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Mungbean Yellow Mosaic India and Bean Common Mosaicviruses Induced Severe Epidemic of Yellowing and Mosaic Vein Banding Diseases, Respectively, on Yardlong Bean in Bali, Indonesia

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Abstract
Outbreak of yellowing and mosaic vein banding diseases emerged simultaneously during 2013 in the main yardlong bean (*Vigna unguiculata* sub sp. S*esquipedalis* L.) growing regions of Bali, Indonesia. Mosaic disease was characterized by mosaic pale green with vein banding on the leaves and could simply be distinguished from yellowing disease symptoms in which bright yellow mosaic was much more pronounced. Reverse transcription or/and polymerase chain reaction (PCR) using primers specific to genus *Begomovirus* and *Potyvirus* successfully amplified an expected DNA band if subjected to total nucleotide derived from yellowing and mosaic vein banding leaves respectively, but not vice versa. Pairwise comparison of sequences obtained from PCR products with corresponding nucleotide sequences in the Gen Bank confirmed the association of *Mungbean yellow mosaic India virus* (MYMIV) with yellowing and *Bean common mosaic virus* strain *Blackeye cowpea mosaic virus* (BCMV-BlCMV) with mosaic vein banding diseases. Nucleotide sequence analyses of MYMIV or BCMV-BlCMV isolates from the bean showed high sequence identities (more than 95%) and phylogeneticaly clustered with respective virus isolates from Java. This report provide first evidence of MYMIV and BlCMV infections in yardlong bean from Bali region of Indonesia.
Keywords: Begomovirus, disease outbreak, identification, nucleotide sequence, Potyvirus.

INTRODUCTION

Yardlong bean (*Vigna unguiculata* sub sp. s*esquipedalis* L.) is a native of Southeast Asia and one of the most widely grown protein-producing food legumes in the area. The crop is known as *kacang panjang* in Indonesia and extensively cultivated in Bali region. The viral diseases of leguminous bean are common.Some viruses may cause significant economic losses, where as,the others are of minor importance.

Among leguminous-infected viruses, two genus virus, *Begomo* - and *Potyviruses* are the most common. Up to now, there were four *Begomoviruses* have been reported infecting leguminous species cause yellowing disease *i.e*., *Mungbean yellow mosaic virus* (MYMV),*Mungbean yellow mosaic India virus*(MYMIV), *Dolichos yellow mosaic virus* (DoYMV) and *Horsegram yellow mosaic virus* (HgYMV).All these,referredas legume yellow mosaic viruses (LYMVs), are transmitted by whitefly (Ilyas *et al*. 2010).

Among Potyvirus, *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) cause one ofthe world's mostserious diseases of beans.The viruses are seed-borne and easily spread by aphid vectors.They occursinall part sof the world where susceptible bean varieties are grown (Pudashini *el al*. 2013).

During 2013, extensive surveys conducted in the main yardlong bean-growing regions of Bali found two different typical viral diseases, yellowing and mosaic vein banding, emerging simultaneously. Mosaic disease was characterized by mosaic pale green with vein banding on the leaves and could simply be distinguished from yellowing disease symptoms in which bright yellow mosaic was much more pronounced (Figure 1). These two viral diseases commonly emerge in the same field, but in the rare case, one disease may not accompanied by the other.

The presence of whiteflies (*Bemisiatabaci*) and aphids (*Aphis craccivora*) as potential virus vectors (Figure 2) were also observed in the fields affected the diseases, suggesting possible *Gemini virus* and *Potyvirus* infections. In this article,were portthe infections of MYMIV and BlCMV were responsible for outbreak of yellowing and mosaic vein banding diseases, respectively, on yardlong bean in Bali.

MATERIALS AND METODS

Survey and Sample Collection

Surveys were conducted in Bali during 2013. Leaf samples were collected using purposive sampling method based on typical symptoms as described by Damayanti *et al.* (2009) and Ilyas *et al.* (2010), i.e. leaf yellowing and mosaic vein banding. Fresh tissue was directly subjected for virus detection and the remainings were stored at -80^{o}C as isolate collection in the laboratory.

Total RNAs and DNAs Extraction

Total viral RNA was isolated from infected leaf following manufacturer protocol (Thermo Scientific – Lithuania). Fresh tissue (0.1 g) was ground in liquid Nitrogen to powder, 500 µl of Plant RNA Lysis Solution was added and the sap was transferred to 1.5 ml clean tube, and then was vortexed 10 – 20 s thoroughly. The sap was incubated in water bath at 56 ºC for 3 min, then centrifuged at 14 000 rpm for 5 min. The supernatant was pipetted to 1.5 ml clean tube, 96% ethanol was added and mixed by pipetting. The liquid was transferred to a purification column inserted in a collection tube and was centrifuged at 12 000 rpm for 1 min and then discarded flow-through. The purification column was added 700 µl of Wash Buffer WB 1 then was centrifuged at 12 000 rpm for 1 min and then discarded flow-through. The purification column was placed into a clean 2 ml collection tube and was added 500 µl Buffer 2 then was centrifuged at 12 000 rpm for 1 min. The solution was discarded flow-through and repeat the additional of 500 µl Buffer 2 using maximum speed of centrifugation. The collection tube was discarded flow-through and the purification column was transferred to aRNase-free 1.5 ml collection tube. The purification column was added 50 µl of nuclease free water to elute the RNA. The column was centrifuged at 12 000 rpm for 1 min and then discarded the purification column. The RNA which was kept on a collection tube was used as template in RT-PCR.

Total viral DNA was isolated from infected leaf following a procedure described by Doyle and Doyle (1987) with minor modification. Fresh tissue (0.1 g) was ground in liquid Nitrogen to powder, 500 µl of CTAB buffer (10% Cetyl-trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5 M NaCl, 1% β-mercapto-ethanol) was added, and the sap was transferred to 1.5 ml clean tube. The sap was incubated in water bath at 65º C for 1 hr, then shaked every 10 min to separate lipid and protein. 500 µl of chloroform/ iso-amyl alcohol (24:1, v/v) was added to the liquid, then tube was vortexed for 5 min, and centrifuged at 14 000 rpm for 15 min. The supernatant was pipetted to 1.5 ml clean tube, 3 M ammonium acetate and isopropanol of 1/10 and 2/3 volume supernatant, was added respectively. The liquid was mixed gently then incubated overnight at -20 ºC or 4 hr at room temperature. After incubation, the liquid was centrifuged at 12 000 rpm for 10 min to precipitate DNA and then discarded flow-through. The pellets were washed with 500 µl of 70% ethanol, centrifuged at 8000 rpm for 5 min and dried under room temperature after discarding the flow through. Dried pellets containing total DNA were dissolved in 50 to 100 µl of nuclease free water or TE buffer (pH 8) and the DNA was ready for amplification.

Reverse transcription-polymerase chain reaction (RT-PCR)

Amplification was conducted using one-step RT-PCR method. RT-PCR reaction contains 12.5 µl Go Taq Green PCR master mix (Fermentas, US), 10 µM each of primer, 2.0 µl DTT 50 mM, 0.1 µl RNAse Inhibitor, 0.1 µl MMuLV, 0.5 µl MgCl, 2.0 µl RNA total, and the reaction was adjusted to 25 µl with nuclease free water. Amplifications was performed in GeneAmp PCR System 9700 machine with 60 min at 42.0 ºC and 2 min at 94.0º C for RT, 5 min at 94.0 ºC for pre-heating, followed by 30 cycles of denaturation (1 min at 94.0º C), annealing (1 min at 50.0º C), and extension (3 min at 72.0º C). The last cycle was ended at 72.0º C for 3 min and cooled down to 4.0º C. Electrophoresis was done using 1% Agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, run at 50 V for 50 min. Following electrophoresis, agarose gel then was soaked on to 0.1% EtBr for 5 min, washed with H_2O, and visualized under UV transilluminator.

Polymerase chain reaction (PCR)

Amplification of viral DNA-A and DNA-B was conducted following method described by Rojas *et al.* (1993) to confirm *Begomovirus* infection (Table 2). PCR reaction contains 10 x PCR Buffer, 25 mMMgCl, 2.5 mMdNTPS, 10 µM each of primer, *Taq* polymerase (5 U/µl), 1 µl of DNA, and the reaction was adjusted to 25 µl with nuclease free water. Amplifications was performed in GeneAmp PCR System 9700 machine with 5 min at 94.0 ºC for pre-heating, followed by 30 cycles of denaturation (1 min at 94.0 ºC), annealing (1 min at 50.0 ºC), and extension (3 min at 72.0 ºC). The last cycle was ended at 72.0º C for 3 min and cooled down to 4.0 ºC. Electrophoresis was done using 1% Agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, run at 50 V for 50 min. Following electrophoresis, agarose gel then was soaked on to 0.1% EtBr for 5 min, washed with H_2O, and visualized under UV transilluminator.

Table 1 Degenerate primers for amplification of *Begomovirus* using polymerase chain reaction (Rojas *et al.* 1993)

Target	Primer	Sequence
DNA-A	PAL1v 1978	5'-GCATCTGCAGGCCCACATYGTCTTYCCNGT-3'
	PAR1C 715	5'-GATTTCTGCAGTTDATRTTYTCRTCCATCCA-3'
DNA-B	PBL1v2040	5'-GCCTCTGCAGCARTGRTCKATCTTCATACA-3'
	PCRc1	5'-CTAGCTGCAGCATATTTACRARWATGCCA-3'

DNA Sequencing

Viral DNA fragments obtained from PCR amplification were sent to PT. Genetika Science, Indonesia for DNA sequencing. Sequence data were compared with other sequences from GenBank (NCBI 2013) and analyzed using software programs BioEdit V.7.0.5, CLC Sequence Viewer 7, and MEGA 6.06.

RESULT AND DISCUSSION

Yellowing and Mosaic Vein Banding Diseases on Yardlong Bean in Bali

Yardlong bean was being cultivated in small scale around all districts of Bali Island. The crop was commonlyplanted in mix with other crop such as chilipepper, tomato, cucumber, eggplant, *etc*.Surveys conducted during 2013in the area found two different kind of viral diseases, *i.e*., yellowing and mosaic vein banding. Mosaic disease was characterized by mosaic pale green with vein banding on the leaves and could simply be distinguished from yellowing disease symptoms in which bright yellow mosaic was much more pronounced (Figure 1). The most common symptom found in every field was yellowing. Incidence of yellow disease was very high of about60% to 80% in most growing areas. In one field, yellowing disease may accompanied by mosaic vein banding disease in separate individual plant.Incidence of mosaic vein banding disease was much lower than the yellow disease, varying from 1% to 15%.

Figure 1 Yellowing and mosaic vein banding diseases outbreak simultaneously on yardlong bean fields in Bali (left) observed during 2013. The typical yellowing (upper right) and mosaic (below right) symptoms on yardlong bean leaves.

During survey, it also found that many individual yardlong bean plants colonized by insects, mainly by whitefly (*Bemisiatabaci*) or by aphids (*Aphis craccivora*) as displayed in Figure 2. These two insects may have a role in spreading the diseases in the fields.

Figure 2 Whitefly (*Bemisiatabaci*, left) and aphids (*Aphis craccivora*, right) colonized the yardlong bean fields affected by yellowing and/or mosaic vein banding diseases in Bali, suggested as thepotential virusvectors.

The results of PCR using *Begomovirus* universal primer and RT-PCR using *Potyvirus* universal primer applied to sampled plants, as displayed in Table 2, suggested that yellowing disease on yardlong bean in Bali was associated with infection of *Begomovirus* and mosaic vein banding disease was associated with *Potyvirus* infection, but not *vice versa*.

Table 2 Detection of *Potyvirus* and *Begomovirus* from leaf samples collected from various locations using reverse transcription-polymerase chain reaction (RT-PCR) and PCR, respectively[a]

District of Bali	Symptoms on plant sample	RT-PCR	PCR
		Potyvirus	*Begomovirus*
Badung	Yellowing	-	+
	Mosaic vein banding	+	-
Bangli	Yellowing	-	+
	Mosaic vein banding	+	-
Buleleng	Yellowing	-	+
	Mosaic vein banding	+	-
Giayar	Yellowing	-	+
	Mosaic vein banding	+	-
Karangasem	Yellowing	-	+
	Mosaic vein banding	+	-
Klungkung	Yellowing	-	+
	Mosaic vein banding	+	-
Tabanan	Yellowing	-	+
	Mosaic vein banding	+	-

[a] -, not detected; +, detected

***Begomovirus* Associated with Yellow Disease on Yardlong Bean**

PCR using *Begomovirus* universal primer successfully amplified a DNA band of 912 bpif subjected to total DNA extracted from yardlong bean showing yellowing, but not from the bean showing mosaic vein banding symptoms (Figure 3). This evidencegive a clue that the virus species is a member of *Begomovirus*. All the leguminous-infecting *Begomoviruses* are bipartite and they have geminate(twin) particles,18–20nmindiameter,30nm long, apparently consisting of two in complete icsahedra joined together in astructure with 22 penta mericcap someres and110i dentical protein sub units(Qazi*etal*.,2007).

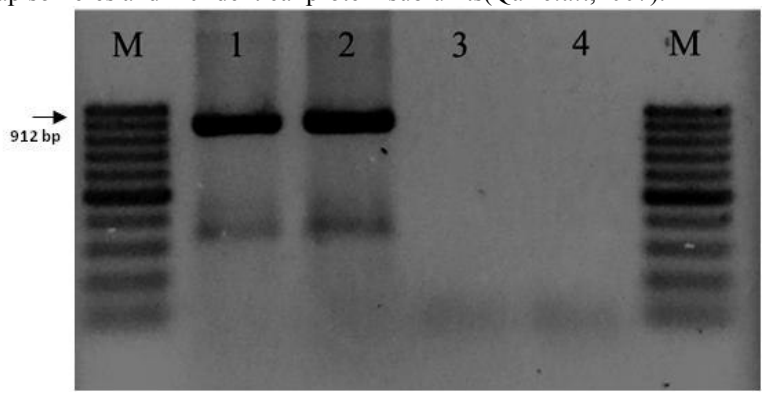

Figure 3 Detection of *Geminivirus* by polymerase chain reaction using primer SPG1/ SPG2 (Li *et al.* 2004) to total DNAs extracted from yardlong bean leaves showing yellowing (line 2) and mosaic vein banding (line 3) symptoms following Doyle and Doyle (1987) method. The primer target was DNA A of the virus and the amplified DNA size was about 912 bp. Line M: marker 100 bp (Thermo Scientific, US); Line 1: total DNA extracted from yardlong bean showing yellowing symptom obtained from West Java; Line 4: total DNA extracted from yardlong bean with no symptom (healthy).

Bipartite genome of the virus isolate in this research was successfully verified by PCR using a primer pair PBL1v2040/PCR1c to amplified DNA-B fragment of ±400 bp (Figure 4). The size of DNA-B fragment was different with those reported previously by Rojas *et al.* (1993), however it was still acceptable as explained by Rojas *et al*. (1993). The same result was also found by Nurulita (2014) for MYMIV Java isolate.

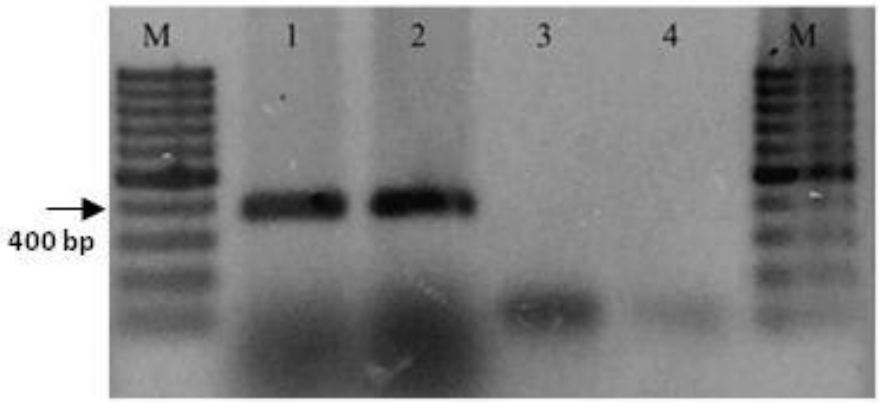

Figure 4 Polymerase chain reaction using primer PBL1v2040/ PCR1c (Rojas *et al.* 1993) targeted to *Geminivirus* DNA B extracted from yardlong bean leaves showing yellowing (Line 2) and mosaic vein banding (Line 3) symptoms. The expected amplified DNA size was about 400 bp. Line M: marker 100 bp (Thermo Scientific, US); Line 1: total DNA extracted from yardlong bean showing yellowing symptom obtained from West Java; Line 4: total DNA extracted from yardlong bean with no symptom (healthy).

Nucleotide sequences were obtained directly from PCR product. Analysis of their identity by comparing to sequences on the Gen Bank showed their high homology with MYMIV from Brebes and Bogor (Java) and other countries (Table 3). Their homology to MYMV, another virus causing yellow mosaic disease in South Asia, is only about 83% and to other *Begomovirus* reported from Indonesia is even lower, i.e. < 70%. This result indicated that the species of *Begomovirus* inducing yellow disease on yardlong bean in Bali was MYMIV and designated as MYMIV_Bali.

Table 3 Homology (identities) of nucleotide sequences of *Begomovirus* isolated from yardlongbean showing yellowing symptom in Bali (MYMIV_Bali) to viruses reported in GenBank*.

No	Virus Isolate	Percent Identity
01	MYMIV_Bangladesh	96.3
02	MYMIV_Pakistan	94.3
03	MYMIV_Nepal	95.4
04	MYMIV_India	97.5
05	MYMIV_Bogor	99.0
06	MYMIV_Brebes	99.2
07	MYMV_India	82.9
08	PepYLCIV	67.1
09	ToLCV_Korea	69.6
10	TLCJV	68.2
11	BCMV_Bogor	23.9

*Data of nucleotide sequences available in Gen Bank used in the matrix were: *Mungbean yellow mosaic India virus* from Bangladesh (MYMIV _Bangladesh) with accession no. AF314145, MYMIV _Pakistan AM992618, MYMIV _Nepal AY271892, MYMIV _India AY937197, MYMIV _Bogor JN368432, MYMIV _Brebes JN368436, MYMIV _India KC911721, *Pepper yellows leaf curl virus* (PepYLCV) AB246171, *Tomato leaf curl virus* from Korea (ToLCV _Korea) FJ434943, *Tomato leaf curl Japan virus* (TLCJV) AB100304, and *Bean common mosaic virus* from Bogor (BCMV _Bogor) FJ653916.

Further phylogenetic analyses to study their relationship showed that all *Begomoviruses* infecting mungbean (MYMIV and MYMV) belong to similar cluster and they are separated from *Begomoviruse s*infecting other crops (PepYLCIV, TLCIV, ToLCJV) (Figure 5). The figure was also showed that MYMIV from Bali has very close genetic relationship to MYMIV from Java.This suggest that MYMIV outbreak in Bali might be come from Java.

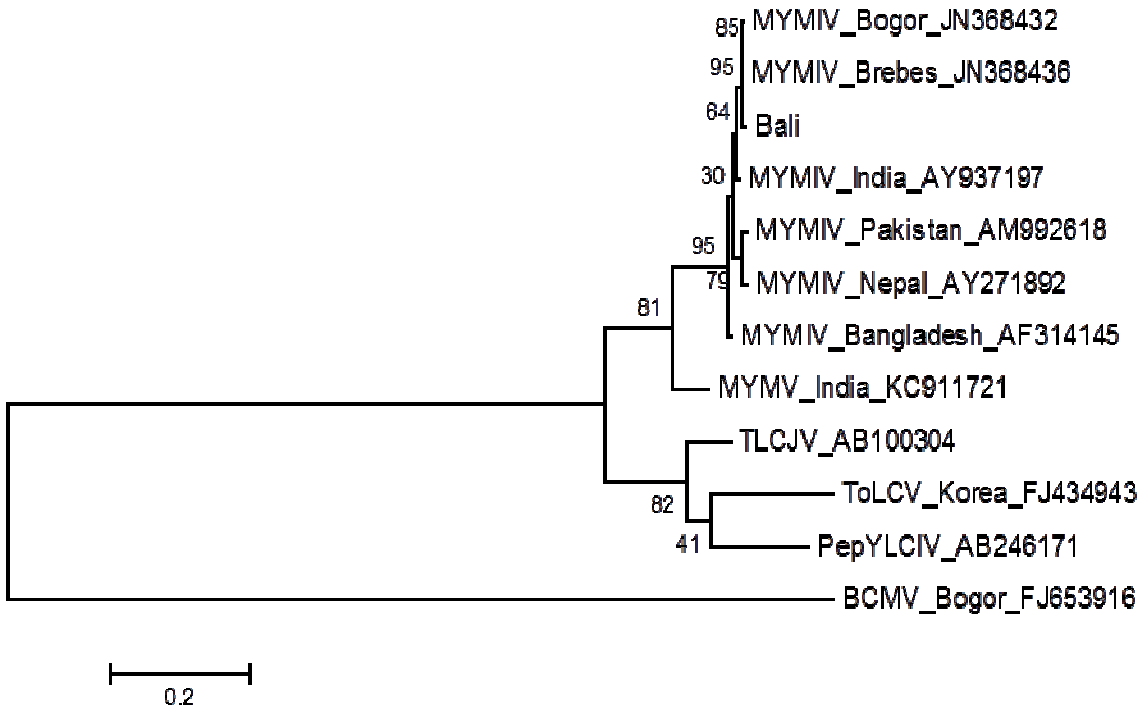

Figure 5 Phylogenetic analysis based on alignment of partial nucleotide sequences of the DNA-A of *Begomoviruses* using Mega 6.06 (Algorithm Neighbour Joining with 1000 bootstraps replicates)

***Potyvirus* Associated with Mosaic Vein Banding Disease on Yardlong Bean**

On the other hand, RT-PCR using *Potyvirus* universal primer successfully amplified an expected DNA band of 650 bp if subjected to total RNA from the bean leaves showing mosaic vein banding symptom, but not from leaves with yellowing symptom (Figure 6). In Java, mosaic vein banding symptom caused by *Potyvirus* infection has been described previously by Damayanti *et al.* (2009) and Melinda (2013).

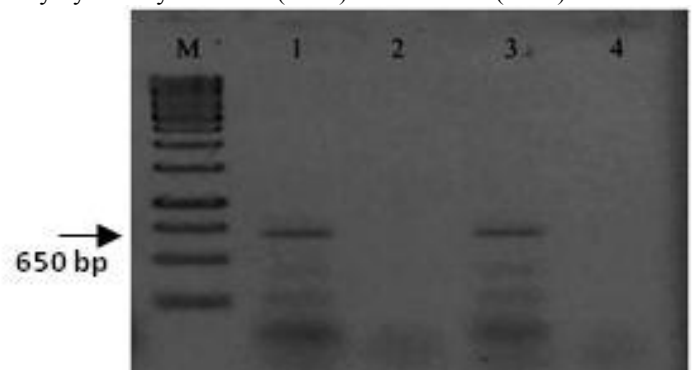

Figure 6 Detection of *Potyvirus* by reverse transcription-polymerase chain reaction using primer CI-For/ CI-Rev to total RNAs extracted from yardlong bean leaves showing yellowing (Line 2) and mosaic vein banding (Line 3) symptoms. The expected amplified DNA size was about 650 bp. Line M: marker 1 kb (Thermo Scientific, US); Line 1: total RNAs extracted from yardlong bean showing mosaic vein banding symptom obtained from West Java; Line 4: total RNAs extracted from yardlong bean with no symptom (healthy).

Nucleotide sequences were obtained directly from RT-PCR product. Analysis of their identity by comparing to sequences on the Gen Bank showed their high homology with BCMV from Indonesia and BlCMV from Taiwan (Table 4).According to Damayanti*et.al*. (2009), BCMV from Indonesia is a strain of BlCMV isolated from Java. Their homology to BCMV, another *Potyvirus* causing mosaic symptom from around the world, is only 81 to 90% and to*Soybean mosaic virus* reported from India is even lower, *i.e.* < 70%. This result indicated that the species of *Potyvirus* inducing mosaic vein banding symptom on yardlong bean in Bali was *Bean common mosaic virus* strain *Blackeye cowpea mosaic virus* (BCMV-BlCMV).

Table 4 Matrix identities of nucleotide sequences of *Potyvirus* isolated from yardlongbean showing mosaic vein banding symptom in Bali (Bali) and viruses reported in GenBank*.

No.	Virus Isolate	Percent Identity
1	BCMV-BlCMV_Indonesia	97.3
2	BICMV_Taiwan	95.7
3	BCMV_China_Soybean	90.9
4	BCMV_China	88.9
5	BCMV_Colombia	84.5
6	BCMV_England	81.4
7	SMV_India	73.7
8	PStV_US	8.09

*Data of nucleotide sequences available in Gen Bank used in the matrix were: *Blackeye cowpea mosaic virus*(BlCMV) infecting yardlong bean from West Java (BCMV _Indonesia) with accession no. FJ653926, *Bean common mosaic virus* (BCMV) from China infecting soybean (BCMV _China _Soybean) KC832501,BCMV_China AJ312438, BCMV _Colombia DQ666332, BCMV _England AY112735, *Soybean mosaic virus* from India (SMV _India) KJ001224, *Blackeye cowpea mosaic virus* from Taiwan (BICMV _Taiwan) AY575773, *Peanut stripe virus* from US (PStV _US) U34972.

Further phylogenetic analyses to study their relationship showed that the virus isolate (BlCMV) isolated from mosaic vein banding-showing yardlong bean in Bali has very close relationship with BlCMV from Java and also from Taiwan, and still close to BCMV from other countries (Figure 7). This figure suggested that BlCMV present in Bali might be come from Java.

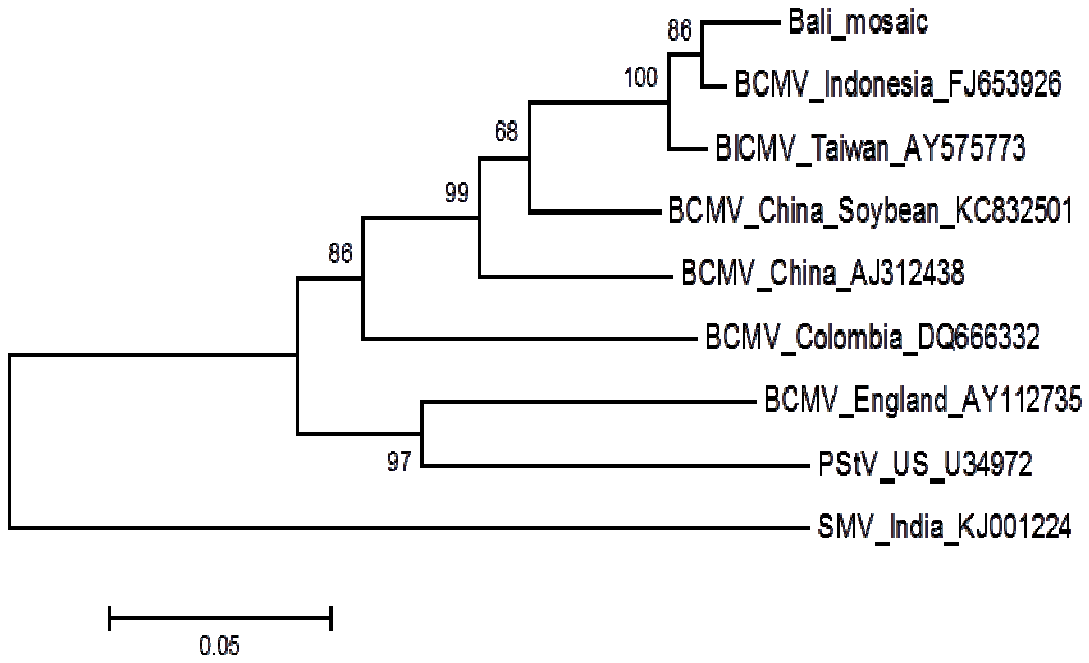

Figure 7 Phylogenetic analysis based on alignment of partial nucleotide sequences of the DNA-A of *Potyviruses* using Mega 6.06 (Algorithm Neighbour Joining with 1000 bootstraps replicates)

CONCLUSSION

Two different diseases, yellowing and mosaic vein banding, on yardlong bean were outbreak simultaneously during 2013 growing season in Bali. Based on nucleotide sequence analyses, yellowing disease was found to be induced by MYMIV and mosaic vein banding caused by BCMV-BlCMV infections . Phylogenetic analyses suggested that the two viruses spread from Java to Bali Island.

LITERATURE CITED

Damayanti TA, Alabi OJ, Rauf A, Naidu RA. 2009. Severe outbreak of yellow mosaic disease on the yard long bean in Bogor; West Java. *HAYATI J Biosci.* 16(2):78-82.

Ilyas M, Qazi J, Mansoor S, Briddon RW. 2010. Genetic diversity and phylogeography of begomoviruses infecting legumes in Pakistan. *J Gen Virol.*[Internet].[2012 Apr 24]; 91:2091-2101.

DOI:10.1099/vir.0020404-0.

Naimuddin, Akram M, Pratap A. 2011. First report of natural infection of *Mungbean yellow mosaic India virus* in two wild species of *Vigna.New Dis Rep.* [Internet].[2012 Apr 17]; 23:21. DOI: 10.5197/j.2044-0588.2011.023.021.

Nurulita S. 2014. Molecular Characterization of *Begomovirus* Infecting Yard Long Bean (*Vignaunguiculata* subsp. *sesquipedalis* L.) and Construction of Its Specific Primers [thesis]. Bogor (ID): Graduate School Bogor Agricultural University.

Pudashini BJ,Shahid M S, Natsuaki K T. 2013. First Report of *Bean common mosaic necrosis virus* (BCMNV) Infecting Sweet Bean in Nepal. Plant Diseases 97 (2): 290

Qazi J, Ilyas M, Mansoor S, Briddon RW. 2007. Legume yellow mosaic viruses: genetically isolated begomoviruses. *Mol Plant Pathol.* [Internet]. [2012 Apr 17]; 8(4):343-348. DOI: 10.1111/J.1364-3703.2007.00402.X.

Rojas MR, Gilbertson RL, Russel DR, Maxwell DP. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted Gemini viruses. *Plant Dis.* [Internet]. [2012 May 14]; 77(4):340-347. Available on: http://www.apsnet.org/publications/plantdisease /backissues/Documents/1993Articles/PlantDisease77n04_340.pdf