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RESEARCH PAPERS

Phenotypic and molecular characterization of *Rhizobium vitis* strains from vineyards in Turkey

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Summary. Crown gall-affected grapevine samples were collected during 2009–2010 from major vineyards, located in different Turkish provinces. One hundred and three bacterial strains were obtained from 88 vineyards and 18 grapevine varieties; they were tumorigenic when inoculated in tobacco, sunflower and *Datura stramonium* plants and were identified as *Rhizobium vitis* using biochemical and physiological tests as well as PCR and specific primers. Nineteen *R. vitis* strains presented a number of anomalous biochemical and physiological characters. PCR and opine-specific primers revealed the presence of octopine/cucumopine-type plasmid in 82 *R. vitis* strains, nopaline-type plasmids in 18 strains and vitopine-type plasmids in three strains. Clonal relationship of strains was determined using Pulsed Field Gel Electrophoresis following digestion of genomic DNA with the restriction endonuclease *Pme*I. The greatest genetic diversity was found for the strains from Denizli, Ankara and Nevşehir provinces. Nopaline and vitopine-types of *Rhizobium vitis* were detected for the first time in Turkey.

Key words: *Rhizobium vitis*, opine, PCR, PFGE, similarity rate.

Introduction

Grapevine crown gall is an important bacterial disease in viticultural regions worldwide, and is predominantly caused by the validated species *Rhizobium vitis* (= *Agrobacterium vitis*) and rarely by *Rhizobium radiobacter* (= *Agrobacterium tumefaciens*) and *Rhizobium rhizogenes* (= *Agrobacterium rhizogenes*) (Panagopoulos *et al*., 1978; Süle, 1978; López *et al*., 2008). It is probable that the taxonomic position of these species will change, because the *Rhizobium* genus is extremely heterogeneous. In a recent phylogenetic study, based on multilocus sequence analysis of four housekeeping genes, Mousavi *et al*. (2015) proposed to transter *R. vitis* to the *Allorhizobium* genus, as *Allorhizobium vitis*.

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Rhizobium vitis is a Gram-negative soil-borne bacterium which is specific to *Vitis* spp. Tumorigenic *Rhizobium* spp. are unique in carrying a tumor-inducing (Ti) plasmid, which bear genes essential for crown gall development. Virulence requires genes located on different regions of the Ti plasmid, including the transferred DNA (T-DNA) and the virulence (*vir*) genes. Upon infection, the T-DNA is transferred and expressed in the grapevine genome resulting in host cell proliferation and gall formation. The bacterium is able to utilize specific amino acid derivatives, called opines, as selective nutritional sources in gall tissues (Petit and Tempé, 1995). Opine synthase genes are also carried on the Ti plasmids of the strains. Agropine and mannopine are produced by *R. radiobacter,* but not by *R. vitis*, while octopine/cucumopine (O/C) and nopaline can be produced by both species. Thus far, vitopine is produced only by *R. vitis* in grapevine. *Rhizobium vitis* can produce and catabolize three types of opines, namely octopine/cucumopine,

nopaline, and vitopine (Szegedi *et al.*, 1988; Paulus *et al.*, 1989; Burr *et al.*, 1998; Szegedi, 2003).

Previous studies on *R. vitis* in Turkey were reported by Argun *et al.* (2002) and Kusek (2007). Strains of the bacterium studied by Kusek (2007) in south eastern Anatolia were all of the O/C-type. Argun *et al.* (2002) reported that the strains from Nevsehir and Ankara provinces differed, based on their T-DNA structures and according to ITS fingerprint analysis, and all strains were identified as the O/C-type. This is the only detailed but province-limited study of Turkish *R. vitis* strains.

Characterization of *Rhizobium* spp. may be achieved using several technologies including biochemical tests, fatty acid analysis and molecular methods (Moore *et al.*, 2001). Methods based on DNA restriction profiles generated by rare cut endonucleases and resolution of fragments from a few kilobases to megabase pair in size by Pulsed Field Gel Electrophoresis (PFGE; Sambrook *et al.*, 1989; Arbeit, 1997; Tang *et al.*, 2000) yield particularly sensitive restriction profiles, useful for typing bacterial strains, including *Rhizobium* spp. (Aujoulat *et al.*, 2011).

The aim of the present study was to characterize strains of *Rhizobium* spp. isolated from crown galls on grapevines in the major viticulture areas of Turkey, using phenotypic tests and molecular analyses, and to determine the genomic relatedness between the strains using PFGE technique.

Materials and methods

Surveys

Extensive surveys were carried out in major Turkish vineyard provinces between July and September 2009–2010. One hundred and three crown gall-infected grapevines were sampled from nine different cities in different geographical regions of Turkey (Figure 1), and a total of 103 bacterial isolates were obtained from 18 different grape varieties (Table 1). *Rhizobium radiobacter* (C58) and *R. vitis* (Tm4, AT1, S4) strains were used as positive controls for biochemical and molecular analyses.

Bacterial isolation

Galls collected from infected grapevines were washed under running water, surface disinfected in 10% sodium hypochlorite and rinsed three times with sterile water. Gall tissue samples were each ground into sterile water with a pestle and mortar, placed in a sterile tube and left for 30 min in suspension so that bacteria could diffuse out of the tissue. The bacterial suspension was then streaked onto a semi-selective Roy and Sasser (RS) medium (Roy and Sasser, 1983) and incubated for 7 d at 28°C. Colonies with smooth margins, red pigmented centres and white transparent halos were selected for further characterization.

Biochemical and physiological tests

All isolates were subjected to the following biochemical and physiological tests, described by Ophel and Kerr (1990) and Moore *et al.* (2001): 3-ketolactose production; acid production from dulcitol, arabitol, melezitose and sucrose; alkali production from L-tartrate and malonic acid; reaction on litmus milk; growth at 35°C and 2% NaCl. Each test was replicated three times. Results were subjected to SPSS (IBM, Version: 22.0) cluster analysis.

Pathogenicity tests

To determine tumorigenicity, all isolates were inoculated on 3–4 week-old seedlings of *Nicotiana tabacum* var. Samsun, *Helianthus annuus* and *Datura stramonium*. Seedling stems were each punctured with a sterile syringe needle, and 5 µL of bacterial suspensions at a concentration of 10^8 cell mL^{-1} were applied to the wounds. The inoculations were replicated three times for all strains for each test plant species. Inoculated test plants were maintained in a growth chamber (Digitech, P33) for 6 weeks. Isolations were made from galls that formed on the plants by plating on RS medium and identification of the pathogen was confirmed by PCR.

Molecular analyses

PCR and specific primers were used to identify the bacterial isolates and to determine their opine types. As template DNA for PCR reactions, bacterial cells lysed according to Abolmaaty *et al*. (2000) were used. Cells were incubated in 0.1M Tris-HCl buffer (pH 8.0) including 2% Triton X and 2.5 mg mL^{-1} sodium azide at 100°C for 10 min, and 3 µL of lysed cells were used as template.

For the identification of the bacterial isolates, PCR and the PGF/PGR primers were used, which amplify the polygalacturonase (*peh*A) gene and spe-

Figure 1. Turkish provinces surveyed for the presence of *Rhizobium vitis* in vineyards.

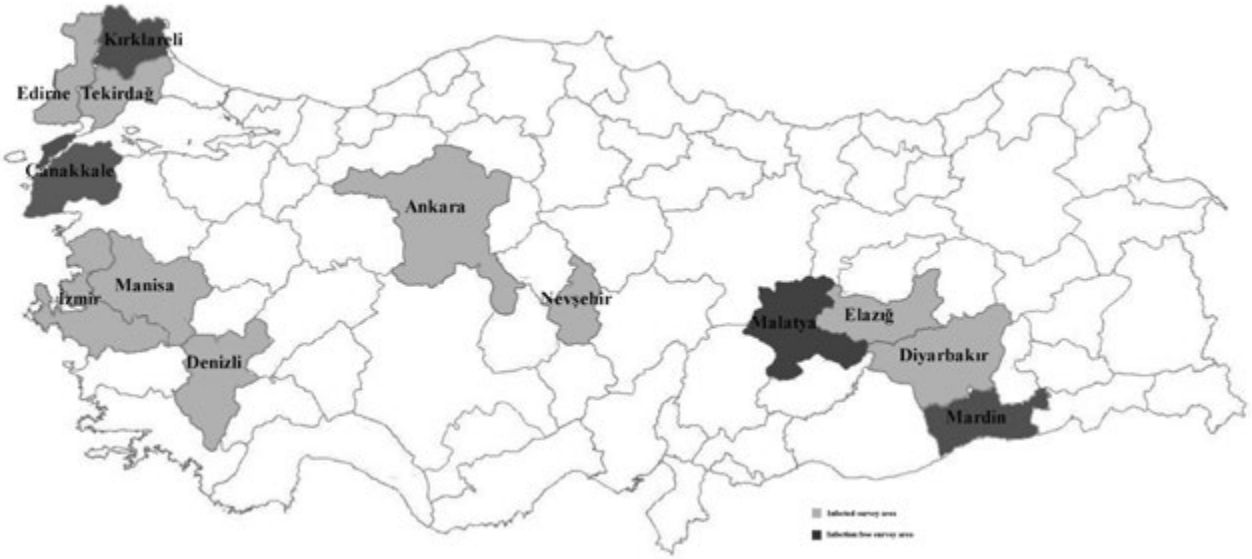

Table 1. Strain information and opine types of *Rhizobium vitis* used in this study.

Isolate	Province	Variety	Opine type
Gaş1, Gaş3	Denizli	Cabernet Frank	Octopine
DM1, DM2, DM3, DM4, DM5, MG3		Boğazkere	
MG1		Öküzgözü	
Goç1		Çalkarası	
Dnzl1, Dnzl2, Dnzl3, Dnzl4, Dnzl5, Dnzl6		Unknown	
Gaş2		Cabernet Frank	Nopaline
MG2		Öküzgözü	
Gaş4, Gaş5		Cabernet Frank	Vitopine
Ak1, Ak2	Manisa	Alphonse	Octopine
Ak3		Razaki	
Srh1		Alicante	
Srh2, Srh3		Syrah	
Gmm1		Unknown	
Al1		Alphonse Lavallée	Nopaline
Srh4		Syrah	
Mnd1, Mnd2	İzmir	Alphonse Lavallée	Octopine

(Continued)

Table 1. (Continued).

Isolate	Province	Variety	Opine type
Klck1, Klck2, Klck3, Klck4, Klck5, Klck6, Klck7	Ankara	Kalecik Karası	Octopine
Aky1, Aky2		Boğazkere	
Aky3		Cabernet Frank	
Aky4, Aky6, Aky7		Emir	
Aky8, Aky9, Aky10		Syrah	
Bp1, Bp2		Unknown	
Aky11, Aky12		Syrah	Nopaline
Çbk1, Çbk2, Çbk3 Çbk4, Çbk5, Çbk6		Unknown	
Aky5		Emir	Vitopine
Kr1, Kv1, Kv2, Kv9, Ürg3, Ürg4, Ürg5	Nevşehir	Emir	Octopine
Kv3, Kv5, Kv6		Syrah	
Kr2, Kr3		Pinot Noir	
Kv7		Chardonnay	
Kv8		Öküzgözü	
Kv10		Merlot	
Ürg1, Ürg2		Narince	
Kv4,		Syrah	Nopaline
Kv11		Merlot	
Ürg3		Emir	
Dc1, Dc2	Diyarbakır	Şıralık	Octopine
Çer1, Çer2, Çer3, Çng2		Öküzgözü	
Çng1		Boğazkere	
Çng3, Çng4		Boğazkere	Nopaline
Mdn1, Mdn2	Elazığ	Boğazkere	Octopine
Alck1, Alck2		Tahannebi	
Alck3		Vanni	
Alck4		Vanni	Nopaline
Uzk1, Uzk2, Uzk3, Uzk4, Uzk5, Uzk6, Uzk7, Uzk8	Edirne	Syrah	Octopine
Tkr1, Tkr2	Tekirdağ	Merlot	Octopine
C58 (*R. radiobacter*)			
S4 (*R. vitis*)			VitopineO/C Nopaline
Tm4 (*R. vitis*)			
AT1(*R. vitis*)			

cifically *R. vitis* strains but not other *Rhizobium* species (Szegedi and Botka, 2002). The VirD2A/VirD2C primer pair was used to distinguish *R. radiobacter-R. vitis* based on different annealing temperatures (Bini *et al*., 2008); opine specific primers were used for determining the presence of each opine (Table 2).

PCR amplifications were each conducted in a reaction volume of 25 µL containing 1× PCR buffer (10×, Promega), 2.5 mM $MgCl_2$ (25 mM, Promega), 2.5 mM dNTPs (100 mM, GeneMark), 0.6 mM of each primer, 5% DMSO (Merck), 1.2 U Taq DNA polymerase (GoTaq Flexi, Promega), and 14.5 µL sterile water. The PCR temperature profiles was performed using the following protocol: predenaturation step at 94°C for 4 min, 32 cycles consisting of denaturation at 92ºC for 1 min, annealing at different temperatures according to the primers (see Table 2) for 1 min, extension at 72ºC for 90 s, and final extension step at 72ºC for 3 min. The PCR products were visualized following electrophoresis in 1% agarose and staining with ethidium bromide (Syngene).

Pulsed field gel electrophoresis (PFGE)

Bacterial cells were grown in the following liquid medium described by Moore *et al.* (2001): sucrose 10 g L^{-1}, casein acid hydrolysate 8 g L^{-1}, yeast extract 4 g L^{-1}, K_2HPO_4 2 g L^{-1}, $MgSO_4.7H_2O$ 0.3 g L^{-1}. A shaking incubator was used at 28°C for 24 h for bacterial cell growth. Bacterial concentration was spectrophotometrically adjusted at OD_{600} = 1.0. Plug preparation and lysis of bacterial cells in plugs were performed as described by Durmaz *et al.* (2007). Briefly, bacterial cells mixed with 10 µL of proteinase K and an equal volume of 1.6% low melting point agarose were pipetted into plug molds. Solid plugs were then inserted in cell lysis buffer containing 10 µL of proteinase K and incubated at 50°C overnight in a water bath. They were then washed twice with distilled water and five times in Tris-EDTA (Tris-HCl, pH: 8.4, 0.5 M EDTA) buffer and stored at 4°C until further use. Six different rare cut restriction endonucleases (RE) were utilized along with different pulse times for the molecular typing of the isolates by PFGE (Table 3). Reaction mix consisted of 20 U enzyme, 10 µL 1× NEB reaction buffer 4, 1U BSA, 87 µL sterile water per isolate for a total 100 µL volume. PFGE gel results were analysed using Bionumerics software version 6.01 (Applied Maths, Belgium). Dice coefficient and the unweighted pair group method was used to generate a dendrogram based on 1.5% tolerance value and 1% optimization setting. Strains were grouped based on the similarity with a coefficient greater than 85% to clonal relationships.

Results

Biochemical and physiological tests

The majority of the strains tested showed biochemical and physiological test characteristics consistent with those previously reported (Moore *et al*.,

Table 2. Primer pairs used for differentiating *Rhizobium* spp. and for determining opine type.

Primer	Sequence	Amplicon size (bp)	Annealing temperatures (°C)	Reference
VirD2A/ VirD2C	5′-ATG CCC GAT CGA GCT CAA GT-3′ 5′-TCG TCT GGC TGA CTT TCG TCA TAA-3′	224	54°C	Haas *et al.* (1995)
PGF/PGR	5′- GGG GCA GGA TGC GTT TTT GAG-3′ 5′- GAC GGC ACT GGG GCT AAG GAT-3′	466	56°C	Szegedi and Botka (2002)
OCTF/ OCTR	5′GAA TAT GAG AAA TCC GTC TCG-3′ 5′-ACT CAG AGC TCG TGG CCT TG-3′	475	52°C	Bini *et al*. (2008)
NOPF/ NOPR	5′-GCA AAC GTA AGT GTT GGA TC-3′ 5′- CAA GCG AAT ACT CGA GAC G-3′	394	52°C	Bini *et al*. (2008)
VisF/VisR	5′-CCG GCC ACT TCT GCT ATC TGA-3′ 5′-CCA TTC ACC CGT TGC TGT TAT T-3′	561	55°C	Szegedi and Botka (2002)

Table 3. Rare-cut restriction endonucleases and working conditions used for PFGE (Voltage: 6 V, temperature: 14ºC, enzyme concentration: 20U)

Enzyme	Start Pulse (s)	Finish Pulse (s)	Time (h)
*Ase*I	10	85	22
	5	45	20
*Dra*I	5	45	20
*Pac*I	10	85	22
	5	45	20
*Pme*I	5	45	20
*Spe*I	10	85	22
	5	45	20
*Swa*I	10	85	22
	5	45	20
*Xba*I	4	10	22

2001), while 19 strains gave anomalous results (Table 4). The DM1 isolate from the Denizli province was the most divergent strain as it produced atypical results in four tests. Cluster analysis based on biochemical and physiological test results revealed that the *R. vitis* population here studied separated into three clusters (Figure 2). The first cluster consisted of 80 strains, which in turn is divided into two groups, and each group comprised two subgroups. Seventy-two strains belong to the first subgroup, 70 of which showed 100% similarity with the reference strain S4 of *R. vitis*. In this group, Alck 2 was differentiated from the other strains by alkali production from malonic acid and acid production from sucrose; strain Alck 3 differed from others in acid production from sucrose (Table 4). The second subgroup of the first branch consisted of eight strains. The second cluster consisted of 17 strains, 15 of which showed the same profile. K2 was differentiated by alkali production from malonic acid and K7 by reaction on litmus milk (Table 4). The third cluster consisted of eight strains. DM 1 was the most divergent strain based on alkali production from L-tartrate and malonic acid, reaction on litmus milk and acid production from arabitol (Table 4). The reference strain of *R. radiobacter* (C58) was located on this branch and showed a unique profile compared to the other strains.

Table 4. Divergent strains of *Rhizobium vitis* as indicated by biochemical and physiological tests.

Isolate	Province	Alcali production from L-tartrate	Alcali production from malonic acid	Acid production from melezitose	Acid production from dulcitol	Acid production from arabitol	Acid production from sucrose	Reaction on Litmus milk
Gaş 1	Denizli	+	+	-	-	+	+	+
Gaş 4	Denizli	+	+	+	+	-	+	+
Gaş 5	Denizli	+	+	+	+	+	+	+
DM 1	Denizli	-	-	-	-	+	+	-
DM 3	Denizli	-	-	-	+	-	+	+
DM 4	Denizli	+	+	+	-	-	+	+
MG 1	Denizli	-	+	+	-	-	+	-
MG 3	Denizli	+	+	+	-	+	+	+
Kr 1	Nevşehir	-	+	-	-	-	+	+
Kr 2	Nevşehir	-	+	-	-	-	+	+
Kr 3	Nevşehir	-	+	-	-	-	+	+
Kv 1	Nevşehir	-	+	-	-	+	+	+
Kv 2	Nevşehir	+	-	-	-	-	+	+
Kv 7	Nevşehir	+	+	-	-	-	+	-
Çng 4	Diyarbakır	-	+	-	-	-	+	+
Mdn 2	Elazığ	+	+	-	-	+	+	+
Alck 1	Elazığ	+	+	-	-	+	+	+
Alck 2	Elazığ	+	-	-	-	-	-	+
Alck 3	Elazığ	+	+	-	-	-	-	+

In grey are highlithed the anomalous results.

Pathogenicity tests

All strains were found to be tumorigenic on test plants. Galls were observed, respectively, on sunflower, tobacco and datura plants at 4, 5 and 6 weeks after the inoculation. Koch's postulates were fulfilled, as *R. vitis*, identified by colony morphology

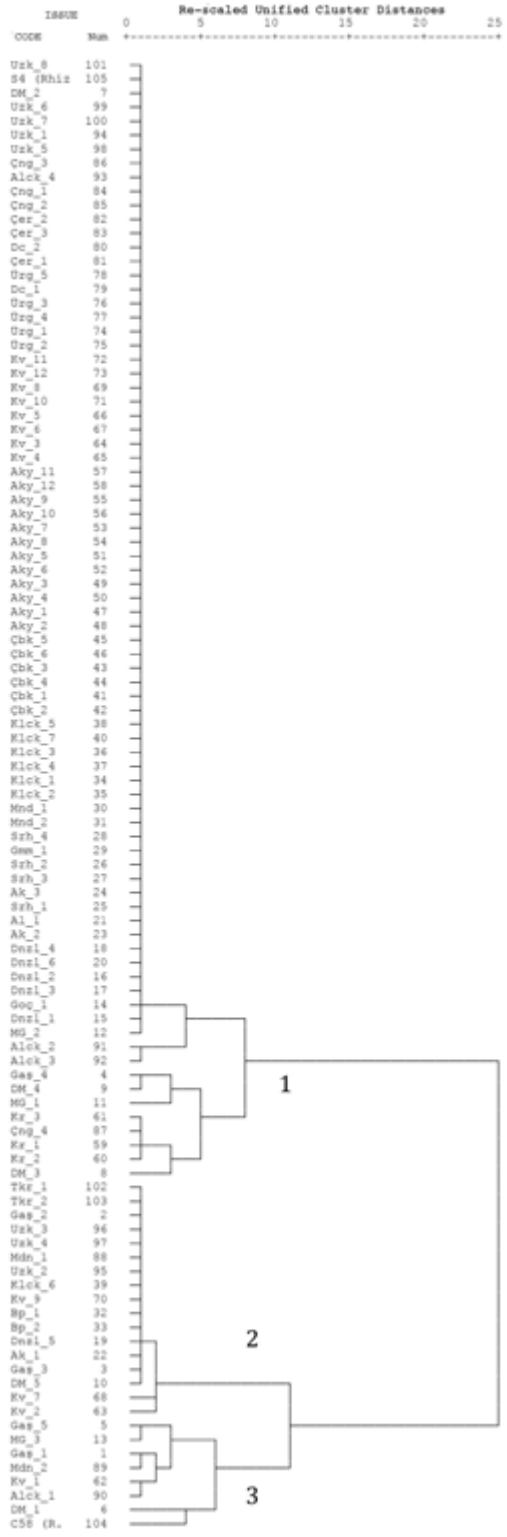

Figure 2. SPSS cluster analysis of *Rhizobium vitis* strains based on phenotypic and physiological test results.

and PCR, was re-isolated from galls of the inoculated plants.

Molecular analyses

All strains gave a 466-bp product by PCR and the primer pair PGF/PGR, indicating that they belong to *R. vitis* (Figure 3). In contrast, none of the strains amplified a product with VirD2A/VirD2C primers at 54°C annealing temperature (data not shown).

Opine types of the strains were determined with primers specific for each gene on *R. vitis* Ti plasmids (Table 2), and all opine type PCR results are shown in Figure 3 representatively. OCTF/OCTR primers amplified a 475-bp product for 82 strains and the reference O/C-type *R. vitis* Tm4 strain. NOPF/NOPR primers amplified a 394-bp product for 18 of the strains and the reference nopaline-type *R. vitis* AT1 strain. VisF/VisR primers amplified a 561-bp product for three strains and from the reference vitopine-type *R. vitis* S4 strain.

PFGE

Preliminary experiments revealed that restriction endonucleases *Ase*I, *Dra*I, *Pac*I and *Swa*I did not generate any restriction patterns for the strains tested. Among *Pme*I, *Spe*I and *Xba*I, *Pme*I was found the most effective RE based on the distinguishable restriction patterns (Figure 4). Preliminary test results of *Spe*I and *Xba*I showed indistinguishable and unrepeatable patterns on PFGE. Therefore, all strains were digested with *Pme*I and differences between

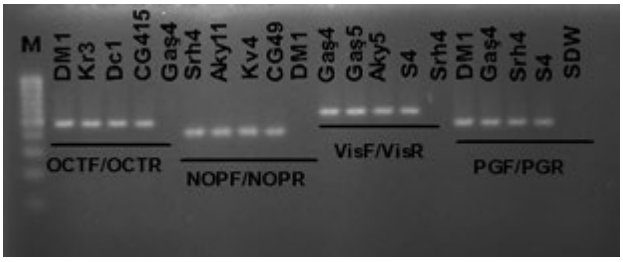

Figure 3. Representative 1% agarose gel of different opine types (M: Thermo scientific 100bp DNA ladder, DM1, Kr3, Dc1 are O/C-type strains, CG415: O/C-type reference strain; Srh4, Aky11, Kv4 are nopaline-type strains, CG49: Nopaline- type reference strain; Gaş4, Gaş5, Aky5 are vitopine-type strain, S4: Vitopine- type reference strain, SDW: sterile distille water).

the strains were illustrated in a dendrogram according to their restriction patterns (Figure 5). Due to six strains having indistinguishable PFGE patterns that did not allow a clear evaluation, 97 strains were evaluated. The dendrogram revealed the presence of 19 clusters, 11 unique profiles and 30 genotypes. Total genotypes were calculated by "clusters + unique profiles". A cluster is defined as the phylogenetic group that consists of at least two indistinguishable strains. A unique profile is characterized as unrelated to other strains and shows an individual profile. The dendrogram divided into two main groups. The first group consists of eight clusters and three unique profiles and the similarity rate of the strains in this group is 36.33%. The second group consists of 11 clusters and six unique profiles for which the similarity rate of the strains is 43.34%.

Strains from Denizli province showed the most genotypic heterogeneity with for unique profiles and 13 genotypes among 20 strains. Vitopine-type isolates, Gaş4 and Gaş5, exhibited restriction patterns that were different from all other strains. In this region, except unique profiles, another nine strains located under four different clusters. Strains from Denizli-Denizler (six strains) were the most heterogenic subgroup within four genotypes. Strains from Ankara were the second most heterogenic subgroup depicted on the dendrogram with seven different genotypes. The similarity rate of the Thrace region isolates was 92.34%. Strains from eastern Anatolia region had indistinguishable profiles except for two strains, Çng 4 from Elazığ and Alck4, from Diyarbakır. These two strains showed unique profiles on the dendrogram. The dendrogram revealed that 71 of 97 strains had 100% similarity. *R. radiobacter* reference strain C58 located separately on the dendrogram and the reference strain was distinguishable from the other strains by its unique profile.

Discussion

Turkey is known as a centre of origin of *Vitis vinifera* L. and viticulture. Furthermore, vineyards are very important in this country, comprising 468.8 ha of vineyards producing more than 4 million t of grapes per year (Anonymous, 2014). In a previous study of *R. vitis* characterization in Turkey, Argun *et al.* (2002)

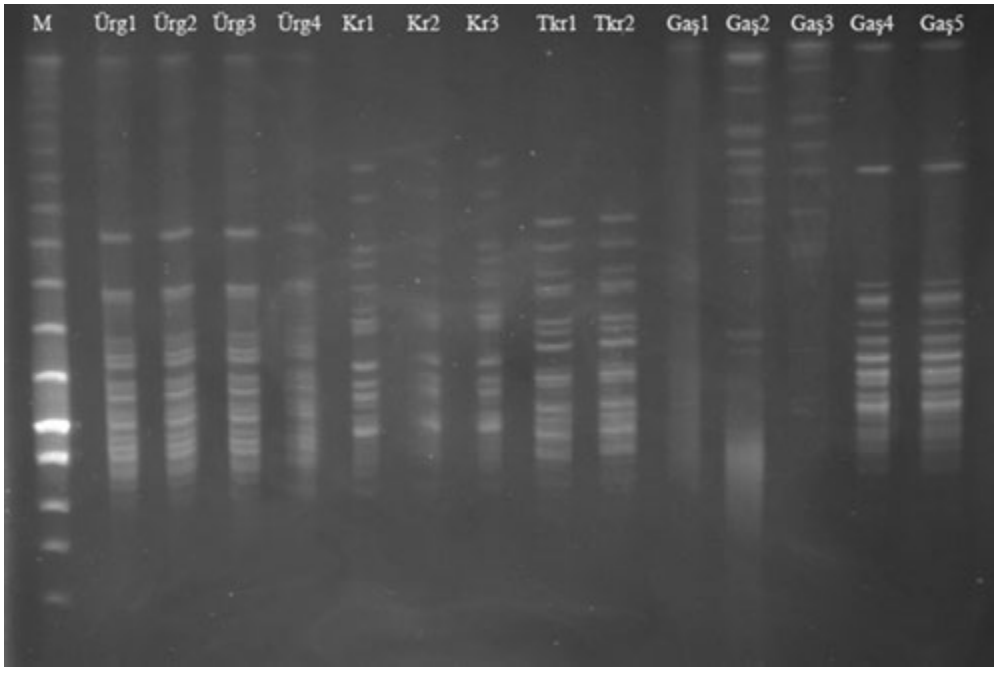

Figure 4. PFGE patterns of chromosomal DNA restriction fragments resolved in 1.6% pulsed field grade agarose for *Rhizobium vitis* DNA digested with *Pme*I (Marker: NEB Low Range PFGE Marker, Working conditions: 5sec→45sec, 6V, 14ºC, 20h). The size of the fragments is indicated in kilobase.

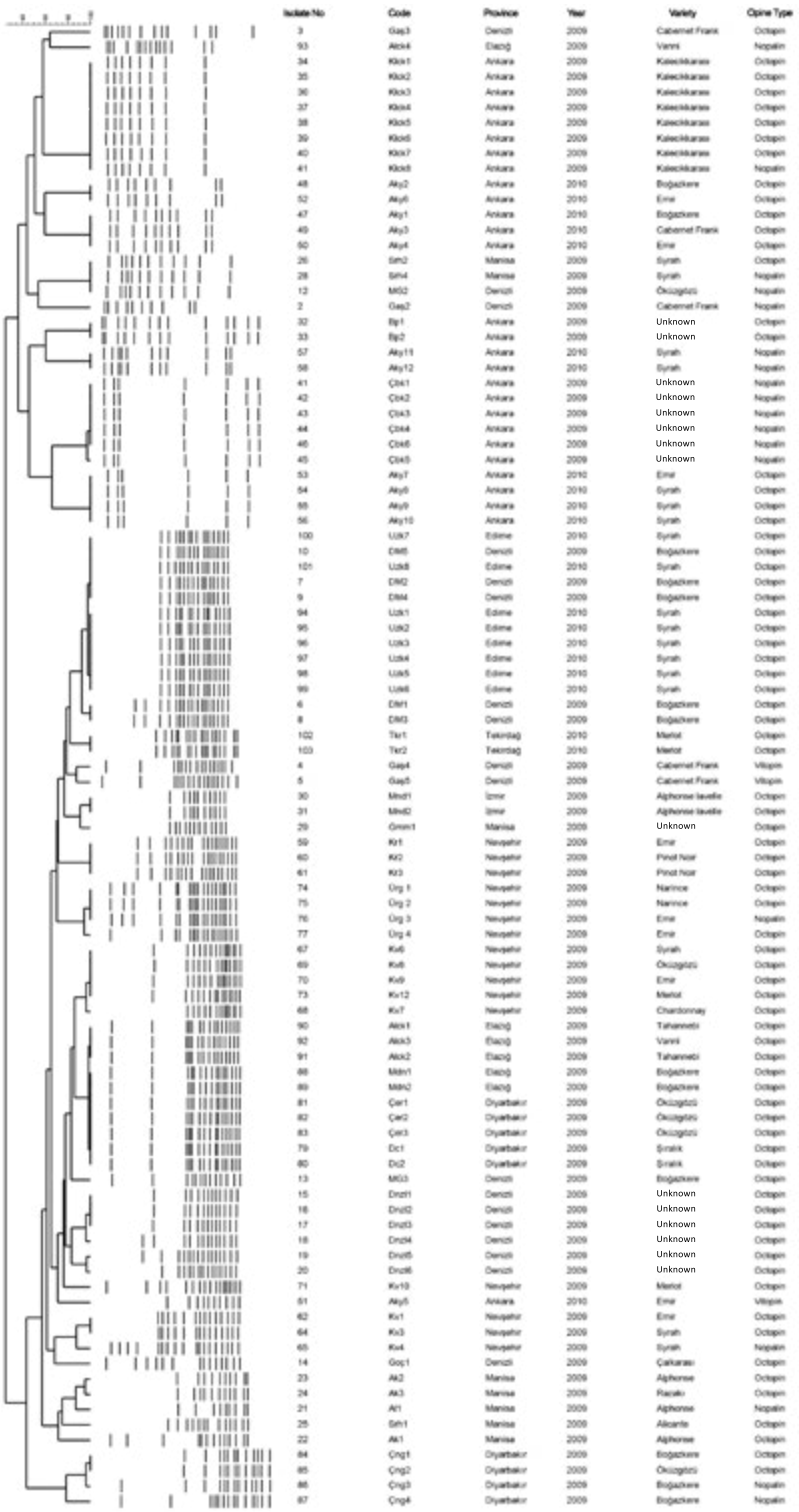

Figure 5. Dendrogram displaying genotypic differences of a Turkish *Rhizobium vitis* population. Percent similarity was calculated by the Dice similarity of PFGE (*Pme*I) restriction endonuclease digestion, constructed using UPGMA algorithm (Bionumerics version 6.01 software) based on 1.5% tolerance value and 1% optimization setting.

characterised strains of the pathogen from Nevsehir and Ankara provinces based on their T-DNA structures and according to ITS fingerprint analysis. This is the only detailed but province-limited study for Turkish *R. vitis* strains. For the study reported here, we aimed to more clearly understand the diversity of *R. vitis* strains from all of the main Turkish viticulture provinces.

Grapevine crown gall was observed in nine of the 13 provinces, that is in the major viticulture areas of Turkey. Bacterial isolates were collected between July and September from young soft cream-coloured galls. All the 103 strains were found to be tumorigenic, based on inoculation of test plants. *Rhizobium* infection may be initiated at wounds on canes and trunks, caused by freezing temperatures. The majority of the surveyed area, especially central and eastern Anatolia, are affected by winter and late spring frosts, that may have contributed to development of the disease.

Although it has been reported that VirD2A/VirD2C primers used at an annealing temperature of 54°C gave a characteristic amplicon only with *R. radiobacter* and the vitopine-type *R. vitis* strains of Bini *et al.* (2008), our vitopine-type strains did not produce this amplicon. However, we confirmed that our strains belong to *R. vitis,* using the specific primer pair PGF/PGR (Eastwell *et al.*, 1995; Szegedi and Bottka, 2002) and using other biochemical and physiological tests. It is possible that the virD2 sequence of our vitopine strains differs slightly resulting in this lack of amplification. Bini *et al.* (2008) also reported that results from the VirD2A/VirD2C primer pair did not always give reproducible results at different annealing temperatures.

Although most of the strains conformed to predicted results for *R. vitis* in biochemical and physiological tests, 19 of the 103 strains showed atypical responses. All strains were tumorigenic and amplified the characteristic *pehA* product with primers PGF/PGR. Eight out of the 19 different strains were L-tartrate negative. Utilization of L-tartrate is a plasmid-borne feature of *R. vitis* strains (Ridé *et al.*, 2000; Szegedi *et al.*, 2005), and these strains may therefore not carry the plasmid. Similarly, Kusek *et al.* (2005) reported that two out of 21 tumorigenic *R. vitis* strains from the east Mediterranean region of Turkey were negative for alkali production from L-tartrate and malonic acid.

Seventeen of the *R. vitis* strains analysed were found to be nopaline-types, and three were vitopine-types. Similarly, in other studies characterizing *Rhizobium* strains, it was reported that 60–70% of *R. vitis* strains were of O/C, 20–30% of nopaline and 5–10% of vitopine-type (Burr and Otten, 1999; Ridé *et al.*, 2000). In the present study, all of the strains were found to amplify at least one opine gene by PCR. Argun (2001) studied the opine types of 50 *R. vitis* isolates from Central Anatolia according to methods of Hooykaas *et al.* (1979), and found they were the O/C type. It was reported that all the strains carried the same type of Ti plasmid (Argun *et al.*, 2002). Kusek *et al.* (2005) and Kusek (2007) also reported the O/C type of *R. vitis* on their study of how plant growth promoting rhizobacteria affect *R. vitis* in Turkey.

Strains Gas4 and Gas5 from Denizli province and strain Aky5 from Ankara demonstrated five different PFGE banding patterns, and these strains showed unique profiles in the dendrogram. These strains were found to have vitopine-type Ti plasmids. Opine production is another plasmid-borne feature of *Rhizobium* spp. (Ridé *et al.*, 2000). Schrammeijer *et al.* (1998) reported that O/C-types of *R. vitis* have the *vir*F gene on their Ti plasmid, which may be related to the differences we observed in PFGE profiles of nopaline-type and vitopine-type strains. To the best of our knowledge, the current study is the first report of nopaline and vitopine-types of *R. vitis* in Turkey.

PFGE is a DNA-based typing method that has been employed for studying pathogen epidemiology (Peters, 2009). It has a high segregation capacity and is highly repeatable. *Rhizobium vitis* strain S4 has two circular chromosomes (Jumas-Bilak *et al*., 1998; Slater *et al.*, 2009). The larger chromosome (chromosome I) contains an origin of replication that is similar to other chromosomal origins within the *Alphaproteobacteria*, while chromosome II has a repABC origin of replication typical of the large plasmids within the *Rhizobacteriaceae*. Slater *et al.* (2009) also reported that strain S4 has five plasmids, rRNA operons and the extensive sets of essential metabolic genes on the second chromosome. Therefore, PFGE was chosen to check and compare the whole genome of the strains and find links between the epidemiology of *R. vitis* in different parts of Turkey. Schulz *et al.* (1993) analyzed the genome of *R. vitis* by PFGE for evolutionary relationships of 42 strains. They identified six genomic groups with RE patterns of the six genome types. *Xba*I, *Sfi*I and *Spe*I patterns of 42 strains designated six genomic groups. One opine type was found dominant and subgroups were designated as three octopine, three

vitopine groups. They did not identify any nopaline subgroup. Our results show that Turkish *R. vitis* strains consisted of 19 genomic groups, comprising 11 unique profiles according to *Pme*I restriction patterns. Vitopine-type strains and most of the nopaline-type strains appear in separate groups. It may be that as a plasmid-borne feature, opine type of the strains shows different restriction patterns because of their differences on Ti-plasmids. Tanaka *et al.* (2006) used PFGE to investigate the physical and chromosomal *vir* genes of K-Ag-1 *R. vitis* strain which was isolated from kiwifruit. They compared a kiwifruit strain of *R. vitis* K-Ag-1 with eight *R. vitis* strains, and reported that three of five Japanese strains were closely related. Two Japanese strains and three from other countries were found different according to PFGE patterns. Similarly, we did not find correlation between the province where strains were taken and genotypic relatedness of the strains. For example Ankara strains had six different profiles. Some of the strains from Ankara, such as Kalecik strains, are indistinguishable according to restriction patterns, but Ankara-Akyurt strains had three different patterns. Edirne and Denizli provinces are located in different regions of Turkey. However DM2, DM4, and DM5 strains from Denizli province appear in the same genomic group with Uzk7 and Uzk8 strains from Edirne province. This report supports our result that there are significant genomic differences between strains isolated from the same region.

The internal transcribed spacer (ITS) region located between 16S-23S rRNA genes shows variability in size and sequence within bacterial strains. The ITS region has been used for grouping *R. vitis* strains by Kuzmanović *et al.* (2014), and they reported five genetic groups. Argun *et al.* (2002) also reported there are differences on the restriction patterns of the ITS region of the O/C-type Turkish *R. vitis* strains. It shows that Ti plasmid and chromosomal structure can be different and these data support our results on unique and different profiles as expressed in Figure 5. The IGS region of the ribosomal DNA of *R. vitis* also has different chromosomal and Ti DNA plasmid characteristics between strains (Otten *et al.*, 1996). Those data show that Ti plasmid and chromosomal structure can be different, and support our results on unique and different profiles as shown in the dendrogram in Figure 5.

Since Turkey is known as a centre of origin of *V. vinifera*, it is important to understand variability of the pathogen strains, whether they are from one source and the importance of imported propagation material as sources of the tumorigenic *R. vitis.* Vineyards in Turkey still grow local varieties in many areas. Boğazkere, Öküzgözü, Çal karası and Vanni are some of these local and important grape varieties of Turkey. When we examined the grouping of strains on the dendrogram, most strains from local varieties in one region showed identical patterns. However, some strains were different, such as Çng 4-Bogazkere (from Diyarbakır) and Alck 4-Vanni (from Elazığ). These two varieties are grown in eastern Anatolia and both show unique profiles although other strains from the same region have similar patterns. On the other hand, Denizli is another important grape production area with 44,000 ha of vineyards. This was where we discovered the greatest genetic diversity of *R. vitis*. Most of the wine varieties in Denizli are imported from abroad which is also likely to impact diversity of *R. vitis* in this province.

An understanding of the variability of the crown gall-causing bacterial strains is important for several reasons. This will facilitate examination of the future spread of *R. vitis* in Turkey. This approach can also be used to investigate suspected introductions of the pathogens into the country on plant material or by other means. In addition, divergent strains may also differ in levels of virulence and thus cause differing economic impacts of crown gall. Future research is warranted to determine how the divergent groups of *R. vitis* that we have identified differ in these and other important characteristics.

This is the first detailed analysis of *R. vitis* strains using PFGE to identify the distribution and diversity of O/C, nopaline and vitopine-type strains in Turkey, from 18 different grapevine varieties and 88 vineyards from main viticulture provinces of Turkey.

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