Characterization of Bois Noir Isolates by Restriction Fragment Length Polymorphism of a Stolbur-Specific Putative Membrane Protein Gene

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ABSTRACT

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Bois noir phytoplasma (BNp), widespread in wine-producing areas of Europe and endemic in France and Italy, is classified in the 16SrXII-A subgroup, whose members are referred to as Stolbur phytoplasmas. The 16S rDNA gene of Stolbur phytoplasma shows low variability, and few non-ribosomal genes are available as markers to assess variation among isolates. We used the Stolbur-specific *stol-1H10* gene, encoding a putative membrane-exposed protein, to investigate genetic diversity of French and Italian BNp isolates from plants and insects. Amplification of *stol-1H10* from infected grapevines, weeds, and *Hyalesthes obsoletus* produced fragments of three sizes, and restriction fragment length polymorphism analysis divided these amplicons further into 12 profiles (V1 to V12). French BNp isolates were more variable than Italian ones, and different profiles were present in infected grapevines from France and Italy. Isolate V3, most abundant among Italian affected grapes but present among French ones, was found in one *Urtica dioica* sample and in all *H. obsoletus* collected on this species. Four Italian-specific profiles were represented among infected *Convolvulus arvensis*, the most frequent of which (V12) was also detected in *H. obsoletus* collected on this species. Most of the variability in the *stol-1H10* sequence was associated with type II on the *tuf* gene.

Phytoplasmas in the 16SrXII-A taxonomic subgroup (suggested name '*Candidatus* Phytoplasma solani') are associated with Stolbur disease in a wide range of wild and cultivated plants in continental and Mediterranean Europe, causing severe losses of annual crops (16). Important perennials also suffer from Stolbur phytoplasma diseases such as grapevine yellows (GY) Vergilbungskrankheit (VK) in Germany (13), also known as Bois noir (BN) in most European wine-producing Regions (2). BN has been present in Italian and French vineyards for a long time but there are recent reports of increased spread of the disease in Italy (1,9,22,23).

In the Euro-Mediterranean Region, phloem-feeding Hemiptera of the Cixiidae family are the vectors of Stolbur phytoplasmas (29). The planthopper *Hyalesthes obsoletus* is responsible for BN transmission to grapevine (26). Adult *H. obsoletus* can feed on various herbaceous plants but only a few species are selected to complete its life cycle. In Germany, *Convolvulus arvensis*, *Calystegia sepium*, and *Urtica dioica* are reported as preferred hosts for reproduction (8) whereas, in Italy, *H. obsoletus* nymphs have so far been found only on *U. dioica* (12).

Restriction fragment length polymorphism (RFLP) and sequence analyses of the 16SrDNA show low variability among Stolbur isolates from different regions and host plants (16). However, biological and genomic diversity exist. Periwinkle plants, experimentally infected with different isolates, can show different symptoms (20), and variation in genome size has also been reported (15). Based on polymerase chain reaction (PCR)-RFLP analysis of the *tuf* gene, three *tuf* types (I–III) have recently been characterized in Germany in infected grapevines as well as in the vector *H. obsoletus* and in weeds. Each type was specifically associated with different herbaceous species and vector populations, suggesting the presence of three natural cycles of VK phytoplasma in German vineyards (8).

Analysis of the *tuf* gene has been successfully adopted to investigate the epidemiology of BN phytoplasma (BNp) in Italian vineyards. Preliminary results showed the presence of two BNp isolates corresponding to *tuf* types I and II (1,9,22). *Tuf* I was also found in vectors collected on *U. dioica* in vineyards where this is the predominant weed (3,12).

Recently, a new approach based on molecular subtraction hybridization was used to obtain enriched genomic libraries of the French Stolbur isolate PO (4). The *stol-1H10* gene, encoding a putative membrane protein absent in '*Ca.* P. asteris' but sharing low similarity with a variable surface lipoprotein of *Mycoplasma agalactiae*, was used as genetic marker to assess the variability of different periwinkle-maintained Stolbur isolates, using PCR-RFLP (20). In the present work, we used this gene as a marker to investigate the genetic diversity of French and Italian BNp isolates from plants and insects.

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MATERIALS AND METHODS

Plant and insect samples. In 2004, 2005, and 2006, 28, 43, and 60 leaf samples, respectively, were collected from BN-infected grapevines of several cultivars in seven Italian traditional grapevine-growing regions. We also included in our survey 111 BN isolates from grapevines collected in 2004 from vineyards in 10 different areas in France (Table 1). Herbaceous weeds were also collected in BN-infected vineyards in northwestern Italy (Piemonte and Valle d'Aosta) during summer 2005 and 2006. In each vineyard, either *C. arvensis* or *U. dioica* were the predominant weeds. Eleven *C. arvensis* samples were collected from plants showing stunting and strong leaf reduction in six Piemonte vineyards in 2005 and 2006. Yellows and stunting were also observed on four *U. dioica* plants collected in two BN-infected vineyards in Piemonte in 2005 and one sample in Valle d'Aosta in 2006.

In all, 30, 55, and 17 adult *H. obsoletus* individuals were collected in a vineyard in Lazio and in several vineyards in Valle d'Aosta and Piemonte, respectively, during June to July 2006. All insects were collected from nettle, except three adults taken on bindweed in one vineyard in Piemonte.

A Sardinian strain of Stolbur from tomato (T2_92) maintained in periwinkle in the collection of the Istituto di Virologia Vegetale, CNR (19) was used as reference isolate and positive control in PCR experiments.

Total DNA extraction and phytoplasma identification. Total DNA was extracted from 1.5 g of fresh grapevine and *U. dioica* midribs and from leaves, stems, and petioles of symptomatic *C. arvensis* plants following a modified cetyltrimethylammonium bromide protocol (17). The DNA was finally suspended in 100 µl of sterile distilled water (SDW).

Total DNA was extracted from single *H. obsoletus* individuals, fresh or stored under 70% ethanol at 4°C using the method of Marzachì et al. (18). The purified DNA was suspended in 100 µl of SDW.

To confirm the presence of BNp in single infection, 2 µl of each DNA extract was used in PCR assays with the universal ribosomal primers P1/P7 (25). Of the 1:40 diluted products of the first PCR, 2 µl was used as template for nested PCRs with the group-specific ribosomal primers R16(I)F1/R1 and R16(V)F1/R1 (11). PCR mixtures and cycling conditions were as described in the original articles. Taq DNA polymerase (1 U) (Polymed, Firenze, Italy) was used in each assay. PCR products were analyzed by electrophoresis through 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer along with a 1-kb-plus DNA size marker (Gibco BRL, Paislay, UK). Gels were stained with ethidium bromide and visualized on a UV transilluminator. To confirm each isolate as a member of the 16SrXII-A subgroup, nested R16(I)F1/R1 PCR amplicons were digested with 2.5 U of *Mse*I (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The restriction fragments, together with the 1-kb-plus DNA size marker (Gibco BRL), were separated by 8% polyacrylamide gel electrophoresis in 1× TBE buffer and stained and visualized as before.

TABLE 1. Geographic origin of Bois noir-affected grapevines

Country of origin, region	Number of isolates
France	
Alsace	17
Aquitaine	27
Bourgogne	16
Centre	3
Languedoc-Roussillon	10
Midi-Pyrénées	7
Pays de la Loire	5
Poitou-Charente	10
Provence-Alpes-Côte d'Azur	7
Rhône-Alpes	9
Italy	
Lazio	13
Liguria	1
Piemonte	75
Sardegna	1
Sicilia	8
Toscana	13
Valle d'Aosta	20

Characterization of BN isolates. Following identification, BN isolates were characterized by PCR-RFLP analysis of two non-ribosomal genomic regions: *tuf* (8) and *stol-1H10*. Primers fTufAY/rTufAY (24) were used to amplify a 940-bp fragment of the *tuf* gene from Italian BN isolates. Of the 1:40 diluted products of the first PCR, 2 µl was used as template for nested PCRs driven by primers fTufu/rTufu (24), using 1 U of Taq DNA polymerase (Polymed). The fTufu/rTufu amplicons (850 bp) were digested with 2.5 U of endonuclease *Hpa*II (Invitrogen) according to manufacturer's instructions.

Stolbur-specific primers H10F1 (5′AGGTTGTAAAATCTTTTATGT3′) and H10R1 (5′GCGGATGGCTTTTCATTATTTGAC3′) overlapping the start and stop codons, respectively, of the *stol-1H10* gene were used to amplify the complete *stol-1H10* gene. PCRs were performed in a final mixture of 40 µl containing 10 to 100 ng of total DNA, 0.5 µM each primer, 200 µM dNTPs, 2 mM $MgCl_2$, and 1 U of Taq polymerase (Polymed) in the buffer supplied. Cycling conditions were as follows: denaturation for 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min 45 s at 72°C; and a post-dwell period of 10 min at 72°C. The first PCR was followed by a nested PCR with internal primers H10F2 (5′TGTCACAGGGAAACAGACAG3′) and H10R2 (5′CACAAACATGATGATTATCAACGA3′). These reactions were carried out in the same conditions as the first PCR using 0.5 µl of the first PCR product as template. The products of the nested PCR (2 to 4 µl) were singly digested with 2.5 U of *Rsa*I or *Alu*I restriction enzymes (Invitrogen) according to the manufacturer's instructions. PCR products were analyzed by electrophoresis through 1% agarose gel, then stained and visualized as above. Restriction fragments were always separated by electrophoresis on 8% polyacrylamide gels in 1× TBE buffer and visualized as detailed above, and 1-kb-plus DNA (Gibco BRL) was used as size marker.

RESULTS

BN detection. Stolbur-specific RFLP profiles were identified on the 16S rDNA amplicons of all the symptomatic Italian grapevine samples, 10 of 11 *C. arvensis* samples, 1 of 5 *U. dioica*, and 65 of 102 *H. obsoletus* individuals (not shown). No Flavescence dorée-specific amplicons were detected following amplification of DNA from the infected grapevines with R16(V)F1/R1 primers (11). No amplification was obtained from healthy grapevines or symptomless weeds.

Diversity of the *tuf* gene in Italian BN isolates from grapevine, weeds, and *H. obsoletus*. Fragments of the expected size (840 bp) were amplified in nested PCR assays from all Stolbur-affected Italian grapevines, 9 *C. arvensis*, 1 *U. dioica*, and 51 *H. obsoletus* individuals. No amplification was obtained from healthy grapevines or symptomless *C. arvensis* and *U. dioica*. RFLP analysis showed the presence of *tuf* type I and II (8) in BN-infected grapevines from Piemonte (52 and 48%, respectively) and Valle d'Aosta (Table 2). Only *tuf*II was detected in BN-infected grapevines from Sicilia, Sardegna, and Toscana, whereas only *tuf*I was found in BN-infected grapevines from Lazio and Liguria.

*Tuf*I was found in *U. dioica* in Valle d'Aosta whereas only *tuf*II was detected in *C. arvensis* in Piemonte. *H. obsoletus* from nettle in Piemonte and Valle d'Aosta harbored only *tuf*I, whereas *H. obsoletus* collected on *C. arvensis* in Piemonte contained *tuf*II. Only *tuf*I was present in insects from Lazio (Table 2).

Diversity of the *stol-1H10* gene in BN isolates from grapevine. Nested PCR with primers H10F2/H10R2 amplified specific bands from 48 of 111 French BN isolates and 102 of 131 Italian BN isolates. No amplification was obtained from healthy grapevines. Amplicons of 1,570 bp were obtained from 3 French grapevine isolates whereas the remaining 45 produced a specific band of 1,820 bp. In all, 1 Italian isolate generated a PCR product of 1,570 bp and 80 isolates produced bands of 1,820 bp, whereas a larger amplicon (2,070 bp) was obtained from the remaining 21 samples collected in Piemonte and Toscana (northwestern and central Italy) (Fig. 1).

BN-specific amplicons restricted with *Rsa*I showed 11 distinct patterns (V types), ranging from V1 to V11 (Fig. 2A and B). Four different V types (V5, V6, V7, and V8) were obtained by digestion of the 1,570-bp band and seven types (V1, V2, V3, V4, V9, V10, and V11) from the 1,820-bp band. The *Rsa*I profile of the 2,070-bp band produced a pattern similar to type V11 due to poor resolution of larger DNA fragments but restriction with *Alu*I resulted in a new V12 profile (Fig. 2C). V3 and V5 profiles were unique to *tuf* type I isolates, V1 was present in both *tuf* types I and II isolates, whereas the remaining V profiles isolated from Italian grapevines were associated with *tuf* type II isolates.

TABLE 2. Distribution of *tuf* type I and II in Bois noir-infected grapevines and *Hyalesthes obsoletus* from different Italian Regions

	Grapevine		*H. obsoletus*	
Origin	*tuf*I	*tuf*II	*tuf*I	*tuf*II
Piemonte	39	36	9	3
Lazio	13	0	8	0
Liguria	1	0	…	…
Sardegna	0	1	…	…
Sicilia	0	8	…	…
Toscana	0	14	…	…
Valle d'Aosta	17	2	31	0
Total	70	61	48	3

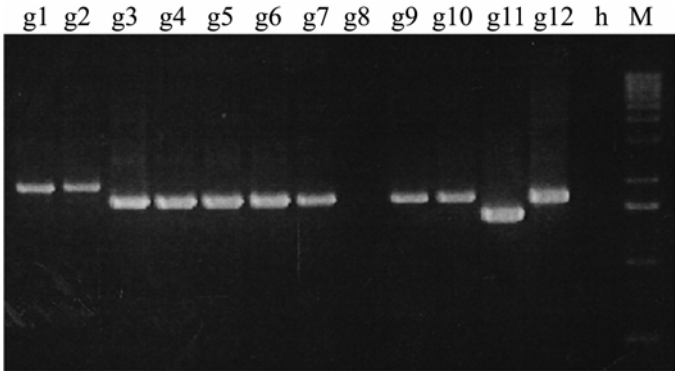

Fig. 1. Agarose gel showing the size polymorphism of polymerase chain reaction products obtained with primers H10F1/R1 followed by primers H10F2/R2 from grapevine (g) Bois noir isolates. M, 1-kb-plus DNA size marker (Gibco BRL, Paisley, UK); g1–g12, grapevine Bois noir isolates from Piemonte; h, healthy grapevine DNA control.

Seven of the RFLP V types (V1, V2, V3, V4, V6, V7, and V8) were detected in French grapevine BN isolates. V1 was the most abundant (39.5%) and V4 was detected in samples from almost all French regions. In all, 10 and 12% of the profiles were V2 and V3 types. V6, V7, and V8 patterns were detected in a few samples from three French regions (Table 3).

Nine V types (V1, V2, V3, V4, V5, V9, V10, V11, and V12) were obtained from Italian grapevine isolates. V5 was only detected in one isolate from Piemonte. V1, V2, and V4 were detected in ≈5% of the samples analyzed. V3 was the most abundant in northwestern regions and Lazio, representing ≈64% of all Italian isolates, whereas it was not detected in isolates from Toscana, Sicilia, or Sardegna. Types V9 to V12 were only detected in Italian isolates; V9 type was present in northwestern regions and in Sicilia, V10 was present only in few grapevines from Piemonte, whereas V11 was present in Sardegna. V12 type was detected in ≈29% of the isolates from Piemonte and in four of five isolates from Toscana (Table 3).

Diversity of the *stol-1H10* gene in BN isolates from weeds and vectors. Specific bands were obtained in nested PCR with primers H10F2/H10R2 from the *U. dioica* sample, 8 *C. arvensis* samples, and 45 *H. obsoletus* individuals. Middle-sized PCR amplicons (1,820 bp) were obtained from the infected nettle, 4 *C. arvensis*, and 42 *H. obsoletus* captured on *U. dioica*. Large amplicons (2,070 bp) were only obtained from four *C. arvensis* isolates from Piemonte and three *H. obsoletus* collected on *C. arvensis* from the same area (not shown). BN-infected *U. dioica* sampled in Valle d'Aosta and 98% of insects collected on *U. dioica* were characterized as V3 (Table 4) and only one individual captured on nettle was characterized as V1. Four *C. arvensis* BN isolates were characterized as V9, 10, or 11 and four were V12 (Table 4). BN isolates from vectors collected on *C. arvensis* were all of type V12 (Table 4).

DISCUSSION

RFLP of 16S ribosomal RNA genes of grapevine BN isolates collected in Italy confirmed our isolates as members of the 16SrXII-A taxonomic subgroup but, as expected, did not allow differentiation among isolates of different years or differ-

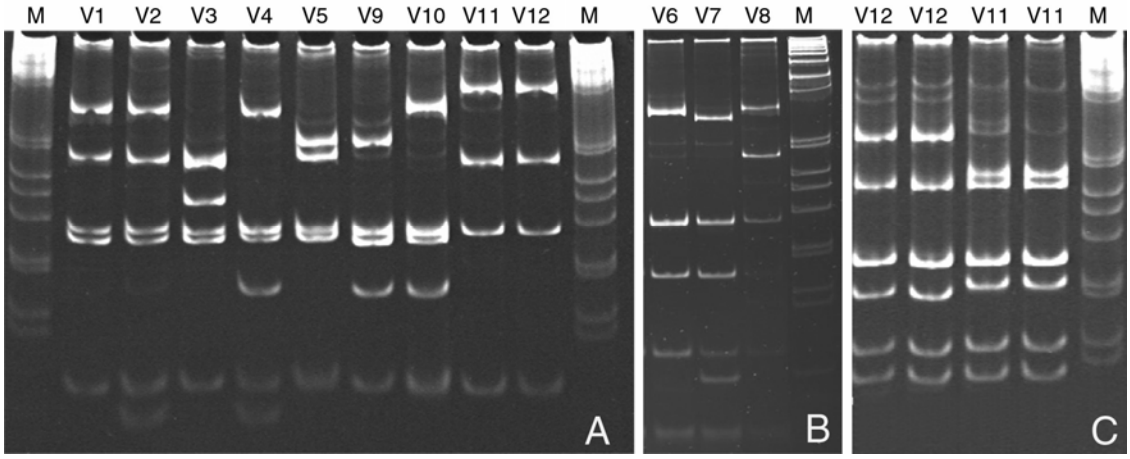

Fig. 2. A and B, *Rsa*I or **C,** *Alu*I restriction profiles obtained following digestion of *stol-1H10* specific nested- polymerase chain reaction amplicons of Bois noir isolates (V1–V12).

ent geographic origins, due to low variability on the 16SrDNA region.

Diversity in the *tuf* gene has been successfully used in Germany for differentiation of phytoplasmas associated with grapevine VK (8). In Piemonte and Valle d'Aosta, we detected both *tuf*I and *tuf*II types, present in affected grapevines and *H. obsoletus* individuals, but associated with nettle or bindweed, respectively. The low number of sampling sites as well as plant or vector samples collected are not representative of the BN situation in central and southern Italy, although our results confirm the prevalence of *tuf* type I in BN-affected grapevines and vectors from Lazio (22). *Tuf* type III, detected in Germany in BN-affected grapevines, in *H. obsoletus*, and in its herbaceous host *Calystegia sepium* (8), was not found in our survey of Italian BN isolates.

Our results show that the *stol-1H10* gene is variable enough to study genetic diversity among BN isolates. We have shown that amplification of the *stol-1H10* gene from field-collected BN-infected grapevines, weeds, and *H. obsoletus* individuals may result in amplicons of three sizes. The largest amplicon was detected only in Italian BN isolates from grapevine and *C. arvensis* and *H. obsoletus* captured on this species, while the medium-size amplicon was the most abundant among isolates collected in Italy and France. The lack of amplification of the *stol-1H10* gene from ≈20% of Italian BN-affected plant samples may be due to lower efficiency of the *stol-1H10*-based primers compared with the ribosomal-based ones, although we cannot exclude sequence mismatches in the template DNA. The negative PCR results obtained from some French grapevines is probably also due to the quality of template DNA tested almost 2 years after extraction and first identification of BN infection.

RFLP analysis of *stol-1H10* amplicons from BN-infected grapevines, weeds, and vectors resulted in 12 profiles, 5 of which (V3, V5, V9, V10, and V12) were not previously described, while the remaining ones were indistinguishable from those of periwinkle-maintained Stolbur isolates from horticultural and other hosts (20). The same analysis showed that different *stol-1H10* profiles were prevalent in France and Italy. Actually, five RFLP patterns were found only in Italy while three were detected among French grapevine BN isolates but were absent in the Italian ones, regardless of the host. Two profiles accounted for >70% of the BN diversity: V3 and V12 in Italy and V1 and V4 in France. Although the number of characterized BN isolates from France was lower than the Italian ones, we have shown that the most representative isolates in Italy and France are different. Our results, therefore, do not support a major role of import of infected material in spreading the disease between the two countries.

RFLP analysis of *stol-1H10* amplicons indicated that four profiles were represented among Stolbur-infected *C. arvensis*, the most frequent of which (V12) was also detected in the *H. obsoletus* individuals collected on this species. We also found that severe growth reduction was clearly associated with BN infection in *C. arvensis*. RFLP analysis of *stol-1H10* amplicons also showed that the V3 profile, the most abundant among Italian BN-affected vines and also present among French isolates, was detected in the majority of *H. obsoletus* collected on *U. dioica* and in the only Stolbur-positive sample of this plant. *U. dioica* hosts high populations of *H. obsoletus* in Northern Italy (3,12) but the percentage of Stolbur-infected plants that we found in our survey was very low. The only Stolbur-infected *U. dioica* plant showed no obvious symptoms. Asymptomatic phytoplasma infections have been reported for several species (10). In these cases, identification of infected plants is difficult; moreover, phytoplasma detection in *U. dioica* is erratic even when DNA enrich-

TABLE 3. Distribution of *stol-1H10* restriction fragment length polymorphism (RFLP) profiles (V) in grapevines from France and Italy

	RFLP type											
Country, region[a]	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
France												
Alsace	2	4	2	2	…	…	…	…	…	…	…	…
Aquitaine	2	…	1	2	…	…	…	…	…	…	…	…
Bourgogne	6	…	…	…	…	…	…	1	…	…	…	…
Centre	…	…	1	2	…	…	…	…	…	…	…	…
Languedoc-Roussilion	…	…	…	1	…	…	1	…	…	…	…	…
Midi-Pyrénées	…	…	2	1	…	1	…	…	…	…	…	…
Pays de la Loire	3	…	…	…	…	…	…	…	…	…	…	…
Poitou-Charente	3	1	…	3	…	…	…	…	…	…	…	…
Provence-Alpes-Côte d'Azur	…	…	…	2	…	…	…	…	…	…	…	…
Rhône-Alpes	3	…	…	2	…	…	…	…	…	…	…	…
Total	19	5	6	15	…	1	1	1	…	…	…	…
Italy												
Lazio	…	…	11	…	…	…	…	…	…	…	…	…
Liguria	…	…	1	…	…	…	…	…	…	…	…	…
Piemonte	…	…	38	…	1	…	…	…	1	2	…	17
Sardegna	…	…	…	…	…	…	…	…	…	…	1	…
Sicilia	…	1	…	2	…	…	…	…	4	…	…	…
Toscana	1	…	…	…	…	…	…	…	…	…	…	4
Valle d'Aosta	1	…	15	…	…	…	…	…	2	…	…	…
Total	2	1	65	2	1	…	…	…	7	2	1	21

[a] Origin of isolate.

TABLE 4. Distribution of *stol-1H10* restriction fragment length polymorphism (RFLP) profiles (V) in *Convolvulus arvensis*, *Urtica dioica*, and *Hyalesthes obsoletus* individuals sampled in different Italian regions

		RFLP type											
Species	Origin	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
C. arvensis	Piemonte	…	…	…	…	…	…	…	…	1	2	1	4
U. dioica	Valle d'Aosta	…	…	1	…	…	…	…	…	…	…	…	…
H. obsoletus	Lazio	…	…	8	…	…	…	…	…	…	…	…	…
H. obsoletus	Piemonte	…	…	6	…	…	…	…	…	…	…	…	3
H. obsoletus	Valle d'Aosta	1	…	27	…	…	…	…	…	…	…	…	…

ment protocols are used (8). All these reasons may explain the low percentage of infected nettles collected in our surveys. The small number of infected plants collected in Sicilia, Sardegna, and Toscana may explain the absence of V3 profiles among grapevine BN isolates from these regions.

We confirm the presence of the *U. dioica–H. obsoletus–Vitis vinifera* epidemiological cycle in northwestern Italy (12) but present strong evidence of the importance of at least a second cycle, involving *C. arvensis–H. obsoletus–V. vinifera*. Stolbur phytoplasma has been detected in individuals of several Cixiidae (21,27) and the role of vectors has been confirmed for some of them (7,29). Moreover, other leafhoppers have been reported as natural hosts of Stolbur phytoplasma in Sardegna (5).

Gene *stol-1H10* represents a genetic marker more variable than other non-ribosomal loci, because at least three *stol-1H10* RFLP profiles are associated with a single profile on the *tuf* gene. Most of the variability on the *stol-1H10* sequence is associated with the *tuf*II profile, which has been reported in South Tyrol (Northern Italy) only since 2004 (1). This BN isolate, linked to the *C. arvensis–H. obsoletus–V. vinifera* cycle, was the most abundant and endemic isolate in Germany (28) until the recent outbreaks of the *tuf*I-type BN isolate. These outbreaks have been linked to the concurrent spread of nettle, harboring this *tuf*I-type isolate, and vector population adapted to this weed (14), although only subtle differences were found between *H. obsoletus* host plant populations (6).

Although more vector individuals as well as weeds need to be tested, only two *stol-1H10* RFLP profiles were present in both a plant host (grapevine and alternative host plant) and *H. obsoletus* tested in Italy. The epidemiological cycles of the remaining isolates are yet to be defined.

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