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Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir

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Bois noir (BN) is an economically important grapevine yellows disease induced by the stolbur phytoplasma and principally vectored by the cixiid *Hyalesthes obsoletus*. This study addresses the involvement of other planthoppers and/or leafhoppers in BN epidemics in the South Banat district of northeastern Serbia, by performing transmission experiments and multilocus typing of stolbur phytoplasma isolates to determine the vector-related characteristics of the disease. Transmission trials were conducted with adults of two cixiid congeners, *Reptalus panzeri* and *R. quinquecostatus*, which were found to harbour stolbur phytoplasma in the vineyards under study. A molecular characterization of stolbur phytoplasma isolates was performed by sequence analysis and/or RFLP typing of the two housekeeping genes *tuf* and *secY* and the two membrane proteins *stamp* and *vmp1*. Transmission trials with naturally infected *R. panzeri* adults from either the BN-infected vineyards or maize redness (MR)-affected maize fields revealed a high stolbur phytoplasma transmission efficiency to grapevines. In contrast, experiments conducted with stolbur-positive *R. quinquecostatus* originating from BN-infected vineyards, provided no evidence for a vector role of this species. Seven stolbur phytoplasma genotypes, all of which were *tuf*-b types, were detected among the grapevine- and insect-associated field samples according to the *tuf*/*secY*/*vmp1*/*stamp* typing. STOLg was the genotype most frequently found in naturally infected grapevine (42%), as well as *R. panzeri* originating from the vineyards (85%) and maize fields (98%). The same genotype was found in all experimental plants inoculated by *R. panzeri*, confirming its vectorship of the disease.

Keywords: grapevine yellows, insect vector, molecular epidemiology, *Reptalus* spp., *stamp* variability, stolbur phytoplasma

Introduction

Bois noir (BN) is an important grapevine yellows disease caused by a phytoplasma that can induce significant economic losses related to wine production. The disease is widespread in Europe, the Mediterranean and the Middle East. Although it is considered to be less epidemic and severe than the flavescence dorée (FD) phytoplasma, BN incidence has increased over the last decade in many regions (Johannesen *et al.*, 2008) and it is becoming an important limiting factor in grape production. Bois noir is associated with the stolbur phytoplasma from the 16SrXII-A subgroup, recently described as '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013).

Phytoplasmas are members of the class Mollicutes and are prokaryotic, phloem-limited plant pathogens that cannot yet be cultured. They multiply within both plant and insect hosts, and they are characterized by obligate transmission by insects or parasitic plants or by grafting (Hogenhout *et al.*, 2008). Insect phytoplasma vectors include leafhoppers, planthoppers and psyllids, which belong to the suborders Auchenorrhyncha and Stenorrhyncha, order Hemiptera.

The stolbur phytoplasma has a broad host-plant range, including various herbaceous and woody host plants of economic importance, as well as numerous weedy hosts acting as pathogen reservoirs. There are two major stolbur phytoplasma genetic clusters involved in the two distinct epidemiological cycles of BN, namely, *tuf*-a and *tuf*-b, which is in accordance with gene for the elongation factor Tu (Langer & Maixner, 2004). The *tuf*-a stolbur type is associated with stinging nettle (*Urtica dioica*), which acts as its primary host plant and pathogen reservoir, and it is the most common in northwestern disease occurrences (Germany, France, Switzerland) where it is an epidemic (reviewed in Johannesen *et al.*, 2012). The *tuf*-b type is predominantly present in the southeastern and eastern disease range, and field bindweed (*Convolvulus arvensis*) is generally recognized as its reservoir, although it infects a number of diverse wild host plants. There are several recent studies of epidemics and the increased spread of the *tuf*-a type in northwestern Europe (Johannesen *et al.*, 2008, 2012; Maniyar *et al.*, 2013), but no studies have focused on *tuf*-b epidemics, even though this type of stolbur phytoplasma has a much broader geographic range and affects a number of economically important crops in addition to grapevine e.g. maize, potato, tomato.

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The cixiid planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae) is considered to be the principal vector of the stolbur phytoplasma that causes BN in both southern and central European vineyards (Maixner, 1994; Sforza *et al.*, 1998). It is primarily a Mediterranean species with its northernmost distribution in southwest Germany and northern France, and the eastern border of the species range expanding into Iran, Kazakhstan, Afghanistan, Tajikistan and Uzbekistan, and Asia Minor to the southeast (Hoch & Remane, 1985). As a consequence, the geographic range of the stolbur phytoplasma vector *H. obsoletus* overlaps with the geographic distribution of stolbur-induced BN grapevine disease. However, several authors have indicated that other polyphagous planthoppers and/or leafhoppers are probably involved in the dispersal of stolbur phytoplasma to grapevines because *H. obsoletus* is absent or its population density is not correlated with BN incidence in some European areas (Trivellone *et al.*, 2005; Pinzauti *et al.*, 2008; Riedle-Bauer *et al.*, 2008). Recent studies in the grape-growing regions of several European countries have shown that between 30 and 40 Auchenorrhyncha species were present in BN-affected vineyards (Palermo *et al.*, 2004; Trivellone *et al.*, 2005; Riedle-Bauer *et al.*, 2008; Cvrković *et al.*, 2011). Polyphagous species such as *Reptalus panzeri*, *R. quinquecostatus*, *Anaceratagalia ribauti* and *Dictyophara europaea* have been found to harbour stolbur phytoplasma, but their ability to transmit the disease to grapevine has not been confirmed. However, the stolbur phytoplasma was found to be naturally transmitted by planthoppers *Pentastiridius leporinus* and *R. panzeri* to sugar beet and maize, respectively (Gatineau *et al.*, 2001; Jović *et al.*, 2007), while *R. quinquecostatus* was demonstrably able to inoculate artificial feeding medium (Pinzauti *et al.*, 2008), suggesting its ability to act as a vector.

The specific interaction of an insect vector and the phytoplasma transmitted by the vector is a central subject of epidemiological cycle investigations of vector-borne plant diseases, along with vector feeding preference and host-plant specificity. A large number of stolbur phytoplasma host plants, noted or supposed insect vectors and several independent epidemiological cycles (e.g. Langer & Maixner, 2004; Bressan *et al.*, 2009; Jović *et al.*, 2009) led to the use of molecular methods to characterize the epidemiological pathways underlying stolbur epidemics and transmission. A number of non-ribosomal stolbur phytoplasma genes were tested and proposed as useful for vector or reservoir plant identification in epidemiological studies (Langer & Maixner, 2004; Cimerman *et al.*, 2009; Fabre *et al.*, 2011). Among them, the antigenic (immunodominant) membrane protein AMP was characterized for its key role in phytoplasma–insect vector interactions (Kakizawa *et al.*, 2006; Suzuki *et al.*, 2006; Galetto *et al.*, 2011). The AMP was shown to form a complex with both the muscle microfilaments surrounding the insect's intestine and ATP synthase of insect midgut and salivary gland cells. In addition, it was found that *amp* is under a strong positive selection pressure, which is crucial for the specific interaction between pathogen and insect vector and for efficient phytoplasma transmission by different, specific vectors (Kakizawa *et al.*, 2006). A stolbur phytoplasma gene, encoding an antigenic membrane protein, named *stamp* was recently characterized and confirmed to be under diversifying positive selection pressure (Fabre *et al.*, 2011).

In the epidemiology of BN in Serbia, a cixiid planthopper *H. obsoletus* is considered to be the major vector, the same as in the other parts of south and southeastern Europe (Sforza *et al.*, 1999). Bois noir is currently reported in all Serbian viticultural regions, including one of the most important regions, which is located in the South Banat district of northeastern Serbia (Cvrković, 2010). An epidemiological survey of Serbian vineyards revealed that *H. obsoletus* was scarce or absent in several BN-infected vineyards. Thus, the estimated population density of *H. obsoletus* did not correlate with the high prevalence of stolbur-infected plants in the southern Banat viticultural region, which prompted a study to evaluate involvement of other planthoppers and/or leafhoppers in the epidemiology of BN.

The main objectives of this study were to: (i) ascertain the diversity and stolbur phytoplasma infection of Auchenorrhyncha species in BN-affected vineyards in the southern Banat viticultural region of Serbia; (ii) characterize the *stamp* and other genes of stolbur phytoplasma isolates in naturally infected grapevines and insects to elucidate the epidemiological system; and (iii) understand the potential vector role of other species aside from *H. obsoletus* in natural BN transmission.

Materials and methods

Survey of potential vectors

Potential phytoplasma vectors in the suborder Auchenorrhyncha were collected from mid-June to mid-September during 2010 and 2011. The survey was carried out in three neighbouring vineyards on a site in the southern Banat viticultural region (45°06′N, 21°21′E) of northeastern Serbia, where stolbur phytoplasma-induced BN symptoms were previously recorded (Cvrković, 2010).

Potential hemipteran vectors, including leafhoppers and planthoppers, were collected from grapevines along the inter-rows with sweep nets and mouth aspirators. Insects were placed in 2 mL plastic vials (Sarstedt) containing 96% ethanol and stored at 10°C until their arrival at the laboratory where they were subsequently identified to the species level using taxonomic keys provided by Holzinger *et al.* (2003) and Biedermann & Niedringhaus (2004). In the laboratory, the insects were stored in ethanol at –20°C until DNA extraction.

Grapevine sampling

In late August of 2011, grapevine plants expressing characteristic symptoms of phytoplasma infection, such as downward-rolled leaf margins and partial interveinal discoloration, were randomly sampled for molecular typing of the disease-inducing stolbur phytoplasma strain. Twelve samples were taken from each of the three vineyards. Prior to DNA extraction, the leaf

veins and petioles of each grapevine sample were frozen in liquid nitrogen and stored at −20°C.

Stolbur phytoplasma identification in insects and grapevine

The total DNA from each individual hemipteran specimen was isolated by applying a modified CTAB method according to Gatineau *et al.* (2001) or by using the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. Total DNA was extracted from the grapevines with symptoms by following the CTAB protocol of Angelini *et al.* (2001).

Stolbur phytoplasma identification in plant and insect material was performed using the stolbur-specific Stol11 nested PCR protocol with primers F2/R1 and F3/R2 (Clair *et al.*, 2003). DNA amplification was performed in 20 μL using reaction conditions according to Radonjić *et al.* (2009). The PCR products were separated on 1% agarose gels, stained with ethidium bromide and visualized under a UV light. DNA extracts of stolbur phytoplasma-infected maize (Jović *et al.*, 2009) were used as positive controls in all amplification reactions.

Transmission experiments

Grapevine seeds (cv. Plovdina) were sown in sterile soil (Klasmann TS 1, Klassman Dielmann) and individually potted in containers (10 cm diameter, 15 cm height) following germination. The plants were maintained in a growth chamber at 24 ± 1°C and 16/8 h light/dark period for 3 months prior to the experiments. By the time they were exposed to potential vectors in the transmission experiments, the grapevine seedlings were at a six-to-eight-leaf stage. Prior to the experiments, the potted plants were transferred into polyvinyl chloride cylinders with 10 cm base diameter × 35 cm edge height, which were ventilated by a hole on the top covered with mesh gauze.

The transmission trials of stolbur phytoplasma to grapevine seedlings were performed with adults from two cixiid species that harboured the pathogen and occurred in significant numbers in the vineyards under study, namely *R. panzeri* and *R. quinquecostatus* (Table 1). Experiments were performed with either eight or 12 replicates (plants). The number of specimens per plant was influenced by the population size of each *Reptalus* species and included 30 *R. panzeri* and 50 *R. quinquecostatus* (Tables 2 & 3). In addition, the transmission experiments with *R. panzeri* were set up with specimens collected from maize redness (MR)-affected maize fields in Samoš from the South Banat district (45°11′N, 20°45′E), which is a 50 km straight line from the vineyards under study. The symptoms of stolbur-induced MR and the presence of naturally stolbur-infected *R. panzeri* individuals were previously recorded in these fields (Jović *et al.*, 2009).

Insects were collected at the beginning of July 2011 in BN-affected vineyards and MR-affected maize fields, confined in cylinders with experimental plants immediately after field capture and carried to the laboratory at 15°C. Transmission tests were carried out under laboratory conditions, wherein insect-exposed grapevine seedlings were kept in a growth chamber for 2 days at 24 ± 1°C (16/8 h light/dark period). The cylinders were checked daily for insect survival and feeding activities. Dead cixiids were immediately collected and stored in 96% ethanol. After the experiments ended, the surviving adults were collected from each of the exposed plants, individually placed in 2 mL vials with 96% ethanol and kept at −20°C for subsequent phytoplasma detection. All insects were submitted to stolbur phytoplasma identification using the Stol11 protocol as described above.

Table 1 Stolbur phytoplasma incidence in Auchenorrhyncha species collected in bois noir (BN)-infected vineyards in the South Banat district of northeastern Serbia

	No. of field-collected/stolbur positive specimens[a]		
Auchenorrhyncha species	Vineyard 1	Vineyard 2	Vineyard 3
Cixiidae			
Hyalesthes obsoletus	0/0	2/0	3/1
Reptalus panzeri	161/36	70/15	76/16
Reptalus quinquecostatus	236/41	242/46	214/38
Reptalus cuspidatus	9/0	3/0	8/0
Delphacidae			
Laodelphax striatella	10/0	16/0	12/0
Dictyopharidae			
Dictyophara europaea	6/0	4/0	2/0
Aphrophoridae			
Philaenus spumarius	28/0	19/0	23/0
Cicadellidae			
Anaceratagallia ribauti	15/0	18/0	23/0
Cicadella viridis	10/0	12/0	9/0
Zyginidia pullula	18/0	12/0	25/0
Fieberiella septentrionalis	10/0	18/0	16/0
Neoaliturus fenestratus	20/0	28/0	19/0
Doratura impudica	12/0	15/0	18/0
Euscelis incisus	58/0	65/0	72/0
Psammotettix alienus	85/0	72/0	95/0
Errastunus ocellaris	27/0	33/0	45/0

[a]Number of collected/stolbur positive specimens per Auchenorrhyncha species in each of the three surveyed vineyards in a site in the South Banat district (45°06′N, 21°21′E). Stolbur phytoplasma identification was performed using the stolbur-specific Stol11 nested PCR protocol.

After the transmission tests ended, 28 exposed grapevine seedlings were kept in insect-proof greenhouse conditions and monitored for symptom development over the subsequent 13 months (i.e. until late August of the following year), after which the leaves were sampled and subjected to stolbur phytoplasma identification analysis using the Stol11 protocol. Tissue samples from four healthy grapevines grown in the same conditions as the exposed plants were used as a negative control in DNA extraction and amplification.

Multilocus typing of stolbur phytoplasma isolates

The molecular characterization of stolbur phytoplasma field-collected and experimentally obtained isolates was performed by sequence and/or RFLP analysis of two housekeeping genes *tuf* and *secY* and membrane protein genes *stamp* and *vmp1*. The isolates subjected to characterization were as follows: (i) grapevines with symptoms from the field, (ii) *R. panzeri* collected in vineyards and used in transmission trials (six isolates per trial replicate, whenever possible), (iii) *R. quinquecostatus* collected in vineyards and used in transmission trials (six isolates per trial replicate, whenever possible), (iv) *R. panzeri* collected from maize and used in transmission trials (six isolates per trial replicate), and (v) experimentally infected grapevines. All these isolates were characterized by *stamp* sequence analysis and *tuf* and *vmp1* gene RFLP analysis. Sequencing of *vmp1* and *secY* genes was performed with representative isolates of the *stamp* genotypes associated with different hosts (Table 4).

Table 2 Experimental transmission of stolbur phytoplasma to grapevine by *Reptalus panzeri*. Stolbur phytoplasma identification was performed using the stolbur-specific Stol11 nested PCR protocol

		R. panzeri from vineyard		*R. panzeri* from maize field	
Experimental plant	Replicate	Stolbur-positive[a]	Transmission[b]	Stolbur-positive[a]	Transmission[b]
Grapevine	1	11/30	+	12/50	+
	2	5/30	+	15/50	+
	3	11/30	+	16/50	+
	4	10/30	+	12/50	+
	5	5/30	+	15/50	+
	6	8/30	+	13/50	+
	7	0/30	–	13/50	+
	8	0/30	–	12/50	+
Total no. of stolbur-positive specimens		50/240	6/8	108/400	8/8
Percentage		21		27	

[a]Number of stolbur-positive *R. panzeri*/number of specimens used per replicate.
[b]Efficacy of stolbur phytoplasma transmission to grapevine determined by symptom expression and phytoplasma identification in experimental plants. +, successful stolbur transmission; –, no transmission.

Table 3 Experimental transmission of stolbur phytoplasma to grapevine by *Reptalus quinquecostatus*. Stolbur phytoplasma identification was performed using the stolbur-specific Stol11 nested PCR protocol

		R. quinquecostatus from vineyard	
Experimental plant	Replicate	Stolbur-positive[a]	Transmission[b]
Grapevine	1	10/50	–
	2	6/50	–
	3	8/50	–
	4	10/50	–
	5	7/50	–
	6	9/50	–
	7	8/50	–
	8	4/50	–
	9	4/50	–
	10	10/50	–
	11	6/50	–
	12	9/50	–
Total no. of stolbur-positive specimens		91/600	0/12
Percentage		15·2	

[a]Number of stolbur-positive *R. quinquecostatus*/number of specimens used per replicate.
[b]Efficacy of stolbur phytoplasma transmission to grapevine determined by symptom expression and phytoplasma identification in experimental plants. –, no transmission.

The *tuf* gene, which encodes translation elongation factor Tu, was amplified in nested PCR with primers Tuf1f/r, followed by TufAYf/r according to the protocol described by Langer & Maixner (2004). Nested PCR amplicons were subjected to restriction analysis using *Hpa*II endonuclease (Fermentas) by following the manufacturer's instructions. The restriction products were separated by vertical electrophoresis through a 13% polyacrylamide gel, stained in ethidium bromide and visualized with a UV transilluminator. The DNA of stolbur phytoplasma *tuf*-a and *tuf*-b types isolated from naturally infected *H. obsoletus* from the Middle-Rhine and Mosel regions of Germany, respectively (provided by M. Maixner, Bernkastel-Kues) were used as the reference controls for comparing restriction profiles.

The stolbur phytoplasma antigenic membrane protein gene *stamp* was amplified by nested PCR with StampF/R0 followed by StampF1/R1 primers using reaction conditions specified by Fabre *et al.* (2011).

A fragment of the *vmp1* gene, which encodes a putative stolbur phytoplasma membrane protein, was amplified with primer pair StolH10F1/R1 (Cimerman *et al.*, 2009), followed by the inner primer pair TYPH10F/R (Fialová *et al.*, 2009). The PCR conditions were those specified by Fialová *et al.* (2009). The TYPH10F/R amplicons of all characterized isolates were digested with *Rsa*I restriction enzyme, and an additional *Taq*I and *Alu*I or single *Alu*I digestion was performed for some of the resulting profiles. The digestion products were separated on polyacrylamide gels as described above.

The amplification of the *secY* gene, which encodes a translocase protein, was performed using PosecF1/R1, followed by PosecF3/R3 primer pair as previously described (Fialová *et al.*, 2009).

Reference sequences from representative *stamp*, *vmp1* and *secY* gene isolates were obtained by sequencing in both directions, and *stamp* was thereafter sequenced with the forward primer only. The sequencing was performed by Macrogen Inc. and the sequences were deposited in the GenBank database under accession numbers KC703009–22 for *stamp*, KC703023–36 for *vmp1* and KC703037–50 for *secY*.

Molecular data analysis

The *stamp*, *vmp1* and *secY* gene sequences were assembled and edited using FinchTV v.1.4.0 (http://www.geospiza.com). Multiple alignments and comparisons with reference stolbur phytoplasma strains for each of the genes (Cimerman *et al.*, 2009; Murolo *et al.*, 2010; Fabre *et al.*, 2011) were performed using clustalW integrated into mega5 software (Tamura *et al.*, 2011).

A *vmp1* reference sequence of representative stolbur strains associated with different hosts were subjected to virtual digestion with *Rsa*I endonuclease using pDRAW32 software, v. 1.1.114 (AcaClone Software, http://www.acaclone.com) to verify the accuracy of identified PCR-RFLP profiles, and with *Alu*I and *Taq*I endonucleases to additionally differentiate *vmp1* RFLP pattern types. The same bioinformatic software was used to generate *in silico Rsa*I restriction patterns as identified by RFLP analysis in

Table 4 The s*tamp* genotypes of stolbur phytoplasma naturally associated with grapevines, *Reptalus panzeri* and *R. quinquecostatus* or experimentally transmitted to grapevine, with corresponding molecular typing on *vmp1*, *secY* and *tuf* gene markers

stamp genotyping								
stamp genotype[a]	Host	Isolate[b]	GenBank acc. no.	No. of isolates of each genotype per host/no. of isolates per host (%)[c]	*vmp1* profile[d]	*secY* sequence[e]	*tuf* profile[f]	Comprehensive genotype[g]
STOL	*R. panzeri* ex grapevine	Rpg39	KC703009	29/34 (85%)	V2-TA	≡ STOL	tuf-b	STOLg
	R. panzeri ex maize	Rpm34	KC703010	47/48 (98%)	V2-TA	≡ STOL	tuf-b	STOLg
	R. quinquecostatus ex grapevine	Rqg60	KC703011	22/68 (32%)	V2-TA	≡ STOL	tuf-b	STOLg
	Vitis – vineyard	Vv21	KC703012	15/36 (42%)	V2-TA	≡ STOL	tuf-b	STOLg
	Vitis experiment – Rp ex grapevine	Vexp_Rpg11	KC703013	6/6 (100%)	V2-TA	≡ STOL	tuf-b	STOLg
	Vitis experiment – Rp ex maize	Vexp_Rpm5	KC703014	8/8 (100%)	V2-TA	≡ STOL	tuf-b	STOLg
Rpm35	*R. panzeri* ex maize	Rpm35	KC703015	1/48 (2%)	V14	≡ STOL	tuf-b	Rpm35g
BG4560	*R. quinquecostatus* ex grapevine	Rqg42	KC703016	17/68 (25%)	V7-A	≡ BG4560	tuf-b	BG4560g
Rqg31	*R. quinquecostatus* ex grapevine	Rqg31	KC703017	17/68 (25%)	V2-TA	≡ GGY	tuf-b	Rqg31g
	Vitis – vineyard	Vv17	KC703018	7/36 (19%)	V2-TA	≡ GGY	tuf-b	Rqg31g
Rqg50	*R. quinquecostatus* ex grapevine	Rqg50	KC703019	12/68 (18%)	V4	≡ STOL	tuf-b	Rqg50g
	R. panzeri ex grapevine	Rpg47	KC703020	5/34 (15%)	V4	≡ STOL	tuf-b	Rqg50g
	Vitis – vineyard	Vv5	KC703021	11/36 (31%)	V14	≡ STOL	tuf-b	Vv5g
Vv24	*Vitis* – vineyard	Vv24	KC703022	3/36 (8%)	V14	≡ GGY	tuf-b	Vv24g

[a]*stamp* genotypes represented by the reference strains (Fabre *et al.*, 2011) or by the reference isolate of the new genotypes detected in this study (designated in bold).

[b]Representative isolates of each *stamp* genotype detected in this study.

[c]Number of each *stamp* genotype per total number of isolates detected in each of the six host types: *R. panzeri* ex grapevine (34 isolates), *R. panzeri* ex maize (48 isolates), *R. quinquecostatus* ex grapevine (68 isolates), *Vitis* ex vineyard (36 isolates), *Vitis* ex experiment with *R. panzeri* ex grapevine (6 isolates), *Vitis* ex experiment with *R. panzeri* ex maize (8 isolates).

[d]*vmp1* RFLP profiles according to Murolo *et al.* (2010). Profiles V4 and V14 were determined by sequence comparison and single digestion using *Rsa*I endonuclease. New profiles V2-TA and V7-A were determined by sequence comparison with reference strains STOL and PO, respectively, and digestion using *Rsa*I and *Taq*I + *Alu*I enzymes (aberration -TA) or *Rsa*I and *Alu*I (aberration -A).

[e]Stolbur genotypes according to a comparison of 726 bp of the *secY* gene with reference strains. ≡, identity of the isolate sequence with the sequence of the indicated reference strain (Cimerman *et al.*, 2009; Fabre *et al.*, 2011).

[f]*tuf Hpa*II RFLP profiles according to Langer & Maixner (2004).

[g]Genotypes according to *tuf*/*secY*/*vmp1*/*stamp* typing.

isolates from this study as well as reference isolates (Cimerman *et al.*, 2009; Murolo *et al.*, 2010).

For the gene analysed in most detail, *stamp*, a maximum parsimony (MP) analysis was performed in order to determine the relatedness and genealogy of stolbur phytoplasma genotypes involved in BN epidemiology. An MP phylogeny was reconstructed with MEGA5 software using the close neighbour interchange algorithm at search level 1, in which the initial trees were obtained with the random addition of sequences (250 replicates) and 500 bootstrap replicates were performed to assess branch support in the resulting tree topology. Abbreviations and country of origin references for stolbur phytoplasma strains (Fabre *et al.*, 2011) were used to construct the phylogenetic tree as indicated in Figure 1. In order to obtain better visualization of the *stamp* divergence among detected genotypes, TCS v. 1.21 (Clement *et al.*, 2000) was employed to infer the genotype network (reticulated graph) using statistical parsimony (Templeton *et al.*, 1992). Isolates of the *tuf*-b-II and *tuf*-b-III *stamp* clusters inferred by MP phylogeny analysis were used to construct the network. A 93% parsimony probability was applied for the plausible connections in the resulting network. Furthermore, *stamp* was tested for positive selection in the newly detected stolbur phytoplasma genotypes by calculating the dN/dS relative to previously identified genotypes (Fabre *et al.*, 2011). Testing was performed using the Nei–Gojobori method in a codon-based *Z* selection test by which the null hypothesis H_0: dN = dS was tested (the number of non-synonymous substitutions per non-synonymous site equals the number of synonymous substitutions per synonymous site). The analysis was conducted in MEGA5, and the variance of the difference was computed using the bootstrap method (500 replicates). It was expected that if the dN/dS value was >1 there was positive selection underlying the diversification, and if the value was <1 it suggests a purifying selection process.

Results

Planthoppers and leafhoppers present in BN-affected vineyards

A survey carried out in three neighbouring BN-affected vineyards in the southern Banat viticultural region during the 2010 and 2011 season allowed the collection of >2000 Auchenorrhyncha specimens belonging to 37 species from six families: Cicadellidae (24), Cixiidae (4), Delphacidae (4), Aphrophoridae (3), Dictyopharidae (1) and Issidae (1). Among all the specimens, only 15 species were collected in numbers >10 (Table 1). The predominant species were cixiid planthoppers *R. quinquecostatus* and *R. panzeri* (*c.* 700 and *c.* 300 specimens collected, respectively), followed by leafhoppers *Psammotettix alienus*, *Euscelis incisus* and *Errastunus ocellaris*, which contributed over 100 collected individuals per species. For the remaining 10 species, the number of collected specimens varied between 70 for *Philaenus spumarius* and 12 for *D. europaea*. Principal BN vector, *H. obsoletus*, was

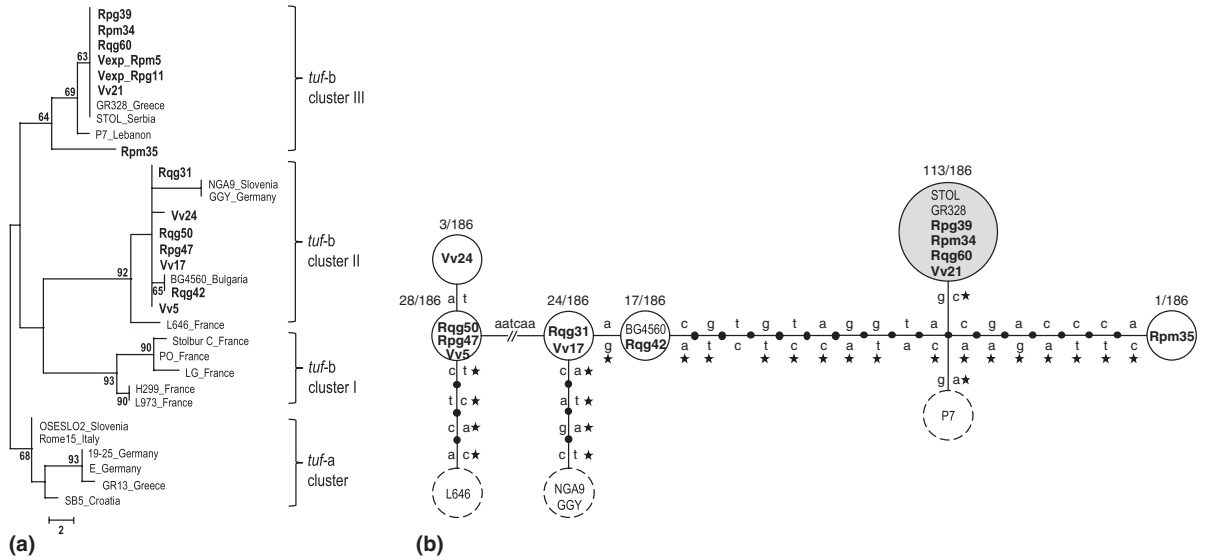

Figure 1 Parsimony analysis of stolbur phytoplasma *stamp* sequences isolates detected in grapevines and putative vectors. (a) One of the 1358 most-parsimonious phylogenetic trees for 14 isolates detected in *Reptalus panzeri* ex grapevine (Rpg), *R. panzeri* ex maize (Rpm), *R. quinquecostatus* ex grapevine (Rqg), *Vitis* ex vineyard (Vv), *Vitis* ex transmission experiments with *R. panzeri* ex grapevine (Vexp_Rpg), *Vitis* ex experiments with *R. panzeri* ex maize (Vexp_Rpm) and 18 reference strains (Fabre *et al.*, 2011). The bootstrap values (>50%) for 500 replicates are shown on the branches. Isolates originating from this study are marked in bold, and the GenBank accession numbers for each isolate are listed in Table 4. Each reference strain aberration and country of origin is indicated according to the data of Fabre *et al.* (2011). Clusters corresponding to *tuf* genotypes are indicated. (b) The statistical parsimony network of the *stamp* genotypes corresponding to the *tuf*-b clusters II and III under 93% parsimony criterion. Strain aberrations are as explained above. Circles represent each genotype. The frequencies of detected genotypes among 186 field-collected samples are indicated above the circles. Dashed circles represent reference genotypes not detected in this study. Each line connecting the circles is one mutational difference. Small black circles represent mutational differences between genotypes. Nucleotide substitutions are indicated. Asterisks indicate amino acid substitutions. The deletion of six nucleotides from genotype Rqg50 to Rqg31 is indicated. The most frequent *stamp* genotype is represented with a double-sized circle and shadowed, with grey indicating a strain transmitted by *R. panzeri* to grapevines.

recorded in negligible numbers from two out of the three studied vineyards (Table 1). The species collected in numbers >10, along with the specimens of *H. obsoletus*, were tested for stolbur phytoplasma.

Stolbur phytoplasma detection in insects and grapevine

PCR analysis with stolbur-specific Stol11 primers revealed the presence of stolbur phytoplasma in three out of 16 tested Auchenorryncha species, all belonging to the family Cixiidae (Table 1). *Reptalus quinquecostatus*, which was the most abundant cicada species, was found to be PCR-positive for stolbur phytoplasma at a rate of approximately 18% (125 stolbur-positive specimens out of 692 collected; Table 1). For the second most abundant species, *R. panzeri*, about 22% of analysed specimens in all of the three inspected vineyards were found to carry stolbur phytoplasma (67 stolbur-positive out of 307 specimens). Only five individuals of the third stolbur-positive cixiid species *H. obsoletus* were collected, and only a single specimen was found to be stolbur-infected.

All 36 field-collected grapevine plants with symptoms from the three studied vineyards in the southern Banat viticultural region tested positive for stolbur phytoplasma.

Transmission of stolbur phytoplasma to grapevine by *Reptalus panzeri* and *R. quinquecostatus*

To determine whether the two cixiid congeners *R. panzeri* and *R. quinquecostatus* could be vectors of stolbur phytoplasma transmission in BN-affected vineyards, adults naturally harbouring stolbur phytoplasma were allowed to feed on grapevine seedlings for 48 h. The feeding activity on experimental plants was confirmed for both species by visual inspection. The survival rate after 48 h on grapevine seedlings was above 90% for *R. panzeri* specimens collected in the vineyards and in the maize fields. A similar survival rate was recorded for *R. quinquecostatus* originating from the vineyards.

After the experiments, the *R. panzeri* and *R. quinquecostatus* specimens used in the transmission trials were analysed for stolbur phytoplasma. Among the *R. panzeri* specimens collected in BN-affected vineyards and MR-affected maize fields, 21 and 27% tested positive for stolbur phytoplasma, respectively (Table 2). The stolbur-infected specimens of *R. panzeri* from vineyards were recorded in six out of eight trial replicates. Stolbur-containing insects were found in all eight replicates in the second experiment using *R. panzeri* adults from maize fields. Among the *R. quinquecostatus* used in the transmission trials, 15% tested positive for stolbur phytoplasma and specimens carrying the phytoplasma were identified in all 12 trial replicates (Table 3).

Approximately 12 months after the experiments ended, six out of eight grapevine seedlings exposed to *R. panzeri* adults from the BN-affected vineyards, as well as all eight grapevine seedlings exposed to *R. panzeri* from MR-affected maize fields, developed typical phytoplasma infection symptoms. The first symptoms appeared as interveinal discoloration followed by leaf vein and lamina reddening. Grapevine plants inoculated by *R. panzeri* from vineyards and maize fields expressed symptoms in a similar manner. The subsequent analysis of plant DNA using Stol11 primers confirmed that all plants with symptoms were infected with stolbur phytoplasma. The two symptomless plants and four plants not exposed to insects tested negative for stolbur phytoplasma.

Experiments conducted with *R. quinquecostatus* individuals revealed that this cixiid did not transmit stolbur phytoplasma to grapevines. Although at least four stolbur-positive *R. quinquecostatus* specimens were feeding on each of the test plants, no symptoms appeared on any of the grapevine plants within 13 months after experiments ended. The PCR results from all 12 grapevine seedlings were negative (Table 3).

Stolbur phytoplasma genotypes in naturally infected grapevines

Information on the specificities of the stolbur phytoplasma genotypes inducing BN in grapevines was expected to help trace these genotypes to the insect vectoring the pathogen, and confirm the vectorship characteristics of the disease. Therefore, multilocus typing of grapevine-associated stolbur phytoplasma isolates was performed from the three BN-affected vineyards in the southern Banat viticultural region.

RFLP analysis of the *tuf* gene revealed that all 36 grapevine samples were infected by the *tuf*-b type of stolbur phytoplasma (Table 4). Characterization of the other three genes enabled pathogen genetic diversity detection. Two genotypes were identified for each *secY* and *vmp1* gene, and four *stamp* genotypes were distinguished among the characterized isolates (Fig. 1; Table 4). A sequence comparison of the two *secY* genotypes with the reference strains resulted in 100% identity with either the STOL strain (GenBank acc. no. AM992086) or the GGY strain (AM992093). These genotypes differ from one another by only a single nucleotide. Two *vmp1* genotypes were designated as V14 and V2-TA according to the RFLP and sequence analyses. Both genotypes had the same TYPH10F/R nested PCR products of about 1450 bp (Fig. 2a), which generated distinguishable *Rsa*I RFLP patterns corresponding to V14 and V2 profiles (Fig. 2b,c). Isolates exhibiting a V14 *Rsa*I profile had a *vmp1* sequence (1302 characters) identical to the Mp46 reference strain (HM008606), which was previously identified as a V14 representative (Murolo *et al.*, 2010). Sequence comparison and virtual *Taq*I and *Alu*I digestion more accurately attributed the second profile to the V2-TA type. Isolates exhibiting a V2 *Rsa*I profile had a *vmp1* sequence identical to the STOL reference strain (AM992103). Because V2 has a profile associated with reference strain GGY (X. Foissac, INRA, Bordeaux-France, personal communication), the STOL genotype can be distinguished from the GGY V2 profile by *Taq*I and *Alu*I digestion (data not shown), and the corresponding RFLP profile is thereby designated as V2-TA.

The sequence variability between the resulting V14 and V2-TA genotypes was 6·5%.

Four genotypes were identified for the putative vector-dependent marker *stamp*, with a maximum variability of 2·6%. Sequence comparison and phylogenetic analysis with maximum parsimony revealed that three out of four *stamp* genotypes detected in grapevines (designated here as Rqg31, Rqg50 and Vv24; Table 4) are unique in comparison to the reference strains and are clustered within the *tuf*-b at cluster II (Fig. 1a). The fourth genotype was clustered within *tuf*-b at cluster III and had sequence characteristics identical to reference strain STOL (FN813261). A statistical parsimony network enabled visualization of additional *stamp* variations among detected genotypes represented by a 6 bp insertion at position 172 of the gene previously recorded for some of the *stamp tuf*-a strains and a single *tuf*-b strain named L646 and associated with *Lavandula angustifolia* in France (Fabre *et al.*, 2011). The *stamp* genotypes Rqg50 and Vv24 had this insertion and a single synonymous mutational difference between them, and the only difference between genotypes Rqg31 and Rqg50 was the above mentioned insertion. The qualitative nucleotide change characteristics in the new *stamp* genotypes were assessed by noting the abundance of non-synonymous mutations. Most of the mutational differences in these genotypes (nine out of 10) within *tuf*-b cluster II were non-synonymous by type inducing amino acid substitutions with respect to one another and reference strains (Fig. 1b). The ratio of non-synonymous over synonymous mutations (dN/dS) was calculated for all pairs (compared genotypes) used in the parsimony network analysis. The values of dN/dS for new *stamp* genotypes compared to the reference strains indicated positive selection pressure on *stamp* in Rqg31 and Rqg50 genotypes in comparison to L646 (dN/dS = 2·163, $P = 0{\cdot}033$) and NGA9 (dN/dS = 2·006, $P = 0{\cdot}047$), and in the Vv24 genotype in comparison to the new Rpm35 genotype associated with *R. panzeri* originating in maize fields (dN/dS = 2·205, $P = 0{\cdot}029$).

Overall, four stolbur phytoplasma genotypes were detected in the BN-affected field-collected grapevine plants according to the *tuf*/*secY*/*vmp1*/*stamp* typing as follows: (i) *tuf*-b/STOL/V2-TA/STOL, the most frequent genotype infecting 42% of analysed samples, hereafter referred to as the STOL genotype (STOLg); (ii) *tuf*-b/STOL/V14/Rqg50 infecting 31% of samples, hereafter referred to as the Vv5 genotype (Vv5g); (iii) *tuf*-b/GGY/V2-TA/Rqg31 infecting 19% of samples, hereafter referred to as the Rqg31 genotype (Rqg31g); and (iv) *tuf*-b/GGY/V14/Vv24 detected in 8% of samples, hereafter referred to as the Vv24 genotype (Vv24g) (Table 4).

Stolbur phytoplasma genotypes associated with *R. panzeri*

Out of the 50 stolbur phytoplasma isolates associated with *R. panzeri* originating from vineyards which were used in transmission trials (Table 2), 34 were subjected to multilocus typing (Table 4) following a predefined parameter to characterize the six isolates per trial replicate, whenever possible. For the isolates associated with *R. panzeri* from maize fields, 48 were subjected to characterization by following the same methodology.

Among the isolates from *R. panzeri* collected in the vineyards, two stolbur phytoplasma genotypes were identified according to the *tuf*/*secY*/*vmp1*/*stamp* typing. The first was the genotype most frequently found in grapevines, STOLg (tuf-b/STOL/V2-TA/STOL). This genotype was predominantly found in *R. panzeri* as well (85%; Fig. 1; Table 4). The second genotype *tuf*-b/STOL/V4/Rqg50 (hereafter referred to as Rqg50g) was found in five *R. panzeri* individuals originating from grapevine (15%) and its genotype was most similar to Vv5g found in grapevines. These two genotypes have the same *tuf* type, *secY* and *stamp* sequence characteristics, but differ in their *vmp1* profiles. All Vv5g isolates had a V14 *vmp1*

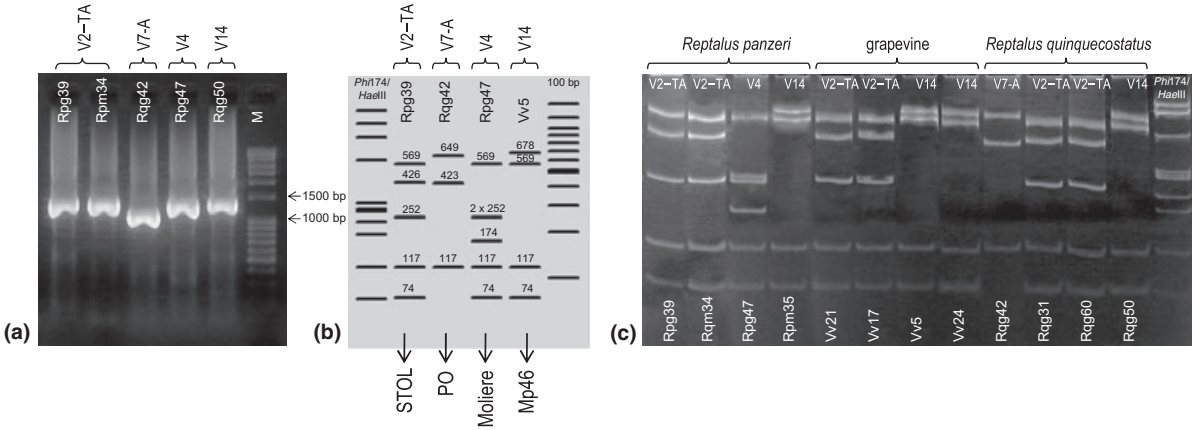

Figure 2 Variability of *vmp1* amplicon lengths and *Rsa*I RFLP profiles. (a) Amplicon sizes obtained with primers StolH10F1/R1 and TYPH10F/R; M, 100 bp DNA marker (Serva); (b) virtual *Rsa*I RFLP profiles of representative strains detected in this study and corresponding reference strains indicated with arrows; (c) actual *Rsa*I digestion profiles of representative isolates associated with different hosts; ϕ174/*Hae*III, DNA marker (Fermentas).

profile, while members of Rqg50g had a V4 restriction pattern (Fig. 2b,c). The *vmp1* gene sequence of isolates exhibiting a V4 profile was identical to the sequence of reference isolate B51 (HM008600), which was previously identified as a representative of the V4 type profile (Murolo *et al.*, 2010).

Stolbur isolates associated with *R. panzeri* from maize fields all belonged to the genotype STOLg with the exception of one (Table 4). The second genotype *tuf*-b/STOL/V2-TA/Rpm35 (Rpm35g) was found in a single *R. panzeri* specimen and differed from STOLg only in its *stamp* sequence. The Rpm35 *stamp* sequence was most closely related to the P7 and STOL reference strains (Fig. 1) and differed at eight non-synonymous mutations when comparing both strains and dN/dS values, indicating a positive selection underlying diversification (the P7 strain dN/dS = 3·106, $P = 0{\cdot}002$; the STOL dN/dS = 2·768, $P = 0{\cdot}007$).

Stolbur phytoplasma genotypes associated with *R. quinquecostatus*

Among 600 *R. quinquecostatus* individuals originating from the BN-affected vineyards used in the transmission trials, 91 were found to be stolbur positive (Table 3). Sixty-four of these isolates where subjected to phytoplasma genotyping. According to the *tuf*/*secY*/*vmp1*/*stamp* typing, the following four stolbur phytoplasma genotypes were detected among *R. quinquecostatus* specimens: (i) STOLg was identified in 22 specimens (32%); (ii) Rqg50g was identified in 12 specimens (18%); (iii) Rqg31g was identified in 17 specimens (25%); and (iv) a new genotype, *tuf*-b/BG4560/V7-A/BG4560 (BG4560g), was detected solely in *R. quinquecostatus* and was present in 17 specimens (25%; Table 4). BG4560g had *secY* and *stamp* marker sequence characteristics identical to the BG4560 reference strain (FN813271, FN813252) originating from grapevines in Bulgaria and associated with the *tuf*-b RFLP profile, as previously noted for this strain (Fabre *et al.*, 2011). A BG4560g *vmp1* marker exhibited a different fragment size for TYPH10F/R nested PCR products of about 1200 bp (Fig. 2a) which generated an *Rsa*I profile corresponding to the V7 (Fig. 2b,c) associated with the PO reference strain (X. Foissac, INRA, Bordeaux, France, personal communication). However, the *vmp1* sequence characteristics were most similar to the STOL strain and showed substantial genetic divergence from the PO strain. A virtual digestion using *Alu*I enabled the recognition of a new *vmp1* profile associated with BG4560g (data not shown), which is designated here as V7-A profile (Table 4).

Stolbur phytoplasma genotype transmitted by *R. panzeri* to grapevines

In two transmission experiments using stolbur-infected *R. panzeri* individuals originating from vineyards and maize fields, six and eight grapevine seedlings were successfully inoculated and expressed symptoms typically associated with BN. All 14 isolates were subjected to multilocus typing in order to determine the stolbur phytoplasma genotype transmitted by *R. panzeri*.

A single genotype was found in all experimental plants, namely STOLg (Table 4). The majority of genotypes identified among field-collected grapevines (42%) and *R. panzeri* originating from the studied vineyards (85%) also corresponded to STOLg, confirming its vectorship of the disease.

Discussion

The epidemiology of vector-borne plant pathogens is directly dependent on the vector species and its life strategy such as its hibernation stage, host specificity, feeding preferences and dispersal capacity (Johannesen *et al.*, 2012 and references therein). However, the fundamental starting point in resolving the epidemiological cycle of any vector-borne disease is an assertive identification of the vector species. For more than 60 years, *H. obsoletus* was first considered a tentative source and was later considered the sole vector of stolbur phytoplasma-induced BN disease in grapevines (Fos *et al.*, 1992 and references therein; Maixner, 1994). Although other planthopper and leafhopper species were considered alternative or main vectors in some parts of the BN-affected grape growing areas of Europe during the last decade (Palermo *et al.*, 2004; Trivellone *et al.*, 2005; Pinzauti *et al.*, 2008; Riedle-Bauer *et al.*, 2008; Cvrković *et al.*, 2011), no experimental confirmations were obtained for any of the species under consideration. In light of a general effort to determine the other hemipteran vector species of BN, the discovery of another cixiid, *R. panzeri*, which naturally transmits stolbur phytoplasma to grapevines in the vineyards of southeastern Europe, is changing the understanding of this disease on a large scale.

Laboratory experiments performed with naturally infected *R. panzeri* adults collected in either BN-infected vineyards or MR-affected maize fields have revealed a high stolbur phytoplasma transmission efficiency by this cixiid to grapevines. In all cases in which infected *R. panzeri* specimens were feeding on experimental plants, they transmitted the phytoplasma (Table 2), and the plants expressed BN symptoms. Although *R. panzeri* was previously proven to be a major vector of stolbur-induced maize redness disease of maize in Serbia (Jović *et al.*, 2007, 2009), a newfound role of this vector in transmission and epidemiology of BN grapevine disease identified this species as a possible vector to other crops as well. The biology, host plant associations and feeding preferences of this cixiid are not well documented, except for its presence in the maize fields of the South Banat district in Serbia (Jović *et al.*, 2009). According to Holzinger *et al.* (2003), *R. panzeri* usually lives on the xerothermic waysides, sunny hillsides or on plateaus up to 500 m elevation. The adults are polyphagous in the shrub and herb layer and the nymphs are subterranean feeders, as are all cixiids. The geographic range of this species is assumed to be restricted to the southern parts

of central Europe, southeastern Europe, the Mediterranean region, Asia Minor and the Caucasus. It is worth noting that in these areas, the increased incidence or emergence of BN induced by stolbur *tuf*-b type phytoplasma was reported in recent years (Riedle-Bauer *et al.*, 2008; Radonjić *et al.*, 2009; Cvrković, 2010).

In addition to the experimental transmission, another significant part of this study included a multilocus typing of the stolbur phytoplasma genotypes, which was performed to determine the vector characteristics of BN epidemics in the South Banat district of Serbia. Overall, seven genotypes in the stolbur phytoplasma were detected among the grapevine- and insect-associated field-collected samples on the basis of *tuf*/*secY*/*vmp1*/*stamp* typing. Four genotypes were found to be associated with grapevines, of which two were solely restricted to grapevines (genotypes Vv5g and Vv24g), with one genotype present in both *R. panzeri* and *R. quinquecostatus* (STOLg), the two most abundant species recorded in studied vineyards, and one found only in *R. quinquecostatus* as a possible vector (Rqg31g). The stolbur phytoplasma genotype STOLg was the most prevalent in both planthoppers and grapevines, and the same genotype was found in all plants experimentally infected by *R. panzeri*. Throughout the literature, this genotype is represented as the historical reference strain of the stolbur phytoplasma originating from pepper in Serbia (e.g. Quaglino *et al.*, 2013) and is associated with isolates from southeastern Europe (e.g. Fabre *et al.*, 2011).

Unlike the *R. panzeri*, which was predominantly carrying (and successfully transmitting) STOLg, *R. quinquecostatus* was found to harbour four diverse stolbur phytoplasma genotypes in more or less equal proportions. However, none of these genotypes were transmitted by the *R. quinquecostatus* to grapevine seedlings in the transmission trials. Although it could be assumed that *R. quinquecostatus* could also play an important role as a stolbur phytoplasma vector to grapevines in BN-affected vineyards, given the abundance of the tentative vector, an infection rate (15%) and corresponding pathogen genotypes originating from naturally affected plants and insects, on the basis of transmission experiments it was concluded that this is not the case. *Reptalus quinquecostatus* was previously considered to be a possible stolbur phytoplasma vector (Trivellone *et al.*, 2005) and was later found to be capable of inoculating the pathogen to artificial feeding medium (Pinzauti *et al.*, 2008), further suggesting its vector capability. However, the second study referred to the *tuf*-a stolbur phytoplasma type, and thus parallels cannot be made with the findings here, which indicate the inability of this planthopper to transmit *tuf*-b type disease to grapevines. Additionally, the possibility that the *R. quinquecostatus* specimens used in the experiments performed here could transmit phytoplasma to an artificial medium or some plant species other than grapevine cannot be ruled out, because the ability of the insect to inoculate plants with phytoplasma depends on complex interactions and coevolutionary adaptations between the vector, pathogen and host plant (Hogenhout *et al.*, 2008).

It can be assumed that different polyphagous vectors involved in the diverse transmission cycles of stolbur phytoplasma could share some of the same host plants and the same stolbur phytoplasma genotype. This assumption would explain the presence of the same genotype STOLg in both *R. panzeri* and *R. quinquecostatus*. However, the ability of the vector to acquire and carry the phytoplasma strain does not imply its ability to transmit the strain. This caveat is especially true when considering an insect's capability to transmit phytoplasma to diverse plant species. The single stolbur phytoplasma genotype Rqg31g was found in 19% of naturally BN-affected grapevines and 25% of the stolbur-positive *R. quinquecostatus* originating from these vineyards, suggesting a role for this cixiid in spreading and increasing the incidence of this pathogen genotype. Because *R. quinquecostatus* was unable to transmit Rqg31g to grapevines under experimental conditions, one can only speculate that it could be a vector of this genotype to some of the weedy plants surrounding the vineyards. If so, *R. quinquecostatus* could act as a vector in an intermediate epidemiological cycle, leading to an increased pathogen incidence in the environment of the vineyard ecosystem and reservoir-plants for subsequent acquisition by some other undetermined vector that is able to transmit the Rqg31g to grapevines. The same could be supposed for BG4560g, a genotype previously detected in BN-affected grapevine from Bulgaria (Fabre *et al.*, 2011), and associated solely with *R. quinquecostatus* in the present study. However, these assumptions require additional comprehensive studies involving detailed genotyping of stolbur phytoplasma strains infecting weedy plants in the areas adjacent to the vineyards.

The diversity of the detected stolbur phytoplasma genotypes in both plant and insect material was highest in the membrane protein genes in comparison to the housekeeping genes. This finding is in agreement with a previous assumption that phytoplasma genes encoding surface proteins, such as *stamp* and *vmp1*, could be involved in the interaction with insect vectors and that they consequently evolve more rapidly than the rest of the genome (Cimerman *et al.*, 2009; Fabre *et al.*, 2011). The isolates genotyped in this study were not diverse in relation to geographic origin, but according to the hosts. Consequently, it was found that the new *stamp* genotypes associated with either *R. panzeri* or *R. quinquecostatus* (Rpm35 and Rqg31, respectively) or both species (Rqg50) are under positive selection pressure, confirming previous results on the variability of *stamp* (Fabre *et al.*, 2011). It remains unclear why the stolbur phytoplasma genotypes sharing the same *stamp* genotype Rqg50 have different *vmp1* profiles when present in the two planthoppers (V4, comprehensive genotype Rqg50g) and naturally infected grapevines (V14, Vv5g). It could be assumed that the *vmp1* gene is involved in adapting to the plant host as well as the insect host, which could lead to the different *vmp1* profiles observed in the putative vectors and host plants.

For *R. panzeri*, it can be concluded that *stamp* genotype STOL of *tuf*-b cluster III is the most common genotype infecting this cixiid and is efficiently transmitted by the insect. Nevertheless, this genotype is also found in *R. quinquecostatus*, so no congruent conclusion could be made about the strict association of a single *stamp* genotype or overall *tuf/secY/vmp1/stamp* genotype of stolbur phytoplasma with specific insect species.

For the epidemiology and emergence of a *tuf*-b type of stolbur phytoplasma-inducing BN disease of grapevine, the new discovery of the *R. panzeri* vector role is an essential contribution with far-reaching consequences for disease management. This development raises new questions related to the host plants and plant reservoirs, and it underlines the need to determine all the details of the BN epidemiological cycle as vectored by *R. panzeri*. In general, phytoplasma transmission by cixiids relies on phytoplasma acquisition by nymphs, because of the short life span of cixiid adults. This short life span disables the acquisition, multiplication and transmission of phytoplasma by the adult life stage. In maize fields, *R. panzeri* early instar nymphs and adults preferably feed on maize, but overwintering nymphs feed on the roots of the winter wheat because of maize–wheat crop rotation as well as on Johnsongrass (*Sorghum halepense*), which is the most common weed in maize crops (Jović *et al.*, 2009). All three plants are proven phytoplasma sources for *R. panzeri* nymphs. Given these findings, future epidemiological studies should determine the host plants of *R. panzeri* nymphs in vineyard ecosystems as well as the source plants of stolbur phytoplasma. This study should be more easily performed following the precise characterization of the transmitted strain from the present study.

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