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MONITORING OF TREEHOPPER (HEMIPTERA: MEMBRACIDAE) POPULATIONS IN NORTHERN CALIFORNIA VINEYARDS

By

MICHAEL LEWIS BOLLINGER
Thesis

Submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Entomology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2021

Monitoring of Treehopper (Hemiptera: Membracidae) Populations in Northern California

Vineyards

Abstract

Two studies were conducted in a project aimed at increasing knowledge of potential

treehopper (Hemiptera: Membracidae) vectors of grapevine red blotch virus (family:

Geminiviridae) in Northern California vineyards.

In the first study, I collected insects identified as *Tortistilus* species from purple vetch

plants in a California vineyard representing individuals with either brown or green coloration and

with prominent pronotal suprahumeral horns or without horns. The horned insects were initially

thought to be *Tortistilus albidosparsus* (Stål 1860) and the unhorned insects *Tortistilus wickhami*

(Van Duzee 1908) and the specimens collected appeared in both green and brown color forms.

However, their occurrence and feeding on the same host on the same date seemed curious and

raised concern about their classification. To ascertain their taxonomic status precisely, two

insects of each morphotype: brown horned, brown unhorned, green horned and green unhorned,

were subjected to shotgun DNA sequencing using Illumina Hiseq4000. Initial analysis on the

assembled contigs of cytochrome oxidase I (COI) indicated that all eight insects were in the

same phylogenetic group and had <2% difference with *T. wickhami* sequences in GenBank.

There were no reference sequences for *Tortistilus albidosparsus* in Genbank. Examination of

genitalia of the males did not reveal any morphological differences among the four morphotypes.

Matings of combinations of horned and unhorned individuals resulted in F1 adults of both

morphotypes.

The second study involved monitoring the three-cornered alfalfa hopper, *Spissistilus festinus* (Say), (Hemiptera: Membracidae) in California vineyards for two years during which the timing and within-plant distribution of its feeding on grapevines was characterized. *Spissistilus festinus* feeding results in a distinctive ring around the entire stem and petiole, referred to as a girdle. Thirty grapevines from two vineyards in Napa and Solano Counties were thoroughly searched for the presence of girdles every two weeks following budbreak and continuing through leaf fall. Once girdles were counted, each girdled stem and petiole was removed to prevent them from being counted again later. Results suggest that *S. festinus* begins to feed on grapevines in late May through June, and that feeding on petioles relative to stems differs throughout the year. This information will be useful for Integrated Pest Management decision-making by indicating the best times of the year to apply control measures to prevent feeding damage and help stop the spread of grapevine red-blotch virus by this species.

Seasonal and Within-Plant Distribution of Feeding Damage to *Vitis vinifera* caused by

Spissistilus festinus (Hemiptera: Membracidae)

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Short version of title: Feeding Damage by *Spissistilus festinus*

Abstract:

A two-year field study of the three-cornered alfalfa hopper, *Spissistilus festinus* (Say),

(Hemiptera: Membracidae) in California vineyards was conducted to characterize timing and

distribution of its feeding on grapevines. *Spissistilus festinus* feeding results in a distinctive ring

around the entire stem and petiole, referred to as a girdle. Thirty grapevines from two vineyards

in Napa and Solano Counties were thoroughly searched for the presence of girdles every two weeks following budburst and continuing through leaf fall for two years. Once girdles were counted, each girdled stem and petiole was removed to prevent them from being counted again later. Results suggest that *S. festinus* begins to feed on grapevines in late May through June, and that feeding on petioles relative to stems differs throughout the year. This information will be useful for Integrated Pest Management decision-making in order to determine the best times of the year to apply control methods to prevent feeding damage and help stop the spread of grapevine red-blotch virus by this species.

Keywords: grapevine red-blotch virus, grape, treehopper, girdling

Introduction

The three-cornered alfalfa hopper, *Spissistilus festinus* (Say) (Hemiptera: Membracidae), is best known for the economic injury that it causes when feeding *en masse* on alfalfa and other legume crops such as cowpeas (Wildermuth 1915), soybean (Moore and Mueller 1976), and peanut (Beyer *et al.* 2017) in the United States where it is believed to have originated (Caldwell 1949). This species has been reported to feed on alfalfa and grapevines in California (Wistrom *et al.* 2010) and is described as an occasional pest of grape (Smith 2013). Feeding injury by the three-cornered alfalfa hopper and other treehoppers occurs when the host plant's epidermal tissue is pierced by the hoppers needle-like stylets in order to obtain nutrients from the phloem. Two common types of visible feeding damage to plant stems are documented. One type of feeding wound occurs as singular punctures causing tiny necrotic spots while a second, and more pronounced, type of damage occurs when contiguous punctures result in a pronounced ring or "girdle" around the entire stem (Wildermuth 1950). On grapevines, treehopper girdles can be found on both leaf petioles and shoots. These girdles inhibit the transportation of photosynthate through the phloem and leaves distal to a girdle. Leaves of red-fruited cultivars often turn red as a result. Carbohydrates and certain amino acids accumulate above the girdle in peanut (Anderson *et al.* 2002). This tissue has been shown to be a preferred feeding site for *S. festinus* in soybean (Mitchell and Newsom 1984), but this behavior has not been studied in grape. Similarly, yield impact of girdling has not been studied in grape, so a damage threshold relating to economic injury has not been proposed. Other treehopper species reported to feed on grapevines in California include *Stictocephala bisonia* (Kopp and Yonke 1977) (Smith 2013) and *Tortistilus albidosparsus* (Stål), (Kopp pers. Comm.). The status of *S. festinus* as a grape pest increased dramatically when it was found to vector grapevine red-blotch virus (GRBV), the

causal agent of grapevine red-blotch disease, in a greenhouse transmission study (Bahder *et al.* 2016), and transmission was subsequently confirmed in the greenhouse by Flasco et al. (2021). Furthermore, Cieniewicz *et al.* (2018) determined that the association of red-blotch disease's spatial patterns with GRBV-positive *S. festinus* adults caught on yellow sticky traps implicated this membracid as the most likely vector of significance to virus epidemiology. Grapevine red-blotch virus threatens the United States wine industry, devaluing wine by reducing Brix, prominent phenolic compounds, and affecting primary metabolites (Pereira *et al.* 2021). Preto *et al.* (2019) sampled *S. festinus* seasonal abundance in a two-year sweep-net study of vineyard floor vegetation in a Napa Co. vineyard and found that adult density peaked between mid-June and Mid-July in both years. It was noted that the decline in treehopper abundance occurred when the vineyard floor vegetation began to dry in summer and girdles began to appear on the vines. They posited that when preferred feeding hosts become scarce that *S. festinus* adults migrate into the grape canopy to feed and may acquire GRBV from infected vines at that time. Cieniewicz *et al.* (2018) found that *S. festinus* adults first tested positive for GRBV in June in both years of their Napa Co. trapping study. The purpose of our study was to characterize the seasonal abundance and distribution of girdles on grapevines resulting from *S. festinus* feeding in two California vineyards. Knowledge of its temporal and spatial feeding on grapevines will lead to improved management actions to prevent feeding injury and potential GRBV spread within vineyards.

Materials and Methods

Two study sites were chosen for a two-year study, and these sites include the following descriptors: The first study site was located near Oakville, Napa County, California. This conventionally-managed Cabernet Sauvignon vineyard consisted of 337 vine rows running east

to west with 20 vines per row and was planted in 2000. Row spacing was 3.66 m with vine

spacing of 1.52 m within the row. The second study site was located at the UC Davis Plant

Pathology Armstrong Tract, Solano County, California. This Cabernet Sauvignon research

vineyard consisted of 18 vine rows running east to west with 55 vines per row and was planted

on Freedom rootstock in 2015. The study area within both vineyards consisted of six grapevine

rows and five vines that were sampled bi-weekly for *S. festinus* girdles. Girdle counts began

approximately 3 weeks after bud break which occurred in late April at both sites and continued

until leaf fall in November. All green tissue on the stems and petioles of each vine was

examined entirely for girdles. Damage was categorically assigned as being on either petiole or

green stem. As *S. festinus* does not feed on brown lignified portions of the vine, only the green

tissues of vines were assessed. After each girdle was recorded, the petiole or the green stem

where girdles occurred were removed to prevent them from being counted again in subsequent

bi-weekly girdle counts. Data for each sampling date were summarized as a mean and $\pm$ SEM

number of girdles per vine (n=30 vines) using Microsoft Excel 2016 for Windows.

Results

Symptoms of treehopper feeding was expressed in two distinct forms. The less damaging

appeared as tiny necrotic spots that resulted from the insertion of the mouthparts into green tissue

and appeared similar to that of other insects with piercing/sucking mouthparts that feed in a

similar fashion. The more extensive and most characteristic *S. festinus* feeding damage was the

girdle, formed when the insect feeds around the entire circumference of the petiole or green

stem. Girdles caused by *S. festinus* appear as a rather narrow dark-brown or black band that is

slightly sunken with respect to the immediately adjacent tissue (Fig. 1 left). Another type of

girdle found in vineyards appears as a large swollen ring that is easily distinguished from that

resulting from *S. festinus* feeding as its appearance is noticeably larger and swollen relative to adjacent tissue (Fig. 1 right). The swelling may be accompanied by raised bumps around the circumference of the ring, and the epidermal layer may crack as the damaged tissue dries which is especially associated with stem-feeding. On California grapevines, this more robust swollen region results from feeding by another membracid, *Tortistilus sp.* that is occasionally found feeding in the same vineyards as *S. festinus*. Few of the larger *Tortistilus sp.* girdles were found in the vineyards we sampled, and these were excluded from our girdle analysis as *Tortistilus sp.* treehoppers have not been found to vector GRBV to date.

The first seasonal occurrence of girdles at the Napa site was in late May of 2017 and early June in 2018 (Figure 2). Girdles were found exclusively on petioles on each sampling date until mid-August in 2017 and early July in 2018, when green stem girdles were first recorded. Mean number of petiole girdles exceeded that of green stem girdles on 3 of the 13 sampling dates when girdles were found in 2017, and on all 12 sampling dates when girdles were found in 2018. Peak numbers of petioles and green stems girdles occurred in mid-September and again about 8 weeks later (mid-November) in 2017. A similar seasonal pattern of *S. festinus* girdling was observed in 2018 with two distinct peaks recorded. However, the first peak of petiole and green stem girdling occurred in early July with the second peak occurring about 12 weeks later in late September. New girdles were found until leaf fall in November in both years. The mean number of new girdles per vine were similar for both peaks within a year, but more girdles were recorded in 2018 than in 2017.

The seasonal pattern of new girdles found at the Solano Co. site was different than observed at the Napa Co. site with only a single peak occurring late September in 2017 and mid-October in 2018 (Figure 3). The total number of girdles was greater at the Solano Co. site as well with

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1436 and 565 counted on the 30 vines in 2017 and 2018, respectively. In contrast, only 91 and 271 girdles were counted at the Napa Co. site in the same years. Girdles appeared somewhat later at the Solano Co. site than was observed at the Napa Co. site, with the first girdles appearing in mid-June in 2017 and in mid-July in 2018. All of the girdles recorded on the first three sampling dates in 2017 were on petioles, while both petiole girdles and green stem girdles appeared on the mid-July sampling date and thereafter for the remainder of the season. Petiole girdles comprised 71% of total *S. festinus* girdles in 2017 and 73% of total girdles in 2018 at the Napa Co. site, but only 42% and 51% of total girdles in two years at the Solano Co. site.

Discussion

The status of *S. festinus* as a vineyard pest has increased with the confirmation of its ability to transmit GRBV, the causal agent of grapevine red-blotch disease, to grapevines in greenhouse studies (Bahder *et al*. 2016, Flasco *et al*. 2021). This has created the need for a more thorough inquiry concerning the biology of this species in vineyards. Its seasonal occurrence in Napa Co., California vineyards has been determined both by sweep-netting vineyard ground cover (Preto *et al*. 2019) and from yellow sticky cards hung on trellis wires in the canopy (Cieniewicz *et al*. 2018, Wilson et al. 2020). Both of these sampling methods have limitations. While sampling vineyard floor ground-cover by sweep-netting facilitates detection of adults in a vineyard, the method does not, by itself, confirm their presence or feeding on the vines themselves. Wistrom *et al*. (2010), in a survey of multiple crop hosts including grapes in California's southern San Joaquin Valley, reported that few *S. festinus* were captured on yellow sticky cards relative to beat-sweeps that consisted of striking the plant canopy being sampled with a stick to dislodge insects that fell into a sweep-net held beneath. Preto *et al*. (2019) did not continue yellow sticky card sampling for *S. festinus* beyond the first year of their study due to lack of captures, and

Cieniewicz *et al.* (2018) noted that sticky cards may not provide a reliable estimate of adult abundance. Since it is impractical to sweep-net insects from the grape canopy due both to the structure of the grapevine and resulting fruit damage, determining the timing and relative abundance of *S. festinus* feeding on grapevines by another method is necessary. Since feeding by Membracidae on grapevines results in visible damage referred to as girdles, the presence of girdles can serve as a direct measure of adult insect feeding in the grapevine canopy.

In our study, we described characteristics that distinguished girdles resulting from feeding by *S. festinus* from those caused by *Tortistilus sp.,* the other treehopper that is most likely to be encountered in northern California vineyards. We then monitored the seasonal abundance of girdles resulting from *S. festinus* feeding in two California Cabernet Sauvignon vineyards over two years and recorded the total number of girdles found on petioles and on green stems.

Although the total number of girdles differed between years, more girdles were recorded in both years at the Solano Co. site. The seasonal pattern of girdles was somewhat similar at each site in both years, but it differed between the two sites. Girdles were found earlier at the Napa Co. site and two seasonal peaks of girdles were observed while only a single large peak of girdles occurred at the Solano Co. site, and the first girdles were recorded somewhat later at the Solano Co. site than at the Napa Co. site. Petiole girdles were relatively more common than green stem girdles at the Napa Co. site while the number of petiole and green stem girdles were roughly equivalent at the Solano Co. site.

Although grape variety, trellising, pruning and irrigation type were similar at both study sites, there were differences in terms of location and pest management practices. These factors could have contributed to the observed differences in girdling. One site was a conventionally-managed vineyard near Oakville in California's Napa Valley that is nearby the riparian habitat present

along the Napa River, and is representative of other conventionally-managed vineyards in the area in terms of pesticide and fertility practices. A vegetated cover was maintained between the rows and alternate rows were mowed or tilled in spring. The other site was a research vineyard at UC Davis in Solano Co. in California's central valley that was located next to an alfalfa planting and in an area dominated by field crops. The vineyard remained untreated with insecticides during the two-year course of this study, minimal nitrogen fertilizer was applied, and row middles were tilled to reduce weed growth during the season.

The proximity of the Solano Co. vineyard to alfalfa, a favored reproductive host of *S. festinus,* likely facilitated adult migration to the grapevines as the resident population increased in the alfalfa during the season, whereas external sources of adults in Napa Co. vineyards originate from riparian and weedy areas as well as internally on hosts in vegetated row middles. Preto *et al.* (2019) reported finding *S. festinus* nymphs and first-generation adults on vegetation in vine rows in late June and early July. The external rather than the largely internal source of *S. festinus* at the Solano Co. site could explain the somewhat later occurrence and single peak of girdles seen there as compared to the Napa Co. site. While the greater number of girdles found at the Solano Co. site might also be attributed to migration from the adjacent alfalfa planting, insecticides applied to control mealybugs and leafhoppers at the Napa Co. site undoubtably suppressed *S. festinus* as well.

Previous studies from north coast California vineyards indicate that girdles tend to increase with the seasonal decline in groundcover quality (Preto *et al.* 2019, Wilson *et al.* 2020). The former study, which included girdling observations from a single site and year, reported results similar to those from our Napa Co. site. The latter study reported total petiole girdles averaged from four study sites, and the first girdles being reported in mid-July. Our study confirms that *S.*

festinus feeding as indicated by the presence of girdles indeed increases in vineyards with ground cover present as the ground cover senesces. However, we found the first girdles in late May and early June which was earlier than was reported in the previous studies. It is also clear that green stems are an important feeding site for *S. festinus*, and these should not be excluded when assessing vines for presence and number of girdles.

The Solano Co. site did not have resident ground cover that would enable a resident population of *S. festinus* to reproduce within the vineyard since survival beyond the second instar is dependent on a suitable developmental host (Preto *et al.* 2018). The source of adults in similar vineyards would be dependent on proximity to external sources of this insect. Our results suggest that in similar situations the first incidence of *S. festinus* feeding would occur somewhat later than in vineyards with vegetated row middles, even when the source of migrants is a favored host such as alfalfa that is adjacent to the vineyard.

Our study of the seasonal occurrence and within-plant distribution of *S. festinus* girdles on grapevines expands on prior studies of the biology of this insect in grape vineyards, suggesting that different seasonal patterns of feeding may occur depending on factors such as proximity to alternate reproductive hosts and presence of reproductive hosts within the vineyard. Monitoring *S. festinus* feeding by sampling petioles and green shoots for girdles also provides an indication of when control actions must commence in order to prevent *S. festinus* feeding on grapevines, thereby reducing risk of direct feeding damage and spread of GRBV from infected vines.

Conclusion

This study characterized the seasonal abundance and distribution of girdles on grapevines resulting from *S. festinus* feeding in north coast (Napa Valley) and central valley vineyards.

Knowledge of how to distinguish *S. festinus* feeding from that of other species when *S. festinus* girdles first occur in vineyards, and their relative seasonal density on both petioles and green stems can assist in development of monitoring and management strategies to reduce direct damage resulting from their feeding as well as the spread of grapevine red-blotch virus.

Citations

Andersen PC, Brodbeck BV, and Herzog DC. 2002. Girdling-induced nutrient accumulation in above ground tissue of peanuts and subsequent feeding by *Spissistilus festinus*, the three-cornered alfalfa hopper. Entomol. Exp. Appl. 103(2):139-149.

Bahder BW, Zalom FG, Jayanth M, and Sudarshana MR. 2016. Phylogeny of Geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. Phytopathology. 106: 1223-1230.

Beyer BA, Srinivasan R, Roberts PM, and Abney MR. 2017. Biology and management of the three-cornered alfalfa hopper (Hemiptera: Membracidae) in alfalfa, soybean, and peanut. J. Integr. Pest Manage. 8(1): 10.

Bick EN, Kron CR, and Zalom FG. 2020. Timing the implementation of cultural practices for *Spissistilus festinus* (Hemiptera: Membracidae) in California vineyards using a stage-structured degree-day model. J. Econ. Entomol. 113: 2558-2562.

Caldwell JS. 1949. A generic revision of the treehoppers of the tribe Ceresini in America north of Mexico, based on a study of the male genitalia. Proc. U S Nat. Mus. 98: 491-521.

Cieniewicz EJ, Flasco M, Brunelli M, Onwumelu A, Wise A, Fuchs MF. 2019. Differential Spread of Grapevine Red Blotch Virus in California and New York Vineyards. Phytobiomes J. 2019. 3: 203-211.

Cieniewicz EJ, Pethybridge SJ, Loeb G, Perry K, Fuchs M. 2018. Insights into the ecology of Grapevine red blotch virus in a diseased vineyard. Phytopathology 108:94-102.

Flasco M, Hoyle V, Cieniewicz EJ, Brandon GR, McLane HL, Perry KL, Loeb G, Nault B, Heck M, Fuchs M. 2021. Grapevine red blotch virus is transmitted by the three-cornered alfalfa hopper in a circulative, non-propagative mode with unique attributes. Phytopathology https://doi.org/10.1094/PHYTO-02-21-0061-R

Kopp DD and Yonke TR. 1977. Taxonomic Status of the Buffalo Treehopper and the Name *Ceresa Bubalus*. *Annals of the Entomological Society of America*, Volume 70, Issue 6, 15 November 1977, Pages 901–905, https://doi.org/10.1093/aesa/70.6.901

Mitchell PL, and Newsom LD. 1984. Histological and behavioral studies of three-cornered alfalfa hopper (Homoptera: Membracidae) feeding on soybean. Ann. Entomol. Soc. Amer.-77(2):174-181.

Moore GC and Mueller AJ. 1976. Biological observations of the three-cornered alfalfa hopper on soybean and three weed species. J. Econ. Entomol. 69(1): 14-16.

Pereira GE, Padhi EMT, Sudarshana MR, Bello-Fialho F, Medina-Plaza C, Giradello RC, Seng T, Bruce RC, Erdmann JN, Slupsky CM and Olberholster A. 2021. Impact of grapevine red blotch disease on primary and secondary metabolites in Cabernet Sauvignon grape tissue. Food Chem. Vol. 342, 16 April 2021, 128312.

Preto CR, Sudarshana MR, Bollinger ML, and Zalom FG. 2018. *Vitis vinifera* (Vitales: Vitaceae) As a reproductive host of *Spissistilus festinus* (Hemiptera: Membracidae). J. Insect Sci. 18(6): 1-7.

Preto CR, Sudarshana MR, Bahder BW, Bick EN, and Zalom FG. 2019. Seasonal dynamics of *Spissistilus festinus* (Hemiptera: Membracidae) in a Californian vineyard. J. Econ. Entomol. 112(3): 1138-1144.

Smith R. 2013. Three-cornered alfalfa hopper, pp. 286-287. In L. J. Bettiga (ed.), Grape Pest Management, 3rd ed. Univ. Calif. Agric. Nat. Res. Publ. 3343, Oakland, CA.

Wildermuth VL. 1915. Three-cornered alfalfa hopper. J. Agric. Res. 3: 343-364.

Wilson H, Yazdani AS, Daane KD. 2020. Influence of Riparian Habitat and Groundcovers on the Three-cornered Alfalfa Hopper (Hemiptera: Membracidae) Populations in Vineyards. J. Econ. Entomol. 113(5), 2020, 2354-2361.

Wistrom C, Sisterson MS, Pryor MP, Hashim-Buckey JM, and Daane KM. 2010. Distribution of glassy-winged sharpshooter and three-cornered alfalfa hopper on plant hosts in the San Joaquin Valley, California. J. Econ. Entomol. 103: 1051-1059.

Figure Captions

Figure 1. Girdles resulting from *Spissistilus festinus* feeding on grape petiole (left) and *Tortistilus sp*. feeding on grape stem (right).

Figure 2. Mean $\pm$ SEM *Spissistilus festinus* girdles per Cabernet Sauvignon grapevine (n=30) at the Oakville, CA site in 2017 (left) and 2018 (right).

Figure 3. Mean $\pm$ SEM *Spissistilus festinus* girdles per Cabernet Sauvignon grapevine (n=30) at the Solano Co., CA site in 2017 (left) and 2018 (right).

Figure 1. Girdles resulting from *Spissistilus festinus* feeding on grape petiole (left) and *Tortistilus sp*. feeding on grape stem (right).

Figure 2. Mean $\pm$ SEM *Spissistilus festinus* girdles per Cabernet Sauvignon grapevine (n=30) at the Oakville, CA site in 2017 (left) and 2018 (right).
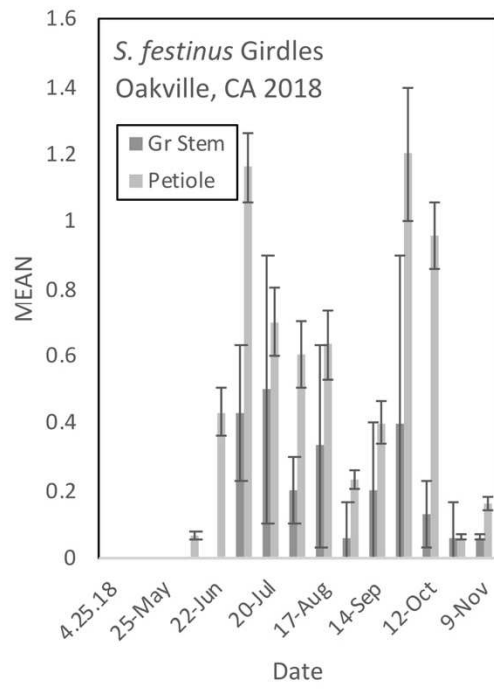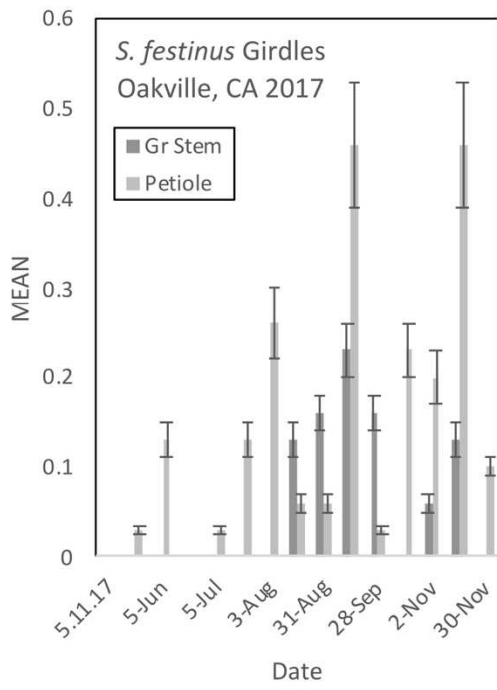
Figure 3. Mean ± SEM *Spissistilus festinus* girdles per Cabernet Sauvignon grapevine (n=30) at the Solano Co., CA site in 2017 (left) and 2018 (right).
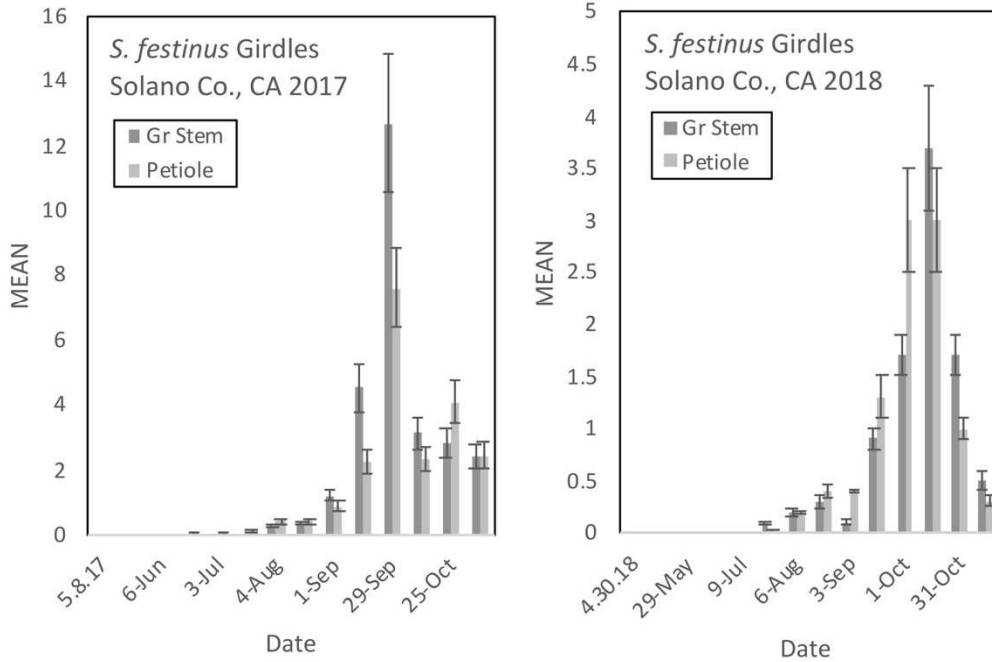
Four Morphotypes of a *Tortistilus* Treehopper (Hemiptera: Membracidae) in a California Vineyard Belong to a Single Species

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Abstract

In a study aimed at identifying potential vectors of *Grapevine red blotch virus* (family: Geminiviridae), insects identified as *Tortistilus* species were found feeding *en masse* on infected grapevines in a California vineyard where virus spread was suspected. The *Tortistilus* included individuals with either brown or green coloration and with prominent pronotal suprahumeral horns or without horns. The horned insects were initially thought to be *Tortistilus albidosparsus* (Stål 1860) and the unhorned insects *Tortistilus wickhami* (Van Duzee 1908) and appeared in both green and brown color forms. However, their occurrence and feeding on the same host on the same date raised a question about their classification. To ascertain their taxonomic status more precisely, two insects of each morphotype: brown horned, brown unhorned, green horned and green unhorned, were subjected to shotgun DNA sequencing using Illumina Hiseq4000. Initial analysis on the assembled contigs of cytochrome oxidase I (COI) indicated that all eight insects were in the same phylogenetic group and had <2% difference with *T. wickhami* sequences in GenBank. Examination of genitalia of the males did not reveal any morphological differences among the four morphotypes. Matings of combinations of horned and unhorned individuals resulted in F1 adults of both morphotypes.

Keywords: Ceresini, subhumeral horns, grape, *T. albidosparsus, T. wickhami*, CO1, sequencing

Introduction

A recently described DNA virus, *Grapevine red blotch virus* (GRBV), genus *Grablovirus*, family *Geminiviridae*, has garnered much attention from the North American grape industry because of its association with grapevine red blotch disease (GRBD) that causes significant impact on wine quality (Sudarshana et al. 2015, Ricketts et al. 2017). All known geminiviruses are transmitted by insects, either aphids, leafhoppers, treehoppers or whiteflies (Rojas et al. 2018). Prior to 2016, the only member of Membracidae known to vector a geminivirus was *Micrutalis malleifera* (Fowler) which transmits *Tomato pseudo curly top virus* in Florida (Simons and Coe 1958).

Since the discovery of GRBV (Krenz *et al.* 2012, Al Rwahnih *et al.* 2013), a great deal of research has been conducted on its genome, ecology and epidemiology. The virus has been shown to spread in vineyards in a manner consistent with transmission by a motile insect vector (Cieniewicz *et al.* 2017, Dalton *et al.* 2019, Sudarshana *et al.* 2015), and attempts have been made to identify potential vectors of the virus. It was initially thought that GRBV was transmitted by the Virginia creeper leafhopper, *Erythronuera ziczac* (Walsh) (Poojari *et al.* 2013). However, observations of vineyard landscapes failed to establish the presence of this species in California vineyards where GRBV spread had been observed, and greenhouse studies failed to demonstrate transmission by *E. ziczac* and two other species of the same genus, *E. elegantula* and *E. variablis,* that are more commonly found in vineyards (Bahder *et al.* 2016, Wilson *et al.* 2020). Successful GRBV transmission was achieved using three-cornered alfalfa hopper, *Spissistilus festinus* (Say), (Hemiptera: Auchenorrhyncha: Membracidae) (Bahder *et al.* 2016, Flaco *et al.* 2021), and in a spatial study, recovery of GRBV-positive *S. festinus* adults on sticky traps correlated well with the distribution of GRBV in a California vineyard (Cieniewicz

et al. 2018). The successful transmission of GRBV was observed using *S. festinus* collected from alfalfa fields where the insects are often found in substantial numbers (Bahder *et al.* 2016), or offspring of insects originating from alfalfa (Flaco *et al*. 2021). However, field transmission of GRBV using *S. festinus* has yet to be confirmed. This may be due to the possibility of genetic diversity among populations of *S. festinus* as has been shown for the existence of cryptic species in morphologically similar *Bemisia tabaci* (Gennadius) a vector of begomoviruses (Xu *et al.* 2010), and aphids (Rebijith *et al*. 2013). Such intraspecific genetic diversity has also been noticed in Membracidae (Foottit *et al*. 2014).

Prior to the discovery of *S. festinus* as a vector of GRBV in the greenhouse, it was considered to be a relatively minor pest of grapevine due to its habit of creating shoot or leaf petiole girdles as a result of its feeding (Smith 2013), but its geographic distribution and biology in California vineyards had been little studied.

In our surveys of California vineyards for GRBV incidence, we observed *S. festinus* is either absent or rarely seen in some of the infected vineyards located on hillsides and at slightly higher elevations in the north coast wine grape production areas. Often in these vineyards, Rather, members of the membracid genus, *Tortistilus,* are often seen feeding on grapevines and/or other nearby plants in the landscape including woody hosts such as oak (*Quercus* spp.) and herbaceous hosts including vetch (*Vicia* spp.) and others. These *Tortistilus* include individuals with prominent pronotal suprahumeral horns as well as others without pronotal horns, commonly found feeding in mixed assemblages on the same hosts and on the same observation dates. The horned insects were initially identified as *Tortistilus albidosparsus* (Stål 1860) and the unhorned insects as either *T. wickhami* (Van Duzee 1908) or *T. pacificus* (Van Duzee 1908) based on external morphology, primarily using the presence or absence of

prominent suprahumeral horns. In the family Membracidae, ten species of *Tortistilus* have been described (Deitze and Wallace 2012). Of these, four species, *T. albidosparsus, T. inermis* (Fabricius 1775), *T. pacificus* and *T. wickhami* have been reported from California (Deitz and Wallace 2012). *Tortistilus albidosparsus* was first described as *Ceresa albidosparsa* by Stål (1860) who designated a specimen from San Francisco, CA as its holotype. We now believe that the reference to 'buffalo treehopper' as a minor pest of north coast California grapes (Smith 2013) was probably *T. albidosparsus* instead based on the presence of pronotal horns.

Because *Tortistilus* is present in GRBV-infected vineyards where *S. festinus* is uncommon and the taxonomic status of both these genera in the Auchenorrhyncha tribe Ceresini, we are attempting to determine the status of *Tortistilus* as GRBV vectors. However, the presence of several morphotypes, horned and unhorned, green and brown, in our field collections, led us to question their genetic relatedness as a single species or multiple species. Here we attempt to provide morphological and genetic evidence to establish their taxonomic relationship as we initiate studies to determine their status as GRBV vectors.

Materials and Methods

Insect collection and voucher preparation.

The *Tortistilus* adults used in this study were collected on June 2, 2017, from purple vetch (*Vicia americana*) plants growing along the northern border of a Pope Valley grape vineyard where GRBV spread was reported. The Napa County, California, vineyard was located at geographical coordinates 38.37'23.69" N –122.22'06.04" W and elevation 242m using a heavy duty 38 cm diameter insect sweep net (Bioquip-7625HS; BioQuip Products, Rancho

Dominguez, CA USA). The insects were aspirated out of the sweep net using a glass aspirator (Bioquip-1135B, BioQuip Products, Rancho Dominguez, CA USA) and the screw lids were placed onto the aspirator containers. Then, they were placed into a small handheld ice cooler containing two frozen blue ice packs and brought to our laboratory at UC Davis. The adult treehoppers were also found on the same date on nearby grapevines and blue oak (*Quercus douglasii*) growing in the adjacent riparian area.

In the laboratory, all aspirator containers were removed from the ice cooler and completely filled with 75% ethanol. An hour later, individual aspirator containers were emptied into separate 150 mm x 15 mm sterile polystyrene petri dishes (Thermo Fisher Scientific, Waltham, MA, USA). These petri dishes were then placed under a Leica MZ 12.5 dissecting microscope to facilitate sorting of the four morphotypes. Two males randomly selected from each sorted morphotype were individually placed inside 1.5 ml centrifuge tubes containing 20 ml commercial bleach mixed with 80 ml deionized water. All eight centrifuge tube lids were closed and vortexed (Vortex Genie 2, Thermo Fisher Scientific, Waltham, MA, USA) on high speed for five sec. All insects were then placed inside new 1.5 ml centrifuge tubes filled with deionized water and vortexed on high speed for five sec. Individual males were then removed from centrifuge tubes using separate, autoclaved, #4, straight forceps (Bioquip 1431, BioQuip Products, Rancho Dominguez, CA USA) and all six legs from each insect were completely removed using similarly sterilized forceps and placed into 75% ethanol in a new 1.5 ml centrifuge tube.

Genomic DNA extraction and sequencing.

The DNA from the six legs removed from each specimen was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), following manufacturer's instruction. Tissue lysing by Proteinase K was facilitated by vortexing and maceration with microtube pestles before and during overnight incubation. After extraction, DNA was further purified using Zymo DNA Clean & Concentrator™-5 (Zymo Research, Irvine, CA) following manufacturer's instructions and eluted in 30 ul buffer. The purified DNA was submitted to the DNA Technologies and Expression Analysis Core at the University of California, Davis for RNase treatment and shotgun DNA sequencing. Paired end sequencing was performed to obtain 150 bp reads on an Illumina HiSeq4000 sequencer (Ilumina Inc, San Diego, CA, USA).

Assembly of Illumina reads and bioinformatics analysis.

After trimming the Illumina barcodes, the reads were filtered using a reference COI gene (GenBank: KR564392.1) and assembled using CLC Genomics Workbench (Qiagen, Germantown, MD, USA). The assembled contigs were subjected to multiple sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and pairwise identity scores were obtained. Subsequently, a phylogenetic tree was constructed using the COI sequences from eight insects from Pope Valley and the COI sequences from the *Tortistilus* species that were available in the GenBank (Table 1).

Examination of male genitalia.

Single, randomly chosen male *Tortistilus* representing each of the four morphotypes were individually placed onto sterile microscope slides under a Leica MZ 12.5 dissecting microscope, and genitalia were removed using Bioquip #3 insect pins (Bioquip-1208B3; Bioquip Products,

Rancho Dominguez, CA USA).   Afterwards, *Tortistilus* genitalia were individually placed into separate 1.5 ml centrifuge tubes that contained 180uL ATL buffer and 20uL proteinase K and incubated at 80C for 40 min. (The ATL and proteinase K incubation period was necessary to remove excess tissues from the genitalia that would have otherwise obstructed the field of view during microscopy). After 40 min incubation, genitalia were removed and placed into separate 1.5 mL centrifuge tubes containing 95% ethanol for storage awaiting microscopy. The genitalia were then removed from the 1.5 mL centrifuge tubes, placed under a dissecting microscope Leica MZ 12.5 (1 Pearl Ct., Allendale, NJ) and individually placed onto a pea-sized piece of Prismacolor Kneaded Eraser that was completely submerged under 95% ethanol in a 95 mm x 15 mm petri dish (Fisher Scientific-FB0875714G). The genitalia were then strategically positioned into the Kneaded Eraser with #3 insect pins. This method facilitates the positioning of very small objects by suspension in a clear fluid. Once the desired position of the genitalia was realized, the images were captured at 110X magnification with the mounted camera (JVC KY-F75, 2201 East Dominguez St. Long Beach, CA), with the Syncroscopy AutoMontage Program (Synoptics Inc. 5123 Pegasus Court Suite Q Frederick MD).

Mating study.

In May 2018, 152 third and fourth instar, sexually immature, *Tortistilus* nymphs were collected from purple vetch (*Vicia americana*) growing adjacent to the same Pope Valley vineyard where the original 2017 collection was made. The nymphs were collected using a 38cm diameter heavy-duty sweep-net (Bioquip #7625HS, Bioquip Products Inc. Rancho Dominguez, CA). The nymphs were transported to UC Davis where they were transferred individually into separate 2.5 cm diameter clip cages attached to mature purple vetch shoots

grown from seed in UC Davis mix soil in a single gallon pot that was placed inside a collapsible

insect rearing cage (Bioquip #1450L). The surviving nymphs matured to become adults in mid-

June. As adults, the *Tortistilus* in individual clip cages were then classified as horned and

unhorned, and sex was determined by presence of either an aedeagus or ovipositor. Finally, the

adults were combined into six groups consisting of three males and five females based on the

presence or absence of suprahumeral horn. One group consisted of horned males and horned

females, one group of unhorned males and unhorned females, and four groups consisted of either

horned males and unhorned females or vice versa. Each of the six groups were then placed into

six separate nylon insect mesh cages (Bugdorm-mesh cage #DC3148, Bioquip Products Inc.,

Rancho Dominguez, CA) fastened onto a potted blue oak tree (*Quercus douglasii*) with purple

vetch (*Vicia americana*) growing at its base. The blue oak seedlings were grown from acorns

collected next to the Pope Valley vineyard in February 2018. Each of the six potted blue oak

trees were then placed into a larger mesh insect cage and transferred into a UC Davis greenhouse

and were not disturbed other than being watered twice a week. Adults from this breeding study

emerged in the cages from April to May 2019.

Results

 The 574 adult *Tortistilus* collected on June 2, 2017, from the Pope Valley, CA, grape vineyard

consisted of 47 individuals with prominent pronotal suprahumeral horns and 530 hornless

specimens. Both horned and unhorned morphs included brown and green color morphs (Figure

1. A-C). DNA barcoding of randomly chosen individuals from this collection representing the

four possible combinations of these color and horn/no horn morphotypes using primers described

by Foottit *et al*. (2014) failed to distinguish the insects as belonging to two (or more) species. On

the contrary, sequencing of the amplified products indicated near identity of the amplicons (data not shown). Because the specimens were collected from a single location, on a single host plant, and on the same date, we suspected the morphological diversity observed may be due to morphotypes of a conspecific single species. To examine this hypothesis, a study of the molecular diversity of the four morphotypes was initiated.

DNA sequences of a shot gun library were obtained from DNA extracted from six legs of eight individual insects representing the brown and green color forms of both horned and unhorned specimens (Table 2). On average, sequencing of the eight DNA samples produced reads equivalent to 13.6 ± 0.31 billion base pairs. Reads corresponding to the COI gene were filtered by using a COI sequence (GenBank: KR564392.1) obtained from the *T. wickhami* voucher in Canada (Hebert *et al.* 2016) and assembled. The 608 n contigs obtained for the COI of each of the eight insects were aligned along with those of 23 other COI sequences attributed to *T. imermis*, *T. minutus*, *T. pacificus*, and *T. wickhami*, Pairwise identity scores were determined and phylogenetic relationships were established. Both analyses indicated that all eight insects belonged to a single species, and that they were not different from the sequences attributed to *T. wickhami* in the GenBank (Figure 2, Table 3). A difference of <2% identity was noticed between the COI genes of eight insects from Pope Valley and the GenBank COI gene barcodes of *T. wickhami*. These sequences were in turn different from those of the other three *Tortistilus* species present in the GenBank (Figure 2; Table 3).

Traditional identification of *Tortistilus* species is made primarily by morphological characteristics of male genitalia. However, taxonomic determination of the *Tortistilus* of concern in California vineyards as possible vectors of GRBV has been challenging due to the occurrence of the four morphotypes. All of the morphotypes appear to have identical male

genitalia, yet the presence of pronotal suprahumeral horns that are consistent with *T. albidosparsus* are lacking on many of the insects collected on the same grapevines that might otherwise have been identified as *T. wickhami.* Figure 3 (A-H) presents high resolution automontage images of the eight specimens corresponding to the CO1 sequences previously presented. The similarities of these male genitalia are striking, and characteristics that would distinguish the genitalia of these specimens from other *Tortistilus* species are lacking. It seems possible that the crude hand-drawings (Caldwell-1949) used in differentiating the male genitalia of these species may have been subordinated to the presence or absence of the more prominent suprahumeral horn characteristic in identification of these insects.

Matings of all combinations of horned and unhorned morphotypes collected as sexually immature nymphs in late spring 2018 produced offspring that matured to adults in spring 2019. Of the six mating groups, three produced both horned and unhorned progeny (Table 4).

Discussion

Members of several Hemipteran families including Aphidae, Cicadellidae, and Aleyrodidae are vectors of geminiviruses, however, the only member of Membracidae known to vector a geminivirus is *M. malleifera* which transmits tomato pseudo curly top virus (Simons and Coe 1958). The recent discovery of GRBV transmission by the membracid *S. festinus* to grapevines under laboratory conditions (Bahder *et al*. 2016, Falco et al. 2021) has prompted the search for other potential treehopper species present in vineyards that may also serve as GRBV vectors.

Our survey of north coast California vineyards where GRBV is present has revealed the sympatric presence of four distinct *Tortistilus* morphotypes consisting of brown and green

colored insects and both color forms with and without pronotal suprahumeral horns (Figure 1.).

However, our initial attempts to barcode these insects failed to distinguish them as belonging to

more than a single species. Using classical morphological traits, the horned morphs were easily

identified as *T. albidosparsus*, originally described as C*eresa albido-sparsa* by Stål in (1860).

However, male genitalia of the unhorned morphs were identical to those of *T. albidosparsus,*

even though they lack the suprahumeral horns described by Stål. Thus, we initiated a study not

only to identify the species of the morphotypes, but to provide additional insight into the genome

of Membracidae which contains species exhibiting diverse morphological, behavioral, ecological

and distribution patterns (Deitz and Wallace 2010).

Analysis on the assembled contigs of CO1 for the four morphotypes we selected

indicated that all eight insects are in the same phylogenetic group, and their pairwise identity

scores when compared with the other *Tortistilus* species represented in the GenBank clearly

show that these sequences are very close to the sequences attributed to *T. wickhami.*

Furthermore, there appears to be two clades of *T. albidosparsus* with eight California specimens

representing one clade. All of these T. albidosparsus sequences are distinct from those of the

three additional *Tortistilus* species (*T. inermis, T. minutus and T. pacificus*) available in the

GenBank.

The genomes described herein were deposited at NCBI under bioproject

(BIOPROJ00090900) as the first genomic resource for *T. albidosparsus*.

Finally, crosses of males and females of the horned and unhorned morphotypes produced

offspring with both horns and no horns. Further analysis of the genome supported by future

mating experiments may unravel if the origin of the observed morphotypes were due to some

epigenetic phenomenon or to inheritable genetic changes. We also anticipate morphological

examination of male voucher specimens along with genome sequencing data will clarify the validity of current systematics within the genus *Tortistilus.*

Disclaimer

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References cited

Al Rwahnih, M.A., A. Dave, M.M. Anderson, A. Rowhani, J.K. Uyemoto, and M.R. Sudarshana. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopath. 103: 1069–1076.

Bahder, B.W., M.L. Bollinger, M.R. Sudarshana, and F.G. Zalom. 2015. Preparation of mealybugs (Hemiptera: Pseudococcidae) for genetic characterization and morphological examination. J. Insect. Sci. 15:104: https://doi.org/10.1093/jisesa/iev086

Bahder B.W., F.G. Zalom, M. Jayanth, and M.R. Sudarshana. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. Phytopath. 106: 1223-1230.

Cieniewicz, E.J., S.J. Pethybridge, A. Gorny, L.V. Madden, H. McLane, K.L. Perry, and M. Fuchs, 2017. Spatiotemporal spread of grapevine red blotch-associated virus in a California vineyard. Virus Res. 241: 156-162.

Cieniewicz, E.J., S.J. Pethybridge, G. Loeb, K. Perry, and M. Fuchs. 2018. Insights into the ecology of *Grapevine red blotch virus* in a diseased vineyard. Phytopath. 108:94-102.

Dalton, D.T., R.J. Hilton, C. Kaiser, K.M. Daane, M.R Sudarshana, J. Vo, F.G. Zalom, J.Z. Buser, and V.M. Walton. 2018. Spatial associations of vines infected with grapevine red blotch virus in Oregon vineyards. Plant Dis. 103: 1507-1514.

Caldwell, J.S. 1949. A generic revision of the treehoppers of the tribe Cerisini in America north of Mexico based on a study of the male genitalia. Proc. U.S. Nat. Mus. 98: 3234.

Deitz, L.L., and M.S. Wallace. 2012. Richness of the Nearctic treehopper fauna (Hemiptera: Aetalionidae and Membracidae). Zootaxa 3423: 1-26.

Flasco M, V. Hoyle, E.J. Cieniewicz, G.R. Brandon, H.L. McLane, K.L. Perry, G. Loeb, B. Nault, M. Heck, and M. Fuchs. 2021. Grapevine red blotch virus is transmitted by the three-cornered alfalfa hopper in a circulative, nonpropagative mode with unique attributes. Phytopath. https://doi.org/10.1094/PHYTO-02-21-0061-R

Foottit, R.G., E. Maw, and P.D.N. Hebert. 2014. DNA barcodes for Neartic Aucheonorrhynca (Insecta: Hemiptera). PLoS ONE 9(7): e101385. https://doi.org/10.1371/journal.pone.0101385.

Hebert, P.D.N., S. Ratnasingham, E.V. Zakharov, A.C. Telfer, V. Levesque-Beaudin, M.A. Milton, S. Pedersen, P. Jannetta, and J.R. deWaard. 2016. Counting animal species with

DNA barcodes: Canadian insects. Phil. Trans. R. Soc. B 371: 20150333.

http://dx.doi.org/10.1098/rstb.2015.0333

Krenz, B., J.R. Thompson, M. Fuchs, and K.L. Perry. 2012. Complete genome sequence of a new circular DNA virus from grapevine. J. Virol. 86: 7715.

Poojari, S., O.J. Alabi, V.Y. Fofanov, and R.A. Naidu. 2013. A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. PLoS ONE. 8: e64194.

Rebijith, K.B., R. Asokan, N.K. Krishnakumar, and V. Krishna. 2013. DNA barcoding and elucidation of cryptic aphid species (Hemiptera: Aphididae) in India. Bull Ent. Res. 103: 601-610.

Ricketts, K.D., M.I. Gómez, M.F. Fuchs, T.E. Martinson, R.J. Smith, M.L. Cooper, M.M. Moyer, and A. Wise. 2016. Mitigating the economic impact of grapevine red blotch: optimizing disease management strategies in US vineyards. Amer. J. Enol. Vit. 68: 127-135.

Rojas, M.R., M.A. Macedo, M.R. Maliano, M. Soto-Aguilar, J.O. Souza, R.W. Briddon, L. Kenyon, R.F.R. Bustamante, F.M. Zerbini, S. Adkins, J.P. Legg, A. Kvarnheden, W.M. Wintermantel, M.R. Sudarshana, M. Peterschmitt, M. Lapidot, D.P. Martin, E. Moriones, A.K. Inoue-Nagata, and R.L. Gilbertson. 2018. World management of geminiviruses. Ann. Rev. Phytopath. 56: 637-677.

Simons, J.N., and D.M. Coe. 1958. Transmission of pseudo-curly top virus in Florida by a treehopper. Virology 6: 43-48.

Smith, R.J. 2013. Treehoppers. In Grape pest management, Bettiga, L.J., ed. Univ. Calif. Agric. Nat. Res. Publ. 3343, pp. 341-345. Richmond, CA.

Stål, C. 1859. Hemiptera. Species novas descripsit. Kongliga Svenska Fregatten Eugenies Resa Omkring Jorden. Kongliga Svenska Vetcnskaps-Akad. Zool. 4: 219-298.

Sudarshana, M.R., K.L. Perry, and M.F. Fuchs. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. Phytopath. 105: 1026-1032.

Wilson, H., A.S. Yazdani, and K.D. Daane. 2020. Influence of riparian habitat and groundcovers on the three-cornered alfalfa hopper (Hemiptera: Membracidae) populations in vineyards. J. Econ. Entomol. 113(5): 2354-2361.

Xu, J., P.J. De Barro, and S.S. Liu. 2010. Reproductive incompatibility among genetic groups of *Bemisia tabaci* supports the proposition that the whitefly is a cryptic species complex. B. Entomol. Res. 100: 359-366.

Figure Captions

Figure 1. (A) Adult *Tortistilus* infesting a Sauvignon blanc grapevine in a California vineyard. Note both green and brown morphs of *Tortistilus* insects on young shoots. (B) Treehopper oviposition scars on a Cabernet sauvignon grapevine shoot, and (C) horned and unhorned morphs of *Tortistilus albidosparsus* on a Cabernet sauvignon grape leaf in Pope Valley, Napa Co., CA.

Figure 2. Phylogenetic tree constructed using the CO1 gene sequence of eight *Tortistilus albidosparsus* morphotypes from a collection made from a grape vineyard in Pope Valley, Napa Co., CA on June 2, 2017, and additional CO1 sequences of *Tortistilus* species available in the GenBank. CO1 sequences for the 8 *T. albidosparsus* morphotypes from the Pope Valley collection are labeled as 01 to 08.

Figure 3. Figures A, C, E and G are profiles of male *Tortistilus albidosparsus* aedeagus posterior and anterior arms. Figures B. D, F and H are caudal views of male *T. albidosparsus* posterior aedeagus and posterior style arms. Figures A and B are horned brown, C and D are horned green, E and F are unhorned brown and G and H are unhorned green morphotypes. All male genitalia were dissected and lysed using 180uL ATL buffer and 20uL proteinase K in a 1.5 ml centrifuge tube at an incubation temperature of 80C for 40 minutes. Images were taken with a digital JVC camera mounted onto a Leica MZ 16A dissecting microscope at 110X magnification.

Figure 1.

Figure 2.
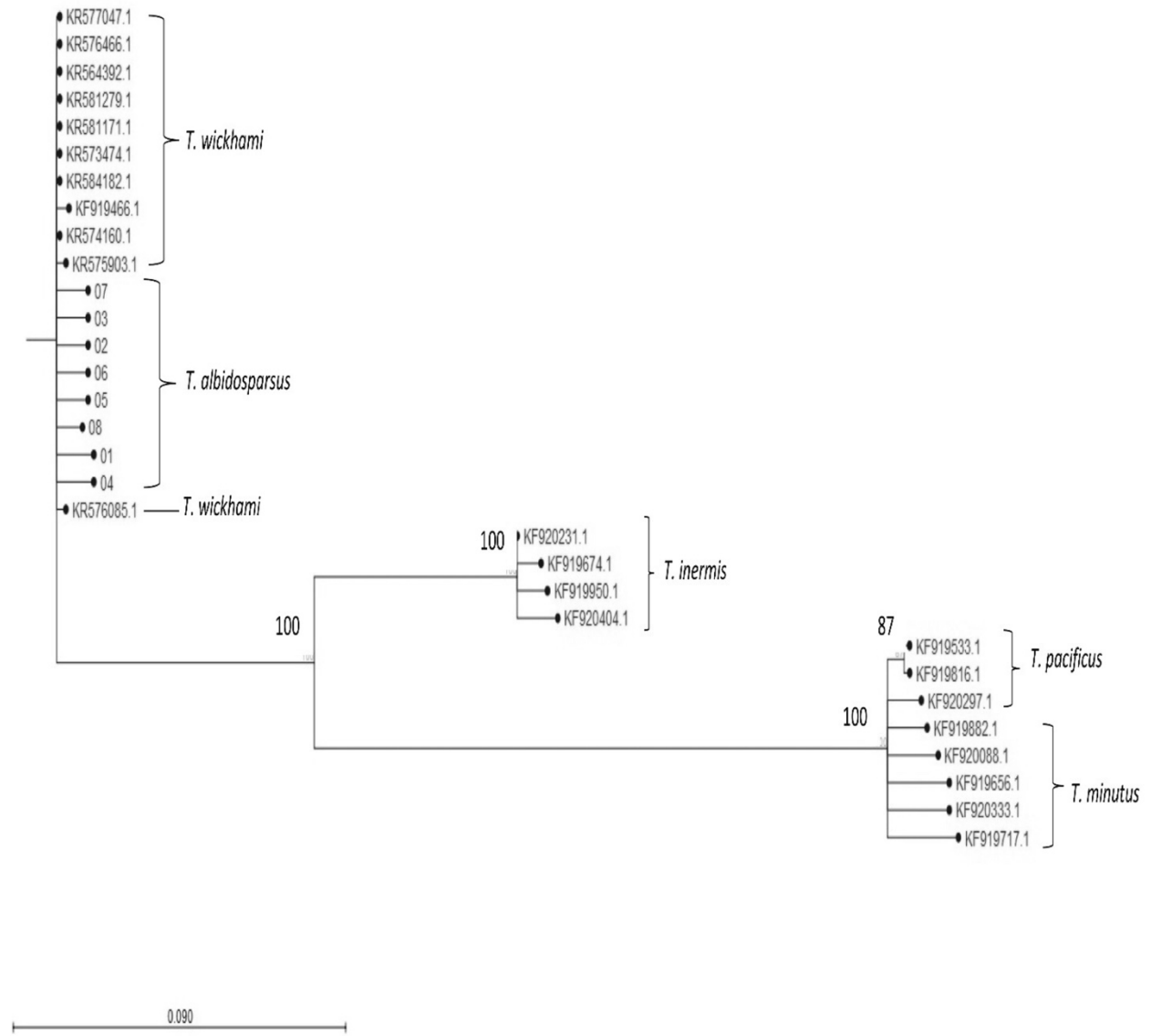
Figure 3.
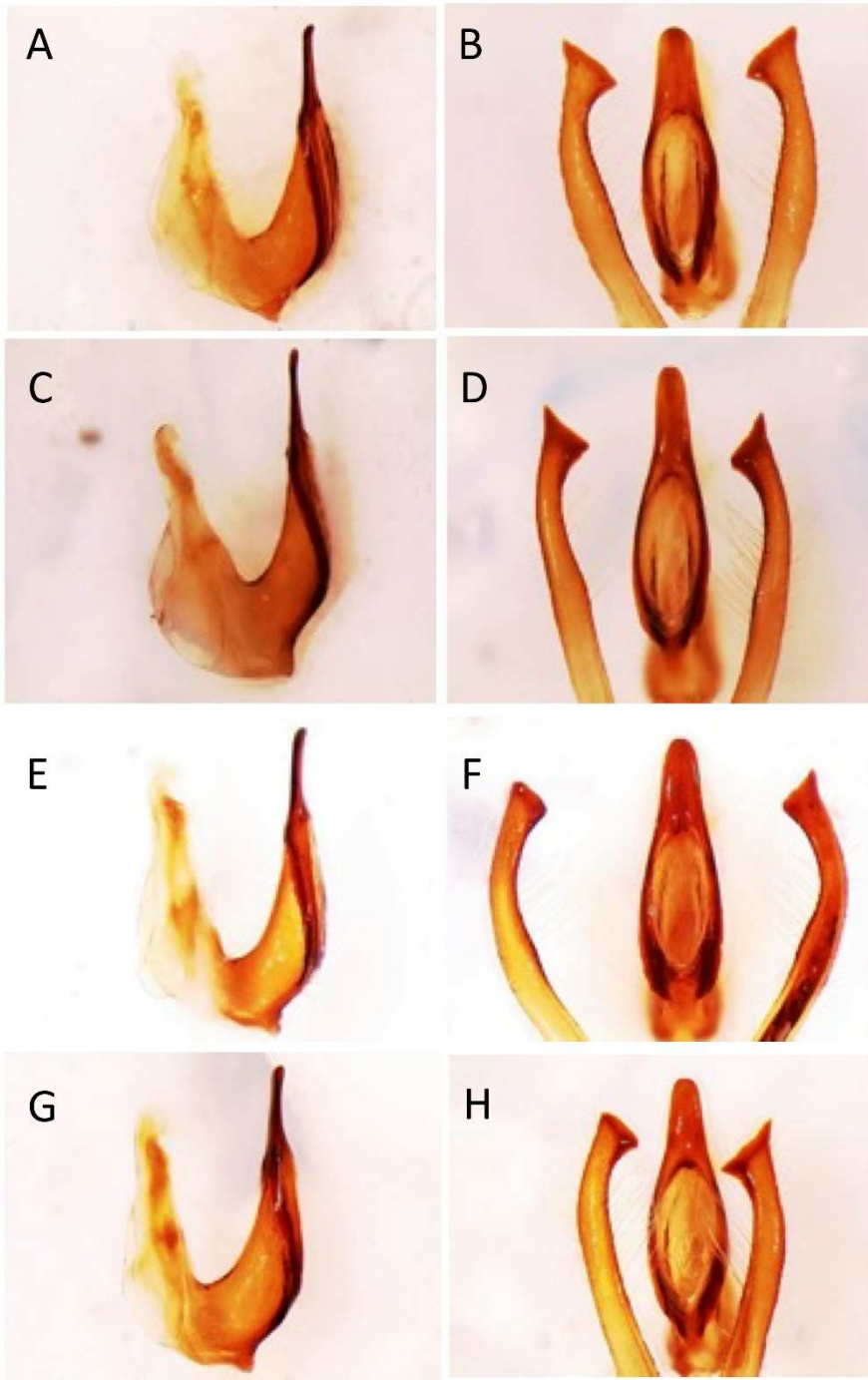
Table 1. COI sequences of *Tortistilus* species in GenBank database and the location where the voucher specimens were collected.

GenBank Accession #	Species	Location where collected
KF919674.1	*T. inermis*	Canada, Ontario, 13 km E Little Current
KF919950.1	*T. inermis*	USA, North Dakota, 5 km SE Balta
KF920231.1	*T. inermis*	USA: Colorado, 6.5 km W Golden
KF920404.1	*T. inermis*	USA: Wisconsin, Grantsburg
KF919656.1	*T. minutus*	Canada: Manitoba, Morden
KF919717.1	*T. minutus*	USA: Wisconsin, Kenosha Co., Chiwaukee Prairie
KF919882.1	*T. minutus*	Canada: Manitoba, Morden
KF920088.1	*T. minutus*	Canada: Manitoba, Morden
KF920333.1	*T. minutus*	Canada: Manitoba, Winnipeg
KF919533.1	*T. pacificus*	USA: Colorado, 7.2 km N
KF919816.1	*T. pacificus*	USA: Minnesota, 7 km SW Rothsay
KF920297.1	*T. pacificus*	USA: Colorado, 7.2 km N
KF919466.1	*T. wickhami*	USA: Wyoming, Jackson
KR564392.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR573474.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR574160.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR575903.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR576085.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR576466.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek

Table 1. Continued.

COI sequences of *Tortistilus* species in GenBank database and the location where the

voucher specimens were collected (continued).

GenBank Accession #	Species	Location where collected
KR577047.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR581171.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR581279.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek
KR584182.1	*T. wickhami*	Canada: Alberta, Waterton Lakes NP, Coppermine Creek

Table 2. Sample information and sequence reads obtained by Illumina sequencing of DNA from four morphs of insects originally identified as *Tortistilus* sp., and collected in a grape vineyard in Pope Valley, Napa County, CA on June 2, 2017.

Sample ID	Code	Color	Horned	Illumina reads	Coverage (million Gbp)
DS17-01	BH-	Brown	No	46.0	13.8
DS17-02	BH-	Brown	No	44.8	13.4
DS17-03	GH-	Green	No	47.3	14.2
DS17-04	GH-	Green	No	45.8	13.7
DS17-05	BH+	Brown	Yes	43.9	13.2
DS17-06	BH+	Brown	Yes	45.9	13.8
DS17-07	GH+	Green	Yes	44.5	13.4
DS17-08	GH+	Green	Yes	45.3	13.6

Table 3. Pairwise identity scores between CO1 sequences of *Tortistilus* species in GenBank database and those sequenced in our study.

No[1] GenBank #	Identity score (%)																										
2: KF919533.1	**99.7**	**100**																									
3: KF920297.1	**98.5**	**98.5**	**100**																								
4: KF919717.1	**99.0**	**99.0**	**100**	**100**																							
5: KF920088.1	**98.3**	**98.3**	**99.2**	**99.5**	**100**																						
6: KF919656.1	**98.6**	**98.6**	**99.5**	**99.8**	**99.7**	**100**																					
7: KF920333.1	**98.8**	**98.8**	**99.8**	**99.8**	**99.8**	**100**	**100**																				
8: KF919882.1	**98.3**	**98.3**	**99.2**	**99.8**	**99.7**	**99.7**	**100**	**100**																			
9: KF919674.1	82.4	82.1	82.4	81.3	82.2	82.2	81.1	82.5	**100**																		
10: KF920231.1	80.9	80.9	80.7	80.6	80.2	80.4	80.4	80.4	**99.3**	**100**																	
11: KF920404.1	82.4	82.1	82.1	80.6	81.9	81.9	80.4	82.2	**99.1**	**99.5**	**100**																
12: KF919950.1	80.9	80.9	80.7	80.4	80.2	80.5	80.2	80.7	**99.1**	**99.3**	**99.8**	**100**															
13: KR575903.1	80.6	80.9	81.2	79.5	81.1	81.1	79.5	81.2	88.9	89.8	88.4	89.3	**100**														
14: KR576466.1	80.6	81.0	81.3	79.6	81.1	81.1	79.7	81.3	88.7	89.3	88.2	88.9	**99.7**	**100**													
15: KR577047.1	80.6	80.9	81.2	79.5	81.1	81.1	79.5	81.2	88.6	89.2	88.1	88.8	**99.7**	**100**	**100**												
16: KR576085.1	81.0	81.3	81.6	80.2	81.4	81.4	80.2	81.6	89.0	89.8	88.5	89.4	**99.7**	**99.7**	**99.7**	**100**											
17: KR574160.1	80.8	81.1	81.4	79.9	81.3	81.3	79.9	81.4	88.8	89.6	88.3	89.1	**99.8**	**99.8**	**99.8**	**99.8**	**100**										
18: KR573474.1	80.8	81.1	81.4	79.9	81.3	81.3	79.9	81.4	88.8	89.6	88.3	89.1	**99.8**	**99.8**	**99.8**	**99.8**	**100**	**100**									
19: KR584182.1	80.8	81.1	81.4	79.9	81.3	81.3	79.9	81.4	88.8	89.6	88.3	89.1	**99.8**	**99.8**	**99.8**	**99.8**	**100**	**100**	**100**								
20: KR581279.1	80.7	81.1	81.4	79.8	81.2	81.2	79.8	81.4	88.8	89.5	88.3	89.0	**99.8**	**99.8**	**99.8**	**99.8**	**100**	**100**	**100**	**100**							
21: KR581171.1	80.7	81.1	81.4	79.8	81.2	81.2	79.8	81.4	88.8	89.5	88.3	89.0	**99.8**	**99.8**	**99.8**	**99.8**	**100**	**100**	**100**	**100**	**100**						
22: KR564392.1	80.8	81.1	81.4	79.9	81.3	81.3	79.9	81.4	88.8	89.6	88.3	89.1	**99.8**	**99.8**	**99.8**	**99.8**	**100**	**100**	**100**	**100**	**100**	**100**					
23: KF919466.1	79.9	79.9	80.2	80.1	79.9	80.2	80.2	80.2	89.6	89.6	89.3	89.3	**99.4**	**99.4**	**99.4**	**99.4**	**99.7**	**99.7**	**99.7**	**99.7**	**99.7**	**99.7**	**100**				
24: DS17-01	80.7	81.0	81.3	80.1	81.0	81.2	80.2	81.3	88.2	88.8	87.7	88.5	**98.8**	**98.9**	**98.8**	**98.9**	**99.0**	**99.0**	**99.0**	**99.0**	**99.0**	**99.0**	98.8	**100**			
25: DS17-03	81.2	81.5	81.8	80.9	81.5	81.6	80.9	81.8	88.6	89.6	88.2	89.2	**99.0**	**99.0**	**99.0**	**99.3**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	99.0	**99.5**	**100**		
26: DS17-07	81.2	81.5	81.8	80.9	81.5	81.6	80.9	81.8	88.6	89.6	88.2	89.2	**99.0**	**99.0**	**99.0**	**99.3**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	99.0	**99.5**	**100**	**100**	
27: DS17-05	81.2	81.2	81.8	80.6	81.5	81.6	80.7	81.8	88.6	89.3	88.2	89.0	**99.0**	**99.0**	**99.9**	**99.0**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	**99.2**	99.3	**99.5**	**99.7**	**99.7**	**100**
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27

[1]Sequences 1 to 3 are from *T. pacificus*, 4 to 8 are from *T. minutus*, 9 to 12 are from *T. inermis*, and 13 to 23 are from *T. wickham*i. DS17-01 to 07 are from this study, from insects identified as *T. albidopsarsus*.

Table 4. Number of *Tortistilus albidosparsus* progeny resulting from the mating of 3 males and 5 females of the designated combinations of morphotypes in cages containing a blue oak, *Quercus douglasii*, seedling and purple vetch, *Vicia americana*.

Group	Male parent with horns?	Female parent with horns?	Male progeny Horns	Male progeny No horns	Female progeny Horns	Female progeny No horns
1	Y	Y	11	none	3	None
2	Y	N	None	none	none	None
3	Y	N	1	8	none	8
4	N	Y	None	none	1	1
5	N	Y	None	none	none	None
6	N	N	None	none	1	2