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SHORT COMMUNICATION

Sequence diversity among badnavirus isolates infecting black pepper and related species in India

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Abstract The badnavirus, piper yellow mottle virus (PYMoV) is known to infect black pepper (*Piper nigrum*), betelvine (*P. betle*) and Indian long pepper (*P. longum*) in India and other parts of the world. Occurrence of PYMoV or other badnaviruses in other species of *Piper* and its variability is not reported so far. We have analysed sequence variability in the conserved putative reverse transcriptase (RT)/ribonuclease H (RNase H) coding region of the virus using specific badnavirus primers from 13 virus isolates of black pepper collected from different cultivars and regions and one isolate each from 23 other species of *Piper*. Of these, four species failed to produce expected amplicon while amplicon from four other species showed more similarities to plant sequences than to badnaviruses. Of the remaining, isolates from black pepper, *P. argyrophyllum, P. attenuatum*, *P. barberi*, *P. betle*, *P. colubrinum*, *P. galeatum*, *P. longum*, *P. ornatum*, *P. sarmentosum* and *P. trichostachyon* showed an identity of >85 % at the nucleotide and >90 % at the amino acid level with PYMoV indicating that they are isolates of PYMoV. On the other hand high sequence variability of 21–43 % at nucleotide and 17–46 % at amino acid level compared to PYMoV was found among isolates infecting *P. bababudani*, *P. chaba*, *P. peepuloides, P. mullesua* and *P. thomsonii* suggesting the presence of new badnaviruses. Phylogenetic analyses showed close clustering of all PYMoV isolates that were well separated from other known badnaviruses. This is the first report of occurrence of PYMoV in eight *Piper* spp and likely occurrence of four new species in five *Piper* spp.

Keywords *Piper yellow mottle virus* · Sequence variability · *Piper* spp. RT/RNase region

The genus *Badnavirus*, which belongs to the family *Caulimoviridae*, have bacilliform shaped virions and a circular non-covalently closed double stranded DNA genome of 7–8 kb. The genomes of all described badnaviruses have a similar organization and contain 3–6 open reading frames (ORFs) [7, 10, 16]. Badnaviruses are mainly transmitted by vegetative propagation, mealy bug and through seeds [10, 12, 16]. There are more than 30 badnaviruses reported all around the world, infecting a wide range of economically important tropical crops including banana, black pepper, cacao, citrus and sugarcane. Many of the badnaviruses were shown to be highly variable at the genomic level [6, 11, 13, 16] while a few such as citrus yellow mosaic virus (CYMV) were shown to be less variable [1, 2]. The guidelines for demarcation of species within the genus *Badnavirus* have been reported. The criteria for defining species are <80 % identity (in the nucleotide sequence) or <89 % identity (in the amino acid sequence) in the conserved reverse transcriptase (RT)/ribonuclease H (RNase H) coding region [3].

Black pepper (*Piper nigrum* L.), which originated in the tropical evergreen forests of the Western Ghats in India is used for a variety of purposes including medicinal applications [14]. The first badnavirus reported on black pepper was *Piper yellow mottle virus* (PYMoV) causing yellow mottle disease, from Malaysia, the Philippines, Sri

Sequences reported in this paper have been submitted to the GenBank database and have been assigned the accession numbers KJ195468 to KJ195494.

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Table 1 Geographical origin and accession numbers of the badnavirus isolates used in the study

Sl.no	*Piper* species	Geographical origin	Cultivar/Variety	Accession number	Abbreviation used
1	*P. nigrum* L.	Kozhikode, Kerala	Subhakara	DQ836227	nigrum-1
2	*P. nigrum* L.	Idukki, Kerala	Jeerakamundi	DQ836229	nigrum-2
3	*P. nigrum* L.	Wynad, Kerala	Wynadan	DQ836231	nigrum-3
4	*P. nigrum* L.	Kodagu, Karnataka	Panniyur 1	DQ836232	nigrum-4
5	*P. nigrum* L.	Kozhikode, Kerala	Karimunda	KC808712	nigrum-5
6	*P. nigrum* L.	Hassan, Karnataka	Panniyur 1	KJ195468[a]	nigrum-6
7	*P. nigrum* L.	Visakhapatnam, Andhra Pradesh	Sreekara	KJ195469[a]	nigrum-7
8	*P. nigrum* L.	Kassargod, Kerala	Karimunda	KJ195470[a]	nigrum-8
9	*P. nigrum* L.	Kannur, Kerala	Karimunda	KJ195471[a]	nigrum- 9
10	*P. nigrum* L.	Udupi, Karnataka	Panniyur 1	KJ195472[a]	nigrum-10
11	*P. nigrum* L.	Chikkmagalur, Karnataka	Panniyur 1	KJ195473[a]	nigrum-11
12	*P. nigrum* L.	Palakkad, Kerala	Arakulamunda	KJ195474[a]	nigrum-12
13	*P. nigrum* L.	Kozhikode, Kerala	Subhakara	KJ195475[a]	nigrum-13
14	*P. nigrum* L.	Kozhikode, Kerala	IISR Girimunda	KJ195476[a]	nigrum-14
15	*P. nigrum* L.	Kozhikode, Kerala	IISR-Thevam	KJ195477[a]	nigrum-15
16	*P. nigrum* L.	Kozhikode, Kerala	Pournami	KJ195478[a]	nigrum-16
17	*P. nigrum* L.	Kozhikode, Kerala	IISR-Shakthi	KJ195479[a]	nigrum-17
18	*P. betle* L.	Kasaragod, Kerala	–	DQ836235	betle -1
19	*P. betle* L.	Kozhikode, Kerala	–	KJ195480[a]	betle-2
20	*P. longum* L.	Kozhikode, Kerala	–	DQ836237	longum-1
21	*P. longum* L.	Kozhikode, Kerala	–	KJ195481[a]	longum-2
22	*P. argyrophyllum* Miq	Palakkad, Kerala	–	KJ195482[a]	argyrophyllum
23	*P.attenuatum* Herb.ex Link	Tirunelveli, Tamil Nadu	–	KJ195483[a]	attenuatum
24	*P. barberi* Gamble	Nagarcoil, Tamil Nadu	–	KJ195484[a]	barberi
25	*P. colubrinum* Link	Kannur, Kerala	–	KJ195485[a]	colubrinum
26	*P. galeatum* (Miq.) C.DC	Palakkad, Kerala	–	KJ195486[a]	galeatum
27	*P. ornatum* N.E.Br	Wynad, Kerala	–	KJ195487[a]	ornatum
28	*P. sarmentosum* Roxb.	Andamans	–	KJ195488[a]	sarmentosum
29	*P. trichostachyon* (Miq) C.DC	Idukki, Kerala	–	KJ195489[a]	trichostachyon
30	*P. bababudani*	Chikkmagalur, Karnataka	–	KJ195490[a]	bababudani
31	*P. chaba* Hunter	Kozhikode, Kerala	–	KJ195491[a]	chaba
32	*P. peepuloides* Roxb.	Jalpaiguri, West Bengal	–	KJ195492[a]	peepuloides
33	*P. mullesua* Buch. Ham ex D.Don	Ooty, Tamil Nadu		KJ195493[a]	mullesua
34	*P. thomsonii* (C.DC) Hook.f	Jalpaiguri, West Bengal	–	KJ195494[a]	thomsonii

[a] Sequences obtained in this study

Lanka and Thailand based on electron microscopy, mealybug transmission studies, genomic characterization and partial genomic sequencing [12]. Subsequently PYMoV was also reported from India [9]. Yellow mottle disease of black pepper is characterized by mottled and small leaves, short internodes, and stunted vines. Recently complete genome sequence of an isolate of PYMoV showed that it possesses four ORFs [8]. Like other badnaviruses, the ORF 3 of PYMoV encodes a polyprotein consisting of viral movement protein, trimeric dUTPase, Zinc finger, retropepsin, RT-LTR and RNase H [8]. In addition to black pepper, occurrence of PYMoV in other two economically important *Piper* species such as betelvine (*Piper betle*) and Indian long pepper (*P. longum*) are also known [12, 15].

Studies on variability in PYMoV or occurrence of other badnaviruses infecting black pepper, betelvine and Indian long pepper is not available so far. Similarly there is no report of occurrence of badnaviruses in other *Piper* species. In the present study we have analysed sequence variability in the conserved putative RT/RNase H coding region of the virus using specific badnavirus primers from 13 isolates of black pepper collected from different cultivars and regions and one isolate each from 23 other species.

Diseased leaves from black pepper showing mild to severe virus-like symptoms were collected from different cultivars/varieties and regions of India (Table 1). Isolates from 23 related species originally collected from different regions of India (Table 1) and maintained at the Experimental Farm of Indian Institute of Spices Research, Kozhikode, Kerala, India were also used. Total nucleic acid was extracted from leaf tissue using the method described previously [9]. Two degenerate primers, Badna FP (5′ ATGCCITTYGGIITIAARAAYGCICC 3′) and Badna RP (5′ CCAYTTRCAIACISCICCCCAICC 3′) [16] were synthesized to amplify 580 bp region comprising of reverse transcriptase (RT)/ribonuclease H (RNase H) coding region of badnaviruses. The PCR reaction contained 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 µM of each primer, 2.5 U *Taq* polymerase, 1 µl of template DNA and sterile water to a final volume of 50 µl. The thermal cycler was programmed for initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, synthesis at 72 °C for 30 s and a final extension for 10 min at 72 °C. Total DNA isolated from a known healthy plant was used as negative control in PCR. Total DNA isolation and PCR tests were repeated for all plants that gave negative results. The reaction products were analysed on 0.8 % agarose gel along with 1 kb DNA ladder. The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Cell Biosciences, Santa Clara, USA).

The PCR products obtained was eluted from the gel using GenElute Gel Elution kit (Sigma-Aldrich, Bangalore, India), cloned into pTZ57R/T cloning vector (Fermentas, Glen Burnie, USA) and transformed into competent *E. coli* strain DH5α using InsTAclone PCR cloning Kit (Fermentas, Glen Burnie, USA) following manufacturer's instructions. Recombinant clones were identified by PCR as well as restriction endonuclease digestion and selected clones were sequenced in both orientations at the automated DNA sequencing facility available at Chromous Biotech, Bangalore, India. Percent nucleotide and amino acid identities were determined using Bioedit program version 5.0.9. The corresponding nucleotide and amino acid sequences of other badnavirus isolates used for comparison were obtained from GenBank. The BLAST programme was used to identify related sequences available from GenBank database. Multiple sequence alignments were made using ClustalW2 programme. Phylogenetic analysis was performed using different methods such as Bayesian analysis (MrBayes version 3.1), maximum parsimony (MEGA5), maximum likelihood (GARLI version 2.0) and neighbor-joining method (MEGA5) each for a bootstrap analysis of 10000 replicates and the consensus tree was identified using consense package of Phylip 3.69. *Rice tungro bacilliform virus* (RTBV) with an accession no. X57924 was designated as outgroup for the selected sequences.

Using primers Badna FP and Badna RP, products of an approximately size of 580 bp were amplified from all virus isolates collected from black pepper, and there was no amplification from negative (badna-free) control. Of the 23 related species used, all except four (*P. arboreum, P. magnificum, P. silentvalleyense and P. sugandhi*) gave expected amplicon of 580 bp. All the amplicons were cloned and their nucleotide sequence determined. BLAST analysis of the sequence from *P. hamiltoni*, *P. hapnium*, *P. hymenophyllum* and *P. sylvaticum* showed highest identity with different plant genomes including retrotransposon (Ty3-gypsy) while their identity with known badnaviruses was quite low (43–55 %). This suggests that they may represent either host genome or retrotransposon and hence were not used in the present analysis. When the nucleotide sequence of all remaining isolates was compared including the published sequence of six PYMoV isolates (four from black pepper, one each from betelvine to Indian long pepper), a high identity of 85–100 % was observed among all 17 virus isolates from black pepper and many *Piper* spp indicating that they are isolates of PYMoV (Table 2). On the other hand, sequences from *P. bababudani*, *P. chaba*, *P. mullesua, P. peepuloides* and *P. thomsonii* showed low nucleotide sequence identity with PYMoV isolates (57–79 %) and other badnavirus species (57–69 %) suggesting the occurrence of new badnaviruses in these *Piper* species. Of these, virus isolates from *P. bababudani* and *P. chaba* showed an identity of 99 % among each other suggesting that they belong to one badnavirus species (Table 2). BLAST analysis of nucleotide sequence from *P. bababudani* and *P. chaba* showed high identity with PYMoV (77–79 %) suggesting that they are closely related to PYMoV. The virus isolates from *P. mullesua, P. peepuloides* and *P. thomsonii* indicated their distinctiveness as they shared only <71 % identity in the nucleotide sequence between them. The BLAST analysis of nucleotide sequences from *P. mullesua, P. peepuloides* and *P. thomsonii* showed highest identity (68 %) with different badnaviruses such as *Musa acuminata* endogenous badnavirus, *Taro bacilliform virus* and *Canna streak virus* respectively with sequence coverage up to 99–100 % suggesting occurrence of new badnaviruses in each of these species.

To examine whether each of the sequences contained functional protein coding sequence, they were translated. Cloned region of all isolates except the *P. peepuloides* was 579 bp long (predicted to code for 193 amino acids), the expected size for a badnavirus. The virus sequence from *P. peepuloides* was 573 bp long coding for 191amino acids. Analysis of the deduced amino acid sequences showed conserved region (YILDDILV) present in badnaviruses. As

Table 2 Percent nucleotide and deduced amino acid sequence (values shown in bracket) identities in the 579 bp coding region of the RT/RNase H of badnaviral isolates from different species of *Piper* from India

Isolates[a]	1–29	30–31	34	32	33	Other badnaviruses[b]
1–29	85–100 (90–100)	77–79	61–65	60–64	57–60	59–68
30–31	(79–83)	99 (99)	64	62	59	59–69
34	(60–65)	(65–66)	100 (100)	71	63	60–65
32	(59–65)	(64–65)	(77)	100 (100)	59	58–66
33	(54–59)	(57–58)	(60)	(58)	100 (100)	57–63
Other badnaviruses[b]	(54–73)	(58–72)	(59–69)	(57–67)	(52–62)	57–69 (52–73)

Designation used for each of the isolates is given in Table 1

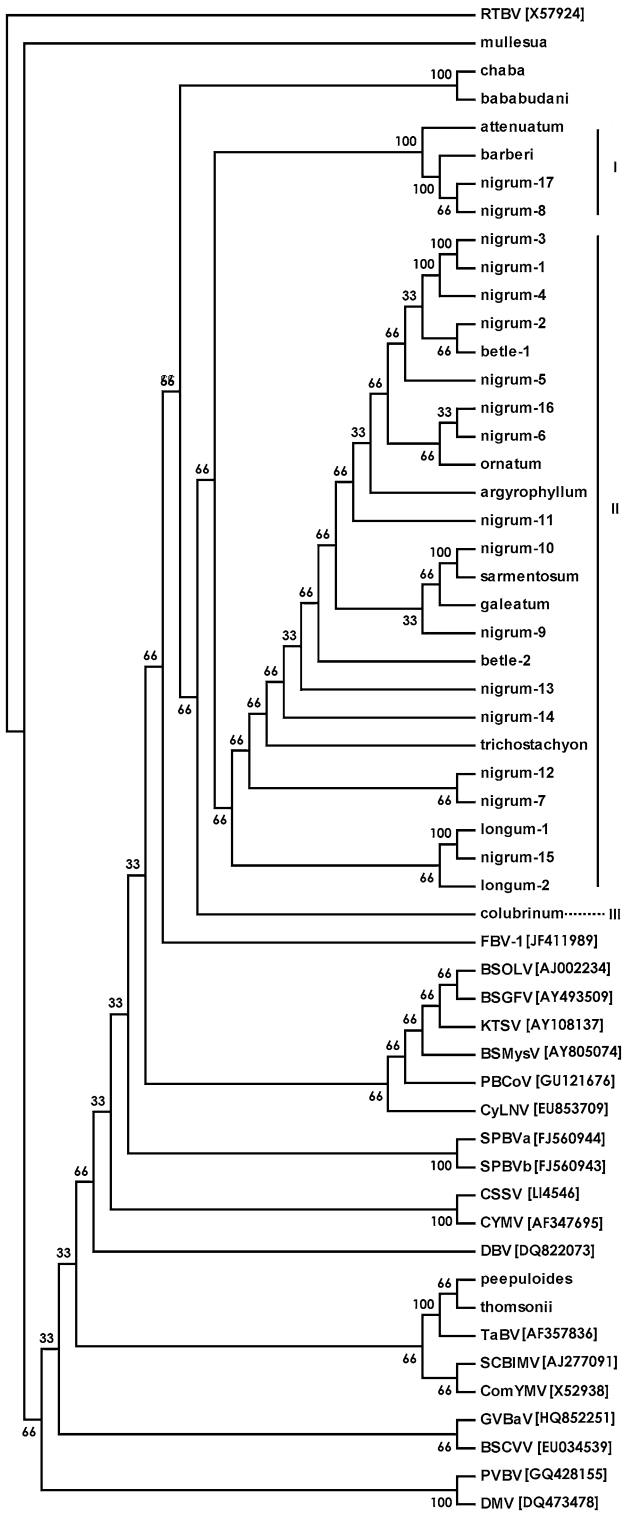
[a] For details of isolates, refer to the Sl. No in Table 1. Isolates 1–17: nigrum-1 to nigrum-17; 18–19: betle-1 to betle-2; 20–21: longum-1 to longum-2; 22: attenuatum; 23: barberi; 24: colubrinum; 25: argyrophyllum; 26: galeatum; 27: ornatum; 28: sarmentosum; 29: trichostachyon; 30: bababudani; 31: chaba; 32: peepuloides; 33: mullesua and 34: thomsoni

[b] Retrieved from GenBank, their acronyms, accession numbers and full names are provided in Fig. 1

observed with nucleotide sequence, high levels of amino acid sequence identity (90–100 %) with PYMoV were detected between the 17 virus isolates of PYMoV from black pepper and many other *Piper* spp confirming that they are isolates of PYMoV (Table 2). On the other hand, isolates from *P. bababudani*, *P. chaba*, *P. mullesua*, *P. peepuloides,* and *P. thomsonii* showed low amino acid sequence identity with PYMoV isolates (54–83 %) and other badnavirus species (52–72 %) suggesting the presence of new badnaviruses. Amino acid sequence identities confirmed high identity between virus isolates from *P. bababudani* and *P. chaba* suggesting the occurrence of a new badnavirus in these species which is most closely related to PYMoV isolates. Further, amino acid sequence identities also suggested likely occurrence of three new badnaviruses in *P. mullesua*, *P. peepuloides,* and *P. thomsonii* as they shared only 57–77 % identity between them (Table 2). The alignment of deduced amino acid sequence of RT/RNase H region of all isolates produced 159 variable and 34 conserved sites.

The consensus phylogenetic tree of the fifty-four sequences including one outgroup based on four methods confirmed results of sequence identity and showed distinctiveness of all PYMoV isolates from black pepper and related species that formed a group well separated from other known badnavirus species (Fig. 1). The relationships between the sequences were supported by high bootstrap values. Phylogenetic analysis of PYMoV isolates revealed that the 29 isolates formed three subgroups; subgroup 1 consisting of virus isolates from *P. attenuatum*, *P. barberi* and two isolates from *P. nigrum* (nigrum-17 and nigrum-8); subgroup 2 consisting 24 isolates and subgroup 3 consisting of only one isolate from *P. colubrinum*. The subgroup 2 was further branched into several subsubgroups (Fig. 1). The virus isolates within subgroup 1 showed 97–99 % amino acid identity while it was 92–100 % among isolates of subgroup 2. The only isolate in subgroup 3 showed an amino acid sequence identity of 94–96 % and 93–95 % respectively with subgroup 1 and subgroup 2 isolates. The badnavirus isolates from *P. bababudani* and *P. chaba* showed very close relationship with PYMoV isolates while badnavirus isolates from *P. mullesua*, *P. peepuloides* and *P. thomsonii* showed very distant relationship with PYMoV (Fig. 1). *Fig badnavirus 1* (FBV-1) was found to be the closest badnavirus species to PYMoV isolates. Badnavirus isolate from *P. peepuloides* and *P. thomsonii* showed close phylogenetic relationship with *Taro bacilliform virus* (TaBV) while virus isolate from *P. mullesua* showed close relationship with *Pelargonium vein banding virus* (PVBV) and *Dracaena mottle virus* (DMV).

The presence of conserved region observed in the amino acid sequences of the RT/RNaseH region of the badnavirus isolates infecting black pepper and related species in this study confirms that all virus isolates in this study belong to badnavirus genome [5]. The 34 virus isolates from black pepper and other *Piper* species from India analysed in this study can clearly be distinguished into five virus species. Of these, 29 virus isolates collected from *P. nigrum* (black pepper), *P. longum* (long pepper) and *P. betle* (betelvine), *P. argyrophyllum, P. attenuatum*, *P. barberi*, *P. colubrinum*, *P. galeatum*, *P. ornatum*, *P. sarmentosum* and *P. trichostachyon* belong to PYMoV, a known badnavirus species as they showed >90 % identity in the amino acid sequence in the coding region of RT/RNaseH [4, 8, 9, 15]. The remaining five isolates collected from *P. bababudani*, *P. chaba*, *P. mullesua*, *P. peepuloides* and *P. thomsonii* did not show any significant sequence identity with any of the known badnaviruses described so far suggesting the likely occurrence of new badnaviruses in these species. Occurrence of PYMoV in black pepper, betelvine and Indian long pepper is known [4, 8, 9, 12, 15] while its occurrence in other *Piper* spp and its variability was not known so far.



◀**Fig. 1** Consensus tree generated using consense in the Phylip package with Neighbor joining, Bayesian, Maximum Likelihood and Maximum Parsimony methods based on the multiple alignment of the deduced amino acid sequences of RT/RNaseH region of badnavirus isolates from black pepper and related species with known badnaviruses. Details of the badnavirus isolates from *Piper* species and their GenBank accession number is provided in Table 1. Corresponding sequences from other badnavirus species used were retrieved from GenBank, their acronym and accession number is indicated in the figure. *BSGFV* Banana streak GF virus, *BSMysV* Banana streak Mysore virus, *BSOLV* Banana streak OL virus, *BSCVV* Bougainvillea spectabilis chlorotic vein-banding virus, *CSSV* Cacao swollen shoot virus, *CYMV* Citrus yellow mosaic virus, *ComYMV* Commelina yellow mottle virus, *CyLNV* Cycad leaf necrosis virus, *DBV* Dioscorea bacilliform virus, *DMV* Dracaena mottle virus, *FBV-1* Fig badnavirus 1, *GVBaV* Gooseberry vein banding virus, *KTSV* Kalanchoe top-spotting virus, *PVBV* Pelargonium vein banding virus, *SCBIMV* Sugarcane bacilliform IM virus, *SPBVa* Sweetpotato badnavirus A, *SPBVb* Sweetpotato badnavirus B, *TaBV* Taro bacilliform virus. *Rice tungro bacilliform virus* (RTBV) was taken as outgroup. Bootstrap values are shown at the nodes

The present study for the first time clearly showed the occurrence of PYMoV in eight species of *Piper*. Of the different species, *P. nigrum*, *P. betle* and *P. longum* are commercially cultivated on a large scale in India, Brazil and many south-east Asian countries while *P. colubrinum* is used as root stock for grafting black pepper as it is known for its resistance to *Phytophthora capsici*, an important fungus causing foot rot disease of black pepper [14]. A sequence variability of 0–15 % and 0–10 % in the nucleotide and amino acid respectively was observed among PYMoV isolates infecting black pepper and other *Piper* species. The sequence variability observed among the isolates may be attributed to the inaccurate replication by reverse transcription reported for members of the badnavirus. Similar kind of sequence variability was reported among isolates of *Citrus yellow mosaic virus* (CYMV) infecting different citrus species such as *Citrus aurantifolia, C. grandis, C. jambhiri, and C. sinensis* [1, 2] and different species of *Dioscorea* [5]. As per the criteria for classification of badnaviruses [3], the present study also suggested likely occurrence of four new badnaviruses in *P. bababudani*, *P. chaba*, *P. mullesua*, *P. peepuloides* and *P. thomsonii*. Occurrence of more than one badnaviruses infecting banana, cacao, dioscorea, sugarcane and taro were reported [5, 6, 11, 13, 16]. This forms the first report of likely occurrence of four new badnaviruses in different *Piper* spp. Further studies on complete genome sequencing

of these virus isolates are needed to confirm whether or not they represent new badnavirus species.

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