Identification and Detection *Odontoglossum ringspot virus* on Native Orchids Collection of Nurserys in Java, Indonesia

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ABSTRACT

Nature orchid are one of original floral in Indonesia. Virus infection is one of the limiting factor in the cultivation of orchid. Infection Odontoglossum ringspot virus (ORSV) was reported infets native orchids collection in Indonesia. The purpose of this study was to identification and ORSV that infects native orchid nurserys collection in Java, Indonesia. Symptomatic samples were collected from 5 nurserys collections, i.e. Rumah Bunga Rizal (Bandung), Bali Tanaman Hias (Cianjur), Borobudur Orchids Center (Magelang), Kebun Anggrek Bungarinte (Yogyakarta), and Titi Orchids (Yogyakarta). Detection and identification was conducted by serological test using ORSV specific antisera, RT-PCR and DNA sequencing. The serological test using ORSV antisera showed that 3 of 11 sampels reacted positively against ORSV antiserum i.e *Phalaenopsis amabilis* (Cianjur.1, Cianjur.2, and Magelang). RT-PCR of the 3 samples using specific primer of ORSV coat protein (CP) gene amplified a DNA with size ± 474 bp. Homology analysis of those 3 Indonesian isolates showed highest index similiarity (IS) was 99.8% with corresponding sequences from 10 other ORSV isolates. Phylogenetic analysis showed that ORSV Cianjur.1 and Cianjur.2 isolates clustered in separated group far from ORSV isolates in other countries.

Keywords: ORSV, *coat protein*, native orchid, Indonesia

INTRODUCTION

Native orchids have an essential function as a parent crossing in plant breeding (Mahfut and Daryono, 2014). Disease infection becomes an obstacle in cultivating and developing the potential of this plant (Kumalawati et al., 2011). The type of virus that is reported to infect orchids and has a wide spread in the world, including in Indonesia is *Odontoglossum ringspot virus* (ORSV) (Kumalawati et al., 2011). This viral infection causes a decrease in plant vigor, flower quality (Koh et al. 2014), and the ability of plant photosynthesis (Mahfut et al., 2017[b]; Mahfut et al., 2019).

Areas of spread of ORSV infections include natural forests (Kumalawati et al., 2011; Mahfut and Daryono, 2014; Mahfut et al., 2016[a]; Mahfut et al., 2016[b]), botanical gardens (Mahfut et al., 2016[a]; Mahfut et al., 2016[b]; Mahfut et al., 2017[a]), dan nurserys (Mahfut et al., 2016[a]; Mahfut et al., 2016[b]). Based on survey of five orchid nurseries, i.e Rumah Bunga Rizal (Bandung), Balai Tanaman Hias (Cianjur), Borobudur Orchids Center (Magelang), Kebun Anggrek Bungarinte (Yogyakarta), and Titi Orchids (Yogyakarta), some native orchids was found with symptoms of virus infection that are thought to be caused by ORSV. Therefore, the ORSV identification and detection research needs to be done to update the health status of the natural orchid nursey collection in Indonesia. The application of the results of this study is one of the potential efforts to support the concept of conservation of natural orchids in Indonesia through efforts to protect plants.

MATERIALS AND METHODS

Detection of Protein Using Serology

Serological detection was carried out using the DAS-ELISA method on 11 of the most representative orchid leaf sample samples based on infection symptoms from each location. ELISA uses ORSV specific antiserum (Agdia Inc.). Samples were read using an ELISA-reader (BioTek) at a wavelength of 405 nm. If the absorbance value approaches the positive control value or 2-3 times the control buffer then the sample is said to be positive (Daryono and Natsuaki, 2009).

Detection of Nucleic Acid Using RT-PCR

RNA isolation was performed on positive samples infected with ORSV by ELISA using a Total RNA isolation kit (SBS Genetech Co., Ltd., China). Amplification of RNA by RT-PCR was done by separate methods using specific primers, namely ORSV CP-F1 (5'-ATGTCTTACACTATTACAGACCCG-3') and ORSV CP-R1 (5'-GGAAGAGGTCCAAGTAAGTCC-3') (Mahfut and Daryono, 2014).

The RT reaction was carried out at 37ºC for 60 minutes, followed by incubation at 96ºC for 5 minutes and ended at 4ºC. CDNA amplification begins with the stage of predenaturation at 95ºC for 5 minutes, followed by 34 cycles, including denaturation at 95ºC for 30 seconds, annealing at 50ºC for 45 seconds, and extension at 70ºC for 1 minute (Mahfut et al., 2016[a]). PCR products were analyzed using electrophoresis on 2% agarose gel. The DNA bands were then visualized on a UV transluminator (Bio-Rad Transilluminator 2000) and documented using a digital camera.

DNA Sequencing and Phylogenetic Analysis

The amplified DNA is traced by its nucleotide sequence by sending DNA to the *1st Base*, Malaysia. Nucleotide sequences are analyzed and combined with *Suite for Sequence Analysis DNA STAR Lasergene DM Version* 3.0.25 software. Alignment analysis of nucleotide sequence ORSV isolates from Indonesia was performed on sequences registered at Genbank using *Basic Local Alignment Search Tool* (BLAST) (www.ncbi.nlm.nih.gov). Selection based on the distribution of selected regions obtained 4 registered ORSV isolates from Indonesia and 10 ORSV isolates from other countries (Singapore, China, India, Germany, South Korea, Argentina, and Brazil). TMV-Yunnan

isolates are used as a comparison outside the group (outgroup). Phylogenetic analysis was performed using *Molecular Evolutionary Genetics Analysis* (MEGA) software with *Neighbor Joining* (NJ) method dan Kimura-2 parameter model for distance estimation. The bootstrap value used is 1000 repetitions.

RESULTS AND DISCUSSION

Virus Detection

Based on the results of serology detection, the incidence of virus infection was 27.3%. A total of 3 out of 11 total samples reacted positively to the ORSV antiserum with an average absorbance value of 1,125-1,152. The 3 positive samples are 2 samples from the Balai Tanaman Hias (Cianjur.1, Cianjur.2) and 1 sample from Borobudur Orchids Center (Magelang). The overall positive orchid sample is *Phalaenopsis amabilis* L. (Blume). RT-PCR results on 3 positive ORSV samples showed DNA fragments measuring ±474 bp amplified using the CP-specific ORSV gene primer (Figure 1).

Analysis of Nucleotide Sequences

The results of nucleotide sequencing obtained total nucleotide genes CP isolates ORSV-Cianjur.1, Cianjur.2, and Magelang measuring 475-480 nucleotides. BLAST analysis showed that 3 ORSV isolates had a homology of 99% with ORSV isolates from other countries in Asia, Africa, America and Europe. The results of the analysis of 10 other ORSV isolates showed up to 99.8% homology with ORSV isolates from Indonesian nurseries.

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107 **Phylogenetic Tree of CP Gene ORSV**

108 The results alignment of the nucleotide sequence show a point mutation in the
109 form of substitution and insertion in ORSV isolates in Indonesia. Magelang isolates
110 experienced the most mutations, namely insertion 4 times, while substitution in 3 isolates
111 occurred 17 times each. This causes these isolates to separate from other Indonesian
112 isolates. The effect of mutations that occur is able to cause changes in the triplet codon
113 coding for amine acids. Magelang isolates show differences in the frequency of the amino
114 acids Cys and Leu which have increased respectively 0.5% and 1.2% and a decrease in
115 Ser and Val of 1.4 % and 0.3%. In contrast to isolates Cianjur.1 and Cianjur.2 which
116 experienced a decrease in the amino acids Cys and Leu by 0.2% and 0.1%, as well as an
117 increase in Ser 0.8% and Asn 0.6% (Table 1). The nucleotide sequence of ORSV CP gene
118 isolates in this study not found deletion.

119 Phylogenetic analysis showed that 3 ORSV isolates from Indonesian nurseries had
120 a very close kinship. The results of the phylogenetic tree analysis divided ORSV isolates
121 into two main groups, namely the Bogor and German isolate groups separated from the
122 second group of 10 other isolates. This second group is divided into 2 subgroups, namely
123 the first group consisting of Magelang isolates, 3 Indonesian isolates that have been
124 registered at Genbank, and 5 isolates from other countries, while the second subgroup
125 consists of 2 nursery isolates (Cianjur.1 and Cianjur.2). When compared between
126 branches, isolates Cianjur.1 and Cianjur.2 were separate from ORSV isolates from other
127 countries (Figure 2). Although all isolates formed several groups, kinship between
128 isolates was still very close. This can be seen in the phylogenetic tree which only forms
129 sub groups.

The ORSV findings that infect orchids in Indonesian nurseries show a lack of effort to maintain collection plants by the botanical garden. Some of the nucleotide mutations that occur cause two ORSV isolates from nursery in Indonesia, namely Cianjur.1 and Cianjur.2 apart from other isolates. The process of nucleotide mutations in each isolate causes changes in amino acids that are formed in the composition of the viral genome. Changes in amino acids will change the function of genes arranged so that the infectivity also changes (Mahfut et al., 2016).

The CP gene is conserved so it has the capability of the proffreading mechanism like most other nuclear genes. But with a relatively small size of the viral genome, a slight error will significantly influence the mutation rate. The rate of mutation will produce a genetic variation of the virus thereby increasing the probability of faster evolution (Matthews, 1992). Long branches in isolates Cianjur.1 and Cianjur.2 also indicate that the virus has evolved and can even lead to speciation.

Indonesian ORSV allegedly originated from Germany. BPPP (2005) noted that Germany was ranked 14th as an importer of seeds and orchid plants to Indonesia from 1997-2001, in addition to the United States, Brazil, India, Singapore, South Korea, China, Japan, Taiwan, and several West Asian countries. Based on this, another effective way to protect and maintain the health status of natural orchids in Indonesia is to limit and control the importation of orchids from other countries.

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199 **Table 1.** The frequency of amino acid CP ORSV gene from nursery in Indonesia

Origin of Isolates	Frequency Of Amino Acid (%)																			
	Ala	**Cys**	**Asp**	**Glu**	**Phe**	**Gly**	**His**	**Ile**	**Lys**	**Leu**	**Met**	**Asn**	**Pro**	**Gln**	**Arg**	**Ser**	**Thr**	**Val**	**Trp**	**Tyr**	**Total**
Indonesia-Cianjur.1	1,42	0,00	3,55	4,26	4,96	3,55	2,13	8,51	3,55	19,86	4,26	3,55	2,84	5,67	5,67	9,22	4,26	7,09	2,84	2,84	141,00
Indonesia- Cianjur.2	1,42	0,00	3,55	4,26	4,96	3,55	2,13	8,51	3,55	20,57	4,26	2,84	2,84	5,67	4,96	9,22	4,96	7,09	2,84	2,84	141,00
Indonesia-Magelang	2,05	0,68	3,42	3,42	5,48	3,42	2,74	7,53	3,42	21,23	4,11	3,42	2,74	5,48	5,48	8,22	4,79	6,85	2,74	2,74	146,00

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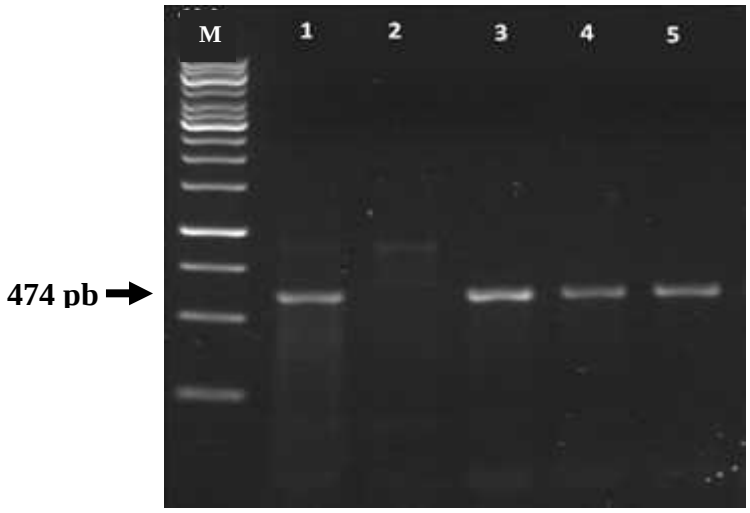

Figure 1. Results of RT-PCR visualization of several ORSV isolates in agarose gel 2%. (M) Marker 1 kb (Rainbow invitrogen), (1) positive control, (2) negative control of healthy plants, (3-4) ORSV from Balai Tanaman Hias (Cianjur.1 and Cianjur.2), dan (5) ORSV from Borobudur Orchid Center (Magelang)

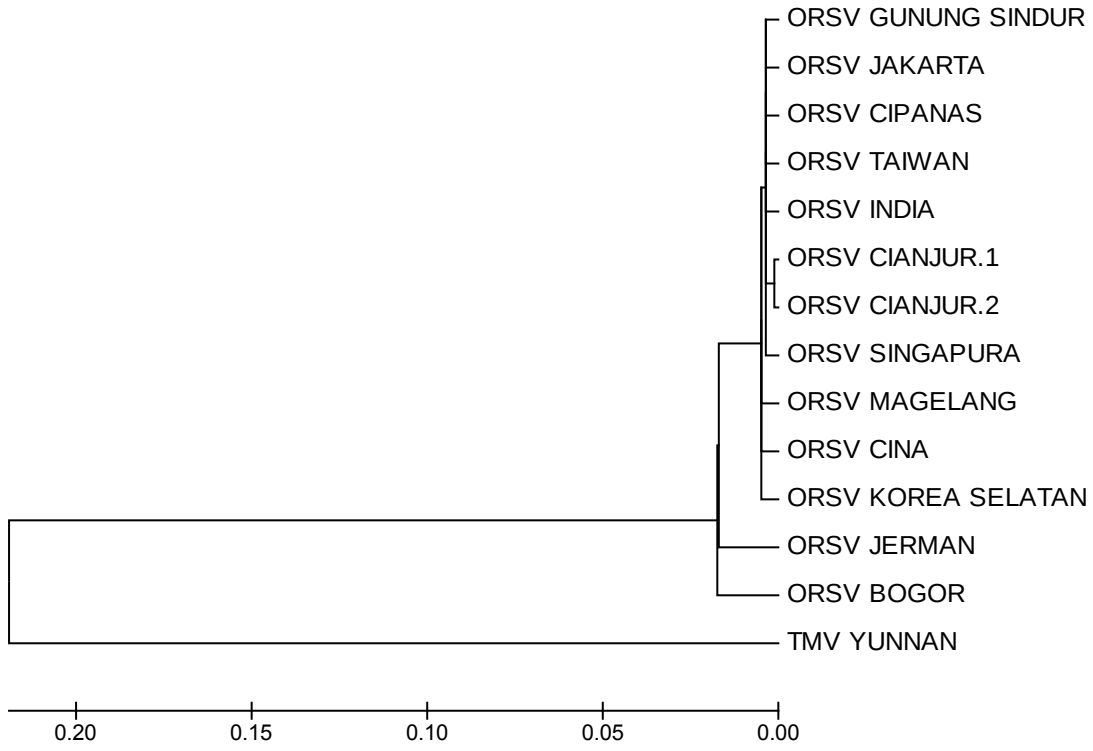

Figure 2. The phylogenetic tree of ORSV isolates based on the nucleotide sequence of CP 3 gene isolates from Indonesian nurseries compared to isolates from other countries. TMV-Yunnan is used as a comparison outside the group