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A MULTI-GENE PHYLOGENY OF *CERATOCYSTIS MANGINECANS* INFECTING MANGO IN PAKISTAN

ASMA RASHID[1*], SHAZIA IRAM[2] AND IFTIKHAR AHMAD[1]

[1]*PARC Institutes of Advanced Studies in Agriculture, National Agricultural Research Center, Islamabad, Pakistan*
[2]*Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan*
[*]*Corresponding author's email: asma_friend@yahoo.com; 0334-5296793*

Abstract

Mango trees (*Mangifera indica* L.) are affected by a serious wilt disease, recognized as mango sudden death first time reported in Muzafargargh Punjab, Pakistan in 1995. Its prevalent is in almost all mango growing areas with severity ranged from 2-5% in Punjab and 5-10% in Sindh. Survey and sampling was conducted during the year 2011-12, on mango orchids in different distracts of Punjab and Sindh and no location was found free from this Disease. For molecular identification, DNA was successfully extracted and was then amplified by using ITS, BT, TEF (600-800)primers through Polymerase Chain Reaction (PCR) assay andnucleotide evidence of Pakistani isolates (45 for each gene) exhibiting the maximum genetic homology with *Ceratocystis manginecans* (99-100%) followed by *C. fimbriata* (97%) and *C. omanensis* (80%) respectively. On the basics of morphological tools and comparison of nucleotide evidence of multi-genes, *C. manginecans* is different from *C. fimbriata* and *C. omanensis* which infect mango in Pakistan. The availability of disease-free planting material and management in combination with fertilization and proper irrigation system would help in improving orchard management system.

Key words: Mango, ITS, BT, TEF, Phylogeny, *Ceratocystis manginecans*.

Introduction

Mangifera indica L. belonging to dicotyledonous family *Anacardiaceae*, generally recognized as mango, is a popular, predominant seasonal fruit originated primarily in the tropical and subtropical regions of the world. Pakistan produces approximately 8.5% of the world's total production and mostly exports to Iran, Middle East, Japan, Germany and China assembling its important contribution as a significant foreign exchange earning fruit crop (Anonymous, 2007).In Pakistan, soil and climatic conditions support production of mango in terms of quality and yield but the country is unable to attain the desired results. Numerous issues contribute towards the low production of mango. In Pakistan mango is susceptible to many abiotic and biotic diseases including mango malformation, mango decline, anthracnose and powdery mildew (Asad *et al*., 2010). Different species of *Ceratocystis* have been reported for canker and fruit rot, vascular discoloration, root and stem root to many trees in different parts of the world. Some of the known *Ceratocystis* species associated with fruit trees mortality were *C. salbifundus, C. pirilliformis, C. fimbriata, C. manginecans, C. fagacearum, C. omanensis* and *C. paradoxa* (Engelbrecht & Harrington, 2005; Wyk *et al*., 2007). Whereas *C. fimbriata, C. manginecans* and *C. omanensis* were associated with mango tree (Wyk *et al*., 2007). This fungus is also known to cause sudden death or "seca" in many area of Brazil (Ploetz, 2004).

On the basis of morphological characteristics species of *ceratocystis* were redefined, like spores, ascospores and perithecia size and shape (Fateh *et al*., 2006). Distinguishing different species of Ceratocystis were not vagued by the usefulness of morphological features and were not have treasured for phylogenitical information and it is defined by molecular analysis with the help of rDNA and internal transcribed spacer (Verkley&Starink-Willemse, 2004). The exertion of resolving relationships among Ceratocystis has been underlined by using single gene phylogenies (Crous *et al*., 2001). Unavailability of reliable phylogenetic information in reconstruction of trees, resulting in poorly resolved trees (Verkley *et al*., 2004; Shinwari *et al*., 1994; Shinwari, 1995).

Sequenced data was used from ITS, beta tubulin (BT), transcription elongation factor (TEF) and ribosomal gene in combination to form phylogenic histories (Moore, 1995). Nuclear and mitochondrial genes are unlinked to each other and therefore, overruled for making phylogenetic histories (Cummings *et al*., 1989). High rate of nucleotides substitutions was found in Ribosomal DNA and it would be helpful in providing additional intuition into relationships among closely related fungus (Moncalvo *et al*., 2000).

In this study, the causal organism of MSD was confirmed through morphological characteristics and nucleotides evidence of multi-genes including ITS, BT, TEF and mtSSU.

Material and Methods

Symptomatic surveys of mango orchids was conducted in mango growing areas of Punjab and Sindh as described by Al Adawi *et al*. (2006).Morphological characteristics were compared with Domsch *et al*., 1980 and pathogenicity tests were used for further confirmation. Totalnucleic acid of local isolate was extracted with modified protocol of Barnes *et al.* (2001) and used as a template (2µl) in PCR assay. PCR reactions (50µl) were comprised of 10X PCR reaction buffer (5µl), 10mM mixed dNTPS (1µl), 25mM $MgCl_2$ (3µl), 5unit/µl Taq DNA polymerase (1µl) and 20 pM (5µl) of each sense and antisense primer. Amplifications of ITS and EF-1α region of local isolates was done by using primers ITS1(5` TCCGTAGGTGAACCTGCG G 3`), ITS4 (5` TCCTCCGCTTATTGATATGC 3`), EF1-728F(5'

CATCGAGAAGTTCGAGAAGG 3') and EF1-986R (5' TACTTGAAGGAACCCTTACC3') and PCR conditions were same as described by White *et al*. (1990). A portion of beta tubulin gene was amplified using primer βt1a (5` TTCCCCCGTCTCCACTTCTTCATG 3`) and βt1b (5' GACGAGATCGTTCATGTTGAACTC 3') and modified PCR conditions were as follows: initial denaturation at 95°Cfor 1 min, followed by 30 cycles of denature (95°C),annealing (55°C) and extension (72°C) for 1 min each with final elongation at 72°C for 7 min. PCR amplified products were visualized in agarose gel (1.5%)with ethidium bromide size of amplified fragment was estimated with 1kb ladder (Fermentas). These products were further purified with standard protocol of PCR purification kit (Fermentas Germany)and directly sequined from commercial lab (Macrogenlbc Korea) for sense and antisense direction. The final sequences of each gene were submitted at DNA data Bank of Japan (DDBJ) and provided accession numbers. Alignments were initially compiled by using Bio Edit and phylogenetic analysis was inferred with the maximum parsimony (MP) optimality criteria using PAUP ver.4.b10. Parsimony tree was generated by using heuristic searches with random stepwise addition (1000 replications) and branch swapping algorithm using TBR (Tree bisection-reconnection). For each analysis, stability of clades was evaluated by using bootstrap (1000) replications. P-value was obtained from partition homogeneity test to each IMF index to determine data partition combinability. By using PAUP software congruency of data was set for sequence of ITS, BT and TEF.

Results

Dead branched, turned brow leaves, brown streaks in vascular region, yellow to brown gum-like substance, bark splitting, gummosis and wilting was observed in all orchids of Punjab and Sindh. Spores were hyaline, cylindrical with truncated ends and ranged from 18-30µm and 13-15.6µm in length and width respectively. Hyaline, hat like ascospores (2-3.1µm length and 3.2-6µm width) was also observed. *Ceratocystis* was homothallic, and all the isolates derived from MSD produced perithecia. The ascomata bases were globose with brown to black and long neck. Ascomata base ranged from 192-252 µm in diameter. Whereas ascomata necks were dark brown in colour and ranged from 515-648 µm in length.

Internal Transcribed Spacer (ITS1 and ITS4) and BT (Bt 1a and Bt 1b) regions amplified approximately 600 to 650bp while 700 to 800bp fragments were observed in TEF (EF1-728 and EF1-986) regions (Fig. 1a, b, c). The final sequence of 45 isolates was submitted on DDBJ and provided the accession numbers AB818966 to AB819010, AB889749 to AB889793, AB894189 to AB894207 and LC000686 to LC000686forITS, β-tubulin, TEF. Nucleotide evidence of local isolates exhibiting the maximum genetic diversity with *C. manginecans* (20%), *C. fimbriata* (2%) and *C. omanensis* (1%) respectively. Parsimony analysis resulted 3 (steps=63, CI = 0.974, RI = 0.851), 1 (step = 74, CI = 0.785, RI = 0.956), 1 (steps = 82, CI = 0.825, RI = 0.872) and 3 (steps= 47, CI = 0.981, RI = 0.964) clades for ITS, BT, TEF and mtSSU respectively. Alignment of the ITS sequence create 636 characters and which 25 (4%) characters were parsimony informative. All 45 isolates of the *Ceratocystis* grouped these subclades with strong (100%) bootstrap support. Whereas C. *bhutanensis, C. moniliformis* and *C. moniliformopsis* respectively included in other subclades (Fig. 2). Assembly of BT contigs and their alignment resulted in a data set of 763 characters, of which 137(7.2%) were parsimony informative (Fig 3). Alignment of TEF sequence generated in a data set of 2.1224 characters, of which 354 (8.5%) were parsimony informative. Bootstrap support value for this group was 98%. Whereas assembly of mtSSU sequences of all *Ceratocystis* isolates resulted in a data set of 685 characters, of which 13 (2.4%) were parsimony informative. The major clade 1 and 2 includes all the *Ceratocystis* isolates with strong bootstrap value (95%) (Fig. 4). Partition homogeneity test for the data set of ITS, BT, TEF and mtSSU gave P-values (0.02) greater than the acceptable level value (P=0.05) and they could thus be combined (Barker *et al*., 2001).The combined sequences data of the multi gene areas resulted in a total of 1953 characters, including gaps. The data set contained 1087 characters, 62 parsimony uninformative characters and 682 parsimony-informative characters. Tree length is about 1874 steps, with a consistency index (CI) of 0.97, homoplasy index (HI) of 0.35, a retention index (RI) of 0.85 and a rescaled consistency index (RC) of 0.68.

Discussions

The mortality of young and adult plants by MSD has become a severe thread for the mango grower in Pakistan. To identify and charactize the most reported pathogen of MSD *Ceratocystis* sp. the samples were collected from Multan, Shujabad, Khanewal, Rahim Yar Khan, Hyderabad, Sanghar, Tando Allah Yah, Matiyari and Muzaffar Garh showing 5 to 15% severity in orchards (Fateh *et al*., 2006). First symptoms observed in MSD were dropping of leaves which are mainly caused due to blockage of xylem vessels by the invading fungus as the nutrients and minerals cannot be transported to the arial parts of the plants therefore the loss of turgidity occur and leaves droop. Next symptoms that appeared was bark splitting which may occur due to exposure to direct sunlight or by fungal invasion in the xylem vessels. The nutrients keep moving from soil to the collar portion of stem and accumulate their making the air pockets inside as the turgidly of cell increases rupturing of cells takes place and the thick dark liquid oozes out from the Splitted bark of the affected tree. The production of gums by the affected trees results from the internal break down or damage and varies in colours according to the severity of the disease. The fungus mostly prevails in the soil of infected orchards and when the irrigation is done the spores travel from one place to another entering the wounded roots of healthy trees. Symptomolgy is not the reliable criteria for the confirmation of MSD because symptom develops may occur due to abiotic factors but it can play a vital role for disease diagnosis (Kazmi *et al*., 2007).

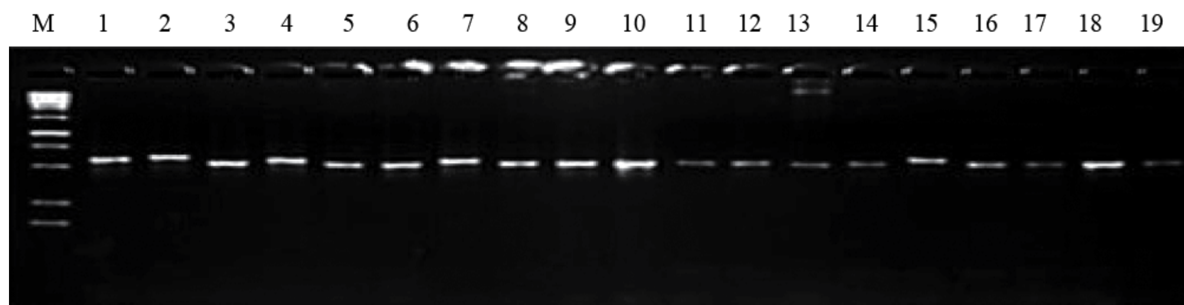

Fig. 1a. DNA amplification of *Ceratocystis* isolates with ITS primers generating 500-600bp fragments.

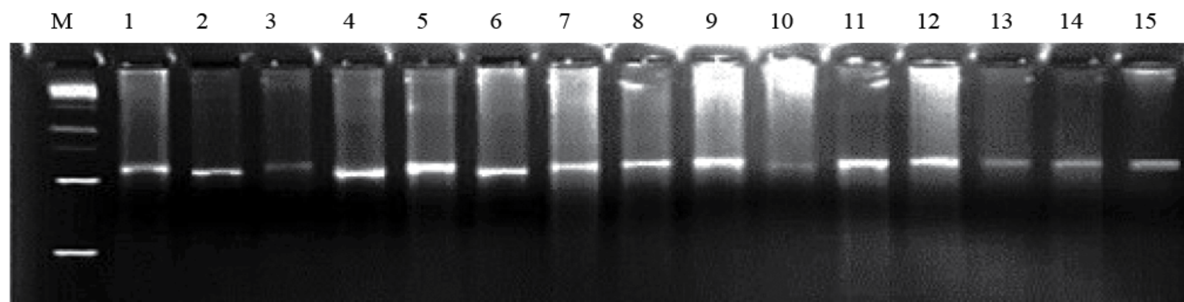

Fig. 1b. DNA amplification of *Ceratocystis* isolates with BT primers generating 500-600bp fragments.

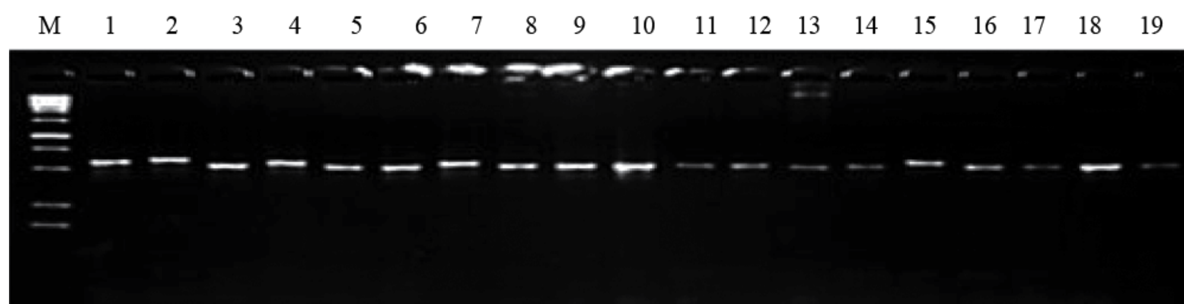

Fig. 1c. DNA amplification of *Ceratocystis* isolates with TEF primers generating 700 -800 bp fragments.

The isolation of *ceratocystis* isolates from infected xylem sample's confirmed the MSD disease. Investigated *Ceratocystis* isolates was morphologically similar to *C. moniliformis*, *C. moniliformopsis*, *C. omanensis*, and *C. bhutanensis* though some differences were also observed. The perithecial and ascomata necks were in range of *C. manginecans* but they are noticeably shorter than *C. moniliformopsis* and *C. moniliformis*. Hence, there are some morphological differences between investigated *Ceratocystis* isolates and its close relatives. It is exhibited that the sub-populations are not always expressed in teens of morphological divergence. Closely related species therefore, lack of taxonomically useful morphological differences long after the initial speciation event. In Pakistan, *Ceratocystis* species were identified through morphologically and no nucleotide evidence was available previously. Pathogenicity tests and nucleotide evidence are reliable tools for reporting new species in Pakistan (Abbas *et al*., 2014).

Multi-genes (nuclear and mitochondrial) based phylogeny for 45 local isolates of *Ceratocystis* exhibited unprecedented resolution between close *Ceratocystis* species resulting from combined use of genes. It provides sufficient evidence for confirmation of the main hypothesis and further assisting in the delimitation of closely related species in the genus *Ceratocystis*. With these evidences, it is confirmed that the agreement between the main results of the multigene phylogeny presented here and the previously published *Ceratocystis* phylogeny (Engelbrecht & Harrington, 2005; Shinwari, 1998).

By using separate data set analysis, these DNA partitions were of limited utility in supporting relationship with *Ceratocystis*. Only ITS did not provide the sufficient support resolution for many nodes except those nodes which are found within groups of related species. It is confirmed here that the limited utility of this gene for supporting phylogenetic resolution between and within *Ceratocystis* (Goodwin & Zismann, 2001. Identification from lower to intermediate taxonomic level, both nuclear and mitochondrial sequencing (ITS, BT, TEF and mtSSU-rDNA) reveals similar character variation and exhibited the identical phylogenetic (Barnes *et al*., 2003). These studies supported the data insufficiently resolution upto the inadequate number of informative sites found (>40%) comparatively to the large number of closely related taxa examined.

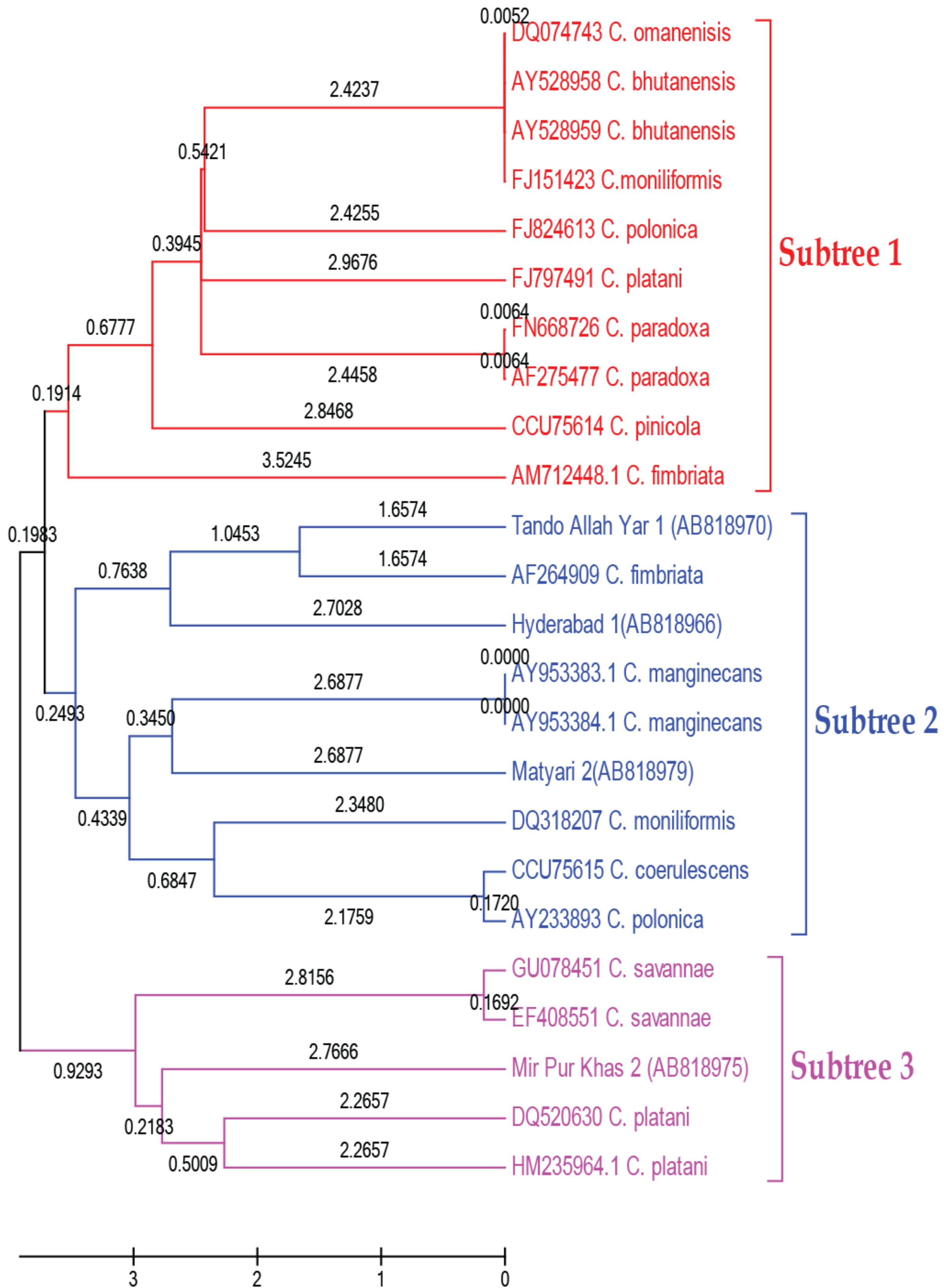

Fig. 2. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from 5.8S r DNA sequences. Tree was generated using the neighbor- joining method. Bootstrap value (more than70 %), expressed as percentage of 1000 replicons, are indicated at the nodes.

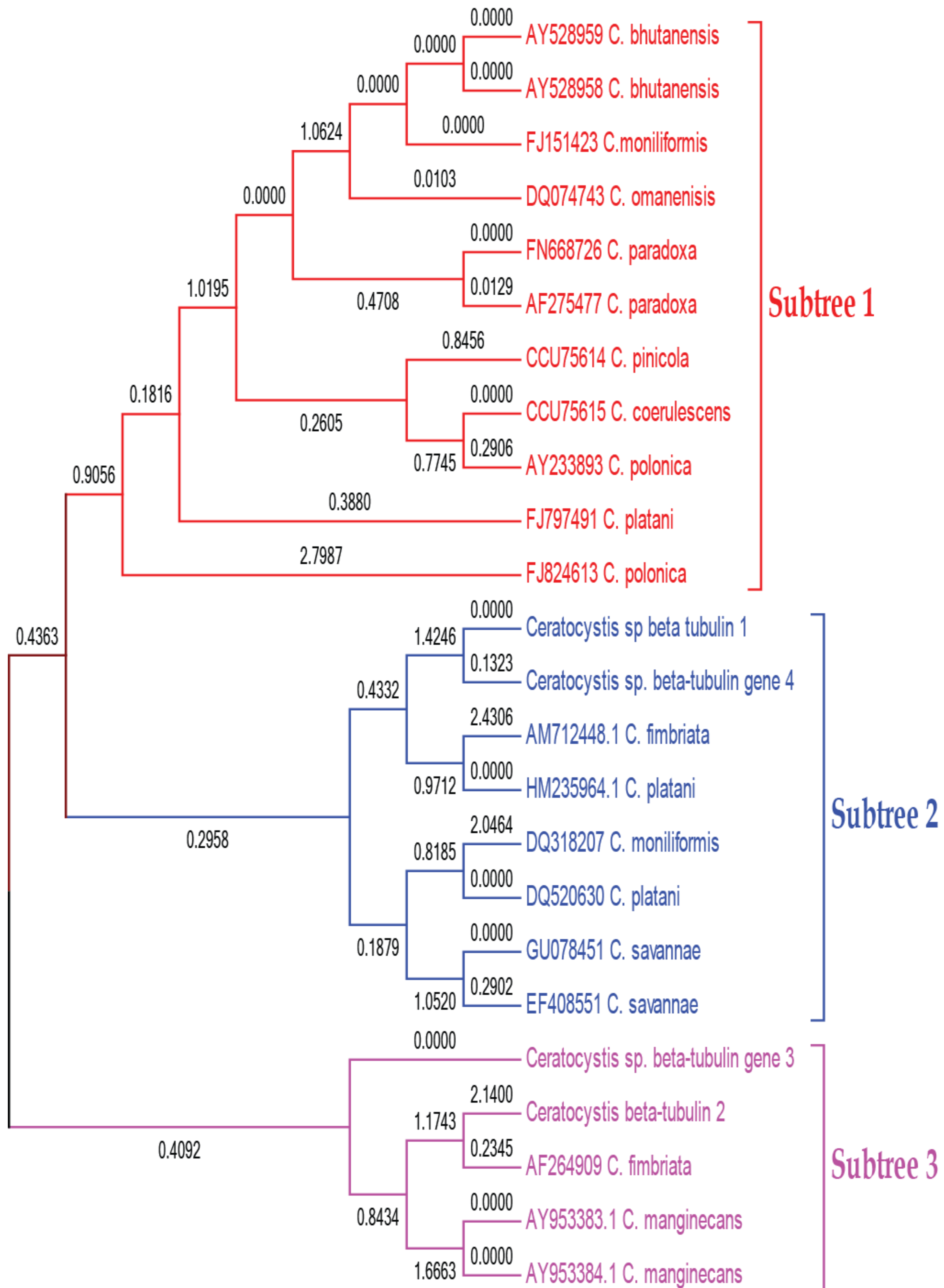

Fig. 3. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from BT sequences. Tree was generated using the neighbor- joining method. Bootstrap value (more than70 %), expressed as percentage of 1000 replicons, are indicated at the nodes.

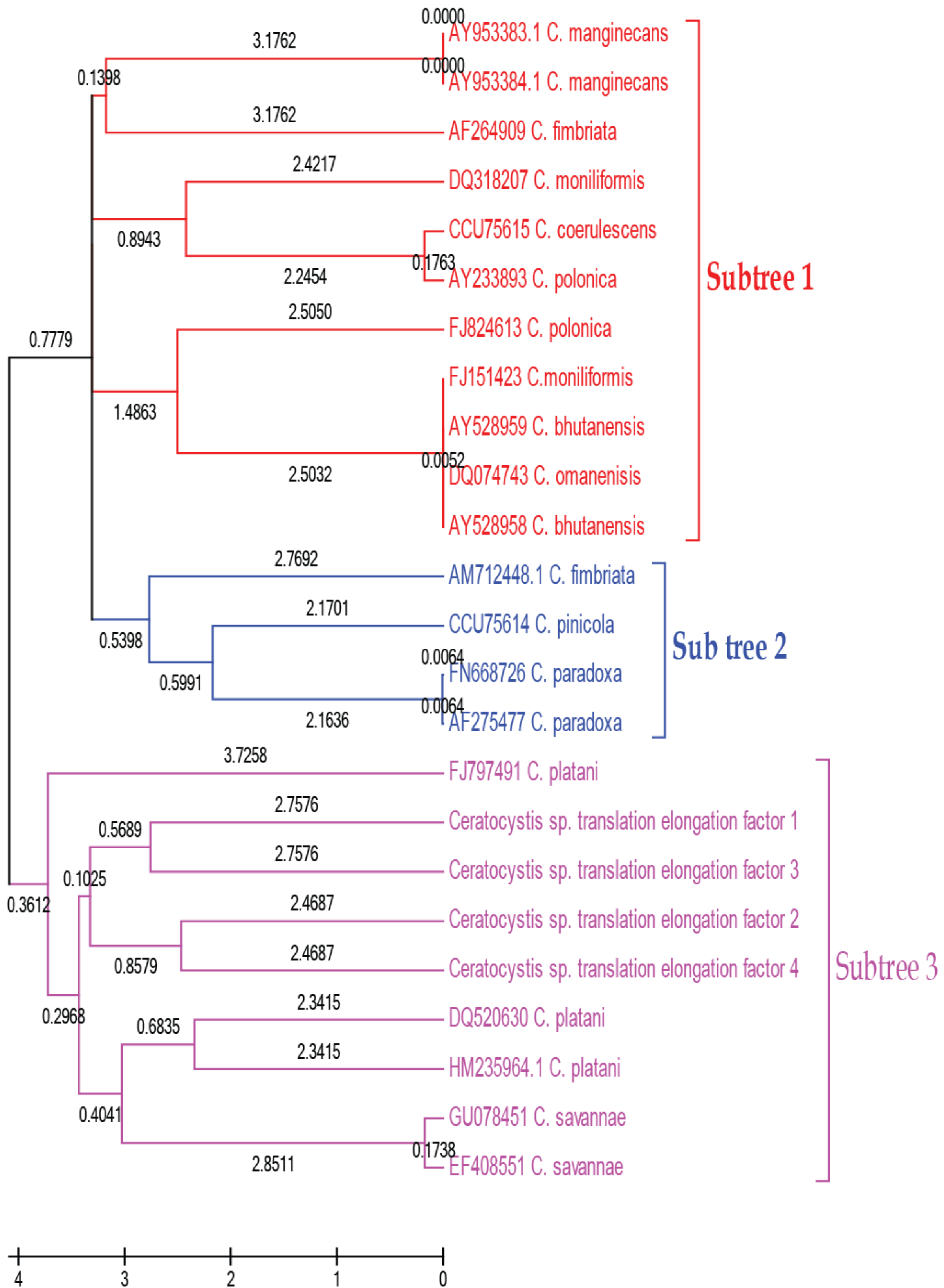

Fig. 4. Phylogenetic tree showing inter relations of *Ceratocystis* spp. with closely related species inferred from TEF sequences. Tree was generated using the neighbor- joining method. Bootstrap value (more than70%), expressed as percentage of 1000 replicons, are indicated at the nodes

Conclusion

Morpho-molecular characteristics support the first report of *Ceratocystis manginecans* infecting mango in Pakistan.

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