Isolation and phylogenetic relationship of orchid-mycorrhiza from *Spathoglottis plicata* of Papua using mitochondrial ribosomal large subunit (mt-Ls) DNA

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ABSTRACT

Suapa S, Agustini V, Suharno. 2012. Isolation and phylogenetic relationship of orchid-mycorrhiza from Spathoglottis plicata of Papua using mitochondrial ribosomal large subunit (mt-Ls) DNA. Biodiversitas 13: 59-64. All terrestrial mycorrhiza have mutual symbiotic with mycorrhizal fungi in order to gain nutrient from surrounding environment. This study was done to isolate and to identify mycorrhiza orchid that associates with *Spathoglottis plicata* and were collected from Cagar Alam Pegunungan Cycloops (CAPC), Jayapura. Isolation of mycorrhizal orchid came after the modified method of Manoch and Lohsomboon (1993). The result showed that based on the morphological characteristic, there were presumably 14 isolations. However, only 2 isolations have been known, namely *Rhizoctonia* sp. and *Tulasnella* sp., while the rest were not identified yet. Among them, the DNA of the 11 isolations were able to be extracted for further analysis. The constructed phylogenetic tree performed that those species could be grouped into 4 major clusters. Two species, *Rhizoctonia* sp. and *Tulasnella* sp. were in different clusters.

Key words: *Spathoglottis plicata*, orchid-mycorrhiza, *Rhizoctonia*, *Tulasnella*, Papua

INTRODUCTION

*Spathoglottis plicata* Blume (ground orchid) is one of the orchid species that is widely distributed in the world, including in the area of New Guinea (Papua New Guinea, PNG and Papua, Indonesia). According to Agustini et al. (2008, 2009) Cycloop Mountains Nature Reserve (CAPC) is one of the conservation area in Papua. This species of orchid is also found here. One of buffer zone areas of this nature reserve is a University of Cenderawasih (Uncen) campus forest in Waena, Jayapura. It has plenty of *S. plicata* orchids. This orchid is easy to grow and has purple-white flowers and bloom throughout the year.

In nature, for the germination and early growth of orchid seed and its development, it needs an association of orchid roots and mycorrhizal fungi (orchid-mycorrhiza) (Warcup 1981; Cameron et al. 2006; Suarez et al. 2006; Agustini et al. 2009). This is because orchid seeds are very small, smooth and soft and have no cotyledon which is a food reserve in the early growth of seeds (Sarwono 2002; Agustini and Kirenius 2002). The association to mycorrhizal fungi helps the orchid in providing the nutrients needed for the process of germination of its seeds (Agustini and Kirenius 2002; Agustini 2003). The hyphae of mycorrhizal fungi penetrate to the cell walls of roots and then will be developed in the root cortex as dense coiled called peloton. Mycorrhizal hyphae are very delicate; therefore they will absorb water and certain minerals (Suarez et al. 2006; Agustini et al. 2009).

The role of mycorrhizal fungi is very interesting in the life cycle of orchids. In the early growth, all orchid species are heterotrophic, so it requires association with mycorrhizal fungi to obtain the needed nutrients (Brandrett et al. 2003; Taylor et al. 2004; Wu et al. 2010). Orchids that have low levels of heterotrophic are dependants on the presence of mycorrhizae. Based on morphological characteristics, most of the fungi associated with orchids are members of the form of anamorphic (asexual)-genus *Rhizoctonia* (Athipunyakom et al. 2004) and generally come from families of Basidiomycetes. Several studies of terrestrial orchid-mycorrhiza in temperate-climate areas have been done a lot, but the studies on the tropics are very few (Otero et al. 2002). In Papua, researches and publications on ground orchid-mycorrhiza, especially *S. plicata* are very small in number. Therefore, there is a need for the study on the diversity of orchid-mycorrhiza associated with ground orchids *S. plicata*. Furthermore, according to Kristiansen et al. (2001) to find out the phylogenetic of the obtained isolates, the DNA sequencing analysis is required. Therefore, the purpose of this study was to isolate and to find out the phylogenetic relationship of the orchid-mycorrhiza of *S. plicata* species using mitochondrial sequences of ribosomal large subunit (mt-Ls) DNA.
**MATERIALS AND METHODS**

**Time and area study**

The research was carried out for 10 months in 2008. The isolation of orchid-mycorrhiza was done in the laboratory of Plant Tissue Culture and Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Cenderawasih (Uncen), Jayapura, Papua, Indonesia. The maintenance and regeneration of the isolates were carried out at the same place.

**Isolation of orchid-mycorrhiza**

According to Manoch and Lohsomboon (1992), isolation of orchid-mycorrhiza was done by the modified method of Masuhara and Katsuya (1989). The roots of S. plicata was cut for about 1 cm, and sterilized with 10% of Clorox for 30 seconds, and then washed with aquadest and rinsed with distilled water, then sterilized again with 70% alcohol. The sterilized pieces of roots were sliced transversely using a sterile knife (razor blade) in the LAF (Laminar Air Flow). The thickness of the slice was ± 200-300 µm or 3-4 pieces in 1 mm slice. The slices then were planted in the media of potato dextrose agar (PDA) in petri dish. Each dish were filled with 1-3 pieces of roots and then incubated at 28°C. Mycorrhizal mycelia start to appear within 2-3 days.

After mycorrhizae grew, isolation was conducted if, through a morphology observation, there was a difference in shapes and growth patterns, to separate each of these groups, because there possibly was more than one species of media. In the same medium, the Isolates were carried out again if in the growing up process, the different shape and growth patterns can still be found, until finally a really pure and free from contamination-free isolate was obtained (each mycorrhizal was cultured separately). In 2-3 times of isolation, the isolates obtained were really pure.

**Identification of orchid-mycorrhiza**

The identification of the species of orchid-mycorrhiza was done by looking at the morphological and microscopic characteristics. Morphological features, including the diameter of colony growth, mycelia morphology and other characteristics. Microscopic characteristics include presence or absence of septum and spores shape or conidia. Some of the literatures used in the identification of orchid-mycorrhiza were Athipunyakom et al. (2004) and Karl-Franzens (2006).

**Isolates of orchid-mycorrhiza and phylogenetic relationship**

*The isolates culture and DNA extraction*

The mycelia of each isolate was dissolved in 2 µL of aquadest and transferred into 0.5 mL Eppendorf tubes containing 6 µL of dd H2O and 2 µL of 10X PCR (polymerase chain reaction) buffer (0.35 M Sorbitol, 0.1 M Tris, 5 mM EDTA pH 7.5). The solution was then heated at 95 °C for 10 minutes in a water bath. The DNA of these isolates was extracted using the method of hexadecyltrimethylammonium bromide (CTAB) (Gardes and Bruns 1993). The DNA quality was checked by electrophoresis.

**DNA amplification by PCR**

Of the extracted Isolates, 8 µL was used as a template for PCR amplification. ML5/ML6 regions of the genes of mitochondrial large submit (mt-Lr) RNA was amplified with a PCT100 thermocycler (MJ Research Inc., MA). PCR solution which was used for each isolates contains 2 µL of 10X PCR Buffer, 2 µL (mM) for each primer, 6 µL ddH2O, 8 µL dNTPs (2 mM) and 0.1 µL Taq polymerase. PCR was conditioned according to the method of Gardes and Bruns (1993): initial denaturation at temperature of 95°C for 1 minute 35 second, followed by 13 cycles of denaturation for 35 seconds, temperature 94 °C, primer annealing for 55 seconds at a temperature of 55 °C, and polymerization for 45 seconds at 72°C. The addition of nine cycles of the polymerization was done by extending to 2 minutes. The 9 cycles consisting of 3 min of extension, and ending with 10 min of polymerization at a temperature of 72°C. Primer used was ML5 (CTCGGCAAATATCCCTCATAAG), MLIN3 (CGACACAGGTCTGATTAGT) and MI6 (CAGTAGAAAGCTCATAGGTC).

**DNA sequencing**

The results of PCR amplification was purified with QIA Quick PCR extraction kit (Qiagen GmbH GE). DNA was dissolved in 30 µL of sterile H2O and was being sequenced with a primer ML5/ML6 using ABI 377 automatic sequencer (Perkin Elmer) (Kristiansen et al. 2001). The sequencing uses the dideoxy chain termination procedure (Sanger et al. 1977).

**RESULTS AND DISCUSSION**

**Isolation of orchid-mycorrhiza**

The results of orchid-mycorrhiza isolation associated with S. plicata on eight sites are 13 isolates. Of these 13, 2 of them morphologically are identified as Rhizoctonia sp. and Tulasnella sp., while 11 other species of orchid-mycorrhiza, namely sp.1, sp.2, sp.3, sp.6, sp.7, sp.8, sp.9, sp.10, sp.11, sp.12, and sp.13 isolates are associated with S. plicata which is only from 1 different location (Table 1) not all of them can be identified based on differences in morphological features (Figure 1). However, the results of phyllogenetic analysis (Table 2, Figure 2) shows that there are 4 clusters that are likely encountered two other species of orchid-mycorrhiza.

The results of this isolation adds the data of isolation result of the initial survey conducted by Agustini et al. (2009) in the campus of Faculty of Mathematics and Natural Sciences, Cenderawasih University, located in the buffer zone of CAPC. In her research, she discovered two species of orchid-mycorrhiza, namely Rhizoctonia sp. and Tulasnella sp which are isolated from orchids S. plicata and several other species of orchids. In this research, at least there are two unknown species of orchid-mycorrhiza. Both species are different from Rhizoctonia sp. and Tulasnella sp.
In a study, Athipunyakom et al. (2004) isolated orchid-mycorrhiza of *S. plicata* obtained from Chiang Mai, Chanthaburi, Nakhon Ratchasima and Bangkok and found three genera and four species of orchid-mycorrhiza which has successfully been identified based on morphological characteristics, namely *Epulorhiza repens*, *Rhizoctonia globularis*, *Rhizoctonia* sp., and *Sebacina* sp.

Table 1 show that *S. plicata* can be associated with more than one species of mycorrhiza-orchid. Kristiansen et al. (2001) showed that the orchid can be associated with more than one species of mycorrhizal fungi. In addition, Handayanto and Hairiah (2007) also said that some mycorrhizal fungi can colonize one root of the plant. This is because identification of orchid-mycorrhiza fungi in conventional way is still difficult to conduct. According to Rasmussen (2004), Hollick et al. (2004) and Kristiansen et al. (2001) the characteristic of the fungus which never reach sexual phase or sterile in a culture condition made it difficult for identification process so that the identification with molecular techniques needs to be done.

**Description of orchid-mycorrhiza species**

Orchid-mycorrhiza orchids isolated from *S. plicata*, showing the development of a variety. The most rapid growth of the colony diameter reached by isolates sp.2 with a diameter of 9 cm in 3 days, followed by *Rhizoctonia* sp., *Tulasnella* sp., sp.12 and sp.1. While other species of orchid-mycorrhiza with colony growth of more than 15 days showed a very slow growth, whereas the optimum diameter of colony growth for orchid-mycorrhiza is 15 days. The growth rapidity of this colony affects the speed of nutrients absorption in nature. According to Smith and Read (2008), the growth rapidity of mycelia/hyphae potentially increased the extent of nutrients absorption surface in the soil during symbiosis process with the plant root system. Thus, the plants quickly obtained nutrients needed through hyphae.
Table 2. Description of the morphology of orchid-mycorrhiza species associated with *S. plicata*.

| Mycorrhiza (isolates) species | Growth on PDA                                                                 | Morphology                                                                 |
|-------------------------------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| *Rhizoctonia* sp.             | The growth of the colony very fast, within 4 days.                           | Branched hyphae, less dense                                                |
|                               | Mycelia are translucent white and thin. There is a set of dark green granules | At the end of hyphae branch, it is formed small sphere-shaped granules,    |
|                               | forming a round shape in the middle.                                          | which in turn form a collection of granules. The presence of globulus oil  |
|                               |                                                                               | is found around it.                                                        |
| *Tulasnella* sp.              | Growth of colonies is rather slow, 8 days.                                   | Branched hyphae with dense septate.                                        |
|                               | Mycelia are white and thicker.                                                | The presence of conidia, in a group or scattered, and with a magnification |
| sp.1                          | Colony growth is slow.                                                        | of 1000x, the conidia seems like bananas, in sectional.                   |
|                               | Achieving a diameter of 9 cm in 12 days.                                      |                                                                             |
|                               | Mycelia are whitish-green brown.                                              |                                                                             |
|                               | Mycelia are thick and round.                                                  |                                                                             |
| sp.2                          | The colony growth is very fast, within 3 days                                | Branched hyphae, more than 1 core, dense septate.                           |
|                               | Blurred white mycelia                                                         | Spores are brown and on the tip of hyphae.                                 |
| sp.3                          | The colony growth is very fast, within 5 days                                | The hyphae are long and round and in the shape of chain, and the conidia  |
|                               | Mycelia are white to yellow and have tree-like branches when mature.         | are round, semi round, ramiform (like kidney shapes), allantoidal (like   |
| sp.6                          | Colony growth is very slow, reaching only Ø 9 cm in 22 days                  | cashew nut shape with no-sharp edge), and crystal look-like.                |
|                               | Mycelia are yellowish green, thick and form concentric zones. On the edge    |                                                                             |
|                               | side, mycelia are thin and white.                                             |                                                                             |
| sp.7                          | Slow colony growth, 15 days                                                  | The hyphae are separte and dense.                                          |
|                               | Mycelia are white with dark brown sides that form a circle in the middle and |
|                               | thick.                                                                        | At the tip of the hyphae, there are round and brown spores, and also in   |
|                               |                                                                             | “V” like shape in brown color with parallel lines in it.                   |
| sp.8                          | Colony growth is slow, 17 days                                                | Branched hyphae with dense septate.                                        |
|                               | Mycelia are white with brown color in the middle, and appear thicker         | Spores are clustered and dispersed small round shape. Some tips of hyphae  |
|                               |                                                                             | form many branches covered by the collection of spores.                    |
| sp.9                          | Colony growth is very slow 20 days                                            | The hyphae are branched with non-close sepatate.                           |
|                               | At the beginning, Mycelia grow clearly white and thick. As the growth       | Conidia’s shape are like crystals and resembles a figure of number eight   |
|                               | continues, white mycelia appear thickner in the middle.                      | are scattered and there is a chain composed ellipse.                       |
| sp.10                         | Growth of colony is very slow, 24 days.                                      | Hyphae are long with sepatate.                                             |
|                               | Mycelia are white with blackish brown in the middle and it looks thicker.    | Conidia are round and crystals-shaped with elongated dots which are       |
|                               |                                                                             | arranged like a chain.                                                     |
| sp.11                         | Slow growth colony, 16 days                                                  | The presence of large round-shaped spores is found on the tip of hyphae.   |
|                               | Mycelia are thick and white forming concentric zone.                         |                                                                             |
| sp.12                         | Colony growth is rather slow, 8 days                                         | Hyphae are septate and branched.                                           |
|                               | Mycelia are blackish green and thick.                                        | Some hyphae appear thickened and there are many globular attached to the  |
| sp.13                         | Growth of colonies is very slow; it needs more than a month to reach a      | hyphae. Among hyphae, there are conidia in crystal shape.                   |
|                               | diameter of 9 cm.                                                            |                                                                             |
|                               | Mycelia are white and thick.                                                  |                                                                             |
|                               |                                                                             | Hyphae are branched with non-close sepatate.                               |
|                               |                                                                             | Many spores are black round-shaped and are found on the tip of hyphae.    |
|                               |                                                                             | Some hyphae are coil-shaped.                                               |
|                               |                                                                             | Hyphae have long septate and a lot of branches.                           |
|                               |                                                                             | There are many scattered elliptical conidia and are attached to the       |
|                               |                                                                             | hyphae. In addition, there is presence of globular attached to the       |
|                               |                                                                             | hyphae.                                                                    |
Table 3. Phylogenetic relationship of 11 isolates of orchid mycorrhiza based on molecular approach (DNA) isolated from orchid *S. plicata*.

| Isolate | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10     | 11     |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|
| 1       | ****  | 0.6667| 0.3333| 0.3333| 0.3333| 0.6667| 1.0000| 0.6667| 0.6667| 0.3333 | 0.3333 |
| 2       | 0.4055| ****  | 0.6667| 0.6667| 0.6667| 1.0000| 0.6667| 1.0000| 1.0000 | 0.6667 | 0.6667 |
| 3       | 1.0986| 0.4055| ****  | 0.3333| 1.0000| 0.6667| 0.3333| 0.6667| 0.6667 | 0.3333 | 1.0000 |
| 4       | 1.0986| 0.4055| 1.0986| ****  | 0.3333| 0.6667| 0.3333| 0.6667| 0.6667 | 1.0000 | 0.3333 |
| 5       | 1.0986| 0.4055| 0.0000| 1.0986| ****  | 0.6667| 0.3333| 0.6667| 0.6667 | 0.3333 | 1.0000 |
| 6       | 0.4055| 0.0000| 0.4055| 0.4055| 0.4055| 0.6667| 0.6667| 0.3333| 0.3333 | 0.3333 | 0.3333 |
| 7       | 0.0000| 0.4055| 1.0986| 1.0986| 1.0986| 0.4055| 0.6667| 0.6667| 0.3333 | 0.3333 | 0.3333 |
| 8       | 0.4044| 0.0000| 0.4055| 0.4055| 0.4055| 0.0000| 0.4055| 0.4055| 0.4055 | 0.4055 | 0.4055 |
| 9       | 0.4055| 0.0000| 0.4055| 0.4055| 0.4055| 0.0000| 0.0000| 0.0000| 0.0000 | 0.0000 | 0.0000 |
| 10      | 1.0986| 0.4055| 1.0986| 0.0000| 1.0986| 0.4055| 0.4055| 0.4055| 0.4055 | 0.4055 | 0.4055 |
| 11      | 1.0986| 0.4055| 0.0000| 1.0986| 0.0000| 0.4055| 1.0986| 0.4055| 0.4055 | 1.0986 | ****  |

**Phylogenetic relationship of orchid-mycorrhiza of *Spathoglottis plicata***

In Table 2, the isolates number of mycorrhizal orchid which were isolated from *Spathoglottis* plants are 13 species, but only 11 species that can be isolated their DNA. Therefore, phylogenetic analysis (Table 3) would only be done on these 11 species that have been successfully analyzed. The results of DNA analysis shows that there are four major groups (clusters) of 11 species of orchid-mycorrhiza isolated from *S. plicata* (Figure 2). With the method of UPGMA (unweighted pair group method with arithmetic mean), cluster 1 shows the phylogenetic ties among sp.1 and sp.7 isolates. Cluster 2 shows the relationship among the four species, namely, sp.2, sp.6, sp.8 and sp.9 isolates. Cluster 3 shows that there are 3 isolates having a very close relationship, namely sp.3 isolates, *Tulasnella* (*Tul*) and sp.11 isolates. Whereas cluster 4 shows that there are two species of isolates having a close relationship i.e. the species of *Rhizoctonia* (*Rhi*) and sp.10 isolates. And also, the results show that each isolates of those four clusters have the same index value of relationship. *Rhizoctonia* has a relationship index equal to 100% to isolates sp.10, *Tulasnella* to isolates sp.3 and sp.11, so it can be said that these isolates have the same species.

The growth rate of the colonies did not affect the nearby distant phylogenetic relationship in one cluster. It can be seen from the *Tulasnella*, which grows slowly (8 days/9 cm), is different from sp.11, which has a diameter of colony growth on PDA medium for up to 16 days/9 cm, although morphologically the relationship among species and isolates within a cluster is very close. It is similar with *Rhizoctonia* case, morphologically, it grows very fast (4 days) on PDA medium, whereas sp.10 grows very slow and it takes about 24 days to reach a diameter of 9 cm. However, microscopically both are similar in its morphology (Table 1). The same thing occurs in clusters 1 and 2. For the species group of the two clusters, the DNA relationship is closer to cluster 3 of *Tulasnella* sp. and is further to *Rhizoctonia* sp. group.

**CONCLUSION**

Based on morphological and microscopic character, there were 13 isolates of orchid mycorrhizal fungi has been isolated from *S. plicata*’s root. Among them, isolates has been known as *Rhizoctonia* sp and sp *Tulasnella*. The constructed phylogenetic tree of 11 isolates performed that those species could be grouped into 4 major clusters. Two species, *Rhizoctonia* sp. and *Tulasnella* sp. were in different clusters.

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