Insect Vector and Reservoir Plant of '*Fragaria* × *ananassa*' Phyllody Phytoplasma (16SrXIII-F) in Central Region of Chile

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Abstract

Strawberry phyllody has emerged as a prevalent disease affecting Chilean strawberry in recent years. The causal pathogen, '*Fragaria* × *ananassa*' phyllody phytoplasma (StrPh), is categorized within the 16S ribosomal group XIII that is exclusively found in the Americas. In the context of economically significant crops, hemipteran insect vectors and alternative host plants play a pivotal role in their natural dissemination. This study comprehensively examined the key epidemiological facets of StrPh in the central region of Chile: the insect vector and alternative hosts. Through field surveys, we identified an abundance of an insect species, *Cixiosoma* sp., in an StrPh-infected strawberry field and confirmed its role as a vector of this phytoplasma through subsequent transmission assays. Moreover, we found a spontaneous weed species, *Galega officinalis*, to be infected with StrPh, raising the possibility of it being a potential alternative host plant for this phytoplasma. StrPh was also detected in cold-stored strawberry runners purchased from a nursery that supplies the local strawberry cultivation, suggesting a potential source of this phytoplasma in Chile. Collectively, these findings provide a significant epidemiological source of StrPh dissemination in central Chile.

Keywords: *Cixiosoma*, epidemiology, Fulgoroidea, *Galega officinalis*, host plant, strawberry

Strawberry (*Fragaria* × *ananassa* Duch.) crop in Chile faces a multitude of challenges from a range of pathogens, including nematodes, fungi, bacteria, and viruses, all of which pose significant threats to the crop (Correa and Alarcón F. 2015). In recent years, a new disease associated with phytoplasma has emerged in key strawberry cultivation regions of Chile, including Valparaíso, Metropolitana, and O'Higgins in the central part of the country, affecting major cultivars including 'Albion', 'Camarosa', 'Monterrey', 'Portola', and 'San Andreas'. Affected strawberry plants exhibit severe symptoms such as phyllody of fruits, achenes' hypertrophy, leaf reddening, and chlorosis (Cui et al. 2019). The associated pathogen was identified as '*Fragaria* × *ananassa*' phyllody phytoplasma (StrPh), which belongs to the 16SrXIII-F subgroup (Mexican periwinkle virescence group). Members of this subgroup have also been identified as the cause of strawberry red leaf disease in Argentina (Fernández et al. 2015). Interestingly, this subgroup of phytoplasmas has been found on both sides of the Andes mountains and has so far only been reported in Chile and Argentina. On the other hand, the distribution of 16SrXIII-F extends even further south, reaching the southernmost region of Magallanes and Antártica of Chile, where it affects calafate (*Berberis microphylla* G. Forst) (Madariaga and Ramírez 2019). This underscores the wide-ranging impact and geographical spread of this subgroup and the diseases associated with it in the South Cone.

The 16SrXIII group was first detected in Madagascar periwinkle (*Catharanthus roseus* [L.] G. Don, 1837) in Mexico (Gundersen et al. 1996). It was subsequently observed in central Florida, U.S.A., where infected strawberry plants displayed symptoms of stunting, abnormally small leaves, and green petals (Harrison et al. 1997; Jomantiene et al. 1998). Initially classified within the 16SrI group, these phytoplasmas were later reclassified as a distinct group, with '*Candidatus* Phytoplasma hispanicum' as the representative '*Candidatus*' species (Davis et al. 2016). Since then, numerous members of this group have been reported. Currently, the 16SrXIII group contains 11 subgroups, all exclusively found in the Americas (Arneodo et al. 2007; Cui et al. 2019; Eckstein et al. 2013; Fernández et al. 2015, 2016; Harrison et al. 2003; Jomantiene et al. 1998; Melo et al. 2013, 2018; Pérez-López and Dumonceaux 2016; Santos-Cervantes et al. 2010). Notably, at least four subgroups, 16SrXIII-B, 16SrXIII-F, 16SrXIII-J, and 16SrXIII-K, have been found associated with strawberry diseases (Cui et al. 2019; Fernández et al. 2015; Jomantiene et al. 1998; Melo et al. 2018). From a phylogenetic perspective, the 16SrXIII group shares close evolutionary ties with the 16SrI and 16SrXII groups (Cui et al. 2019), both of which have a global distribution including the Americas. Interestingly, the 16SrXII group contains members infecting strawberry worldwide but has been detected only in plants other than strawberry in the Americas; meanwhile, the 16SrI group has been reported to infect strawberry exclusively in the Americas (Jomantiene et al. 1998, 2002; Padovan et al. 2000; Terlizzi et al. 2006; Valiunas et al. 2006). On the other hand, another group of phytoplasmas, the 16SrXVIII, which is closely related to the 16SrXII group and associated with a potato purple top wilt disease complex, has recently been reported to infect strawberry plants in the United States (Lee et al. 2006; Nikolaeva et al. 2020). This distribution pattern suggests a potential overlap in ecological niches between 16SrI, 16SrXVIII, and 16SrXIII in the Americas, although no mixed infection has been reported so far.

Phytoplasmas, belonging to the class Mollicutes, are obligate pathogens residing exclusively in the phloem of infected plants. While they can be transmitted through plant grafting, their primary mode of field transmission involves insect vectors in a persistent and propagative manner (Alma et al. 2019). Insects with piercing-sucking mouthparts feed on infected plants' phloem sap and, after a latency period, transmit phytoplasmas to healthy hosts. In competent vectors, acquired phytoplasmas circulate in the insect's body, reaching the salivary glands for inoculation into the next host plant during feeding (Weintraub and Beanland 2006). Sustained transmission between

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host plants and insect vectors is crucial for phytoplasma establishment and spread in a region. For phytoplasmas infecting crops and fruit trees, other plants such as weeds also play an essential role as alternative hosts, facilitating the completion of epidemiological cycle (Alma et al. 2019).

To date, insect vectors responsible for transmitting phytoplasmas have been classified into three Hemiptera superfamilies: Membracoidea, Psylloidea, and Fulgoroidea (Jarausch et al. 2019; Jović et al. 2019; Weintraub et al. 2019). Regarding 16SrXIII phytoplasmas, the smoke-tree sharpshooter (*Homalodisca liturata* Ball.) is the only known insect vector reported so far (Servín-Villegas et al. 2018). This insect belongs to the Cicadellidae family and is distributed across the Arizona-California-Central America region (Burks and Redak 2003). Notably, in the Baja California peninsula, a geographically isolated region, this insect has been observed carrying and transmitting various subgroups of phytoplasmas, including 16SrXIII-D, -B, and -I (Servín-Villegas et al. 2018).

In the central region of Chile, we conducted a comprehensive survey in search for insect vectors that transmit the '*Fragaria* × *ananassa*' phyllody phytoplasma and spontaneous plant species that could potentially serve as alternative hosts for this phytoplasma in the field. We also analyzed plant samples from a strawberry nursery aiming at uncovering the potential source of this phytoplasma in Chile. This is the first systemic epidemiological study of a 16SrXIII-F phytoplasma. The results not only contribute to a deeper understanding of the distribution and dissemination of the 16SrXIII group of phytoplasmas but also shed light on the broader context of the origin and evolution of this group.

Materials and Methods

Field survey and sample collection

From April 2019 to July 2023, surveys were conducted in four strawberry fields located in Litueche, O'Higgins region, Chile (Table 1). Hemiptera species were captured using sweep nets directed at the weeds and strawberry crops in the fields as well as in the spontaneous plants at the peripheral areas. In spring and summer, the captures were carried out in the morning, while in autumn and winter, the captures were made around midday, when there was more flight of insects. The captured insects were placed in glass specimen tubes with cotton balls. In the dry seasons (October to April), a leaf blade of weed was inserted into the tube to maintain the humidity. The tubes containing the insects were kept in a cooler with icepacks and transferred to the laboratory. Leaves and fruits from strawberry plants showing symptoms of phyllody and achenes' hypertrophy as well as from asymptomatic plants were collected. A total of 20, five, one, and five strawberry plants (cultivar Monterrey) were collected from Fields A, B, C, and D, respectively. Weeds and spontaneous plants, including herbaceous plants and shrubs that showed symptoms of yellowing, leaf reddening, decreased leaf size, leaf deformation, shortened internodes, witches' broom, shoot proliferation, and phyllody, were also collected.

Morphological identification of insects and plants

The insects were observed and photographed using a LEICA S6D stereoscope. Specimens from Membracoidea, Psylloidea, and Fulgoroidea superfamilies were separated and identified by morphological characters (Fiore et al. 2015; Longone et al. 2011; Quiroga et al. 2019). Specifically, to identify the Fulgoroidea species, the description by Fennah (1965) was followed. Plant species were identified by morphological characters.

Sampling of strawberry runners

Cold-stored strawberry runners were obtained from a commercial nursery supplier, with five boxes acquired for each of the two strawberry varieties, 'Monterrey' and 'San Andreas'. Each of these boxes contained approximately 20 individual runners in a dormant state. To prepare the samples for DNA extraction, a pooling method was employed. Specifically, five individual runners were combined to form a single sample. Within each sample, the crowns of the runners were isolated from the rest of the plant. These crown segments were finely chopped into small cubes that were approximately 3 mm in length per side. Subsequently, the chopped crown segments were thoroughly mixed, and 1 g from each mixed sample was used for the DNA extraction process.

DNA extraction

DNA extraction from insects was performed using a modified CTAB-based method. One insect was ground in 500 µl of extraction solution containing 2% hexadecyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, pH = 8, 100 mM Tris-HCl, pH = 8, and 3 mM β-mercaptoethanol. The homogenized sample was incubated at 60°C for 1 h with intermittent agitation. The homogenate was extracted with equal volume of chloroform:isopropanol (24:1) and centrifuged at 4°C for 20 min at 15,000 × *g*. The aqueous layer was mixed with 0.7 volume of isopropanol, and the DNA was precipitated by 10 min of incubation at room temperature and 20 min of centrifugation at 4°C. The DNA pellet was washed with 70% ethanol before drying. The pellet was suspended in 100 µl of TE buffer. Plant DNA was extracted from midribs, petioles, phloem tissues, and crown of runners using the CTAB-based method previously described (Cui et al. 2019).

PCR, sequencing, and sequence analysis

PCR of cytochrome oxidase subunit 1 (COI) was performed using the universal primer pair LCO1490 and HCO2198 (Folmer et al. 1994). Amplicons of approximately 710 bp were purified from the gel and sent to Psomagen (Maryland, U.S.A.) for Sanger sequencing using the LCO1490/HCO2198 primer pair. Nested PCR for the 16S rRNA gene was performed using the universal primer sets P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) and R16F2n/R2 (Gundersen et al. 1996). PCR was repeated twice for each positive result. Amplicons of approximately 1.25 kb were purified from the gel and sent for direct sequencing using the R16F2n/R2 primer pair. PCR products from insects and plants were visualized in 1% agarose gels. A selection of amplicons was cloned into pGEM-T Easy vector

Table 1. Locations and dates of field surveys and numbers of *Cixiosoma* sp. captured from the fields in Litueche

Field code	Coordinates	Dates	Insect number (captured/tested/positive)
A	34°02′40.0″S 71°43′44.6″W	April 2019	12/12/8
		May 2019	50
		June 2019	103/103/26
		July 2019	130
		August 2019	73/73/13
		September 2019	0
		October 2019	0
		November 2019	0
		December 2019	0
		January 2020	0
		February 2020	0
		March 2020	0
B	34°02′50.6″S 71°43′32.6″W	January 2020	0
		February 2020	0
		March 2020	0
		February 2021	0
		March 2021	0
		May 2021	150/41/0
		June 2021	114/23/0
		April 2022	106/95/4
		June 2022	130/60/4
		July 2022	70
		July 2023	114/74/4
C	34°02′41.3″S 71°43′38.7″W	January 2020	0
		November 2020	0
D	34°07′43.2″S 71°42′40.2″W	July 2021	1/1/0

(Promega), and the plasmids were transformed into TOP10 competent cells. Three individual colonies for each sample were selected, and the plasmids were extracted and sent for Sanger sequencing using the T7/SP6 primer pair. Sequence assembly and alignment was performed using BioEdit version 7.7.1. The assembled sequences were analyzed using BLASTn for identification. Ribosomal groups and subgroups were assigned using *i*PhyClassifier (Zhao et al. 2009). Phylogenetic analysis was performed using MEGA7 (Kumar et al. 2016).

Transmission assays

Periwinkle plants were obtained from commercially purchased seeds. The plants were cultured in soil and maintained at 22°C under 16/8-h day/night conditions. Each plant was examined with nested PCR before transmission to ensure they were free from phytoplasma infection. Insects captured from Field A (Table 1) were encaged with a 2-month-old periwinkle plant. A total of 17 plants were used in the transmission trials, and eight to 11 *Cixiosoma* sp. individuals were transferred to each plant (Table 2). Four plants without insects were used as negative controls. The plants were maintained under the same condition as described above, and the contact period lasted for 7 to 17 days. At the end of the transmission, the insects were collected from each plant and stored in 70% ethanol at 4°C before DNA extraction.

Grafting of periwinkle plants

Grafting was performed following established procedures (Hodgetts et al. 2013). Specifically, symptomatic periwinkle branches were selected as scions. A 5- to 10-cm branch was carefully cut using a sterile scalpel blade, with adjacent leaves near the cut end removed to leave a 2-cm bare stem. The lower part of the stem was trimmed from both sides to expose the inner structure. Two-month-old periwinkle plants that had been tested negative for phytoplasma served as rootstocks. Using a sterile scalpel blade, leaves and side branches within 3 cm of the main stem's growing point were removed. The growing point was horizontally cut off. A vertical 2-cm split, creating a V shape, was made at the center of the main stem. The scion was inserted into the rootstock, and the cut area was securely wrapped with Parafilm. The grafted plant was maintained under the same conditions as previously described.

Results

Detection of '*Fragaria* × *ananassa*' phyllody phytoplasma in *Cixiosoma* sp.

From April 2019 to March 2020, monthly surveys were conducted in a field heavily infested with insects located in Litueche, where a significant proportion of strawberry plants exhibited severe symptoms of phyllody and achenes' hypertrophy (Table 1, Field A; Fig. 1A and B). To confirm the presence of phytoplasma infection, 20 symptomatic strawberry plants were sampled in April 2019 for DNA extraction. Subsequent nested PCR targeting the 16S rRNA gene generated a 1,244-bp band, and sequencing confirmed that all collected samples were infected with StrPh.

During the initial survey in April 2019, a particular insect species from the Cixiidae family was found to be highly abundant in the field (Supplementary Table S1). Twelve insects were collected for DNA extraction, and eight of them tested positive for the 16S rRNA gene through nested PCR. Sequencing and a BLASTn search indicated that the amplicons shared a 99.84 to 100% identity with the StrPh strains (Supplementary Table S2), and *i*PhyClassifier assigned all eight samples to the 16SrXIII-F subgroup. This insect species was visually identified as a *Cixiosoma* sp. based on taxonomic keys (Fig. 1C and D). PCR targeting COI produced a 709-bp band in all individual specimens, with BLASTn results revealing an 87.21 to 87.37% identity with *Cixius praecox*, also belonging to the Cixiidae family (Supplementary Table S2). There was no specific record of this exact species, either morphologically or molecularly. Indeed, there has been no publicly available molecular data for the genus *Cixiosoma* to date, making this insect the first to be documented in the public nucleotide database.

Subsequent surveys in the same field involved capturing another 356 specimens of *Cixiosoma* sp., of which 176 were analyzed. StrPh was detected in 39 of these specimens. To observe the occurrence of this insect in relation to changing seasons, the number of captured insects was plotted against the local precipitation and temperatures. Interestingly, the abundance of this insect exhibited a negative correlation with the temperatures and a positive correlation with the precipitation (Fig. 1E).

From January 2020 to July 2023, 11 surveys were conducted in another field located about 450 m from Field A and its peripheral areas (Table 1, Field B). While strawberry plants in this field had previously displayed symptoms of phyllody, they had been promptly removed. At the time of the surveys, no symptomatic plants were observed. Five strawberry plants were randomly collected, all of which resulted negative for phytoplasma. *Cixiosoma* sp. was captured between May 2021 and July 2023 but not between January 2020 and March 2021. In Field B, no phytoplasmas were detected from the insects captured in 2021. However, in 2022 and 2023, insects positive for 16SrXIII-F were found.

Two subsequent surveys were conducted in a field adjacent to Field A, one in January and the other in November 2020 (Table 1, Field C). During these surveys, one symptomatic strawberry plant was collected, which tested positive for StrPh. No *Cixiosoma* sp. was found. Instead, several insect species from the Cicadellidae family were captured. These specimens were identified based on morphological characteristics (Supplementary Table S1). Nested PCR targeting the 16S rRNA gene yielded negative results for all these specimens, indicating that none of these Cicadellidae species resulted positive to the phytoplasma 16SrXIII-F.

In another survey conducted in July 2021, in a field located approximately 9 km away from Field A (Table 1, Field D), symptomatic strawberry plants had been removed before the survey similar to Field B. Five strawberry plants were randomly collected, and all of them exhibited negative results for phytoplasma. One single *Cixiosoma* sp. specimen and several Cicadellidae species were collected. However, all these insects tested negative for phytoplasma.

Table 2. Transmission assays

Plant code	Total insects (dead insects)[a]	Positive insects (dead insects)[a]	Days of feeding	Occurrence of symptoms (dpt[b])
G	11	6	9	70
H	10 (1)	2	16	112
I	9	2	16	56
J	11	2	16	56
K	10 (2)	4 (1)	16	76
L	10 (10)	2 (2)	16	76
M	9	0	16	NO[c]
N	9 (1)	3	16	70
O	9	1	17	76
P	10	3	17	76
Q	9 (2)	1 (1)	17	NO[c]
R	9 (5)	2	7	59
S	9	2	10	80
T	10	3	10	59
U	10	1	10	NO[c]
V	8	1	10	46
W	9	0	10	NO[c]
Total	153 (21)	35 (4)		

[a] Number of insects dead by the end of the contact period.
[b] dpt = days post the start of transmission trial.
[c] NO = not observed.

The *Cixiosoma* sp. transmits StrPh to periwinkle

To investigate whether *Cixiosoma* sp. indeed functioned as a vector for StrPh, live insects were captured from Field A for transmission assays. Throughout the entire contact period, the insects were observed actively feeding on leaves and stems (Supplementary Fig. S1). By the conclusion of the contact period, 21 out of the 162 insects were dead (Table 2), being primarily trapped in the water droplets due to the high humidity within the enclosed container. The remainder of the insects continued to exhibit vigorous movement,

much like their behavior in the field, suggesting that this insect species could thrive on periwinkle plants.

In the transmission assays, a total of 162 insects were employed, and by the end of the experiment, 35 of these insects tested positive for StrPh (Table 2). Notably, plants labeled as "M" and "W" that came into contact with insects showed no evidence of phytoplasma infection, as all the insects tested negative, and these plants displayed no symptoms associated with phytoplasma infection. For the remaining 15 plants, with each in contact with one to six positive insects, symptoms began to appear between 46 and 112 days from the initiation of the transmission trials. Thirteen of these plants exhibited symptoms associated with phytoplasma infection. There was no observable correlation between the number of positive insects per plant and the timing of symptom manifestation. Additionally, control periwinkle plants that were not inoculated with infected insects showed neither symptoms nor tested positive for phytoplasmas.

For each infected periwinkle plant, the symptoms initially manifested as virescence, characterized by a portion of the petals turning green (Fig. 2A). Within approximately 6 days, the newly formed flowers opened entirely green (Fig. 2B), ultimately failing to produce any seed pods. As the symptoms progressed, the petals increasingly resembled leaves, and shoots began to emerge from the center of the flowers (Fig. 2C). Around 130 days into the transmission trial, the plants ceased flower production, and the new leaves were small and narrow with shortened internodes, indicating typical phyllody symptoms (Fig. 2D). Subsequent nested PCR targeting the 16S rRNA gene confirmed that all the symptomatic plants were indeed infected with StrPh. These findings substantiate that *Cixiosoma* sp. serves as a bona fide vector for the '*Fragaria* × *ananassa*' phyllody phytoplasma.

Graft transmission of StrPh

After successfully transmitting the phytoplasma, the symptoms in the infected periwinkle plants gradually worsened, leading to the death of these plants between 190 and 307 days into the transmission trial. To preserve the infected plant lines, we grafted branches displaying symptoms onto healthy periwinkle plants. A total of five plants were used as rootstocks, and approximately 2 months after grafting, three of these plants developed phytoplasma-associated symptoms. Subsequent nested PCR tests on these symptomatic plants confirmed the presence of StrPh, demonstrating that this phytoplasma could be transmitted through grafting in periwinkle. The other two rootstocks exhibited no symptoms associated with phytoplasma and consistently tested negative until their disposal, which occurred approximately 1 year after grafting.

Alternative host plant of '*Fragaria* × *ananassa*' phyllody phytoplasma

Throughout the surveys conducted in Litueche, the most prevalent weeds growing in the strawberry fields included wild blackberry (*Rubus ulmifolius* Schott), bindweed (*Convolvulus arvensis* L.), and various species of the Asteraceae family (Supplementary Table S3). In the peripheral areas, the predominant spontaneous plant was galega (*Galega officinalis* L.), which was extensively distributed across the field where specimens of *Empoasca* sp. and *Cixiosoma* sp. were captured (Supplementary Table S1). Samples were collected from 26 plant species and subjected to analysis using nested PCR

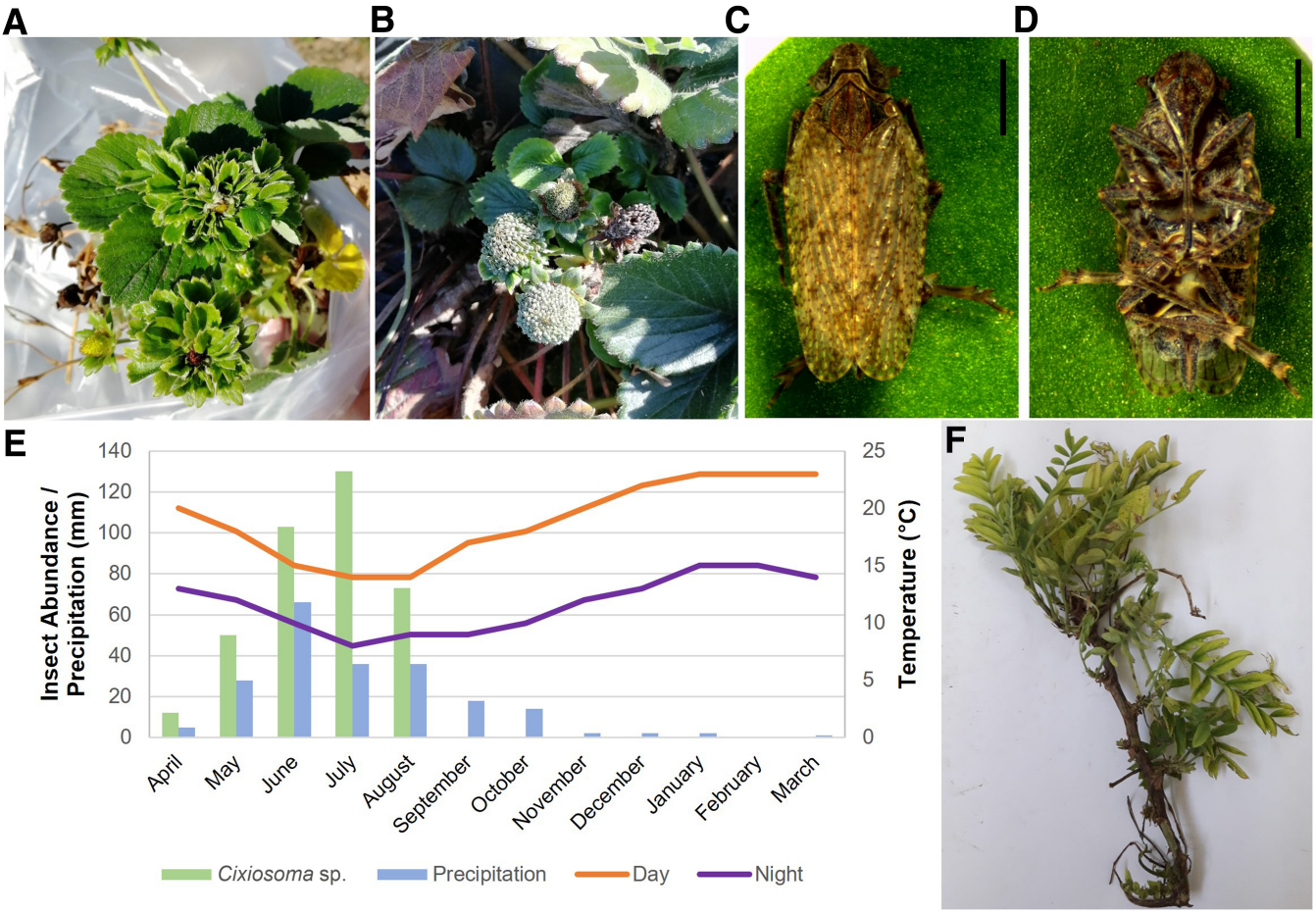

Fig. 1. The insect vector and potential alternative host plant of '*Fragaria* × *ananassa*' phyllody phytoplasma. **A and B,** Symptomatic strawberry plants from a heavily infested field located in 34°02′40.0″S, 71°43′44.6″W. Plants displayed symptoms of A, phyllody or B, achenes' hypertrophy. The *Cixiosoma* sp. in **C,** dorsal and **D,** ventral view. **E,** Population trends of *Cixiosoma* sp. and climatic factors during each month in the strawberry field. Temperature and precipitation data were taken from https://www.cuandovisitar.cl/chile/litueche-4024657/. **F,** The spontaneous plant, *Galega officialis*, infected with '*Fragaria* × *ananassa*' phyllody phytoplasma. Size bars = 1 mm.

targeting the 16S rRNA gene. Among these samples, only one galega plant tested positive for StrPh (Supplementary Table S3). This infected plant exhibited characteristic symptoms of phytoplasma infection, including chlorosis and shortened internodes (Fig. 1F), suggesting that galega might serve as an alternative host for StrPh. All other plant samples tested negative for phytoplasma.

'*Fragaria* × *ananassa*' phyllody phytoplasma was detected in strawberry runners from nursery

A total of 19 samples of 'Monterrey' and 19 samples of 'San Andreas' were examined for phytoplasma infection using a nested PCR targeting the 16S rRNA gene. Among the 'San Andreas' samples, four (S3, S5, S10, and S15) resulted positive for phytoplasma, while all 'Monterrey' samples showed negative results. Sequencing analysis unveiled that the three clones from sample S3 exhibited identical sequences. In contrast, samples S5 and S10 yielded two and three distinct sequences, respectively (Supplementary Table S4). BLASTn search results indicated that all these sequences shared a 99.60 to 100% identity with StrPh strains, confirming the infection of this phytoplasma in samples S3, S5, and S10. Intriguingly, in the case of sample S15, two of the sequences displayed a 99.92% identity with StrPh, while the third sequence exhibited a 99.60% identity with '*Candidatus* Phytoplasma pruni' clone PYWB-ArgG2, which belongs to the 16SrIII-J subgroup (Supplementary Table S4). These results demonstrated that the cold-stored strawberry runners of 'San Andreas' were infected with phytoplasma, with infection rates ranging from 4 to 20%. The detection of both 16SrXIII-F and 16SrIII-J phytoplasmas raises the possibility of coinfection in strawberry plants by these two phytoplasma subgroups.

Phylogenetic analysis

To explore the genetic relationships among the phytoplasmas present in the collected samples, a phylogenetic tree was constructed using a 1,244-bp segment of the 16S rRNA gene (Fig. 3). All samples utilized in this study, except for S15-3, formed a distinct cluster within the tree. This cluster also included the five previously identified StrPh strains (StrPh-CL1, 3, 5, 6, and 7, as reported by Cui et al. 2019). This clustering pattern suggests that the phytoplasmas infecting the insects and plants from the surveyed fields, as well as those from the nursery, are closely related.

Discussion

This study delved into three key epidemiological aspects of '*Fragaria* × *ananassa*' phyllody phytoplasma: insect vectors, alternative host plants, and the source. The insect *Cixiosoma* sp. was confirmed as a competent vector of StrPh by transmission assays. In contrast, only a solitary sample of *G. officinalis* was found to be infected by StrPh, and the extent of its role in the natural dispersion of this phytoplasma remains to be determined. Furthermore, this study uncovered one potential source of StrPh in Chile, shedding light on the path of its dissemination across the Americas.

No specific record of the *Cixiosoma* sp. serving as a vector for phytoplasmas was reported earlier for vector of phytoplasmas, although several similar individuals have been documented as endemic to Chile, Argentina, and Uruguay (Fennah 1965). Our findings raise the possibility that this insect species might be a new one not reported earlier in insect taxonomic databases. As shown in the correlation between insect abundance of this species and local temperature and precipitation patterns, this species thrived in cooler conditions characterized by high humidity, which contrasted with many known vectors from the Cicadellidae family.

The cultivation practices in the surveyed fields of Litueche followed these steps: young plants propagated from runners were purchased from nurseries and subsequently transplanted into freshly plowed fields. These crops were nurtured for a period spanning 2 to 3 years, during which the fruits were harvested. After this productive phase, when yields began to decline, the crops were removed from the fields. Subsequently, the fields were allowed to remain fallow for a period of 1 to 2 years before the soil was prepared for cultivation of new crops. Fields with crops established for over 1 year tended to gradually become infested with weeds, and spontaneous plants also proliferated in peripheral areas. These plants created environments

Fig. 2. Periwinkle plants showing symptoms associated with phytoplasma infection after transmission assay. **A,** Virescence, showing partial corolla turning green (indicated by arrowhead). **B,** Virescence, showing a whole flower turning green. **C,** Phyllody, showing leaf-like petals and branches emerging from the center of a would-be flower (indicated by arrow). **D,** Severe phyllody stage, showing complete disappearance of flower. **E,** Closed-up view of a would-be flower. **F,** Healthy periwinkle plant.

conducive to insect breeding and could potentially serve as alternative hosts.

Cixiosoma sp. thrived in the strawberry field heavily infected with phytoplasma (Field A), as well as in the surrounding peripheral areas colonized by a galega community (Field B). Remarkably, even after the strawberry crops from Field A were removed and the field remained fallow for over 2 years, StrPh continued to be detected in *Cixiosoma* sp. dwelling in the galega community within the peripheral areas. In contrast, almost no specimens of *Cixiosoma* sp. were captured in the other two strawberry fields where symptomatic crops had been promptly removed and no galega community had been established in the peripheral areas. These observations suggest that the galega community may serve as a natural habitat for the *Cixiosoma* sp., which potentially represents an endemic species. When strawberry crops infected by phytoplasma were planted in the nearby field, the insect was drawn to the infected plants, subsequently carrying the pathogen back to the galega community where it persisted for several years. This speculation is reinforced by multiple experiments demonstrating that insect vectors tend to reproduce more on plants infected with phytoplasma under both natural and laboratory conditions (MacLean et al. 2014; Orlovskis and Hogenhout 2016; Queiroz et al. 2016; Sugio et al. 2011). Alternatively, the phytoplasma may have been preexisting in the galega community, and *Cixiosoma* sp. carrying StrPh could have transmitted it to the strawberry plants. However, this scenario appears less likely because only one out of 26 galega samples tested positive for StrPh, whereas the rate of symptomatic strawberry plants was considerably higher. The successful transmission of StrPh from strawberry to galega signifies a crucial step in the establishment of this phytoplasma in the area.

The origin of StrPh in Chile presents a compelling question. The fact that 16SrXIII phytoplasmas have exclusively been detected in the Americas strongly suggests that this ribosomal group likely originated on these continents, likely stemming from the 16SrI group (Cui et al. 2019). Its initial detection in Mexico (Gundersen et al. 1996) and the notable diversity found in the Baja California peninsula (Servín-Villegas et al. 2018) provide additional indications that Central America could be the epicenter of this group's origin. It is reasonable to speculate that the ancestor of 16SrXIII dispersed in

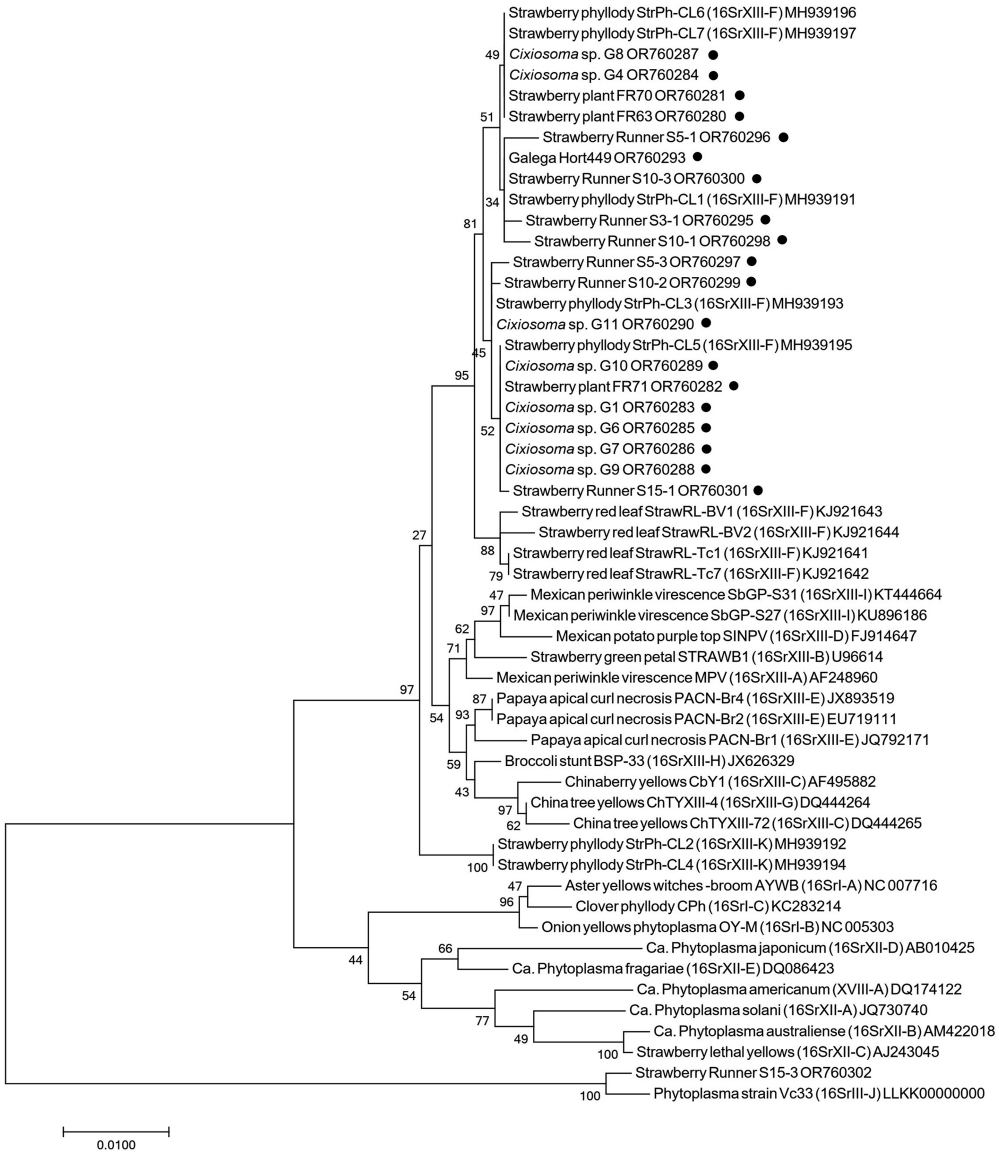

Fig. 3. Phylogenetic tree constructed using 16S rRNA gene sequences, employing the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) with MEGA7. A bootstrap test with 1,000 replicates was performed, and the resulting tree with the highest log likelihood is presented. The tree is depicted to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the related taxa formed clusters is indicated alongside the branches. The samples obtained in this study are marked with dots.

both north and south directions across the Americas; however, the spread of 16SrXIII-F may have taken a more expedient route. Assisted by a widely distributed insect vector such as the smoke-tree sharpshooter, this phytoplasma could have easily propagated northward to California, which is home to numerous strawberry nurseries. Infected mother plants may have given rise to infected runners, which were subsequently cold-stored and shipped to other states and subdistributors in South America. Supporting this hypothesis, there have been instances where strawberry plants in West Virginia tested positive for 16SrI phytoplasma, and these plants were originally procured from a Californian nursery (Jomantiene et al. 2002). In our present study, StrPh was indeed detected in cold-stored strawberry runners, further substantiating the notion that this phytoplasma could have disseminated through logistical supply chains. Notably, the same nursery from which these samples were purchased also supplies strawberry plants to Argentina. Consequently, 16SrXIII-F was also detected in strawberry plants in Argentina (Fernández et al. 2015), although infection in other plants has not yet been reported. To conclusively validate this potential path of dissemination, extensive investigations into phytoplasma infections in nurseries across various locations would be imperative.

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