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# Occurrence of Thraustochytrids on fallen mangrove leaves from Pagbilao Mangrove Park, Quezon Province

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**Abstract**

Thraustochytrids are marine protists that can be abundantly found on fallen mangrove leaves. These organisms are drawing attention from scientists and commercial manufacturers alike primarily because they are able to produce omega-3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in copious amounts within their cells. Scientists from Brazil, Malaysia, Taiwan, and Thailand have capitalized on their country's thraustochytrids; yet, in the Philippines only a limited number of researches have been conducted on these organisms. Thus, the significance of this research is to further investigate, supplement additional information, and add to the number of existing studies on the thraustochytrids from Philippine mangroves. This research isolated, purified, and characterized thraustochytrids present in yellow, yellow brown, and brown leaves from two (2) mangrove species—*Avicennia* and *Rhizophora* spp. in Pagbilao Mangrove Forest. The thraustochytrids from each leaf sample of the two mangrove species were isolated and purified on GYPSA (Glucose Yeast Peptone Sea Salt Agar) media. Morphological characterization was done through microscopy for partial identification. The isolates present on fallen mangrove leaves from Pagbilao, Quezon were preliminarily identified as morphologically indicative to either be *Aurantiochytrium, Hondaea, or Monorhizochytrium*.

**Keywords:** marine protists, heterotrophs, Thraustochytriaceae, Philippines

## Introduction

Mangrove ecosystems are one of the most vital ecosystems in tropical countries (Dangan-Galon *et al*., 2016; Gevaña *et al*., 2019). They are considered as a link between terrestrial and marine life, forming the base of numerous marine food sources since they transfer organic matter and energy from land to sea (Gevaña *et al*., 2019; Lee *et al*., 2014; Nagelkerken *et al*., 2008). In mangrove habitats, there are many species of microorganisms that thrive, and one of these are the thraustochytrids (Leaño, 2001; Phuphumirat *et al*., 2016; Raghukumar *et al*., 1994; Saravanakumar *et al*., 2016).

These heterotrophic eukaryotes belong to the *Thraustochytriacae* of the Straminipila (Dick, 2001; Bennett *et al*., 2017; Marchan *et al*., 2018; Tsui *et al*., 2009). They are easily found in mangrove detritus because of the substrates' contact with brackish water, containing trace minerals required for their survival, along with rich organic material (Fan *et al*., 2002; Nagano *et al*., 2013; Raghukumar, 2002; Raghukumar *et al*., 1994; Saravanakumar *et al*., 2016; Shabala *et al*., 2009; Ueda *et al*., 2015; Wong *et al*., 2005). Moreover, they have also been observed to be just as abundantly associated in organic material in marine environments (Naganuma *et al*., 1998; Ramaiah *et al*., 2005; Sharma *et al*., 1994). Their reproduction and nutrient scavenging occur through the production of ectoplasmic nets (that may be very pronounced in some species), biflagellated zoospores and/or amoeboid cells (Bennett *et al*., 2017; Dellero *et al*., 2018; Iwata & Honda, 2018; Raghukumar, 2002; Yokoyama & Honda, 2007).

For about two decades now, they have consistently drawn attention from scientists due to their ability to produce large amounts of fatty acids, which may be of benefit to human and animal development as well as well-being maintenance (Arafiles *et al*., 2011, 2014; Fan *et al*., 2007; Furlan *et al*., 2017; Huang *et al*., 2001; Leaño & Liao, 2004; Raghukumar, 2008; Singh *et al*., 2016; Yamasaki *et al*., 2006). Examples of these fatty acids are the polyunsaturated fatty acids (PUFA), which are traditionally

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sourced from deep ocean fish like tuna. With threats to global fish populations, research has consistently veered towards single-cell sources of PUFA such as the thraustochytrids to provide for demands in the nutritional supplement and aquaculture industries (Béligon *et al*., 2016; Gupta *et al*., 2012). Even researchers from temperate countries such as Australia, Canada and Japan have placed great efforts in studying thraustochytrids. (Aki *et al*., 2003; Furlan *et al*., 2017; Huang *et al*., 2001; Singh *et al*., 2016; Ugalde *et al*., 2018; Yamasaki *et al*., 2006). Despite the rich mangrove habitats in the Philippines, there are but a handful of researches conducted here (Arafiles *et al*., 2011; Atienza *et al*., 2012; Dela Peña *et al*., 2016; Estudillo-Castillo *et al*., 2009; Leaño, 2001; Leaño *et al*., 2003; Ludevese -Pascual *et al*., 2016; Oclarit & Hepowit, 2007; Uba *et al*., 2016). This research further investigates, supplements additional information, and adds to the number of researches on thraustochytrids in the country.

This study aims to characterize the morphological features, and to provide a partial identification of the species of thraustochytrids present in different colors on the leaves of *Avicennia* and *Rhizophora* spp. mangrove trees in Pagbilao Mangrove Forest, Quezon.

## Materials and Methods

### *Sampling, Collection, and Media Preparation*

Leaf samples (yellow, yellow brown, and brown) from *Avicennia* spp. (local name "bungalon") and *Rhizophora* spp. (local name "bakauan lalaki") were collected from Pagbilao, Quezon (13.9753°N, 121.7255°E) in February 2019 when temperatures ranged between 25-32ºC. In particular, yellow, yellow-brown and brown leaves were chosen because they were expected to be in advanced stages of decay. Raghukumar *et al*. (1994) determined that detritus aged >21 d old harbored more cells of the thraustochytrid *Aurantiochytrium mangrovei* than those <21 d old. Since more fungi and oomycetes are also expected to be present in freshly fallen leaves, leaves that had already turned yellow or brown were preferred over green ones (Raghukumar *et al*., 1994; Saravanakumar *et al*., 2016; Wong *et al*., 2005)

Leaf samples were placed in a resealable plastic bag halfway filled with seawater, stored in an icebox, and brought to the Laboratory of Pure and Applied Microbiology (PAM) at the Thomas Aquinas Research Complex (TARC), University of Santo Tomas for processing.

Glucose-Yeast-Peptone-Seawater Agar (GYPSA) composed of 0.3% glucose, 1.25 g yeast, 1.25 g peptone, 15 g agar, 50% seawater (salinity = ~18 ppt), and 50% distilled water, was used in the experiment. In order to inhibit the growth of contaminants, 250 mg/L-medium of streptomycin and penicillin, and nystatin were added (Leaño, 2001).

### *Isolation, Purification, and Characterization*

The leaves were initially washed with running tap water to remove attached debris. Leaves were cut into 15-mm wide strips and washed in 50% sterile seawater three times with an interval of 1 h between washes (Fan & Kamlangdee, 2003). The leaves were blot-dried on tissue paper and placed on the GYPSA media. A drop of 50% SSW was placed on the side of each leaf to promote primary sporulation of the thraustochytrids. The plates were incubated at room temperature (~30ºC) for 24-48 h. The periphery and the bottom surface of the leaves that were in direct contact with the agar were checked for colonies of the organism (Leaño *et al*., 2003). Colonies were picked with an inoculating loop and then subcultured and purified on GYPSA agar. Pure thraustochytrid cultures were incubated for 3-4 d and were characterized by microscopy.

## Results and Discussion

Twelve (12) thraustochytrid isolates were isolated from mangrove leaves in Pagbilao Mangrove Park. Their colony morphologies were observed after culturing on GYPSA and revealed white to pale orange colonies (Figure 1). Despite being sampled from fallen leaves of different trees, the appearance of their colony forms, elevation, surface and opacity were similar. However, there were two isolates (RY-2, RB-1) from leaves of *Rhizophora* sp. that formed undulate margins (Table 1).

Although most of the isolates appeared white to cream-colored, it is worthwhile to mention that 25% of all the isolates have somewhat pale orange appearance, which may signify carotenoid or xanthophyll production (Aki *et al*., 2003; Atienza *et al*., 2012). Thus far, there has only been one report of carotenoids in Philippine thraustochytrids (Atienza *et al*., 2012). This study, therefore, can be an encouraging new starting point for those interested in studying about carotenogenous Philippine thraustochytrids and their prevalence in the country.

Micrographs of the cell morphology and mode of reproduction of all isolates are presented in Figures 2 & 3. All isolates had a circular cell shape ranging between 12-20 μm in diameter (Figure 2). The cells slightly glistened green, which is indicative of their oleaginous nature. The cells exhibited partitioning from a central point, rather than the release of amoeboid or zoosporic cells; moreover, their ectoplasmic nets were also inconspicuous. These observations along with that of the colonial features pointed to the morphologically similar genera *Aurantiochytrium, Hondaea* or *Monorhizochytrium* as a

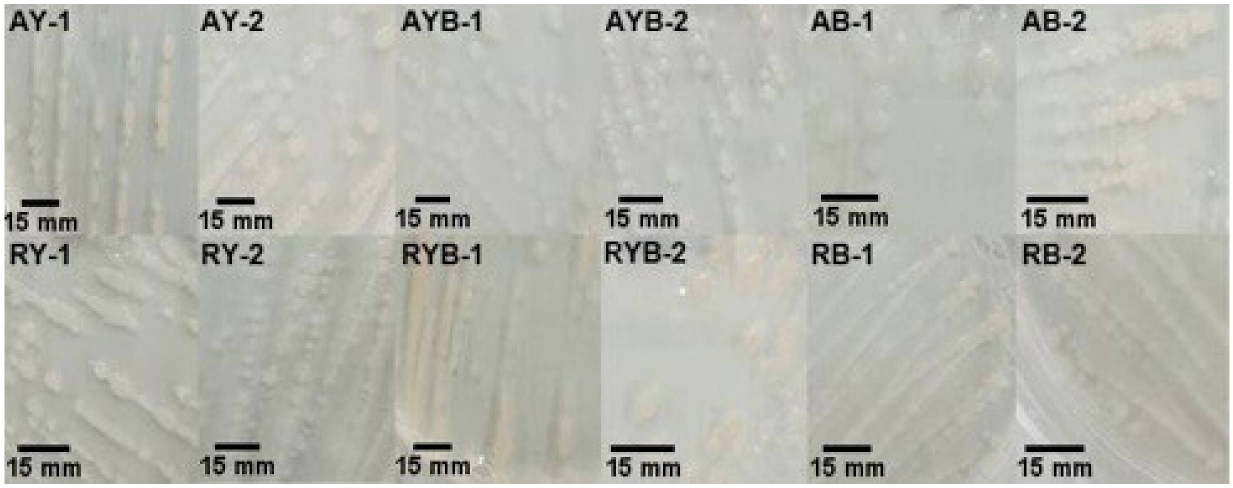


**Figure 1.** Colony appearance and size of thraustochytrids isolated from fallen leaves of *Avicennia* sp. (top) and *Rhizophora* sp. (bottom)

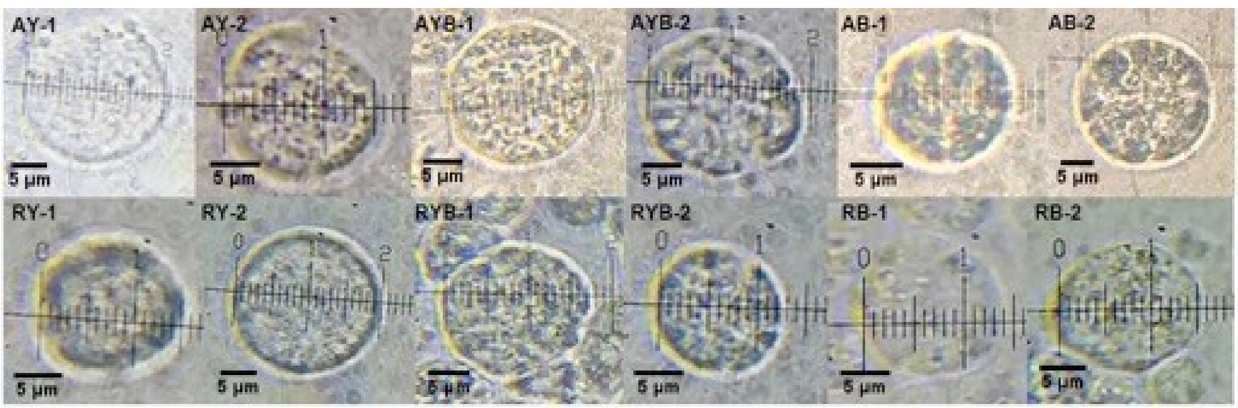


**Figure 2.** Cell size of thraustochytrids isolated from fallen leaves of *Avicennia* sp. (top) and *Rhizophora* sp. (bottom). Magnification: 100x

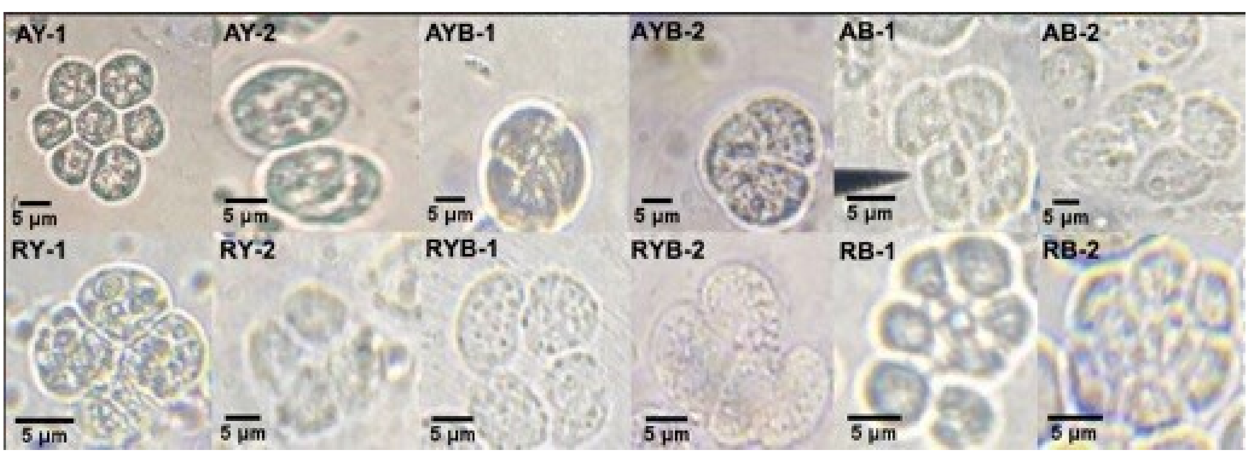


**Figure 3.** Cell morphological features of thraustochytrids isolated from fallen leaves of *Avicennia* sp. (top) and *Rhizophora* sp. (bottom). Magnification: 100x

 

**Table 1.** Description of colony morphologies of thraustochytrids isolated from fallen leaves of decaying *Avicennia* sp. and *Rhizophora* sp. leaves

| Leaf species | Leaf color | Isolate code | Colony description | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Form | Elevation | Margin | Surface | Opacity | Color |
| *Avicennia* | Yellow | AY-1 | Circular | Flat | Entire | Glistening | Opaque | Pale orange |
| | | AY-2 | Circular | Flat | Entire | Glistening | Opaque | White |
| | Yellow brown | AYB-1 | Circular | Flat | Entire | Glistening | Opaque | White |
| | | AYB-2 | Circular | Flat | Entire | Glistening | Opaque | White |
| | Brown | AB-1 | Circular | Flat | Entire | Glistening | Opaque | White |
| | | AB-2 | Circular | Flat | Entire | Glistening | Opaque | White |
| *Rhizophora* | Yellow | RY-1 | Circular | Flat | Entire | Glistening | Opaque | White |
| | | RY-2 | Circular | Flat | Undulate | Glistening | Opaque | White |
| | Yellow brown | RYB-1 | Circular | Flat | Entire | Glistening | Opaque | Pale orange |
| | | RYB-2 | Circular | Flat | Entire | Glistening | Opaque | Pale orange |
| | Brown | RB-1 | Circular | Flat | Undulate | Glistening | Opaque | White to pale orange |
| | | RB-2 | Circular | Flat | Entire | Glistening | Opaque | White to pale orange |

highly possible classification of the 12 isolates (Dellero *et al*., 2018; Doi & Honda, 2017; Yokoyama & Honda, 2007).

*Aurantiochytrium* possesses a globose thallus with a thin wall and underdeveloped ectoplasmic net. Cell diameters range from 10-20 µm and undergo continuous bipartitioning. Colonies are off-white in color and became pale-orange upon prolonged incubation due to the presence of carotenoids (Ganuza *et al*., 2019; Yokoyama & Honda, 2007) *Hondaea* and *Monorhizochytrium* are also similar morphologically to *Aurantiochytrium* and have been classified together by Dellero *et al*. (2018) as a superclade within the *Thraustochytriacae* due to their 18S rRNA sequences having more than 97% similarity to each other.

*Aurantiochytrium* is indisputably the most studied among the thraustochytrids for their great promise as fatty acid and carotenoid producers for industries (Aasen *et al*., 2016; Aki *et al*., 2003; Arafiles *et al*., 2014; Furlan *et al*., 2017; Yamasaki *et al*., 2006). However, the recently isolated *Hondaea fermentalgiana* from the Indian Ocean is expected to be a promising source of sterol production based on pathway mapping (Dellero *et al*., 2018). To conclusively point the identity of the 12 isolates to a particular genus, more data composed of chemotaxonomic features and 18S rRNA sequences will be necessary.

Yet with the morphologically similar isolates found in this study, this is no assurance that the environment of Pagbilao Mangrove Park only harbors this group of genetically similar thraustochytrids. There are thraustochytrids such as *Oblongochytrium* that are known to be most abundant in ecosystems but are unable to grow well in agar media (Ueda *et al*., 2015). This genus is characterized by thin-walled, globose, pale yellow thalli and well-developed ectoplasmic nets (Yokoyama & Honda, 2007; Ganuza *et al*., 2019). A close taxonomic relative of the thraustochytrids, the labyrinthulids, have exhibited the use of their ectoplasmic nets to forage on diatoms for nutrition (Hamamoto & Honda, 2019). The strains in this study may have unwittingly been given an advantage over oligotrophic thraustochytrids due to the culture media and conditions provided. For all the *Thraustochytriacae* to be detected and well-represented, non-culture-based methods will have to be employed and are strongly recommended for future thraustochytrid diversity research.

**Conclusion and Recommendations**

Twelve (12) thraustochytrid isolates from fallen mangrove leaves in Pagbilao Mangrove Park, Quezon were identified as possible members of the genera *Aurantiochytrium, Hondaea* or *Monorhizochytrium.* All isolates appeared similar; thraustochytrids were also present in all the leaf samples despite the latter in different states of degradation as indicated by their color. Since this study has only employed colonial and microscopic observations to identify the isolates, it is highly recommended to subject the isolates to 18S rRNA sequencing to fully determine their identity to the species level and to de-replicate isolates, if possible. Their fatty acid and carotenoid compositions can also be characterized along with further observations of their zoospore size, shape, counts and formation/release process. There were promising isolates that appeared pale orange to the naked eye. Thus, these isolates may warrant further study by microbial biotechnologists for possible industrial applications.

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**Author Contributions**

J.G. Perez, A.K. Mappala, C.K. Icaro & A.M. Estrada conducted the leaf sampling, thraustochytrid isolation, results documentation and writing of the initial versions of this manuscript. K.H. Arafiles & G.R. Dedeles contributed to later versions and proofread this manuscript.

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