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SHORT COMMUNICATION

Phylogenetic relationship between different race representative populations of *Fusarium oxysporum* f. sp. *ciceris* in respect of translation elongation factor-1α, β-tubulin, and internal transcribed spacer region genes

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Abstract Genetic diversity of 70 isolates of *Fusarium oxysporum* f. sp. *ciceris* originated from various states of India representing eight races causing wilt in chickpea (*Cicer arietinum*) was analyzed using translation elongation factor-1α (TEF-1α), β-tubulin, and internal transcribed spacer (ITS) gene regions. TEF-1α, β-tubulin, and ITS gene-specific markers produced ~720-, ~500-, and ~550-bp amplicons, respectively, in all the isolates of the pathogen. A phylogenetic tree constructed from the sequences generated in the present study along with the sequences of foreign isolates of *Fusarium* species available in NCBI database sharing more than 90 % nucleotide sequence similarity grouped the isolates into two major clusters. Most of the isolates of the present study showed more or less similar grouping pattern in case of the three gene sequences. Each group had the isolates representing different races as well as place of origin indicating low level of diversity among the isolates in respect of these gene sequences. Except TEF-1α, the groups generated by β-tubulin and ITS gene sequences did not correspond to the state of origin and races of the pathogen. However, the groups of TEF-1α partially corresponded to the place of origin as well as races of the pathogen. The isolates did not show any race-specific grouping patterns; however, most of the isolates representing race 1 clustered separately.

Keywords Genetic diversity · TEF-1α · β-tubulin · ITS · Chickpea · *Fusarium oxysporum* f. sp. *ciceris*

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Introduction

Chickpea (*Cicer arietinum* L.) is an important pulse crop cultivated worldwide. Wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo and K. Sato is one of the major factors responsible for low productivity of the crop. The disease is distributed in all the chickpea-growing areas of the world including India. Eight races of *F. oxysporum* f. sp. *ciceris* were identified by reactions on a set of differential chickpea cultivars. Of these, 4 races, namely 1A, 2, 3, and 4, were reported from India (Haware and Nene 1982), while races 0, 1B/C, 5, and 6 were reported from Mediterranean region and the USA (Phillips 1988). In addition to differences in races, the Indian populations of the pathogen produce typical vascular wilt symptoms in chickpea. Honnareddy and Dubey (2006) reported the occurrence of more than one race from each state of India along with a few new races. Further, it was suggested that some of the new chickpea cultivars showing clear-cut differential reaction for Indian population of the pathogen possibly due to the development of new variants in a field should be incorporated into earlier reported set of differentials (Dubey and Singh 2008; Dubey et al. 2010).

Different molecular markers were used for the characterization of genetic diversity of the pathogen and the evolutionary origin of a particular forma specialis (Jimenez-Gasco et al. 2002; Dubey and Singh 2008). The phenotypic and genetic characterization of an area-specific pathogenic population of *F. oxysporum* f. sp. *ciceris* is essential for efficient disease management. Different genetic markers such as ITS or intergenic spacer regions of ribosomal DNA (rDNA) and the TEF-1α gene have been used to resolve evolutionary relationships in *Fusarium* species and for developing PCR (polymerase chain reaction)-RFLP assays for the differentiation of *Fusarium* at the species

level (Gurjar et al. 2009). The TEF-1α gene (Stengleina et al. 2010) and β-tubulin gene sequences have been used for phylogenetic analysis in fungi (Thon and Royse 1999). Jimenez-Gasco et al. (2002) analyzed 7 *F. oxysporum* f. sp. *ciceris* isolates representing four Indian races for TEF-1α and β-tubulin gene sequences. Gurjar et al. (2009) used ITS-RFLP, ISSR, and AFLP for distinguishing Indian races of the pathogen. In both the studies, less number of isolates were used and the virulence analysis of these isolates has not been done for race determination as now it has been proved that each state of India has more than one race (Dubey and Singh 2008) and new distribution pattern of the race in India has been determined (Dubey et al. 2012). The isolates included in Jimenez-Gasco et al. (2002) study may be representative of the same race, because earlier the race distribution was area/state specific, but presently, it has been observed that each state/area has more than one race (Dubey et al. 2012). The three genes TEF-1α, β-tubulin, and ITS sequences of Indian populations of *F. oxysporum* f. sp. *ciceris* representing 8 races have not been analyzed earlier.

The aims of the present study were to analyze the genetic diversity of new Indian populations of *F. oxysporum* f. sp. *ciceris* originating from different chickpea-growing areas on the basis of TEF-1α, β-tubulin, and ITS gene sequences and to examine whether the variation in the sequences of these genes could be correlated with their geographic origin (state) and/or races of the pathogen.

Materials and methods

Fungal cultures and DNA extraction

Seventy isolates of *F. oxysporum* f. sp. *ciceris* collected from 13 states of India were included in the present study (Table 1). These isolates are representative populations of 640 isolates of the pathogen, which have already been characterized for their morphological feature as well as virulence (Dubey and Singh 2008; Dubey et al. 2010). The isolates were maintained at 4 °C on potato dextrose agar. The single spore pure cultures of *F. oxysporum* f. sp. *ciceris* isolates were multiplied in potato dextrose broth (20 g l^{-1}; Hi-media) at 25 ± 1 °C on shaking incubator for 120 rpm for 7 days. Harvested mycelial mats were used for DNA extraction following modified CTAB method (Murray and Thompson 1980; Dubey and Singh 2008) and stored at −20 °C in small aliquots.

PCR amplification and sequencing

The genomic DNA of 70 isolates was analyzed with gene-specific primers, namely TEF-1α, β-tubulin, and ITS. PCR mixture (25 μL) contained 50 ng template DNA, 1.0 U *Taq* DNA polymerase, 1.5 mmol/L of $MgCl_2$, 0.6 mmol/L of each dNTPs (Bangalore Genei, India), 10 pmol each of primers for TEF-1α and β-tubulin, and 5 pmol each for ITS in 10× reaction buffer. The PCR was performed using Eppendorf ep™ gradient thermal cycler with initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for TEF-1α, 62 °C for β-tubulin and 55 °C for ITS for 1 min, and extension at 72 °C for 1 min with final extension at 72 °C for 7 min followed by cooling at 4 °C. Amplification products were electrophoresed on 1.25 % agarose gel in 1x TAE buffer subsequently eluted, purified using *Genei Pure*™ gel extraction and purification kits (Bangalore Genei, India), and sequenced (Xcelris Labs Limited, Ahmedabad, India). The nucleotide sequences were subjected to BLAST analysis (http://www.ncbi.nih.gov/index.html) and submitted to *GenBank* at NCBI and accession numbers (JN231134 to JN231186 for TEF-1α gene, JN231187 to JN231236 for β-tubulin gene, and JN400675 to JN400720 for the ITS region) obtained. The multiple sequence alignment and pairwise alignment were made using BioEdit version 7.0.5 (Hall 1999). The phylogenetic tree was constructed by CLUSTALW 1.8 sequence alignment and bootstrap neighbor joining by MEGA 4.1 program with 1,000 bootstrap replicates.

Results

TEF-1α analysis

The genomic DNA of 70 isolates of *F. oxysporum* f. sp. *ciceris* was successfully amplified with a set of TEF-1α gene-specific primers EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) (O'Donnell et al. 1998) and obtained ~720-bp amplicons. The nucleotide sequences of 53 representative isolates of *F. oxysporum* f. sp. *ciceris* varied from 630 to 737 bp and shared more than 90 % nucleotide sequence similarity with the TEF-1α sequences of *F. oxysporum* available in *GenBank*. The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 53 isolates of the present study along with 11 isolates of *F. oxysporum* from other countries clearly grouped the isolates into two major clusters (Fig. 1). Thirty-nine isolates of the present study representing eight races were grouped into first major cluster along with 11 isolates from other countries. Second major cluster had 14 isolates from the present study. Out of 12 isolates of race 1, 8 were placed into this cluster along with two isolates of race 6 and one isolate each from race 2, 3, 5, and 7. The both major clusters were further subdivided into two subclusters.

Table 1 The details of the isolates of *Fusarium oxysporum* f. sp. *ciceris* and their *GenBank* accession numbers for various genes used in the present study

S. No.	Accession no.	Place of collection/district	States	Race	*GenBank* accession no		
					TEF-1α	β-tubulin	ITS
1	Foc 118	ICRISAT, Hyderabad	Andhra Pradesh	1	JN231134	JN231190	JN400678
2	Foc 168	DOR, Hyderabad	Andhra Pradesh	1	–	–	–
3	Foc 169	Hyderabad	Andhra Pradesh	1	JN231135	JN231187	JN400675
4	Foc 143	Guntur	Andhra Pradesh	1	JN231136	JN231188	JN400676
5	Foc 144	Nellore	Andhra Pradesh	1	JN231137	JN231189	JN400677
6	Foc 149	Warangal	Andhra Pradesh	1	JN231180	JN231207	JN400691
7	Foc 127	Bilaspur	Chhattisgarh	6	JN231138	JN231192	JN400680
8	Foc 162	Bilaspur	Chhattisgarh	6	–	–	–
9	Foc 161	Raipur	Chhattisgarh	6	JN231139	JN231191	JN400679
10	Foc 53	IARI New Delhi (MB-4C)	Delhi	4	JN231140	JN231193	–
11	Foc 108	IARI New Delhi	Delhi	4	JN231141	JN231194	JN400681
12	Foc 123	Anand	Gujarat	7	JN231142	JN231196	JN400684
13	Foc 163	Anand	Gujarat	7	–	–	–
14	Foc 122	Junagadh	Gujarat	7	JN231143	JN231197	JN400682
15	Foc 164	Porbandar	Gujarat	7	JN231144	JN231195	JN400683
16	Foc 41	Hisar	Haryana	4	JN231145	JN231198	–
17	Foc 66	Rohtak	Haryana	4	–	–	–
18	Foc 33	Shikohpur	Haryana	4	JN231146	JN231199	JN400685
19	Foc 23	Dumka	Jharkhand	6	JN231147	JN231201	JN400688
20	Foc 100	Darisi	Jharkhand	6	JN231148	JN231203	JN400686
21	Foc 28	Ranchi	Jharkhand	6	JN231149	JN231200	JN400687
22	Foc 46	Ranchi	Jharkhand	6	–	–	–
23	Foc 97	Ranchi	Jharkhand	6	JN231150	JN231202	–
24	Foc 98	Ranchi	Jharkhand	6	–	–	–
25	Foc 126	Bangalore	Karnataka	1	JN231185	JN231208	JN400692
26	Foc 121	Dharwad	Karnataka	1	–	–	–
27	Foc 152	Dharwad	Karnataka	1	JN231184	JN231209	JN400694
28	Foc 150	Gulbarga	Karnataka	1	JN231183	JN231204	JN400693
29	Foc 151	Shimoga	Karnataka	1	JN231182	JN231205	JN400689
30	Foc 148	Raichur	Karnataka	1	JN231181	JN231206	JN400690
31	Foc 155	Jabalpur	Madhya Pradesh	6	JN231179	JN231214	JN400717
32	Foc 156	Indore	Madhya Pradesh	6	JN231178	JN231210	JN400714
33	Foc 157	Rewa	Madhya Pradesh	6	JN231177	JN231211	JN400715
34	Foc 158	Sehore	Madhya Pradesh	3	JN231176	JN231212	JN400716
35	Foc 153	Narsinghpur	Madhya Pradesh	7	JN231175	JN231215	JN400718
36	Foc 160	Tikamgarh	Madhya Pradesh	7	JN231174	JN231213	JN400719
37	Foc 170	Jabalpur	Madhya Pradesh	6	–	–	–
38	Foc 124	Badnapur	Maharashtra	7	JN231173	JN231218	JN400711
39	Foc 171	Badnapur	Maharashtra	7	–	–	–
40	Foc 128	Satara	Maharashtra	7	JN231172	JN231219	JN400710
41	Foc 165	Amravati	Maharashtra	7	JN231171	JN231216	JN400712
42	Foc 166	Dhule	Maharashtra	4	JN231170	JN231217	JN400713
43	Foc 19	Faridpur	Punjab	8	JN231169	JN231232	JN400708
44	Foc 31	Firozpur	Punjab	3	–	–	–
45	Foc 45	Ludhiana	Punjab	3	JN231168	JN231233	–
46	Foc 62	Ropar	Punjab	3	JN231167	JN231234	–

Table 1 continued

S. No.	Accession no.	Place of collection/district	States	Race	*GenBank* accession no		
					TEF-1α	β-tubulin	ITS
47	Foc 89	Dumewal	Punjab	4	–	–	–
48	Foc 93	Gurdaspur	Punjab	3	JN231166	JN231235	JN400709
49	Foc 18	Abohar	Punjab	8	–	–	–
50	Foc 4	Jaipur	Rajasthan	5	JN231165	JN231222	–
51	Foc 6	Jaipur	Rajasthan	5	–	–	–
52	Foc 11	Alwar	Rajasthan	5	JN231164	JN231223	JN400702
53	Foc 50	Udaipur	Rajasthan	5	JN231163	JN231224	–
54	Foc 68	Sri Ganganagar	Rajasthan	8	JN231162	JN231225	JN400703
55	Foc 80	Hanumangarh	Rajasthan	3	–	–	–
56	Foc 87	Churu	Rajasthan	5	JN231161	JN231220	JN400704
57	Foc 36	Sikar	Rajasthan	5	JN231160	JN231221	JN400705
58	Foc 58	Suratgarh	Rajasthan	5	JN231159	–	JN400706
59	Foc 79	Jetsar	Rajasthan	3	JN231158	–	JN400707
60	Foc 119	IIPR Kanpur	Uttar Pradesh	2	JN231157	JN231229	JN400700
61	Foc 129	Jhansi	Uttar Pradesh	2	JN231156	–	JN400695
62	Foc 130	Gorakhpur	Uttar Pradesh	2	JN231155	JN231230	JN400701
63	Foc 137	Shahjahanpur	Uttar Pradesh	4	–	–	–
64	Foc 140	Lucknow	Uttar Pradesh	4	–	–	–
65	Foc 142	Meerut	Uttar Pradesh	2	JN231154	JN231226	JN400696
66	Foc 167	Kanpur	Uttar Pradesh	4	JN231153	JN231231	JN400697
67	Foc 133	Lalitpur	Uttar Pradesh	2	–	–	–
68	Foc 134	Mahoba	Uttar Pradesh	2	JN231151	JN231228	JN400699
69	Foc 141	Jaunpur	Uttar Pradesh	2	JN231152	JN231227	JN400698
70	Foc 125	Dholi	Bihar	2	JN231186	JN231236	JN400720

– Not sequenced

β-Tubulin analysis

β-Tubulin gene-specific primers (Jimenez-Gasco et al. 2002) successfully amplified the genomic DNA of 70 isolates and produced ~500-bp amplicons. β-Tubulin nucleotide sequences of 50 area/state representative isolates of the present study ranged from 479 to 517 bp. The β-tubulin gene sequences of the isolates used in the present study shared more than 90 % nucleotide sequence similarity with β-tubulin sequences of *F. oxysporum* available in *GenBank*. The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 50 isolates of the present study representing eight races along with eight foreign isolates grouped them into two major clusters. The first major cluster had all 50 isolates of the present study along with one isolate from Spain. The remaining seven foreign isolates were grouped into the second major cluster (Fig. 2).

ITS analysis

PCR amplification with specific primers ITS1 and ITS4 given by White et al. (1990) yielded a single DNA fragment ~550 bp in size present in all the isolates. Nucleotide sequences of ITS region of 46 area representative isolates ranged from 528 to 564 bp. The phylogenetic tree of 46 *F. oxysporum* f. sp. *ciceris* isolates of the present study and 10 isolates of *F. oxysporum* originated from other parts of the world grouped the isolates into two major clusters. All Indian isolates representing 8 races of the present study were placed in the first major cluster along with 8 isolates of other countries. The second major cluster had only two isolates one each from Japan and Greece (Fig. 3).

Discussion

The phylogenetic tree analysis of TEF-1α, β-tubulin, and ITS gene sequences of *F. oxysporum* f. sp. *ciceris* showed that the each clade had the isolates from different states of India representing different races of the pathogen, indicating considerable variability in some of the isolates belonging to the same areas. The sequence analysis of TEF-1α gene clearly indicated that more than 50 % Indian populations representing 8 races of the pathogen clustered

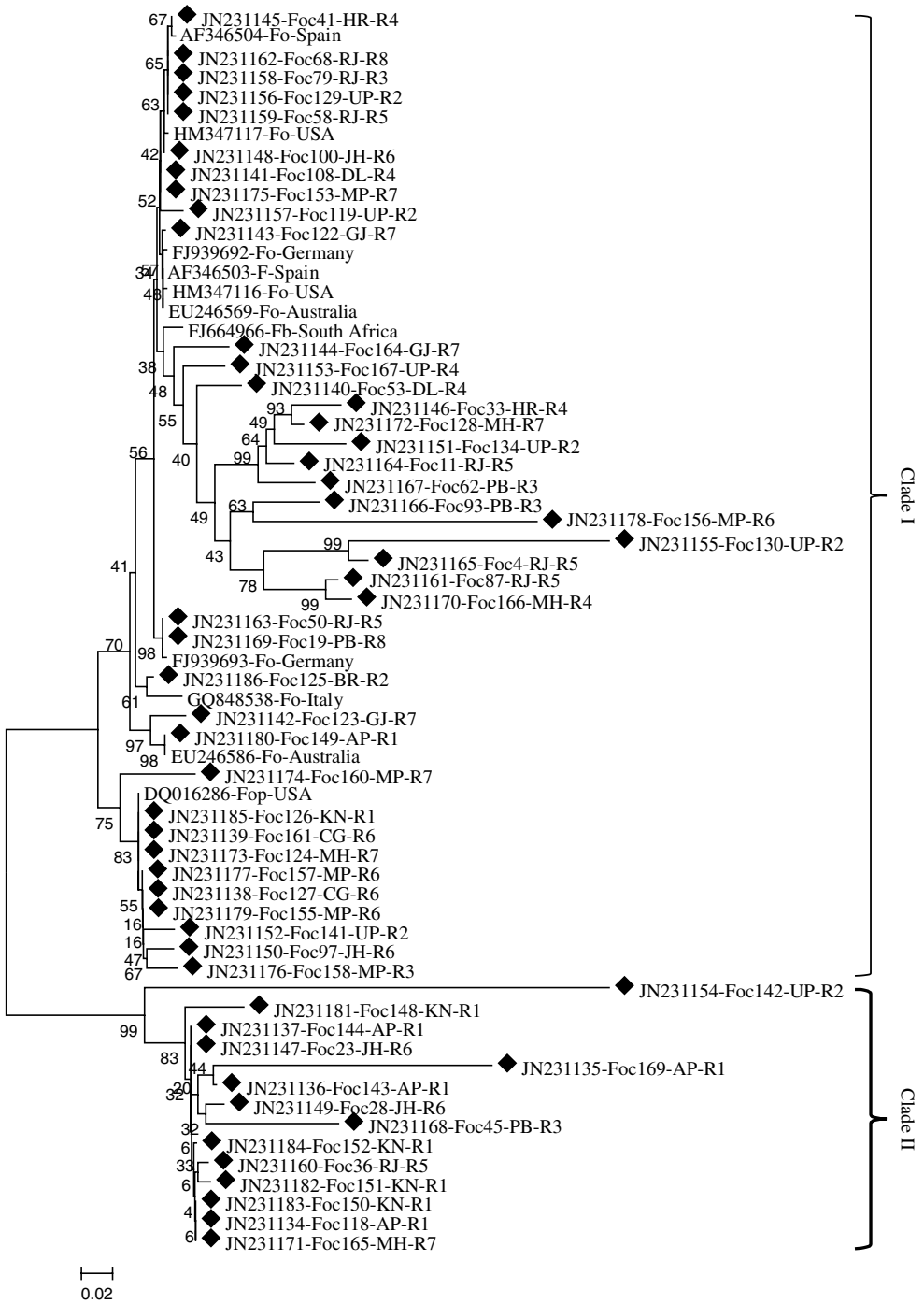

Fig. 1 Neighbor-joining tree showing the phylogenetic relationships among the isolates of *F. oxysporum* f. sp. *ciceris* (Foc) based on their TEF-1α sequences. The sequences generated during this study were labeled (labeled *diamond*) Fo- *F. oxysporum*, Fb- *F. oxysporum* f. sp. *cubense*, F- *Fusarium*, Fop- *F. oxysporum* f. sp. *phaseol,* and R-race number

along with 11 foreign isolates of *Fusarium*. The remaining Indian populations (14 isolates) representing 6 races of the pathogen are considered distinct from others in respect of TEF-1α gene sequences and predominated by the population of race 1, which was prevalent mainly in southern part of India. Earlier to this, TEF-1α gene region was used for variability and identification of various *Fusarium* species (Yli-Mattila et al. 2011). The sequence analysis of β-tubulin gene clearly showed high level of similarity, and all the isolates were clustered with an isolate of Spain in a single group. The isolates belonging to other forma specialis were clustered separately; therefore, in respect of

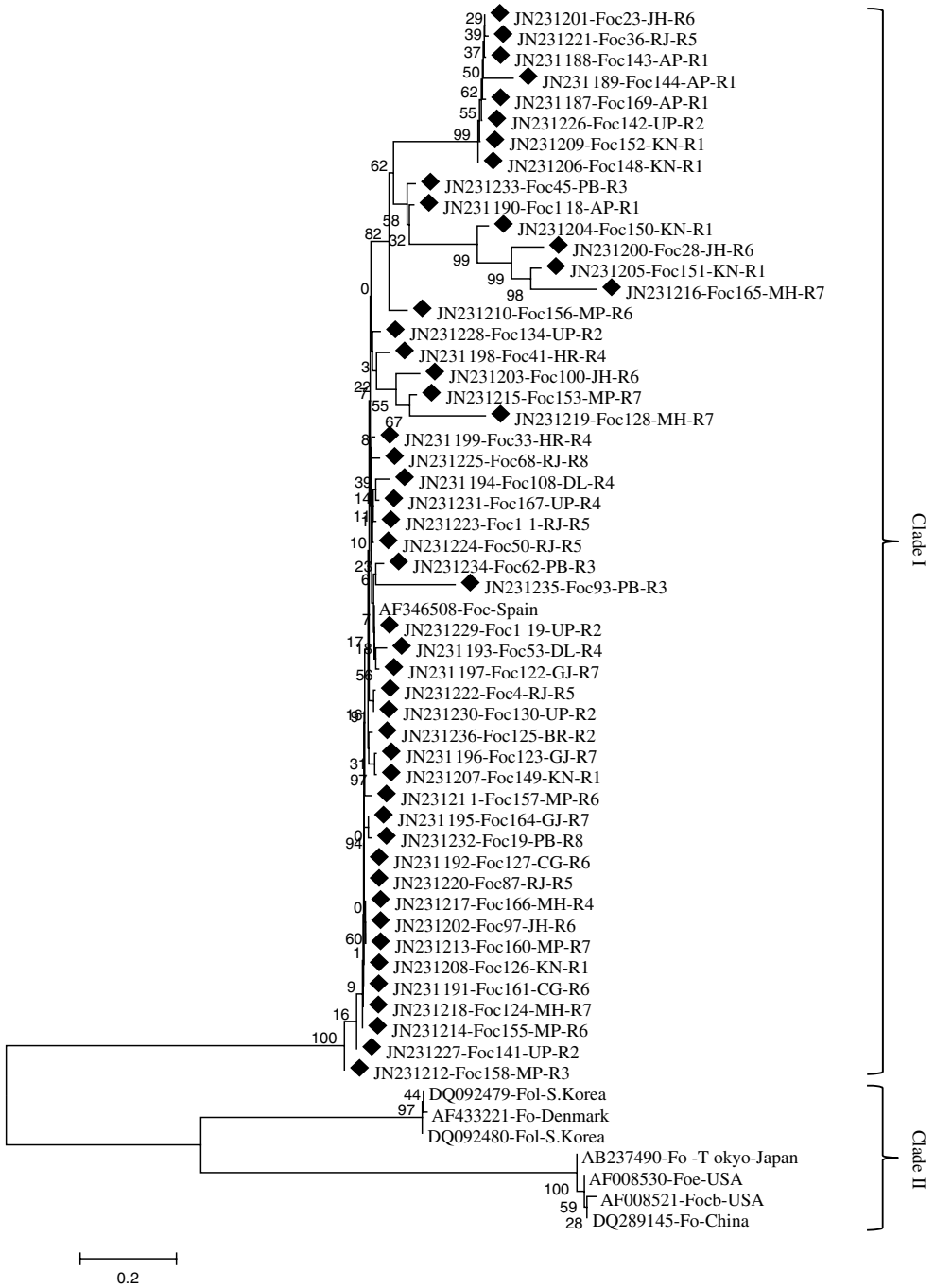

Fig. 2 Neighbor-joining tree showing the phylogenetic relationships among isolates of *F. oxysporum* f. sp. *ciceris* (Foc) based on their β-tubulin sequences. The sequences generated during this study were labeled (labeled *diamond*) Fol- *F. oxysporum* f. sp. *lycopersici*, Fo- *F. oxysporum*, Foe- *F. oxysporum* f. sp. *erythroxyli*, Focb- *F. oxysporum* f. sp. *cubense* ,and R-race number

β-tubulin gene, forma specialis-specific grouping could be made. The present findings are in accordance with the earlier observation that the β-tubulin genes are potentially useful in phylogeographic investigations and characterization at species level (O'Donnell and Cigelnik 1997; Chung et al. 2008). The β-tubulin gene sequences did not correspond to the place of origin and race of the isolates. Similarly, Hill et al. (2010) while working on *F. oxysporum* from sugar beet concluded that the phylogenetic group did not correlate with pathogenicity and/or geographic origin. The phylogenetic tree from ITS sequences showed a high level of similarity and grouped into a single cluster in spite of the

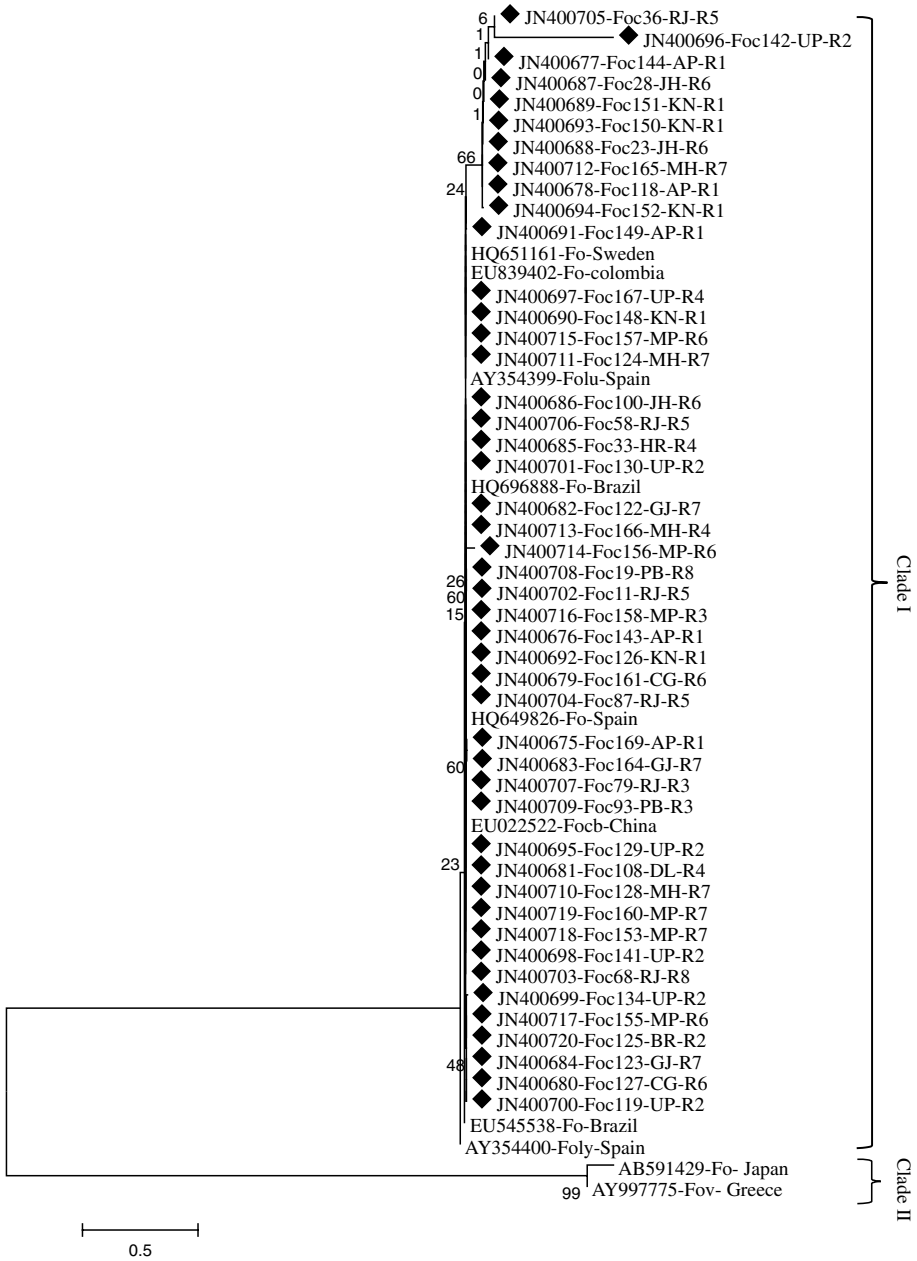

Fig. 3 Neighbor-joining tree showing the phylogenetic relationships among isolates of *F. oxysporum* f. sp. *ciceris* (Foc) based on their ITS sequences. The sequences generated during this study were labeled (labeled *diamond*) Fo- *F. oxysporum*, Focb- *F. oxysporum* f. sp. *cubense*, Folu- *F. oxysporum* f. sp. *luffae*, Foly- *F. oxysporum* f. sp. *lycopersici,* Fov- *F. oxysporum* f. sp. *vasinfectum,* and R-race number

differences in the places of origin of the isolates and the races of the pathogen. Similarly, Hill et al. (2010) while working on *F. oxysporum* concluded that the phylogenetic group did not correlate with pathogenicity and/or geographic origin.

The high level of sequence similarity (90 %) in respect of these three gene regions is attributed to the origin of *F. oxysporum* f. sp. *ciceris* from a small founder population that was pathogenic to *Cicer* spp. (Jimenez-Gasco et al. 2002). The present findings are in accordance with the observations of Jimenez-Gasco et al. (2002) that the isolates representing the two pathotypes and the geographic range of the pathogen showed identical sequences for TEF-1α, β-tubulin, histone 3, actin, and calmodulin. Their observations were based on seven typical wilt producing *F. oxysporum* f. sp. *ciceris* isolates representing 4 Indian races, but presently, the racial scenario of the pathogen has been entirely changed, and a new set of differential cultivars were identified for race profiling of the pathogen (Dubey et al. 2012). The isolates included in Jimenez-Gasco et al. (2002) study were not tested for their virulence to determine races, and however, earlier the race distribution was area/state specific, and now in each state/area, more than one race was present (Dubey et al. 2012). The phylogenetic analysis of β-tubulin sequences only supported the theory of monophyletic origin of the pathogen given by Jimenez-Gasco

et al. (2002). The present finding is differ with the results of Jimenez-Gasco et al. (2002) in respect of phylogenetic analysis of the sequences of TEF-1α and ITS, which grouped the other *F. oxysporum* isolates separately. This difference might be due to less number of isolates included in the earlier studies. Earlier to this, Gurjar et al. (2009) identified the race 3 of *F. oxysporum* f. sp. *ciceris* as *F. proliferatum* based on phylogenetic analysis with EF-1α sequences without determining the races of the isolates used in the study. The EF-1α sequences generated from the original race 3 representative isolates deposited in ITCC, New Delhi, India, by Haware and Nene (1982) showed sequence similarity to *F. oxysporum* f. sp. *ciceris* isolates of EF-1α. Therefore, the identity of the culture represented race 3 of *F. oxysporum* f. sp *ciceris* used by Gurjar et al. (2009) might be relooked. However, recently, the Indian populations have been characterized into eight races based on a new set of differential cultivars of chickpea (Dubey et al. 2012).

The critical analysis of the sequences of TEF-1α, β-tubulin, and ITS genes revealed that most of the isolates shared more than 90 % similarity among themselves for each gene sequence. The isolates showed less variability in respect of gene-specific regions, indicating the development of variants due to introduction of large numbers of area-specific resistant varieties of chickpea in the recent past. The phylogenetic groups generated in the present study did not correspond to the origin of the isolates and races of *F. oxysporum* f. sp. *ciceris* in respect of β-tubulin and ITS sequences, whereas it was partially corresponded in case of TEF-1α sequence. This is might be due to introduction of various race representative populations of the pathogen through infected seeds and cultivation of chickpea cultivars having different genetic background in an area. The markers/genes to determine virulence factors for different races of the pathogen have to be worked out. The low level of variability observed among the populations in respect of these three gene sequences could be used for designing *F. oxysporum* f. sp. *ciceris*-specific markers. The β-tubulin gene showed advantage over other two genes studied due to high level of similarity and could be more suitable for *F. oxysporum* f. sp. *ciceris*-specific studies. The information generated in the present study on *F. oxysporum* f. sp. *ciceris* is new because it is based on Indian populations representing 8 new races of the pathogen recently characterized using new set of chickpea differentials (Dubey et al. 2012). The information based on TEF-1α, β-tubulin, and ITS genes generated in the present study is useful for understanding of evolutionary relationship of wilt producing populations of the pathogen and development of specific diagnostics.

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