Ectomycorrhiza Status of *Castanopsis buruana* Miq. in The Field

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**Abstract.** *Castanopsis buruana* Miq. is one of the important species, growing in Indonesian lowland forest. The plant is used as source of wood and highly nutritious nuts. *Castanopsis* is reported to be associated with ectomycorrhiza, although the *C. buruana* Miq. which grows in Indonesian tropical rain forest has not been known for its ectomycorrhiza status. The objective of this research was to study the ectomycorrhiza status of *C. buruana* Miq. which grows in Sulawesi forest as endemic species. Samples were collected from *C. buruana* Miq. in Grand Forest Park Nipa-Nipa, Kendari. Collection of samples in the field was conducted with line transect technique using root tracing method. Ectomycorrhiza morphotype, root anatomy and root colonization analyses used method of Colour Atlas of Ectomycorrhizae, microtome and gridline intersection, respectively. Analysis results showed that roots of *C. buruana* Miq. formed eight different root tip morphotypes. Percentage of ectomycorrhiza colonization of the eight morphotypes varied and were categorized into 3 groups, namely <5%, 5-10% and > 50%. **Unramified black** root tip morphotype exhibites the highest colonization percentage (53.76%) compared to the others root tip morphotype observed.

**Keywords:** ectomycorrhiza, diversity, *Castanopsis buruana* Miq.

1. **Introduction**

Indonesian forests constitute the third largest tropical forest in the world after Brazil and Congo Democratic Republic, and possess high biological richness. *Castanopsis*, from family Fagaceae is one of the forest plants existing in Indonesian tropical forest. Genus *Castanopsis* comprise 120 species and has distribution range from north-east India to west of China, Korea, Japan, and all over Malesia (Indonesia, Malaysia, Philippines, Burma, Thailand and Papua New Guinea) [1]. In Malesia region, 34 species were reported, and the largest number of species was found in Indonesia, namely around 24 species. *Castanopsis* grow and are distributed from western to eastern part of Indonesia, such as in Kalimantan, Sumatera, Java, Sulawesi and Maluku [2,3].

*Castanopsis buruana* Miq. is one of the important species which grows in Indonesian lowland forest and is distributed in Sulawesi and Maluku [4,5]. Locally, *C. buruana* Miq. had ever been dominant in South Sulawesi, whereas in Southeast Sulawesi, *C. buruana* Miq. was abundantly found in Buton island, regency of Muna, Konawe, South Konawe and Kolaka [6]. This species grows in
primary and secondary forest in hilly areas which range from low elevation, up to 1000 m asl [1], and occurs in evergreen forests, particularly in Latosol and Oxisol soils with rainfall of 2,000-3,000 mm/year and dry season less than 6 months [7]. As one of the indigenous species, C. buruana Miq. has an important role in mountain ecosystem, in high elevation forests which function as life supporting system, particularly for maintaining hydrology system for the high and lower elevation areas.

Besides ecological benefits, C. buruana Miq. also possesses very important economic value. This plant produces wood and non wood forest products. The wood is traded with local name berangan, and categorized as wood with strength class II-III and durability class III [5], so that it is suitable for medium to heavy construction. The C. buruana Miq. also produces edible nuts, which are highly nutritious and free from gluten. Bark of the stem tree produces tannin and natural coloring substance. [1]. At present, there is excessive exploitation of this plant species through illegal logging, mining activities, and expansion of plantation and agriculture, which threaten the existence of this species in natural forest. In nature, C. buruana Miq. is categorized as having difficulties in regeneration and growth because the seeds of this species have low viability, which range between 20-75% and its growth and distribution depend on ectomycorrhizal fungi.

It has been reported that growth and development of the Castanopsis is highly dependent on ectomycorrhiza, this could also be the case in C. buruana Miq. [8]. Several Castanopsis species which associate with ectomycorrhiza fungi were among others C. fargesii grown in subtropical forest of China [9], C. tribuloides in mixed evergreen forest of Thailand [10], C. cuspidata in Japan [11-13], C. dentata in Western Wisconsin of America [14], and C. sieboldii [15]. Fungi which were reported to form ectomycorrhiza were among others Lactarius sp., Russula sp., Boletus sp. [9], Thelephora sp. [16], Tricholoma fulvocastaneum [10], Scleroderma citrinum [14,13], Hebeloma albicola [12] and Tricholoma matsutake [15]. However, up to present, there has been no report of association and status of ectomycorrhiza for C. buruana Miq. in Indonesian tropical forest.

Research on status and diversity of ectomycorrhiza fungi for C. buruana Miq. is very important to be conducted because ectomycorrhizal fungi can play a key role in nutrient cycle [17] and affect the diversity and composition of tropical rain forest [18]. Ectomycorrhiza fungi play important in tropical forest ecology, because they provide water and nutrients for the host plant and increase protection from various environmental stress factors [19]. Therefore, this symbiosis increases C. buruana Miq. seedlings growth and population in nature. The objective of this research was studying the ectomycorrhiza status of C. buruana Miq. which grows in Sulawesi, as endemic in Indonesia.

2. Study sites and methods

2.1. Sample plots
Collection of root tip samples used survey method with line transect techniques. Each line transect comprised 3 plots measuring 20 m x 20 m each. Starting point of transect line was determined purposively, namely on the basis of elevation and representativeness of plant population. Coordinates of each line transect were determined with GPS. Grand Forest Park Nipa-Nipa is geographically positioned between 03°54'05"- 03°58'00" S and 122°29'38"-122°04'25" E, and is administratively positioned in two regency/municipal territories, namely regency of Konawe and city of Kendari, in Southeast Sulawesi Province. Grand Forest Park Nipa-Nipa is located at elevation of 25-100 m asl with level and very steep topography (mountainous). Slopes range between 8% and more than 40% with soil types Cambisol and Podsolic.

2.2. Collection of ectomycorrhizal roots in the field
Roots were collected using root tracing method. As many as 3 trees of C. buruana Miq. were selected randomly from each plot. Root samples were taken from 3 points of each tree by digging the main roots at depth of 15 – 20 cm at crown tip region. Samples were put into plastic container and were given information on plot number, tree identity, and collection location, and were afterwards put into
box which contain dry ice. After arrival at laboratory, the samples were put inside refrigerator until used.

Root samples were washed with flowing water above a sieve and were cut into pieces of 5 cm long. Fine roots were cleaned with brush, using the aid of stereoscopic microscope. Cleaned roots were labeled for morphotype analysis root anatomy and root colonization.

Root samples were observed under stereoscopic microscope, and roots which were colonized by ectomycorrhiza were separated, and their morphotype were characterized by Agerer method [20], while color of the colonized root was determined by using book of Standard Soil Color Charts. Percentage of root colonization was analyzed using method of gridline intersection [21]. Percentage of colonization was calculated as follows:

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K = \frac{\text{Number of colonized root}}{\text{Total number of roots observed}} \times 100\%
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Root anatomy was analyzed using root cross section prepared with microtome YiDi (YD 202A) for observing mantle structure and hartig net. Before being dissected, roots were prepared with processes of fixation, dehydration, preparaffination and paraffination the root preparation following steps: fixation by soaking the root segments in FAA solution (formalin : glacial acetic acid : alcohol 100% = 5 : 5 : 90 v/v) for 12-24 hours. Dehydration was performed by soaking root segments in alcohol with the following steps: soaking in alcohol 40% for 3 times with each miless was taken 5 minutes, alcohol 60% 3 times with each miless was taken 5 minutes and alcohol 80% 3 times with each miless was taken 5 minutes. Preparaffination was performed by soaking root segments in solution of alcohol-xylol with the following steps: alcohol : xylol 4 : 0 for 3 times with each miless was taken 5 minutes, alcohol : xylol 3 : 1 for 3 times with each miless was taken 5 minutes, alcohol : xylol 2 : 2 for 3 times with each miless was taken 5 minutes, alcohol : xylol 1 : 3 for 3 times with each miless was taken 5 minutes and alcohol : xylol 0 : 4 for 3 times with each miless was taken 5 minutes. This proces was performed in glass jar in oven with temperature of 55°C. Paraaffination was performed by soaking root segments in solution of xylol-parafin with the following steps: xylol : paraffin 4 : 0 for 3 times with each miless was taken 5 minutes, xylol : paraffin 1 : 3 for 3 times with each miless was taken 5 minutes, xylol : paraffin 2 : 2 for 3 times with each miless was taken 5 minutes, xylol : paraffin 1 : 3 for 3 times with each miless was taken 5 minutes and xylol : paraffin 0 : 4 for 3 times with each miless was taken 5 minutes. Dissection of root tissue used microtome. Paraffin blocks were dissected with thickness of 0.5 µm. Staining (coloring) was performed by using staining solution of safranin and alcian blue [22].

2.3. Data analysis

Percentage of root colonization was analyzed using Duncan’s multiple-range test (DMRT) for analyzing significant difference, with significance level of 5% using software SPSS version 23.

3. Results

3.1. Ectomycorrhiza morphotype variation of C. buruana Miq.

There were 1,545 root tips of C. buruana Miq. ectomycorrhiza obtained which comprise 8 morphotypes from 27 collected samples from 9 plots. The eight morphotypes of ectomycorrhizal were as follows:

Root tip morphotype type I simple branching characteristic, or without branching (unramified), mantle surface resembled woolly, mantles had gray color (5YR 6/1), branch tip ended straight and acuminate (figure 1a). Rhizomorph resembled hairy, had black color (5YR 2.5/1) and emerged at certain parts of the mantle surface (figure 1a). Cross section of root tips which were colonized by type I showed gray colored mantle which was formed with thickness of 15.6 – 65.2 µm (figure 1b). Hartig net was formed between epidermal cells and the formation continued further surrounding 1-2 rows of cortical cells (figure 1b).
Root tip morphotype type II has solitary characteristic (not ramifying) and monopodial pinnate. Mantle surface was smooth, mantle had olive brown color (2.5Y 4/6), branch tip ended crooked and acuminate (figure 1c). Rhizomorph was not seen in the mantle surface (figure 1c). Cross section of roots colonized by type II showed light brown colored mantle (7.5 YR 6/4) which was formed with thickness of 8.6 – 34.6 µm (figure 1d). Hartig net was formed between epidermal cells and the formation continued further surrounding 1-2 rows of cortical cells (figure 1d).

Root tip type III has simple branching, or without branching unramified characteristic. Mantle surface resembled cottony, mantle white color (5 YR 8/1), branch tip ended crooked and acuminate (figure 1e). Rhizomorph was in the form hyphal fans and had brownish yellow color (10 YR 6/8), emerging at certain parts of the mantle surface (figure 1e). Cross section of root tips showed brown colored mantle (7.5 YR 5/4) which was formed with thickness of 1.5 – 2.6 µm (figure 1f). Hartig net was formed between epidermal cells and the formation continued further surrounding 1-2 rows of cortical cells (figure 1f).

Root tip type IV has dichotomous characteristic. Mantle surface resembled woolly structure, mantle black color (5Y 2.5/1), branch tip ended crooked and acuminate (figure 1g). Rhizomorph hairy and black color, emerging at certain parts of mantle surface (figure 1g). Cross section of root tips showed black colored mantle which was formed with thickness of 7.5 – 16.5 µm (figure 1h). Hartig net was formed between epidermal cells, but was not formed between cortical cells (figure 1h).

Root tip type V has monopodial-pinnate characteristic. Mantle surface was reticulate, mantle has light yellowish brown (10 YR 6/4), branch tip ended straight and acuminate (figure 1i). Rhizomorph is in the form of hyphal fans and brownish yellow (10 YR 6/8) and emerged at certain parts of the mantle surface (figure 1i). Cross section of root tips showed light brown colored mantle (7.5 YR 6/4) which was formed with thickness of 4.4 – 11.9 µm (figure 1j). Hartig net was formed between epidermal cells and the formation continued further surrounding 1-2 rows of cortical cells net was formed between epidermal cells and the formation continued further surrounding 1 row of cortical cells (figure 1j).

Root tip type VI has monopodial-pinnate characteristic. Mantle surface was reticulate, mantle had dark gray color (10 YR 5/1), branch tip ended crooked and acuminate (figure 1k). Rhizomorph was in the form of hyphal fans and had dark gray color and emerged at certain parts of the mantle surface (figure 1k). Cross section of root tips showed light gray colored mantle (10 YR 7/1) which was formed with thickness of 20.9 – 29.9 µm (figure 1l). Hartig net was formed between epidermal cells and the formation continued further surrounding one to one and half rows of cortical cells (figure 1l).

Root tip type VII has monopodial-pyramidal characteristic. Mantle surface cottony, mantle has white (10 YR 1/1), branch tip ended crooked and acuminate (figure 1m). Rhizomorph is in the form of hyphal fans and gray (10 YR 7/1) and emerged at certain parts of the mantle surface (figure 1m). Cross section of root tips showed dark brown colored mantle (10 YR 3/3) which was formed with thickness of 15.3 – 29.7 µm (figure 1n). Hartig net was formed between epidermal cells and the formation continued further surrounding one row of cortical cells (figure 1n).

Root tip type VIII has irregularly-pinnate characteristic. Mantle surface is smooth, mantle has brown to dark brown (7.5 YR 4/6), branch tip ended straight and acuminate (figure 1o). Rhizomorph was not seen in the mantle surface (figure 1o). Cross section of the root tips VIII showed brown colored mantle (7.5 YR 5/6) which was formed with thickness of 1.2 - 6.1 µm (figure 1p). Hartig net was formed between epidermal cells and the formation continued further surrounding one row of cortical cells (figure 1p).
Figure 1. Root tip morphotype and root anatomy *Castanopsis buruana* Miq. ectomycorrhiza characteristics: a,b) unramified black; c,d) unramified olive brown; e,f) unramified brownish yellow; g,h) dichotomous black;
Figure 1. Root tip morphotype and root anatomy *Castanopsis buruana* Miq. ectomycorrhiza characteristics: i,j) monopodial-pinnate brownish yellow; k,l) monopodial-pinnate dark gray; m,n) monopodial-pyramidal light gray; o,p) irregularly–pinnate brown. EH: External Hyphae, M: Mantle, EP: epidermis, HN: Hartig net, C: cortex. Bar a, c, g, k 200 μm; i 500 μm; m 1 mm; o 100 μm; b, d, f, h, j l, n, p 20 μm.
3.2. Percentage of ectomycorrhizal root colonization
Ectomycorrhiza colonization percentage in *C. buruana* Miq. in each morphotype showed varying degrees. *Root tip* type I (*unramified black*) was morphotype which possessed the highest colonization percentage, namely 53.76%, followed consecutively by morphotype V (*monopodial-pinnate brownish yellow*), III (*unramified brownish yellow*), II (*unramified olive brown*), IV (*dichotomous black*) and VII (*monopodial-pyramidal light gray*) which were respectively 10.58%, 9.62%, 8.64%, 7.27% and 5.46%. On the other hand, morphotype VI (*monopodial-pinnate dark gray*) and VIII (*irregularly-pinnate brown*) were morphotypes with lowest colonization percentage, namely 2.62% and 2.05% respectively (figure 2).

Therefore, ectomycorrhiza colonization percentage in *C. buruana* Miq. on the basis of morphotype can be categorized into 3 categories as follows: Category I morphotype with percentage < 5% were *monopodial-pinnate dark gray* morphotype (type VI) and *irregularly-pinnate brown* morphotype (type VIII). Category II with percentage 5-10% were *unramified olive brown* morphotype (type II), *unramified brownish yellow* morphotype (type III), *dichotomous black* morphotype (type IV), *monopodial-pinnate brownish yellow* morphotype (type V) and *monopodial-pyramidal light gray* morphotype (type VII). On the other hand, category III with percentage > 50% was *unramified black* morphotype (type I).

![Figure 2. Ectomycorrhiza colonization percentage of Castanopsis buruana Miq. for each morphotype: I. unramified black; II. unramified olive brown; III. unramified brownish yellow; IV. dichotomous black; V. monopodial-pinnate brownish yellow; VI. monopodial-pinnate dark gray; VII. monopodial-pyramidal light gray; VIII. Irregularly-pinnate brown.](image)

4. Discussion
This is the first description of ectomycorrhiza root tip formed by *C. buruana* Miq. in low land forest in Indonesia. In this research, we confirmed that *C. buruana* Miq. consistently forms symbiotic root association with ectomycorrhiza fungi. All samples of *C. buruana* Miq. root tip contained ectomycorrhiza with morphological and anatomical characters as shown in figure 1. *C. buruana* Miq. forms eight ectomycorrhiza root tip morphotype, namely *unramified black*, *unramified brownish yellow*, *dichotomous black*, *monopodial-pinnate brownish yellow*, *monopodial-pinnate dark gray*, *monopodial-pyramidal light gray*, and *irregularly-pinnate brown*. 
Unramified black morphotype possessed pseudoparenchymatous mantle structure. The same phenomenon also occurred unramified olive, unramified brownish yellow, monopodial-pinnate dark gray, and monopodial-pyramidal light gray in morphotype. Although the five morphotypes mentioned before possessed similar mantle structure, if they were analyzed on the basis of their mantle types, it appeared that the mantle structures exhibited differences. Unramified black morphotype was pseudoparenchymatous type P, with cells tended to rounded with hyphal net. Unramified olive brown morphotype possessed pseudoparenchymatous type L mantle structure, with cells tended to be rounded and constituted the characters of fungi ectomycorrhiza Tometella sp [9]. Unramified brownish yellow morphotype was pseudoparenchymatous type K, with angular cells and with round cells in the mantle surface. On the other hand, monopodial-pinnate dark gray, and monopodial-pyramidal light gray morphotype were pseudoparenchymatous type L, with cells tended to be rounded [23].

On the other hand, dichotomous black, monopodial-pinnate brownish yellow, irregularly–pinnate dark brown, and irregularly–pinnate brown morphotype possessed pletenchymatous mantle structure. Dichotomous black morphotype was pletenchymatous type G, because the hypha were arranged like star and were attached to each other. The mantle structure of monopodial-pinnate brownish yellow was pletenchymatous type D, with cystidia being shaped like awl. On the other hand, morphotype irregularly–pinnate dark brown and irregularly–pinnate brown possessed similar mantle structure, namely pletenchymatous type A because the hypha were irregular [23].

The eight morphotypes formed by the four orders possessed hartig net formed between epidermal cells. Cortical hartig net is a unique character for fungi which colonize Angiospermae plants [24,8].

Black colored simple morphotype without branching exhibited the highest percentage of colonization (53.76%). These research results were different from being reported by Lee et al. [25] which showed that dichotomous morphotype was dominant in C. buruana Miq. This differences may due to different environmental condition. This was in agreement with opinion of Kovacs et al. [26], who reported that the morphotype being obtained could be affected by environmental condition, such as water, nutrition, pH, temperature, humidity and attack by pathogen. Furthermore, Teredesoo et al. [27] repeated that global diversity of ectomycorrhiza fungi was mostly affected by temperature and rainfall.

Structure differences in root tip morphotype could also indicate that fungi which from symbioses with the roots are from different species. Therefore, it can be suggested that higher diversity of root morphotypes, indicate would higher diversity of fungal species being associated [28].

This research obtained eight morphotypes, which was fewer as compared with research results by Castanopsis fargesii [9] 19 morphotypes. This was possibly due to lower size of sample taken in our experiment. Therefore, more comprehensive research covering large samples will be needed to understand these phenomena [29].

5. Conclusion

C. buruana Miq. is mycotropic plant forming eight ectomycorrhiza root tip morphotypes. Five out of eight morphotypes, namely unramified black, unramified olive brown, dichotomous black, monopodial-pinnate brownish yellow and monopodial-pinnate dark gray (type I, II, IV, V, and VI) were commonly found in various species of Castanopsis. Whereas the three other morphotypes, namely unramified brownish yellow, monopodial-pyramidal light gray and irregularly–pinnate brown (type III, VII and VIII) were uniquely found in C. buruana Miq. Unramified black morphotype (type I) was the dominant ectomycorrhiza found in C. buruana Miq.

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