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Phylogenetic analysis of *Rhizoctonia solani* AG-4 isolates from common beans in Black Sea coastal region, Turkey, based on ITS-5.8S rDNA

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Abstract: In this study, 114 *Rhizoctonia solani* isolates were obtained from diseased common bean (*Phaseolus vulgaris* L.) plants from the Black Sea coastal region of Turkey. Genetic variation was determined among *R. solani* anastomosis group 4 (AG-4) subgroups (AG-4 HG-I and AG-4 HG-II). ITS-5.8 rDNA sequences of these isolates were aligned with other known *R. solani* sequences from GenBank, and distance and parsimony analysis were used to determine phylogenetic relationships. The *R. solani* AG-4 isolates were placed in 2 main lineages, corresponding to AG-4 HG-I and AG-4 HG-II. Based on phylogenetic analysis, genetic variations were shown in the AG-4 subgroups (especially AG-4 HG-I). This study represents the first reported use of rDNA-ITS sequence data to examine AG-4 HG-I and HG-II in *P. vulgaris* from Turkey. The results also suggest that the Giresun isolates are closely related to isolates obtained from Italy.

Key words: rDNA-ITS phylogeny, *Rhizoctonia solani*, AG-4, *Phaseolus vulgaris*, common bean

1. Introduction

Rhizoctonia solani Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a soil-borne fungus that causes economically damaging diseases in many crop species (1). The species consists of many related but genetically isolated groups (2). Based on hyphal anastomosis reaction, *R. solani* can be divided into 13 anastomosis groups (AGs) (3). Some AGs of *R. solani* have been further divided into subgroups based on cultural morphology, host range, virulence, and molecular techniques (4–6). Isolates within the same AG, or within the same subgroup, may have similar characteristics, such as host preference and disease symptoms (7).

Most available molecular techniques are based on the detection and typing of genomic polymorphisms at several levels. Sequencing of ribosomal DNA (rDNA) has been widely employed in recent years to reconstruct phylogenetic relationships between different organisms of the form genus (8). Techniques in molecular biology have contributed to determining genetic diversity and taxonomic classification within fungal species (9–12). Currently, rDNA internal transcribed spacer region [(ITS) composed of ITS1, 5.8S, and ITS2 regions] sequence information seems to offer the most accurate method of establishing the taxonomic and phylogenetic relationships for *Rhizoctonia* spp. (13). Sequence analysis of the genomic regions encoding the rDNA-ITS is convenient for AG determination and has become increasingly common with the accumulation of sequences from different isolates in databases (14,15). Both within and between the various AGs, rDNA-ITS sequencing has been used to analyze the genetic diversity of *R. solani* and to examine the relationships of the AGs to one another.

AG-4 has been divided into HG-I, HG-II, and HG-III subgroups. Separation of the AG-4 subgroups is not possible by hyphal anastomosis reactions because anastomosis occurs within members from the same AG of *R. solani*. To differentiate isolates within AG-4, biochemical and molecular techniques such as fatty acid analysis (16), DNA base sequence homology (17), restriction fragment length polymorphism of ITS (11), random amplified polymorphic DNA (18,19), and rDNA-ITS nucleotide sequences (9) have been used.

AG-4 is one of the most widely recognized causes of disease incited by *R. solani*. Isolates of *R. solani* identified as AG-4 cause seed rot, postemergence damping off, and root rots in many important crop plants (16).

The common bean is the most important legume crop in the Black Sea coastal region. However, it is affected by *R. solani*, a widespread soil-borne pathogen of common bean that causes damping-off, root rot, hypocotyl rot, and web blight, which can result in significant economic losses to producers.

The current study aims to determine the genetic variation of *R. solani* isolates from the common bean growing areas of the Black Sea coastal region of Turkey.

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2. Materials and methods

2.1. Sampling, isolation, and identification of *Rhizoctonia isolates*

Samples of bean seedlings with root disease symptoms were collected from 4 provinces located on the coast of the Black Sea region (Figure 1). The data for the 16 sampling areas in the study were presented in a previous paper (20).

2.2. DNA extraction and PCR of ITS1-5.8S-ITS2 region

DNA extraction from *R. solani* AG-4, selected on the basis of AG and cultural morphology, was carried out following the procedure of Pascual et al. (21). Two primers [ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)] were used to amplify the DNA region encoding ITS1-5.8S-ITS2 of DNA samples (22). The amplification for 50 µL of PCR product was performed using ≤1 µg genomic DNA, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 2.5 mM dNTP mix, 1X PCR buffer, and 50 pmol of each primer. For amplification, a MWG Primus thermal cycler was used with the following temperature profile: an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 49 °C for 2 min, and 72 °C for 3 min, and a final extension at 72 °C for 7 min. The PCR products were electrophoresed on 1% agarose gel (Amresco; Solon, OH, USA) in 1X TBE buffer, stained with ethidium bromide, and visualized with the GeneGenius Bio imaging system.

2.3. DNA sequencing and data analysis

The DNA sequencing of the selected isolates (on the basis of anastomosis group, cultural morphology, and locality) was performed by Macrogen, South Korea, using an ABI 3730 XL DNA sequencer. Accession numbers of the *R. solani* samples isolated and sequenced in this study as well as the isolates used in the phylogenetic analysis of the different AG-4 isolates are given in the Table. Nucleotide alignments of ITS sequences were assembled by using ClustalX (23) and were optimized manually. Data were analyzed by distance and parsimony methods with PAUP v. 4.0b10 (24). Neighbor-joining (NJ) and maximum parsimony (MP) were employed to evaluate phylogenetic relationships among isolates. The Bayesian test (BAY) and Akaike information criterion (AIC) (25) were applied using MODELTEST v. 3.7 (26) to establish the most appropriate model of DNA substitution for the data.

Parsimony analysis was performed with a heuristic search approach using the tree bisection and reconnection swapping algorithm and 10 random repetitions. A majority rule consensus tree was constructed from multiple equally parsimonious MP trees. Bootstrap analysis of the MP tree was conducted with 1000 replications using 10 random repetitions for each replication. Only nodes with bootstrap values over 50% were considered significant. Bootstrapping (10,000 replications) for the NJ trees was carried out with the substitution models selected by MODELTEST. For both analyses, trees were rooted using isolate *Thanatephorus cucumeris* AG-1-IB (accession no. AY154302.1) as the outgroup. The resulting trees were visualized with Treeview 1.6.6. All new sequences were deposited in the EMBL database (accession nos. HE805668–HE805691).

3. Results

PCR amplification of the DNA of *R. solani* AG-4 common bean isolates using universal primers ITS1 and ITS4 generated a fragment of approximately 700 bp long for each isolate. The whole ITS region (ITS1, 5.8S rDNA, and ITS2) of AG-4 common bean isolates was sequenced in both directions with the same primers used for PCR amplifications. Sequences of these isolates were aligned with other known *R. solani* sequences from the NCBI GenBank (Table), and distance and parsimony analysis were used to obtain phylogenetic trees.

The heuristic search conducted on the entire rDNA-ITS region data matrix resulted in 46 equally parsimonious trees of 75 steps, CI = 0.827, RI = 0.772, and HI = 0173. Only the majority rule consensus tree obtained from

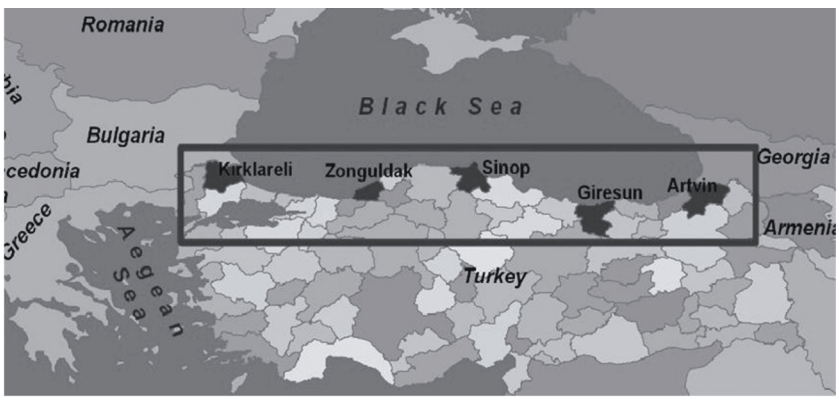

Figure 1. The 5 provinces of the Black Sea region of Turkey where samples were collected.

Table. *Rhizoctonia solani* isolates from different places and hosts used for phylogenetic analysis.

Isolate number	Host	GenBank accession number	Origin
M47ZD (2)	Common bean	HE805690	Zonguldak-Devrek
M48KP (3)	Common bean	HE805668	Kırklareli-Pınarhisar
M60SA (9)	Common bean	HE805669	Sinop-Ayancık
M51KM (4)	Common bean	HE805678	Kırklareli-Demirköy
M52GD (5)	Common bean	HE805677	Giresun-Dereli
M54ZG (6)	Common bean	HE805687	Zonguldak-Gökçebey
M56KD (7)	Common bean	HE805673	Kırklareli-Dereköy
M57KM (8)	Common bean	HE805670	Kırklareli-Demirköy
M46ZA (1)	Common bean	HE805689	Zonguldak-Alaplı
M62ZG (10)	Common bean	HE805671	Zonguldak-Gökçebey
M63ZC (11)	Common bean	HE805682	Zonguldak-Çaycuma
M64ZG (12)	Common bean	HE805675	Zonguldak-Gökçebey
M67ZA (13)	Common bean	HE805686	Zonguldak-Alaplı
M69ZC (14)	Common bean	HE805676	Zonguldak-Çaycuma
M70ZG (15)	Common bean	HE805672	Zonguldak-Gökçebey
M72GP (16)	Common bean	HE805688	Giresun-Piraziz
M74GK (18)	Common bean	HE805674	Giresun-Kovanlık
M75GP (19)	Common bean	HE805683	Giresun-Piraziz
M76GK (20)	Common bean	HE805679	Giresun-Kovanlık
M86GD (21)	Common bean	HE805680	Giresun-Dereli
M88GE (22)	Common bean	HE805685	Giresun-Espiye
M89GE (23)	Common bean	HE805684	Giresun-Espiye
M91KK (24)	Common bean	HE805691	Kırklareli-Kıyıköy
M93KD (26)	Common bean	HE805681	Kırklareli-Dereköy
Str45	*Fragaria ananassa*	DQ102448	Israel
SJ05	Soybean	AY270002	Brazil
RhFag	Common bean	DQ021450	Italy
RS99	*Poa annua*	AF222798	Unknown
Ankara	Pepper	AF478450	Turkey
Adana	Strawberry	AF478449	Turkey
EP-1	*Spinacia oleracea*	AY152697	Brazil
Rs20	Strawberry	DQ102449	Israel
BD	Tomato	AY154308	Brazil
BS	Soybean	AY270005	Brazil
HI521-21	Soil	AB000020	Japan
AH-1	Peanut	AB000012	Japan

(..): Isolate numbers from previous study (20).

the parsimony method is given (Figure 2). The Bayesian information criterion test supported the TrN + G (Tamura-Nei) model with a gamma correction (G) frequency of 0.1580. The AIC test supported the TIM1 + G (transition) model, with frequency of gamma shape at 0.1570. The NJ tree produced from this substitution model (TrN + G) is shown in Figure 3; the highest bootstrap values were generated with this model.

Phylogenetic analysis by distance and parsimony methods supported very similar phylogenetic trees, with bootstrap values of 100%. In both the NJ and MP trees, there were 2 major groupings, with 1 corresponding to AG-4 HG-III. Within the second group there were 2 sublineages, 1 corresponding to AG-4 HG-I and 1 to AG-4 HG-II. In the NJ tree and MP tree, AG-4 HG-I was a sister to the AG-4 HG-II subgroup, supported by a bootstrap

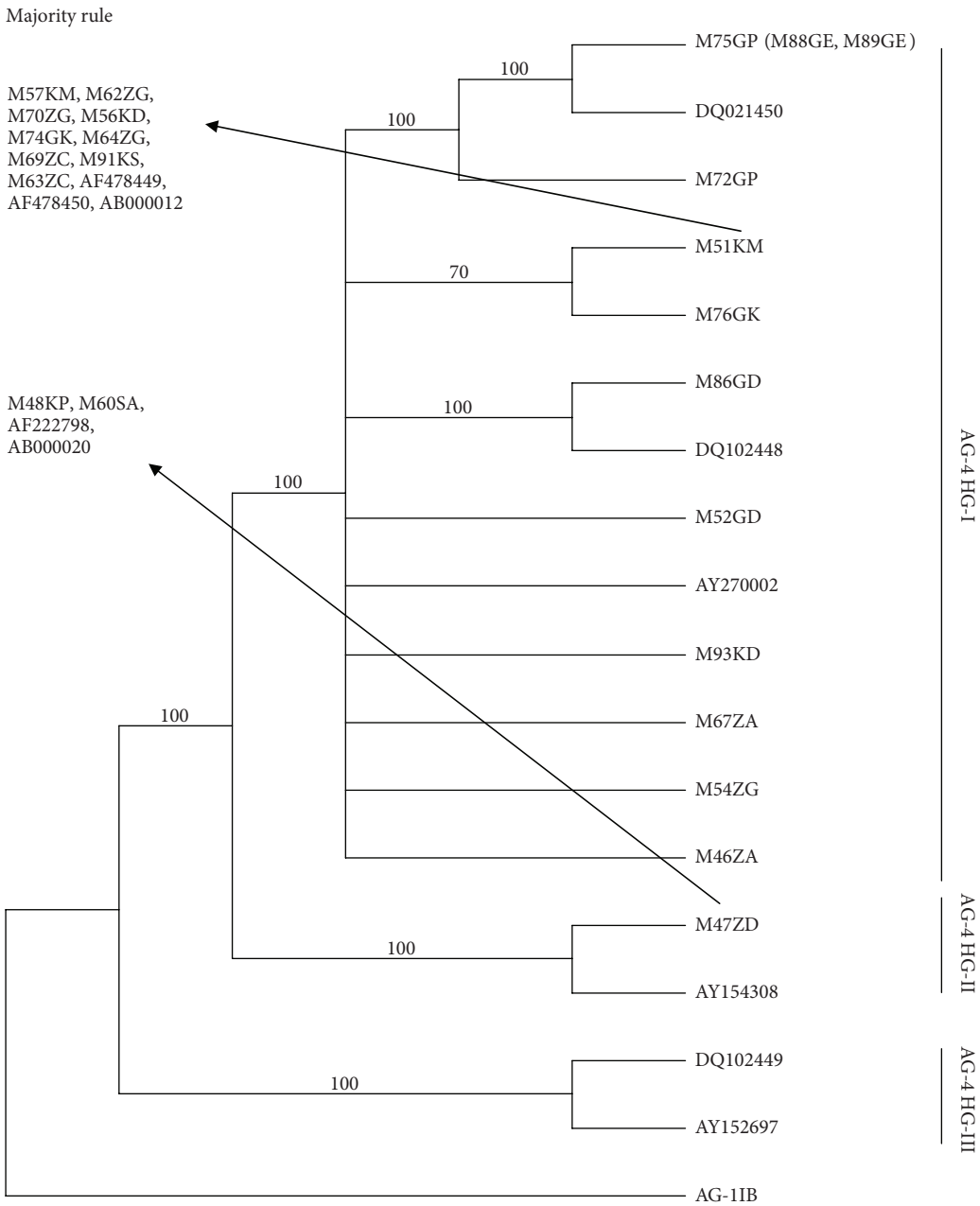

Figure 2. Majority rule consensus of 46 equally parsimonious trees with 75 steps (CI = 0.827, RI = 0.772, and HI = 0173) based on rDNA-ITS sequence data from 24 common bean isolates of AG-4 and 12 known AG-4 isolates from GenBank.

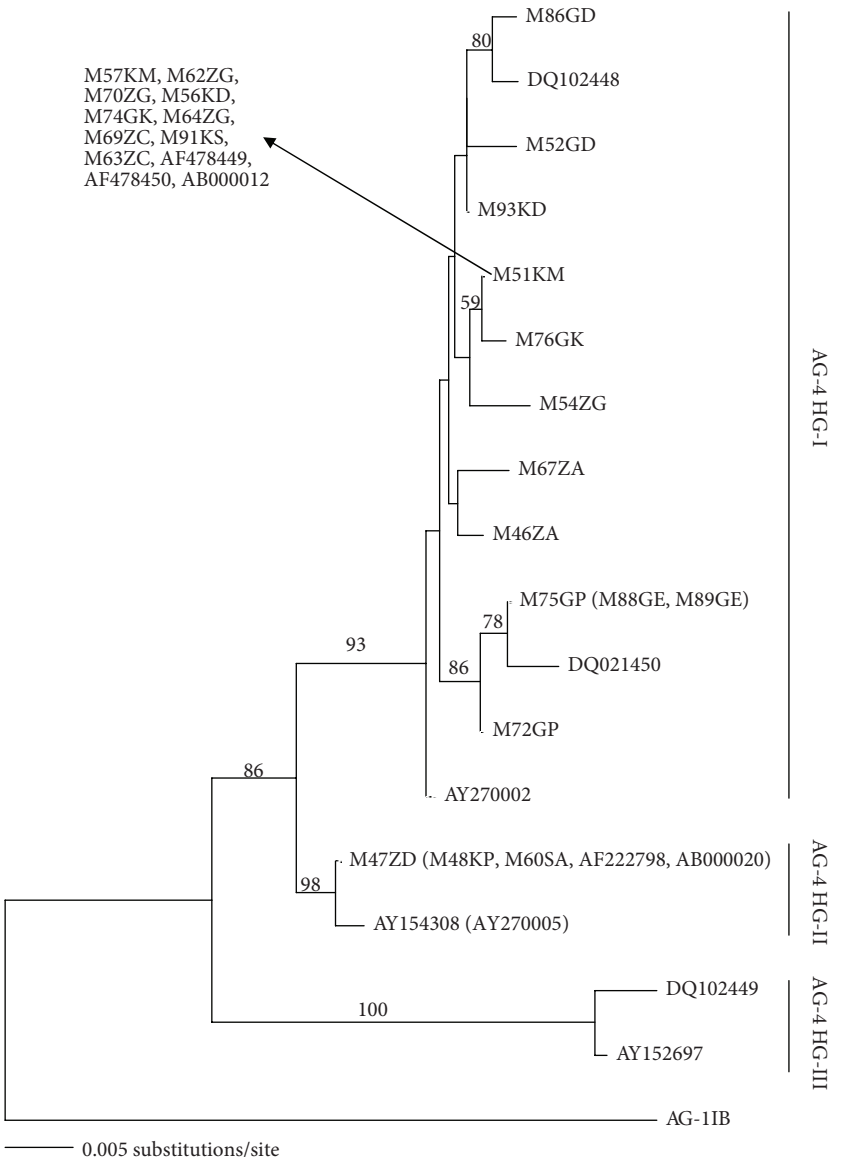

Figure 3. NJ tree of 24 representative common bean isolates of AG-4 and 12 known AG-4 isolates from GenBank. Tree based on TrN + G distances and rooted with AG-1-IB.

value of 86% in the NJ tree and a value of 100% in the MP tree, and AG-4 HG-III was sister to group with AG-4 HG-I and AG-4 HG-II.

When the MP and NJ trees were analyzed, M75GP, M88GE, and M89GE in the AG-4 HG-I lineage were located in the same position as DQ021450 from the Italian isolate, supported by a bootstrap value of 100% in the MP tree and 78% in the NJ tree, indicating a close relationship between these isolates. These isolates were also sisters to the M72GP isolate. Additionally, the M51KM and M76GK isolates were located in the same lineage, supported by a bootstrap value of 59% in the NJ tree and 70% in the MP tree. Furthermore, M86 and DQ102448 isolates were located in the same lineage, supported by a bootstrap value of 80% in the NJ tree and 100% in the MP tree.

4. Discussion

The present study aimed to determine the genetic diversity and phylogeny of *R. solani* AG-4 isolates obtained from *P. vulgaris* in the Black Sea coastal region. These isolates were characterized (with colony morphology, anastomosis reactions, pathogenicity, and rDNA-ITS RFLP) in a previous study (20). According to the previous study, all AG-4 isolates anastomosed with both AG-4 HG-I and AG-4 HG-II testers at low frequencies. Subgroups of *R. solani* AG-4 isolates were not differentiated with

anastomosis reactions. Our results also supported the proposition that anastomosis reactions are insufficient for determining AG-4 subgroups. Therefore, PCR-RFLP patterns in the rDNA-ITS were used for the subgrouping of AG-4 isolates. Consequently, it is recommended that sequence analysis be carried out to determine whether there is further genetic diversity (20).

Previous studies have reported several anastomosis groups in bean including AG-4, AG-1 IA, AG-1 IB, AG-1 IE, AG-1 IF, AG-2 1, and AG-2 2 from places such as Brazil, Cuba, Central and South America, New York, Costa Rica, and Puerto Rico (27–37). In Turkey, some *R. solani* AGs (AG-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-9, AG-10, and AG-11) and binucleate *Rhizoctonia* (AG-A, AG-B, AG-E, AG-F, AG-G, AG-I, and AG-K) have been identified in bean species (38–42). Congruent with this assignment, a previous study (20) identified 114 *R. solani* isolates as AG-4 in accordance with hyphal anastomosis, colony morphology, and pathogenicity. In addition to subgroupings of AG-4, isolates were determined using PCR-RFLP patterns of rDNA-ITS. The same study determined 3 rDNA-ITS RFLP patterns, and in the present study we sequenced rDNA-ITS genes for the representative isolates for each pattern and found that 2 patterns were related to AG-4 HG-I and 1 was related to AG-4 HG-II. As mentioned above, although previous studies have reported AG-4 from *P. vulgaris* in Turkey, the subgroup separations are unclear because they lack molecular information. The present study is the first report on the determination of AG-4 HG-I and AG-4 HG-II with rDNA-ITS sequencing from *P. vulgaris* in Turkey.

In the present study, NJ and MP trees obtained from phylogenetic analysis showed the genetic relatedness within and between the anastomosis subgroups. In recent years, findings based on molecular data have provided additional support for AG-4 as a distinct species (10). The present results demonstrated the presence of considerable genetic variation. However, further studies with more genes are required to confirm the phylogenetic position of AG-4.

The isolate DQ021450, obtained from Italy, was located in the same sublineage (AG-4 HG-I) as Giresun (Espiye and Piraziz) isolates (M75GP, M89GE, M88GE, and M72GP). According to these results, isolates from Italy were closely related to Giresun isolates.

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