Detection of Multiple Phytoplasmas in Perennial Fruit Trees with Decline Symptoms in Italy

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ABSTRACT

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Nested polymerase chain reaction assays with two universal and four phytoplasma (formerly called mycoplasmalike organism) 16S rRNA group-specific primer pairs were employed to investigate etiologies of diseases associated with pear (decline), plum (leptonecrosis), nectarine (chlorotic leaf roll), and apricot (chlorotic leaf roll and decline) fruit crops grown in northern Italy. Restriction fragment length polymorphism analyses of phytoplasma 16S rDNA sequences amplified with various combinations of these primer pairs revealed that two to four distinct types of phytoplasmas affiliated with phytoplasma 16S rRNA group I (aster yellows phytoplasma and related strains), group III (peach X-disease and related phytoplasmas), group V (elm yellows phytoplasma and related strains), and group X (apple proliferation and related phytoplasmas) were associated with most diseases. Predominant phytoplasma strains associated with pear decline, apricot chlorotic leaf roll, and plum leptonecrosis were identified as members of group X (subgroups A and B). Phytoplasma strains associated with nectarine chlorotic leaf roll were members of group I. Minor phytoplasma strains (one or more distinct types) were also detected in each infected plant, except for those affected by apricot decline.

Phytoplasmas (formerly called mycoplasmalike organisms) are known to be associated with diseases of many perennial fruit crops on the European continent (1–4,8,9,11–13,15,18,19,25–28, 30,31). For stone fruit trees, the major phytoplasma group detected in diseased field samples from some European countries contains the apple proliferation phytoplasma and related strains (1). This phytoplasma group was classified as restriction fragment length polymorphism (RFLP) group V by Schneider et al (30) on the basis of restriction analysis of the amplified 16S rDNA with *Alu*I and was designated by Lee et al (23) as 16S rRNA group X on the basis of extensive RFLP analysis of 16S rDNA with 15 restriction enzymes. In Italy, evidence indicates that decline of several fruit crops, such as pear, apricot, plum, and grapevine, can be attributed to phytoplasma infections (2,3,5,9,11–13,19,25–27). Previous attempts to determine the identity of the phytoplasma or phytoplasmas associated with each disease have yielded inconsistent results. For instance, at least two distinct types of phytoplasmas were reported to be associated with grapevine yellows in Italy (3,9,28), and at least four distinct phytoplasma groups are associated with this disease worldwide (8,28). Types of phytoplasmas detected in periwinkle plants experimentally infected by dodder or through insect vector transmission were not consistent with those present in the original, natural hosts (1,30).

The possibly complex etiologies of diseases associated with perennial fruit crops may be compounded by the recent findings that a single host plant might be doubly or multiply infected by different phytoplasma groups (3,22). Hence, two or more phytoplasmas may simultaneously contribute to a disease associated with perennial fruit crops. In northern Italy, it is common to grow mixed-fruit crops at the same location. This unique ecosystem composed of overlapping vegetative phases provides vast opportunities for diseases to spread from one crop to another, provided that both crops are potential hosts of the associated phytoplasmas of the diseases and the insect vectors. The etiologies of several prominent diseases, such as pear decline, apricot chlorotic leaf roll, apricot decline, plum leptonecrosis, and nectarine leaf roll, have not been clarified, and whether mixed-phytoplasma infections are involved in these diseases has not been investigated. A lack of reliable information on the etiology has hindered studies of the epidemiology of these diseases.

The objective of the present study was to systematically investigate whether phytoplasma or phytoplasmas are associated with these diseases and what types are present. Specially designed nested polymerase chain reaction (PCR) assays that included the universal primer pairs and four phytoplasma group-specific primer pairs, which detect four major phytoplasma groups most commonly present in Italy, were employed to detect and identify the associated phytoplasmas. This study has revealed that two to four types of phytoplasmas are associated with each particular disease and that a single plant can be doubly or multiply infected with different phytoplasmas.

MATERIALS AND METHODS

Sources of phytoplasmas and phytoplasma-infected tissues. The reference phytoplasma strains for the four major phytoplasma 16S rRNA groups (I, III, V, and X) and subgroups (14,23) included in this study are Maryland aster yellows (AY-1) (group I-B), tomato big bud (BB) (group I-A), clover phyllody (CPh) (group I-C), Canada peach X-disease (CX) (group III-A), clover yellow edge (CYE) (group III-B), elm yellows (EY1) (group V), and apple proliferation (AP-A) (group X-A). Strain AY-1 was originally field collected in diseased periwinkle (*Catharanthus*

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roseus (L.) G. Don) at Beltsville, Maryland. Other phytoplasma strains were from the following researchers, who provided each strain separately in periwinkle or in other hosts as indicated: BB (J. Dale, University of Arkansas, Fayetteville); CPh, CYE in clover, and CX (L. N. Chiykowski, Agriculture Canada, Ottawa, Ontario); EY1 (W. A. Sinclair, Cornell University, Ithaca, NY); and AP-A (L. Carraro via A. Bertaccini). Asymptomatic and symptomatic tissues of apricot, pear, Nashi pear, Japanese plum, and nectarine trees were collected from several orchards in the Emilia-Romagna region of northern Italy, where chlorotic leaf roll (apricot [ACLR], nectarine [NCLR]), decline (apricot [AD], pear [PD], Asian Nashi pear [PD]), and leptonecrosis (Japanese plum [PLN]) occur.

Primer pairs and PCR conditions. The universal primer pairs R16F2/R2 (23), modified R16F2/R2 (designed in this study), and R16F1/R0 (10,23) (designed on the basis of an AY phytoplasma) were used in PCR assays for general detection of various phytoplasmas. Three phytoplasma 16S rRNA group-specific primer pairs (22), R16(I)F1/R1, R16(III)F2/R1, and R16(V)F1/R1, and one primer pair, R16(X)F1/R1 (designed in this study), were based on the unique sequences of phytoplasma 16S rDNA in the region (about 1.2 kb) amplified with the universal primer pair R16F2/R2 among representative members of four phytoplasma 16S rRNA groups, I, III, V, and X. These sequences were identified by direct PCR sequencing with standard dideoxy termination reactions (fmol kit, Promega, Madison, WI) (14). A specific DNA fragment approximately 1.1 kb in size was amplified by using primer pairs R16(I)F1/R1, R16(V)F1/R1, and R16(X)F1/R1; a fragment of approximately 0.8 kb was amplified by primer pair R16(III)F2/R1. The oligonucleotide sequences of the new primer pairs are i) R16F1/R0: R16F1, 5′-AAG ACG AGG ATA ACA GTT GG-3′ and R16R0, 5′-GGA TAC CTT GTT ACG ACT TAA CCC C-3′; ii) modified R16F2/R2: modified R16F2, 5′-ACG ACT GCT AAG ACT GG-3′ and R16R2, 5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′; and iii) R16(X)F1/R1: R16(X)F1, 5′-GAC CCG CAA GTA TGC TGA GAG ATG-3′ and R16(X)R1, 5′-CAA TCC GAA CTG AGA CTG T-3′.

For PCR, total nucleic acid was extracted from asymptomatic and symptomatic tissues, as described elsewhere (21). Nucleic acid samples were diluted in sterile deionized water to give a final concentration of 20 ng per µl. PCR assays were performed as previously described (23,29), with 20 ng each of total nucleic acid, 200 µM deoxynucleoside triphosphate (dNTP), and 0.4 to 1.0 µM primer pair. Thirty-five PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following parameters were used: 1 min (2 min for the first cycle) denaturation step at 94°C, annealing for 2 min at 50°C, and primer extension for 3 min (10 min in final cycle) at 72°C. Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. PCR products were analyzed by electrophoresis through a 1% agarose gel and stained in ethidium bromide. DNA bands were then visualized with a UV transilluminator.

Nested PCR assays for detection of phytoplasmas associated with perennial fruit crops. Nested PCR assays with the universal primer pair R16F1/R0 followed by another universal primer pair, R16F2/R2 or modified R16F2/R2, or with the universal primer pair R16F2/R2 followed by one of the four phytoplasma group-specific primer pairs, R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1, or R16(X)F1/R1, were employed to detect phytoplasmas associated with diseased apricot (chlorotic leaf roll and decline), nectarine (chlorotic leaf roll), plum (leptonecrosis), and pear (decline). PCR products initially amplified by using the universal primer pair R16F1/R0 or R16F2/R2 were diluted (1/40) with sterile deionized water and used as template DNA for a subsequent series of 35 PCR cycles in which reaction mixtures contained the second universal or a group-specific primer pair. In some cases, the second PCR amplifications, with the primer pair R16(I)F1/R1, were performed under an annealing temperature of 60°C. Samples were run at least twice to evaluate the consistency of this assay procedure.

RFLP analyses of PCR products. Five to fifteen microliters of each selected PCR product (phytoplasma 16S rDNA sequence) was digested separately with selected restriction endonucleases, e.g., *Alu*I, *Rsa*I, *Hpa*II, *Hha*I, *Hae*III, *Kpn*I, *Taq*I (GIBCO BRL, Gaithersburg, MD), and *Mse*I (New England Biolabs, Beverly, MA). The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel and stained in ethidium bromide. DNA bands were visualized by using a UV transilluminator (23).

RESULTS

Phytoplasma 16S rDNA sequences amplified by PCR assays with universal primer pairs. Direct PCR amplifications with universal primer pairs R16F1/R0 and R16F2/R2 yielded visible PCR products (specific phytoplasma 16S rDNA sequences and, in some samples, additional nonspecific DNA fragments) when total nucleic acid samples prepared from symptomatic or symptomless tissues from trees with diseases (chlorotic leaf roll [apricot and nectarine], decline [pear and Asian pear], and leptonecrosis [plum]) were used. Few visible products were obtained with the primer pair R16F1/R0 (Fig. 1A), but several samples (e.g., Apc1, PrNa1, PrNa2, PrNa3, PrNa4, and PrCe2) were amplified with R16F2/R2 (Fig. 1B). Nested PCR assays with the primer pair R16F1/R0 followed by the primer pair R16F2/R2 or the modified R16F2/R2 yielded PCR products from nearly all (Fig. 1C) or all (Fig. 1D) the nucleic acid samples, including those prepared from symptomless tissues. The nested PCR assays in which the two universal primer pairs were used generally detected the predominant phytoplasmas present in each sample (Table 1).

Mixed-phytoplasma infections detected by nested PCR assays. Nested PCR assays with one of the four phytoplasma group-specific primer pairs revealed that more than one type of phytoplasma could be detected in tissue from a single diseased plant (Fig. 2A–D and Table 1). Phytoplasmas affiliated with phytoplasma 16S rRNA groups I (Fig. 2A), III (Fig. 2B), V (Fig. 2C), and X (Fig. 2D) were all detected among the samples from symptomatic or symptomless apricot, nectarine, pear, Asian pear, and Japanese plum trees. Types of phytoplasmas detected by nested PCR assays varied with each plant, and two or more types were often found (Table 1). Phytoplasmas affiliated with group III were found less commonly in these samples.

RFLP analyses of phytoplasma 16S rDNA sequences and identification of mixed phytoplasmas. On the basis of reference RFLP patterns (23; I.-M. Lee, *unpublished*), RFLP profiles (Fig. 3) of phytoplasma 16S rDNA sequences amplified by nested PCR with universal primer pairs R16F1/R0 and R16F2/R2 indicated that predominant phytoplasmas associated with these fruit crop diseases are strains belonging to phytoplasma 16S rRNA group X (apple proliferation and related phytoplasmas) (14,23) (e.g., Fig. 3A and C, lane PrNa2). This was verified by nested PCR with phytoplasma group X-specific primer pair R16(X)F1/R1 (Fig. 2D) and was verified in part by direct PCR assays with primer pair R16F2/R2 (Fig. 1B), which required more phytoplasma DNA template to yield detectable PCR products. RFLP analyses of PCR products visible on gels (six samples [Fig. 1B] and 19 samples [Fig. 2D]) indicated that all phytoplasma strains detected in these samples belonged to phytoplasma 16S rRNA group X (data not shown). The group X phytoplasma strains were associated with samples collected from all Asian Nashi pear and plum trees and some pear and apricot trees (Table 1 and Fig. 4). The second major phytoplasma type associated with these fruit crops belonged to phytoplasma 16S rRNA group I, which was detected in samples prepared from apricot, plum, nectarine, and some pear trees, including several symptomless trees (e.g., Fig.

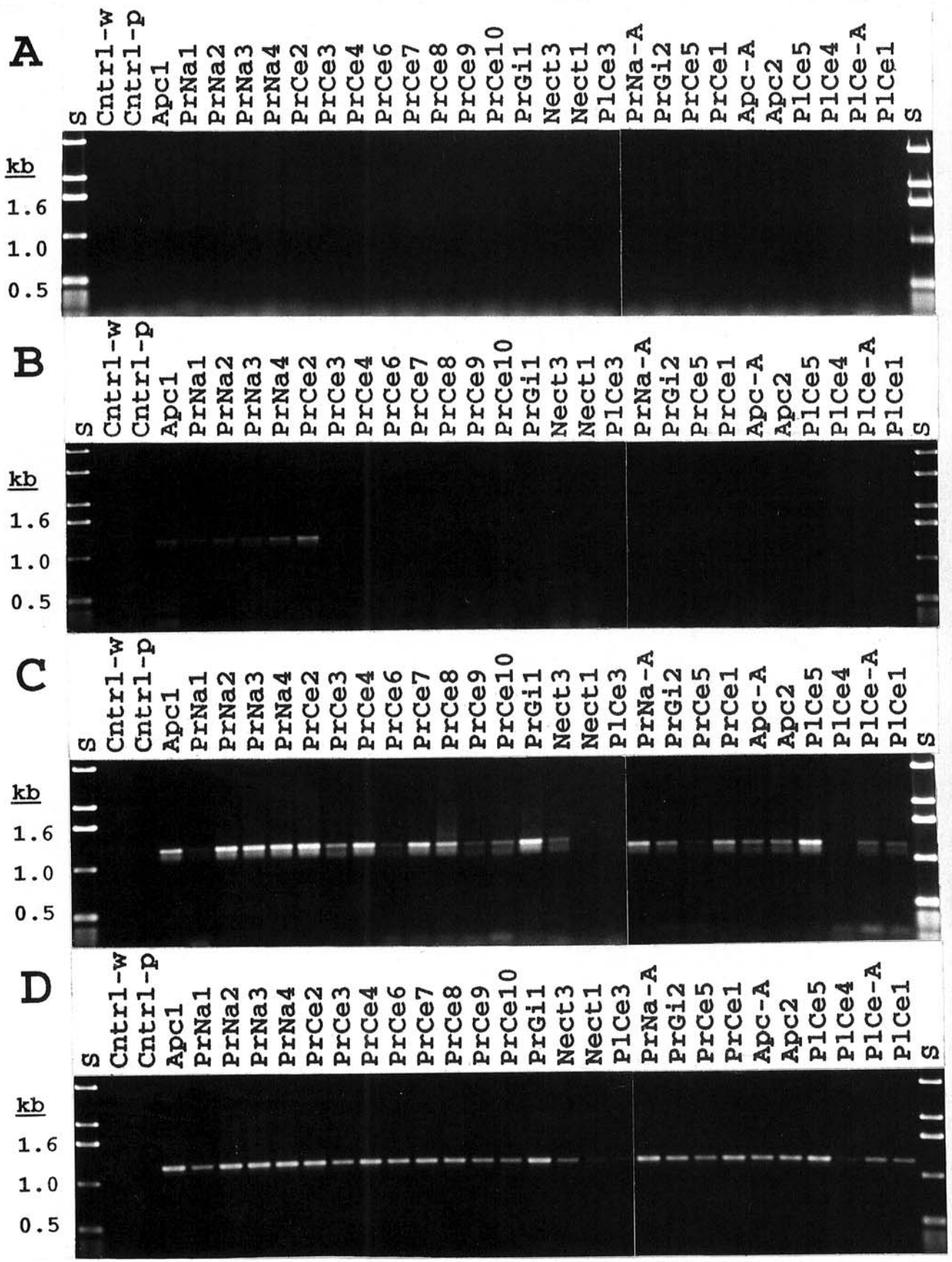

Fig. 1. Direct and nested polymerase chain reaction (PCR) amplification of phytoplasma 16S rDNA sequences from naturally infected fruit trees in northern Italy. Direct PCR amplification with **A,** the universal primer pair R16F1/R0 and **B,** the universal primer pair R16F2/R2 and nested PCR assays with **C,** the primer pair R16F1/R0 followed by R16F2/R2 and **D,** the primer pair R16F1/R0 followed by modified R16F2/R2. S = DNA fragment sizes standard; Control-w = water control; and Control-p = healthy control (periwinkle plant). All other sample abbreviations are defined in Table 1.

3A and C, lane Apc2). Mixed RFLP profiles were observed in some samples (e.g., Fig. 3A and C, lanes PrCe8 and PlCe-A), indicating the presence of two predominant types of phytoplasmas with similar titers.

RFLP analyses of the amplified 16S rDNA sequences from these diseased fruit tree samples further identified subgroup affiliations of phytoplasma strains within each phytoplasma 16S rRNA group (Table 1 and Fig. 4). Two subgroups (X-A and X-B) and at least two subgroups (I-B and I-G) (32) were identified among phytoplasma strains that were categorized as phytoplasma 16S rRNA group X and group I, respectively. RFLP patterns of the PCR product obtained from direct PCR with universal primer pair R16F2/R2 or nested PCR with primer pairs R16F1/R0 and R16F2/R2 or R16F2/R2 and one of the group-specific primer pairs were analyzed after digestion with endonucleases *Alu*I, *Mse*I, *Rsa*I, *Hpa*II, *Hha*I, *Kpn*I, *Taq*I, and *Hae*III. The RFLP profiles (e.g., Fig. 3, those analyzed with *Mse*I and *Rsa*I) indicated that the primary phytoplasmas detected in Asian and other pear were similar to one another and to that of the apple proliferation (AP-A) phytoplasma. A phytoplasma associated with apricot chlorotic leaf roll (ACLR) (sample Apc1) and with a plum leptonecrosis (PLN) sample (PlCe5) was closely related to, but distinct from, the AP phytoplasma. The same type of phytoplasma was found to be associated with each of the samples (Table 1) with symptoms of apricot decline (AD), including early sprouting of leaves and witches'-broom growth of branches, collected from another orchard in the Rimini area (Emilia-Romagna) (2). The ACLR (Apc1), AD (all samples), or PLN (PlCe5) phytoplasma represents a subgroup, 16S rRNA group X-B (14), within phytoplasma 16S rRNA group X on the basis of a unique RFLP pattern revealed by using *Rsa*I (Fig. 3C, lanes Apc1 and PlCe5, and Fig. 4B, lanes Apc1, PlCe5, and Apc5). RFLP profiles (analyzed with *Mse*I, *Alu*I, *Rsa*I, and *Hpa*II) of 16S rDNA PCR products from representative samples in which group X phytoplasmas were detected are shown in Figure 4. Phytoplasmas in samples Apc1, PlCe5, and Apc5 belong to 16S rRNA group X-B. The remaining phytoplasmas belong to 16S rRNA group X-A, like the type strain AP-A. Phytoplasmas associated with many samples from diseased pear and nectarine (e.g., PrCe5, Nect3, PrCe3, PrCe6, PrCe9, and PrCe10) were identified as 16S rRNA subgroup I-G on the basis of unique RFLP patterns, distinct from phytoplasma AY-1 (16S rRNA group I-B), by using *Taq*I (data not shown) and *Mse*I (Fig. 3A and B). Phytoplasmas associated with apricot and nectarine (e.g., Apc2, Apc-A, and Nect1) were identified as 16S rRNA group I-B (type strain AY-1) (Fig. 3A and B).

DISCUSSION

Phytoplasmas have been suspected of being associated with pear decline, apricot chlorotic leaf roll or apricot decline, plum leptonecrosis, and nectarine chlorotic leaf roll diseases of fruit crops grown in northern Italy (2,11–13,25–27). The lack of sensitive assays has hindered the identification of phytoplasmas associated with these diseases. In the present study, nested PCR with two universal primer pairs detected phytoplasma infections in all samples, including asymptomatic ones, collected from pear, apricot, plum, and nectarine trees. Because of an inability to obtain pure cultures of phytoplasmas, their pathogenicity has never been unequivocally demonstrated (20,24) However, inoculation experiments through dodder or insect vectors strongly indicated that phytoplasmas alone can cause numerous diseases, including the fruit diseases investigated in this study (1,5,8,13, 15–17,24,32). Detection of phytoplasmas in apparently asymptomatic fruit trees underscores the need to reassess the role of phytoplasmas in the chronic decline of these perennial fruit crops in northern Italy. The percentage of phytoplasma-infected fruit trees may be much higher than previously thought. Whether phytoplasma infection alone or, in some cases, multiple phytoplasmas collectively contribute to most cases of decline of these fruit crops cannot be determined without long-term epidemiological studies of these phytoplasma-associated fruit tree diseases. Nested PCR assays with phytoplasma group-specific primer pairs developed in this work should provide sensitive means to monitor phytoplasma titers in the infected trees and help to determine the roles of phytoplasma complexes in these fruit decline diseases.

Because phytoplasmas are not culturable in vitro (20,24), these putative plant pathogens have been named and classified according to disease symptoms and associated hosts (2,4,7,11–13,16–18,24). It was generally conceived that one type of phytoplasma contributed to each disease. The identities of associated phytoplasmas had never been clarified until recently, when molecular technologies enabled identification and classification of phytoplasmas on the basis of molecular characteristics (1,6,8,9,15,19, 23,28,30,31). Recently, studies on etiologies of diseases associated with some fruit crops in Europe and North America have proved that the concept that each disease is caused by one type of phytoplasma is not generally applicable (3,9,16,28,30). For example, three discrete groups of phytoplasmas were detected in grapevines with yellows symptoms. Phytoplasmas that caused

TABLE 1. Mixed phytoplasmas detected by nested polymerase chain reaction (PCR) assays in pome and stone fruits in northern Italy and phytoplasma group affiliations[a]

Template DNA	Associated disease,[c] host	Primer pairs[b] U1 + U2	Primer pairs[b] U2 + I, III, V, or X
Apc1	ACLR, apricot	X-B[d]	I-B, V, X-B
Apc2	ACLR, apricot	I-B	I-B, III, V
Apc-A	Asymptomatic, apricot	I-B	I-B, V
Apc3	AD, apricot	X-B	ND[e]
Apc4	AD, apricot	X-B	ND
Apc5	AD, apricot	X-B	ND
Apc6	AD, apricot	X-B	ND
Apc7	AD, apricot	X-B	ND
Nect1	NCLR, nectarine	I-B	I-B, V
Nect3	NCLR, nectarine	I-B, I-G	I-B, I-G, V
PlCe1	PLN, Japanese plum	I-B, X-A	I-B, V, X-A
PlCe3	PLN, Japanese plum	X-A	I, III, V, X-A
PlCe4	PLN, Japanese plum	I-B, X-A	I-B, V, X-A
PlCe5	PLN, Japanese plum	X-B	III, V, X-B
PlCe-A	Asymptomatic, Japanese plum	I-B, X-A	I-B, III, V, X-A
PrNa1	PD, Nashi pear	X-A	I, V, X-A
PrNa2	PD, Nashi pear	X-A	I, III, X-A
PrNa3	PD, Nashi pear	X-A	I, V, X-A
PrNa4	PD, Nashi pear	X-A	I, V, X-A
PrNa-A	Asymptomatic, Nashi pear	X-A	I, V, X-A
PrCe1	PD, pear	X-A	I, V, X-A
PrCe2	PD, pear	X-A	I, V, X-A
PrCe3	PD, pear	I-G	I-G, III, V
PrCe4	PD, pear	X-A	I, V, X-A
PrCe5	PD, pear	I-G	I-G, V
PrCe6	PD, pear	I-G	I-G, V
PrCe7	PD, pear	X-A	I, V, X-A
PrCe8	PD, pear	I-G, X-A	I-G, V, X-A
PrCe9	PD, pear	I-G	I-G, III, V
PrCe10	PD, pear	I-G	I-G, III, V
PrGi1	PD, pear	X-A	III, V, X-A
PrGi2	PD, pear	X-A	III, V, X-A

[a] Affiliation of phytoplasma 16S rRNA group was based on restriction fragment length polymorphism (RFLP) analyses of amplified 16S rDNA sequences.

[b] U1 = R16F1/R0; U2 = modified R16F2/R2; I = R16(I)F1/R1; III = R16(III)F2/R1; V = R16(V)F1/R1; and X = R16(X)F1/R1. Nested PCR with primer pairs U1 + U2 detected the predominant phytoplasma(s) in each sample. Nested PCR with U2 + I, III, V, or X detected, in addition to the primary phytoplasma, secondary or minor phytoplasmas in each sample.

[c] ACLR = apricot chlorotic leaf roll; AD = apricot decline; NCLR = nectarine chlorotic leaf roll; PLN = plum leptonecrosis; and PD = pear decline.

[d] I, III, V, and X = phytoplasma 16S rRNA groups; A, B, and G = phytoplasma 16S rRNA subgroup affiliations based on RFLP of amplified DNA.

[e] Not determined.

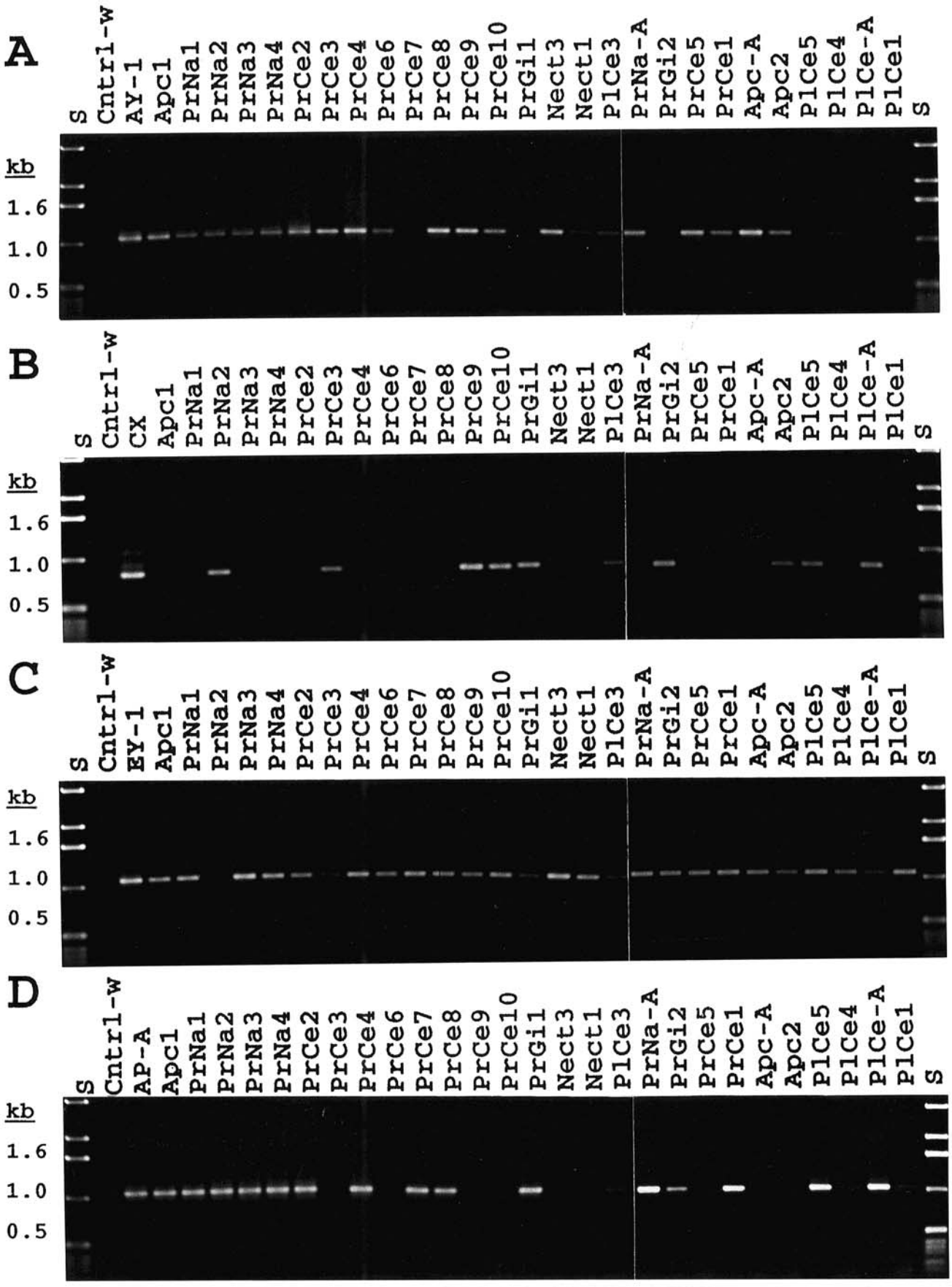

Fig. 2. Nested polymerase chain reaction amplification of phytoplasma 16S rDNA sequences from naturally infected fruit trees in northern Italy with the universal primer pair R16F2/R2 followed by phytoplasma group-specific primer pairs: **A,** R16(I)F1/R1; **B,** R16(III)F2/R1; **C,** R16(V)F1/R1; and **D,** R16(X)F1/R1. Positive controls AY-1, CX, EY-1, and AP-A were tissue samples infected with Maryland aster yellows, peach X-disease, elm yellows, and apple proliferation phytoplasmas, respectively. All other sample abbreviations are defined in Table 1.

decline in peach in North America and Europe belonged to two discrete groups (3,8,28,30). Determining the causal agent or agents of a particular disease has been further complicated by the discovery that secondary phytoplasmas are commonly present in a single host plant (3,22). Results from the present study provided evidence that as many as four discrete types of phytoplasmas could be detected in samples from a single diseased plant. Primary phytoplasmas associated with a particular disease varied with individual plants, even those from the same orchard.

Predominant phytoplasmas detected in stone and pome fruit trees were strains belonging to 16S rRNA group X (apple proliferation and related phytoplasmas) and group I (aster yellows and related phytoplasmas) (Table 1). It is interesting to note that phytoplasma strains belonging to 16S rRNA group I (subgroups I-B and I-G) were readily detected in many samples. Thus far, group I (corresponding to RFLP group I in Schneider et al [30]) phytoplasmas have never been detected from field samples of diseased stone or pome fruit trees, with the exception of samples from cherry trees infected with Molières disease (1,15). However, this group of phytoplasmas was readily detected in several periwinkle plants experimentally infected with apricot chlorotic leaf roll disease from Spain and leptonecrosis of Japanese plum from Italy (1). Phytoplasmas belonging to group I-G were also found associated with grapevine yellows in northern Italy (32). The role of group I phytoplasmas in the decline of these fruit crops warrants further investigation. Evidence that multiple phytoplasmas can be detected in four fruit crops in Italy may explain the inconsistency of previous reports on etiologies of associated

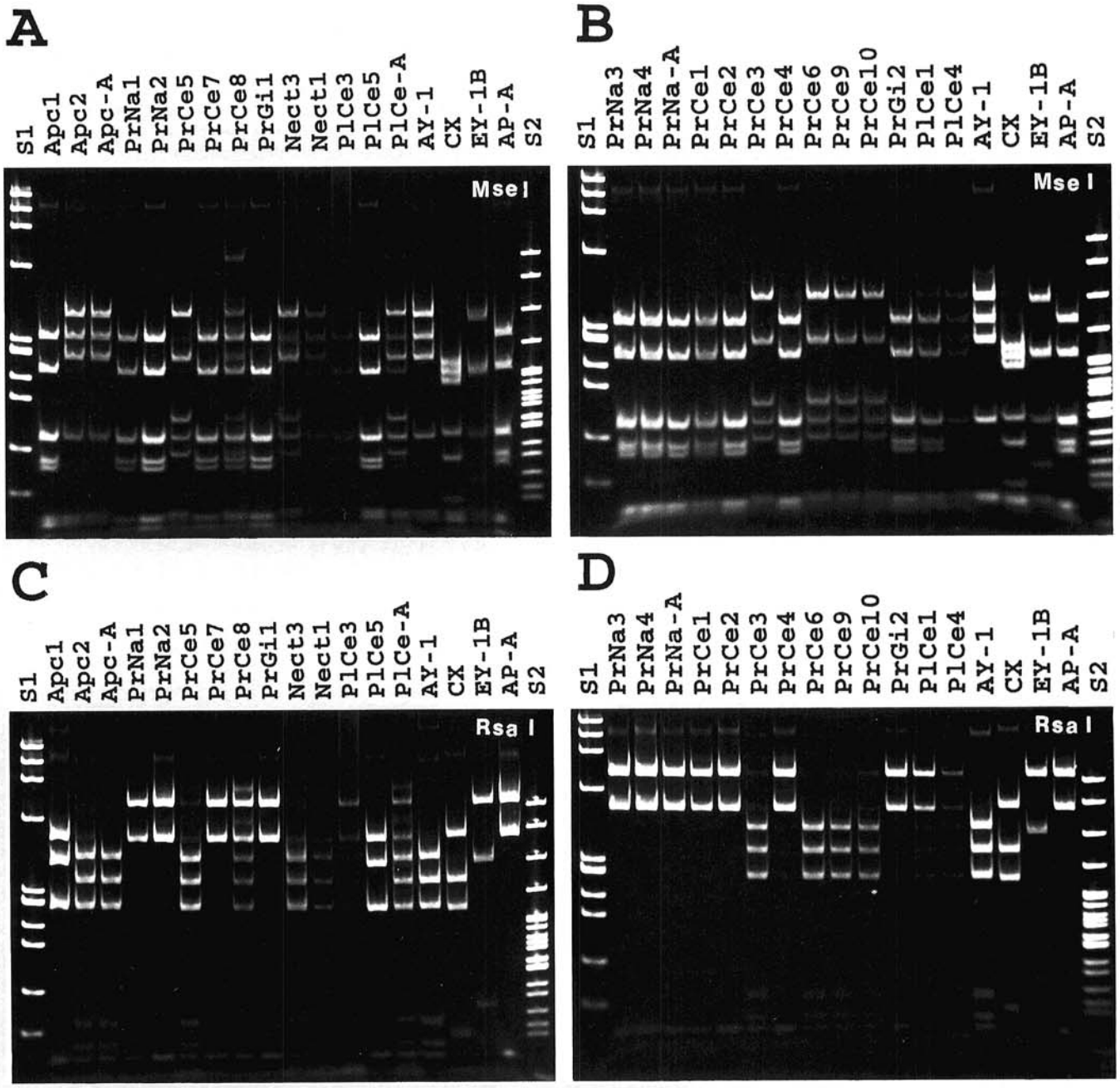

Fig. 3. Restriction fragment length polymorphisms of phytoplasma 16S rDNA amplified by nested polymerase chain reaction with two universal primer pairs, R16F1/R0 and modified R16F2/R2. DNA products were digested with restriction enzymes **A and B,** *Mse*I and **C and D,** *Rsa*I and separated by electrophoresis through a 5% polyacrylamide gel. Lane S1, ϕX174 RFI DNA *Hae*III digest; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; and lane S2, pBR322 DNA *Msp*I digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9. Reference strains: AY1 (16S rRNA group I-B), CX, (group III-A), EY-1B (group V-A), AP-A (group X-A). All other sample abbreviations are defined in Table 1.

diseases. Certain phytoplasma types might have been preferentially transmitted through dodder or insect vectors into indicator plants (periwinkle) where the associated phytoplasma was detected (1).

The concept of phytoplasma etiology is changing. For some phytoplasma-associated diseases, it no longer seems appropriate to assume the identity of a phytoplasma on the basis of the associated host plant and disease symptoms. Instead of a single type of phytoplasma, dual or multiple phytoplasmas should be considered as putative causal agents for some diseases associated with perennial fruit crops. Geographical distribution of phytoplasma types should be taken into consideration in determination of the predominant phytoplasmas associated with a particular disease. For example, strains of 16S rRNA group III-A phytoplasma (peach X-disease phytoplasma) that are associated with peach, cherry, and pear declines in North America (16,23) are rare or absent on the European continent (30,31), whereas strains of 16S rRNA group I-G (related to the aster yellows phytoplasma) that cause grapevine yellows in some regions of northern Italy have not been detected in North America (28). Because naming phytoplasmas according to their associated hosts and the disease symptoms they induce now appears to be invalid, it becomes necessary to establish a more consistent classification system that can be applied to accurately distinguish among various phytoplasmas. In this study, the associated phytoplasmas were identified and referred to as members of phytoplasma 16S rRNA groups or subgroups (14,23), since a formal taxonomy for phytoplasmas has not been established.

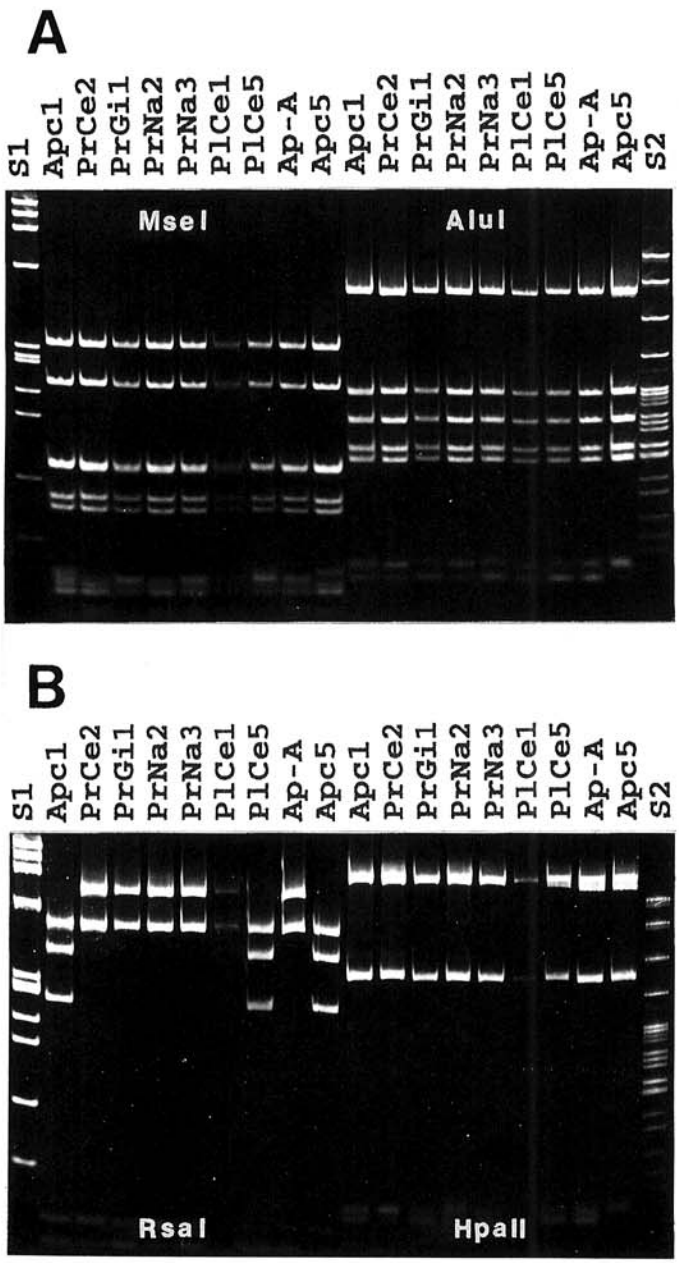

Fig. 4. Restriction fragment length polymorphisms of 16S rDNA amplified from selected samples containing phytoplasmas belonging to 16S rRNA group X. DNA products were digested separately with restriction enzymes **A,** *Mse*I and *Alu*I and **B,** *Rsa*I and *Hpa*II. Phytoplasma strain AP-A is the type strain of 16S rRNA group X-A. Lane S1, ϕX174 RFI DNA *Hae*III digest; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; and lane S2, pBR322 DNA *Msp*I digest; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9. All other sample abbreviations are defined in Table 1.

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