	China	1669	AJ311372	Chen et al. (2003)
NYSV	NaKM1-CL7	*Narcissus tazetta*	Japan	1738	LC158481	Ohshima el al. (2018)
NYSV	NsN14-CL6	*Narcissus tazetta*	Japan	1738	LC158491	Ohshima el al. (2018)
NYSV	NaN14-CL2	*Narcissus tazetta*	Japan	1738	LC158490	Ohshima el al. (2018)
INP	Lucknow 2	*Narcissus tazetta*	India	2912	EU888298	Yadav and Khan (2008)
NLSYV	NaF1-CL4	*Narcissus tazetta*	Japan	1737	LC158449	Ohshima el al. (2016)
NLSYV	Hangzhou 2	*Narcissus tazetta*	China	1668	AJ493579	Chen et al. (2003)
NLSYV	–	*Narcissus* cv. Missouri	UK	1608	EU887015	Monger and Nixon (2008)
INP	Lucknow	*Narcissus* spp.	India	1558	DQ991145	Yadav and Khan (2008)
NDV	NBRI-NDV1	*Narcissus tazetta*	India	1577	MK572806	Under study
NDV	NaKM9-CL5	*Narcissus* spp.	Japan	1612	LC158497	Ohshima el al. (2016)
NDV	NaSG8-CL3	*Narcissus tazetta*	Japan	1612	LC158500	Ohshima el al. (2016)
NDV	NV-3	*Narcissus tazetta*	China	1566	EU200456	Wylie and Jones (2012)
NDV	NaN14-CL8	*Narcissus tazetta*	Japan	1615	LC158506	Ohshima el al. (2016)
NDV	NaF1-CL16	*Narcissus tazetta*	Japan	1612	LC158495	Ohshima el al. (2016)
NDV	NaKM9-CL18	*Narcissus tazetta*	Japan	1612	LC158498	Ohshima el al. (2016)
CEVA	NBRI-2	*Narcissus tazetta*	India	1601	KF430815	Under study
CEVA	NBRI-1	*Narcissus tazetta*	India	1601	KF430816	Under study
CEVA	NBRI-4	*Narcissus tazetta*	India	1601	KM066974	Under study
CEVA	NBRI-3	*Narcissus tazetta*	India	1601	KM066973	Under study
CEVA	WA-1	*Cyrtanthus elatus virus*	Australia	1056	GU812282	Wylie et al. (2010)
CEVA	NaN19-CL3	*Cyrtanthus elatus*	Japan	1825	LC158493	Ohshima el al. (2016)
CEVA	–	*Vallota speciosa*	New Zealand	1602	DQ417604	Unpublished
CEVA	–	*Narcissus sp.*	UK	1560	FJ032248	Unpublished
CEVA	WA-2	*Cyrtanthus elatus*	Australia	1048	GU812283	Wylie et al. (2010)
OrMV	Hangzhou	*Narcissus*	China	1667	AJ493580	Chen et al. (2003)
JYMV	J2	*Japanese yam*	Japan	876	AB027009	Fuji et al. (2000)

NYSV: *narcissus yellow stripe virus*; INP: *Indian narcissus virus*; NLSYV: *narcissus late season yellow virus*; CEVA: *cyrtanthus elatus virus-A*; NDV: *narcissus degeneration virus*; JYMV: *Japanese yam mosaic virus*; OrMV: *ornithogalum moasic virus*

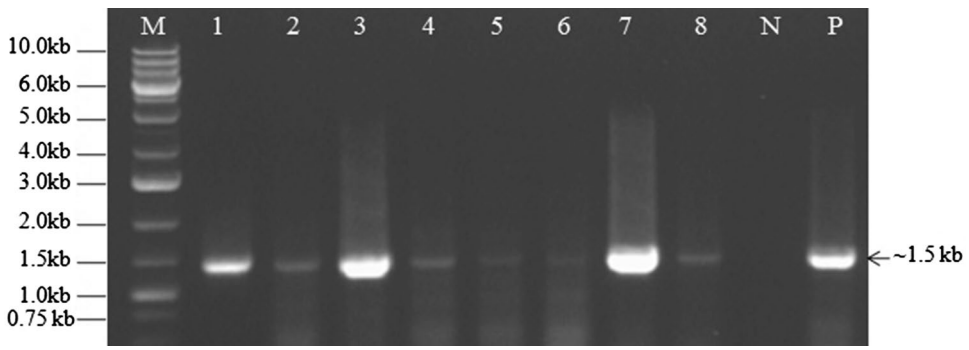

Fig. 1 Gel electrophoresis of RT-PCR products obtained by Potyvirus generate primers (Gibbs and Mackenzie 1997) showing successful amplification of expected size ~ 1.5 kb band in all eight infected narcissus samples (lanes 1–8) similar to a positive control (lane P) but no such amplicon was obtained in the healthy narcissus sample (lane N) taken as negative control. *M* = 1.0 kb DNA ladder marker

LC158505), NDV-NaN9-CL9 (Japan, LC158503) and NDV-NaF1-CL14 (Japan, LC158494) isolates.

Furthermore, the sequence analysis using *Genomatix DiAlign* tool employing NAR-1 sequence revealed its highest 91% and 85% identity at nucleotide (nt) and amino acid (aa) levels, respectively, with NAR2 sequence. It also showed 86–87% and 83–84% identities at nt and aa levels, respectively, with other isolates of NYSV (LC158490, LC158491, LC158481, AJ311372) reported from Japan and China (Table 2). The identities were 77% and 82% at nt and aa levels, respectively, with *Indian narcissus virus* (INV, EU888298). While, the identities were lesser, 54–57% at nt and 60–62% at aa level with NLSYV isolates reported from China and the United Kingdom (Table 2). The NBRI-1 sequence revealed highest 97% and 98% identity at the nt and aa levels, respectively, with CEVA isolate (DQ417604) reported from New Zealand. It also showed 94% identity at nt and 97–98% identities at the aa levels with NBRI-3 and NBRI-4 sequences under study while, identities were 80% at nt and 92% at aa with NBRI-2 sequence. It also showed identities were 75–80% at nt and 80–92% at aa with other isolates of CEVA reported from Japan (NaN19-CL3, LC158493) and Australia (WA-1, GU812282) (Table 2). The analysis of NBRI-NDV1R sequence revealed identity from 94 to 95% and 96 to 97% at nt and aa levels, respectively, with NDV isolates reported from Japan (NaKM9-CL5: LC158497, NaSG8-CL3: LC158500) and China (NV-3: EU200456) (Table 2). BLASTn and *Genomatix DiAlign*-based sequence analyses of NBRI-1, NBRI-2, NBRI-3, NBRI-4; NAR-1, NAR-2; and NBRI-NDV1R sequences under study with the published available sequences in GenBank putatively identified them as potyvirus isolates belonging to CEVA, NYSV and NDV, respectively.

Phylogenetic analysis of all sequences under study was performed both at nucleotide (Fig. 2) and amino acid (Fig. 3) sequence levels with the selected sequences of NYSV, CEVA and NDV reported worldwide along with some other closely related potyvirus as an out group sequences by MEGA tool. Phylogeny revealed more or less similar results both at nucleotide and amino acid sequence levels for NYSV and CEVA sequences under study (Figs. 2, 3). The NAR-1 and NAR-2 sequences clustered together and showed closest phylogenetic relationships with 99% bootstrap value with NYSV-Zhangzhou reported from China (Chen et al. 2003) and other NYSV isolates (LC158481, LC158490, and LC158491) reported from Japan (Ohshima el al. 2018). They also showed a close homology with NLSYV isolates reported from China (AJ493579, Chen et al. 2003) and UK (EU887015). While, they showed distant relationships with INV (EU888298) reported from India (Yadav and Khan 2015), and other potyviruses considered in the present study (Figs. 2, 3).

During analysis of CEVA sequences under study both at nucleotide and amino acid levels with the sequences of other potyvirus isolates considered for study, the NBRI-1, NBRI-3 and NBRI-4 showed close relationships and clustered with CEVA-New Zealand isolate (DQ417604). Contrary to this, NBRI-2 showed closest relationship and clustered with CEVA-WA-1 isolate (GU812282) reported from Australia (Wylie et al. 2010) and CEVA-NaSG8-CL3 isolate (LC158493) from Japan (Ohshima el al. 2016). These results of phylogeny analyses both at the level of nucleotide and amino acid sequences clearly indicate sequence diversity among sequences under study (Figs. 2, 3).

The NBRI-NDV1R sequence clustered with reported NDV isolates and revealed closest relationships with NDV-NV-3 isolate (EU200456) from China (Wylie and Jones 2012) and *Indian narcissus virus* (DQ991145) from India which was subsequently identified as *lycoris virus* (Yadav and Khan 2008). Our sequence also showed close relationships with other isolates of NDV: NaSG8-CL3 (LC158500), NaKM9-CL5 (LC158497) and NaKM9-CL18 (LC158498) reported from Japan (Ohshima el al. 2016), whereas distant relationships with other potyviruses considered in the present study at nucleotide and amino acid levels (Figs. 2, 3). Based on pair-wise sequence identity and phylogenetic analyses, the NBRI-1, NBRI-2, NBRI-3 and NBRI-4; NAR-1 and NAR-2 and NBRI-V1R sequences from narcissus were identified as isolates of CEVA, NYSV and NDV, respectively.

Discussion

The yellow stripe symptoms on leaves were observed in several *N. tazetta* var. Paperwhite plants growing in cultivated field, garden beds and experimental plots at CSIR-NBRI, Lucknow. The exhibited disease symptoms in narcissus were found similar to those described earlier for potyvirus in India (Aminuddin et al. 1999; Khan and Yadav 2008; Kumar et al. 2015) and from abroad (Wylie et al. 2014; Ohshima et al. 2018) hence infection of potyvirus was suspected. Therefore, detection of potyvirus was attempted by RT-PCR using Pot I and Pot II degenerate potyvirus primers capable of amplifying conserved 3′-UTR to partial Nlb region and as used for detection of a variety of potyviruses (Gibbs and Mackenzie 1997).

The analyses of nucleotide and amino acid sequence data of cloned PCR products resulted in identification of three potyvirus species: NYSV, CEVA and NDV in accordance to the ICTV species demarcation criteria suggested for potyvirus speciation that coat protein (CP) coding region sequences were considered usually to be < 80% amino acid and < 76% nucleotide sequence identities (Adams et al. 2005; Zerbini et al. 2012). The pair-wise sequence identity

Table 2 Pair wise percent identities of NYSV (NAR-1: JQ686724), NDV (NDV1R: MK572806) and CEVA (NBRI1: KF430816) sequences under study at nucleotide (nt) and its amino acid (aa) with respective sequences of other potyviruses available in GenBank using *DiAlign* tool

Gene Bank Accession	Virus	Isolate	Location	Percent identity nt (aa)
Sequence identities of NYSV NAR-1 (JQ686724) with other potyvirus isolates				
KM066972	NYSV	NAR-2	India	91 (90)
AJ311372	NYSV	Zhangzhou	China	87 (89)
LC158481	NYSV	NaKM1-CL7	Japan	86 (89)
LC158491	NYSV	NsN14-CL6	Japan	86 (89)
LC158490	NYSV	NaN14-CL2	Japan	86 (89)
LC158449	NLSYV	NaF1-CL4	Japan	58 (68)
AJ493579	NLSYV	Hangzhou 2	China	57 (69)
EU887015	NLSYV	–	UK	54 (67)
DQ991145	INP	Lucknow	India	32 (44)
EU888298	INP	Lucknow 2	India	77 (85)
AJ493580	OMV	Hangzhou	China	34 (49)
AB027009	JYMV	J2	Japan	51 (53)
Sequence identities of NDV NBRI-NDV1 (MK572806) with other potyvirus isolates				
LC158497	NDV	NaKM9-CL5	Japan	94 (96)
LC158500	NDV	NaSG8-CL3	Japan	94 (96)
EU200456	NDV	NV-3	China	95 (97)
LC158506	NDV	NaN14-CL8	Japan	94 (97)
LC158495	NDV	NaF1-CL16	Japan	95 (96)
LC158498	NDV	NaKM9-CL18	Japan	94 (96)
LC158449	NLSYV	NaF1-CL4	Japan	37 (53)
AJ493579	NLSYV	Hangzhou 2	China	38 (54)
EU887015	NLSYV	–	UK	38 (52)
DQ991145	INP	Lucknow	India	96 (97)
EU888298	INP	Lucknow 2	India	35 (50)
AJ493580	OMV	Hangzhou	China	38 (49)
AB027009	JYMV	J2	Japan	48 (51)
Sequence identities of CEVA NBRI-1 (KF430816) with other potyvirus isolates				
KF430815	CEVA	NBRI-2	India	75 (80)
KM066973	CEVA	NBRI-3	India	94 (97)
KM066974	CEVA	NBRI-4	India	94 (98)
DQ417604	CEVA	–	New Zealand	97 (98)
LC158493	CEVA	NaN19-CL3	Japan	78 (89)
GU812282	CEVA	WA-1	Australia	80 (92)
FJ032248	CEVA	–	UK	77 (86)
GU812283	CEVA	WA-2	Australia	79 (92)
LC158449	NLSYV	NaF1-CL4	Japan	39 (54)
AJ493579	NLSYV	Hangzhou 2	China	37 (53)
EU887015	NLSYV	–	UK	37 (52)
DQ991145	INP	Lucknow	India	57 (70)
EU888298	INP	Lucknow 2	India	35 (51)
AJ493580	OrMV	Hangzhou	China	35 (53)
AB027009	JYMV	J2	Japan	45 (50)

NYSV: *narcissus yellow stripe virus*, NLSYV: *narcissus late season yellows virus*, CEVA: *cyrtanthus elatus virus* A, INP: *Indian narcissus virus*, JYMV: *Japanese yam mosaic virus*, NDV: *narcissus degeneration virus*, OrMV: *ornithogalum mosaic virus*

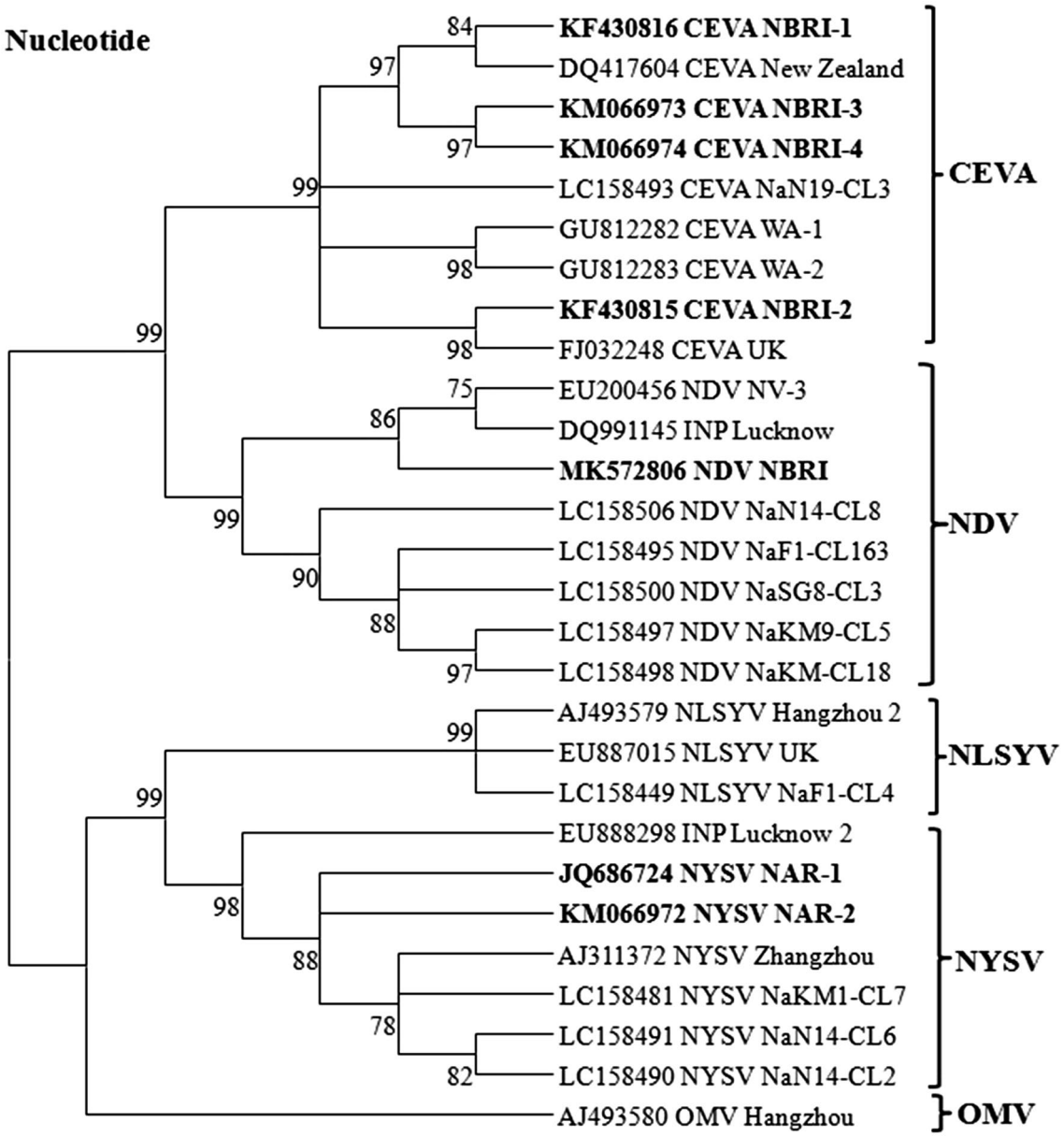

Fig. 2 Phylogenetic tree showing relationships of sequences under study: NYSV (KM066972 and JQ686724); CEVA (KF430816, KM066974, KM066973, and KF430815) and NDV (NBRI-NDV1R: MK572806) at nucleotide sequence level with the respective sequences of other selected potyvirus isolates reported worldwide (listed in Table 1). The tree was constructed employing the MEGA v7.0 tool and the Maximum Likelihood method with 1000 bootstrap replicates. The tree is drawn to scale with branch lengths measured in the number of substitutions per site and the percentage of trees in which the associated taxa clustered together shown next to the branches

and phylogenetic analysis of NYSV and CEVA isolates revealed that sequences under study were genetically diverse and showed approximately 33% nucleotide sequence diversity among them. NYSV and CEVA were reported early in 1908 for the first time in narcissus in the UK (Darlington 1908) and India (Kumar et al. 2015), however, NDV has been detected in narcissus for the first time in India.

In the present study, Indian NYSV sequences showed highest sequence identities at nucleotide and amino acid levels, and close phylogenetic relationships with NYSV isolates reported from China (Chen et al. 2003) and from Japan (Ohshima et al. 2016). This may be due to the migration of virus through some human activity, through transport of any planting material and import of the bulbs from Japan and China to India at initial stages of establishment of narcissus cultivation in Indian continent. However, we could not trace any evidence if infected narcissus bulbs were imported to India from China or Japan.

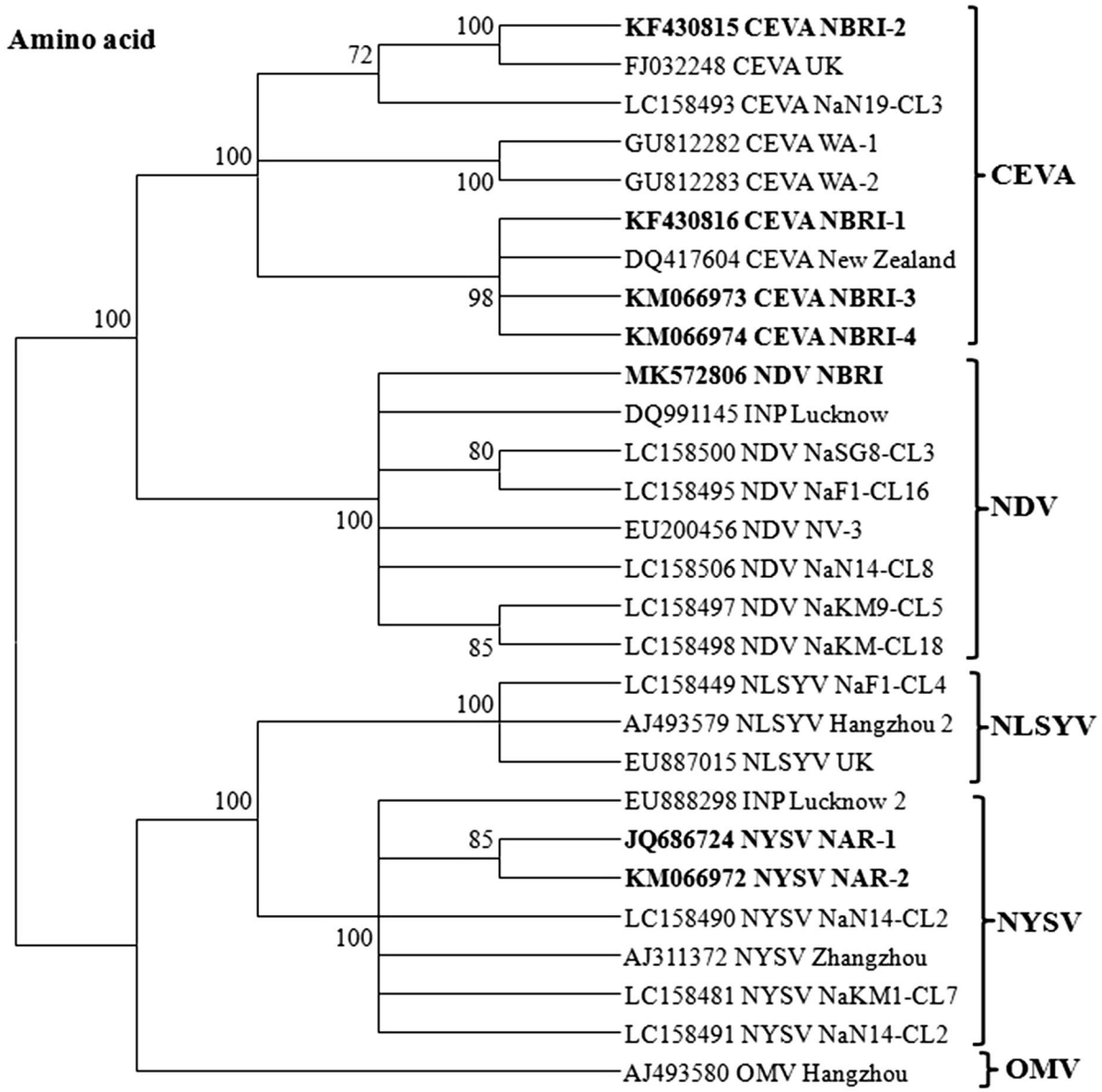

Fig. 3 Phylogenetic tree showing relationships of sequences under study: NYSV (KM066972 and JQ686724); CEVA (KF430816, KM066974, KM066973, and KF430815) and NDV (NBRI-NDV1R: MK572806) at amino acid sequence level with the respective sequences of other selected potyvirus isolates reported worldwide (listed in Table 1). The tree was constructed employing the MEGA v7.0 tool and the Maximum Likelihood method with 1000 bootstrap replicates

The occurrence of *lycoris virus* (Yadav and Khan 2008), *Indian narcissus virus* (Yadav and Khan 2015), CEVA (Kumar et al. 2015) and a distinct potyvirus (Chandel et al. 2010) having close resemblance with NLSYV and NYSV are already reported on narcissus from India based on analyses of their partial genome sequences. The complete genome sequences of CEVA and NYSV infecting narcissus have been published recently by us (Raj et al. 2018, 2019). We report here the existence of three potyvirus species: CEVA, NYSV and NDV in *N. tazetta* var. Paperwhite. The probable reasons for occurrence of three species at NBRI, Lucknow may be because of mixing and pooling of bulbs from infected/symptomatic narcissus plants collected from all the plots during their harvest and storage.

Conclusion

In this study, the existence of CEVA, NYSV and NDV has been investigated in Indian *N. tazetta* var. Paperwhite exhibiting leaf yellow stripe symptoms based on sequence analyses of about 1.5 kb genomic fragments obtained from

seven infected samples by RT-PCR amplification using potyvirus degenerate primers. The study suggests prevalence of diverse potyvirus species present in the vicinity and probability of emergence of new recombinant species is high. Therefore, information may be useful for understanding virus epidemiology and designing the disease management strategies in interest of the narcissus growers.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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