

Research Article

The Use of DNA Barcoding and Phylogenetic Analysis to Improve Identification of *Usnea* spp. Based on ITS rDNA

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ABSTRACT

Lichen of the genus *Usnea* is quite common being used as a traditional herbal remedy. This genus is characterized by thallus, which is very similar among the species, leads to some difficulties in distinguishing them. In Indonesia, such research report on the availability of this genus based on their morphological characteristic is minimal. This might be due to too high morphological similarities among them. The molecular character, which is based on the DNA Barcode of Internal Transcribed Spacer (ITS) rDNA sequences, with its conserved region (5.8S) and varied region (ITS1 and ITS2), are becoming essential characters on identifying as well as analyzing the phylogenetic. The current study then proposed to identify and draw the species dendrogram of species within the *Usnea* genus obtained from Mount Lawu Forest of Central Java and Turgo Forest of Yogyakarta based on their phylogenetic and phenetic analysis. The dendrogram was constructed with UPGMA using the simple matching coefficient, whereas the phylogenetic tree was constructed with Maximum Likelihood (ML) using Kimura-2 parameter with 1000 bootstrap. The data were unable to draw phenetic relationships among the subgenus *Usnea* and *Eumitria* members. The phylogenetic tree shows the primary two clades, distinguishing the subgenus *Usnea* and *Eumitria*. The ITS rDNA sequence was able to identify most of the *Usnea* species.

Keywords: *Usnea*, DNA Barcode, ITS rDNA, Phylogenetic

INTRODUCTION

Lichen is an outstanding successful group of symbiotic organisms comprised of algae (phycobiont) and fungal (mycobiont) (Zachariah & Varghese 2018). Lichen of the genus *Usnea* is used as traditional herbal remedies in Solo and Yogyakarta, with of local name of "Kayu Angin". The local people collect the thallus from the forests of Turgo Hill and Mount Lawu. They use *Usnea* as one component of all herbal medicines, such as cholesterol, diabetes, gout, maternity, high blood pressure, skin, clods, and heart disease (Jannah 2019a).

Usnea (Parmeliaceae) has been distinguished with approximately 600 species worldwide (Hawksworth et al. 1995). Articus (2004) grouped the

Usnea members as inadequate taxonomy since they have many similarities among the species. The thallus of this genus is very similar among species. At this level, there is exceptionally high plasticity on their morphological characters to respond to environmental factors, which leads to a very complicated effort in drawing a clear boundary among the species (Clerc 1998). The situation leads to species identification limitation based on their morphological characters but not on the genus' family.

Divakar et al. (2006) stated there were sister species cases due to high similarities between two species of *U. florida* and *U. subfloridana*. These two species could only be distinguished on the presence or absence of their reproductive organs where *U. florida* has more sexual organs than another. The reproductive organ, such as apothecia, is an essential character for species-level identification, but the prolonged growth during the life phase becomes an obstacle to identification (Clerc 1998; Swinscow & Krog 1978). Based on the above facts, molecular analysis with DNA Barcodes is needed to strengthen and support species identification quickly, precisely, and accurately.

Research on the identification of *Usnea* in Indonesia using DNA barcodes from ITS rDNA has not been reported yet. However, the ITS rDNA region has been used extensively in the study of lichenized fungi overseas, including in assessing the species boundaries as well as testing the correlation between genetic and morphological diversity in species complexity (White et al. 1990; Korabecna et al. 2007; Del-Prado et al. 2010; Kelly 2011; Jannah 2019b). The molecular characters based on Internal Transcribed Spacer (ITS) rDNA sequences that have conserved regions (5.8S) and varied regions (ITS1 and ITS2) are needed to strengthen and support the identification and phylogenetic analysis (Articus et al. 2002; Ohmura 2002). The objective of this research was to carry out a phylogenetic analysis of *Usnea* species.

MATERIALS AND METHODS

Materials

The samples used in this research were fresh thallus obtained through the explorative method. A total of 16 examined specimens in the present study were collected at Mount Lawu Forest (LW) (East Java) and Turgu Hill Forest (I) (Yogyakarta) (Table 1).

Table 1. List of *Usnea* spp. examined and location of origin.

| No. | Species | Accession Number | Origin |
|-----|------------------------|------------------|-------------------|
| 1 | <i>U. himalayana</i> | LW1 | Mount Lawu Forest |
| 2 | <i>U. himalayana</i> | LW2 | Mount Lawu Forest |
| 3 | <i>U. pectinata</i> | LW3 | Mount Lawu Forest |
| 4 | <i>U. rubrotincta</i> | LW4 | Mount Lawu Forest |
| 5 | <i>U. himalayana</i> | LW5 | Mount Lawu Forest |
| 6 | <i>U. fragilesceus</i> | LW6 | Mount Lawu Forest |
| 7 | <i>U. baileyi</i> | LW7 | Mount Lawu Forest |

Table 1. Contd.

| No. | Species | Accession Number | Origin |
|-----|--------------------------|------------------|-------------------|
| 8 | <i>U. nidifica</i> | LW8 | Mount Lawu Forest |
| 9 | <i>U. fragilescens</i> | LW9 | Mount Lawu Forest |
| 10 | <i>U. nidifica</i> | LW10 | Mount Lawu Forest |
| 11 | <i>U. himalayana</i> | LW11 | Mount Lawu Forest |
| 12 | <i>U. baileyi</i> | T1 | Turgo Hill Forest |
| 13 | <i>U. bismolliuscula</i> | T2 | Turgo Hill Forest |
| 14 | <i>U. baileyi</i> | T3 | Turgo Hill Forest |
| 15 | <i>U. bismolliuscula</i> | T4 | Turgo Hill Forest |
| 16 | <i>U. bismolliuscula</i> | T5 | Turgo Hill Forest |

Methods

The *Usnea* thallus' total DNA was extracted using a modified CTAB method that we developed by adding High Salt-TE. It is challenging to get pure DNA from the *Usnea* thallus since there has never been a manuscript on DNA isolation from lichens in Indonesia. The following is the DNA isolation method that we developed. Total DNA of lichen was extracted from the thallus using a modified CTAB method. As many as 0.05 gr of *Usnea* thallus was powderized with additional liquid nitrogen. The powders are transferred into a 1.5 ml tube, added 400 µl of pre-heated 2X CTAB buffer, and incubated for 65 °C for 10 minutes. Then, added 400 µl of chloroform/isoamyl alcohol solution 24:1, homogenized gently for 5 minutes at room temperature, then centrifuged at 12,000 rpm for 1 minute. The supernatant, in turn, was added with 250 µl isopropanol and centrifuged speed of 12,000 rpm for 1 minute. The supernatant was removed, added 100 µl of High Salt-TE, incubated at 65 °C until dissolved, then added 800 µl 100% ethanol, incubated at -20°C for 15 minutes, centrifuged 15,000 rpm 15 minutes at 4°C and the supernatant removed and the pellet dried. After the pellet was added with 300-500 µl of 70% ethanol added, centrifuged at 15,000 rpm for 3-5 minutes 4°C, the supernatant was removed, and the pellet was drained. A volume of 300-500 µl 70% ethanol was added into the pellet, centrifuged 15,000 rpm for 3-5 minutes at 4°C, and the pellet is dried again. Finally, the pellet was dissolved by adding 50 µl of buffer pH TE 8. The purified DNA is stored at -20 °C.

Fungal nuclear ITS rDNA was amplified using the primer ITS 1 and ITS 4 (White et al. 1990). The PCR amplifications using KAPPA 2G™ Fast ReadyMix (2x) were performed with a program of initial denaturation for 2 min at 95°C, followed by 25 cycles of 0.5 min at 95°C, 0.5 min at 56.3°C, 1 min at 72°C, and a final elongation for 1 min at 72°C. The PCR product was electrophoresed in 1% agarose gel stained with 1 µl good view at 50 Volt for 40 minutes and visualized through UV-trans illuminator. The sequencing process was carried out at First Base, Singapore, through the PT Genetika Science Indonesia service.

The ITS rDNA sequence was analyzed and edited using Bioedit and DNA Baser. The homology level was determined through BLAST online. The sequence alignment was analyzed using Clustal-X and nucleotide similarity was executed through Phytidit. The phylogenetic tree reconstruction was carried out using Maximum Likelihood (ML) with Kimura-2 parameter and 1000 bootstrap replications on MEGA-5.05. The Automatic Barcode Gap Discovery (ABGD) was carried out through <http://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html> using Jukes-Cantor (JC69) distance.

RESULTS AND DISCUSSION

Seven species were found based on morphological characters, namely *U. pectinata* (LW3), *U. rubrotincta* (LW4), *U. himalayana* (LW1, LW2, LW5, LW11), *U. fragileszens* (LW6, LW9), *U. nidifica* (LW8, LW10), *U. baileyi* (LW7, T1, T3), and *U. bismolliuscula* (T2, T4, T5). These species of *Usnea* come from subgenus *Usnea* (Figure 1) dan *Eumitria* (Figure 2).

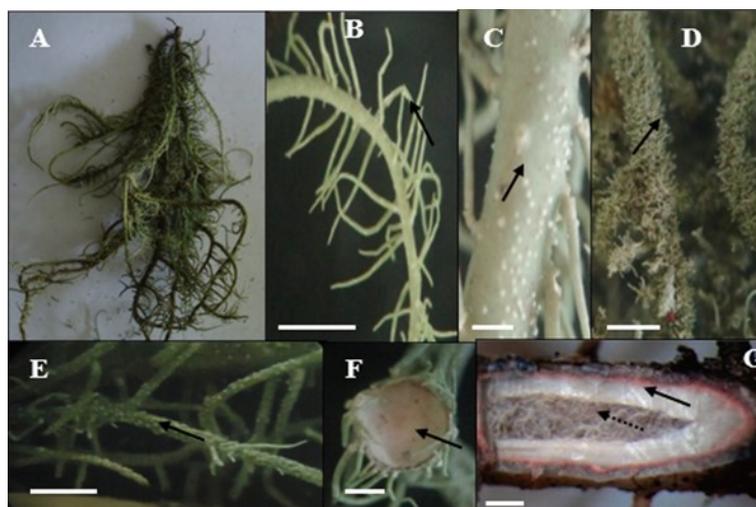


Figure 1. Morphological structure of *U. baileyi* (subgenus *Eumitria*). A. thallus, B. fibril, C papillae, D. isidia, E. soralia, F. apothecia, G. compact medulla & fistulose central axis. Bar=0.5 mm.

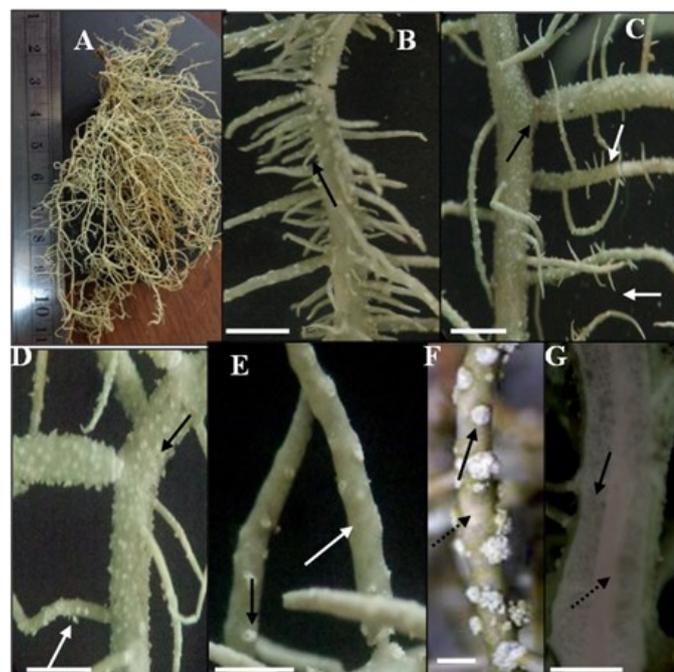


Figure 2. Morphological structure of *U. fragileszens* (subgenus *Usnea*). A. thallus, B. fibril, C. branch, D. white isidia, and black papillae, E-F. soralia, F. apothecia, G. loose medulla & solid central axis. Bar=0.5 mm.

The dendrogram generated from morphological characters showed that all specimens are divided into two large clades (clade A and B), separating *U. baileyi* (clade A) and the other *Usnea* species (clade B) (Figure 3). Morphologically, *U. pectinata* is closely related to *U. bismolliuscula*, *U. rubrotincta* is closely related to *U. fragilescens*, *U. himalayana* is closely related to *U. nidifica*, and *U. baileyi* is distantly related to those clustered in clade B.

The morphological character distinguishing both clades is the central axis type. Clade A consists of *U. baileyi* with a fistulose central axis, while the clade B member has a solid central axis. The morphological differences between the subgenus *Usnea* and *Eumitria* are not always apparent in some species. *Usnea pectinata* and *U. baileyi* belong to the subgenus *Eumitria* but both are separated into distinct clades because *U. pectinata* having a solid central axis. The clustering result based on morphological characters in this study could not reflect the overall relationship among *Usnea* species. It can not separate the member of subgenus *Usnea* and *Eumitria* accordingly.

Sokal et al. (1963), stated that the phenetic approach in determining the relationship between individuals is based on the existing similarity of characteristics without comparing the characters that are homology (characters inherited from ancestors to their descendants) and homoplasy (characters obtained as a result of the adaptation to the same environment). The existence of convergent evolution is obtained because of the adaptation process to the same habitat conditions and divergent evolution that makes the gains different forms from the same ancestor due to different environmental habitats resulting in the phenetic method almost unable to describe the true relationship among species (Campbell et al. 2008).

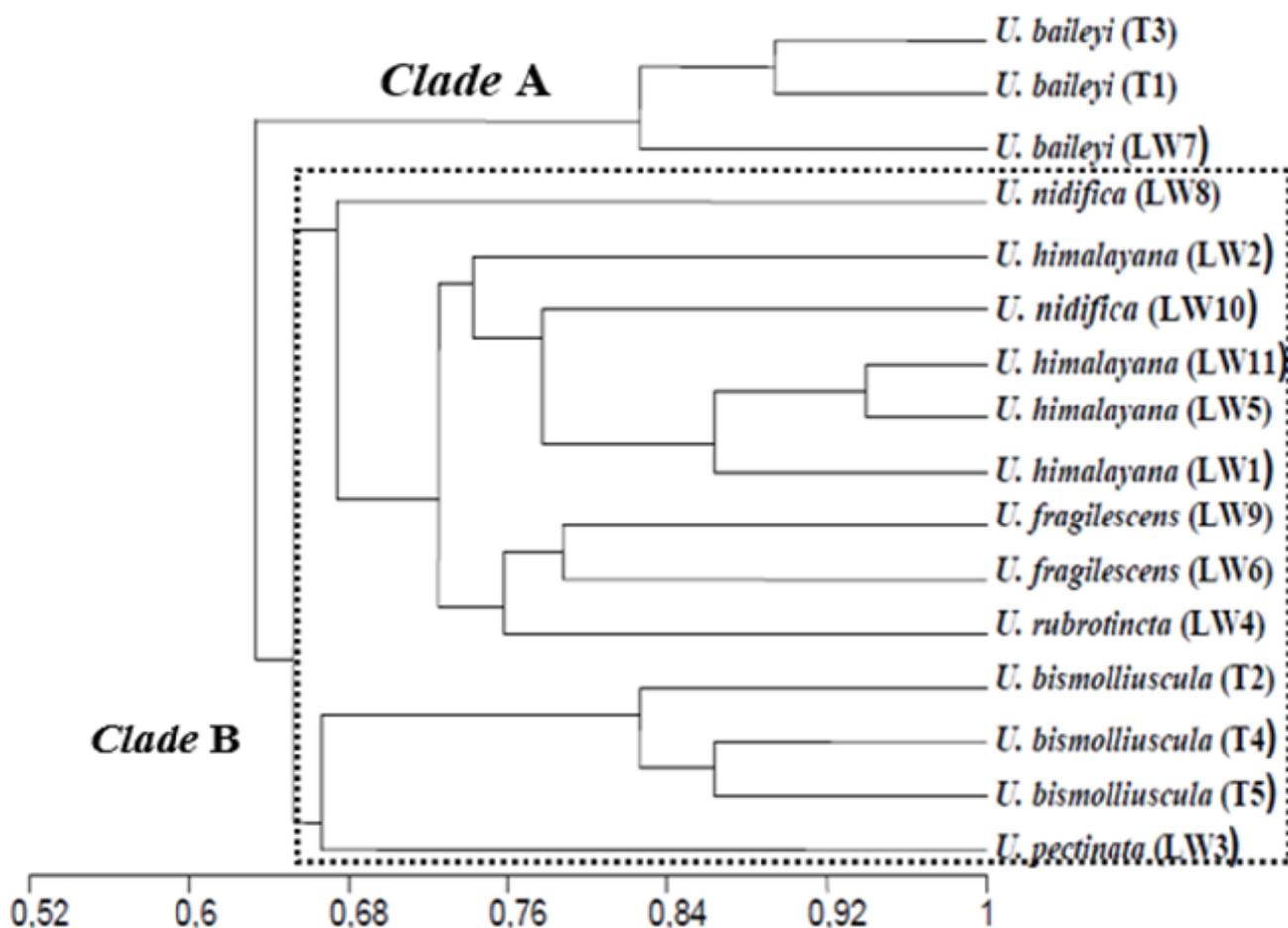


Figure 3. The dendrogram of *Usnea* is based on the UPGMA method using Simple Matching Coefficient.

The identification of *Usnea* used by the people around Yogyakarta and Solo, which comes from Turgo Hill forest and Mount Lawu forest, was carried out based on molecular data inferred from the Internal Transcribed Spacer (ITS) rDNA sequence. ITS rDNA sequences are widely used for species-level identification, capable of differentiating inter and intra-species, and determine the relationship of species through the differences of the conserved region and the similarity of variable region (White et al. 1990; Korabecna et al. 2007; Del-Prado et al. 2010; Kelly 2011).

The amplification of ITS rDNA from the *Usnea* genome was successful. The result of PCR visualized using agarose shows a clear strong band, which means that the ITS rDNA has been successfully amplified. ITS sequence length is ± 559 bp. The results of the PCR products that have been obtained and are under the target sequence length (ITS rDNA) are then sequenced to read the ITS rDNA nucleotide sequence from each sample (Figure 4). The sequencing results were then analyzed with BLAST software on the NCBI Gene Bank to determine the homology level of the ITS rDNA sequences obtained from the Gene Bank database, besides being able to show that the sequence obtained was true ITS rDNA. This can be proven if the homology level of the ITS rDNA sequence obtained with the ITS rDNA sequence in Gene Bank shows high compatibility between 94-99%. This high compatibility means that the target sequence obtained is the ITS rDNA sequence lichen genus *Usnea*.

The phylogenetic tree was constructed using Maximum Likelihood (ML) and 1000x bootstrap methods. This method is used to identify differences in genetic distance and analyze the similarity between samples. The ITS rDNA sequence was able to identify most of the *Usnea*, but do not support the separation of some species in *Usnea*. The topology of the phylogenetic tree that was formed showed that ITS rDNA was able to separate species in each sub-genus into groups to form one clade. In the results of the phylogenetic tree reconstruction, it is clear that the species included in the *Usnea* subgenus are grouped into one clade (monophyletic), and separate from the *Eumitra* subgenus (Figure 4). The results of this study indicate that the genetic distance between *Usnea* species is meager. The lowest genetic distance of 0 is found in *U. pectinata* and *U. baileyi* (T1 and T3) (Table 2).



Figure 4. Results of 496 bp ITS rDNA sequences (1-188 bp for ITS1, 189-346 5.8S, & 347-496 for ITS2) of *U. baileyi*.

Table 2. Genetic distances of *Usnea* spp. based ITS rDNA sequences.

| No | Specimen | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|----|-------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | <i>U. himalayana</i> (LW1) | | | | | | | | | | | | | | | |
| 2 | <i>U. himalayana</i> (LW2) | 0,002 | | | | | | | | | | | | | | |
| 3 | <i>U. pectinata</i> (LW3) | 0,123 | 0,121 | | | | | | | | | | | | | |
| 4 | <i>U. himalayana</i> (LW11) | 0,006 | 0,008 | 0,131 | | | | | | | | | | | | |
| 5 | <i>U. bismolliuscula</i> (T5) | 0,079 | 0,077 | 0,138 | 0,086 | | | | | | | | | | | |
| 6 | <i>U. himalayana</i> (LW5) | 0,000 | 0,002 | 0,123 | 0,006 | 0,079 | | | | | | | | | | |
| 7 | <i>U. baileyi</i> (LW7) | 0,113 | 0,111 | 0,032 | 0,116 | 0,128 | 0,113 | | | | | | | | | |
| 8 | <i>U. fragilesceus</i> (LW9) | 0,066 | 0,063 | 0,125 | 0,063 | 0,059 | 0,066 | 0,110 | | | | | | | | |
| 9 | <i>U. baileyi</i> (T1) | 0,123 | 0,121 | 0,000 | 0,131 | 0,138 | 0,123 | 0,032 | 0,125 | | | | | | | |
| 10 | <i>U. bismolliuscula</i> (T2) | 0,079 | 0,077 | 0,138 | 0,086 | 0,000 | 0,079 | 0,128 | 0,059 | 0,138 | | | | | | |
| 11 | <i>U. baileyi</i> (T3) | 0,123 | 0,121 | 0,000 | 0,131 | 0,138 | 0,123 | 0,032 | 0,125 | 0,000 | 0,138 | | | | | |
| 12 | <i>U. bismolliuscula</i> (T4) | 0,079 | 0,077 | 0,138 | 0,086 | 0,000 | 0,079 | 0,128 | 0,059 | 0,138 | 0,000 | 0,138 | | | | |
| 13 | <i>U. nidifica</i> (LW8) | 0,004 | 0,002 | 0,123 | 0,010 | 0,079 | 0,004 | 0,113 | 0,065 | 0,123 | 0,079 | 0,123 | 0,079 | | | |
| 14 | <i>U. nidifica</i> (LW10) | 0,019 | 0,017 | 0,123 | 0,025 | 0,084 | 0,019 | 0,113 | 0,063 | 0,123 | 0,084 | 0,123 | 0,084 | 0,019 | | |
| 15 | <i>U. fragilesceus</i> (LW6) | 0,066 | 0,063 | 0,125 | 0,063 | 0,059 | 0,066 | 0,110 | 0,000 | 0,125 | 0,059 | 0,125 | 0,059 | 0,065 | 0,063 | |
| 16 | <i>U. rubrotincta</i> (LW4) | 0,070 | 0,068 | 0,125 | 0,072 | 0,067 | 0,070 | 0,103 | 0,041 | 0,125 | 0,067 | 0,125 | 0,067 | 0,070 | 0,068 | 0,041 |

The ABGD species delimitation method retrieved four “initial partitions” and five “recursive partitions” with all samples of *U. bismolliuscula* are monospecific. Only when values of intraspecific divergence (P) were lower than 0.0046 was a higher number of groups suggested (5) and only by the recursive analysis resulting in the split of *U. nidifica* in two different lineages; both represented the specimens from Lawu (Table 3, Figure 5).

The topology of the phylogeny based on ITS rDNA sequences showed that within the clade of subgenus *Usnea*, *U. himalayana* is closely related to *U. nidifica* and *U. bismolliuscula* is more closely related to *U. rubrotincta* than *U. fragilesceus*. Within the clade of subgenus *Eumitria*, *U. pectinata* is closely related to *U. baileyi* (Figure 6).

In the *Usnea* subgenus clade, it shows *U. himalayana* and *U. nidifica* to form one clade, which means that among these species, they have the same ancestor (monophyletic) supported by a very high bootstrap value of 99%. The bootstrap shows that *U. himalayana* is very closely related to *U. nidifica*. The phylogenetic topology shows *U. himalayana* (LW 2) separated from its fellow species and forming a clade with *U. nidifica* (LW8), which is also separated from fellow species *U. nidifica* (LW 10). This indicates that *U. himalayana* (LW2) and *U. nidifica* (LW 10) began to navigate from their fellow

Table 3. The specific composition of the four and five groups hypotheses derived from ABGD analysis.

| Group | Four group hypothesis species composition | Group | Five group hypothesis species composition |
|-------|--|-------|---|
| 1 | <i>U. himalayana</i> (LW1), <i>U. himalayana</i> (LW2), <i>U. himalayana</i> (LW11), <i>U. himalayana</i> (LW5), <i>U. nidifica</i> (LW8), <i>U. nidifica</i> (LW10) | 1 | <i>U. himalayana</i> (LW1), <i>U. himalayana</i> (LW2), <i>U. himalayana</i> (LW11), <i>U. himalayana</i> (LW5), <i>U. nidifica</i> (LW8) |
| 2 | <i>U. pectinata</i> (LW3), <i>U. baileyi</i> (LW7), <i>U. baileyi</i> (T1), <i>U. baileyi</i> (T3) | 2 | <i>U. pectinata</i> (LW3), <i>U. baileyi</i> (LW7), <i>U. baileyi</i> (T1), <i>U. baileyi</i> (T3) |
| 3 | <i>U. bismolliuscula</i> (T5), <i>U. bismolliuscula</i> (T2), <i>U. bismolliuscula</i> (T4) | 3 | <i>U. bismolliuscula</i> (T5), <i>U. bismolliuscula</i> (T2), <i>U. bismolliuscula</i> (T4) |
| 4 | <i>U. fragilesceus</i> (LW9), <i>U. fragilesceus</i> (LW6), <i>U. rubrotincta</i> (LW4) | 4 | <i>U. fragilesceus</i> (LW9), <i>U. fragilesceus</i> (LW6), <i>U. rubrotincta</i> (LW4) |
| | | 5 | <i>U. nidifica</i> (LW10) |

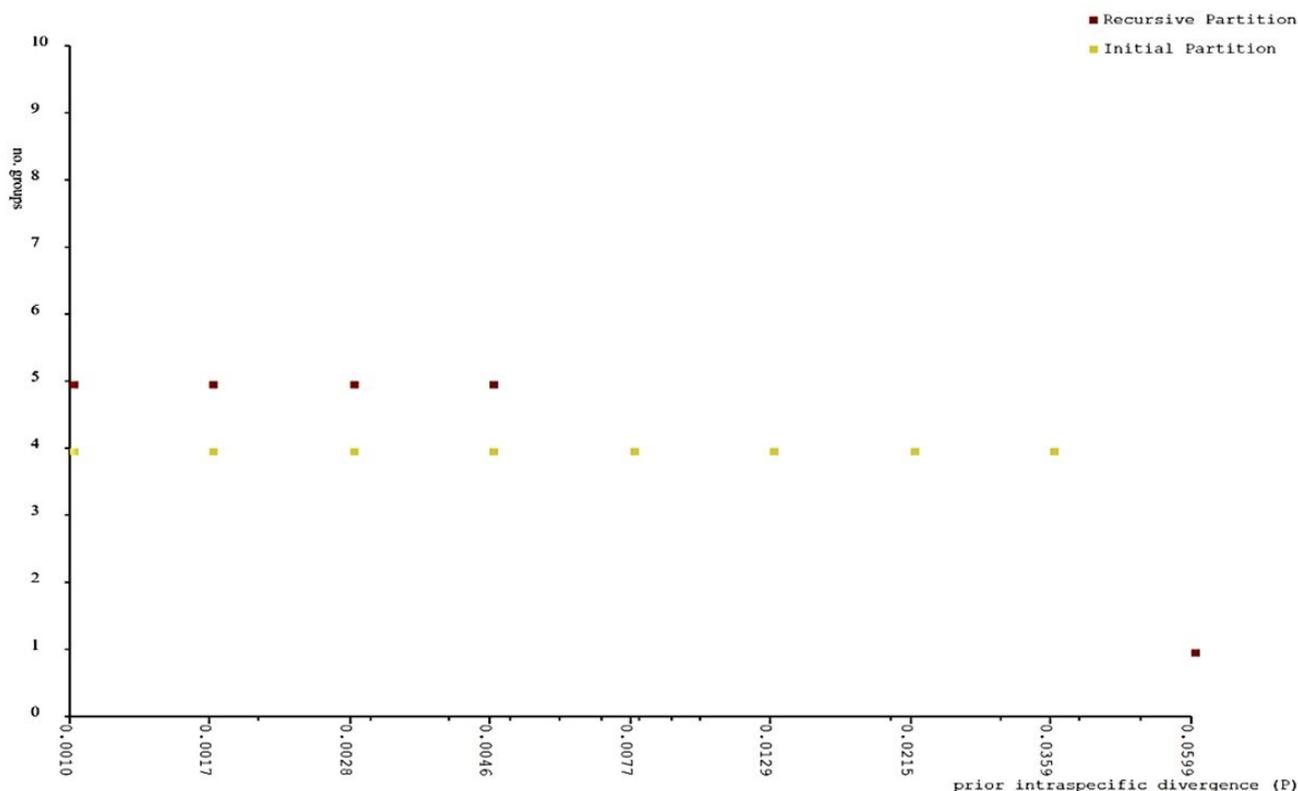


Figure 5. Results of the Automatic Barcode Gap Discovery (ABGD) analysis showing the four partitions (initial) also 5 and 1 (recursive) recovered. Nearly all partitions suggested the different number of species (\neq No of groups) with all representatives of *U. bismolliuscula* rendered monospecific. Only recursive partitions rendered a higher number of species (5) when P values were ≤ 0.0046 .

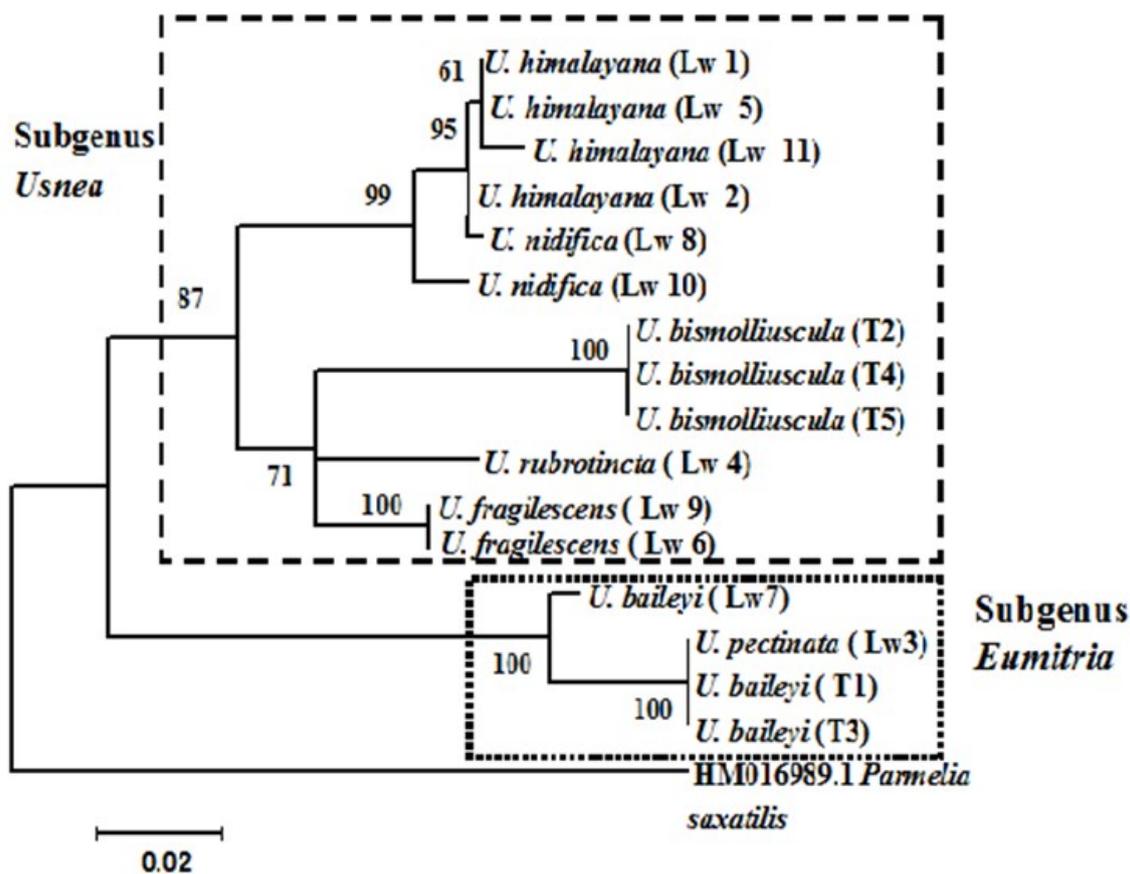


Figure 6. Phylogram obtained from a Maximum Likelihood analysis, evolution model Kimura-2 parameter with 1000 bootstrap replications, showing the phylogenetic relationship among *Usnea* based on internal transcribed spacer (ITS) rDNA.

species. This is in line with the opinion of Ohmura (2012) which stated that *U. himalayana* and *U. nidifica* have very close genetic relationships both morphologically and molecularly. The morphology between *U. himalayana* and *U. nidifica* is only distinguished by the absence of soralia in *U. himalayana*.

The alignment results of 515 bp ITS rDNA of *U. himalayana* with *U. nidifica* showed that there were substitutions, namely ten transitions and three transversions (72, 486, and 490 base positions) (Table 4). The alignment results showed the insertion of 12 nucleotide *U. himalayana* (LW 1) (TTCTACGTCGGT) in the 79th to 90th base positions (Table 5).

The subgenus *Eumitria* clade consists of 4 specimens (LW3, LW7, T1, and T3), which are divided into two species, namely *U. pectinata* and *U. baileyi*. The phylogenetic tree topology formed shows that *U. baileyi* (T1 and T3) formed one clade with *U. pectinata* (LW3) supported by a very high bootstrap value of 100%. Instead, *U. baileyi* separated from its fellow species and was supported by a value high bootstrap of 100%. It can be said that, based on ITS rDNA, *U. baileyi* (T1 and T3) is more closely related to *U. pectinata*, than to its fellow species (LW7). Ohmura (2002) stated that phylogenetic result based on ITS rDNA sequences strongly suggests that the close relationship between *U. pectinata* and *U. baileyi*.

Table 4. Nucleotide base substitution in ITS rDNA of *U. himalayana* and *U. nidifica*.

| Sample | Variation of Nucleotide Base | | | | | | | | | | | | |
|-----------------------------------|------------------------------|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 57 | 59 | 68 | 71 | 72 | 103 | 107 | 202 | 237 | 351 | 359 | 486 | 490 |
| LW 1 (<i>U. himalayana</i>) | C | G | T | A | C | G | T | G | G | C | T | G | G |
| LW 2 (<i>U. himalayana</i>) | . | . | . | . | . | . | C | . | . | . | . | . | . |
| LW 11 (<i>U. himalayana</i>) | T | . | . | . | . | . | . | . | . | . | C | . | T |
| LW 5 (<i>U. himalayana</i>) | . | . | . | . | . | . | . | . | . | . | . | . | . |
| LW 8 (<i>U. nidifica</i>) | . | . | . | . | . | . | C | . | . | . | . | T | . |
| LW 10 (<i>U. nidifica</i>) | . | A | C | G | G | A | C | A | A | T | . | . | . |

Note: green shows transition and red shows transversion.

Table 5. Insertion in ITS rDNA nucleotide bases of *U. himalayana* and *U. nidifica*.

| Sample | Variation of Nucleotide Base | | | | | | | | | | | | |
|--------|------------------------------|----|----|----|----|----|----|----|----|----|----|----|--|
| | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | |
| LW 1 | T | T | C | T | A | C | G | T | C | G | G | T | |
| LW 2 | - | - | - | - | - | - | - | - | - | - | - | - | |
| LW 11 | - | - | - | - | - | - | - | - | - | - | - | - | |
| LW 5 | - | - | - | - | - | - | - | - | - | - | - | - | |
| LW 8 | - | - | - | - | - | - | - | - | - | - | - | - | |
| LW 10 | - | - | - | - | - | - | - | - | - | - | - | - | |

Table 6. Nucleotide similarity in ITS rDNA of *U. baileyi* and *U. pectinata*.

| | <i>U. pectinata</i> (LW3) | <i>U. baileyi</i> (LW7) | <i>U. baileyi</i> (T1) | <i>U. baileyi</i> (T3) |
|---------------------------|---------------------------|-------------------------|------------------------|------------------------|
| <i>U. pectinata</i> (LW3) | --- | 15/499 | 0/499 | 0/499 |
| <i>U. baileyi</i> (LW7) | 96.99 | --- | 15/499 | 15/499 |
| <i>U. baileyi</i> (T1) | 100.00 | 96.99 | --- | 0/499 |
| <i>U. baileyi</i> (T3) | 100.00 | 96.99 | 100.00 | --- |

Table 7. Nucleotide Base Substitution in ITS rDNA of *U. baileyi* and *U. pectinata*.

| Sample | Variation of Nucleotide Base | | | | | | | | | | | | | |
|---------------------------|------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | 35 | 44 | 51 | 92 | 137 | 334 | 346 | 361 | 378 | 436 | 439 | 443 | 446 | 448 |
| <i>U. pectinata</i> (LW3) | G | C | T | A | A | C | T | G | A | T | T | T | T | T |
| <i>U. baileyi</i> (LW7) | T | T | C | G | G | T | C | A | G | A | C | C | A | C |
| <i>U. baileyi</i> (T1) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| <i>U. baileyi</i> (T3) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

Note: green shows transition and red shows transversion.

This statement is reinforced by the results of the similarity analysis of nucleotide bases, from the compared 494 nucleotide bases ITS rDNA, which shows that between *U. pectinata* and *U. baileyi* (T1 and T3) there is not a single difference in nucleotide bases. At the same time, *U. baileyi* (LW7) and its fellow species only had a 96.99% similarity of nucleotide bases (15 of 499 differences in compared nucleotide) (Table 6).

Usnea baileyi (LW7), which separates from its fellow species, is indicated by a rather long branch in the phylogenetic tree topology, indicating that this species is starting to experience divergence. The alignment result supports the divergence, showing the existence of substitution in the form of 11 transitions and three transversions (G-T, T-A) at the position of the 35th, 436th, and 446th nucleotide bases (Table 7).

The results of this study are in line with what Ohmura (2002) did, that *U. pectinata* and *U. baileyi* shared a common ancestor to form one clade, which was supported by a boost value of 99%. A similar case was reported by Articus et al. (2002), the unclear separation between *U. florida* and *U. subfloridana* also in *U. rigida* and *U. barbata*. Based on a phylogenetic approach using β -tubulin and ITS-LSU, *U. florida* and *U. subfloridana* clustered in one clade supported with high bootstrap values. This also happened to *U. barbata* and *U. rigida*, so the concept of species pair was proposed among them.

Based on the results of this study, it shows that in some *Usnea* species such as *U. himalayana* with *U. nidifica*, also in *U. pectinata* and *U. baileyi* indicates the starting of species divergence. It is necessary to carry out further comprehensive research so that the position of the taxon can be clearly seen. In the genus *Usnea*, only a few morphological characters are distinguished from one species to another, and most of them are only distinguished by one character. Some of them are only distinguished by their reproductive organs, whereas the environment very much influences the appearance of reproductive organs. The determination of the species name in the genus *Usnea* follows the concept of species presented by Motyka et al. (1936-1938)

in Clerc (1998), that was “A species is a strong character that has a little variation between them (1 character = 1 species)”. The result of this study needs further and thorough observation related to the nomenclature in the genus *Usnea*.

CONCLUSION

The ITS rDNA sequence can be utilized to strengthen the identification and investigation of relationships within the *Usnea* genus. The ITS rDNA sequence was able to identify most of the *Usnea* spp. However, it cannot distinguish between *U. baileyi* and *U. pectinata*. So that further research is needed by using more distinguishable sequences with a faster evolutionary rate so that differences in species of *Usnea* can be solved.

AUTHORS CONTRIBUTION

Contribution of the author: **M.J.** designed the research, collected and analyzed the data, and wrote the manuscript, **M.R.H.** analyzed the data and wrote the manuscript, **R.S.K.** and **N.S.N.H.** supervised all the processes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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