Virus Genes (2014) 49:304–311
DOI 10.1007/s11262-014-1085-5

Detection, distribution, and genetic diversity of *Australian grapevine viroid* in grapevines in India

Charith Raj Adkar-Purushothama · Poornachandra Rao Kanchepalli · Sreenivasa Marikunte Yanjarappa · Zhixiang Zhang · Teruo Sano

Received: 28 February 2014 / Accepted: 5 May 2014 / Published online: 23 May 2014

Abstract *Australian grapevine viroid* (AGVd) is a viroid specific to grapevine with the least records in the world till date. Here, we report for the first time the presence of AGVd in grapevines in Indian sub-continent. The overall infection rate of AGVd in major grapevine producing areas in India was 9.3 %, which is conspicuously higher than the other regions of the world except for Tunisia and Iran. To understand the AGVd diversity in India, the genetic divergence was examined based on the disparity in the cultivars and the locations. Nucleotide sequence analysis revealed the existence of five major AGVd variants in India besides other 44 minor variants implying the "quasi-species" nature. Further, sequence alignment of all the Indian AGVd variants along with Australian type species underscored the presence of eleven mutation points which are archetypal for Indian AGVd, irrespective of the region, and cultivar of grapevines. Plotting of Indian AGVd sequence variants against Australian type species unveiled that all these eleven mutations are distributed on upper and lower left terminal and pathogenicity regions of the molecule. Phylogenetic analysis divulged all the major Indian AGVd variants formed two distinct clusters, suggesting the two separate evolutionary lineages of AGVd in Indian viticulture.

Keywords AGVd · Viroid · *Australian grapevine viroid* · Apscaviroid · Grapevine viroids

C. R. Adkar-Purushothama · T. Sano (✉)
Plant Pathology Laboratory, Faculty of Agriculture and Life Sciences, Hirosaki University, Hirosaki, Aomori 036-8561, Japan
e-mail: sano@cc.hirosaki-u.ac.jp

Present Address:
C. R. Adkar-Purushothama
Département de Biochimie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, 3201 rue Jean-Mignault, Sherbrooke, QC, Canada

P. R. Kanchepalli · S. M. Yanjarappa
Department of Studies in Microbiology, University of Mysore, Manasagangothri, Mysore 570 006, India

Z. Zhang
State Key Laboratory of Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

Introduction

Viroids are smallest known plant pathogens. They are naked, single stranded, highly structured circular RNA molecule with 246–401 nucleotides (nt) in length [1]. They do not code any peptides; hence they rely entirely on host factors for their replication [2]. Broadly viroids are classified into two families, i.e., *Pospiviroidae* family, whose members replicate in nucleus and contain five conserved structural/functional domains, and the *Avsunviroidae* family members replicate in chloroplast and exhibit ribozyme activity [3]. Viroid infection is often accompanied by wide variety of symptoms like stunting, epinasty, leaf distortion, localized veinal chlorosis, and necrosis [4].

Grapevine (*Vitis vinifera* L.) belonging to family *Vitaceae* is a commercially important fruit crop of India. India is among the first ten countries in the world in the production of grape as per records of Department of Horticulture, Government of India [5]. India's 70 % of grapes are grown in the states of Karnataka and Maharashtra [6, 7]. Five viroids, *Hop stunt viroid* (HpSVd), *Grapevine yellow speckle viroid-1* (GYSVd-1), *Grapevine yellow speckle viroid-2* (GYSVd-2), *Citrus exocortis viroid* (CEVd), and *Australian grapevine*

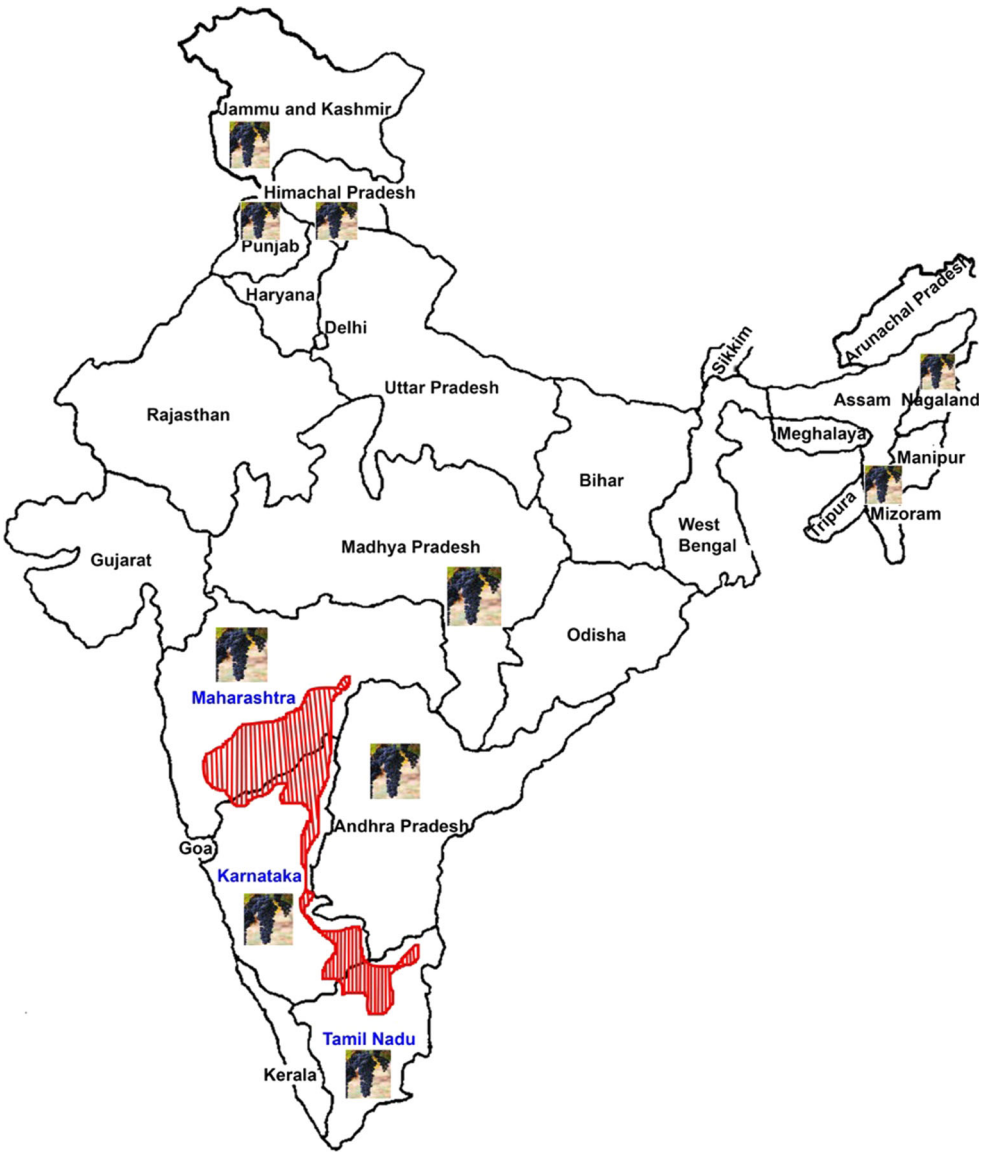

Fig. 1 A map showing area used for grapevine production regions in India. Samples were collected from Maharashtra, Karnataka, and Tamil Nadu states, which account for 70 % of gross domestic grapevine production. Samples were collected from the *red colored* region (Color figure online)

viroid (AGVd) have been reported to infect grapevines [8–12]. These grapevine-infecting viroids are belong to three different genera of *Pospiviroidae*; namely, CEVd belongs to genus *Pospiviroid*, HpSVd belongs to the genus *Hostuviroid*, while GYSVd-1, GYSVd-2, and AGVd belong to the genus *Apscaviroid*. Of these five viroids, only GYSVd-1 and GYSVd-2 are known to produce symptoms on grapevines [12, 13]. Recently, GYSVd-1 and HpSVd has been reported from grapevines grown in the Karnataka state of India [14].

AGVd is 369 nt in length and was first reported from Australia in 1988 [10]. It contains the entire central conserved region of the *Apple scar skin viroid* (ASSVd) group and is a member of the genus *Apscaviroid*, family *Pospiviroidae* [15]. It appears to have originated from extensive RNA recombination involving GYSVd, CEVd, and ASSVd. Further, it has only been isolated from grapevines which are frequently mix-infected with one or some of HpSVd, GYSVd-1, GYSVd-2, and CEVd [15, 16]. Till date, AGVd has only been reported from Australia, China, USA, Tunisia, and Iran [11, 16–19]. Here in this manuscript, we have detected AGVd for the first time from grapevines cultivating in India and found that the infection rate is conspicuously high (i.e., ca. 10 %) in major cultivars grown in the major grapevine production areas. Genetic diversity of Indian isolates of AGVd was underscored and their unique nucleotides were plotted against AGVd type species.

Materials and methods

Plant material

To investigate the presence of AGVd, 24 grapevine leaf samples including normal green-colored leaf as well as

Table 1 Details of grapevine samples collected from three states in India and the result of AGVd detection by RT-PCR and northern hybridization

Cultivar	Place of sample collection	Total number samples	Number of positive samples		Percentage infection[a]	Percentage infection
			RT-PCR	Northern assay		
Thompson seedless	Maharashtra	15	2	2	13.3	9.4
	Karnataka	18	2	2	11.1	
	Tamil Nadu	20	1	0	5	
Anab-e-Shahi	Maharashtra	12	2	2	11.1	10.4
	Karnataka	18	2	2	11.1	
	Tamil Nadu	18	1	1	5.6	
Sharad	Maharashtra	18	2	1	11.1	7.9
	Karnataka	12	1	1	8.3	
	Tamil Nadu	8	0	0	0	
Bangalore Blue	Maharashtra	12	2	2	16.6	10.6
	Karnataka	19	2	2	10.5	
	Tamil Nadu	16	1	1	6.3	

[a] Based on PCR analysis

pale-colored ones were randomly collected from Karnataka state, India in May 2012. To further study the distribution of AGVd in grapevines in India, 186 fully expanded leaf samples were collected from four cultivars of vine trees approximately 10–20 years old; namely, Thompson Seedless, Anab-e-Shahi, Sharad seedless, and Bangalore Blue (Fig. 1; Table 1). Sampling has been done similarly in various states of Maharashtra, Karnataka, and Tamil Nadu states of India during July (early–mid growing season) 2012 to January (early harvest season) 2013.

RNA extraction

Total nucleic acids were extracted using a modified CTAB method [20]. Briefly, 0.2 g of leaf sample was homogenized in 1.5-mL microfuge tube using CTAB buffer (2 % CTAB, 2 % PVP-40, 100 mM Tris–HCl, 2.0 M NaCl, 20 mM EDTA, and 2 % β-mercaptoethanol) using pestle and silica. The homogenate was incubated at 65 °C for 15 min. During incubation, mixture was shaken vigorously several times. Nucleic acids were extracted with equal volume of phenol:chloroform (1:1). Finally, low molecular weight RNA was enriched by 2 M LiCl fractionation [21].

Amplification and sequencing of viroid cDNA

Complementary DNAs were synthesized by *M-MuLV* reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) using random hexamer according to the manufacturer's description. The fragment of viroid cDNA was then amplified by polymerase chain reaction (PCR) using LA *Taq* polymerase (TaKaRa Bio, Shiga, Japan) in the presence of primer pair PBCV100C/PBCV194H as described previously [22]. Resulting products were analyzed on 2.0 % agarose gel electrophoresis. Amplicon of expected size was cloned using a pCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA). Sequencing was performed using automated DNA sequencer (ABI 3750XL DNA analyzer) and analyzed in BioEdit software version 7.0.9.0 [23], and assembled by using CLC Free Workbench version 4.6 software (http://www.clcbio.com/index.php?id=28). Sequence comparisons against international GenBank databases were performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the partial sequence obtained, a new reverse transcription-PCR (RT-PCR) primer set was synthesized (Table 2) and remaining portion of the viroid was amplified. Provisional nucleotide sequence of the entire viroid was confirmed using a third set of PCR primers, i.e., F3 and R4 [18]. Amplified cDNA fragment was cloned and sequenced as described earlier.

Northern hybridization

Northern hybridization was performed using digoxygenin-labeled riboprobes for *Apple fruit crinkle viroid* (AFCVd), which has 85 % sequence similarity with AGVd [21].

Secondary structure prediction for analyzing genetic variation

All the sequences obtained were aligned along with AGVd type member (GenBank Acc. No. X17101) and mismatches were noted. Possible secondary structure of AGVd type member was predicted in m-fold (http://mfold.rna.albany.edu/?q=mfold) and sequence variants identified in India were plotted against AGVd secondary structure.

Table 2 Details of the primers used in the present study for the detection and amplification of AGVd

Primer name	Sequence	Description	Expected size (nt)	References
PBCV100C	AGACCCTTCGTCGACGACGA	*Apscaviroid* genus-specific primer	225	[21]
PBCV194H	TGTCCCGCTAGTCGAGCGGA			
AGV-60RT	TTTTTCTTCCTAGCTTCGCG	Reverse transcription		This work
AGV-77F	TAAGACTCACCTGGCGACTC	PCR	330	This work
AGV-37R	TCGGTGAGTACCACAGGAAC			
F3	TTGGATCCTCCAGCGGAGGACTGAAG	PCR	369	[17]
R4	TTGGATCCGGACCCCTAGCGTTCCTG			

Underlined: *Bam*HI recognition site

Phylogenetic analysis

The sequences were aligned with those of the other AGVd deposited in the GenBank database using the ClustalW (Ver 1.83) program of Bioedit [23] and phylogenetic analysis were performed using neighbor-joining with Jukes–Cantor model of MEGA6 software [24]. HpSVd was used as out-group.

Results

Detection of AGVd in India

The DNA fragment with the expected size ca. 225 bp was successfully amplified by RT-PCR using a universal primer set for *Apscaviroid* from six of the 24 samples collected from Karnataka state in India (Fig. 2a). They were cloned for sequencing. The BLAST homology search revealed the sequences amplified were all similar to AGVd, suggesting the existence of AGVd in India. Further, all these samples were also revealed to harbor either GYSVd-1 and/or HpSVd, indicating the mixed infection. The information of partial sequence data obtained for AGVd was used for designing new primers and the other half of the molecule was amplified by RT-PCR using AGV-60RT for RT and AGV-77F/AGV-37R for PCR. The DNA fragment with the size ca. 330 bp was amplified from all the six samples (Fig. 2b). Further analysis on the six RT-PCR-positive samples by northern hybridization using digoxigenin-labeled riboprobe for AFCVd confirmed that they were all positive (Fig. 2c), supporting the presence of AFCVd-like viroid in Indian grapevines; i.e., AGVd and AFCVd shared ca. 85 % sequence identity and are cross hybridized with each other in this hybridization conditions [18]. Since partial AGVd sequence was detected, the six AGVd-positive samples were used for RT-PCR amplification of the full-length cDNA copy using F3/R4 primer set. As the result, the DNA fragment with the size ca. 370 bp was amplified from all the six samples (Fig. 2d). They were recovered from the gel and used for cloning and sequencing. The sequences obtained were aligned with the former partial sequences and reconstructed a complete nucleotide sequence, which was consisted of 369 nt in length and showed 98 % sequence homologies to AGVd isolate ZV1 (GenBank Acc. No. EU743606) reported in China, stipulating that the viroid is AGVd.

Distribution of AGVd in India

A total of 186 grapevine leaf samples were collected from three major grapevine producing regions of India (Table 1). The RNA was extracted and examined the infection of AGVd by RT-PCR using AGVd-specific primer set F3 and R4 [18]. Of them 18 showed amplification of DNA fragment with ca. 370 bp, indicating the presence of AGVd in all the examined regions of India. To re-confirm the result, RT-PCR assay was repeated once again on the 18 AGVd-positive samples by AGV-77F/AGV-37R primer set using freshly prepared RNA samples. Presence of the amplicon with ca. 330 bp in length was shown clearly in all the 18 samples, and was further confirmed by sequencing and BLAST analysis. Northern blot hybridization analysis using DIG-labeled AFCVd-cRNA probe also showed 16 of 18 was positive, further confirmed the presence of AGVd in all the three states of India.

Focusing on the domestic regional disparity, of 57 samples collected from Maharashtra state 8 (14 %) showed positive, of 67 from Karnataka state 7 (10.4 %) showed positive, and of the remaining 62 from Tamil Nadu state 3 (5 %) showed positive; namely, samples from Maharashtra and Karnataka showed higher infection rate (Fig. 3). Next, focusing on the disparity in grapevine cultivars, AGVd was detected from 5 (9.4 %), 5 (10.4 %), 3 (7.9 %), and 5 (10.6 %) samples of 53 Thompson seedless, 48 Anab-e-Shahi, 38 Sharad, and 47 Bangalore Blue, respectively (Table 1).

Furthermore, more detailed analysis has been done on the combinations of cultivars and locations. Among the four cultivars in Maharashtra, Bangalore Blue recorded the

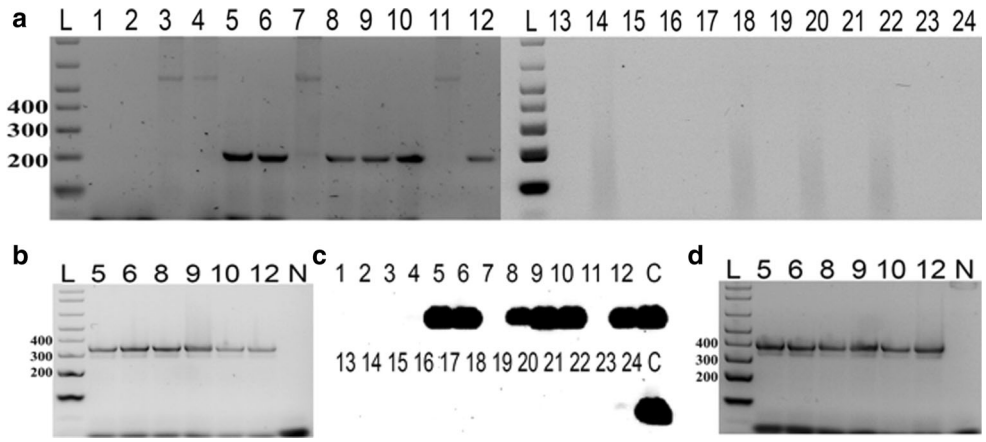

Fig. 2 Detection of AGVd in 24 samples using **a** *Apscaviroid* universal primer set PBCV100C/PBCV194H on 2.0 % agarose gel electrophoresis; **b** amplicons obtained from six positive samples by newly designed AGVd-specific primer set (AGV-60RT, AGV-77F/AGV-37R); **c** northern blot hybridization reaction with digoxygenin-labeled riboprobes for AFCVd; and **d** full-length amplification of AGVd in six positive samples by using F3/R4 primers. *Lane L* 1 kb plus molecular marker (Invitrogen, Carlsberg, CA), *lane 1–24* grapevine sample number, *lane N* negative control and *lane C* is AFCVd (positive control)

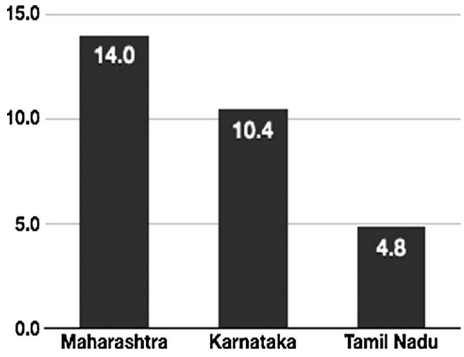

Fig. 3 Comparison of percentage distribution of AGVd-positive rate among three states of India. *Numerical numbers* on the *bars* are given in percentage.

highest incident of AGVd, accounting for 16.6 %, whereas the least was the cultivars Anab-e-Shahi and Sharad, both accounting for 11.1 % (Table 1). Significant difference could not be seen in the four cultivars collected from Karnataka state, where incident of AGVd is accounting for 8.3–11.1 % (Table 1). In case of Tamil Nadu, cultivar Sharad recorded zero percent of incident out of eight samples tested, whereas cultivar Bangalore Blue showed 6.3 % (Table 1).

Genetic diversity of AGVd isolates

To understand the genetic diversity of AGVd in India, all the 18 RT-PCR positives amplified by AGVd-specific primer set F3/R4 were cloned for sequencing. At least ten clones per sample, a total 193 independent cDNA clones were sequenced. Moreover, the sequence of the primer-binding site (i.e., corresponding to the position 18–96 in the reference sequence GenBank Acc. No. X17101) was examined by direct sequencing of freshly prepared 18 RT-PCR products using AGV-77F/AGV-37R primer pair. As the result, AGVd populations in India contained five major sequence variants consisting of varieties of minor sequence variants with high overall sequence homology, indicating that AGVd forms "quasi-species." Nucleotide sequence of the five major Indian AGVd variants was deposited in DNA bank by the accession, Ind-1 (GenBank Acc. No. KJ019300), Ind-2 (GenBank Acc. No. KJ019301), Ind-3 (GenBank Acc. No. KJ019302), Ind-4 (GenBank Acc. No. KJ019303), and Ind-5 (GenBank Acc. No. KJ019304).

Genetic array on AGVd secondary structure

By aligning all the 193 (independent cDNA clones) plus 18 (direct sequencing RT-PCR product by AGV-77F/AGV-37R) sequences obtained in the present study against the genome sequence of AGVd type member (GenBank Acc. No. X17101), we have detected 49-sequence variants. Furthermore, we used the sequences, which revealed a mutation at least in two different clones. Out of these 49-sequence variants, five are most abundant, i.e., Ind-1 (8/193 cDNA clones), Ind-2 (15/193 cDNA clones), Ind-3 (11/193 cDNA clones), Ind-4 (9/193 cDNA clones), and Ind-5 (13/193 cDNA clones). All these 49-sequence variants were plotted with different color codes against predicted secondary structure of AGVd (Fig. 4). In detail, red color nucleotides are the most abundant variants and are present in all the AGVd sequences detected in the present study, whereas blue color nucleotides were detected in more than two clones.

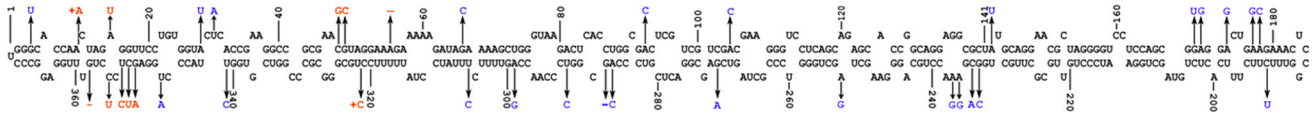

Fig. 4 Locations and changes of sequence variations found in 18 Indian AGVd isolates on a secondary structure model of AGVd type species (GenBank Acc. No. X17101). *Red color* nucleotides are the most abundant variants and are found in all 49-sequence variants, whereas *blue color* nucleotides were detected in more than two clones (Color figure online)

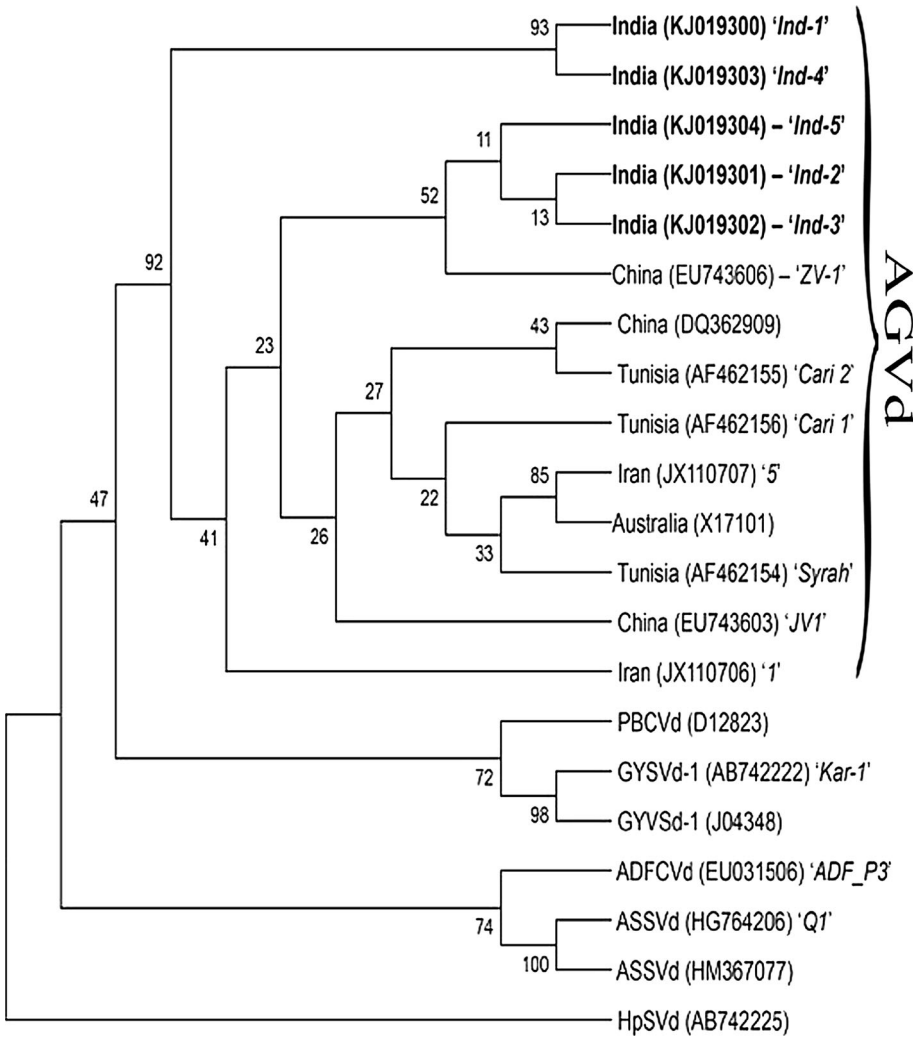

Fig. 5 Phylogenetic relationship of AGVd sequence variants obtained from India and the representative isolates from Australia, China, Iran, and Tunisia

Phylogenetic analysis

A phylogenetic analysis was carried out on the five paramount sequence variants isolated from Maharashtra, Karnataka, and Tamil Nadu together with the other nine representative AGVd sequences; i.e., the type isolates from Australia, three predominant isolates from China, three isolates from Tunisia, and two isolates from Iran. The selected members of *Apsca-* and *Pospiviroid* were also included for comparison. As exhibited in Fig. 5, all the Indian variants formed two distinct clusters, one along with the previous report from China in cultivar "Zaoyu"; i.e., ZV1 variant, accession EU743606 [25], although bootstrap support was not high.

Discussion

Identification of AGVd in India

Two grapevine viroids, GYSVd-1 and HpSVd, had already been detected from grapevines cultivated in India [14], hence it was suspected that the other viroid species such as GYSVd-2 or AGVd could be also in India. As AGVd

infection is not known to induce symptoms [16, 26], leaf samples were randomly collected without considering symptoms from vines trees of 10–20 years old. As expected, initial inspection of 24 grapevine samples by RT-PCR using *Apscaviroid*-specific primer set revealed the presence of *Apscaviroid* in India. The species was further narrowed by northern blotting using AFCVd-cRNA probe and RT-PCR amplification by a newly designed primer set specific to AGVd. The nucleotide sequencing of the full-length copy of viroid genome finally unveiled the presence of AGVd in India.

Dissemination and genetic disparity of AGVd in India

Since AGVd was detected, we planned to look into distribution of AGVd and its genetic divergence in the main grapevine growing regions of India. For this purpose, 186 samples were further collected and were subjected for RT-PCR analysis using AGVd-specific primer sets. Out of these, 18 samples showed amplification of DNA fragment with ca. 370 bp, the size expected to be amplified from AGVd. The specificity of RT-PCR result was verified by northern assay and confirmed that 16 of 18 RT-PCR positives were positive by northern. The reason why two RT-PCR-positive samples were negative by northern assay will be attributed to either of low AGVd titer in these samples and lower sensitivity of northern assay comparing to RT-PCR [27]. Further analysis by sequencing followed by BLAST confirmed AGVd infection in these samples, meaning that 9.3 % of overall Indian grapevine samples were AGVd-positive. So far, it was generally accepted that AGVd infection rate is quite low comparing to the other grapevine viroids except for CEVd [26]; i.e., among the relatively large-scale surveillance and combined data reported previously [26], the infection rate was 3.1 % in China, 0 % in Japan, 0 % in EU, and 8.3 % in USA, although the number of samples examined was only limited in USA. Interestingly, exceptionally high infection rate of AGVd was recorded recently from Tunisia underscoring 26 % [16] and 18.7 % infection rate was reported from Iran [28]. Further, in case of grapevines in Australia, two separate studies were conducted in 1989 [29] and in 1999 [30]. According to the previous report, none of the 27 tested samples were positive for AGVd infection whereas, 100 % incident of AGVd was observed in five samples examined, but the number of samples used in these studies was not significant to extrapolate AGVd incident in Australia. In conclusion, the infection rate of AGVd in Indian grapevine is apparently higher than those in East Asian countries (China and Japan) but a little lower comparing to those in West Asian (Iran) and African (Tunisia) countries, when considering the fact that we did not do any bias sampling because AGVd does not show symptoms on grapevines.

Concentrating on the domestic regional disparity, AGVd infection rate was 14 % in Maharashtra state, 10.4 % in Karnataka state, whereas 5 % in Tamil Nadu state. After all, regional disparity looked significant than cultivar disparity. It is very interesting to note that the infection rate was the highest in Maharashtra and gradually became lower toward Tamil Nadu, indicating that the infection rate was as low as the southern part in India, although it is essential to collect more AGVd-positives to lead a certain conclusion. Since these states are major grapevine producing areas in India, it is necessary to pay full-attention to the production and distribution of scions and root-stocks for viticulture in these regions. Moreover, infection rate of AGVd in the four grapevine cultivars examined did not show significant difference, suggesting that all these cultivars have similar level of susceptibility to AGVd infection.

Sequence analysis of 193 clones obtained from 18 RT-PCR positive samples revealed at least 49-sequence variants underscoring "quasi-species" nature of AGVd in India. To avoid the possible error caused by polymerase during RT-PCR, we used high-fidelity *Taq* DNA polymerase. Further, to eliminate any incorrect information withdrawn by possible mistakes of *Taq* polymerase, only the sequences found in two clones at least were taken into account for further analysis. Among these 49-sequence variants, five found to be more abundant, namely Ind-1, Ind-2, Ind-3, Ind-4, and Ind-5. In detail, all the five major variants were detected in Maharashtra and Karnataka states, whereas Tamil Nadu region recorded only two variants Ind-2 and Ind-5. Taking account of all the 49-sequence variants identified in the present work and plotting against AGVd type species revealed very interesting data. All the India AGVd sequence variants showed variations at the left terminal region of the predicted secondary structure. This is in agreement with previous report of AGVd sequence variants from China [24]. On the contrary, Indian AGVd isolates showed some sequence variations in the central conserved region (CCR), i.e., at the positions 82–123, and also in the variable and the right terminal regions. More interestingly, it is worth to note that all the Indian AGVd sequence variants unveiled unique nucleotide substitutions at position A15U; C47G, G48C, G351A, C352U, U353C, and C355U, referring to the type sequence (GenBank Acc. No. X17101). Further, analysis of the data combining 193 plus 18 individual sequences identified that two co-variation mutations could be taken place in the TL (i.e., insertion of A between the nucleotide Nos. 10 and 11 and deletion of G from No. 358) and the P (i.e., deletion of A from No. 55 and insertion of C between Nos. 321 and 322) regions. The effect of these mutations especially in P domain is interesting as it might cause different in symptom on certain hosts. Hence, further work

with herbaceous plant is required to see the effect of these mutations in symptom expression.

In the present study, we verified the presence of AGVd in India along with the cultivars specificity and regional disparity. To the best of our knowledge, this is the sixth sequence report of AGVd after Australia, USA, Tunisia, China, and Iran. AGVd infection rate is recorded as high as 12.3 % in Maharashtra state, conspicuously higher rate of AGVd incident. Although any noticeable AGVd-related discrepancy could be seen among the major grapevine cultivars in India, attention has to be paid for the production and distribution of scions and stocks for domestic viticulture. Sequence and phylogenetic analyses emphasized Indian AGVd variants are distinct but some of them are somewhat similar to one of the Chinese variants. Further research is needed to understand the evolution and adaptation of AGVd in Indian viticulture.

Acknowledgments Authors would like to thank Prof. Jean-Pierre Perreault, Department of Biochemistry (PRAC), Université de Sherbrooke, Canada for fruitful discussions during manuscript preparation. This work was supported in part by Japan Society for the Promotion of Science KAKENHI Grant Nos. 24405026 and 24380026. Adkar-Purushothama C.R. received JSPS postdoctoral fellowship for foreign researchers.

Conflict of interest The authors declare no conflict of interest.

References

1. B. Ding, Annu. Rev. Phytopathol. **47**, 105–131 (2009)
2. R. Flores, C. Hernández, A.E. Martínez de Alba, J.A. Daròs, F. Di Serio, F. Annu, Rev. Phytopathol. **43**, 117–139 (2005)
3. R. Flores, J.W. Randles, M. Bar-Joseph, T.O. Diener, Arch. Virol. **143**, 623–629 (1998)
4. R.A. Owens, K.B. Tech, J.T. Shao, T. Sano, C.J. Baker, Mol. Plant Microbe Interact. **25**, 582–598 (2012)
5. S.D. Shikhamany, Grape production in India (RAP publication, 2001), http://www.fao.org/docrep/003/x6897e/x6897e06.htm. Accessed 23 Feb 2014
6. Grape producing states map, maps of India (2013), http://www.mapsofindia.com/indiaagriculture/fruits-map/grapes-producing-states.html. Accessed 23 Feb 2014
7. Grape, http://nhb.gov.in/Horticulture%20Crops%5CGrape%5CGrape1.htm. Accessed 23 Feb 2014
8. T. Sano, K. Ohshima, I. Uyeda, E. Shikata, T. Meshi, Y. Okada, Jpn. Acad. **61**, 265–268 (1985)
9. F. Garcia-Arenal, V. Pallas, R. Flores, Nucleic Acids Res. **15**, 4203–4210 (1987)
10. A.M. Koltunow, M.A. Rezaian, Nucleic Acids Res. **16**, 849–864 (1988)
11. M.A. Rezaian, A.M. Koltunow, L.R. Krake, J. Gen. Virol. **69**, 413–422 (1988)
12. A.M. Koltunow, L.R. Krake, S.D. Johnson, M.A. Rezaian, J. Gen. Virol. **70**, 3411–3419 (1989)
13. J.A. Szychowski, R. Credi, K. Reanwarakorn, J.S. Semancik, Virology **248**, 432–444 (1998)
14. A.B. Sahana, C.R. Adkar-Purushothama, G. Chennappa, Z.X. Zhang, M.Y. Sreenivasa, T. Sano, Plant Dis. **97**, 1517 (2013)
15. M.A. Rezaian, Nucleic Acids Res. **18**, 1813–1818 (1990)
16. A. Elleuch, H. Fakhfakh, M. Plechat, P. Landrt, M.J.P. Marrakchi, J.P. Perreault, Eur. J. Plant Pathol. **1108**, 815–820 (2002)
17. M.A. Rezaian, L.R. Krake, D.A. Golino, Intervirology **34**, 38–43 (1992)
18. R. Guo, T. Sano, Z. Cheng, S.-F. Li, Plant. Pathol. **14**, 9 (2006)
19. M. Hajizadeh, B. Navarro, N.S. Bashir, E.M. Torchetti, F. Di Serio, J. Virol. Methods **179**, 62–69 (2012)
20. Z. Zhang, S. Peng, D. Jiang, S. Pan, H. Wang, S. Li, Eur. J. Plant Pathol. **132**, 9–16 (2012)
21. S. Machida, M. Shibuya, T. Sano, J. Gen. Plant Pathol. **74**, 204–207 (2008)
22. T. Sano, H. Yoshida, M. Goshono, T. Monma, H. Kawasaki, K. Ishizaki, J. Gen. Plant Pathol. **70**, 181–187 (2004)
23. T.A. Hall, Nucleic Acids Symp. Ser. **41**, 95–98 (1999)
24. K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, Mol. Biol. Evol. **28**, 2731–2739 (2011)
25. D. Jiang, S. Peng, Z. Wu, Z. Cheng, S. Li, Virus Genes **38**, 178–183 (2009)
26. D. Jiang, T. Sano, M. Tsuji, H. Araki, K. Sagawa, C.R. Adkar-Purushothama, Z. Zhang, R. Guo, L. Xie, Z. Wu, H. Wang, S. Li, Virus Res. **169**, 237–245 (2012)
27. S. Streit, C.W. Michalski, M. Erkan, J. Kleeff, H. Friess, Nat. Protoc. **4**, 37–43 (2008)
28. M. Zaki-Aghl, K. Izadpanah, A. Niazi, S.A.A. Behjatnia, A.R. Afsharifar, J. Agric. Sci. Technol. **15**, 855–865 (2013)
29. A.M. Koltunow, M.A. Rezaian, Virology **170**, 575–578 (1989)
30. Y.F. Wan Chow Wah, R.H. Symons, J. Phytopathol. **147**, 285–291 (1999)