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Genetic diversity of *Agrobacterium vitis* strains, isolated from grapevines and wild grapes in Bulgaria, assessed by Cleaved Amplified Polymorphic Sequences analysis of 16S-23S rDNA

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Summary

Nineteen tumorigenic *Agrobacterium vitis* strains isolated from commercial vineyards and wild grapes at different locations in Bulgaria were studied in relation to the Ti plasmid type and chromosomal background. The PCR analysis showed that all but one of the strains harbor an octopine/cucumopine type of Ti plasmid and one carries a vitopine type. The genetic diversity among the studied strains and 20 more *A. vitis* strains originating from different geographic regions in Europe, Asia, USA and South Africa was assessed by Cleaved Amplified Polymorphic Sequences (CAPS) analysis of 16S-23S rDNA region. The comparison of the obtained CAPS profiles and performed cluster analysis showed a high level of polymorphism among the studied strains distributed in totally 15 different groups within two main clusters. All Bulgarian strains are located in only three groups within one of the clusters. A high level of correlation between the chromosomal background and type of the carried Ti plasmids was found. The performed CAPS analysis demonstrated that all *A. vitis* strains isolated from wild grapes (*V. vinifera* ssp. *silvestris*) showed CAPS profiles identical with a number of strains isolated from commercial vineyards from different vine-growing regions in Bulgaria. A possible origin of this group of strains from an ancestral *A. vitis* strain, which initially inhabits wild grapes (*V. vinifera* ssp. *silvestris*) and later has been disseminated to cultivated grapevines is proposed.

K e y w o r d s : *Agrobacterium vitis*, genetic diversity, 16S-23S rDNA, Cleaved Amplified Polymorphic Sequences markers, wild grapes, *Vitis vinifera* ssp. *silvestris*,

Introduction

Agrobacterium vitis is the predominant causal agent of crown gall disease of grapevine (Burr *et al*. 1998). The pathogenic strains harbor Ti plasmid, which includes: T-DNA region that is transferred into plant genome, virulence genes responsible for packaging and transport of the T-DNA and genes involved in the synthesis and catabolism of opines. The Ti plasmids of *A. vitis* are classified into three main types: an octopine/cucumopine, a nopaline and a vitopine. Each Ti plasmid type has a specific genetic make-up of the T-DNA structure and is related to a particular type of opine synthase gene(s) (Szegedi *et al*. 1988, Burr and Otten 1999, Szegedi 2003). The opines metabolism of *A. vitis* is not exclusively linked to the Ti plasmids, since existence of non-pathogenic *A. vitis* strain F2/5 harboring an octopine utilization plasmid was reported by Szegedi *et al.* (1999). Following a genetic characterization of diverse pools of tumorigenic strains of *A. vitis*, Momol *et al*. (1998) demonstrate a high correlation between the chromosomal background and the type of Ti plasmids the strains carry. In recent years, the genetic diversity of *A. vitis* strains was evaluated mainly by assessment of the ribosomal DNA sequences (Bouzar *et al*. 1995, Otten *et al*. 1996, Momol *et al*. 1998, Argun *et al*. 2002). While the 16S and 23S genes of the ribosomal operon are highly conserved, the intergenic spacer region (IGS) between them is variable in *Proteobacteria* and thus suitable for analyzing closely related strains. Using "universal" PCR primers, regions of the ribosomal operon including the intergenic spacer could be readily amplified and characterized by Cleaved Amplified Polymorphic Sequences (CAPS) analysis. This approach could be easily applied to study very diverse pools of strains, providing high reproducibility of the results and reliable comparison of the obtained data within different experiments and laboratories.

In a previous study we reported the isolation of *Agrobacterium* strains from grapevines in Bulgarian vineyards and wild grapes and their physiological, biochemical and tumorigenicity characterization (Genov *et al.* 2006). It was demonstrated that tumor bearing grapevines, as well as symptomless wild grapes (*Vitis vinifera* ssp. *silvestris*), contain predominantly pathogenic strains of *A. vitis*.

In this study we determine the Ti plasmid type of 19 pathogenic *A. vitis* strains and apply CAPS analysis of rDNA region for assessment of their genetic diversity together with 20 other *A. vitis* strains originating from different countries. The correlations of the determined Ti plasmid type and chromosomal background, as well as the possible origin of the *A. vitis* strains isolated from the wild grapes are discussed.

Material and Methods

P C R a n a l y s i s o f t h e s t r a i n s : The *A. vitis* strains used in our experiments are listed in the Table. Bacterial DNAs were isolated from overnight cultures using the rapid Triton X-100/sodium azide protocol

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Table

Agrobacterium vitis strains used for CAPS analysis

A. vitis strains	Origin	Presence and type of Ti plasmid[1]	Source[2]/Reference
IG-1, IG-2, IG-3, IG-4, IG-5, IG-6, IG-7, IG-8, IG-9, IG-10, IG-11, IG-12, IG-13, IG-14, IG-15, IG-16, IG-17, B-29;	Bulgaria	OC	ABI, Genov *et al.* 2006
AB-3	Hungary	OC	RIVE, Szegedi *et al.* 1988
CG-415, CG-475, CG-102, CG108;	USA	OC	CU, Otten *et al.* 1996
AA-34	Afghanistan	OC	CU, Ercolani
MD-1, MD-15;	Moldavia	OC	ABI
N-16, N-14;	Turkey	OC	CU, Argun N.
339-6	Spain	OC	CU, Lopez M.
IG-18	Bulgaria	V	ABI, Genov *et al.* 2006
S-4	Hungary	V	RIVE, Szegedi *et al.* 1988
CG-81, CG-78;	USA	V	CU, Otten *et al.* 1996
1860-(3)	Italy	V	CU, Bazzi C.
CG-49, CG-47;	USA	N	CU, Otten *et al.* 1996
AT-1	Hungary	N	RIVE, Szegedi *et al.* 1988
F2/5	South Africa	-	RIVE, Szegedi *et al.* 1999
CG-510	USA	-	CU, Burr T.

[1)] Ti plasmid type: OC - octopine/cucumopine; V - vitopine; N - nopaline; (-) the strain does not carry Ti plasmid

[2)] ABI - AgroBioInstitute, Bulgaria, RIVE - Research Institute for Viticulture and Enology, Hungary; CU - Cornell University, USA

(Abolmaaty *et al*. 2000, Szegedi and Bottka 2002). Three PCR primer pairs were used to determine the Ti plasmid type. T primer pair, TF (5'-TGGCCGAAATTGTTTACTTCCACCC) and TR (5'-CTATGCCGAAAGACGGCTTGACCCT), specifically amplified a 520 bp fragment of the *6b* gene of *A. vitis* octopine pTis (Szegedi *et al*. 2005). N primer pair, NF (5'-TTAACCCAAATGAGTACGATGACGA) and NR (5'-TTATTTCGGTACTGGATGATATTAG) specifically amplified a 570 bp fragment of the *6b* gene of *A. vitis* nopaline pTis (Szegedi *et al*. 2005). Vis primer pair, VisF (5'-CCGGCCACTTCTGCTATCTGA) and VisR (5'-CCATTCACCCGTTGCTGTTATT), specifically amplified a 561 bp fragment of *A. vitis* vitopine synthase (VIS) gene associated with vitopine type Ti-plasmid (Canaday *et al*. 1992, Szegedi and Bottka 2002). The PCR amplifications were carried out in 25 µl reaction mixture, which consists of 1x *Taq* polymerase buffer (Fermentas, USA), 1.8 mM $MgCl_2$, 0.4 µM of each primer, 160 µM of each dNTP, 1.25 unit Taq polymerase (Fermentas, USA) and 2 µl template DNA (about 40 ng). The reaction conditions involved an initial denaturation step at 94 °C for 1 min, followed by 32 cycles at 92 °C, 60 °C for T, 58 °C for N and 54 °C for VIS, 72 °C for 1 min at each temperature and a final extension step at 72 °C for 3 min. The samples were analyzed after electrophoresis in ethidium-bromide stained agarose gels.

C A P S a n a l y s i s o f t h e s t r a i n s: For CAPS analysis, the 16S-23S rDNA region of studied strains was PCR amplified using of FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG) and FGPL132' (5'-CCGGGTTTCCCCATTCGG) primers (Normand *et al*. 1992). The obtained PCR fragments are about 2500-2700 bp long and include a large part (1479 bp) of 16S rDNA, the 16S/23S intergenic spacer and part (132 bp) of 23S rDNA (Ponsonnet and Nesme 1994). The PCR amplifications were carried out in 50 µl reaction mixture, which consists of 1x *Taq* polymerase buffer, 1.8 mM $MgCl_2$, 0.4 µM of each primer, 160 µM of each dNTP, 1.25 unit Taq polymerase (Fermentas, USA). The PCR conditions included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C, 55 °C, 72 °C for 2 min at each temperature and a final extension step at 72 °C for 3 min. The amplified DNA fragments were further digested with *Msp* I, *Rsa* I and *Taq* I (Fermentas, USA). Eight µl of the PCR reaction mixtures were mixed with 10 units of enzyme in a final volume of 16 µl, containing 1x enzyme buffer. The digestion products were size-separated by vertical electrophoresis in 8 % denaturating polyacrylamide gel containing 7 M urea with 1x TBE running buffer (Sambrook *et al*. 1989). Samples prepared as digestion reactions were mixed with equal volume of 40 % formamide / loading dye solution and denaturated at 95 °C for 4 min. Following electrophoresis the gels were stained for 30 min in TBE buffer containing 1.0 $\mu g \cdot ml^{-1}$ ethidium bromide and documented.

D a t a a n a l y s i s: The CAPS patterns of the studied strains for each of the used restriction enzymes were

subject of data analysis, the presence and absence of restriction fragments were recorded as 1 or 0, respectively and treated as discrete character. Pairwise comparison of banding patterns was evaluated using the program package RAPDistance 1.04 (Armstrong *et al*. 1994). The data were analyzed to generate the Nei-Li similarity coefficient (Nei and Li 1979). The similarity coefficient was used to construct a dendrogram using neighbor-joining (NJ) analysis (Saitou and Nei 1987).

Results and Discussion

Nineteen tumorigenic *A. vitis* strains isolated from different vineyards and wild grapes (*V. vinifera* ssp. *silvestris*) in Bulgaria (Genov *et al.* 2006) were tested by PCR analysis for determination of the Ti plasmid type. All but one of the strains showed PCR amplification with T primer pair but not with N and Vis primer pairs (Fig. 1), suggesting that they carry an octopine/cucumopine Ti plasmid (Table). For one of the strains (IG-18), PCR amplification was detected only with Vis primer pair, pointing out that it harbours a vitopine type plasmid. Well studied *A. vitis* strains AB-3, AT-1 and S-4 carrying an octopine/cucumopine, a nopaline and a vitopine type Ti plasmid (Szegedi *et al*. 1988), were used as controls. Our results demonstrate that the octopine/cucumopine type of *A. vitis* is predominantly spread in the commercial vineyards and in wild grapes (*V. vinifera* ssp. *silvestris*) growing in Bulgaria. The Ti plasmid type of several additional strains (AA-34, 1860-(3), N-16, N-14; 339-6) originating from other geographical regions, involved in the presents study was clarified with T, N and Vis primer pairs (data not shown, Table).

The genetic diversity of the analyzed *A. vitis* strains was further evaluated through CAPS analysis of 16S-23S rDNA region. Twenty more *A. vitis* strains derived from different geographic regions and having different Ti plasmid types were included in the study (Table). Two of the *A. vitis* strains (F2/5 and CG-510) are non-tumorigenic and do not harbour Ti plasmids. The PCR amplifications with FGPS6 and FGPS132' primers from the isolated DNAs resulted in obtaining single PCR fragments for all of the studied strains (data not shown). The amplified DNA fragments were digested with *Msp* I, *Rsa* I and *Taq* I enzymes. The separation of the digested DNA fragments by electrophoresis in 8 % denaturating polyacrylamide gels (Fig. 2) rather than in agarose gels, provides better resolution of the obtained fragments and data processing. The dendrogram constructed from the performed CAPS analysis demonstrated that the analyzed 39 *A. vitis* strains form a total of 15 different groups, belonging to two main clusters A and B (Fig. 3). Cluster A consists of the octopine/cucumopine and vitopine type strains and cluster B includes strains from all types: the octopine/cucumopine, the nopaline and the vitopine strains. Following the comparative study of various *A. vitis* strains Momol *et al*. (1998) reported a close correlation between Ti plasmid type and chromosomal background of the strains. In this study, the comparison of plasmid type of the strains and their position in the constructed dendrogram provides further support for such correlation. Indeed, all but one group of strains showing identical CAPS pattern also possess the same type of Ti plasmid (Fig. 3). The only exception is for strain IG-16, which harboured the octopine/cucumopine Ti plasmid but showed a digestion profile typical of the vitopine type strains IG-18 and S-4. Such 'unusual' combination of an octopine/cucumopine-type Ti plasmid carried by a strain with chromosomal background similar to the vitopine strains was reported earlier for the strain CG-474 (Otten *et al*. 1996). Based on rDNA fingerprint and RAPD analysis of chromosomal DNA, the CG-474 strain carrying octopine/cucumopine-type Ti plasmid was classified in the group of the vitopine type *A. vitis* strains by Momol *et al*. (1998). These data suggest a possibility for lower frequency existence of strains with 'vitopine-type' chromosomal background carrying octopine type Ti plasmids. The inclusion of the non-pathogenic octopine utilizing strain F2/5, which doesn't harbor the Ti plasmid, in our study reveals that it has identical CAPS pattern with the pathogenic octopine/cucumopine strain CG-415 and also is part of a larger sub-cluster of pathogenic octopine/cucumopine strains. Szegedi *et al* (1999) reported that the strain F2/5 carries an octopine utilization plasmid. This suggests that the correlation between the Ti plasmid type and the chromosomal background could be further extended also for non-pathogenic strains carrying only non-Ti octopine (opine) utilizing plasmids.

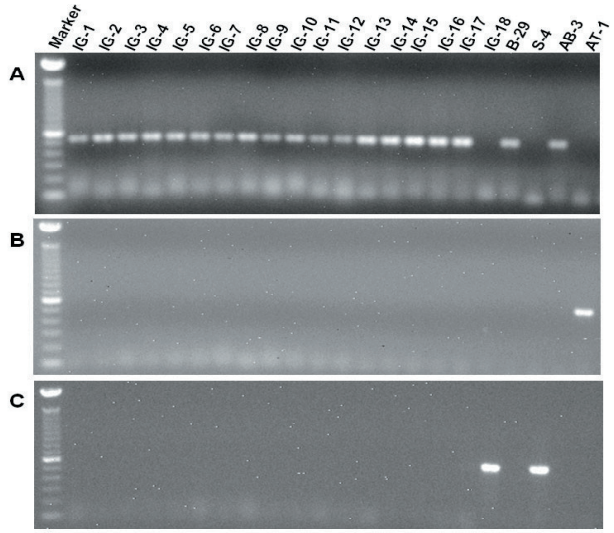

Fig. 1: PCR analysis of the *Agrobacterium* strains: **A,** T primer pair amplifies a 520-bp product from octopine type *A. vitis* strains. **B,** N primer pair amplifies 570-bp product from nopaline type strains. **C,** Vis primer pair amplifies a 561-bp product from vitopine type strains. Names of strains are shown on top. Bands in size-marker lanes represent increments of 100 bp.

The present study does not demonstrate a significant correlation between the CAPS pattern and the geographical origin of the analyzed strains. At the same time all Bulgarian strains are involved in three groups located only in cluster A of the dendrogram suggesting a lower level of genetic diversity compared to the entire pool of strains. Such lower genetic complexity could be due to spreading of small initial groups of strains through occasional contamination of grapevine planting material, propagated at

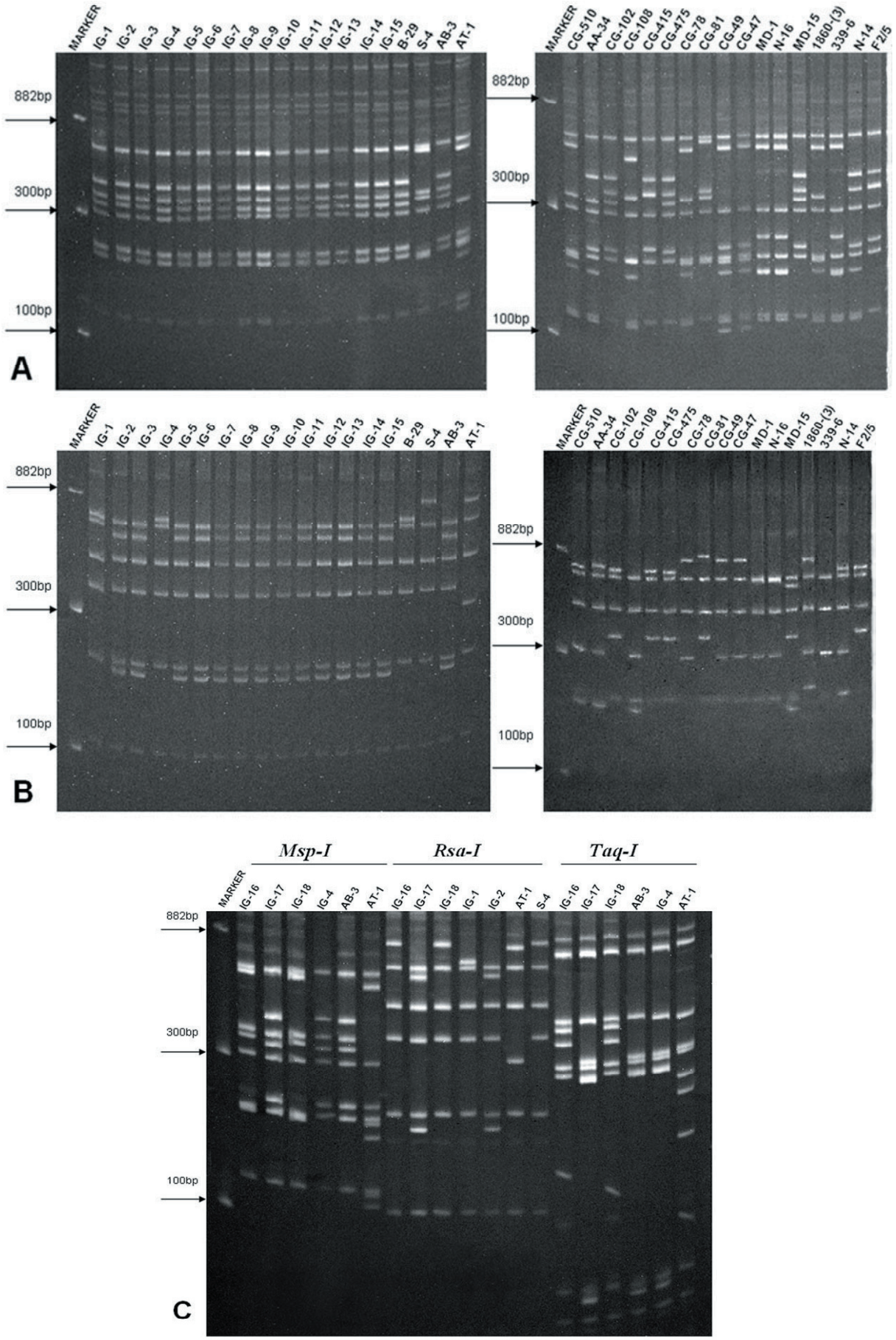

Fig. 2: Digestion profiles of the PCR-amplified 16S-23S rDNA region from studied *Agrobacterium* strains. Names of the strains are shown on top. Bands in size-marker lane represent 100 bp, 300 bp, 882 bp. **A,** Digestion profiles generated with *Msp* I; **B,** Digestion profiles generated with *Rsa* I; **C,** Digestion profiles generated with *Msp* I, *Rsa* I, *Taq* I;

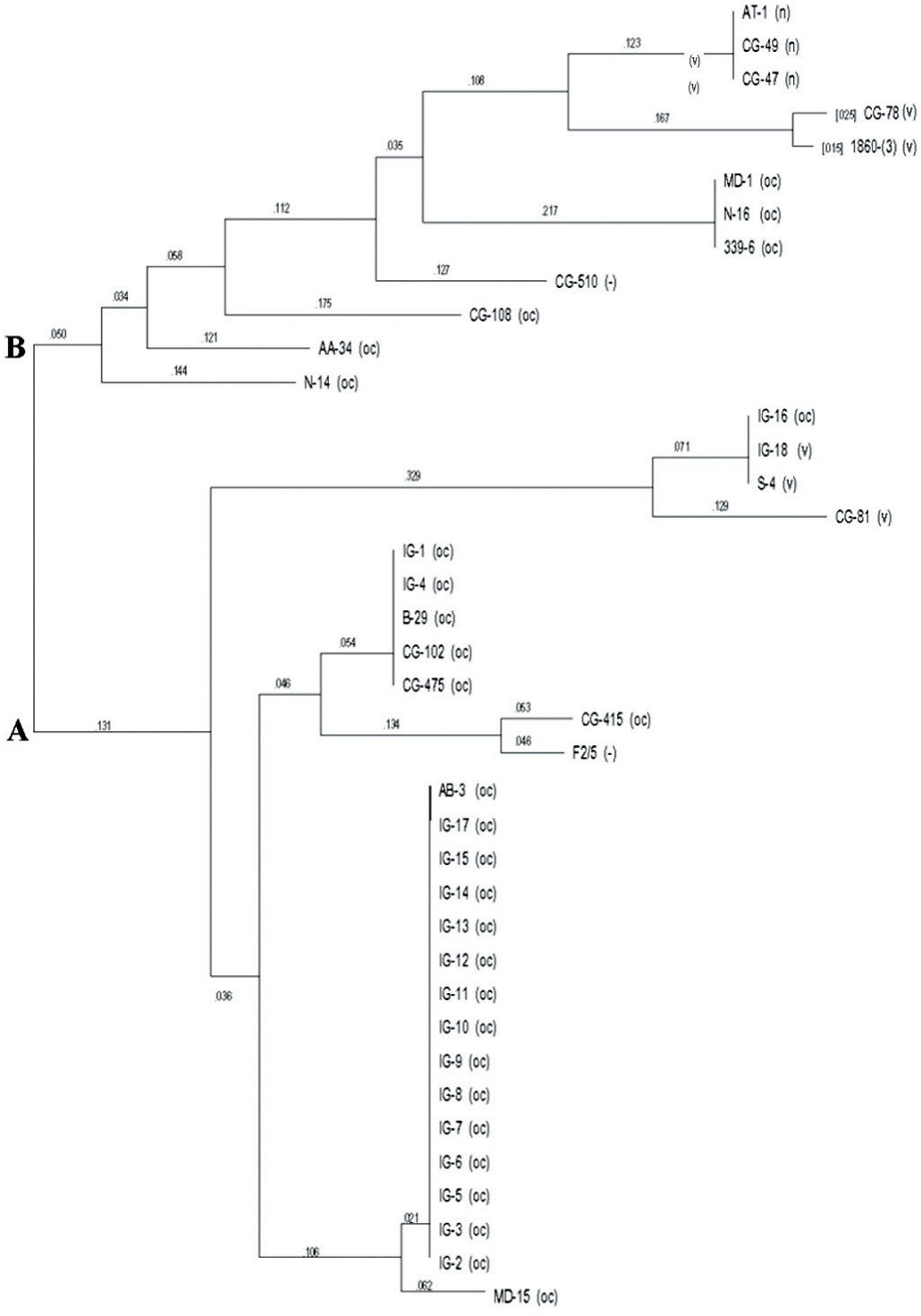

Fig. 3: Dendrogram from neighbor-joining (NJ) analysis of the *Agrobacterium vitis* strains based on Nei-Li similarity coefficients that were determined from the digestion profiles of the PCR-amplified 16S-23S rDNA. The Ti plasmid type of each strain is presented with small letters: (oc) – octopine/cucumopine; (v) – vitopine; (n) – nopaline; (-) the strain does not carry Ti plasmid.

large scale in Bulgaria during the second half of the last century. Another possible explanation is that at least part of the studied strains originate from a common ancestral strain, endogenous for the region. In relation to this, one of the most interesting results of this study is the finding that all *A. vitis* strains (IG-9, IG-10, IG-11, IG-12) isolated from wild grapes (*V. vinifera* ssp. *silvestris*) showed identical CAPS profiles with a number of strains isolated from commercial vineyards in different regions of Bulgaria (IG-2, IG-3, IG-5, IG-6, IG-7, IG-8, IG-13, IG-14, IG-15, IG-17). In a previous study, the strains from these two groups were found to have the same characteristics following physiological, biochemical and virulence tests (Genov *et al.* 2006). Since the sampled wild grapes were located at a significant distance from commercial vineyards contamination is unlikely (Genov *et al.* 2006). Summarizing, this

led to the attractive hypothesis that the strains from pointed group originated from ancestral *A. vitis* strain(s), which initially inhabited wild grapes (*V. vinifera* ssp. *silvestris*) and later may have been disseminated to cultivated grapevines. Further extension of the CAPS analysis and/or sequence comparison to different chromosomal DNA regions of these strains may provide clues for *A. vitis* evolution and the dissemination pattern.

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