

Full Length Research Paper

Morphogenesis of edible gall in *Zizania latifolia* (Griseb.) Turcz. ex Stapf due to *Ustilago esculenta* Henn. infection in India

Swapan Kumar SINHA^{1*}, Alak Chandra DEKA¹ and Raju BHARALEE²

¹The Energy and Resources Institute, Northeastern Regional Centre, Express Highway, Hengrabari, 781036, Guwahati, Assam, India.

²Department of Molecular Biology and Biotechnology, Cotton College State University, Panbazar, Guwahati 781001, Assam, India.

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The *Zizania latifolia* Griseb. Turcz. ex Stapf., a perennial wetland wild rice species get infected by a basidiomycetous fungus *Ustilago esculenta* Henn that produces gall. Here we report the morphogenesis behaviour of this plant during gall transformation. The identity of *Z. latifolia* and *U. esculenta* were confirmed by comparing microsatellite and Internal Transcribed Spacer region of nuclear ribosomal DNA sequence with the public database. *U. esculenta* infection transforms the *Z. latifolia* into two distinct morphotypes such as gall forming and non-gall forming. The non-gall bearing morphotype exhibited pinkish microporous lamellar discs (15-20 numbers) filled with mycelia in the internodal spaces. Whereas the gall bearing morphotype fuses 3-4 nodes into compact spindle shaped hypertrophic tissue measuring 2.5 cm diameter x 8-10 cm length tapering at the tip end where dark brown coloured teliospores are deposited in linear sori. In vitro culture of lamellar discs and teliospores in Potato Sucrose Agar medium initially develops white colony 2.6- 4.0 cm diameter later turned into yellowish brown after 7 -10 days and subsequent sporulation within 8-12 days. Spherical sporangium of 28 µm diameter developed on long sporangiophore 7 µm diameter filled with large number of spores 1-1.5 µm in diameters. The unsuccessful sporulation of *Ustilago* even after infection failed to develop gall in the host plants behave normal life cycle and are not consumed. The spore along with hypertrophic soft tissue is consumed as vegetable in the oriental countries.

Key words: Manchurian wild rice, teliospore, microsatellite.

INTRODUCTION

Manchurian wild rice or water bamboo (*Zizania latifolia* Griseb, Turcz. ex Stapf.) grows luxuriantly in wetland. Its

*Corresponding author. E-mail: sksinha@teri.res.in Tel: +913612334790.

perennial stems are comprised of rhizomes with scale leaves, stolons, and culms with relatively broad leaves. The grains contains high protein, carbohydrate and low anti-nutritional components such as cyanogenic glycosides, phytate, oxalate, saponin and tannin thus makes it more nutritious than any other rice and safe as human food (Umar et al., 2013), grains are used as food and rhizomes as diuretics and as medicines for anaemia, heart disease and liver disease (Stapf, 1909) and preventing obesity and liver lipotoxicity (Han et al., 2012) and the gall due to presence of tricin derivatives for allergy and inflammation treatment (Lee et al., 2015). *Z. latifolia* is one of the four wild rice species indigenous in northeastern India, Burma, China, Japan, in parts of Siberia and Russia. The biomass yield recorded as 109.9 tonnes dry weight ha⁻¹ where 80% are in below ground (Champion and Hofstra, 2006).

Ustilago esculenta Henn, a basidiomycetous fungus has very restricted host range and till date *Z. latifolia* is the only known host. The fungus stimulates enlargement of the host culms into an edible gall that contains ascorbic acid, protein, water soluble pectin and free radical-scavenging properties besides angiotensin-converting enzyme (Qian et al., 2012). *U. esculenta* infection interfere flower initiation thereby affect seed production in *Z. latifolia* (Chan and Thrower, 1980; Yang and Leu, 1978). Two unique strains of *U. esculenta* such as sporidial (T) and mycelial (M-T) strains are reported in *Z. latifolia* (Yang et al., 2014). Cultivars of *Z. latifolia* are divided into green, red, and white according to the colour of the outer skin of the gall.

Identification of fungi through sequence similarity search of nuclear DNA internal transcribed spacer (ITS) region with the reported sequence in the public database is one of the most commonly used method. The ITS region being highly conserved at species level and variable in higher taxa (Bruns et al., 1991) has many advantages in molecular identification of fungi due to their high copy numbers that can easily be amplified and sequenced with universal primers (Bruns et al., 1991; Henson and French, 1993).

Z. latifolia population in India is grows only in and around Loktak Lake of Manipur, a wetland of Ramsar site. This lake has a unique ecosystem called 'phoomdi' (a Manipuri word meaning floating mats of soil and heterogeneous vegetation includes *Z. latifolia*) that forms the Keibul Lamjao National Park (40.5 km²) home to the Sangai the brow-antlered deer (*Cervus eldi eldi*). The nutrient analysis of *Zizania* gall collected from Loktak Lake and its uses by ethnic communities are well described (Jain et al., 2012).

This paper investigates the authenticity of *Z. latifolia* and its pathogen *U. esculenta* found in Loktak Lake of Manipur, through microsatellite sequence alignment for *Z. latifolia* and ITS1-4 sequence alignment for *U. esculenta* and illustrates the features pertaining to gall bearing and

non-gall bearing.

MATERIALS AND METHODS

Sample collection

Zizania plants were collected for both gall bearing and the non-gall bearing from Loktak freshwater wetlands of Manipur, India during August-November 2013. The whole plants were transported to Laboratory stationed at Guwahati, India about 550 km away from the source of collection for further studies and established there.

Gall bearing and non-gall bearing *Z. latifolia*

Both qualitative and quantitative parameters were recorded for leaf, stem, rhizome, stolons using measuring tape and calipers. The non-gall bearing stem were dissected longitudinally and recorded lamellae numbers in internodal space. The lamellae were observed under microscope (Axioscope A1.0, Carl Zeiss). The gall was cleaned by removing the leaves attached to it and dissected transversely into slices and longitudinally thereafter teliospores were collected by gentle tapping. The teliospore morphology was observed under microscope.

Microbial studies

After surface sterilization with 0.2% sodium hypochlorite (Fisher Scientific) for 10 min, followed by four times washing with doubled distilled water thin slices of lamellae from no gall bearing internodal space were inoculated into the PSA (Potato Sucrose Agar) media and incubated at 28°C for 6–10 days in incubator (Max 8000, Thermofisher). On the second day, watery white coloured spot on the lamellae was visible and on 3rd day white mycelium developed that was aseptically transferred to PSA plates and incubated for sporulation. Further, the teliospores from gall tissue were also inoculated into same media and incubated as stated. These fungal cultures were subcultured once every month. PSA media was used for the regular maintenance of the culture and a temperature of 28°C was maintained for all the experiments. The pH of the medium was adjusted to 6.5 and was sterilized at 121°C for 20 min. The solid medium contained 1.7% agar agar (Himedia). After several passages of sub-culturing, the pure culture were obtained and allowed for sporulation in the culture plate.

The spores were germinated in PSA (both in semi-solid and liquid) medium at 28°C within the days of transfer. Sporidia were observed with an optical microscope after 5 days of culture establishment. After establishment of new colonies on PSA media the mycelia were observed microscopically. These strains were re-cultured on shake-cultured method in PS broth and incubated at 28°C (150 rpm) for 8 days and then recorded macroscopic morphological characters. The culture morphology and sporangium, spore morphology are recorded under microscope.

DNA isolation and molecular identification

The young leaf tissues and infected gall of *Z. latifolia* was collected from Loktak freshwater wetlands of Manipur. The infected gall was longitudinally dissected and released spores of *Ustilago esculenta* were collected by tapping the gall on a clean paper. The collected leaf tissues and spores were used for DNA isolation. The DNA was isolated from spores and leaf tissues by CTAB method (Doyle and Doyle, 1990) with slight modifications. The isolated DNA was

checked in a agarose gel and used for downstream work.

For establishment of identity of *Z. latifolia* used in the present study we have searched the public databases for any reported sequences from this species and we were able to find a microsatellite sequence in the gene bank of NCBI reported from China (GenBank accession no. EU798777.1). From this sequence we have designed the possible primers to amplify the same sequence from our fungal species. However, we could design primer pair (Forward- 5'-ACGCGGCCACATAGAAAGAA-3'; Reverse- 5'-TGCGTGTAGACATGGTTGCT-3') which would amplify only a part (356bp) of the reported sequence. Designing primers to amplify the entire reported sequence was not successful due to presence of sequences that did not allow optimum primer design using Primer3 online tool.

For molecular identification of the pathogenic fungus under study we have used universal primers (ITS1F- 5'-CGTAGGTGAACCTGCGGAG-3' and ITS4R- 5'-TCCTCCGCTTATTGATATGC-3') to amplify the ITS region spanning partial ITS1, 5.8S and complete ITS2 region. The amplified product (703bp) was cloned and sequenced. The sequence thus obtained was compared with the sequences reported in the public database of NCBI through BlastN programme for sequence similarity based identification of the fungal species under study. The sequences of reported ITS region of *Ustilago esculenta* showing high similarity with the query sequence were retrieved and aligned (clustalw2) to check the sequence similarity.

Phylogenetic position of isolated fungi within the Genus

The Nucleotide Basic Local Alignment Search Tool (BLASTN) analysis of the query sequence have also shown significant similarity with 22 different fungal species under the genus *Ustilago*. These sequences were retrieved and analysed in MEGA6 (Tamura et al., 2013) to know the evolutionary history using neighbor-joining method (Saitou et al., 1987). By Neighbouring method to know the phylogenetic and evolutionary position of the isolated *Ustilago* species among the reported species under the genus *Ustilago*.

RESULTS

The *Z. latifolia* found in Manipur, India were about 3-4 m height having distinct rhizome, stolon, culm, leaf, roots and adventitious roots. Leaf has 13 cm long sheathing base and 120 cm long lamina about 20 cm wide. Culms were about 50 cm in length with 7-8 nodes (Figure 1a) covered with sheathing leaf base where thick adventitious roots surround the node in a single row.

Non-gall bearing *Z. latifolia*

The culms, leaf, rhizome were normal in shape and size. Culm internode length in the base and tip were shorter and longer in the middle (Table 1, Figure 1a). The cross section of culm revealed 15-20 numbers of soft velvet textured pinkish lamellar discs arranged parallel to each other in the internodal space (Figure 1c and h). The colour of lamellae observed pinkish from 2nd internodes

above the rhizome till tip of the culm. Whereas in the rhizome region 5-10 numbers of thick and white lamellae were found. Lamellae in the apical intermodal region were thin and soft and arranged in close proximity than at the middle culm region (Figure 1e-f). No sign of infusion of culm internode and internodes were detected. The lamella had large number of micro pores 8-10 μ m in sizes that were intermingled with fungal hyphae (Figure 1d).

Gall bearing *Z. latifolia*

Plants were short and stout and at the 3rd or 4th nodes small maize shaped gall covered with leaves was observed (Figure 2a). Galls were 2.5-3 cm wide in the middle and 8-12 cm length tapering at the tip (Figure 2b) and the apical part of gall was spirally coiled (Figure 1g). Rectangular shaped 1 x 10 mm pinkish colour distinct spots were arranged linearly all along the leaf surface (Figure 1b). The cross section of gall in the middle portion revealed about 90-100 numbers of sori irregular in shape ranged between 0.5-2 mm diameters upon removal of teliospores a beautiful network of sori was observed (Figure 2c-d). Each sorus had a thin whitish peridium that holds the dark brown coloured powdery mass of teliospores (Figure 1i). The longitudinal section of gall revealed linearly arranged sori of measuring about 1 mm diameter and 8-16 mm length (Figure 1j).

Teliospore

The teliospores were easily removed by gentle tapping the cut surface of gall. From a gall about 15-20 gm of teliospores were collected. Teleiospores were fine and slippery in textures and dark brown in colour. The teliospores were spherical, oval and ellipsoidal and their surface ornamented with spines around. The diameter of teliospores recorded 6-7 μ m having distinct 1 μ m thickness wall ornamentation with layers of exosporium, middle layer and endosporium (Figure 2e). There were 1-4 numbers lobes of greenish pigmentation observed inside the teliospores.

Microbial studies

On the second day, watery white coloured spot on the lamellae was observed and on 3rd day white mycelium was observed. The surface of the single colony developed form lamellar source was smooth, moist and round in shape. The colony size recorded was 2.6- 4.0 cm and whitish in colour in the initial stage and turned yellowish to yellowish brown after 7 -10 days of culture. Maximum colony size was observed within 7 days of culture in PSA



Figure 1. Morphogenesis of gall in *Z. Latifolia*. a) Normal *Z. latifolia* plant, b) Pigmentation on leaf surface size 1 x 10 mm c) pink lamellae in the internodal space length 9.5 cm d) microporous (diameter 10 μ m) lamella with fungal mycelia e) Apical shoot on non gall forming plants f) Internal view of apical shoot non gall forming g) Apical part of gall. h) Cross section of non gall stem i) Cross section of gall with peridium. j) Cross section and longitudinal section of gall.

media maintained at 28°C. Sporulation developed within 8-12 days of incubation (Figure 2f). The microscopic observation revealed distinct spherical sporangium measuring 28 μ m in diameter developed on long sporangiophore measuring diameter 7 μ m. Large number of spores measuring 1-1.5 μ m in diameters were present in each sporangium (Figure 2g). While the teliospore suspension on PSA the germination observed on very next day. After 3 days of incubation individual colony formation was observed on the surface of the PSA solid culture medium. No difference in colony morphology of lamellar and teliospore source was observed except darker in colour on maturity for the later.

Molecular identification

BLASTN analysis of the isolated microsatellite sequence (356bp) had shown high similarity (98%) with the

microsatellite sequence reported in the database (GenBank accession no. EU798777.1) from where the primers were designed for amplification. This high similarity of the isolated sequences with the *Z. latifolia* microsatellite sequence in the database indicated that the host species for the fungal pathogen under study was also a *Z. latifolia* found in Manipur. The sequence was later submitted to GenBank as partial sequence of microsatellite sequences of *Z. latifolia* with accession no. KF944445.1.

The BLASTN analysis of the ITS sequence isolated (703bp) from the pathogenic fungus under study had shown high similarity (99%) with the ITS sequences of *U. esculenta* reported in the public database with 99% query coverage. The alignment of top 12 blast hit sequences of *U. esculenta* showed high similarity (Figure 3) with the query sequence had helped us to infer that the pathogenic fungus under study belonged to genus *Ustilago* and species *esculenta*. The sequence was

Table 1. Features of *Zizania latifolia* gall bearing and non-bearing.

Features	Unsuccessful gall	Successful gall
Apical shoot	Straight	Spirally coiled
Stem nodes	Total culm length 50 cm with 7 nodes	Transformed into whitish swollen soft tissue 8-10 cm long and 2.5-3 cm diameter
Stem internodes length (cm)	From rhizome to tipward 1.8, 2.3, 9.5, 4.3, 5.6, 4.1, 2.6	Not distinction
Internodal lamellae	15-20 arranged parallel Thin pinkish microporous lamellae	Not found
Leaf	Leaf sheath Length 15 cm, leaf lamina length 120 cm, lamina width 19.62 mm green in colour	On leaf rectangular pinkish tinge
Mycelia	Present in the lamellae	Only spore found
Teliospores	Not found	Dark brown powdery mass 6-7 μm size in the linear sori



Figure 2. Illustration of gall a) Gall bearing on tip of *Z. latifolia* plants b) A bunch of gall sold in market c) Cross section of gall 2.5 cm diameter with dark brown coloured powdery teliospores d) A section of gall after removal of teliospores 2.5 cm diameter e) Teliospores under microscope 6-7 μm size f) *U. esculenta* sporulation in culture plate g) *U. esculenta* sporangium (28 μm) with sporangiophore (7 μm diameter) under culture condition.

Table 2. Features of *Ustilago esculenta* under culture condition.

Features	Observations
Colony shape	Round 2.6-4 cm in diameter
Colony colour	White for lamellar source and white brown for teliospore origin
Sporangium	28 µm diameter red in colour on the top of long sporangiophore
Sporangiophore	7 µm wide,
Spore	1-1.5 µm in diameter

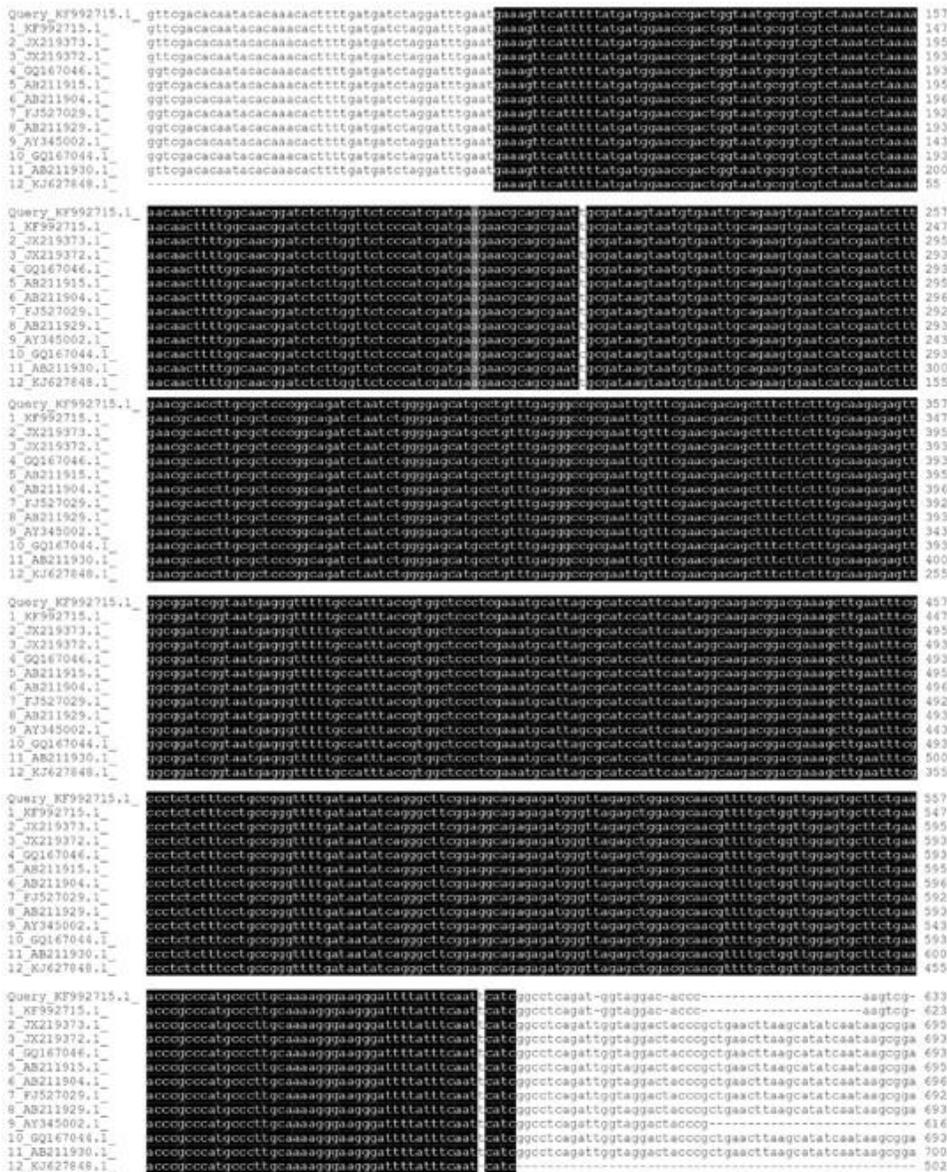


Figure 3. Alignment of isolated ITS region of the pathogenic fungus under study with the top 12 blast hit species of *Ustilago esculenta* showing high sequence similarity with the query sequence. The numbers on the left column of the figures indicates the Gene bank accession numbers of sequences used in the alignment. The bases that are conserved in all sequences under study are indicated by black background while bases not conserved in all sequences are indicated with white background.

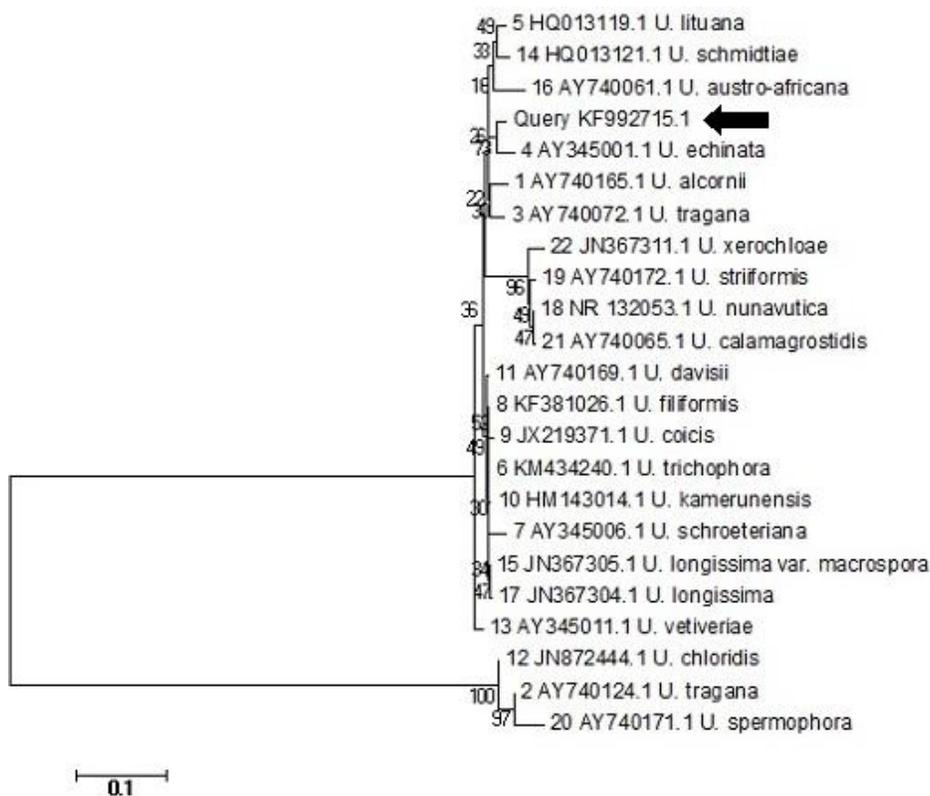


Figure 4. Evolutionary position of isolated fungi based on ITS sequence. The phylogenetic tree constructed in MEGA6 showing the taxonomic position of isolated fungi (indicated by black solid arrow) among the members of the genus *Ustilago*.

submitted to Genbank as ITS region of *U. esculenta* with accession no KF992715.1

The phylogenetic tree in MEGA 6 (Tamura et al., 2013) was constructed to know the taxonomic position of the fungus among other members of the genus *Ustilago*. The optimal tree was constructed with sum of branch length = 1.36901858 (Figure 4). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein et al., 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. The isolated fungus had been clustered with *U. echinata* with a bootstrap value of 73 (Figure 4).

DISCUSSION

Gall formation occurs in *Z. latifolia* sporadically due to *U. esculenta* infection however if mycelia present in the

main stem fails to invade new tillers during the tillering period the plants remained without infection (called 'male' Jiaobai) during cultivation (Ding et al., 1991). Histological studies revealed that *U. esculenta* are distributed throughout the stem tissue except leaves and root tissues of *Z. latifolia* (Yang and Leu, 1978) and even specifically designed primers for the pathogen failed to amplify the DNA extracted from leaves and healthy plant tissue (Chen and Tzeng, 1999). Present studies illustrates that in Indian *Z. latifolia* population non-gall bearing plants has infection of *U. esculenta* on stem tissues that local people called as "Eishing Kambong Laba" (a Manipuri word of male wateroat) that are not eaten by communities thus get selectively discarded. Cell wall degrading enzymes from *U. esculenta* participates in gall formation of *Z. latifolia* (Nakajima et al., 2012). *U. esculenta* infection interfere inflorescence development and subsequent seed production in *Z. latifolia*, which results in an increase in the size and the number of host cells (Chan and Thrower, 1980; Yang and Leu, 1978). Gall formation includes an increase of the size and number of host cells, suggestive of the involvement of a high IAA content within the swelling tissues. The hypertrophy of tissues also implicates

cytokinin involvement in the gall formation (Chan and Thrower, 1980). The imbalance between IAA and cytokinin due to pathogen infections is important for overgrowth, hypertrophy and tumor formation in many plant-microbe interactions (Surico et al., 1985). Although higher levels of IAA and cytokinins are often found in the gall tissues (Chan and Thrower, 1980), the role of these two plant regulators in gall development has not been yet experimentally demonstrated. The mycelia infested pinkish soft lamellae in the intermodal space of culm is the first report from unsuccessful gall forming culm of *Z. latifolia* (Table 1). There may be several abiotic and biotic factors influencing the gall formation even after infection by *U. esculenta* in to the host plant that needs to be investigated. The gall as vegetable is high in fiber, vitamins, essential amino acids and minerals (Jiang and Cao, 2008). The gall is derived from a hypertrophy of host tissues due to colonization of pathogen and usually located in the stem region of 3-4 nodes (Chan and Thrower, 1980; Yang and Leu, 1978). The gall is edible with unique flavour and tendency. Therefore, the crop is cultivated as a vegetable in southern Asia (Guo et al., 2007). In India *Z. latifolia* is not domesticated yet thus collections are made from wild habitat especially in and around Loktak wetland, Manipur, India. Based on the colour of the outer gall sheath, three cultivars (green, white and red) *Z. latifolia* are commonly identified due to infection by *U. esculenta* in Taiwan (Yang and Leu, 1978). In India only green cultivar is found that are harvested during September-October. Further two unique strains of *U. esculenta* such as sporidial (T) and mycelial (M-T) strains are reported in *Z. latifolia* (Yang et al., 2014). In Indian population both strains of *U. esculenta* are also observed.

Since there is very high similarity of the isolated microsatellite sequence of the host with the *Z. latifolia* reported in the database, we can infer that the host plant under study is a *Z. latifolia* plant collected from Manipur. We have also confirmed the identity of the pathogenic fungi as *U. esculenta* by comparing the ITS region. The high similarity of the isolated ITS region of the pathogenic fungi with the *U. esculenta* sequence in the database helped us to infer that the isolated fungus belongs to the genus *Ustilago* under the species *esculenta*. The taxonomic position of the fungus among other species of the genus *Ustilago* have shown that it is very closely related to *U. echinata* and forming a small cluster with bootstrap value of 73. Inclusions of these two species within a single cluster have indicated their close evolutionary relationship. Further, unlike other species of *Ustilago*, these two species has got similar type of host plant, that is, wet land grasses. *U. esculenta* parasitize *Z. latifolia* while *U. echinata* parasitize *Phalaris* sp. or *Glyceria* sp., both are wetland grasses (Matthias et al., 2003). Both species have been reported to infect the stems forcing the plant to enter into a sterile life

phase (Vánky, 1987, 1994). These similarities between these two species have justified their close evolutionary relationship as revealed by the phylogenetic analysis. This is first sequence of *Z. latifolia* and *U. esculenta* deposited in the Genbank database from India.

This study authentically identified the *Z. latifolia* host plants and its pathogen *U. esculenta* using partial microsatellite region and Internal Transcribed Spacer (ITS1-4) region of nuclear ribosomal DNA (nrDNA) sequence alignment respectively. Indian *Z. latifolia* population is described as green cultivars with dark brown coloured teliospores. The non-gall bearing *Z. latifolia* stem has unique microporous pink coloured lamellae in the stem internodal space and same is fused into hypertrophic soft edible tissue as gall that are consumed as vegetables. In India *Z. latifolia* is found only in and around Loktak lake, Manipur and is not cultivated yet thus the collections are made from wild. *Z. latifolia* vegetation along with other species form a unique ecosystem called Phoomdi a floating mass distributed in all throughout the lake and serves as habitat for the Sangai the brow-antlered deer (*Cervus eldi eldi*).

Conflict of Interests

The authors have not declared any conflict of interest.

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