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Research Article

Clone Based Sequence for the Identification and Phylogenetic Study of Lichenized Fungi: A Case Study from *Usnea*

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ABSTRACT

Background: *Usnea* is a large genus in the family Parmeliaceae, with more than 350 species widely distributed in polar, temperate and tropical regions. **Aim:** The *Usnea* genus is recognized based on the fruticose thallus, branches with a cartilaginous central axis and usnic acid in the cortex. **Materials and Methods:** The phylogenetic relationships and the morphological variation among *Usnea* species have studied. The morphological characters traditionally used for species recognition of several European *Usnea* species analysed regarding their reliability. The evolution and distribution of the morphological characters looked to a phylogeny based on sequence data. It is easiest to obtain sequences from fresh *Usnea* material (not older than five years). DNA from the central axis has extracted to minimize the risk of contamination with lichen parasites. Since lichens are a combination of multiple organisms (fungi and algae), obtaining a single organism's DNA is difficult. These lichenized organisms cannot quickly be grown in axenic culture or manually teased apart from their associated microbial communities. It is a common phenomenon to observe multiple DNA bands following PCR caused by DNA from various organisms. **Results:** There is a greater chance of failure in the sequencing process. The cloning approach is the best one to check the sequence. To do the cloning, consider purifying at least five bands separately from the gel electrophoresis. Thus, get five clones to get an accurate picture of which sequences contained in the DNA. That way, even if the sequencing fails, you will still have the cloned products as a backup. Thus, clone-based sequencing is more efficient than that traditional sequencing methods. **Conclusion:** In the present study, we use this method for the phylogenetic interpretation of the genus *Usnea* and the results compared with the global data sets.

Keywords: Lichen, ITS, PCR, Phylogenetic, RAPD, Sequence.

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INTRODUCTION

Lichens are biologically distinct entities composed of fungus (mycobiont) with photosynthetic partners (photobiont), usually green algae or cyanobacterium. The algal partner synthesizes the food by photosynthesis and shares it with a fungal partner; in turn, the fungus protects the algae. They grow in diverse climatic conditions and on various substrates. The ability to quickly absorb and retain water from many sources makes it possible for lichens to live in harsh environments like deserts, Polar Regions and exposed surfaces like bare rocks, walls, roofs and tree branches. The photobiont is not known to reproduce sexually in the lichen state. However, the fungal partner is specific to the lichen taxon, so that the classification of lichens based on the sexual characteristic of the fungal partner.

Characteristic features and diversity of *Usnea*

The genus *Usnea* (Parmeliaceae, Ascomycota) recognized by fruticose (hair-like) thallus with a radial organization (Figure 1). An axis consisting of a cartilaginous strand of longitudinally arranged hyphae gives rise to many branches and the presence of usnic acid in the cortex. The variation in morphological characters such as the colour of the thallus, the thickness of the main branch and the thallus' length makes it difficult to distinguish one species of *Usnea* from another. According to Clerc,[1] morphological feature of *Usnea* that are constant and that do not change with the changing environmental conditions of the geographical area should be used to distinguish species of *Usnea*. Such characters include pigmentation of the basal part of the thallus, cortex and medulla; density of fibrils; the shape of the branches, branching type; and the ratio of Cortex, Medulla and Axis (C/M/A) in longitudinal section.

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Lichen genus *Usnea* is represented by ca. 350 species globally,[2] and India represent 60 species.[3] Motyka published the first world monograph of genus *Usnea* and divided *Usnea* into six subgenera *viz*., *Protousnea* Motyka, *Neuropogon* (Nees and Flot.) Motyka, *Lethariella* Motyka, *Chlorea* (Nyl.) Motyka, *Eumitria* (Stirt.) Motyka and *Euusnea* Jatta. All these subgenera later became independent genera except *Eumitria* (Stirt).[4] Motyka and *Euusnea* Jatta are the two genera combined and considered the genus *Usnea*. Different authors have discussed the delimitation of Usnea and the generic concept changed from time to time.[5-7] Many taxonomic uncertainties remain at the species level. Species identification of *Usnea* thought to be exceptionally difficult by most lichenologists because they are incredibly variable on morphology. The eco-phenotypes of the same species often look radically different.

General remarks on taxonomy and molecular work

The identification and taxonomic positioning of fungi, bacteria, viruses and parasites are essential for biologists interested in their diversity or conservation and others to help distinguish the organism of their interest (*e.g.*, plant pathologists, manufacturers of pharmaceutics). The conventional taxonomic approaches usually based on floral characteristics alone. Traditional methods like environmental sample culturing, laboratory identification by morphology and biochemical tests are still fundamental. Traditional microscopic techniques fail to identify the vegetative hyphae.[8] Such casual observations cause ambiguity in the classification of closely related species and populations. Molecular techniques were used to investigate genetic diversity and relationships among species. These data provide helpful tools for taxon delimitation, especially in plant groups in which the number of diagnostic morphological characters is limited.[9] Most of the biological identifications still carried out using traditional paper-based expertise, which has to be followed manually, although molecular methods are becoming more common.[10] While using this taxonomic key, the user makes a series of choices from a successive species group. This identification is only as good as the expert's observations who have compiled the key and its correct interpretation by the user.

Lichens are complex organisms formed by many fungi and algae species. To determine which one is more dominant or which one is most significant in the taxonomic perspective is a puzzling question generally we the lichenologist ask. Although most of the lichens are identified based upon the lichenized fungi, secondary fungi presence in the form of lichenicolous or as an endolichenic form is not rare, creating confusion performing molecular identification.

Gene sequences in Phylogeny

To infer phylogenetic and taxonomic relationships among lichens, both morphological and molecular data often considered. In recent years advances in technology and knowledge of gene sequences have significantly contributed to angiosperm phylogeny.[11] Biologists have utilized chloroplast, nuclear, mitochondrial genes to elucidate relationships at all levels of taxonomic rank. Molecular approaches for analyzing phylogeny have become increasingly valuable, especially where morphological characters have been insufficient to distinguish taxa at different levels.[12,13]

The application of molecular markers has been the most active research area in animal and plant systematic analysis. The analysis of the genetic changeability within and among populations of the species is crucial for understanding their future maintenance and developing their improvement strategies and conservation programs. Based on polymorphism found in proteins or DNA, molecular markers development has dramatically facilitated research in various disciplines such as taxonomy, phylogeny, ecology, genetics and plant breeding.[14]

There are several DNA-based marker systems for studying phylogeny, each with its pros and cons. These markers are phenotypically stable and are not prone to environmental

Figure 1: A and B Saxicolous and Corticolous *Usnea* species.

change.[15] In recent days, there has been constant recognition that the higher-order structure is fundamental for establishing meaningful structure-function relationships among biological macromolecules.[14-16]

One of the extensively used regions in a different organism's phylogenetic studies is the nuclear ribosomal DNA cistron.[17] The nuclear gene encoding the cytoplasmic ribosomal RNAs (rDNA) are in most eukaryotes organised into transcriptional units with a Small (18S/SSU), 5.8S and a Large (28S/LSU) subunit rDNA region, separated by internal transcribed spacer regions ITS1 and ITS2. The DNA sequences for LSU and SSU rDNA are under stabilising solid selection due to their critical role in ribosome synthesis. The non-coding region, like the ITS rDNA regions, is not under similar functional constraints. Several general features of the ITS region promotes its use for phylogenetic analysis of angiosperms. First, along with the other components of the nrDNA multigene family, the ITS region is highly repeated in the plant nuclear genome. The entire rDNA repeat unit is present in many thousands of copies arranged in tandem repeats at a chromosomal locus or multiple loci. This high copy number promotes detection, amplification, cloning and sequencing of nrDNA. Second and most importantly, this gene family undergoes rapid concerted evolution via unequal crossing over and gene conversion from phylogeny reconstruction. This property promotes Intra genomic uniformity of repeat units, even between nrDNA loci on non-homologous chromosomes. And in general, it also supports the accurate reconstruction of species relationships from these sequences.[17]

Internal Transcribed Spacer (ITS) region is the primary choice for molecular identification of fungi. It is two highly variable spaces ITS1 and ITS2 are usually species-specific, whereas the intercalary 5.8s gene is highly conserved. White *et al.*[18] designed several primers to sequence the ITS region widely used by phylogeneticists. Along with the development and application of molecular techniques, the Internal Transcribed Spacer (ITS) of rRNA has frequently been used to investigate phylogenetic relationships of *Usnea*. Due to the high evolution rate existing as size variation and sequence divergences in the ITS region, it became somewhat difficult to reliably construct alignments reflecting speciation relationships. Hence, some other primers should also be sequenced to get a clear picture.

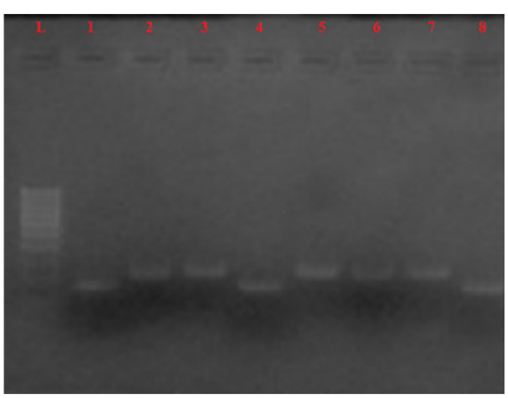

Figure 2: Gel Image Showing the DNA fragments obtained after the purification. L: 100 bp LADDER, Sample1-8.

The quantitative comparison among nucleotide sequences has recently become a useful tool to facilitate species identification.[19,20] However, several factors, such as variable evolution rates, divergent pseudogene copies, highly complex evolution and the ways to construct a phylogenetic tree may confirm the construction of evolutionary relationships. Like other phenotypic characters, rRNAs secondary structure has proposed a molecular marker for identification, classification and phylogeny of fungi.[21,22]

Previous molecular studies of *Usnea* mainly focused on the taxonomic status of some subgenera. The phylogenetic relationships among different species explored. The nucleotide sequence comparison used as the primary analysis method in these studies.[23,24] Here, our work concentrated on the application of morpho-molecular features to delineating phylogenetic groups and species determination. Species with close relationship share similar secondary structures, which indicate that the secondary structure of ITS2 is a helpful character in taxonomy and phylogeny of *Usnea*. The differences of the secondary structure appear relatively easy to be identified in contrast to nucleotide base comparison. Similar to morphological characters, the molecular structure is classified further to explore lichenological systematics.

We used ITS universal primers (ITS4 and ITS5) for the species delimitation, which is ideal for identifying species in lower taxonomic levels. Since lichens are also difficult to culture, the thallus' DNA is used directly for the PCR and sequencing studies. In the case of lichens, due to many organisms within the species, we got multiple bands of uncultured natural isolates in the PCR product. Although we purify the genetic materials using professional DNA purification Kits like Quegen Plant Mini-Kit, complete purification is not assured. In the gel electrophoresis, we got multiple bands indicating impurities or a mixture of the genetic materials. If we use these impure genetic materials for sequencing, there is a greater chance of failure of sequencing or mismatch of sequence. To overcome the sequence difficulties the clone-based sequencing was used. If we get more than one band and want to sequence them to check which one is the correct band, the best approach would be to clone-based sequencing technique.

MATERIALS AND METHODS

Sample collection and DNA isolation

For DNA isolation to identify the taxa and phylogenetic studies, the fruticose lichens were collected with more caution. Samples are collected from different areas in five replicates each. They were kept in plastic zip-lock covers and put in the icebox until bringing it to the lab. After returning to the laboratory, freshly collected materials are washed in distilled water and stored in 1.5 mL Eppendorf tubes in a -20ºC deep freezer until using for DNA extraction. Qiagen DNeasy® Plant Mini Kit isolates the DNA from the foliicolous lichens following the standard protocol (Qiagen-2012). Purification of the DNA obtained is tested by the OD260:OD280 ratios (the OD ratio for pure DNA is 1.8±1). The OD260 value measures DNA concentration (approx. 1.0 reading at OD260 is equivalent to 50 µg/mL).

Gel Electrophoresis

Agarose gel (0.8 g) powder is boiled in 100 mL of 0.5X TBE buffer for 1-2 min. 0.5 µg/mL of ethidium is added to this agarose solution and allowed to cool. Coombes is adjusted to about 0.5-1.0 mm above the plate with a stand in a gel tray to form wells for loading the DNA samples. Agarose solution poured onto the plate and the gels tray is allowed to cool to about a temperature of 40º-50ºC and allowed to polymerise for about 10-15 min (when ready, turns whitish and opaque). Coombes was carefully removed once the gel polymerised. Then the gel is placed in the electrophoresis tank containing 0.5X TBE buffer. About 6 µL of the genomic DNA mixed with 6 µL sterilised water and 2 µL of 10X loading dye and loaded in the wells, against 10 µL of the DNA marker Lamda DNA HindIII-EcoR1 double-digit as a standard to compare the band intensities. The setup is then covered with a lid and connected to a power supply. The DNA is allowed to diffuse with gel and runs for 1-2 hr at a constant voltage (80 V). After the electrophoresis completed power supply is turned off, the gel is removed and visualised and archived using Gel Documentation System. The presence of highly resolved high molecular weight bands confirms the excellent quality of DNA.

PCR Amplification

The ~700 bp Internal Transcribed Spacer (ITS) region amplified using a high-fidelity Polymerase Chain Reaction (PCR) polymerase. PCR amplification was performed by using universal primers (nuITSrDNA: ITS4-5'-GAAACCTTGTT AYGMCTD-3'and ITS5-5'-GGAAGTAAAAGTCGTNASAAGR-3'). For the samples in which full length (~650 bp) PCR does not work, internal ITS primers spanning ~300 bp are used. The PCR amplicon was column purified and cloned into a cloning vector (T-vector). TA cloning is one of the simplest and most efficient methods for the cloning of PCR products. The procedure exploits the terminal transferase activity of specific thermophilic DNA polymerases, including *Thermusaquaticus* (Taq) polymerase. Taq polymerase c has non-template dependent activity, which preferentially adds single adenosine to the 3'-ends of a double-stranded DNA molecule. Thus, most of the molecule's PCR amplified by Taq. Polymerase possesses single 3'-A overhangs. Using a linearised "T-vector" with single 3'-T overhangs on both ends allows direct, high-efficiency cloning of PCR products, facilitated by the complementarity between the PCR product 3'-A overhangs and vector 3'-T overhangs. Eco-RI and Hind-III sites used as identifiable restriction sites.

Master-mix for PCR amplification: DNA: 1 µL (100 ng), forward primer: 400 ng, reverse primer: 400 ng, dNTPs (2.5 mM each) 4 µL, ×10 Taq DNA polymerase assay buffer 10 µL, Taq DNA polymerase Enzyme (3 U/µL) 1 µL, water X µL to make up the total reaction volume: 100 µL. All reagents were of chromous make.

The following PCR cycle times set for the different processes: Initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 45 sec and a final extension at 72ºC for 5 min. The PCR amplified product was subjected to 1.2% agarose gel (with ethidium bromide) electrophoresis for base-pair size analysis.

DNA Cloning

To clone the genetic material, we used a PCR product cloning kit. In this method, we add our PCR product to the vector and ligate it in (the exact procedure varies, but it's straightforward). Effectively we will have cloned all the different products produced by our PCR. Then we perform colony PCR of >5 colonies and identify those who have the correct size product. We can then grow up the plasmid, miniprep it and sequence. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis. Cloning the amplicons on T-vectors or Gateway plasmids is easy. Then take five clones and extract plasmid DNA.

Sequencing

Cleaned DNA products sequenced in Chromous Biotech Pvt Ltd., Bengaluru. The same set of primers as for PCR (nu ITS rDNA: ITS4-5'-GAAACCTTGTTAYGMCTD-3'and ITS5-5'-GGAAGTAAAAGTCGTNASAAGR-3') were used for Sequencing. Sequencing the PCR amplified product was performed on ABI 3500×L Genetic Analyzer of Applied Biosystem Micro Amp, USA, using cycle sequencing kit and Big Dye Terminator Version 3.1. 10 µL of the sequencing analysis mixture contained 4 µL of Big Dye Terminator Ready Reaction Mix, 1 µL of PCR amplified product (100 ng/µL), 2 µL primer (10 pmol/λ) and 3 µLMilli-Q Water.

Analysis conditions for sequencing were programmed to-denaturation at 96ºC for 1 min, followed by 25 cycles of denaturation at 96ºC for 10 sec, hybridisation at 50ºC for 5 sec and elongation at 60ºC for 4 min. The resultant nucleotide sequence was analysed using the software Seq Scape version 5.2, which follows an analysis protocol of BDTv3-KB-Denovo_v 5.2.

Jukes-Cantor corrected distance model was used to generate a distance matrix. A minimum comparable position of 200 ignoring alignments inserts used. The phylogenetic tree was created using Weighbor with alphabet size 4 and length 1000 using the sequences aligned with a system software aligner SeqScape_v 5.2.

Sequence fragments assembled in Sequencher 5.2 (Gene Codes Corp., MD, USA) software. Further, the final phylogenetic tree obtained by performing a heuristic search using maximum likelihood and Bayesian analysis. Strength of the tree branching analysed by 1000 bootstrap repeats. For the statistical analysis and tree building of the sequencing data, Mr Base v 3.1.2, BEAST v 1.7, Fig Tree v 1.3.1 and tree view v 1.6.

RESULTS AND DISCUSSION

Analysing sequence for identification of species

The DNA sequence analysis using universal lichen (fungal) primers, species-specific primers and comparative Gene Bank searches provide specific identification of a more significant number of *Fusarium* and *Aspergillus* species. However, this would require the use of appropriate DNA target regions that display sufficient interspecies sequence variation without it being excessive. The DNA target regions examined for such purposes. In addition, the variable parts at the 5' of the 28S rRNA gene (D1-D2 region) and the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of rDNA have examined by other sequencing targets.[25-27] Once a sequence database is established, it has to be analysed further. As the first step, sequences compared with existing databanks (*e.g.*, Gene Bank, RDP and EMBL) perform a Basic Local Alignment Search Tool (BLAST) search in most cases. BLAST did online via the internet. As a result, it is possible to construe quickly whether the determined sequences need to align or not with other sequences. The public alignment package ClustalW produces good alignments from scratch, but other programs can be used as well.

A case study from *Usnea*

In this study, eight species of *Usnea* subjected to phylogenetic analysed. The DNA sequenced by both the conventional method and the clone-based method. Figure 2, we can see the photography of the gel image taken after running the purified DNA before sequencing. Thus, generated sequence data aligned. The aligned sequences used to create phylogenetic trees.

The cladograms for the eight species of *Usnea* created using conventional DNA sequencing data and clone-based sequence data. In both cladograms, the evolutionary history inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa.

Uses of Clone based sequencing

- They are rapid and require a tiny sample.
- Can be performed for environmental samples, i.e., without culturing.
- Meagre rate of sequence failure.
- Assurance of good quality sequence.
- It can be repeated easily.
- All the symbionts in the system (lichen) sequenced without any confusion or contamination.
- Detection of species is accurate and can get good BLAST result.

CONCLUSION

Although direct sequencing is much cheaper and more comfortable to carry out, comparatively, clone-based sequencing is the most efficient sequencing method. Next-gene sequencing also comes into the limelight, where the organisms' complete genomes are sequenced in the present days. In India, the next-gene sequencing is not yet completely into practice in all places and it is much costlier. Hence, clone-based sequencing is better than the blindfolded direct sequencing of organisms for the time being.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic acid; **rDNA:** Recombinant DNA; **LSU:** Large subunit; **SSU:** Small subunit; **ITS:** Internal Transcribed Spacer; **PCR:** Polymerase chain reaction; **RDP:** Ribosomal Database Project; **EMBL:** European Molecular Biology Laboratory; **BLAST:** Basic Local Alignment Search Tool.

SUMMARY

This study focuses on the Usnea genus, a large group within the Parmeliaceae family, known for its fruticose thallus and usnic acid presence. It examines the phylogenetic relationships and morphological variation across various Usnea species, particularly in Europe, assessing the reliability of traditional morphological characteristics used for species identification. The research highlights the challenges in extracting and sequencing DNA from these lichenized fungi due to their symbiotic nature with algae and susceptibility to contamination. A cloning approach

to sequencing was adopted to improve accuracy, involving the purification of multiple DNA bands from gel electrophoresis and subsequent cloning. This method, compared against traditional sequencing techniques, proves more effective in managing the complexities of lichen DNA and provides a robust framework for understanding the evolutionary and phylogenetic context of the Usnea genus, with findings aligned with global datasets.

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