Phylogenetic studies in *Ravenelia esculenta* and related rust fungi

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Abstract *Ravenelia esculenta* Naras. and Thium. is a rust fungus, which infects mostly thorns, inflorescences, flowers and fruits of *Acacia eburnea* Willd. Aecial stages of the rust produce hypertrophy in infected parts. DNA of the rust fungus was isolated from aeciospores by ‘freeze thaw’ method. 18S rDNA was amplified and sequenced by automated DNA sequencer. BLAST of the sequence at NCBI retrieved 96 sequences producing significant alignments. Multiple sequence alignment of these sequences was done by ClustalW. Phylogenetic analysis was done by using MEGA 3.1. UPGMA Minimum Evolution tree with bootstrap value of 1000 replicates was constructed using these sequences. From phylogenetic tree it is observed that *Ravenelia esculenta* and the genus *Gymnosporangium* share a common ancestry, though *Ravenelia esculenta* is autoecious on angiosperm and the genus *Gymnosporangium* is heteroecious with pycnia, aecia on angiosperm and uredia, telia on gymnosperm. Two major clades are recognized which are based on the nature of aecial host (gymnosperm or angiosperm). These clades were also showing shift from pteridophytes to angiosperms as telial hosts. The tree can be interpreted in the other way also where there is separation of 14 families of Uredinales depending upon nature of teliospores, nature of aeciospores and structure of pycnia. These studies determine the phylogenetic position of *Ravenelia esculenta* among other

rust fungi besides broad separation of Uredinales into two clades. These studies also show that there is phylogenetic correlation between molecular and morphological data. This is first report of DNA sequencing and phylogenetic positioning in genus *Ravenelia* from India.

Keywords Hypertrophy · Rust · Aeciospores · 18S rDNA · Minimum Evolution.

Introduction

Ravenelia esculenta Naras and Thirum is a rust fungus that infects *Acacia eburnea* Willd. causing hypertrophy in leaves, thorns, inflorescences, flowers and fruits¹. The rust is common in dry regions of Asia, Africa and America. The genus *Ravenelia* is a poorly studied genus and has been placed under Raveneliaceae on the basis of compound teliospores¹. The genus *Ravenelia* is the third largest genus of rust fungi with approximately 200 known species² of which about 20 produce abnormal growth on their hosts. *Ravenelia esculenta* is of particular interest as it produces hypertrophy and can represent a source for natural phytohormones.

The relationships between various rust fungi have traditionally been inferred based on morphology, host specificity, symptomatology, histopathology etc². These approaches though primarily important are problematic as relatively few characters delineate species occurring on a particular host. Also there are several anamorphic genera in rusts, like *Uredo*, *Aecidium*, *Peridermium*, *Aecidiolum*, *Caeoma* for which either aecial or telial hosts are unknown². These reasons in addition to the complexity in physiological specialisation, the small number of morphological characters

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and variations in the life cycle patterns found among rust fungi, pose problems for their phylogenetic analysis.

To avoid the impact of these variations, the characters that are independent of rust morphology and host range are required. PCR and sequencing of specific DNA fragment provides such data. The advantages of using DNA data for molecular analysis have been discussed by other authors^{3,4}.

The study was undertaken because the genus *Ravenelia* as a whole and *R. esculenta* in specific have never been analysed before for phylogenetic relationship within the family and with other rust fungi.

This study was undertaken to establish phylogenetic relationship of *Ravenelia esculenta* with other rust fungi.

This is one of the very few reports DNA sequencing in genus *Ravenelia* (www.ncbi.nlm.nih.gov), and the only report in India. The molecular phylogenetic tree for *Ravenelia esculenta* and other related rust fungi producing significant alignments by BLAST is presented here.

Material and Methods

Collection of infected material

Sporulating stages of *Ravenelia esculenta* were collected randomly from the field. The material was kept in brown paper bags till processed.

DNA extraction

Aeciospores scraped under aseptic conditions were used as a substrate for DNA extraction. DNA extraction was done by treating the spores with lyticase followed by 5–6 freeze – thaw cycles. DNA was precipitated by using DNAzol reagent (Gibco BRL).

Polymerase Chain Reaction (PCR)

The PCR reaction mixtures and conditions were as per the method described by White *et al.*⁵ 18S rDNA region was amplified by using NS1 (5'GTAGTCATA TGCTTGTCTC3', Tm 49°C) and NS8 (5'TCCGCAG-GTTACCTACGGA3', Tm 59°C) primers⁵. Primers NS3 (5'GCAAGTCTGGTGCCAGCAGCC3' Tm 68°C), NS4 (5'CTTCCGTCAATTCCTTTAAG3' Tm 56°C) and NS5 (5'AACTTAAAGGAATTGACGGAAG3' Tm 57°C), NS6 (5'GCATCACAGACCTGTTATTGCCTC 3' Tm 65°C) were used as internal primers to check the reliability of the sequence obtained. 30 cycles of PCR were carried out in a programmable thermal controller. The PCR products were checked on 1% agarose gel for amplification.

DNA sequencing

DNA sequencing was done by using dye terminator method in Hitachi 3310 automated DNA analyser.

Sequence analysis

The DNA sequence was blasted at NCBI by using BLASTn (Nucleotide- nucleotide BLAST). The sequences showing similarity with the blasted sequence were retrieved and aligned by ClustalW⁶. For this multi-sequence alignment, progressive alignment method was used. In this, the most similar sequences, that is, those with the best alignment score were aligned first. Then progressively more distant groups of sequences were aligned until a global alignment is obtained. This heuristic approach is necessary because finding the global optimal solution is prohibitive in both memory and time requirements. The algorithm first computes a rough distance matrix between each pair of sequences based on pairwise sequence alignment scores. These scores were computed using the pairwise alignment parameters for DNA sequences. Next, the neighbor-joining method was used with midpoint rooting to create a guide tree, which was used to generate a global alignment. The guide tree serves as a rough template for clades that tend to share insertion and deletion features. This generally provides a close-to-optimal result, especially when the data set contains sequences with varied degrees of divergence, so the guide tree is less sensitive to noise. All these computations, with the specifications, were done by ClustalW⁶. The phylogenetic analysis and construction of Minimum Evolution tree was done by using MEGA3 software⁷.

Results and Discussion

The aligned dataset consisted of 96 taxa and 3205 characters of which 1171 characters were variable and 451 characters were parsim-informative.

Sequenced 18S rDNA was found to be 1704 bp long. The sequence is available online (GenBank Acc. No. DQ145756). The BLAST search at NCBI retrieved 96 sequences of taxa which produced significant alignment with the query sequence. Majority of these sequences are of rust fungi many of which produce hypertrophoid structures on the host at some stage during their life cycle.

The ME tree generated could be divided into two major clades (Fig. 1). These two clades represent all 14 families of Uredinales viz. Melampsoraceae, Cronartiaceae, Coleosporiaceae, Pucciniastraceae, Spherophragmiaceae, Chaconiaceae, Phakosporaceae, Pucciniaceae, Raveneliaceae, Uropyxidaceae, Pileolariaceae, Pucciniosiraceae, Phragmidaceae and Incertae Sedis.

Phylogenetic position of *Ravenelia esculenta* according to this tree falls in Clade II (Fig. 1). *Ravenelia esculenta* (Raveneliaceae) is associated with the genus *Gymnosporangium* which belongs to family Pucciniaceae.

There are major differences between these two genera. First, *Gymnosporangium* is heteroecious rust genus with aecial stages on angiosperms and telial stages on gymnosperms⁸. Second, the teliospores in *Gymnosporangium* are two celled². Third, the genus *Gymnosporangium* produces hypertrophy on its gymnosperm i.e. telial host^{2,9}. As opposed to this *Ravenelia esculenta* is an autoecious rust on angiosperm¹. The teliospores are in the form of compound heads and the genus produces hypertrophy during aecial stages¹. The only similarity between these two genera is production of hypertrophy at some stage during life cycle. Hence the phylogenetic position of *Ravenelia esculenta* is not well supported by its combination with *Gymnosporangium*. Nevertheless both *Gymnosporangium* and *Ravenelia esculenta* are distinct genera. *Gymnosporangium* has telial host as a gymnosperm (Cupressaceae, *Juniperus*, *Calocedrus* etc.). The aecial hosts of the genus are in Rosaceae. The majority of conifer rusts have aecial hosts in other families of gymnosperms. *Ravenelia esculenta* also stands separated from other angiosperm rusts in nature of its teliospores (Compound teliospores) and significant hypertrophy produced during aecial stages with predominant aecial cups easily visible on hypertrophoid organs (Plate 1).

Hence both genera, *Gymnosporangium* and *Ravenelia esculenta* are unrelated to their respective group of rusts (Conifer rusts and angiosperm rusts). Their placement in Clade II is therefore, supported by their distinct characters described above. Recent treatment of Sphaerophragmiaceae being synonym of Raveneliaceae is also supported by this cladistic analysis. Members from Sphaerophragmiaceae viz. *Triphragmium ulmariae* and *Nyssopsora echinata*, are closely associated with *Ravenelia esculenta*. This explains

Plate 1 Hypertrophy in *Acacia eburnea* due to infection of *Ravenelia esculenta*. Note prominent aecial cups.

the affinities of *Ravenelia esculenta* (Fig. 1). In addition, taxa with pteridophytes as telial hosts are grouped in Clade I. These placements indicate that telial hosts are phylogenetically more informative.

As more *Ravenelia sp.* sequences become available, the positioning of *Ravenelia* on evolutionary tree may become clearer.

The tree presented here can be interpreted in two ways to explain the broad phylogenetic relationships of rust fungi.

Firstly, this tree well explains the division of Uredinales into two major clades. Earlier taxonomic treatments also divided Uredinales into two families¹⁰. Monophyletic origin of Uredinales is also supported by this tree¹⁰.

Both these clades contain rust taxa that are either autoecious or heteroecious. But Clade I is represented by genera with pycnia and aecia mostly on gymnosperms. These rusts alternate with angiosperm or pteridophytes. The genera which alternate with pteridophytes, i.e., telial stages on pteridophytes (*Hylopsora*, *Uredinopsis* and *Milesina*)

Table 1 Aecial and telial hosts of rust taxa in clade I

Genus	Life Cycle Pattern	Aecial Host	Telial Host
<i>Melampsoridium</i>	Heteroecious	Gymnosperm	Angiosperm
<i>Cronartium</i>	Heteroecious	Gymnosperm	Angiosperm
<i>Endocronartium</i>	Autoecious	Gymnosperm	Gymnosperm
<i>Coleosporium asterum</i>	Heteroecious	Gymnosperm	Angiosperm
<i>Coleosporium paederiae</i>	Autoecious	Angiosperm	Angiosperm
<i>Chrysomyxa</i>	Heteroecious	Gymnosperm	Angiosperm
<i>Uredinopsis</i>	Heteroecious	Gymnosperm	Pteridophyte
<i>Milesina</i>	Heteroecious	Gymnosperm	Pteridophyte
<i>Hyalopsora</i>	Heteroecious	Gymnosperm	Pteridophyte

Fig. 1 ME tree showing separation of genera according to nature of aecial and pycnial host

Fig. 2 ME tree showing separation of genera according to morphological characters of spores and fruiting bodies

grouped together into two smaller clades basal to Clade I. These genera are predominantly found in temperate (*Milesina*) to North temperate (*Uredinopsis* and *Hylophora*) region of the world. Other taxa in Clade I (*Melampsoridium*, *Cronartium*, *Coleosporium asterum* and *Chrysomyxa*) are heteroecious and alternate between gymnosperm and angiosperm (Table 1). *Endocronartium* is believed to be an autoecious derivative of its closest relative *Cronartium* and infects hard pines (*Pinus radiata*). Rust taxa which are evolutionary more advanced occur on primitive hosts (i.e. Pteridophytes and Gymnosperms). This is in contrast with the argument that the primitive rust genera occur on primitive hosts. This argument is countered by Leppik¹¹ who described these rusts to be tropical or temperate in origin. The molecular phylogenetic tree presented here supports this view.

But there is only one exception of *Coleosporium paederiae* in Clade I (pycnia and aecia mostly on gymnosperm) which is autoecious and completes its entire life cycle on an angiospermic host.

Clade II is distinctly marked by presence of taxa which have aecia and pycnia always on angiosperms.

In Clade II, there are both autoecious and heteroecious taxa. Autoecious taxa are represented by most genera grouped at apical portion of Clade II (eg. *Triphragmium ulmariae*, *Nyssopsora echinata* (only uredial and telial stages are known), *Ravenelia esculenta*, *Racospermyces digitatus*, *Racospermyces koeae*, *Maravalia cryptostagiae*, *Prospodium tuberculatum* (only uredial and telial stages are known)) and *Uromycladium* complex from Pileolariaceae, *Puccinia* complex (for *Puccinia pelargonii-zonalis* only uredial and telial stages are known) and *Uromyces appendiculatus*. It is interesting to note that most of the genera at apical portion of Clade II, whether autoecious or heteroecious, have angiosperm as a host for both aecial and telial stages.

Thus from this first interpretation, it is seen that, heteroecious rust genera are most advanced (Clade I). The telial hosts are shifted from pteridophytes (*Uredinopsis*, *Milesina* and *Hylophora*) to angiosperms in advanced genera like *Melampsoridium* and *Cronartium*. More primitive genera are mostly autoecious with angiospermic host or if heteroecious, both aecial and telial hosts are angiosperms (Clade II) (eg. *Dasturella divina*) with an exception of *Gymnosporangium*.

In the second type of interpretation (Fig. 2), again two major clades can be observed. 14 families of Uredinales are divided in these two clades.

The Clade I has members from Melampsoraceae, Cronartiaceae, Coleosporiaceae and Pucciniastraceae. All of the genera in Clade I have invariably sessile teliospores. The pycnia in genera from Clade I are without bounding structures and aeciospores in these taxa are catenulate (except in

Pucciniastraceae where aeciospores are very rarely pedicillate). One genus from Incertae Sedis is included in Clade I, viz. *Peridermium ephedrae*, which has *Ephedra trifurca* as a telial host. Its cladistic association with *Pucciniastrum epilobii* is worth mentioning, although *Peridermium ephedrae* has gymnosperm as telial host and *Pucciniastrum epilobii* has angiosperm as telial host.

Remaining 10 families are grouped separately in Clade II. These include Sphaerophragmiaceae, Chaconiaceae, Phakosporaceae, Pucciniaceae, Raveneliaceae, Uropyxidaceae, Pileolariaceae, Pucciniosiraceae, Phragmidiaceae and Incertae Sedis. Recent treatment of Sphaerophragmiaceae being synonym of Raveneliaceae is also supported by this cladistic analysis. This explains the affinities of *Ravenelia esculenta* (Fig. 1). Members from Sphaerophragmiaceae viz. *triphragmium ulmariae* and *Nyssopsora echinata*, are closely associated with *Ravenelia esculenta*.

Most of the members from this clade have pedicillate teliospores with the exception of members from Pucciniosiraceae and Phakosporaceae which have sessile teliospores and are borne in chains. Aeciospores are catenulate or pedicillate and pycnia are with bounding structures (except in Uropyxidaceae and Phragmidiaceae).

From the figures, it is seen that *Caeoma torreyae*, forms the most basal group in Clade II. This is particularly anamorphic genus with gymnosperm (*Torreya californica*) as aecial host. The primitive nature of *Caeoma torreyae* has been commented on by Peterson¹².

It can be seen, from this interpretation, that the nature of teliospores (pedicillate or sessile), presence or absence of bounding structures in pycnia and catenulate or pedicillate nature of aeciospores are the characters with phylogenetic importance, in addition to nature of telial host (gymnosperm or angiosperm) and life cycle patterns (autoecious or heteroecious) of rust fungi.

These studies show that the phylogenetic relationships established from molecular data can be well correlated with morphological and taxonomical data. It is also seen from the two types of interpretations (Figs. 1, 2) that molecular data frequently fits well and in accordance with the morphometric divisions of major taxa in case of rust fungi with very insignificant variation in size of the clades generated.

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