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Spiroplasma citri: A Wide Host Range Phytopathogen

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Abstract: Spiroplasmas are helical motile filamentous, wall-less and culturable mollicutes. Thirty six spiroplasma species have been identified. Only *S. citri, S. kunkelii* and *S. phoeniceum* have been identified as plant pathogens. *Spiroplasma citri*, the causal agent of citrus stubborn disease, have a wide host range. *S. citri* infects most citrus species and cultivars and a wide range of non-rutaceous plant species. Citrus srubborn disease widely distributed in the southwestern united states of America, northern Africa and Mediterranean countries. It is naturally transmitted by phloem-feeding leafhopper vectors. *S. citri* can be detected by grafting to citrus indicators, culturing on artificial media, serological, DNA probes, dot-immunobinding assay, Immunocapture Polymerase Chain Reaction (I C- PCR), Polymerase Chain Reaction (PCR) and real-time PCR. There is genetic variability among isolates of *S. citri.*

Key words: *Spiroplasma* species, stubborn, non-rutaceous host, detection

INTRODUCTION

Spiroplasmas (spira: a coil, spiral; plasma: something formed or molded) are motile, filamentous, wall-less and culturable mollicutes (Whitcomb and Tully, 1982; Bove, 1997). Spiroplasmas have helical, tubular and pleomorphic morphology. Extracellular spiroplasmas are more helical and tubular whilst round or flask forms are more dominant in intracellular spiroplasmas (Ammar *et al*., 2004). They were discovered from infected corn (*Zea mays* L.) plants with the corn stunt agent by dark-field microscopy (Davis *et al*., 1972). In 1971, *Spiroplasma citri* was cultured in artificial media (Saglio *et al*., 1971). The genus *Spiroplasma* was stablished in 1973 through studies on motile and helical microorganism associated with corn stunt disease (Davis and Worley, 1973). In 1973, the first spiroplasma species has been named as *Spiroplasma citri* as well (Saglio *et al*., 1973). *S. citri* is the type species of the type genus *Spiroplasma* of the family Spiroplasmataceae. Taxonomically, Spiroplasmas are in the kingdom Bacteria, Phylum Tenericutes, Class Mollicutes, Order Entomoplasmatales, Family Spiroplasmataceae and Genus Spiroplasma (Gasparich, 2002, 2010; Tully *et al*., 1987; Williamson *et al*., 1998). Based on the spiroplasma species concept as determined by the International Research Programme on Comparative Mycoplasmology and the International Subcommittee on the Taxonomy of Mollicutes, 36 spiroplasma species have been identified (Whitcomb *et al*., 1987; Whitcomb, 1977; Wang *et al*., 2010) (Table 1). Only *S. citri, S. kunkelii* and *S. phoeniceum* have been identified as plant pathogens.

The genome size of spiroplasmas ranges from 780 to 2220 kbp (Carle *et al*., 1992, 1995; Williamson *et al*., 2010) and the *S. citri* genome is one of the largest among Mollicutes with a high adenosine-thymidine content (up to 75%) (Melcher and Fletcher, 1999). G+C base composition of spiroplasmas ranges from 24-31% (Carle *et al*., 1983; Gasparich *et al*., 2004). They are helical filaments with 3-15 µm length range and 200-250 nm in width and an amplitude of 0.4 µm (Fletcher *et al*., 2006). Spiroplasmas require sterol for growth. They are resistance to penicillin. In spiroplasmas, UGA is not used as a stop codon. They use both UGA and UGG as tryptophan codons (Citti *et al*., 1992; Renaudin *et al*., 1986; Stamburski *et al*., 1992). In most mollicutes, comprising spiroplasmas, UAA and UAG are possible termination codons (Melcher and Fletcher, 1999).

***Spiroplasma citri*:** *Spiroplasma citri*, the causal agent of citrus stubborn disease, is restricted to the phloem sieve tubes in plants. It is an obligate parasite, surviving in citrus or in a variety of other host plants, with no saprophytic phase. Citrus Stubborn Disease (CSD) was first observed in Washington navel orange trees in California about 1915 (Fawcett *et al*., 1944).

Spiroplasma citri infects most citrus species and cultivars and a wide range of non-rutaceous plant species

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Table 1: Spiroplasmas classification and characterization

Species	Group	Accession No. of type strain	Host	References
Spiroplasma alleghenense	XXVI	AY189125	scorpionfly	Adams *et al.* (1997)
Spiroplasma apis	IV	AY736030	honey bee,flowers	Mouches *et al.* (1983)
Spiroplasma cantharicola	XVI-1	DQ861914	cantharid beetle	Whitcomb *et al.* (1993a)
Spiroplasma chinense	XXIV	AY189126	Calystegia hederaceae	Guo *et al.* (1990)
Spiroplasma chrysopicola	VIII-2	AY189127	Crysops sp. flies	Whitcomb *et al.* (1997a)
Spiroplasma citri	I-1	X63781	plant phloem/leafhopper	Saglio *et al.* (1973)
Spiroplasma clarkii	IX	M24474	Cotinus beetles	Whitcomb *et al.* (1993b)
Spiroplasma corruscae	XIV	AY189128	Ellychnia corrusca beetles/ horse flies	Hackett *et al.* (1996a)
Spiroplasma culicicola	X	AY189129	Aedes mosquitoes	Hung *et al.* (1987)
Spiroplasma diabroticae	XII	M24482	Diabrotica undecimpuntata beetles	Carle *et al.* (1997)
Spiroplasma diminutum	XXV	AY189130	Culex mosquito	Williamson *et al.* (1996)
Spiroplasma eriocheiris			Chinese mitten crab	Wang *et al.* (2010)
Spiroplasma floricola	III	AY189131	Cockchafer, flowers	Davis *et al.* (1981)
Spiroplasma gladiatoris	XXIII	M24475	Tabanus gladiator	Whitcomb *et al.* (1997a)
Spiroplasma helicoides	XXXII	AY189132	horse fly	Whitcomb *et al.* (1997b)
Spiroplasma insolitum	I-6	AY189133	Flowers, Eristalis flies	Hackett *et al.* (1993)
Spiroplasma ixodetis	VI	M24477	Ixodes pacificus ticks	Tully *et al.* (1995)
Spiroplasma kunkelii	I-3	-	Maize/leafhopper	Whitcomb *et al.* (1986)
Spiroplasma lampyridicola	XIX	AY189134	Photuris pennsylvanicus (firefly beetles)	Stevens *et al.* (1997)
Spiroplasma leptinotarsae	XX	AY189305	Leptinotarsa decemlineata (Colorado Potato Beetle)	Hackett *et al.* (1996b)
Spiroplasma lineolae	XXVII	DQ860100	horse fly	French *et al.* (1997)
Spiroplasma litorale	XVIII	AY189306	Tabanus nigrovittatus	Konai *et al.* (1997)
Spiroplasma melliferum	I-2	AY325304	honey bee	Clark *et al.* (1985)
Spiroplasma mirum	V	M24662	rabbit tick	Tully *et al.* (1982)
Spiroplasma monobiae	VII	M24481	Monobia wasps	Whitcomb *et al.* (1993c)
Spiroplasma montanense	XXXI	AY189307	horse fly	Whitcomb *et al.* (1997b)
Spiroplasma penaei	I-9	AY771927	Pacific white shrimp	Nunan *et al.* (2005)
Spiroplasma phoeniceum	I-8	AY772395	Periwinkle/leafhopper	Saillard *et al.* (1987)
Spiroplasma platyhelix	XXVIII	AY800347	Dragonfly	Williamson *et al.* (1997)
Spiroplasma poulsonii	II	M24483	Drosophila	Williamson *et al.* (1999)
Spiroplasma sabaudiense	XIII	AY189308	Aedes mosquitoes	Abalain-Colloc*etal.*(1987)
Spiroplasma syrphidicola	VIII-1	AY189309	Eristalis arbustorum flies	Whitcomb *et al.* (1996)
Spiroplasma tabanidicola	XXXIII	DQ004931	horse fly	Whitcomb *et al.* (1997a)
Spiroplasma taiwanense	XXII	M24476	Culex tritaeniorhynchus	Abalain-Colloc*etal.*(1988)
Spiroplasma turonicum	XVII	AY189310	horse fly	Helias *et al.* (1998)
Spiroplasma velocicrescens	XI	AY189311	Monobia wasp	Konai *et al.* (1995)

(Gumpf and Calavan, 1981; Oldfield and Calavan, 1980; Whiteside *et al*., 1988). Citrus is the main economic host of *Spiroplasma citri*. The name "stubborn" should be restricted to the disease in citrus.

Genome characteristics: G+C content of *S. citri* GII3-3X chromosome is 26.1%. It encodes one single 16S-23S-5S rRNA operon (Carle *et al*., 2010). Several pathways have been identified in *S. citri*, including phosphoenolpyruvate Phosphotransferase System (PTS) to import sugars to synthesize ATP using F_0F_1-ATP synthase, purine and pyrimidine metabolism pathways, pathway for the biosynthesis of a C55 terpenoid, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway to the synthesis of isopentenyl pyrophosphate, glycolytic and lactate dehydrogenase pathways to enhance fermentation. *S. citri* chromosome also encodes essential subunits for ATP synthase and ATP Binding Cassette (ABC) transporters. Whilst 21% of truncated CDS in *S. citri* GII-3X chromosome compared to their bacterial orthologs, reveals an important gene decay. It indicate that the reductive evolution of the spiroplasma genome to smaller genomes may be is still ongoing on the way (Carle *et al*., 2010).

There are many Coding Sequences (CDS) of plectovirus in the *S. citri* chromosome (Carle *et al*., 2010). *Spiroplasma citri* use fructose for pathogenicity and growth in plants (Andre *et al*., 2005; Gaurivaud *et al*., 2000). Spiralin, the most abundant and major membrane lipoprotein of 26 kDa, is essential for transmission of *S. citri* by the leafhopper vector *Circulifer haematoceps* (Duret *et al*., 2003).

Host range: Stubborn is an important disease of citrus. Sweet orange (*C. sinensis* (L.) Osbeck), sour orange (*C. aurantium* L.), mandarin (*C. reticulata* Blanco), grapefruit (*C. paradisi Macfad.*), lemon (*C. limon* (L.) Burm.f.), pomelo (*C. maxima* Merr.), sweet lime (*C. limettioides* Tan.), Rangpur (*C. limonia* Osbeck), Calamondin (*C. madurensis* Lour.), rough lemon (*C. jambhiri* Lush.), satsuma mandarin (*C. unshiu* Marcow), tangelo (*C. paradisi* x *C. reticulata*), kumquat

(Fortunella spp.) and citrange (*C. sinensis* x Poncirus trifoliata) are susceptible to infection. Acid limes, trifoliate orange and trifoliate orange hybrids are tolerant (Calavan, 1980; Whiteside *et al.*, 1988).

Many varieties of sweet orange comprising Washington navel, Valencia, Thomson navel, Frost Navel, Frost Valencia, Washington sanguine, Hamlin, Cadenera, Portugaise, Surprise navel, Beni Selman, Petit Jaffa, local cultivar of Fars and Ramsar Number 4 are infected to *S. citri* with different range of suscebtibility (Childs and Carpenter, 1960; Nejat *et al.*, 2007).

Citrus is not the only host plant of *S. citri*. Many non-citrus plant species throughout the world have been also found to naturally and experimentally infected with *S. citri* (Table 2, 3).

Periwinkles (*Catharanthus roseus* (L.) G. Don) were the first non-rutaceous plants to have been found naturally infected by the *Spiroplasma citri* in California and Arizona (Allen, 1975; Granett *et al.*, 1976) and then has been found in Mediterranean countries including Syria, United Arab Emirates, Oman, Cyprus and Turkey (Bove, 1986) and Malaysia (Nejat *et al.*, 2011).

Symptoms: Stubborn disease can much reduces the quality and quantity of yields. Affected trees by stubborn disease usually are stunted and have a dense or bunchy type and upright position of growth with shortened stem internodes and multiple axillary buds. Typical leaves symptoms of stubborn are small, to call the disease little leaf, cupped shaped with rounded tip and leathery appearance. Infected leaves sometimes indicate a variety of chlorotic or mottled resembling zinc, iron and manganese deficiencies.

Fruits symptoms on stubborn-affected citrus trees is variable and include small, lopsided (curved columella), acorn-shaped with stem-end peel of normal thickness and thin rind at stylar end, stylar-end greening (retention of green color of the stylar end after ripening and become orange color of fruit) and small fruits drop. The taste of the diseased-fruits sometimes is insipid or bitter and show seed abortion (Fig. 1). (Bove, 1995; Calavan, 1968, 1979; Calavan and Carpenter, 1965; Calavan and Oldfield, 1979; Fawcett *et al.*, 1944; Gumpf and Calavan, 1981).

Naturally infected periwinkle showing the following symptoms: Rapid decline in the number and size of the flowers were observed until flowering ceased. The buds and flowers were abscised prematurely with the reduction in leaf size and yellowing of the leaves that starts from the margin and tip, progressing to the center part. General chlorosis starts from down part, proliferation of auxillary buds, stunting and death.

Geographical distribution: Citrus srubborn disease widely distributed in the southwestern united states of America, northern Africa, Mediterranean countries and Southeast Asia including the Arizona, California, Illinois and Maryland in the United States of America, France, Greece, Italy, Spain, Libya, Algeria, Cyprus, Egypt, Iran, Iraq, Jordan, Lebanon, Palestine, Morocco, Oman, Saudi

Table 2: Natural non-rutaceous hosts of Spiroplasma citri

Species	Common name	Family	Country	Reference
Daucus carota L.	Carrot	Apiaceae	United state	Lee *et al.* (2006)
Aster amellus L.	Aster	Asteraceae	Iran	Nejat *et al.* (2004)
Crepis echioides (L.) All.	Ox tongue	Asteraceae	Turkey	Kersting *et al.* (1992)
Tagetes spp. L.	Marigold	Asteraceae	United state	Allen and Donndelinger (1981)
Zinnia sp. L.	Zinnia	Asteraceae	United state	Allen and Donndelinger (1981)
Echium sp. L.	Bristly oxtongue	Boraginaceae	Turkey	Kersting *et al.* (1992)
Armoracia rusticana Gaertn., Mey and Scherb	Horseradish	Brassicaceae	United state	Gumpf and Calavan (1981)
Brassica rapa L.	Turnip	Brassicaceae	United state	Gumpf and Calavan (1981)
B. nigra L.	Black mustard	Brassicaceae	United state	Gumpf and Calavan (1981)
Hirschfeldia incana (L.) Lagr.-Foss. (B. geniculata)	Short pod mustard or wild mustard	Brassicaceae	United state	Gumpf and Calavan (1981)
B. tourneortii Gouan.	Asian mustard	Brassicaceae	United state	Gumpf and Calavan (1981)
B. pekinensis Lour	Chinese cabbage	Brassicaceae	United state	Gumpf and Calavan (1981)
Brassica chinensis L.	Pak choi	Brassicaceae	United state	Gumpf and Calavan (1981)
Raphanus sativus L.	Radish	Brassicaceae	United state	Gumpf and Calavan (1981)
Raphanus raphanistrum L.	Wild radish	Brassicaceae	United state	Gumpf and Calavan (1981)
Sisymbrium irio L.	London rocket	Brassicaceae	United state	Gumpf and Calavan (1981)
Convolvulus arvensis L.	Field bindweed	Convolvulaceae	Iran	Nejat *et al.* (2004)
Cucurbita pepo L.	Squash	Cucurbitaceae	United state	Allen and Donndelinger (1981)
Sesamum indicum L.	Sesame	Pedaliaceae	Iran, Turkey	Salehi and Izadpanah (2002) Kersting *et al.* (1992)
Plantago sp. L.	Plantain	Plantaginaceae	United state	Calavan and Oldfield (1979)
Sorghum halepense (L.) Pers.	Johnsongrass	Poaceae	Turkey	Kersting *et al.* (1993)
Viola sp.	Viola	Violaceae	United state	Oldfield and Calavan (1980)

Table 3: Experimental non-rutaceous hosts of Spiroplasma citri

Species	Common name	Family	Country	Reference
Tetragonia tetragonioides (Pallos) Kuntze	New Zealand Spinach	Aizoaceae	United states	Oldfield and Calavan (1980)
Alium cepa L.	Onion	Alliaceae	United states	Calavan and Oldfield (1979)
Apium graveolens L.	Celery	Apiaceae	United states	Oldfield and Calavan (1980)
Daucus carota L.	Carrot	Apiaceae	Iran	Nejat *et al.* (2006)
Bellis perennis L.	Common Daisy	Asteraceae	United states,	Oldfield and Calavan (1980)
Callistephus chinensis (L.) Nees	China aster	Asteraceae	United state	Calavan and Oldfield (1979) Fletcher (1983)
Chrysanthemum maximum hort.	Shasta daisy	Asteraceae	United states,	Calavan and Oldfield (1979)
Barbarea vulgaris R.Br.)	Yellow rocket	Brassicaceae	United states,	Fletcher (1983)
Brassica kaber (DC.) L. C. Wheeler	Wild mustard	Brassicaceae	United states,	Fletcher (1983)
Brassica napobrassica (L.) Mill.	Rutabaga	Brassicaceae	United states,	Calavan and Oldfeild (1979)
B. oleracea L. var. *botrytis*	Broccoli	Brassicaceae	United state,	Calavan and Oldfield (1979)
B. oleracea L. var *capitata*	Cabbage	Brassicaceae	United state	Calavan and Oldfield (1979)
B. oleracea L. var. *gonglyodes*	Kohlrabi	Brassicaceae	United state	Oldfield and Calavan (1980)
B. Oleracea var. *viridis* L.	Kale	Brassicaceae	United states	Oldfield and Calavan (1980)
Capsella bursa-pastoris (L.) Medik.	Shepherd' purse	Brassicaceae	United state, Iran	Fletcher (1983) Nejat *et al.* (2006)
Descurainia sophia L.	Flixweed	Brassicaceae	United state,	Oldfield and Calavan (1980)
Eruca sativa Mill.	Rocket	Brassicaceae	Iran	Nejat *et al.* (2006)
Erysimum hieraciifolium L.	European wallflower	Brassicaceae	United state	Oldfield and Calavan (1980)
Erysimum repandum L.	Spreading wallflower	Brassicaceae	Iran	Nejat *et al.* (2006)
Lunaria annua L.	Honesty	Brassicaceae	United state	Oldfield and Calavan (1980)
Matthiola incana (L.) W.T.Aiton	Stock	Brassicaceae	United states	Oldfield and Calavan (1980)
Myagrum perfoliatum L.	Bird's-eye cress	Brassicaceae	Iran	Nejat *et al.* (2006)
Sinapis arvensis L.	Charlock mustard	Brassicaceae	Iran	Nejat *et al.* (2006)
Sisymbrium altissimum L.	Tall tumblemustard	Brassicaceae	United state	Oldfield and Calavan (1980)
Dianthus barbatus L.	Sweetwilliam	Caryophyllaceae	United states	Calavan and Oldfield (1979)
Cucumis sativus L.	Cucumber	Cucurbitaceae	United states	Oldfield and Calavan (1980)
Trifolium repens L.	White clover	Fabaceae	United states	Calavan and Oldfield (1979)
Cicer arietinum L.	Chickpea	Fabaceae	United states	Oldfield and Calavan (1980)
Lathyrus odoratus L.	Sweet pea	Fabaceae	United states	Oldfield and Calavan (1980)
Lupinus polyphyllus Lindl.	Bigleaf lupine	Fabaceae	United states	Oldfield and Calavan (1980)
Alcea rosea L.	Hollyhock	Malvaceae	United states	Calavan and Oldfield (1979)
Eschscholzia californica Cham.	California poppy	Papaveraceae	United states	Oldfield and Calavan (1980)
Phlox drummondi Hook.	Annual Phlox	Polemoniaceae	United states	Fletcher (1983)
Prunus avium (L.) L.	Wild cherry	Rosaceae	Illinois	Calavan and Oldfield (1979)
Delphinium sp.	Larkspur	Ranunculaceae	United states	Calavan and Oldfield (1979)
Nigella damascene L.	Love-in-a-Mist	Ranunculaceae	United states	Calavan and Oldfield (1979)
Nigella sativa L.	Black cumin	Ranunculaceae	Iran	Nejat *et al.* (2006)
Schizanthus sp.		Solanaceae	United state	Oldfield and Calavan (1980)
Tropaeolum majus L.	Nasturtium	Tropaeolaceae	United state	Calavan and Oldfield (1979)

Arabia, , Syria, Tunisia, Turkey, Pakistan, Yemen and the United Arab Emirates in Mediterranean and Middle East areas, Mexico and Malaysia (Bove, 1995; Nejat *et al.*, 2011). The disease does not appear to be a problem in cool areas or areas with warm, humid climates (Whiteside *et al.*, 1988). While there is *Spiroplasma citri* in equatorial areas and can cause severe symptoms and lethal disease on periwinkle (Nejat *et al.*, 2011).

Transmission: It is naturally transmitted by phloem-feeding leafhopper vectors in propagative manner: *Circulifer tenellus* Baker, *Scaphytopius nitridus* Delong and *S. acutus delongi* (Order Hemiptera, suborder Homoptera, family Cicadellidae) in California (USA) (Kaloostian *et al.*, 1979; Mello *et al.*, 2009; Oldfield, 1988; Oldfield *et al.*, 1976, 1977), *Neoaliturus haematoceps* Mulsant and Rey (Bove *et al.*, 1986) and *C. tenellus* (Klein *et al.*, 1988) in the Mediterranean area. *S. citri* has been also transmitted experimentally by *Euscelis plejebus* Fallen (Markham and Towsend, 1974; Towsend *et al.*, 1977) and *Macrosteles fascifrons* (O'Hayer *et al.*, 1983). It is graft-transmissible through infected budwood but is neither seed nor mechanically transmissible (Whiteside *et al.*, 1988; Rangel *et al.*, 2005). It can be also transmitted via the parasitic plant, dodder (*Cuscuta compestris* or *C. subinclusa*) (Lee *et al.*, 2001).

***S. citri* detection:** *S. citri* can be detected by grafting to citrus indicators, culturing on artificial media, serological, DNA probes, dot-immunobinding assay, Immunocapture Polymerase Chain Reaction (I C- PCR), Polymerase Chain Reaction (PCR) and real-time PCR.

S. citri can be detected by graft inoculation of indicator plants of which the most suitables are sweet orange cv. Madame Vinous, grapefruit cv. Marsh and tangelo cv. Sexton, respectively (Bove, 1988). Side grafting is more successful than bud grafting to transmit *S. citri* (Rangel *et al.*, 2005).

Phytopathogenic spiroplasmas is often detected by cultivation in artificial media. Several media have been

Fig. 1: Citrus stubborn disease symptoms

used for the cultivation of *S. citi* such as: SMC (Saglio *et al*., 1971, 1973), C-3 (Chen and Liao, 1975), M-1 (Williamson and Whitcomb, 1975); LD8 (Lee and Davis, 1978, 1984); SP4 (Whitcomb, 1983). *S. citri* culturing is time-consuming and 2-3 weeks need to growth *S. citri* in culture media and contamination can cause by non-target microorganisms (Rangel *et al*., 2005).

The Enzyme-linked Immunosorbent Assay (ELISA) with polyclonal and monoclonal antibodies as a sensitive serological method has been applied for identifying spiroplasmas directly in plant or insect material (Archer and Best, 1980; Archer *et al*., 1982; Clark *et al*., 1978; Lin and Chen, 1985; Saillard *et al*., 1980, 1993; Tully *et al*., 1973).

The DNA probes and dot-immunobinding assay (DIMA) were applied to detect the *Spiroplasma citri* in medium, infected plants and insects. DIMA is rapid assay but less sensitive than ELISA while DNA probe has a high level of sensitivity (Fletcher, 1987; Nur *et al*., 1986; Saillard *et al*., 1993).

Immuno-capture (IC)-PCR method has been applied for detection of stubborn (El-Banna *et al*., 2005; Saillard *et al*., 1993, 1996).

Polymerase Chain Reaction (PCR) is useful method for spiroplasma detection in infected plant phloem or insect vectors with 100 -1000 times of sensitivity greater than ELISA and it are also more rapid than serological techniques and culturing (Fletcher *et al*., 2006; Rangel *et al*., 2005). PCR detection of *S. citri* has been used with primers based on gene sequences for spiralin (Foissac *et al*., 1996), 16S rRNA gene in particular spiroplasma infection of carrot in the United States (Lee *et al*., 2006), Putative P89 adhesin and Putative P58 adhesin-like genes (Yokomi *et al*., 2008) (Table 4).

There is genetic diversity among isolates of *S. citri* based on the Crossed Immunoelectrophoresis (CIE) with intermediate gel and polyacrylamide gel electrophoresis, three repetitive extragenic palindromic elements (BOX, ERIC and REP), random amplified polymorphic DNA (RAPD) and spiroplasma bacteriophage insertions as discriminative techniques (Mello *et al*., 2006, 2008; Omar *et al*., 2006).

Control: Stablish *S. citri* –free mother trees and citrus nurseries in locations where very low or no spread of *S. citri* occur. Elimination of brassicaceous weed hosts

Table 4: Several oligonucleotide primers used for spiroplasma detection by PCR and real-time PCR

Primer set	Primer sequence (5'-3')	Location	Expected size of amplicon	Reaction	Reference
ScR16F1	AGGATGAACGCTGGCGGCAT	16S	1800 bp	Conventional	Lee *et al*. (2006)
ScR16R1	GTAGTCACGTCCTTCATCGT			PCR	
ScR16F1A	GCATGCCTAATACATGCAAG	16S	1500 bp	Nested PCR	Lee *et al*. (2006)
ScR16R2	ATCCATCCGCACGTTCTCGTAC				
D	GTATAAAGTAGGGTTAGAAGC	Spiralin	1053 bp	Conventional	Foissac *et al*. (1996)
D'	CCCTTGTGAATCACCACC			PCR	
P89-f	ATTGACTCAACAAACGGGATAA	Putative P89 adhesin gene	707 bp	Conventional	Yokomi *et al*. (2008)
P89-r	CGGCGTTTGTTAATTTTTGGTA			PCR	
P58-1f	CACCGCATAAACCATATACTTTGAAT	Putative P58 adhesin-like gene	701 bp	Conventional	Yokomi *et al*. (2008)
P58-5r	GTAGCAGAATGTAACCCACGAC			PCR	
P58-6f	GCGGACAAATTAAGTAATAAAAGAGC	Putative P58 adhesin-like gene	450 bp	Conventional	Yokomi *et al*. (2008)
P58-4r	GCACAGCATTTGCCAACTACA			PCR	
P58-1f	CACCGCATAAACCATATACTTTGAAT	Putative P58 adhesin-like gene	86 bp	Real-time PCR	Yokomi *et al*. (2008)
P58-2r	TTCGCTCGCATAAGTATCATATCTTC				
P58-3f	GTCCCTAATGCACCGTGAAAA	Putative P58 adhesin-like gene	119 bp	Real-time PCR	Yokomi *et al*. (2008)
P58-4r	GCACAGCATTTGCCAACTACA				

within and around citrus groves. Budwood should be taken from clean propagative materials and healthy trees. Trees that appear diseased and showing symptoms or abnormally stunted should be removed and replaced with healthy replants or more tolerant varieties. Use of trap plants such as sugarbeet which is leafhopper attractive but not host of *S. citri* can be reduced the incidence of stubborn disease in the citrus orchards. Although *S. citri* is highly sensitive to tetracylines in vitro but is not practical (Bowyer and Calavan, 1974; Fletcher *et al*., 2006; Gumpf and Calavan, 1981; Saglio *et al*., 1973; Whiteside *et al*., 1988).

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