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AMPLIFICATION AND SEQUENCING OF INTERNAL TRANSCRIBED REGIONS 1 & 2, AND 5.8S rDNA FROM LOCAL ISOLATES OF *FUSARIUM* SPECIES

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Abstract

Fusarium oxysporum is a phytopathogenic fungus. It is widely distributed around the globe. Conventional classification of *F. oxysporum* is based on phenotypic observations which not only vary highly, but are also sensitive to environment. In *Fusarium* taxonomy this problem is recently being addressed by sequence comparison at different loci. Internally transcribed spacer (ITS) region including 5.8S rRNA coding region in ribosomal DNA is one of the favorite targets for this purpose. The focus of present study was on the genetic diversity analysis of the ITS regions of rRNA gene complex of local isolates of *Fusarium*. The genomic DNA of these isolates was amplified using FoxF, FoxR and FoxIR primers designed at the end, and start of conserved 18S and 28S region and between ITS1 and ITS2 respectively. FoxF and FoxR primer set amplified ~500 bp product from all *Fusarium* strains. The amplified products were sequenced and sequence analyses have shown that *F. oxysporum* f.sp. *ciceri* strains possess a couple of SNPs. Similarly *F. oxysporum* f. sp. *lentis* has shown variations with two strains of *F. oxysporum* f. sp. *ciceri* at two position. Comparison of *F. moniliforme* isolates with *F. oxysporum* isolates have revealed that 5.8S region is identical in all isolates while significant sequence variation was observed in ITS regions of *F. oxysporum* and *F. moniliforme*. Insertions and deletions of many nucleotides were observed at several positions which differentiate *F. moniliforme* from *F. oxysporum*. The phylogenetic analysis revealed no significant difference among local isolates and internationally reported sequences. From a clear grouping of *F. moniliforme* and *F. oxysporum* isolates into different clades it may be evident that ITS regions are useful for classifying *F. oxysporum* isolates at specie level.

Introduction

Genus *Fusarium* was first introduced in 1809 by Link. This genus is notorious for harboring a variety of phytopathogenic fungal species (Zhang *et al*., 2012).Wilt which is an economically important plant disease with worldwide distribution is caused by *Fusarium oxysporum* which colonizes in vascular tissues and causes wilting of plant (Rai *et al.*, 2011). *F. oxysporum* is a specie complex and different host specific individual within this complex are termed as *formae specialis* and abbreviated as "f. sp." (Beckman, 1987). On the basis of cultivar specific pathogenecity of *F. oxysporum* strains, some *formae speciales* are further divided into sub-groups called races (Armstrong & Armstrong, 1981).

Wilt disease is among major yield reducing factors in Pakistan such a wilt epidemic occurred in 1956 and caused more than 75% crop yield losses. In Pakistan, wilt disease causes an estimated annual loss of Rs. 12 million (Sattar *et al*., 1953). Wilt pathogen survives in soil for years (Farhat *et al*., 2010) hence simple methods such as crop rotation is not much effective. Use of resistant crop varieties is a good strategy against wilt but their use is limited due to location specific pathogen races (Singh *et al.*, 2006).

Traditionally classification of *Fusarium* isolates was based on morphological characters like presence/absence of chlamydospores, and size and shape of macro and microconidia (Leslie *et al*., 2007). *Fusarium* isolates were also classified on the basis of vegetative compatibility groups (Puhalla, 1985) and host specificity, nevertheless all these parameters were not persistent to develop a consensus scheme.

With the advancement of molecular biology, fungal classification and phylogenetic studies have shifted to DNA sequence base methods (Bruns *et al*., 1991). These methods play an important role in *Fusarium* identification (Lee *et al.*, 2000) and in understanding of genetic diversity of members of genus *Fusarium* (Bogale *et al.*, 2006).

In present study, genetic characterization of local *Fusarium* isolates was done by using internal transcribed spacer (ITS) sequences of rRNA gene complex. There is a significant consensus about the use of the ITS sequences in mold identification as an initial step and as a default region for species identification by international sub-commission on Fungal Barcodeing (Balajee *et al*., 2009).

Nuclear rDNA is considered an ideal target for specific PCR primers, as each sequence is variable at the family, genus, or species level. It is a complex of tandem repeats containing genic and nongenic or spacer regions (Cullings & Vogler, 1998). Component of this gene complex have different domains that evolved at different rates (Jorgenson & Cluster, 1988). The rRNA genes are highly conserved (Bruns *et al*., 1991) and have been used in addressing broad phylogenetic hypothesis (Lee *et al*., 2000). The ITS regions are more variable and used to analyze interspecies (Vogler & Bruns, 1998) and sometimes intra-species relations (Bruns *et al*., 1991).

In present study, local pathogenic isolates of *Fusarium* were characterized for the first time on genic level. ITS regions of rDNA gene complex were sequenced and compared to determine similarity and diversity of these isolates at genetic level. The study will contribute towards understanding of genetic make-up of local pathogenic *Fusarium* strains and may contribute significantly in crop breeding and disease management.

Materials and Method

All isolates of *Fusarium oxysporum* and *Fusarium moniliforme* were obtained from National Agriculture Research Centre (NARC), which were isolated either from seeds or roots of wilted lentil, chickpea and maize plants from different areas of Pakistan mentioned in Table 1. Total genomic DNA was isolated from all *Fusarium* isolates by the miniprep protocol reported by Cenis, (1992) with minor modifications. Briefly, *Fusarium* strains grown on potato dextrose agar were carefully scraped off the surface of the agar with the help of sterilized spatula and transferred to 1.5 mL eppendorf tube. 500 µL of Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) was added to the mycelial mat and washed by centrifugation at 13,000 rpm for 5 minute. The pelleted mass was homogenized manually for 5-10 min in 500 µL of extraction buffer (0.5% sodium dodecyl sulfate, 200 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 25 mM EDTA). After homogenization, 150 µL of 3 M sodium acetate (pH 5.2) was added and mixture was shifted to an eppendorf and placed at -20°C for 10 min. The tube was then centrifuged for 5 min at 13,000 rpm and supernatant was carefully transferred to a new clean tube. An equal volume of isopropanol was added followed by centrifugation for 10 min at 13,000 rpm to pellet down the DNA. Supernatant was removed and DNA pellet was washed twice with 500 µL of 70% ethanol to remove excess salt. DNA pellet was air dried and resuspended in 50 µL Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Table 1. List of isolates used in present study.

Isolate	Source	Location*
P.FSD1	*Cicer arietinum* Plant	AARI-FSD
P.CHK2	*Cicer arietinum* Plant	Chakwal
P.FSD3	*Cicer arietinum* Plant	NIAB-FSD
P.ISD4	*Cicer arietinum* Plant	NARC-ISD
P.ISD6	*Lens culnaris* Seed	NARC-ISD
P.ISD9	*Zea mays* Plant	NARC-ISD
P.ISD10	*Zea mays* Plant	NARC-ISD

*__AARI-FSD__: AYUB Agricultural Research Institute, Faisalabad
__NIAB-FSD__: Nuclear Institute for Agriculture and Biology, Faisalabad
__NARC-ISD__: National Agricultural Research Centre, Islamabad

Primers were designed manually on conserved ribosomal DNA 18S and 28S region to amplify ITS1- 5.8S-ITS2 regions (Table 2). For this purpose sequences of *F. oxysporum* f. sp. *ciceri* were obtained from NCBI (www.ncbi.nlm.nih.gov) bearing accession numbers GQ856367.1, GQ856366.1, EU442589.1, EU442590.1, EU442588.1, EU442582.1, EU442583.1 and EU442590.1.

Table 2. Primers used for amplification and sequencing.

S. No.	Primers	Sequence (5' to 3')
1.	Fox F	CMT KCG GAG YGA TCA TTA CC
2.	Fox R	TTC CTA CCT GAT CCG AGK TCAA
3.	Fox IR	GCA TTT TGC TGC GTT CTT CATC

Each PCR mixture contained 2.5 µL 10 X Taq polymerase buffer, 2 µL $MgCl_2$ (25mM), 0.5 µL dNTPs mix (10mM), 2 µL of genomic DNA preparation, 25 pM each of forward (Fox F) and reverse (Fox R) primers, 0.4 µL DNA polymerase (5U/ µL) and 16.6 µL of distilled water. Thermal profile used included one cycle of denaturation at 94°C for 3 min, annealing at 51°C for 2 min, and extension at 72°C for 3 min followed by 35 cycles at 94°C, 51°C and 72°C for 20, 20 and 40 seconds each respectively. Final extension was given at 72°C for 20 minute.

Amplified products from all strains were eluted from the gel using Wizard SV Gel and PCR Clean-Up System according to the instruction of manufacturer (Promega). Sequencing was performed using an internal primer (FoxIR) in addition to a forward (FoxF) or a reverse (FoxR) primer. FoxF and FoxR primers were same as used for amplification. Blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homology of consensus sequences obtained from multiple sequence runs, with already reported sequences present in nucleotide database.

ClustalW (http://www.genome.jp/tools/clustalw), a multiple sequence alignment (MSA) tool was employed to score similarity among different isolates. Molecular evolutionary genetic analysis (MEGA v 5.0) program (Tamura *et al*., 2011) was used for phylogenetic analysis and a neighbor joining tree based on nucleotide sequences of ITS regions used in present study was constructed with additional *Fusarium* ITS sequences obtained from GenBank. p-distance method was used to compute evolutionary distances (Nei & Kumar, 2000) while tree reliability was determined by applying bootstrap test analysis (Felsenstein, 1985).

Results

Amplified product of approximate 500 bp was generated from all of the *Fusarium* strains using FoxF and FoxR primers (Fig. 1).

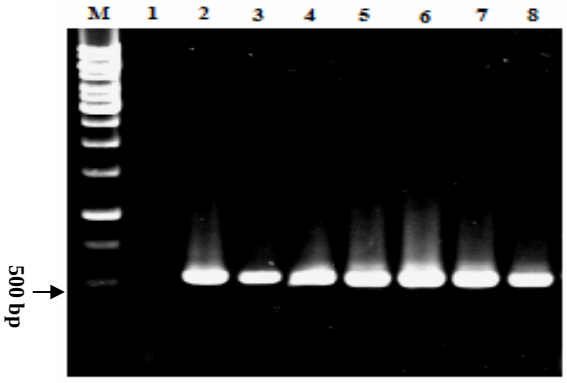

Fig. 1. PCR products of *Fusarium* strains at 51ºC annealing temperature. Amplified PCR product obtained using FoxF and FoxR primers at 51ºC annealing temperature.Lane M:1kbDNAladder (Fermentaz), Lane 1: negetive control, Lane 2: FSD1, Lane 3: CHK2, Lane 4: FSD3, Lane 5: ISD4, Lane 6: ISD6, Lane 7: ISD9, Lane 8: ISD10.A band of 500 bp was observed in lane 2-8.

Amplified products from all *Fusarium* strains were sequenced with FoxF, FoxR and FoxIR primers. Sequence alignment of *F. oxysporum* isolates showed very low sequence diversity among them (Fig. 2). Only single nucleotide variation was observed at two sites in ITS regions. In FSD3 strain, nt T is present at position 438 while in all other *F. oxysporum* f. sp. *ciceri* nt A is present at the same position. Nucleotide T and A both are reported at this position by O'Donnell *et al.,* (2009) & Dong *et al*., (2005) in various isolates worldwide. Another difference among *F. oxysporum* f. sp. *ciceri* strains is the presence of nt A at 75 position in ISD4 instead of nt C which is present in all other *F. oxysporum* f. sp. *ciceri* strains at the same position. Different sequences with nt C and A at the same positon have already been reported from different *Fusarium* isolates (Shrestha *et al.,* 2011).

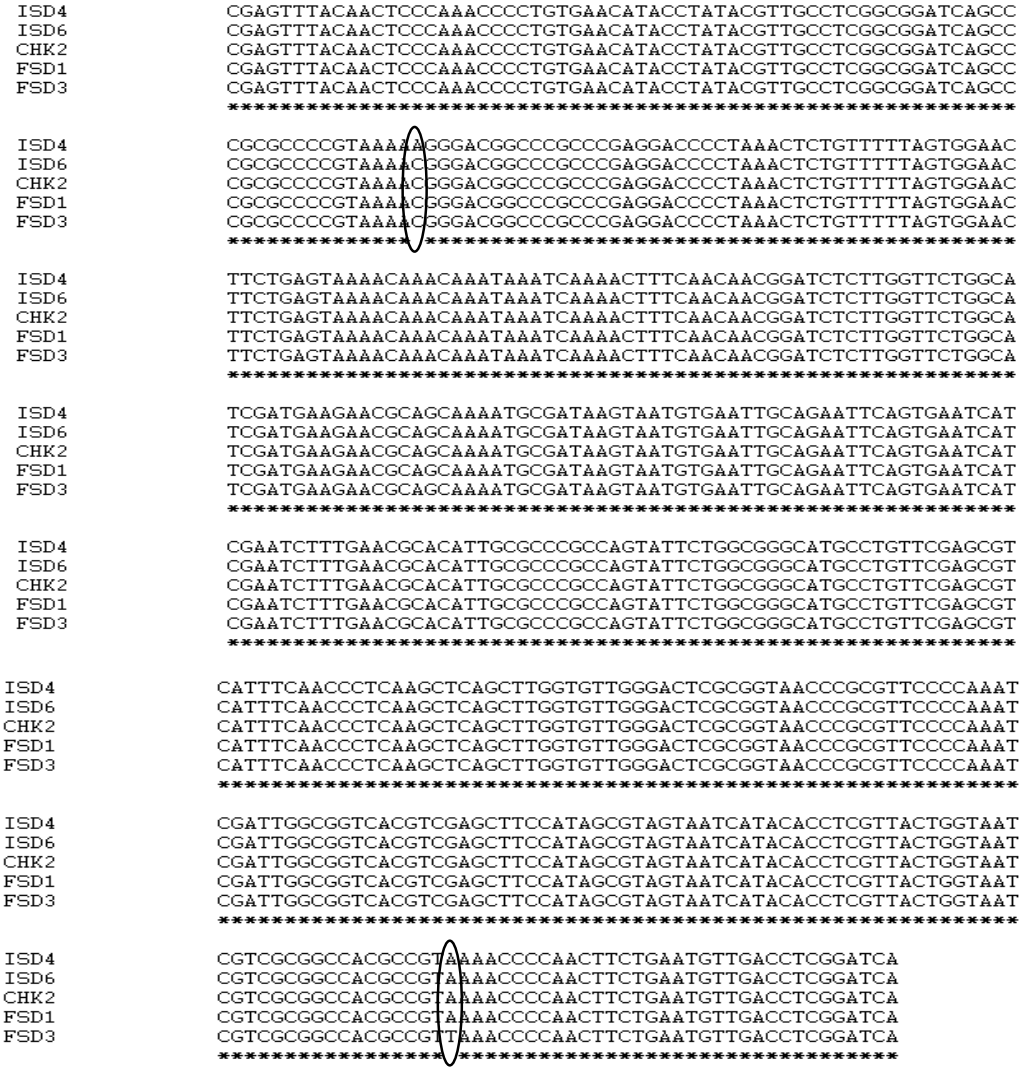

Fig. 2. Sequence alignment of *F. oxysporum* isolates. Regions showing sequence variations are placed in ovals.

Alignment has shown that sequence of *F. oxysporum* f. sp. *lentis* strain is identical to *F. oxysporum* f. sp. *ciceri* strains FSD1 and CHK2 but it has shown variations with FSD3 and ISD4 strains at two positoin (Fig. 2). *F. oxysporum* f. sp. *lentis* strain has nt C at position 75 (GenBank accession number GQ505706) while nt A is present in ISD4 strain. *F. oxysporum* f. sp. *lentis* strain also has sequence variation at 438 position. *F. oxysporum* f. sp. *lentis* has nt A at this positoin while ISD3 has nt T at this position. Sequence variations among strains of same *forma specialis* and sequence similarities of strains belonging to different *formae speciales* have suggested that isolates belong to different *formae speciales* may genetically be more similar than isolates within a *forma specialis* (Kistler, 1997). Alignment of sequences obtained from *Fusarium moniliforme* strains showed that both have identical ITS regions and not even a single nucleotide variation was observed (Fig. 3). Comparison of *Fusarium moniliforme* strains with *Fusarium oxysporum* strains revealed that 5.8S region was identical in all *Fusarium* strains (Fig. 4). Insertion and deletion of many nucleotides were observed at several positions which clearly differentiate *F. oxysporum* from *F. moniliforme*.

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ISD9            CGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCC
ISD10           CGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCC
                ************************************************************

ISD9            GCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAAC
ISD10           GCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAAC
                ************************************************************

ISD9            TTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
ISD10           TTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
                ************************************************************

ISD9            CGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
ISD10           CGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
                ************************************************************

ISD9            GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTC
ISD10           GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTC
                ************************************************************

ISD9            ATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCTCACGGCAAGCCG
ISD10           ATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCTCACGGCAAGCCG
                ************************************************************

ISD9            GCCCCGAAATACAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTAAAACCCTCGC
ISD10           GCCCCGAAATACAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTAAAACCCTCGC
                ************************************************************

ISD9            AACTGGTACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGAT
ISD10           AACTGGTACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGAT
                ************************************************************

ISD9            CA
ISD10           CA
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Fig. 3. Sequence alignment of *F. moniliforme* strains.

Neighbor joining (NJ) tree was constructed using analyzed and internationally reported *Fusarium* sequences taken from GenBank nucleotide database. NJ analysis revealed that *Fusarium* strains were separated into two clades (Fig. 5). Irrespective of their geographic origin all strains of *F. oxysporum* were grouped together in clade 1. Clade 1 further splits into clusters and sub-clusters. P.FSD1, P.CHK2 and P.ISD6 strains, which have identical ITS regions, were isolated in same sub-cluster. Strain P.ISD and P.FSD3, both differed in one nucleotide from other *F. oxysporum* strains and were placed in separate sub-cluster 2 (64% bootstrap support) and cluster 1 (100% bootstrap support) respectively with in clade 1. P.ISD9 and P.ISD10 strain which showed remarkable nucleotide differences compared with *F. oxysporum* strains were grouped into a separate clade 2 on phylogenetic tree.

The evolutionary history was inferredusing Neighbor-Joining method. The optimal tree with the sum of branch length=0.09891304 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates), are shown next to the branches. The tree is drawn to scale, with branch lengths in the same unit as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base difference per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st +2nd +3rd non-coding. All positions containing gaps and missing data were eliminated.

Discussion

There is a significant consensus about use of rRNA gene complex in mold identification (Balajee *et al.,* 2007). rRNA gene complex is a mosaic of conserved and variable regions and has various utilities in phylogenetic analyses. In the present study ITS region of rRNA gene complex was used to evaluate genetic diversity of geographically distributed local *Fusarium* isolates. Sequence specific primers were designed at conserved genic regions of rRNA gene complex to amplify ITS regions. A band of almost 500 bp was amplified from all isolates. Sequence alignment analysis suggested low sequence diversity among all *F. oxysporum* strains. Alignment of *F. oxysporum* strains with *F. moniliforme* strains revealed identical 5.8S region from all strains. 5.8S is gene is a conserved region and can be used to examine important evolutionary events (Samuels & Seifert, 1995). Insertion and deletion of many nucleotides at several positions were observed which differentiate *F. oxysporum* from *F. moniliforme*. It may also be evident from this study that ITS regions are good candidate to classify fungal strains at specie level (Chillali *et al*., 1998).

Phylogenetic analysis indicates that local isolates have low level of genetic diversity among them. From phylogenetic analysis it can be inferred that local isolates do not show any significant genetic variability from internationally reported sequences.

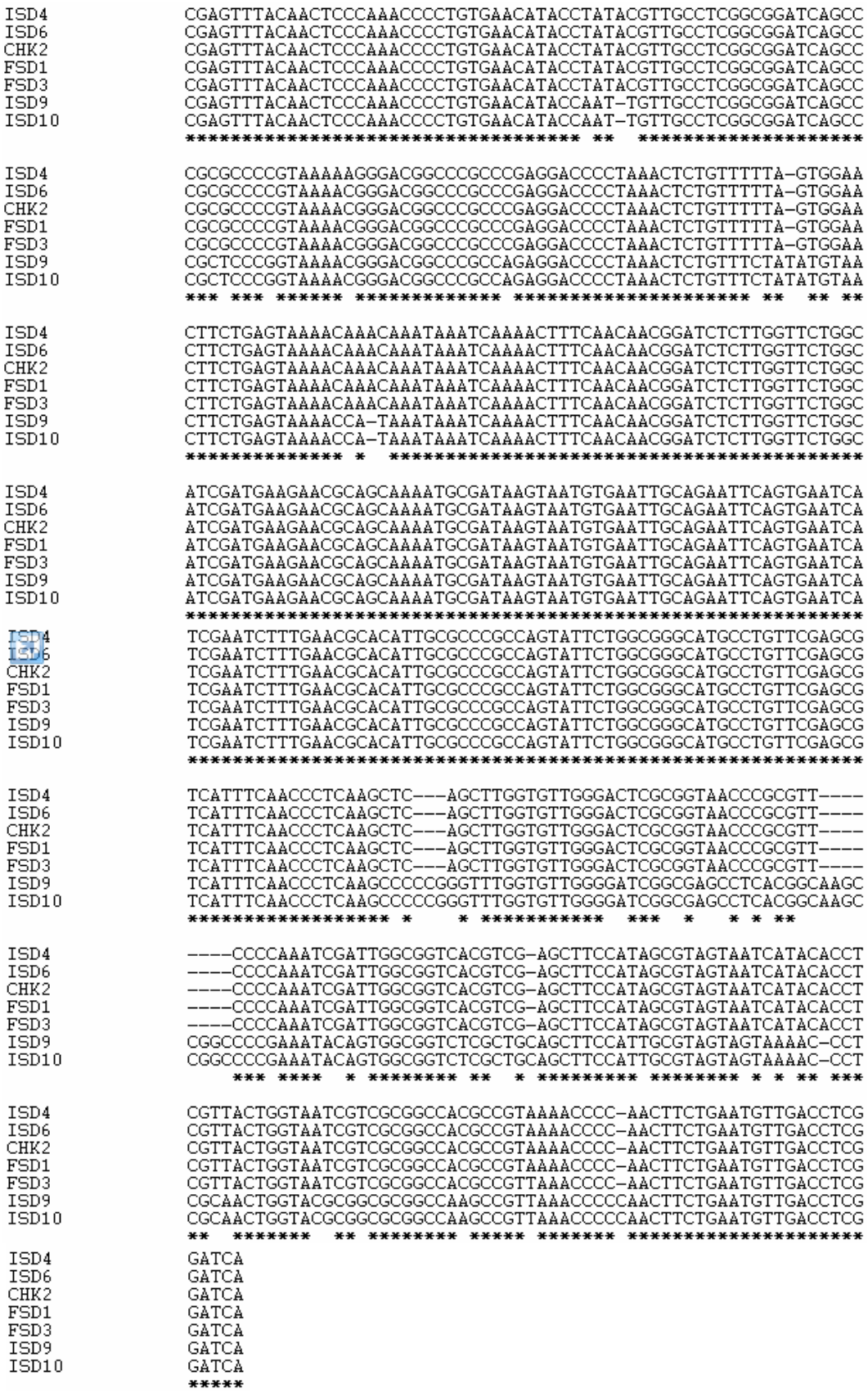

Fig. 4. Sequence alignment of all *Fusarium* isolates.

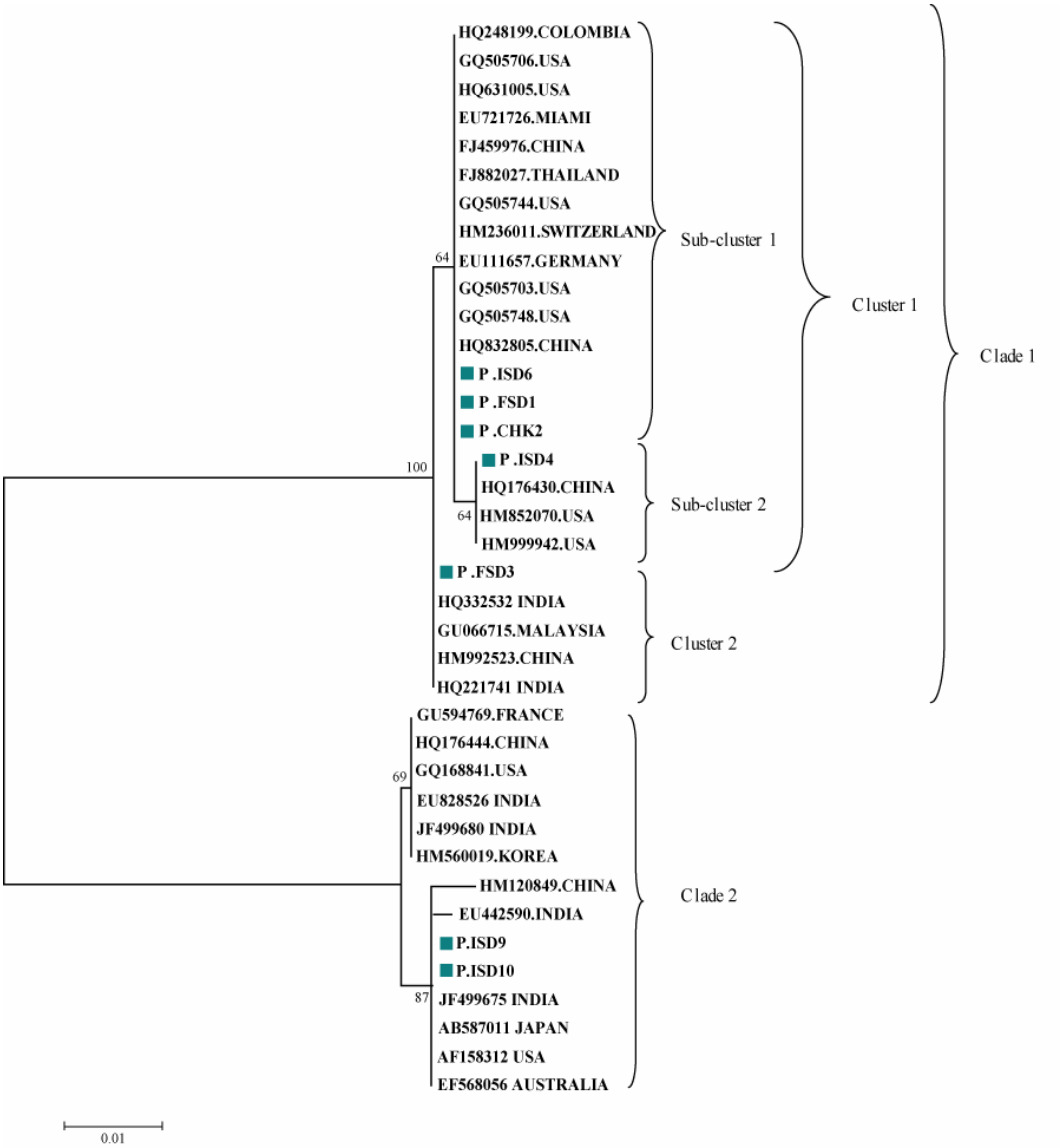

Fig. 5. NJ tree of all *Fusarium isolates*.

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References

Armstrong, G.M. and J.K. Armstrong. 1981. *Formae speciales* and races of *Fusarium oxysporum* and *Alternaria alternata*. In: *Fusarium Diseases, Biology and Taxonomy*. (Ed.): R. Cook. Penn State University Press, University Park, PA. pp. 391-399.

Balajee, S.A., J. Houbraken, P.E. Verweij, S.B. Hong, T. Yaghuchi, J. Varga and R.A. Samson. 2007. Aspergillus species identification in the clinical setting. *Stud. Mycol*., 5(9): 39-46.

Balajee, S.A., A.M. Borman, M.E. Brandt, J. Cano, M. Cuenca-Estrella, E. Dannaoui, J. Guarro, G. Haase, C.C. Kibbler, W. Meyer, K. O'Donnell, C.A. Petti, J.L. Rodriguez-Tudela, D. Sutton, A. Velegraki and B.L. Wicke. 2009. Sequence-Based Identification of *Aspergillus, Fusarium*, and *Mucorales* Species in the Clinical Mycology Laboratory Where Are We and Where Should We Go from Here? *J. Clin. Microbiol*., 47(4): 877-884.

Beckman, C.H. 1987. *The nature of wilt diseases of plants*. The American Phytopathological Society Press, St. Paul, MN.

Bogale, M., B.D. Wingfield, M.J. Wingfield and E.T. Steenkamp. 2006. Characterization of *Fusarium oxysporum* isolates from Ethiopia using AFLP, SSR and DNA sequence analyses. *Fungal Diversity*, 2(3): 51-66.

Bruns, T.D., T.J. White and J.W. Taylor. 1991. Fungal molecular systematics. *Ann. Rev. Ecol. Syst*., 22: 525-564.

Cenis, J.L. 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucl. Acids Res*., 20(9): 2380.

Chillali, M., H. Ighili-Idder, J.J. Guillaumin, C. Mohammed, B.L. Escarmant and B. Botton. 1998. Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European Armillaria. *Mycol. Res*., 10(2): 533-540.

Cullings, K.W. and D.R. Vogler. 1998. A 5.8S ribosomal RNA gene sequence database application to ecology and evolution. *Mol. Ecol*., 7(9): 19-923.

Dong, D., H. Liu, H. Peng, X. Huang, X. Zhang and Y. Xu. 2005. Rapid differentiation of *Fusarium oxysporum* isolates using PCR-SSCP with the combination of pH-variable electrophoretic medium and low temperature. *Electrophoresis*, 26 (22): 4287-4295.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evol.*, 39: 783-791.

Jamil, F.F., M. Sarwar, N. Sarwar, J.A. Khan, M.H. Zahid, S. Yousaf, H.M.I. Arshad and I. Haq. 2010. Genotyping with RAPD markers resolves pathotype diversity in the *Ascochyte Blight* and *Fusarium wilt* pathogenof chickpea in Pakistan. *Pak. J. Bot.,* 42(2): 1369-1378.

Jorgenson, R.D. and P.D. Cluster. 1988. Modes and tempos in the evolution of nuclear rDNA new characters for evolutionary studies and new markers for genetic and population studies. *Ann. Missouri Bot. Gard.,* 7(5): 1238-1247.

Kistler, H.C. 1997. Genetic diversity in the plant-pathogenic Fungus *Fusarium oxysporum*. *Phytophathol.*, 8(7): 474-479.

Lee, Young-Mi, Y. Choi and B. Min. 2000. PCR-RFLP and Sequence Analysis of the rDNA ITS Region in the *Fusarium* spp. *J. Microbiol*., 3(8): 66-73.

Leslie, J.F., L.L. Anderson, R.L. Bowden and Y. Lee. 2007. Inter and specific genetic variation in Fusarium. *Int. J. of Food Microbiol*., 11(9): 25-32.

Nei, M. and S. Kumar. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.

O'Donnell, K., D.A. Sutton, M.G. Rinaldi, C. Gueidan, P.W. Crous and D.M. Geiser. 2009. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the United States. *J. Clin. Microbiol*., 47(12): 3851-3861.

Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Can. J. Bot.*, 63: 179-183.

Rai, G.K., R. Kumar, J. Singh, P.K. Rai and S.K. Rai. 2011. Peroxidase, polyphenol oxidase activity, protein profile and phenolic content in tomato cultivars tolerant and susceptible to *F. oxysporum* f.sp. *lycopersici*. *Pak. J. Bot.,* 43(6): 2987-2990.

Samuels, G.J. and K.A. Seifert. 1995. The impact of molecular characters on systematics of filamentous ascomycetes. *Ann. Rev. Phytopathol.,* 33: 37-67.

Sattar, A., A.G. Arif and M. Mohy-ud-Din. 1953. Effect of soil temperature and moisture on the incidence of gram wilt. *Pakistan J. Sci. Res*., 5: 16-20.

Shrestha, P., T.M. Szaro, T.D. Bruns and J.W. Taylor. 2011. Systematic search for cultivatable fungi that best deconstruct cell walls of Miscanthus and sugarcane in the field. *Appl. Environ. Microbiol*., 77(15): 5490-5504.

Singh, B.P., R. Saikia, M. Yadav, R. Singh, V.S. Chauhan and D.K. Arora. 2006. Molecular characterization of *Fusarium oxysporum* f. sp. *ciceri* causing wilt in chickpea. *African J. Biotech*., 5(6): 497-502.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. *Mol. Biol. Evol.*, 28: 2731-2739.

Vogler, D.R. and T.D. Bruns. 1998. Phylogenetic relationship among the pine stem rust fungi (*Peridermium* spp.). *Mycology*, 90(2): 244-257.

Zhang, S., X. Zhao, Y. Wang, J. Li, X. Chen, A. Wang and J. Li. 2012.Molecular detection of *Fusarium oxysporum* in infected cucumber plants and soil. *Pak. J. Bot.,* 44(4): 1445-1451.

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