Pathogenic Fungal Diversity Of *Carica Papaya* From Marathwada Region

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Abstract:

The fungi associated with the infection of papaya fruits in Osmanabad and Aurangabad city, Maharashtra State were studied. Five varieties of papaya fruit were used in the study the pathogenic fungi with potato dextrose broth and potato dextrose agar as the artificial growth media while the pour plate method was employed in their isolation. The fungi were isolated, and characterized on the basis of their cultural and microscopic features and identified as *Aspergillus sp, Alternaria sp., Colletotrichum sp., Curvularia sp., Fusarium sp.,Penicillium sp*. And *Rhizopus sp*. ITS sequencing was done using ITS 1 and ITS 4 primer pairs and the homology studied to confirm the microbial identification. Diversity analysis was done using PAST which indicated highly diverse fungal isolates. The result of the pathogenicity test carried out on the healthy fruits showed that all the fungal species re-isolated from the healthy samples had the same cultural and microscopic features as the original fungal isolates, indicating that these isolates were responsible for the infection of the papaya fruits examined. These organisms may have gained entrance into the produce from the air, during transportation, processing in an unhygienic environment and through improper handling. The organisms are known to produce potent toxins which have deleterious effects on humans and animals, therefore care must be taken during their handling, transportation and processing of papaya fruits to minimize the entry and proliferation of these organisms in the produce, as well as the health risk their infestation pose to the consumers of the fruits.

Key words: *Carica papaya*, diversity, ascomycota, PAST, phylogeny

1. Introduction:

Papaya (*Carcia papaya*) is a popular fruit plant grown all over parts of the world (Onuorah et al., 2014). It is a herbaceous plant with latex vessels that grows fast but has a short lifespan. It has a stem that is upright, branched or unbranched, and is covered in lead scars all over (Oniha, 2012). The thick, delicious fruit is often green but becomes yellow as it ripens. The main ingredients in the edible component include water, sugars, vitamins A and C, proteins, and ash (Baiyewu, 1994). Over time, there has been a significant rise in the demand for papaya fruits, which may be attributable to the tropics' growing consumption trends (Ivan et al., 2020). Papaya has several medicinal benefits in addition to being used as food (Onuorah et al., 2014). Because of its high levels of nutrients and sugars as well as its low pH levels (Singh et al., 2021). An excessive softness, mycelia development, loss of moisture, bad odour, shrinkage, and complete drying up of the fruit's water content are all signs of infected papaya fruit (Tadele, 2019). Pawpaw fruit is often cultivated in the wild and harvested by local farmers into big open baskets or fibre bags using long sticks (Onuorah et al., 2014). In India, papaya post-harvest losses have been attributed to *Aspergillus Niger, Aspergillus flavus, Rhizopus sp , Curvularialunata, and Fusarium equiseti*(Baiyewu et al., 2007). The papaya fruit markets may incur losses, but the fungi-damaged fruits also pose a threat to consumer health since they produce mycotoxins that can lead to mycotoxicoses in humans (Effiuvwevwere, 2000). Typically, pathogenic or toxic spoilage fungi are both possible(Tournas & Stack, 2001). There have been reports of toxic fungi in papaya (Mons, 2004). On the other hand, pathogenic fungus may result in allergies or infections. Ochratoxin production by *Aspergillus* species is hazardous to both humans and animals (Akinmusire, 2011). Though the consumption of papaya is increasing due to its nutritional value, their infection by fungi needs attention. Therefore, in this study, the fungi associated with the disease of papaya fruits in Osmanabad city were isolated, characterized and identified. The diversity of the fungi and dominance were studied to understand which fungi is the prime cause of fruit deterioration losses.

2. Materials and methods:

2.1. Collection of samples:

The papaya plants were collected from three different locations of Aurangabad and Osmanabad fruit market. Two infected fruits of papaya from each location, and a total of 5 locations were selected for sampling. The infected patches of *Carica papaya* fruit samples were used to isolate pure cultures of pathogenic fungi. The fruits were sampled at 5 places on an average based on the different pathogenic symptoms to avoid redundant sampling.

2.2.   Isolation of fungi:

Small pieces measuring 5 mm ^{2} each of infected tissue, were peeled off from these fruits with the help of a sterile sharp knife. The pieces were separately transferred to sterile potato dextrose agar plates (PDA) and incubated at 28 °C for seven days(Ugwuanyi & Obeta, 1991). Petri dishes was observed daily, and the distinct colonies of fungi were picked. The isolated fungi were purified using single spore technique (Leyronas et al., 2012) and the pure colonies of fungal isolates were maintained on PDA slants.

2.3.   Identification of fungi:

The fungal colonies were grown on PDA and fungal cultures were grown until maturity. The identification of the genus was done by observing the colony characters of fungi. The fungal hyphae, conidia and spores of mature fungi helped in the identification of the fungal genera and grouping similar looking fungi and prevent reselection of same isolate. The confirmation of the fungal isolate was done by genetic identification by partial sequencing of ITS region within the ITS1 and ITS4 primer pairs.

2.3.1 Morphological identification of fungi:

The fungal cultures were used for morphological identification after the sporulation stage. The morphological identification of the fungi was done by staining the fungal hyphae and conidia with Lactophenol Cotton Blue. The stained fungal cultures were observed under a compound light microscope under 40 X and 60X. The conidial structures and spores were compared with the reference manual to have a species-level identification (Barnett & Hunter, 1972).

2.3.2 DNA extraction of fungal isolates:

The fungal cultures at sporulation stage were harvested and taken in eppendorf tube for genomic DNA extraction. The DNA extraction was done using (Doyle, 1991) CTAB-based extraction procedure with slight modifications. The PVP (5 mg) was added as solid in the mycelial mass during crushing with liquid nitrogen. The PCI solution was used rather mere chloroform and isoamyl alcohol solution. The ethanol-based DNA precipitation was done rather than using isopropanol. Isolated DNA was used for the partial sequence amplification of ITS region.

2.3.3 Qualitative and quantitative assessment of fungal genomic DNA

The DNA quality was determined using the Nanodrop technique(Nakayama et al., 2016). 1 μL sampleof DNA was placed on the Thermo fisher ™ Nanodrop ™ and scanned at 260nm and280nm to get a ratio (1.8 is desired ratio for pure DNA). The protein, carbohydrate or RNA contaminations were checked. 2 μL of template DNA was also run on a 0.8 % agarose gel in TBE buffer to check the DNA quality.

2.3.4 Amplification of ITS region of fungal gDNA

Once the DNA was found to be of optimal concentration, the template DNA was assured to be of use for PCR.The universal primers used for fungal amplification were ITS1 (5′TCTGTAGGTGAACCTGCGGG 3′), which hybridizes at the end of 18S rDNA, and ITS4(5′TCCTCCGCTTATTGATATGC 3), which hybridizes at the beginning of 28SrDNA (White et al., 1990).

Total PCR mixture of 25µL, each contained 1µL (100 ng/µL) DNA template, 1µL each primer, 0.33µL (3 units/µL) Taq polymerase, 0.5µL of dNTPs, 2.5 µL of 10× PCR buffer with 25 mM MgCl2 and 18.67µL Milli Q water for each reaction mixture. PCR reaction was performed in Thermo Fisher Scientific Veriti™ 96-Well Thermal Cycler under the conditions: pre-denaturation at 96 °C for 2 min, 35 cycles of 96 °C (denaturation) for 30 sec, 55 °C (annealing) for 15 sec, 60 °C (extension) for 4 min and then a final extension for 4 min at 60 °C. Amplified PCR products were resolved by electrophoresis in a 1.5 % (w/v) agarose gel. The band was stained with ethidium bromide (0.5µg/mL) for visual examination in UV transilluminator.

2.3.2 Molecular identification:

The identified sequences of microbes were subjected to nBLAST analysis tofind the homology. The query sequences were aligned with sequences in the NCBInBLAST database to search for the highest homology with the highest coverage ofquery sequence. The accession numbers and names of microbes after nBLASTanalysis were noted at the species level. Sequences obtained were aligned using ClustalW, and neighbour-joining tree (NJ tree) was constructed to find the phylogenyof the isolates in MEGA-X. The homology analysis provided an assurance of theidentification of isolate when it showed significant sequence coverage andphylogenetic homology with already registered partial genome sequences in GenBankdatabase.

2.4. Phylogenetic analysis:

Sequences obtained were aligned using Clustal W, and neighbour-joining tree (NJ tree) was constructed to find the phylogeny of the isolates in MEGA-11(Kumar et al., 2016; Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown on the branches (Shimodaira & Hasegawa, 2001). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option).

2.5. Diversity analysis:

Species richness, dominance, Simpson index, Shannon–Wiener index and Whittaker's evenness was calculated to express the diversity of endophytes using PAST software (Bardia et al., 2019). Percentage dominance (%D) of each fungi was calculated by the formula %D= n/N×100, where n= Total isolates of fungi, N=Total isolates of all fungi

3. Results:

3.1. Occurrence of fungi:

Isolation of fungi linked to 5 cultivars of infected papaya fruits from the Aurangabad and Osmanabad region using PDA. Morphological identification of the fungi using fungal hyphae and spore structures showed the isolates to be *Alternaria alternata, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Colletotrichum gloeosporioides, Curvularialunata, Fusarium equiseti, Fusarium moniliforme, Fusarium oxysporum, Penicillium digitatum, Rhizopus stolonifer and penicillium islandicum.*Repeated sampling of the fruits didn't find any other diverse fungi.

3.2. Molecular identification:

All the samples of fungi exhibited a 260/280 ratio of 1.8 or less in their extracted genomic DNA. For PCR amplification, the extracted genomic DNA was suitable. The final DNA concentration in each sample was close to 100 ng/l, which was ideal for ITS sequence amplification.

The consensus sequence obtained after Sanger sequencing found gave different sequence lengths of the amplicons. The nBlast analysis gave homology with the closest sequence. The sequences with the highest query coverage and similarity helped in identification of the isolates. The isolate with the highest homology along with the morphological identification data helped in confirming the taxonomy of the isolates. The details of the nBlast analysis are mentioned in Table 1.

Table 1nBlast analysis of amplified ITS sequences of the pathogenic fungal isolates from selected papaya fruits..

Amplicon size	Query coverage	Percent identity	Closest accession	Scientific name
541	100	100	MG214866.1	*Alternaria alternata*
1156	100	100	MT582749.1	*Aspergillus niger*
1182	100	100	MN190286.1	*Aspergillus fumigatus*
576	100	100	KC491415.1	*Aspergillus flavus*
531	100	100	KX066883.1	*Colletotrichum gleosporioides*
576	100	100	MH858245.1	*Curvularialunata*
528	100	100	MK503775.1	*Fusarium equiseti*
548	100	100	MN652648.1	*Fusarium verticillioides*

Amplicon size	Query coverage	Percent identity	Closest accession	Scientific name
1135	100	100	MT453296.1	*Fusarium oxysporum*
845	100	100	MK450692.1	Penicillium digitatum
304	100	100	EU652685.1	Penicillium islandicum
875	100	100	FN401529.1	Rhizopus stolonifer

3.3.   Diversity analysis

The sampling data showed that a total of 7 genera of pathogenic fungi, comprising of *Alternaria, Aspergillus, Colletotrichum, Curvularia, Fusarium, Penicillium* and *Rhizopus* were prevalent in the deteriorating fruits. All the isolates, as shown in the Table 3 made up 1091 isolates out of a total of 1800 samplings. It was commercially inconvenient to sequence all the isolates for molecular identification. Hence, sequencing representative isolates was a convenient mode of fungal identification.

The highest frequency was that of the genera *Aspergillus*, which made up 340 isolates. This was followed by *Fusarium*. The next prevalent generawas*Penicillium*. The least prevalent was found to be *Curvularia*. Aspergillus spp. had the highest dominance percentage (31.2).

Table 2 Frequency distribution, and dominance percentage of the isolated pathogenic fungi from infected papaya fruits.

Fungi	n	Dominance %
Alternaria spp.	117	10.7
Aspergillus spp.	340	31.2
Colletotrichum spp.	127	11.6
Curvularia spp.	57	5.2
Fusarium spp.	226	20.7
Penicillium spp	132	12.1
Rhizopus spp.	92	8.4

Where n is the frequency of the fungal genera,

The occurrence of all the fungi in different locations and in different samples were also observed. The occurrence of *Aspergillus niger* was the highest amongst all the samples (143), followed by *Colletotrichum gloeosporioides* (127). The least prevalent was *Curvularialunata* (57). The detailed frequency distribution of the identified fungal isolates is shown in Table 3.

Table 3 Frequency distribution of fungal isolates on different varieties of infected papaya fruit

Fungal isolate	Co-I						Co-II						Washington						Taiwan						Local						Total (n)	Dominance (%)
	loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3			
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2		
Alternaria alternata	5	4	5	3	4	5	3	4	4	5	3	5	3	4	4	3	5	3	4	3	2	4	3	4	5	4	5	3	4	4	117	10.7
Aspergillus flavus	4	3	3	4	2	3	4	2	3	4	3	3	3	4	3	4	5	4	4	3	4	3	4	3	3	4	4	4	4	3	104	31.2
Aspergillus fumigatus	2	3	3	3	4	2	3	4	3	4	3	4	2	3	4	5	3	4	2	3	3	4	3	3	2	3	3	3	2	3	93	11.6
Aspergillus niger	5	5	5	5	4	5	4	5	4	5	5	5	5	5	5	4	5	5	5	5	4	5	4	5	5	5	4	5	5	5	143	5.2
Colletotrichum gleosporioides	5	4	5	4	5	5	4	5	3	5	4	5	3	4	5	4	4	4	4	5	4	5	4	4	3	4	4	4	4	4	127	20.7
Fusarium oxysporum	3	2	3	3	4	3	3	3	3	4	3	3	3	3	3	4	3	3	3	2	3	4	3	4	3	3	3	4	3	4	95	12.1
Fusarium	2	3	2	2	1	2	1	1	2	1	2	1	3	2	3	2	1	4	3	2	3	3	2	2	3	2	3	2	3	2	65	8.4

Fungal isolate	Co-I						Co-II						Washington						Taiwan						Local						Total (n)	Dominance (%)
	loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3		loc1		Loc 2		Loc 3			
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2		
equiseti																																
Fusarium moliniforme	2	1	2	1	2	1	3	2	3	2	3	2	3	2	3	2	3	2	1	3	2	3	2	1	3	2	3	2	3	2	66	10.7
Penicillium digitatum	2	3	2	1	2	1	2	3	2	3	2	3	4	2	3	2	3	3	2	3	2	3	2	4	3	2	2	3	2	2	73	31.2
Penicillium islandicum	1	1	3	2	3	2	1	2	3	2	1	3	2	3	2	3	2	3	2	3	2	1	2	1	2	1	2	1	2	1	59	11.6
Rhizopus stolonifer	1	2	3	2	4	3	4	3	4	3	2	3	2	4	3	4	3	3	4	3	2	3	2	3	3	4	3	4	4	4	92	5.2
Curvularialunata	1	2	1	2	1	2	1	2	2	1	2	2	2	1	2	1	1	1	3	4	3	2	3	2	3	2	2	2	3	1	57	20.7

Where 'loc' is location of the sampling, 'S1' and 'S2' are the two papaya samples selected from the location. N is the total number of isolates

The dominance indices were found to be quite variable based on the frequency of the fungal isolates (Table 4). There was not much of difference in the number of isolates obtained from the different fruit varieties (205-227). The diversity was also calculated based on the varietiesof papaya under study. The species richness was highest in Co-I as compared to other papaya varieties. The Shannon-Weiner and Simpson Index indicates that there was a greater diversity of in Taiwan as compared to other varieties of papaya. Brillouin diversity index did not have much difference as compared to previous diversity indices but showed slightly higher diversity in Taiwan variety.

The Shanon H index showed that the highest diversity was found in the variety Taiwan. This was also proved by the lower dominance index (0.089) in the same variety. The dominance was highest in Co-1 (0.097) as compared to others. Washington had the lowest dominance. The dominance index suggested either the dominant trait of the fungi to inhibit other fungi, or genetic constraints controlling the growth of fungi. The detailed diversity index calculation based on the varietal differences are shown in Table 4.

Table 4 Diversity analysis of the fungal isolates of different varieties of papaya taken from 3 different location.

Diversity indices	Co-I	Co-II	Washington	Taiwan	Local
Taxa_S	12.000	12.000	12.000	12.000	12.000
Individuals	205.00	216.000	227.000	220.000	223.000
Dominance_D	0.097	0.093	0.090	0.089	0.091
Simpson_1-D	0.903	0.908	0.910	0.911	0.909
Shannon_H	2.406	2.427	2.445	2.451	2.439
Evenness_e^H/S	0.924	0.944	0.961	0.967	0.955
Brillouin	2.289	2.314	2.335	2.339	2.329
Menhinick	0.838	0.817	0.797	0.809	0.804
Margalef	2.066	2.046	2.028	2.039	2.034
Equitability_J	0.968	0.977	0.984	0.986	0.982
Fisher_alpha	2.782	2.740	2.701	2.725	2.715
Berger-Parker	0.142	0.130	0.128	0.127	0.130
Chao-1	12.000	12.000	12.000	12.000	12.000

The graph of Shannon H index against the sampling number showed that there was a gradual increase in the diversity with the sampling number. As the sampling increased, the diversity went on decreasing. After a specific sampling number, the diversity remained stable, despite the increase in the sampling number. This concluded that no diverse fungal isolate would be remaining from the fruit and all

the prevalent fungi were isolated. This was an important step to be confirmed before calculating the diversity indices. The graph of Shannon H index against sampling is shown in Figure 1.
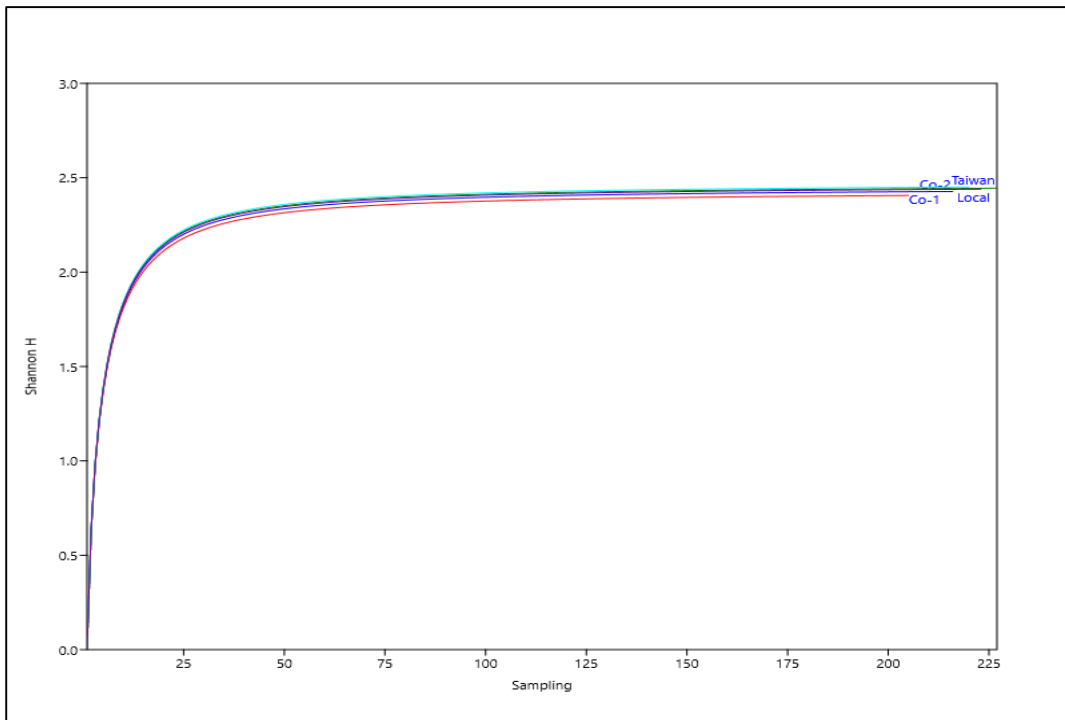

Figure 1 The Shannon H index against sampling, showing that sampling was enough so that no further diversity was observed.

3.4. Phylogenetic analysis:

The nBLAST tool was used to align the querysequences to the sequences in GenBank database. The sequences with highest query coverage and percent identity were the putative sequences to match with the query sequences of the isolates. The nearest phylogenetic neighbour of each common sequence is shown in Figure 2as phylogenetic tree. The relative abundances at the respective levels in the cohort are reflected in the node sizes at those levels. The node length of the isolates and the similarity percent of the ITS sequences were used to distinguish the phylogeny of the 12 isolates found in this research. The percentage of internal node coverage is shown by figures with a percent sign.

The results show that there was a greater similarity found between *the Aspergillus and Penicillium* ITS sequences since it was found in similar clades. Likewise, there was a considerable similarity in the *Fusarium and Colletotrichum* genera.
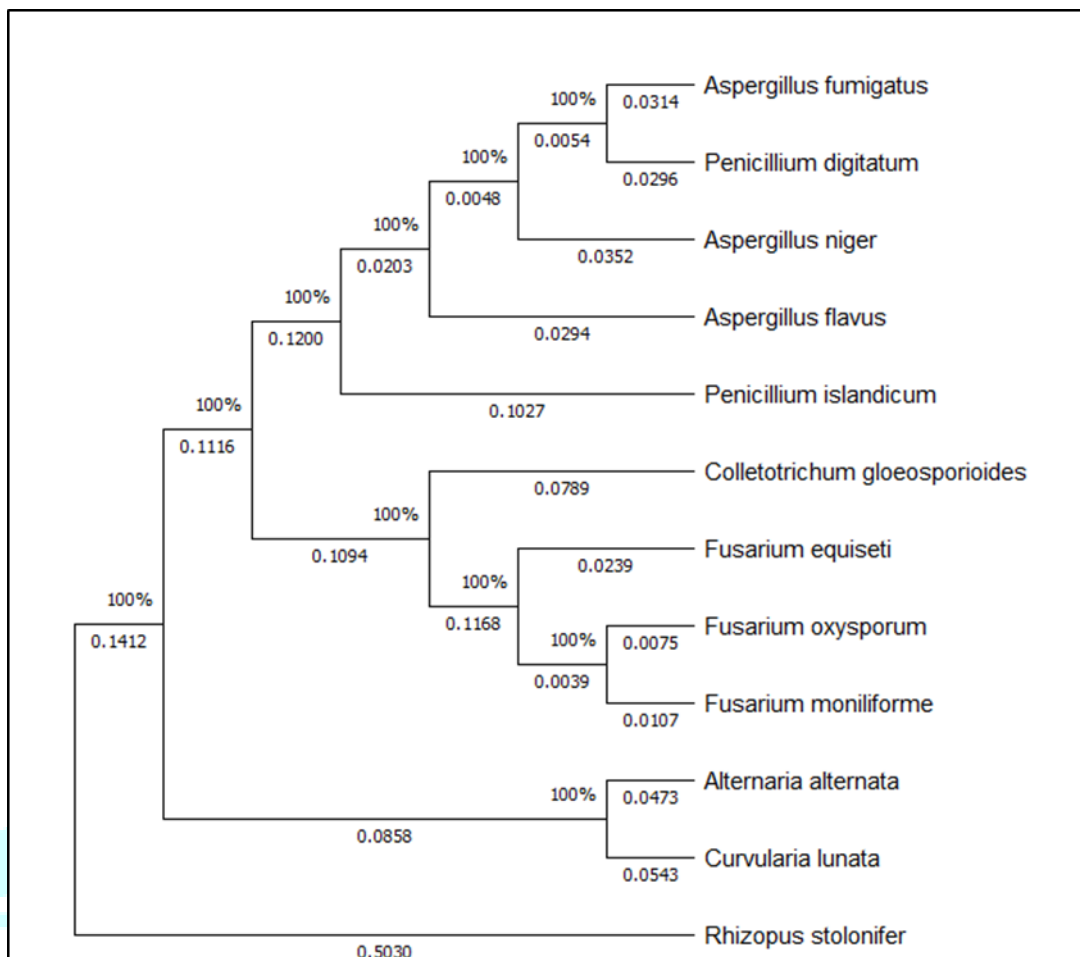
Figure 2 Phylogenetic analysis of the identified fungi using MEGA-X by neighbour joining method.

4. Discussion

Papaya fruits with five varieties namely Co-1, Co-2, Taiwan, Washington and Local which are commonly cultivated in Marathwada regions were screened to know their occurrence of pathogenic fungi by using agar plate method (Nwosu & Okafor, 1995). The results clearly indicate that local variety is more virulent for the pathogen whereas Taiwan is more susceptible. Out of all the isolates, *Alternaria spp.*, *Aspergillus spp. and Fusarium spp.* were dominantly occurred on all the varieties. All the isolated pathogenic fungi showed great variation in diameter, colour and growth pattern of colony on solid media and sporulation. (Zhang et al., 1995) reported that the growth pattern of *Aspergillus species* varies in different growth media, however the physical and nutritional factors also shows variation in sporulation count. There was lesser difference in the prevalence of specific genera among all the isolates. Though, there was difference in the magnitudes of prevalence of different genera on same fruits. There was not much of difference in the total number of isolates from different varieties of papaya. The variation in the frequency of the isolates can be due to the storage parameters or the varietal genetic reason. Albeit, the fruits were kept open at vending stalls, hence always exposed to the fungal spores. Hence, there is a greater chance that Co-1 might harbour fungi that grow either controlling the growth of diverse fungi through mycoparasitic, nutrient competing or antibiosis mode of inhibition. This was proven from the fact that the variety had highest dominance index of pathogenic fungi. The study on varietal reason of fungal

growth or dominance of the fungi through dual culture would have provided better insight on the probable reason.

The Shannon H index was highest in the Taiwan variety, indicating that there was lesser dominance of single fungal isolates. This could correspond to a mutualistic proliferation relation of the fungi, spatial distribution, or reduced matrix competition until the nutrient depletion is observed. The study didn't find the performance of fungi in case of nutrient depletion. It is observed that fungi grow differently when the nutrients deplete or there is a co-existence of bacteria or fungi during their growth (Mille-Lindblom et al., 2006).

The phylogenetic tree of the isolated fungal strains showed 3 clades, the first had homology of *Aspergillus and Penicillium*, the second clade showed homology in *Fusarium and Colletotrichum*, whereas the third was totally different from that of others. This included *Rhizopus and Curvularia*. *Rhizopus* belongs to Zygomycota, whereas *Curvularia* belongs to Ascomycota. Hence, it was evident to be different from the others. The first two clades belonged to Ascomycota. There are evidences that Zygomycota has a lesser spore release range (Fischer et al., 2010) as compared to Ascomycota (Trail, 2007).

The sequencing and phylogenetic analysis was a very good tool to distinguish between the isolates, based on taxonomic hierarchy as well(Badotti et al., 2017). The sequence lengths of the ITS sequences show that the evolutionary period made differences in the gene sequences (nucleotide insertion or deletions) which were discrete for a particular strain. There isevidence that the sequence variations can be found in the same species, denoting diverse strains. This study found that fungi of diverse groups were prevalent, but the fact of sequence transfer or similar sequences of different genera were not observed in any of the fungi, despite being of same class.

5. Conclusion

The diversity of pathogenic fungal isolates from diverse *Carica papaya* varieties was studied from Aurangabad and Osmanabad region, which has never been reported previously. 7 different genera, comprising 12 different fungal isolates were found. *Aspergillus flavus* and *Penicillium digitatum* were found to be dominant fungi among all. The dominance index was highest in variety Co-1, and least in Taiwan variety.The diversity index was highest in Taiwan variety. The ITS based sequencing was a robust method to identify the fungal isolates. The phylogenetic analysis among the isolates grouped the isolates as per the clade, showing a greater homology in the rDNA sequences. Single isolate (*Rhizopus*) of Zygomycota was found. Rest of the isolates were from Ascomycota. The dominance of the Ascomycota class could correspond to the mode of spore release and long range of spore discharge in the environment.

Acknowledgement

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