

MOLECULAR CHARACTERIZATION OF *Ceratocystis manginecans* sp. FROM MANGO IN PAKISTAN

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A serious mango sudden death caused by *Ceratocystis* sp. is an important disease of Pakistan. Symptoms of affected trees include bark splitting, discoloration of the vascular tissue, wilting, gummosis and eventually results in rapid death. Detailed molecular analysis is performed based on nuclear ribosomal DNA *Ceratocystis* sp. Sequencing of nuclear encoded ITS1-5.8 s –ITS2 rDNA region confirmed preceding taxonomic conclusion based on morphology. Evolutionary reconstructions consequences in a phylogenetic tree of internal transcribed spacer. Blast searches showed that isolates from Pakistan grouped within the *C. fimbriata* species complex and were most closely related to *C. manginecans*. Three clades were observed that included *Ceratocystis* sp. with similar morphological features. Unexpectedly sequence analysis revealed genetic variations distinguishing the *Ceratocystis manginecans* from *Ceratocystis fimbriata*. The identified genetic variation in *Ceratocystis* species appears to affect the 5.8s RNA folding as deduced from secondary structure models. It is revealed that *Ceratocystis manginecans* internal transcribed spacer ITS and ITS2 folds into the multi branched closed and open loop with different locations in different species of *Ceratocystis*.

Keywords: Mango sudden death, *Ceratocystis manginecans*, PCR, phylogenetic analysis

INTRODUCTION

Mango (*Mangifera indica* L.) is one of Pakistan's major fruit crop attracting the attention of world markets. Mangoes from Pakistan are being exported to many countries such as Dubai, France, Saudi Arabia, Holland, UK, Germany, Switzerland, Italy, Singapore and Malaysia. Recently, in Pakistan mango has occupied an area of 1987.38 thousand hectares with 1846.0 thousand tones production (Anonymous, 2010). At present mango production has been affected by diverse biotic and abiotic factors. Among the biotic factors, fungi are considered most important. Important mango diseases include mango sudden death (MSD), mango malformation, powdery mildew, die back and anthracnose (Asad *et al.*, 2010). During early 1995, a serious disease of mango sudden death appears in Muzafargargh, Pakistan. The genus *Ceratocystis* comprises of many pathogen and plants Ploetz, 2004. Up till now many species have been described within this genus. This disease has affected 50 % of the tree in major mango growing areas of Punjab and Sindh. Disease symptoms on tree include bark splitting, leaf dropping/drying and gummosis (Iqbal *et al.*, 2007).

In the current study, detailed comparative molecular technique of genus *Ceratocystis manginecans* isolated from mango sudden death tree in Pakistan was performed. The sequence of ITS-5.8S-ITS2 rDNA region was obtained and phylogenetic relationship between the Pakistani isolates of *Ceratocystis manginecans* and all other species belonging to the *Ceratocystis* groups were reconstructed. Detailed

comprehensive analysis of the sequence data revealed an unexpected nucleotide difference of the 5.8s rRNA which appears to influence the structural folding of 5.8 s rRNA, but its biology remain unclear. Detailed examination required for understanding the association of *Ceratocystis* with mango sudden death and the species involved.

MATERIALS AND METHODS

Sampling and isolation of fungus: Sampling was done in different mangoes area of Punjab and Sindh. Tree showing symptoms of leaf dropping/leaf drying, bark splitting and gummosis were selected. Samples were taken from bark of the trees and then stored in polythene bags named with date, area and name of grower. These samples were then brought in the lab for the isolation of required fungus (Al Adawi *et al.*, 2006). Samples were then cut into small pieces and then placed in the MEA (Malt extract Agar). After incubation plates were examined daily to isolate *Ceratocystis* sp.

Genomic DNA extraction: Total genomic DNA was isolated from eight different isolates of *Ceratocystis* sp. by phenol extraction method (Reader and Broda, 1985). Liquid nitrogen was used to crush the mycelial cell wall into a fine liquid Then 500 mL phenol: chloroform: iso-amyl alcohol in (25:24:1) ratio was added to the ground mycelium followed by the addition of 1000 mL DNA extraction buffer. The mixture was first vortexed for uniform mixing and then centrifuged for 10 minutes for 10,000 rpm. The supernatant was carefully collected in the labeled eppendorf. Sodium

acetate 50 mL and isopropanol 500 mL were added, and then mixed gently with hands. DNA was appeared in thread like structure. Centrifuge it again for 10,000 rpm, for 10 in. The supernatant was removed which leaved DNA in the form of a pellet at the bottom. Ethanol 70 % (500 mL) was added in the eppendorf and centrifuges it again at 10,000 rpm for 10 minutes. Next, ethanol was removed leaving pellet at the bottom. RNase was added in the DNA and then incubates it at 35°C for 30 min. Concentration of DNA was estimated by λ DNA standards. The DNA stock was then stored in the refrigerator for future use.

DNA amplification: DNA amplification was done by using ITS 1F (5'- TCC GTA GGT GAA CCT GCG G-G-3') (Gardes & Bruns, 1993) and ITS 4R (5'- TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990) primers at 55°C annealing temperature. Volume of reaction mixture is 50 μ l which contained template DNA of 25 ng, 20 pmol of both primers ITS1 and ITS4, 10Mm of dNTPs, 5 μ l PCR buffer with NH₄(SO₄)₂, MgCl₂ 5 μ l (Fermentas) and Taq DNA Polymerase 1 U (Fermentas Canada). PCR program was set for amplification with denaturation (initial) for 1 minutes at 95°C, followed by 35 cycles of 1 minute each for denaturation at 95°C, annealing at 55°C and extension at 72°C while final extension of 7 minutes at 72°C. PCR products were then run on agarose of 2%. Amplified bands were then compared with fermentas ladder 1kb. Purification of amplified PCR product was purified with PCR purification kit (Fermentas, Canada).

Sequencing of DNA and phylogenetic analysis: PCR purified product was sequenced Macrogen, Korea (<https://dna.macrogen.com/eng>). Sequence editing and contig assembly was done through Software Bio edit (version 7.0.8). Nucleotide sequence of other *Ceratocystis* sp. was attained from NCBI for comparative analysis. Multiple sequence alignment was done in clustal W. Final aligned sequenced were visualized in MEGA 6. Molecular phylogenetic tree was constructed on the basis of multiple alignments using Neighbor-joining (NJ) method in the Mega 6 software (Tamura *et al.*, 2007).

RNA secondary structure: RNA secondary model were inferred as previously explained by Marek *et al.*, 2010. Two dimensional structure (2D) model for 5.8 s were computed by the MFOLD algorithm (Zuker, 2003). Thermodynamically secondary models were built in RNA structure v.4.4 software. This secondary model was then exported from RNA structure model for further refinements and visualization with RNA viz (De Rijk and De Wachter, 1997) software.

RESULTS

Investigated *Ceratocystis* sp. produced an unpleasant banana-oil odour and produced ascomata within a few days of incubation. *C. manginecans* were initially whitish grey

and finally black with age. Cultures were initially white and then turned to mouse grey when older. *C. manginecans* also produced Perithecia within few days, and these could be clearly seen in older cultures, that tended to have sparse aerial mycelium. Spores were cylindrical, hyaline and truncated at the end which ranged from 4.96-6.20X 32.59-51.21X μ m.

Nucleotide sequences of 5.8s rDNA region of eight Pakistani *Ceratocystis* isolates were determined. These sequenced were submitted in DDBJ and AB818966 (Hyderabad), AB818967 (Tando Jam), AB818969 (Tando Allah Yar), AB818973 (Mirpur Khas), AB818975 (Multan), AB818977 (Rahim Yar Khan), AB818976 (Muzzafar garh), AB894231 (Khanewal), AB818981 (Sanghar) and AB894229 (Shujabad) accession number were assigned. Blast searches in Gene Bank indicated that isolates from Pakistan grouped within the *C. fimbriata* species complex and were most closely related to *C. manginecans*. The high similarities (99-100) were observed for all deposited *C. manginecans* isolates. To get insight into the phylogenetic relationship between isolates of *C. manginecans* of Pakistan and other isolates of *Ceratocystis* species, a detailed molecular phylogeny approach was applied. Four clades were obvious in the phylogenetic trees that include *Ceratocystis* sp. with similar morphological characters (Fig. 1 a,b,c,d). The other subclades include included isolates of *C. bhutanensis*, *C. moniliformis* and *C. moniliformopsis*, respectively. Carefully examination of these nucleotide data set used for phylogeny resulted in several substitutions that distinguish *C. manginecans* of Pakistan from other members of *Ceratocystis* species (*C. fimbriata*, *C. bhutanensis*, *C. moniliformis*).

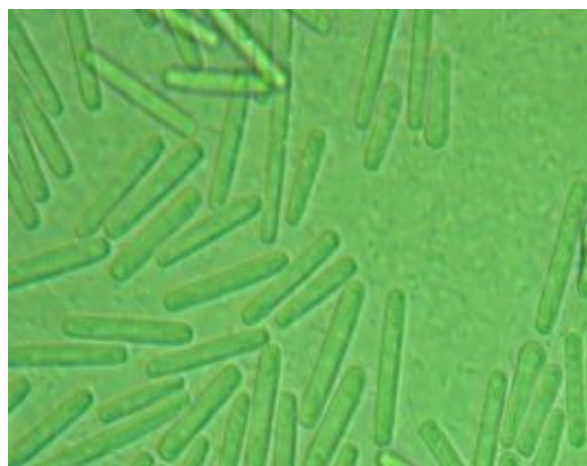


Figure 1a. Spores of *Ceratocystis* sp.

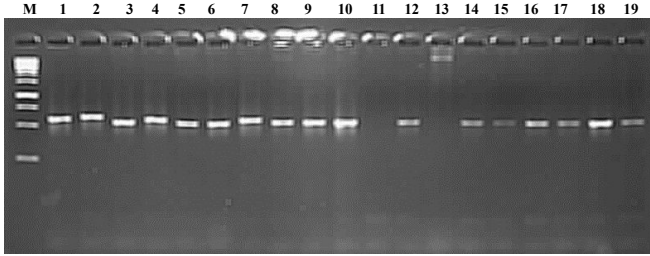


Figure 1b. DNA amplification of *Ceratocystis* isolates with ITS primers generating 500-600bp fragments.

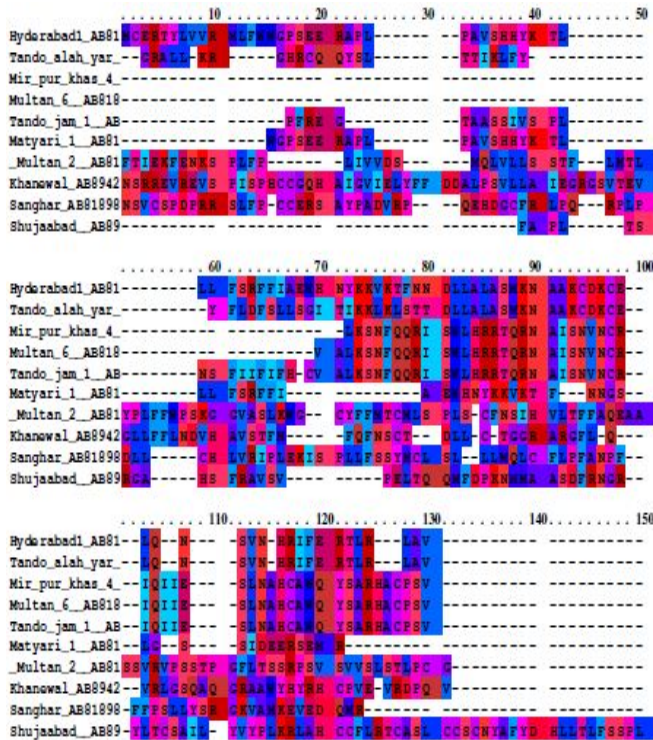


Figure 1c. Amino acid sequences analysis of *Ceratocystis manginecans* from Pakistani isolates

5.8s RNA gene regions are highly conserved between related species due to its structure (Roth *et al.*, 1998). ITS rDNA region is widely used for determining genetic variability within the fungi at specie and sub-specie level (Goodwin & Zismann, 2001 and Karthikeyan *et al.*, 2009). All species of *Ceratocystis* group share an entirely identical sequence motif GAN6TC, where N6 represents six nucleotide positions in the 3' end region of the 5.8 s RNA-coding region. Whereas the *Ceratocystis manginecans* of Pakistan contain a different sequence motif CGN6GT, in the 3' end of the 5.8 s RNA. By using an RNA structure prediction approach it was concluded that the observed nucleotide substitution in the region of 5.8s RNA probably affect it's folding (Fig. 2 a,b,c)

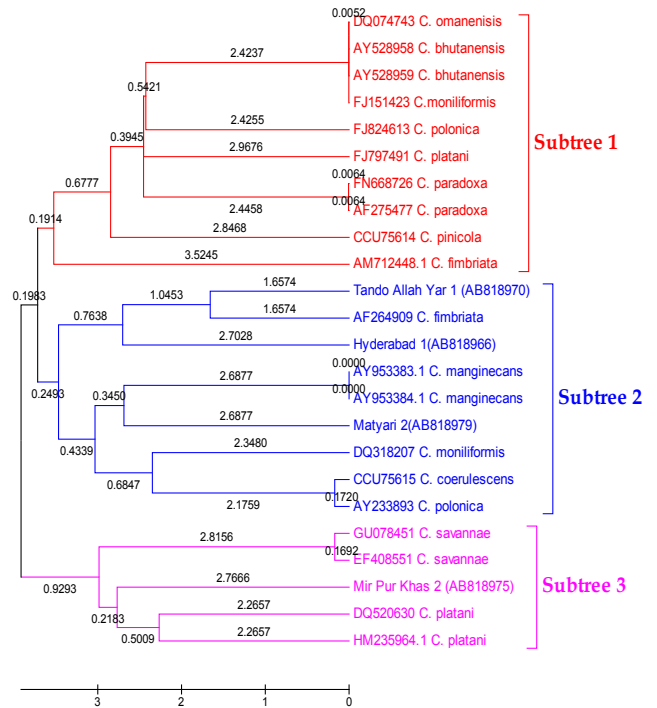


Figure 1d. Phylogenetic tree showing inter relations of *Ceratocystis* sp. with closely related species inferred from 5.8S r DNA sequences. Tree was generated using the neighbor-joining method. Bootstrap value (more than 70%), expressed as percentage of 1000 replicons, and are indicated at the nodes.

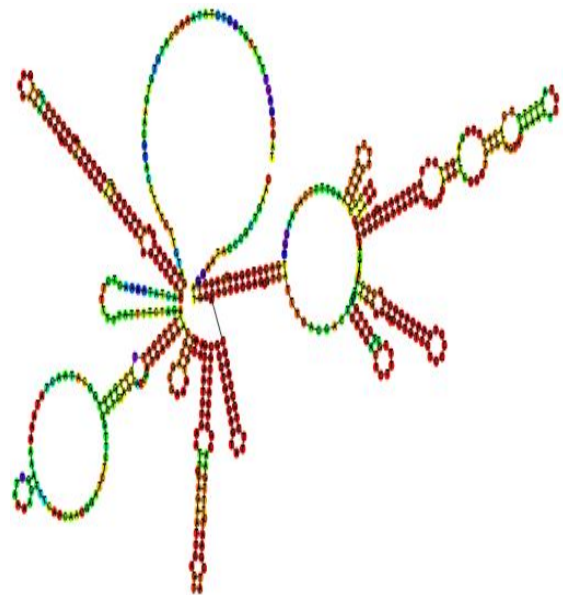


Figure 2a. Secondary structure of *C. manginecans*

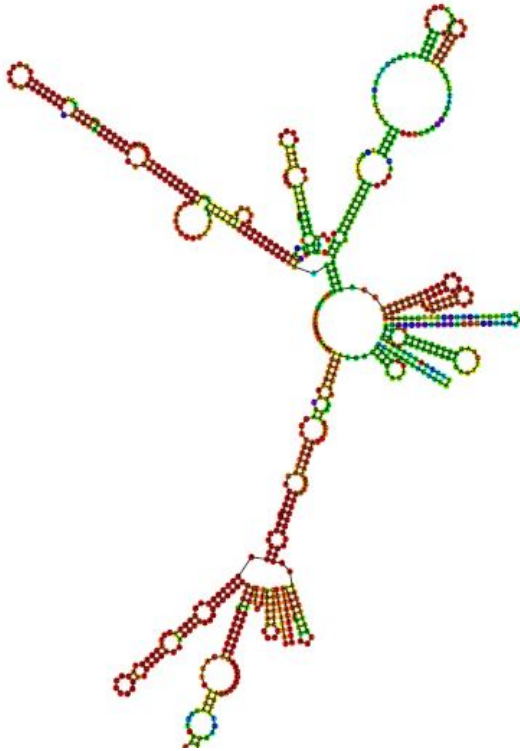


Figure 2b. Secondary structure of *C. omanensis*

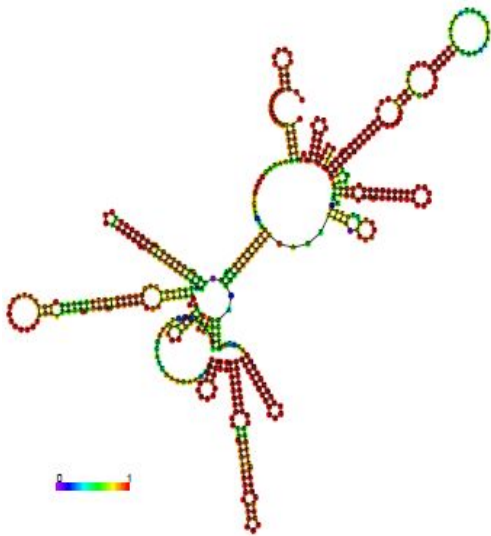


Figure 2c. Secondary structure of *C. fimbriata*

DISCUSSION

This study represents the nucleotide sequence of 5.8 s region of *Ceratocystis manginecans*, a recently identified species of mango sudden death in Pakistan. Comparative molecular study based on the sequenced data was performed between different isolates of *C. manginecans* and other closely related fungus species belonging to *Ceratocystis* group.

A high percentage of sample's infection and frequency of *Ceratocystis* sp. in bark tissues confirms the possible role of the fungus in causing sudden death of mango (Fateh *et al.*, 2006). Micro and macroscopic characterization of *Ceratocystis* sp. samples from sudden death mango tree of Pakistan showed similar characterization agreement with Al-Adawi *et al.* (2006) and Kazmi *et al.* (2005). Coding regions within the fungal rDNA genes 18S, 5.8S, and 28S are quite conserved and evolve slowly in different fungi. While non-coding regions i.e., internally transcribed spacer (ITS1 and ITS2) evolve rapidly so here rates of evolution vary between species of different genera as well as among species of same genus. However, comprehensive sequence analysis identified a nucleotide exchange specific to Pakistani isolates of *Ceratocystis manginecans*. This specific sequence motif (CGN6GT), found at 3'end of 5.8 s RNA startlingly unique for Pakistani isolates of *Ceratocystis manginecans* as a member of *Ceratocystis* groups. All other species belong to *Ceratocystis* group have different sequence motif (CGN6TC) that was found in the *Ceratocystis* members. Due to this difference in the nucleotide molecular evolutionary reconstructions resulted in the formation of phylogenetic tree which showed Pakistani isolates of *Ceratocystis* separated as member clades of other species of *Ceratocystis* (Fig. 3). Pakistani isolates of *Ceratocystis manginecans* was grouped together with other *Ceratocystis manginecans* isolates, thus the sequence characterization results provide new evidence about genetic variation distinguish the isolates of *Ceratocystis manginecans* from previously described *Ceratocystis Omanensis* and *Ceratocystis fimbriata* (AL-Subhai *et al.*, 2006; Prusky, 2000).

Secondary structure modeling showed that the presence of different loops in *C. manginecans* from other members of *Ceratocystis* species. *C. manginecans* consists of both open multibranch loop with several helical domains (Mullineux and Hausner, 2009). Secondary structure predict that ITS stem helix (5.8 s) of Pakistan isolates of *manginecans* had perfect complementary pairing, while the stem helices of other species showed internal bulge. This prominence, differentiate the Pakistan isolates of *C. manginecans* from other closely related members of *Ceratocystis*, and the main reason is the presence of identified sequence variation.

Previously taxonomy of *Ceratocystis* species in Pakistan was based on morphological method and it was much more difficult to distinguish between different species and strains of *Ceratocystis* so comprehensive studies provides here new insight into genetic variability of mango sudden death associated with *Ceratocystis manginecans*. Author's believe that it is the first report of *Ceratocystis manginecans* sp. nov from Pakistan. The most important condition to avoid mango sudden death is good pre- harvest management practices of the orchard. It was observed that farmers make certain mistakes or adapt unrecommended cultural practices which help in infection development.

Acknowledgement: Financial support for this research was provided by Government of Australia through ACIAR under Pakistan Australian Agriculture Sector Linkage Programme (ASLP).

REFERENCES

- Subhi, A.M., A.O. Al Adawi, M. VanWyk, M.L. Deadman and M.J. Wingfield. 2006. *Ceratocystis omanensis*, a new species from diseased mango trees in Oman. *Mycological Res.* 110:237-245.
- Anonymous. 2010. Statistical Year Book of Pakistan. Federal Bureau of Statistics, Islamabad, Pakistan.
- Rijk, P.D. and R.D. Wachter. 1997. RNAVIZ, a program for the visualization of RNA secondary structure. *Nucleic Acids Res.* 25:4679-84.
- Fateh, F.S., M.R. Kazmi, I. Ahmad and M. Ashraf. 2006. *Ceratocystis fimbriata* isolated vascular bundles of declining mango trees in Sindh, Pakistan. *Pak. J. Bot.* 38:1257-1259.
- Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes- application to the identification of mycorrhiza and rusts. *Mol. Ecol.* 2:113-118.
- Goodwin, S.B., L.D. Dunkle and V.L. Zismann. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopath.* 91: 648-658.
- Iqbal, Z., E.E. Valeem, M. Shahbaz, K. Ahmad, Z.I. Khan, M.T. Malik and M. Danish. 2007. Determination of different decline disorders in mango orchards of the Punjab, Pakistan. *Pak. J. Bot.* 39:1313-1318.
- Karthikeyan, M., R. Sandoskumar, S. Mathiyazhagan, M. M. Kumar, V. Valluvaparidasan, S. Kumar and R. Velazhahan. 2009. Genetic variability and aflatoxigenic potential of *Aspergillus flavus* isolates from maize. *Arch. Phytopathol. Plant Protect.* 42:83-91.
- Kazmi, M., R.F.S. Fateh, K. Majeed, A.M. Kashkhely, I. Hussain, I. Ahmad and A. Jabeen. 2005. Incidence and etiology of mango sudden death phenomenon in Pakistan. *Pak. J. Phytopath.* 17:154-158.
- Marek, M., M. Zouhar, O. Douda, J. Mazakova and P. Rysanek, 2010. Bioinformatics-assisted characterization of the ITS1-5.8S-ITS2 segments of nuclear rRNA gene clusters, and its exploitation in molecular diagnostics of European crop-parasitic nematodes of the genus *Ditylenchus*. *Plant Path.* 59:931-43.
- Masood, A., S. Saeed, N. Iqbal, M.T. Malik and M.R. Kazmi. 2010. Methodology for the evaluation of symptoms severity of mango sudden death syndrome in Pakistan. *Pak. J. Bot.* 42:1289-1299.
- Mullineux, T. and G. Hausner. 2009. Evolution of rDNA ITS1 and ITS2 sequences and RNA secondary structures within members of the fungal genera *Grosmannia* and *Leptographium*. *Fungal Genet. Biol.* 46:855-67.
- Ploetz, R.C. 2004. Diseases of Mango. In: R.C. Ploetz (ed.), *Diseases of Tropical Fruit Crops*. CABI Publishing, Wallingford, UK. 544:327-363.
- Prusky, D. 2000. *Colletotrichum* host specificity, pathology and host-pathogen interaction. St. Paul, MN: The American Phytopathological Society.
- Raeder, U. and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* 1:17-20.
- Roth, A., M. Fischer, M.E. Hamid, S. Michalke, W. Ludwi and H. Mauch. 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16s-23s RNA gene internal transcribed spacer sequences. *J. Clin. Microbiol.* 36:139-147.
- White, T., J. Bruns, T.S. Lee and J.W Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (eds.), *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press Increment. pp.315-322.
- Zuker, M. 2003. MFOLD web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31:3406-3415.