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# *Phylogenetic Analysis of liverworts (Marchantiophyta) in Imugan falls, Santa Fe, Nueva Vizcaya, Philippines using rbcL gene marker*

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**ABSTRACT**

*A widely held view of land plant relationships places liverworts as the first branch of the land plant tree. In the past several years, the application of molecular methods to the unraveling of liverwort phylogeny has generated new insights into their evolutionary history and revolutionized their classification. There has been no available substantial sequence of liverworts so far and molecular approach in identifying complex liverworts species was still a challenge. This study is the first attempt to identify and classify the liverworts from Imugan Falls, Sta. Fe, Nueva Vizcaya, Philippines by molecular approach. The liverworts were collected through transect walk along falls and after initial identification using morphological characterization, the genomic DNA of liverworts were extracted and amplified using rbcL, and then PCR products were purified and were sequenced. Sequences were used for BLAST analysis to determine sequence similarity of the sequences available from NCBI GenBank.*

*We have identified two species; these are Dumortiera hirsuta and Targionia hypophylla both with 99% maximum identity. For phylogeny analysis based on the rbcL region, the proximate clade was made up of complex thalloid genera and comprises the monophyletic marchantiaceae, targioniaceae, and wiesnerellaceae in a well-supported sister relationship, it also includes the simpler thalloid monocleaceae. Accessions of Dumortiera hirsuta and Wiesnerella sp. are in a robust sister relationship and sister to the remainder Targionia hypophylla and Monoclea forsteri.*

***Key words:*** *BLAST, phylogeny, thalloid, Dumortiera hirsuta, Targionia hypophylla*

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## INTRODUCTION

Philippines, being a tropical country, has a rich growth of bryophytes such as liverworts [1]. The Philippine forests are now becoming ecologically disturbed because some are converted into agricultural landscape and second-growth forest. These may result to the loss of these species [2] given that 20% of plant diversity has been extinct [3]. There is a need to study its local flora and intensive collections should be conducted before the country's rain forests disappear [4].

The liverworts are non-vascular embryophytes that are well established to occupy the first nodes among extant lineages in the land plant tree of life, existed for several hundreds of million years, and have played a prominent role in subsequent evolution of all forms of plant life on land [5]. They possess a key position in land plant evolution and hold the link between green algal ancestors and vascular plants. The liverworts are the only group of land plants with

a dominant gametophytic generation that share many fundamental structural features and display unifying and innovative reproductive characters [6].

Recently, liverworts (Marchantiophyta) include 8,500 species [7]. Presently, the current state of knowledge of liverwort taxa in the Philippines needs to be explored [2]. A total of 700 species in 228 genera and 55 families were credited in the Philippines. The settled conditions in such regions as northern Luzon, and the greater propensity among recent botanists for climbing mountains have been responsible for most of the additions of known species [4].

Imugan is located at Santa Fe, Nueva Vizcaya, Philippines and has a 1,684-meter mountain peak. It ranks as the third highest mountain in Cagayan Valley and the 180th highest mountain in the Philippines [8]. The degree of morphological similarity and differences were the basis of classification schemes of liverwort that is used in showing relationships among organisms and also reflect evolutionary assumptions. As botanical explorations expanded, new systems of classification were proposed to accommodate the increasing numbers of genera being described [9]. To answer the problem of identification and to quantify global biodiversity, DNA barcoding has been recently proposed [10] which aids species identification and discovery in large assemblage of life by employing sequence diversity in short and standardized gene regions [11].

At the moment, no universal stand-alone DNA barcoding marker for bryophytes such as for liverwort is available [12]; so far, *rbcL* was considered as the marker with the best performance for DNA barcoding bryophytes [13]. Thus, this study was conducted to identify the liverwort present in Imugan Falls, Santa Fe, Nueva Vizcaya, Philippines using *rbcL* gene marker.

## MATERIALS AND METHODS

### *Collection of Liverworts*

The liverwort samples were collected from Imugan Falls, Santa Fe, Nueva Vizcaya, Philippines. A GPS (Global Positioning System) was used to countercheck the field site and to identify the altitude. The collection of samples was conducted by a transect walk (Alpha Taxonomy) for each vegetation type. This was done by collecting and listing all the liverwort seen and along the trail. Samples were collected on different substrates such as on trunk, twigs, logs, rocks, soil, and litters. The collected samples were placed in a zip lock with a field label data: collection number, date of collection, substrate, and other associated habitats. Secondary data were measured and recorded on a data notebook. Photographs and documentation were taken from actual observations in the field as to the species natural habitat.

### *Morphological Characterization of Liverwort*

The specimens collected were identified, classified and described morphologically by their diagnostic characters such as habitat, leaf arrangement, color, size, margin, sporophyte characters, thallus, and rhizoids. Identification was done using the existing herbaria and taxonomic keys from books, scientific articles and journals, based on the most recent classification systems [9] for liverworts.

### *Molecular Identification of Liverwort*

Molecular approach was done to identify the species for further confirmation and proper identification using *rbcL* gene sequences. In extracting DNA samples, CTAB method [14] was conducted. One hundred milligrams of clean liverwort tissue was weighed and placed in liquid nitrogen. After freezing, it was grinded using mortar and pestle and transferred to a centrifuge tube. Then 750 µl of pre-warmed 2x CTAB buffer and 20 µl of 20% sodium dodecyl sulfate (SDS) were added. It was mixed thoroughly by vortexing and incubated in a water bath at 65°C for 30 minutes. After incubation, it was cooled briefly into room temperature then 750 µl of chloroform-isoamyl alcohol (24:1 ratio respectively) was added, then it was vortexed to mix the samples thoroughly. Centrifugation at 10, 000 rpm for 30 minutes was done. Decantation was done, the aqueous was then transferred to a new sterile 1.5 ml centrifuge tube then 600 µl ice-cold isopropanol was added. It was incubated in -20°C deep freezer overnight. Then centrifugation at 10, 000 rpm for 10 minutes was done. Decantation was done and the pellet was washed with 500 µl of 70% ethanol. Centrifugation at 10, 000 rpm for 3 minutes was done then the alcohol was discarded and then drained dry by inverting the centrifuge tube in a paper towel to get rid of the excess liquid. Then the 70% ethanol treatment was repeated.

To check the DNA quality, 1 µl of stock DNA was mixed with 2 µl loading dye and checked on a 1% agarose gel containing 1 µl of gel red (GelRed™Nucleic acid, Biotium) at 100 V for 30 minutes along with standard ladder for scale.

The genomic DNA was diluted 1:100 using sterilized distilled water. To identify the identity of the sample, the chloroplast gene region was amplified using the *rbcL* and *matK* gene marker, while the nuclear gene region was amplified using *nrDNA ITS* gene marker (Table 1) using PCR machine (2720 Thermal Cycler). PCR protocol for Random Amplified Polymorphic DNA Analysis [15] was used, a method to create genomic DNA fingerprinting of plant species, and was modified [16] for improved PCR amplification of liverworts.

PCR amplification products were resolved by horizontal gel electrophoresis to check for quality and molecular weight. A mixture of 2 µl amplification product and 1µl loading dye were loaded onto 1% agarose gel prepared from 1XTris-acetate EDTA buffer along with standard DNA ladder. Electrophoresis was carried out at 100 V for 30 min. The amplified products were checked using gel documentation system (Enduro™GDS).

When the expected size of amplified fragments has been confirmed, amplicons were sent to FirstBASE Laboratory at Malaysia for PCR purification and bi-directional sequencing procedure. The BioEdit software was used for visual presentation of chromatogram to check the sequence quality.

The consensus sequences were used for BLAST (Basic Local Alignment Search Tool) analyses, default search parameters on the standard nucleotide BLAST were used. Related gene sequences were extracted from NCBI GenBank for identification and phylogenetic analysis.

The sequences from different samples were aligned using the ClustalW multiple alignment tool. The neighbor-joining method [17] was used for the construction of phylogeny analysis. The output data was processed using Molecular Evolutionary Genetics Analysis version 6.01 software [18] to render phylogenetic tree.

## RESULTS AND DISCUSSION

*Taxonomy and Morphological Characterization of the Collected Liverworts*

Field inventory of the liverworts in Imugan listed only two species, however there are different bryophytes such as various species of mosses observed in the study site. The specimens were collected with the approval of the barangay officials, these specimens were classified and identified using existing data and related literature. Moreover, other information for each species includes description based on the observed morphology. Sampling sites and other physical parameters of the collected specimens in Imugan falls were also observed.

Based on morphology, Specimen A was identified as *Dumortiera hirsuta* (Figure 1). This species is also known as Dumortier's liverwort or hairy dollar liverwort, and belongs to the class Marchantiopsida, order Marchantiales, and family weisnerellaceae.

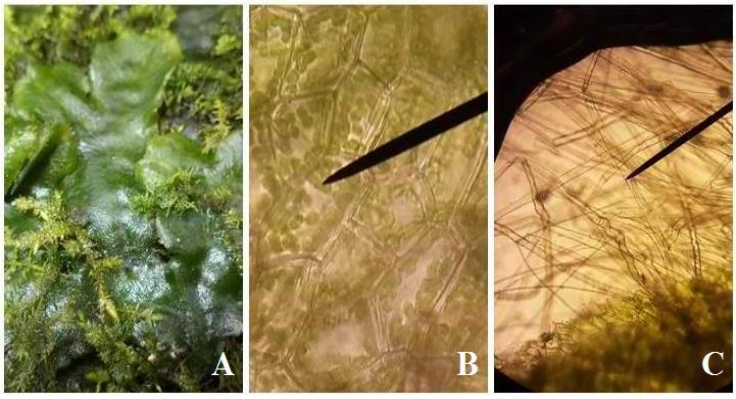

**Figure 1.** Morphological characteristics of Dumortiera hirsuta. Plant habit (A), Light microscopy of oil bodies found in the leaves (B), and rhizoids (C).

The species has a velvety surface on the thallus and this feature separates itself from other thalloid liverworts [19]. It has thallus with broad, flat, semi-translucent, dichotomously branching thalli up to 2 cm wide. The leaf base was plain, with leaf margin entire and leaf apex round. The thallus is light green to dark green. The thallus margins and undersides bear scattered, stiff bristles. The male receptacles are bristly and borne on a very short stalk. Female plants have long-stalked, bristly receptacles borne at the thallus tip; each receptacle is flat-topped with 6 to 12 short, spreading lobes.

*Dumortiera hirsuta* was found on surface of a large stone near the falls, with the location coordinates based from GPS readings of N 16º 11" 38.76 E 121° 6" 36.72" and altitude of 3376 ft. Other physical parameters were also noted such as pH of substrate of 7.0, wind speed of 1.4 kph, and wind temperature of 34 ºC.

On the other hand, Specimen B was identified as *Targionia hypophylla* (Figure 2). This species is also known as Orobus-seed liverwort, and belongs to the class Marchantiopsida, order Marchantiales, and family targioniaceae.

This species has long, narrow, leathery, strongly aromatic thalli, 2–5 mm wide, dark green or slightly bluish-green above with dark purple margins. The upper surface is flat, with weak reticulations and conspicuous dots (air pores). When dry, the thallus rolls up to form a black, worm-like tube [9].

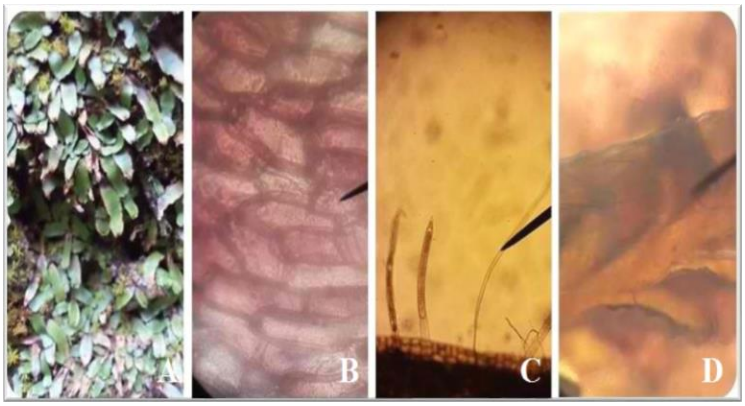


**Figure 2.** Morphological characteristics of Targionia hypophylla. Plant habit (A), parenchymal cells (B), rhizoids (C), and thickened midrib found dorsal in the leaves (D).

*Targionia hypophylla* was found on surface of a large stone along the trail, with the location coordinates based from GPS readings of N 16º 9" 32 E 120° 54" 10" and altitude of 3349 ft. Other physical parameters were also noted such as pH of substrate of 7.0, wind speed of 1.4 kph, and wind temperature of 34 ºC.

*Molecular Identification*

DNA was extracted from eight collected presumably liverwort samples and was subjected to gel electrophoresis in 1% agarose gel. Initially, gene specific primers *rbcL* was used for amplification. Several PCR protocols were tried but could not amplify the target DNA region of the liverwort specimens, to overcome these limitations, the PCR protocol for Random Amplified Polymorphic DNA Analysis [15] was conducted.

A modified PCR conditions were initiated at 92ºC followed by 44 cycles of denaturation at 92ºC for one minute, annealing at 42ºC for one minute, and extension at 72ºC for one minute and thirty seconds, followed by final extension for five minutes. Under the said PCR conditions, 750 base pair fragment of *rbcL* was amplified from liverwort samples A and B using *rbcLaF* and *rbcLaR* primers. BLAST analysis of the sequences showed that the liverwort species collected were identified as follows; Sample A as Dumortiera hirsuta (HQ026271) with 99% identity and sample B as Targionia hypophylla (FJ173734) with 99% identity using *rbcL* gene marker (Table 1).

| Code | Sample Identification | BP | Maximum Identity | Gen Bank Accession Number |
|---|---|---|---|---|
| A | *Dumortiera hirsuta* | 439 bp | 99% | HQ026271 |
| B | *Targionia hypophylla* | 474 bp | 99% | FJ173734 |

*Phylogenetic Analysis*

The phylogenetic tree comprises the nucleotide sequence of liverwort samples A (*Dumortiera hirsuta*) and B (*Targionia hypophylla*) together with 18 related species obtained from GenBank database, only one sequence for each related species was selected. The phylogenetic tree was composed of five main branches that roughly corresponded to five main groups or clades (Figure 3).

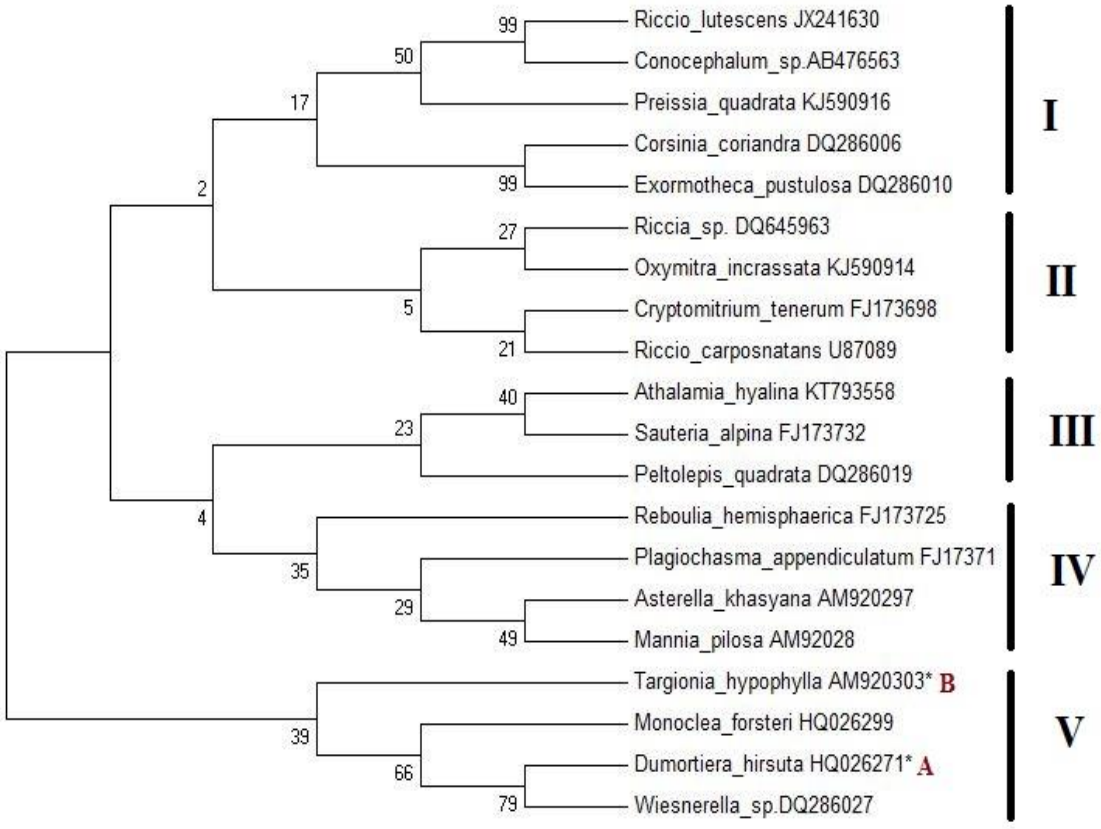


**Figure 3.** Molecular phylogenetic tree showing evolutionary relationships among the clades of liverworts. The topology is from a maximum likelihood analysis of 20 *rbcL* nucleotide sequences.

The proximate clade was made up of complex thalloid genera and comprises the monophyletic marchantiaceae, targioniaceae, and wiesnerellaceae in a well-supported sister relationship, it also includes the simpler thalloid monocleaceae, that is comparable to the results of Boisselier-Dubayle *et al.*, (2002) [20] based on nuclear LSU rDNA

data and on analyses of various chloroplast, nuclear, and mitochondrial markers [21]. Accessions *of Dumortiera hirsuta* and *Wiesnerella sp.* are in a robust sister relationship and sister to the remainder *Targionia hypophylla* and *Monoclea forsteri.*

Liverworts have been identified as the earliest land plants [22] and a widely held view of land plant relationships places liverworts as the first branch of the land plant tree of life [23]. Complex thalloid liverworts are the second diverging lineage within their taxa [24] with rates of *rbcL* sequence evolution much lower than in other liverworts [24- 25].

*Dumortiera hirsuta* is characterized with thallus weakly differentiated into layers, with vestigial air chambers and air pores absent or few near the thallus apex; and ventral scales in 2 rows, without appendages. While, *Targionia hypophylla* has differentiated thallus, with simple air pores; ventral scales in 2 rows, with 1 appendage; sporophytes ventral at the thallus apex; and involucres bivalve. On the other hand, *Monoclea forsteri* with undifferentiated thallus, air chambers and air pores absent; ventral scales absent, but with stalked mucilage papillae ventrally and *Wiesnerella sp.* with differentiated thallus, with simple air pores; ventral scales in 2 rows, with 1 appendage; perigonial chambers aggregated in terminal cushions on the thallus [9].

Genera with elaborate carpocephala were aligned with acarpocephala taxa, as in the case with *Wiesnerella* and *Targionia,* and *Dumortiera* and *Monoclea* [9]. Thus, this shows that there is high degree of incongruence between past morphology-based classification schemes of marchantiales and the phylogenetic relationships based on recent multi-locus analyses [24], with the molecular tree topology predominates in the combined analysis [20].

## CONCLUSION

The study identified and classified liverwort specimens collected from Imugan falls, Santa Fe, Nueva Vizcaya using morphological and molecular approaches. Liverworts collected were identified as *Dumortiera hirsuta* and *Targionia hypophylla*, both with 99% identity. The complexity of liverworts' DNA caused difficulties in PCR protocol optimization and because of this the researcher concluded that this is due to numerous cytosine and guanine present in its DNA sequence.

The phylogenetic tree revealed that the proximate clade was made up of complex thalloid genera and comprises the monophyletic *Dumortiera hirsuta* and *Wiesnerella sp.* in a robust sister relationship and sister to the remainder *Targionia hypophylla* and *Monoclea forsteri.*

The main evolutionary trend of its taxa leads to reduction and simplification of traits based from molecular approach, whereas a trend from simple towards complex traits prevails in the morphological tree. Thus, this shows that there is high degree of incongruence between past morphology-based classification schemes of marchantiales and the phylogenetic relationships based on recent multi-locus analyses, with the molecular tree topology predominated in the combined analysis.

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