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Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows

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Elm yellows group (16SrV) phytoplasmas, which are associated with devastating diseases in elm, grapevine, blackberry, cherry, peach and several other plant species in America, Europe and Asia, represent one of the most diverse phytoplasma clusters. On the basis of phylogenetic analysis of 16S rDNA sequences, elm yellows group phytoplasmas form a discrete subclade within the phytoplasma clade. Three phylogenetic parameters, namely 16S rRNA, ribosomal protein and *secY* genes, have been evaluated for their usefulness in differentiating elm yellows group phytoplasmas. RFLP analysis of 16S rRNA sequences differentiated the elm yellows group phytoplasmas into five subgroups. Twelve RFLP subgroups were differentiated on the basis of ribosomal protein and 13 were differentiated using *secY* gene sequences. Phylogenetic analysis of the ribosomal protein genes and *secY* gene alone or in combination indicated that the subgroups constitute 12 genetically distinct lineages, each of which appears to have evolved under different ecological constraints such as specific vector or plant hosts. On the basis of unique DNA and biological properties, it is proposed that the elm yellows phytoplasma EY1^T represents a novel taxon, '*Candidatus* Phytoplasma ulmi'.

INTRODUCTION

The highly conserved 16S rRNA gene sequence has been used as the primary phylogenetic parameter for classification of phytoplasmas in the emerging phytoplasma taxonomy (Lee *et al.*, 1993b, 1998a; Schneider *et al.*, 1995; Seemüller *et al.*, 1994, 1998). On the basis of extensive RFLP or phylogenetic analyses of 16S rDNA sequences from a wide array of phytoplasma strains, more than 20 distinct phytoplasma groups have been identified (Lee *et al.*, 2000; Seemüller *et al.*, 1998). Each group was proposed to represent at least one species (Gundersen *et al.*, 1994; Seemüller *et al.*, 1998). Because it continues to be impossible to obtain a pure culture of any phytoplasma, a provisional taxonomic system for uncultured bacteria proposed by Murray & Schleifer (1994) was adopted, with some modifications, for the naming of phytoplasma species candidates. So far, nine '*Candidatus* Phytoplasma' species have been proposed (Lee *et al.*, 2000; Jung *et al.*, 2002; Verdin *et al.*, 2003). The elm yellows (EY) group (=16SrV group, delineated using RFLP analysis) represents one of the major phytoplasma groups identified.

The EY phytoplasma (16SrV) group consists of diverse phytoplasma strains, representing the third largest phytoplasma cluster after the aster yellows and X-disease phytoplasma groups (Gundersen *et al.*, 1996; Lee *et al.*, 2000). EY phytoplasma strains (EY1^T, EY125, EY626 and others) have caused a decline in American elms in North America and Eurasian elm species and hybrids in several European countries (Griffiths *et al.*, 1999a; Lee *et al.*, 1993a, 1995; Marcone *et al.*, 1997; Mäurer *et al.*, 1993; Sinclair

Abbreviations: ALY, alder yellows; CLY, cherry lethal yellows; EY, elm yellows; FD, flavescence dorée; HD, hemp dogbane; JWB, jujube witches'-broom; PY-In, peach yellows; RuS, rubus stunt; SpaWB, spartium witches'-broom.

The GenBank accession numbers for the sequences of elm yellows phytoplasma strain EY1^T are AY197655 (16S rDNA), AY197675 (*rpl22–rps3*) and AY197690 (*secY*).

Details of RFLP pattern groups for 16S rDNA and ribosomal protein operons and putative restriction maps from ribosomal protein and *secY* gene sequences of representative EY phytoplasmas are available as supplementary material in IJSEM Online.

et al., 1976). Strain EY1^T is the reference phytoplasma strain for the EY phytoplasma group (Lee *et al.*, 1993b). Other EY group phytoplasmas associated with diseases in diverse plant genera in various geographical regions are as follows: flavescence dorée (FD) and grapevine yellows phytoplasmas in the European grapevine (Bertaccini *et al.*, 1997; Daire *et al.*, 1997; Martini *et al.*, 2002; Seemüller *et al.*, 1994); rubus stunt (RuS) phytoplasmas in wild and cultivated blackberries (*Rubus* spp.) in Europe (Lee *et al.*, 1995; Mäurer & Seemüller, 1994; Marcone *et al.*, 1997); a strain of the cherry lethal yellows (CLY) phytoplasma (CLY5) in the cherry in China (Zhu *et al.*, 1997); peach yellows (PY-In) phytoplasma in peach in India; jujube witches'-broom (JWB) phytoplasma in *Ziziphus* spp. in China and India (Tian *et al.*, 2000; Zhu *et al.*, 1997); alder yellows (ALY) phytoplasmas in alder in several European countries (Marcone *et al.*, 1997; Mäurer *et al.*, 1993); spartium witches'-broom (SpaWB)-EY phytoplasma in *Spartium* sp. in Italy (Marcone *et al.*, 1996a); eucalyptus little leaf phytoplasma in *Eucalyptus* sp. in Italy (Marcone *et al.*, 1996b); and hemp dogbane (HD)-associated phytoplasma (HD1) in *Apocynum cannabinum* in New York state (Griffiths *et al.*, 1999a). Recently, a novel EY group phytoplasma, Virginia creeper phytoplasma, was reported to infect Virginia creeper (*Parthenocissus quinquefolia*) plants in southern Florida (Harrison *et al.*, 2001) and an EY-related phytoplasma was reported to infect tomato plants in Italy (Del Serrone *et al.*, 2001). Many diseases inflicted by EY group phytoplasmas are economically important and are subject to quarantine internationally.

Members of the EY phytoplasma group appear to be homogeneous on the basis of 16S rRNA gene sequences: they share 98·6–99·9 % similarity (Lee *et al.*, 1998a). However, despite their close relatedness (on the basis of 16S rRNA sequences), EY group phytoplasmas apparently constitute several strain clusters that share no common ecological niches (Lee *et al.*, 1998b). In nature, they are transmitted by different insect vectors and are associated with different plant hosts in various geographical regions. For epidemiological studies and for quarantine purposes, it is necessary to have a classification system that allows the differentiation of these ecologically distinct strains in the EY group. EY group phytoplasmas have been classified into five subgroups based on 16S rDNA sequences (Davis & Dally, 2001; Lee *et al.*, 1998a; Martini *et al.*, 1999). This approach was found to be insufficient for differentiating many members of this group, of which several strains (or strain clusters) have been shown to be distinct according to biological or pathological properties. Many studies have focused on finer differentiation of phytoplasma strains associated with FD and grapevine yellows phytoplasma diseases in grapevine (Angelini *et al.*, 2001; Daire *et al.*, 1997; Davis *et al.*, 1997; Martini *et al.*, 1999, 2002). On the basis of analysis of a non-rDNA fragment, FD9, encoding a translocase protein (secY) and a partial ribosomal protein (rpl15) (Reinert, 1999), several distinct subgroups were identified. However, there is no comprehensive classification scheme for differentiation and identification of all members of the expanded EY phytoplasma group.

The objectives of the present study were to investigate phylogenetic relationships among members of the expanded EY phytoplasma group, using combined analyses of the 16S rRNA, ribosomal protein and *secY* gene sequences, and to evaluate new phylogenetic parameters for finer strain differentiation. In this study, we report RFLP subgroups identified on the basis of these gene sequences. Phylogenies based on these three genes have strengthened the notion that the EY phytoplasma group consists of heterogeneous phytoplasma strains that represent several distinct phylogenetic clusters. The phylogenetic and biological criteria are consistent with the concept that the EY phytoplasma group comprises more than one species. In this paper, we propose that the EY phytoplasma strain EY1^T (a member of subgroup 16SrV-A) associated with EY in the American elm represents a novel '*Candidatus*' phytoplasma species, '*Candidatus* Phytoplasma ulmi'.

METHODS

Phytoplasma strains and nucleic acid preparation. All representative EY phytoplasma strains listed in Table 1, except strains FD (Lom/Piem) and Virginia creeper phytoplasma, were used in the present study. Total nucleic acid or DNA was extracted using leaf midribs or other tissues from periwinkle or the original hosts that were infected by representative phytoplasma strains according to the method described by Ahrens & Seemüller (1992) or Lee *et al.* (1993a). These representative phytoplasma strains were previously characterized and identified by RFLP or sequence analysis of 16S rDNA (Lee *et al.*, 1993b, 1998a; Davis & Dally, 2001; Martini *et al.*, 1999; Marcone *et al.*, 1997).

RFLP and putative restriction map analyses. RFLP analyses of three conserved genes, 16S rRNA, ribosomal protein and *secY* (a translocase gene), were evaluated for their usefulness in differentiating EY group phytoplasmas. Partial 16S rDNA sequences were prepared by using a nested PCR with universal primer pair P1/P7 (Schneider *et al.*, 1995) followed by universal primer pair R16F2n/R16R2 (Gundersen & Lee, 1996). R16F2n/R16R2 PCR products (1·2 kb) were digested with restriction enzymes *Mse*I, *Alu*I, *Sau*3AI, *Tha*I, *Hae*III, *Bfa*I, *Rsa*I, *Hpa*II and *Tsp509*I. To differentiate FD-D from other members of the EY group, nearly full-length 16S rDNA (about 1·5 kb) was amplified by using a nested PCR with primer pair P1/P7 followed by P1A (5′-AACGCTGGCGGCGCGCCTAATAC-3′) and 16S-SR (5′-GGTCTGTCAAAACTGAAGATG-3′) (designed in this study). The P1A/16S-SR PCR products were digested with *Taq*I. To prepare the ribosomal protein gene operon, a nested PCR was performed using the ribosomal protein primer pair rp(V)F1/rpR1 specific to the EY phytoplasma group (Lee *et al.*, 1998a; Lim & Sears, 1992) followed by another EY group-specific primer pair, rp(V)F1A (5′-AGGCGATAAAAAAGTTTCAAAA-3′) and rp(V)R1A (5′-GGCATTAACATAATATATTATG-3′) (designed in this study). The nested PCR with primer pair rp(V)F1A/rp(V)R1A yielded a DNA fragment (about 1·2 kb) that covered the region with ribosomal protein genes *s3* and *l22*. Nested PCR products were digested with restriction enzymes *Hpa*II, *Hae*III, *Hha*I, *Dra*I, *Ssp*I, *Taq*I, *Alu*I, *Tsp509*I and *Mse*I. Restriction fragments were visualized according to the procedures described previously (Lee *et al.*, 1993b, 1998a). To prepare *secY* gene sequences, an FD9 DNA fragment (1·4 kb) encoding the 3′-end of *rpl15* and the *secY* gene was amplified using the primer pair FD9f/r reported previously by Daire *et al.* (1997).

Table 1. Designations, associated diseases and origins of representative phytoplasma strains in the EY phytoplasma group (16SrV)

Strain	Disease/host	Insect vector	Origin	RFLP classification subgroup		
				16SrV	rpV	secYV
RuSR19	Rubus stunt, *Rubus caesius*	Unknown	Germany	E	I	
RuS400	Rubus stunt, *Rubus fruticosus*	Unknown	Italy	E	I	
RuS971	Rubus stunt, *Rubus idaeus*	Unknown	Switzerland	E	I	
RUS	Rubus stunt, periwinkle	Unknown	Italy	E	I	I
ALY	Alder yellows, periwinkle	Unknown	Italy	C	H	H
ALY882	Alder yellows, *Alnus glutinosa*	*Oncopsis alni*	Germany	C	K	K
ALY1068	Alder yellows, *Alnus glutinosa*	Unknown	Italy	C	H	
HD1	Hemp dogbane, *Apocynum cannabinum*	Unknown	NY, USA	C	J	J
FD70	Flavescence dorée, *Vitis vinifera*	*Scaphoideus titanus*	France	C	F	F
FD-D	Flavescence dorée, *Vitis vinifera*	*Scaphoideus titanus*	Italy	D	E	E
FD-C	Flavescence dorée, *Vitis vinifera*	*Scaphoideus titanus*	Italy	C	D	D
FD (Lomb/Piem)	Flavescence dorée, *Vitis vinifera*	*Scaphoideus titanus*	Italy	C	G	
VC	Virginia creeper, *Parthenocissus quinquefolia*	Unknown	FL, USA	C		
SpaWB229	Spartium witches'-broom, *Spartium junceum*	Unknown	Italy	C	L	L
SpaWB251	Spartium witches'-broom, *Spartium junceum*	Unknown	Italy	C	L	
EY1^T	Elm yellows, *Ulmus americana*	*Scaphoideus luteolus*	NY, USA	A	A	A
EY125	Elm yellows, *Ulmus minor*	Unknown	Italy	A	A	
EY626	Elm yellows, *Ulmus minor*	Unknown	Italy	A	A	M
EY627	Elm yellows, *Ulmus minor*	Unknown	Italy	A	A	
JWB	Jujube witches'-broom, *Ziziphus jujuba*	*Hishimonoides chinesis*	China	B	C	C
PY-In	Peach yellows, *Prunus persica*	Unknown	India	B	M	N
CLY5	Cherry lethal yellows, *Prunus avium*	Unknown	China	B	B	B

Putative restriction maps of the *secY* DNA fragment (1·2 kb) and the 1·2 kb rp DNA fragment from representative phytoplasma strains were generated by using the DNASTAR program MapDraw option.

Cloning of PCR products and sequencing of DNA. PCR-amplified products of 16S rRNA, ribosomal protein and *secY* genes were cloned and sequenced. To obtain nearly full-length 16S rDNA, P1A/P7A PCR products (about 1·8 kb long and extending from the 5′-end of 16S rRNA, through the intergenic spacer region, to the 3′-end of the 23S rRNA) were cloned. P1A/P7A DNA fragments were amplified using diluted P1/P7 PCR products as templates in a PCR using the universal primer pair P1A/P7A (5′-ACGCTG-GCGGCGCGCCTAATAC-3′/5′-CCTTCATCGGCTCTTAGTGC-3′). The P1A/P7A PCR products, rp(V)F1A/rp(V)R1A PCR products (about 1·2 kb long, containing the *rpl22–rps3* gene sequence) and FD9f/r PCR products (about 1·4 kb long, containing the *secY* gene sequence) were purified using the Qiaquick PCR purification kit (Qiagen) and cloned into *Escherichia coli* by using the TOPO TA cloning kit (Invitrogen) according to the manufacturers' instructions. Sequencing was performed with an automated DNA sequencer (model 377; ABI Prism) at the Center for Agricultural Biotechnology (University of Maryland, College Park, MD, USA). Cloned nucleotide sequences were deposited in GenBank (see Figs 3 and 4 for accession numbers).

Phylogenetic analysis. Phylogenetic interrelationships among strains of the EY group and other phytoplasma groups were assessed on the basis of 16S rRNA gene sequences. Partial sequences of 16S rDNA (1·5 kb) from members of the EY phytoplasma group and representative phytoplasma strains available in GenBank were aligned by using CLUSTAL V (Higgins & Sharp, 1989) and LASERGENE software (DNAStar). Cladistic analyses were performed with PAUP, version 4.0 (Swofford, 1998), on a Power Mac G4. Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by using a heuristic search via random stepwise addition, implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s) (Gundersen *et al.*, 1994). *Acholeplasma laidlawii* JA1 was selected as the outgroup to root the tree. The analysis was replicated 1000 times. Bootstrapping was performed to estimate the stability and support for the inferred clades. Finer phylogenetic relationships among representative members of the EY phytoplasma group were determined on the basis of sequences of a partial ribosomal protein gene operon (1·2 kb, covering the *rpl22* and *rps3* genes) and a translocase protein gene, *secY* (1·2 kb). Deduced amino acid sequences of ribosomal protein (*rpl22* and *rps3*) and *secY* genes from representative subgroups were also compared. Potato witches'-broom phytoplasma (GenBank accession no. AY197683), a member of subgroup 16SrVI-A, was selected as the outgroup for ribosomal protein gene analysis and *Bacillus subtilis* 168 (AL009126) was selected as the outgroup for *secY* gene and ribosomal protein/*secY* genes combined.

RESULTS AND DISCUSSION

Strain differentiation by RFLP analysis

Gene sequences of 16S rRNA and ribosomal protein were compared for efficacy for finer strain differentiation among members of the EY phytoplasma group. Collective profiles obtained from digests of partial 16S rDNA (1·2 kb; a nested PCR product with primer pair R16F2n/R16R2) sequences from all 20 representative members of the EY

phytoplasma group with *Rsa*I, *Hpa*II, *Tsp509*I and *Bfa*I differentiated four distinct subgroups (16SrV-A, 16SrV-B, 16SrV-C and 16SrV-E) of the EY group (Fig. 1, Table 1) (see also Supplementary Table A in IJSEM Online). Sequence alignment of the 1·8 kb P1/P7 DNA fragment, which contains nearly the entire 16S rRNA gene and 16S–23S spacer region, of all 20 EY group phytoplasma strains revealed that additional sequence variations were present in the regions of 16S rRNA outside the portion covered by primers R16F2n/R16R2. One additional 16SrV subgroup (16SrV-D) was recognized on the basis of a unique *Taq*I restriction site present in these regions in strain FD-D (Fig. 1e). Variations were also found in the spacer regions, but these were not used for 16SrV subgroup differentiation since 16Sr groups or subgroups were defined on the basis of 16S rRNA gene sequences. New members of each subgroup were designated on the basis of collective RFLP profiles (Table 1). Strains EY1^{T}, EY125, EY626 and EY627 belonged to subgroup 16SrV-A with profile type A; strains CLY5, JWB and PY-In belonged to subgroup 16SrV-B with profile type B; strains ALY, ALY882, ALY1068, HD1, FD 70, FD-C, SpaWB229 and SpaWB251 belonged to subgroup 16SrV-C with profile type C; strain FD-D belonged to subgroup 16SrV-D with profile type D; and strains RuSR19, RuS400, RuS971 and RUS belonged to subgroup 16SrV-E with profile type E. RFLP patterns of *Mse*I and *Alu*I digests of 16S rDNA were identical among all strains analysed, and the patterns were unique to this group (Lee *et al.*, 1998a) (Fig. 1, Table 1). Patterns of *Tha*I, *Sau*3AI and *Hae*III digests were also identical (data not shown).

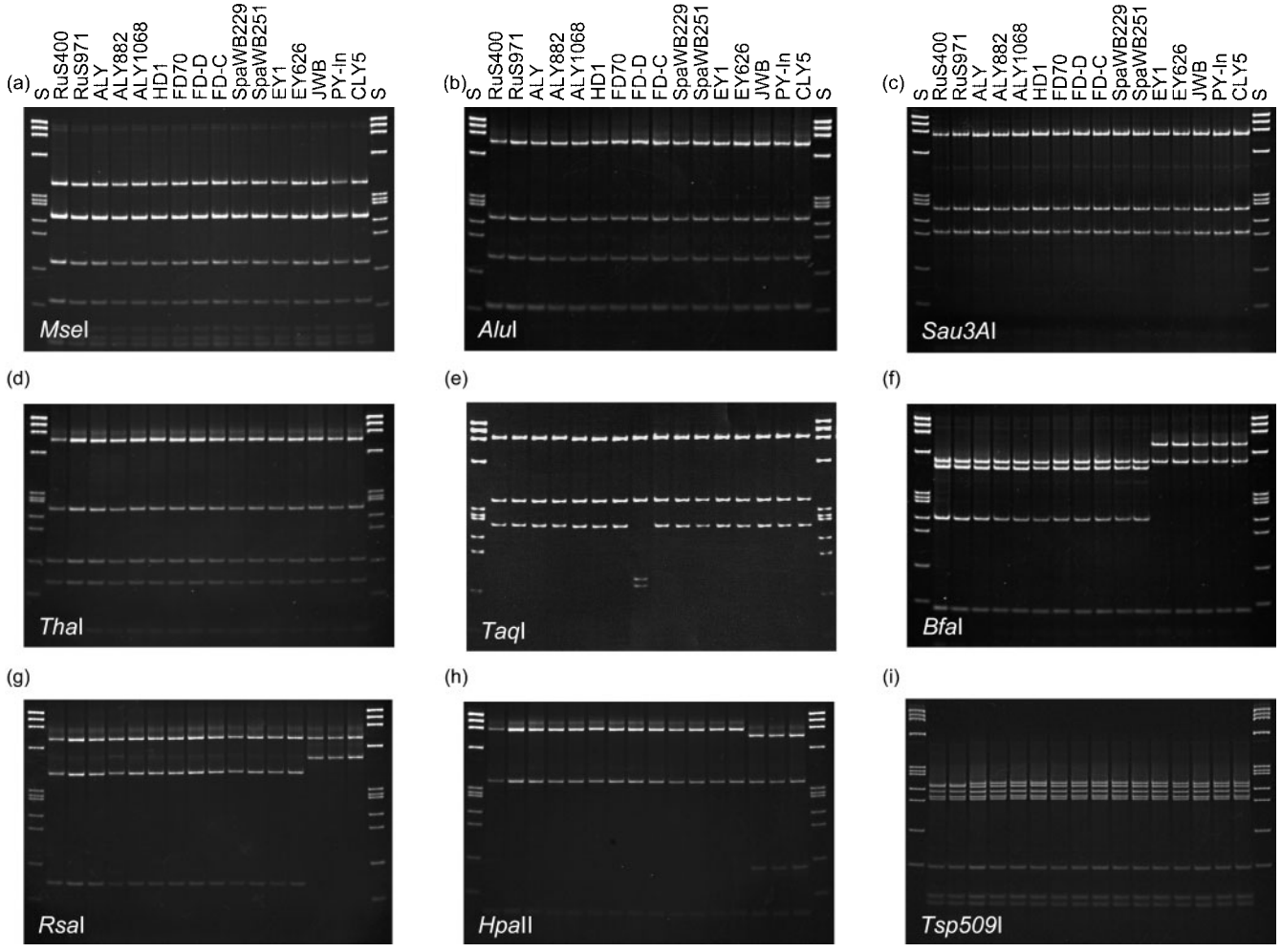

Fig. 1. RFLP profiles of 1·2 kb 16S rDNA (a–d, f–i) amplified by nested PCR with primer pair P1/P7 followed by primer pair R16F2n/R16R2 and 1·5 kb 16S rDNA (e) amplified by nested PCR with primer pair P1/P7 followed by primer pair P1A/16S-SR, from representative phytoplasma strains in the EY phytoplasma group (16SrV). PCR products were digested with *Mse*I (a), *Alu*I (b), *Sau*3AI (c), *Tha*I (d), *Taq*I (e), *Bfa*I (f), *Rsa*I (g), *Hpa*II (h) or *Tsp509*I (i) and separated by electrophoresis through 5 % (12 % for *Tsp509*I digests) polyacrylamide gels. Lanes S, ϕX174 replicating form I DNA *Hae*III digest; fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Other abbreviations are defined in Table 1.

RFLP analyses of ribosomal protein gene sequences resulted in finer differentiation among the 20 EY phytoplasma strains. Collective profiles obtained from digests with restriction enzymes *Mse*I, *Tsp509*I, *Alu*I, *Taq*I, *Hae*III, *Hpa*II, *Hha*I, *Dra*I and *Ssp*I differentiated 12 distinct ribosomal protein subgroups within the EY group (Fig. 2, Table 1). The RFLP pattern types with individual enzymes and ribosomal protein subgroup designations are summarized in Table 1. Seven distinct ribosomal protein RFLP pattern types (subgroups) were differentiated among members of 16SrV-C included in the present study, and three ribosomal protein RFLP pattern types (subgroups) were differentiated among members of 16SrV-B (Supplementary Table B). Eight ribosomal protein subgroups identified in the present study were consistent with those identified by Martini *et al.* (2002).

Subgroups delineated on the basis of putative restriction maps were generally consistent with those based on RFLP analyses (Supplementary Fig. A). Twelve rpV subgroups were differentiated on the basis of putative restriction sites; these subgroups are consistent with subgroups identified on the basis of actual RFLP analysis of the 1·2 kb ribosomal protein operon sequences.

Restriction map analysis of *secY* sequences delineated 13 subgroups, 12 of which coincided with their corresponding ribosomal protein subgroups (Table 1; see also Supplementary

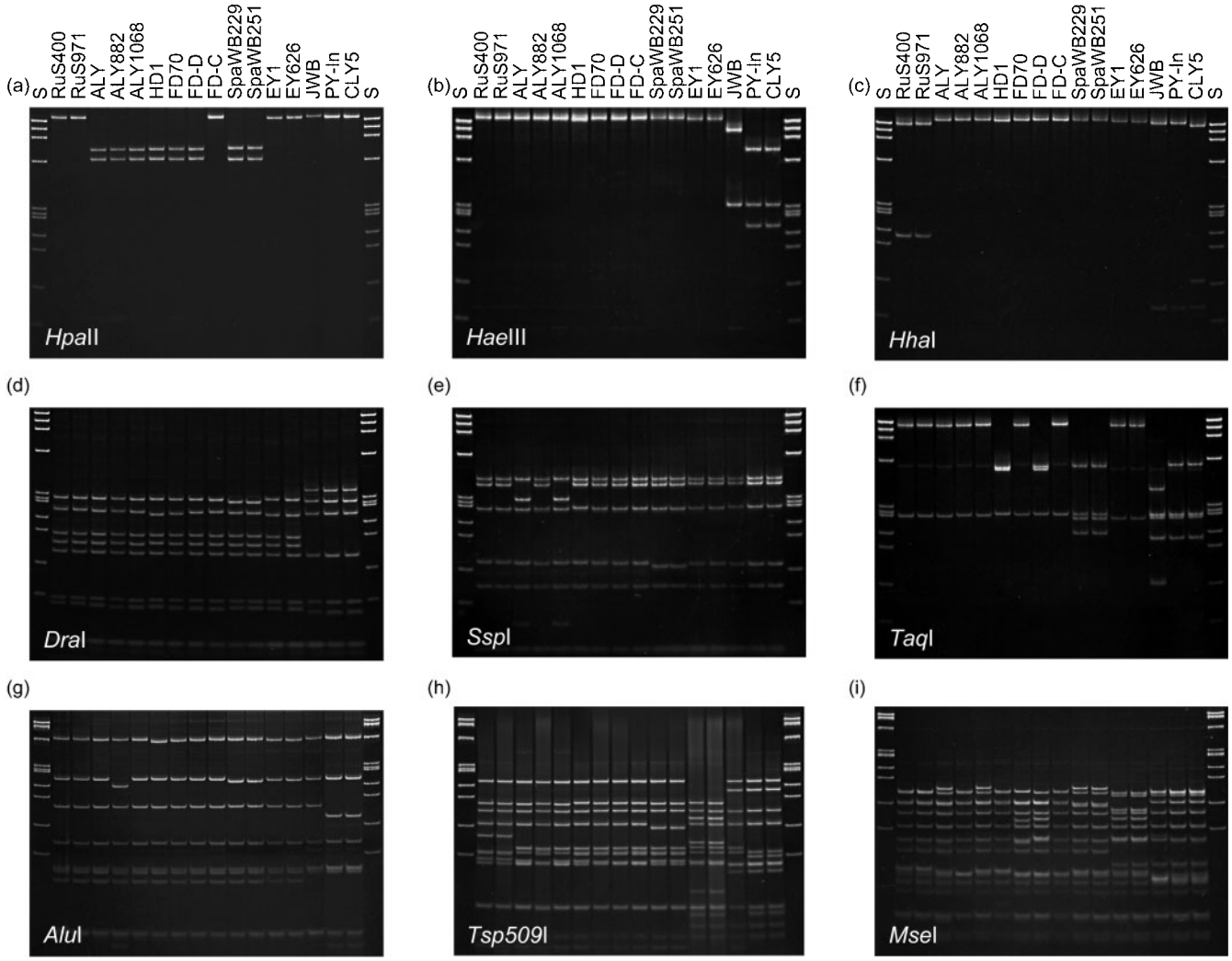

Fig. 2. RFLP profiles of ribosomal protein operon sequence (1·2 kb, contains *rpl22* and *rps3* genes), amplified by nested PCR with primer pair rp(V)F1/rpR1 followed by primer pair rp(V)F1A/rp(V)R1A from representative phytoplasma strains in the EY phytoplasma group (16SrV). (a)–(f) PCR products were digested with *Hpa*II (a), *Hae*III (b), *Hha*I (c), *Dra*I (d), *Ssp*I (e) or *Taq*I (f) and separated by electrophoresis through 5% polyacrylamide gels. Lanes S, ϕX174 replicating form I DNA *Hae*III digest (fragment sizes given in legend to Fig. 1). (g)–(i) PCR products digested with *Alu*I (g), *Tsp509*I (h) or *Mse*I (i) were separated through 12% polyacrylamide gels. Lanes S, ϕX174 replicating form I DNA *Hae*III digest (1353 bp fragment not shown). Other abbreviations are defined in Table 1.

Fig. B). Subgroup 16SrV-A strains were classified into two secYV subgroups, secYV-A (EY1^T) and secYV-M (EY626). The Italian strain EY626 contains additional *Mse*I and *Tsp509*I sites that distinguish it from strain EY1^T from the USA. This finding confirms previous results (based on RFLP analysis of the FD9f/r PCR fragment) reported by Daire *et al.* (1997) and Angelini *et al.* (2001).

Genetic variation assessed by comparative sequence analyses of three different conserved genes

Genetic variation was determined on the basis of three conserved genes, 16S rRNA, ribosomal protein and *secY*. Members of the EY phytoplasma group exhibited different degrees of genetic variability in these three genes. On the basis of the 16S rRNA gene, the mean sequence similarity ranged from 98·6 to 99·0 % among members of the EY phytoplasma group. However, more heterogeneity was evident when ribosomal protein and *secY* genes were analysed. The four EY phytoplasma strains (EY1^T, EY125, EY626 and EY627) in subgroup 16SrV-A, which are associated with EY-infected elms in North America and Europe, shared 99·9 % sequence similarity in the 16S rRNA gene, 99·7 % in the ribosomal protein genes and 99·5 % (based on two strains) in *secY*. The three strains (CLY5, PY-In and JWB) in subgroup 16SrV-B, which are associated with cherry, peach and jujube in Asia, shared 99·9 % sequence similarity in the 16S rRNA gene, 99·1–99·7 % in the ribosomal protein genes and 98·5–99·6 % in *secY*. In contrast, the seven representative strains (ALY, ALY882, HD1, FD70, FD-C, FD-D and SpaWB251) in subgroup 16SrV-C shared 99·7–99·9 % sequence similarity in the 16S rRNA gene, 94·3–99·0 % in the ribosomal protein genes and 91·1–97·2 % in *secY*. More genetic variation was observed when two 16SrV subgroups were compared. Mean sequence similarity among representative 16SrV subgroups ranged from 94·2 to 97·4 % for ribosomal protein genes and from

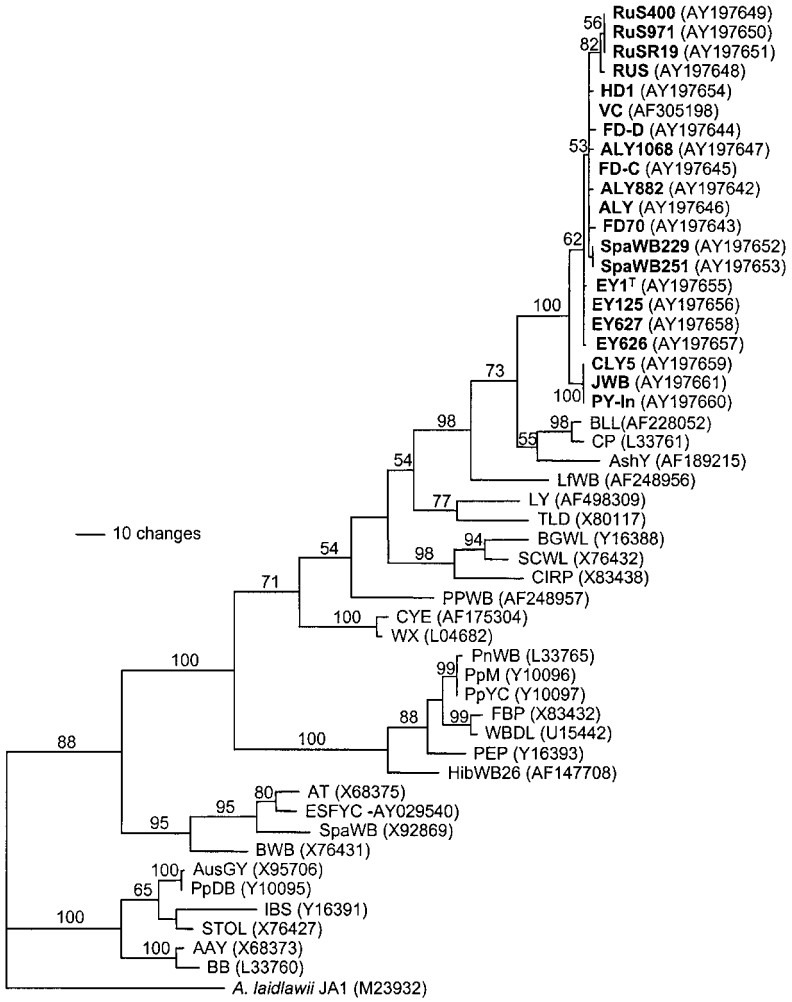

Fig. 3. Phylogenetic tree constructed by parsimony analysis of nearly full-length 16S rDNA sequences from representative phytoplasma strains in the EY group (16SrV) and other 16Sr phytoplasma groups. Sequences were aligned with CLUSTAL V. *A. laidlawii* JA1 was used as the outgroup. Bar, 10 inferred character-state changes. Branch lengths are proportional to the number of inferred character-state transformations. Bootstrap values are shown on branches. Phytoplasmas or '*Candidatus* Phytoplasma' species not included in Table 1 are: BLL, brinjal little leaf; CP, clover proliferation; AshY, ash yellows ('*Candidatus* Phytoplasma fraxini'); LfWB, loofah witches'-broom; LY, palm lethal yellowing; TLD, Tanzanian lethal decline of coconut; BGWL, bermudagrass white leaf; SCWL, sugar-cane white leaf; CIRP, cirsium phyllody; PPWB, pigeon pea witches'-broom; CYE, clover yellow edge; WX, Western X; PnWB, peanut witches'-broom; PpM, papaya mosaic; PpYC, papaya yellow crinkle ('*Candidatus* Phytoplasma australasia'); FBP, faba bean phyllody; WBDL, witches'-broom disease of lime ('*Candidatus* Phytoplasma aurantifolia'); PEP, *Picris echioides* phyllody; HibWB26, hibiscus witches'-broom ('*Candidatus* Phytoplasma brasiliense'); AT, apple proliferation; ESFYC, European stone fruit yellows; SpaWB, spartium witches'-broom; BWB, buckthorn witches'-broom; AUGY, Australian grapevine yellows ('*Candidatus* Phytoplasma australiense'); PpDB, papaya dieback; IBS, Italian bindweed stolbur; STOL, stolbur of pepper; AAY, American aster yellows; BB, tomato big bud/Arkansas. GenBank accession numbers are indicated.

83·2 to 91·2 % for *secY*. For example, the mean sequence similarity between subgroup 16SrV-A strains and strains in other subgroups was 99·46 % for 16S rRNA gene, 96·66 % for ribosomal protein genes and 86·48 % for *secY*. The mean sequence similarity between subgroup 16SrV-A strains and subgroup 16SrV-B strains was 99·3 % for 16S rRNA gene, 95·8 % for ribosomal protein genes and 80·46 % for *secY*. The EY group phytoplasmas exhibited slightly more variation in amino acid sequences deduced from the ribosomal protein and *secY* genes.

Phylogenetic relationships

Phylogenetic analysis using nearly full-length 16S rDNA sequences of 20 EY group phytoplasmas, 26 representative phytoplasmas of distinct phytoplasma groups or subgroups and *Acholeplasma laidlawii* (as an outgroup) resulted in 20 equally parsimonious trees. One of the most parsimonious trees is presented in Fig. 3. The EY group phytoplasmas represented a distinct strain cluster (or subclade) in the phytoplasma clade. The following are close relatives of the EY group: clover proliferation phytoplasma (reference strain for the 16SrVI group), brinjal little leaf phytoplasma and ash yellows phytoplasma, '*Candidatus* Phytoplasma fraxini' (reference strain for the 16SrVII group). Within the EY phytoplasma subclade, strain FD-D (subgroup 16SrV-D) and members of subgroup 16SrV-C clustered together. Flanked with this cluster were members of subgroup 16SrV-E, RuS phytoplasmas, and subgroup 16SrV-A EY phytoplasmas. Members of 16SrV-B (strains CLY5, JWB and PY-In) formed a distinct lineage distantly related to all other members of the EY group. However, because of the high degree of similarity shared by members of the EY phytoplasma group, not all of the 16Sr subgroups identified by RFLP analysis were resolved into distinct phylogenetic lineages based on 16S rRNA gene sequence. Phylogeny based on ribosomal protein genes revealed more phylogenetic divergence in the EY phytoplasma group. One of the seven most parsimonious trees is presented in Fig. 4(a). Twelve distinct phylogenetic lineages were resolved; these were clustered into three major phylogenetic groups, as indicated by high bootstrap values (>96 %): (i) a group including all representative phytoplasma strains in subgroups 16SrV-C, 16SrV-D and 16SrV-E; (ii) a group including four EY strains in subgroup 16SrV-A; and (iii) a group including strains CLY5, PY-In and JWB in subgroup 16SrV-B. The 12 lineages were consistent with designated ribosomal protein subgroups based on RFLP analysis (Table 1). However, the phylogenetic relationships among strains FD, ALY, RuS and SpaWB were not well resolved, as indicated by the low bootstrap value (<50 %). Phylogeny based on the *secY* gene delineated 12 distinct lineages similar to those based on ribosomal protein genes, but the branching patterns of strains FD, ALY, RuS and SpaWB differed (Fig. 4b). The divergence of the 12 lineages became more apparent on the basis of *secY*. Phylogenetic analysis based on combined 16S rRNA and ribosomal protein gene sequences, or based on combined 16S rRNA, ribosomal protein and *secY* gene sequences, resulted in a tree congruent with that inferred by analysis of the ribosomal protein or *secY* gene (data not shown). As indicated by nucleotide sequence analyses, phylogenetic analysis using amino acid sequences deduced from ribosomal protein and *secY* genes showed that strains CLY5, JWB and PY-In formed a distinct cluster highly divergent from all the other strains in the EY phytoplasma group (data not shown).

Conclusions

Sequence analysis of the 16S rRNA gene is probably the most widely used method for the determination of phylogenetic relationships and for molecular classification of micro-organisms (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994; Weisburg *et al.*, 1989; Woese, 1987). This approach has been very useful for the classification of micro-organisms at the genus level or for higher taxonomic ranks. Because of its highly conserved nature, however, the 16S rRNA gene sequence is not very useful for differentiation at the species level (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). Often, additional molecular, biochemical or biological criteria are needed for accurate speciation. We have

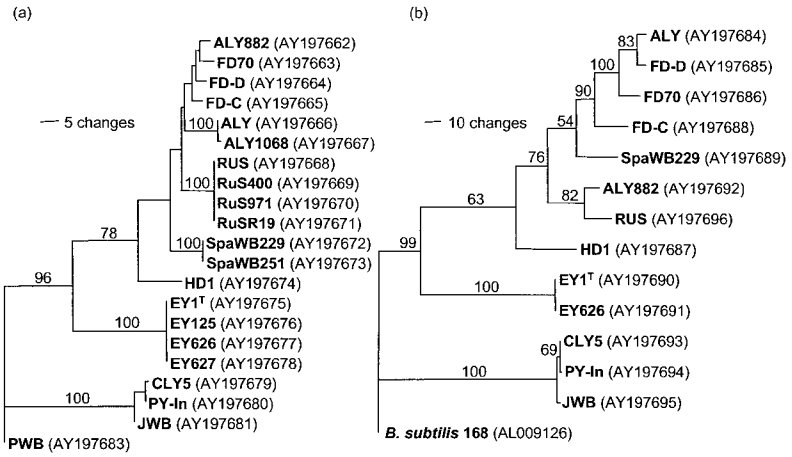

Fig. 4. Phylogenetic trees constructed by parsimony analyses of partial ribosomal protein operon (covering *rpl22* and *rps3* genes) (a) and *secY* gene (b) sequences from representative phytoplasma strains in the EY group (16SrV). Sequences were aligned with CLUSTAL V. Outgroup sequences are detailed in Methods. Bar length represents inferred character-state changes. Branch lengths are proportional to the number of inferred character-state transformations. Bootstrap values are shown on branches. Strain abbreviations are defined in Table 1. GenBank accession numbers are indicated.

encountered a similar situation with respect to phytoplasmal classification. In the past decade, phytoplasmas have been classified primarily on the basis of the 16S rRNA gene sequence (Lee *et al.*, 1998a; Seemüller *et al.*, 1998). More than 20 major phytoplasma groups and more than 40 subgroups have been identified. The difference in 16S rDNA sequence between two groups is 2·3 % or more. There is a consensus that each group should represent at least one separate species (Gundersen *et al.*, 1994; Jung *et al.*, 2002; Lee *et al.*, 1998a; Seemüller *et al.*, 1998). So far, of the nine '*Candidatus* Phytoplasma' species proposed (Davis *et al.*, 1997; Griffiths *et al.*, 1999b; Jung *et al.*, 2002, 2003; Montano *et al.*, 2001; Sawayanagi *et al.*, 1999; Verdin *et al.*, 2003; White *et al.*, 1998; Zreik *et al.*, 1995), two ('*Candidatus* Phytoplasma aurantifolia' and '*Candidatus* Phytoplasma australasia') belong to subgroups in the peanut witches'-broom group (=group 16SrII) according the classification scheme proposed by Lee *et al.* (1998a). The other six are '*Candidatus* Phytoplasma fraxini' (16SrVII-A), '*Candidatus* Phytoplasma australiense' (16SrXII-B), '*Candidatus* Phytoplasma japonicum' (a strain closely related to members of 16SrXII), '*Candidatus* Phytoplasma brasiliense' (16SrXV-A), '*Candidatus* Phytoplasma castaneae' (most closely related to palm lethal yellows group 16SrIV), '*Candidatus* Phytoplasma phoenicium' (16SrIV-B) and '*Candidatus* Phytoplasma ziziphi' (16SrV-B). According to the recently revised scheme, the EY phytoplasma group (=16SrV group) was subdivided into five subgroups on the basis of RFLP analyses of 16S rDNA sequences (Lee *et al.*, 1998a; Davis & Dally, 2001; Martini *et al.*, 1999). The variation in 16S rDNA sequence between two subgroups ranged from 0·2 to 1·4 %. The relatively small variation makes it unclear whether the EY phytoplasma group should represent more than one species. However, biological and ecological differences among these five subgroups strongly suggest that the EY group consists of highly heterogeneous phytoplasma strains.

In the present study, we performed comparative analyses using the 16S rRNA gene and two more variable genes, the ribosomal protein gene operon and the *secY* gene, encoding a translocase protein, for strain differentiation in the EY phytoplasma group. Phylogenetic analysis based on the ribosomal protein genes or *secY* indicated several distinct lineages in the EY phytoplasma group. This result has underscored the need to employ phylogenetic molecular parameters that are genetically more variable for finer strain differentiation within a given phytoplasma group that was defined on the basis of the highly conserved 16S rRNA gene. By including ribosomal protein and *secY* genes for analyses, we have gained some insights into the diversity of the EY group phytoplasmas.

In contrast to subgroups defined by 16S rDNA sequences, the majority of the subgroups differentiated on the basis of ribosomal protein or *secY* gene sequences appeared to be consistent with their specific ecological niches (specific host plants, vectors or geographical regions). For example, subgroup 16SrV-C consists of a rather heterogeneous phytoplasma strain cluster that is associated with diverse plant and insect hosts. The constituent members in subgroup 16SrV-C were subdivided into seven ribosomal protein subgroups, or seven secY subgroups, each of which is associated with a single, closely related plant host. Cross-infection of two or more plant species by members of a given ribosomal protein subgroup or secY subgroup seems to be rare, but a given disease (e.g. FD or ALY) could be attributed to more than one type of phytoplasma. For instance, Italian (strains ALY, ALY1068) and German (strain ALY882) ALY phytoplasma strains represented two distinct lineages on the basis of ribosomal protein or *secY* gene sequences. The phytoplasma strain cluster associated with FD in the grapevine was subdivided into four ribosomal protein subgroups (three identified in this work) (see Table 1): three subgroups occurred in Italy and two in France (Martini *et al.*, 2002). On the basis of an analysis of the FD9 DNA sequence, the German ALY strain 882 (subgroup rp-K) was very similar to grapevine yellows phytoplasma (Palatinate grapevine yellows) strains that were reported to cross-infect black alder and grapevine in Germany (Angelini *et al.*, 2001). Phytoplasma strains that are associated with grapevine yellows disease in grapevine in Germany are transmitted by the alder-feeding *Oncopsis alni* and cannot be transmitted by *Scaphoideus titanus*, the vector of FD phytoplasma strains associated with FD in France and Italy (Maixner *et al.*, 1995). Members of subgroup 16SrV-A, which are associated with *Ulmus* spp., and subgroup 16SrV-E, which are associated with *Rubus* spp., represented two rather homogeneous strain clusters, based on phylogenetic analyses of both 16S rRNA and ribosomal protein genes. Members of subgroup 16SrV-B (consisting of CLY5, PY-In and JWB) were subdivided into three ribosomal protein subgroups or three secY subgroups. The three strains are associated with different plant hosts, and strain JWB is known to be transmitted by *Hishimonoides chinensis* (Tian *et al.*, 2000; Zhu *et al.*, 1997).

Phylogenies based on the ribosomal protein and *secY* genes revealed that phytoplasma strains that constitute the EY group are more heterogeneous than was indicated by analysis based on the 16S rRNA gene. On the basis of combined analyses of 16S rRNA, ribosomal protein and *secY* gene sequences, the EY phytoplasma group clearly consists of at least three genetically distinct strain clusters represented by subgroups 16SrV-A, 16SrV-B and 16SrV-C. The degree of genetic variability among strain clusters increased when analysed using ribosomal protein or *secY* gene sequences. The phylogenetic divergence and ecological constraints specific for each strain cluster in the EY phytoplasma group suggest that each strain cluster should represent one species.

According to the recommendation of the International Committee of Systematics of Prokaryotes Subcommittee on the taxonomy of *Mollicutes*, two phytoplasmas that share more than 97·5 % 16S rRNA gene sequence similarity but

clearly represent ecologically separated populations can be designated as separate '*Candidatus* Phytoplasma' species if they meet the following three criteria: (i) they are transmitted by different vectors; (ii) the two phytoplasmas have a different natural plant host(s); and (iii) there is evidence of molecular diversity between the two phytoplasmas (Anonymous, 2000). All three distinct phylogenetic strain clusters resolved by analysis of the ribosomal protein gene operon or the *secY* gene appear to fulfil the first two criteria and some of the third criterion as well. We propose that each of the three strain clusters should be a potential candidate for a separate '*Candidatus*' species when the third criterion is fulfilled.

A novel '*Candidatus*' species has been proposed for strain JWB, a member of the 16SrV-B subgroup (Jung *et al.*, 2003). In this work, we propose that EY phytoplasma strain EY1^T (a member of the 16SrV-A subgroup) be assigned to a novel '*Candidatus*' species on the basis of three criteria. (i) The 16SrV-A phytoplasmas shared $<97{\cdot}5\,\%$ sequence similarity with all known phytoplasmas belonging to other phytoplasma groups and showed 98·2 % 16S rRNA gene sequence similarity, 93 % similarity in the ribosomal protein genes and 81·0 % similarity in the *secY* gene with respect to strain JWB phytoplasma. (ii) Phylogenetic analyses based on the three genes clearly indicated that the 16SrV-A strain cluster (consisting of strains EY1^T, EY125, EY626 and EY627) represents a distinct lineage divergent from the 16SrV-B strain cluster (consisting of CLY5, PY-In and JWB) in the EY group. Subgroups 16SrV-A and 16SrV-B can be differentiated by PCR using a primer pair specific to subgroup 16SrV-B (designed previously; Zhu *et al.*, 1997). (iii) There are no plant hosts or vectors common to 16SrV-A and 16SrV-B phytoplasmas; strain EY1^T was transmitted by *Scaphoideus luteolus* (Sinclair *et al.*, 1976). On the basis of the guidelines proposed by Murray & Schleifer (1994), the 16SrV-A phytoplasma EY1^T is designated as a novel species with the following description. '*Candidatus* Phytoplasma ulmi': [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession nos AY197655, AY197675, AY197690); oligonucleotide sequences of unique regions of 16S rRNA, *rpl22–rps3* and *secY* genes, as shown in Table 2. P (*Ulmus americana*, phloem); M]. EY1^T is the reference strain. Related phytoplasmas include strains EY125, EY626 and EY627, associated with *Ulmus minor*. 16SrV-A phytoplasma strains cause diseases in *Ulmus* spp. and induce symptoms including epinasty, yellowing, dwarfing and premature casting of leaves, witches'-brooms at the tips of twigs and branches and precocious opening of vegetative buds.

Table 2. Sequences unique to '*Candidatus* Phytoplasma ulmi' in 16S rRNA, *rpl22–rps3* and *secY* genes

Gene	Signature sequence (5′–3′)	Position
16S rRNA	GGAAA	827–835
	CGTTAGTTGCC	1098–1108
rpl22–rps3	TTACGCTTGCC	284–294
	CATTTAATAAAATTGCTATT	739–758
	AAATTCTATTTCTATGGGAAT	910–932
secY	TTTGATCCAATGTTAA	350–365
	GTCTTTCGGTCATGGATTGA	595–614
	ATTTAGTCTAAT	616–627
	CAAATAGAACAA	1053–1064

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