Mealybug Transmission of Grapevine Leafroll Viruses: An Analysis of Virus–Vector Specificity

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ABSTRACT

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To understand ecological factors mediating the spread of insect-borne plant pathogens, vector species for these pathogens need to be identified. Grapevine leafroll disease is caused by a complex of phylogenetically related closteroviruses, some of which are transmitted by insect vectors; however, the specificities of these complex virus–vector interactions are poorly understood thus far. Through biological assays and phylogenetic analyses, we studied the role of vector-pathogen specificity in the transmission of several grapevine leafroll-associated viruses (GLRaVs) by their mealybug vectors. Using plants with multiple virus infections, several virus species were screened for vector transmission by the mealybug species *Planococcus ficus* and *Pseudococcus longispinus*. We report that two GLRaVs (-4 and -9), for which no vector transmission evidence was available, are mealybug-borne. The analyses performed indicated no evidence of mealybug–GLRaV specificity; for example, different vector species transmitted GLRaV-3 and one vector species, *Planococcus ficus*, transmitted five GLRaVs. Based on available data, there is no compelling evidence of vector–virus specificity in the mealybug transmission of GLRaVs. However, more studies aimed at increasing the number of mealybug species tested as vectors of different GLRaVs are necessary. This is especially important given the increasing importance of grapevine leafroll disease spread by mealybugs in vineyards worldwide.

Additional keywords: *Ampelovirus, Closteroviridae*, semipersistent.

The majority of plant viruses are vector-borne. Arthropods, nematodes, and fungi transmit 76% of plant viruses; among these, the most important group of vectors are sap-sucking insects such as aphids which, altogether, transmit 55% of described plant viruses (16,23). The modes of pathogen transmission are generally classified based on characteristics of virus–vector interactions; viruses that replicate within insects are transmitted in a propagative manner and those that do not replicate are nonpersistently, semipersistently, or circulatively (persistently) transmitted (16,24). Virus–vector specificity, the ability of an insect species to transmit a specific virus, varies substantially among vector-borne plant viruses. However, a genus of plant viruses is usually transmitted by vectors from one family of insects and has the same transmission mode, suggesting long-term evolutionary relationships between virus and vector (23). This characteristic of insect-borne plant viruses is consistent across taxa, so much so that virus taxonomy may be partially proposed based on the type of vector (18).

The virus family *Closteroviridae* is composed of several insect-borne plant viruses of economic importance, including *Citrus tristeza virus*, *Grapevine leafroll-associated virus*-3 (GLRaV-3), and *Tomato chlorosis virus*, among others. The family *Closteroviridae* is subdivided into three genera: *Closterovirus*, *Ampelovirus*, and *Crinivirus* (21). These are large, positive-sense, single-stranded RNA viruses with genomes of 15.3 to 19.5 kb in size and virions of 650 to 2,200 nm in length; in addition, the overall structure of the linear genome of closteroviruses is similar (6,21). Among shared molecular characteristics, viruses in the family *Closteroviridae* have a unique heat-shock protein homologue that is useful for across-taxa phylogenetic analysis due to its conserved nature (6). Vectors of many closteroviruses have been identified. Members of the genus *Closterovirus* have been shown to be aphid-borne, while ampeloviruses are mealybug and soft scale-borne and criniviruses are transmitted by whiteflies (18,21). Current evidence indicates that the level of vector–pathogen specificity for different virus species is variable for the family *Closteroviridae*; some viruses have a wide vector range and others have been demonstrated to be transmitted by only one insect species (18).

The identification of vector species for insect-borne plant viruses and the level of vector specificity among these interactions are of epidemiological importance (23). The recognition of trends in transmission biology provides information that drives research in poorly characterized systems because transmission characteristics (not efficiency) are generally shared among viruses belonging to the same family, genus, or species. Among closteroviruses, species in the genus *Ampelovirus* are especially understudied in relation to transmission biology, with the exception of pineapple mealybug wilt-associated viruses and GLRaV-3, for which some information is available (4,8,28,29,31).

GLRaVs cause grapevine leafroll disease as a virus complex, with several viruses sequentially named GLRaV-1, GLRaV-2, GLRaV-3, and so on. All members of this virus complex belong to the genus *Ampelovirus*, with the exception of GLRaV-2 (*Closterovirus*) and GLRaV-7 (unassigned genus). Leafroll disease has become an emerging problem in different grape-growing regions of the world, with awareness about the disease increasing after

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this virus complex, once thought to be only graft transmissible, was found to be spreading within vineyards (3,11,13,14). Mealybugs were first shown to transmit *Ampelovirus* spp. in 1990 (8). Since then, some mealybug (*Pseudococcidae*) and soft-scale (*Coccidae*) species have been shown to transmit different GLRaVs (1,4,8,28,30). Transmission of GLRaVs, based on a limited number of studies, seems to occur in a semipersistent manner (31).

In California, where evidence of leafroll disease spread is recent (11), four mealybug species have been associated with grapevines for decades, including *Pseudococcus longispinus*; while one, *Planococcus ficus*, was introduced into the state in 1994 (5). Direct damage to grapevines due to these mealybug species is primarily associated with infestation of the fruit clusters and growth of sooty molds as a consequence of honeydew excretion. Populations of these species are often kept low due to insecticide applications and natural enemies. However, these approaches are unlikely to be successful in reducing mealybug-vector populations low enough to limit GLRaVs spread. The species used in this study, include *Planococcus ficus*, the sole *Planococcus* sp. important in California vineyards, and *Pseudococcus longispinus*, which is representative of three species in the *Pseudococcus–maritimus* complex. *Planococcus ficus* is present in most grape-growing regions and *Pseudococcus longispinus* is found in cooler coastal regions where GLRaVs may cause greater damage.

Despite growing interest in the biology, ecology, and transmission of closteroviruses associated with grapevine leafroll disease, the degree of vector specificity has not been analyzed for this virus complex. Here, we present data showing that two species of mealybugs transmit different GLRaV species; two of these ampeloviruses have not been previously reported to be vector-borne. In addition, we discuss the degree of vector specificity among mealybugs and GLRaVs in the genus *Ampelovirus*.

MATERIALS AND METHODS

Screening for *Planococcus ficus*-transmissible viruses. *Planococcus ficus* colonies were established on butternut squash (*Cucurbita moschata*) from single females collected in vineyards near Del Rey, CA. Colonies were maintained in a growth chamber at 22 ± 2°C, with a 12:12-h photoperiod. First instars were used in all experiments because this life stage was shown previously to be the most efficient vector of GLRaV-3 (31). Virus source vines used for this study and their infection status are listed in Table 1. We collected the source vines from a virus collection at the University of California (UC) Davis (9) in the fall and brought them to the UC Berkeley for transmission tests. The source vines were rinsed with water to remove dust and blotted dry with Kimwipe papers. Healthy test plants used for mealybug transmission assays were rooted from dormant cuttings of grapevine cv. Cabernet Franc obtained from the Foundation Plant Services (FPS) at UC Davis, and were grown in a greenhouse until they reached ≈20 cm tall with approximately six expanded leaves. We used Cabernet Franc because it is a good biological indicator for leafroll disease (27). All experiments had greenhouse negative controls, grapevines from the same batch of test plants that were not exposed to mealybug vectors.

In the first experiment conducted, mealybugs were allowed to move onto source vine cuttings (20 cm) laid on mealybug colonies. After 2 h, the cuttings were removed from the mealybug colonies and maintained in flasks of water. After an acquisition access period (AAP) of 24 h, insects were gently shaken off the source tissue onto paper disks (0.5 cm in diameter). Potentially viruliferous mealybugs were transferred on paper disks to healthy test plants by caging the insects on leaf blades using clip cages previously described (31). For each treatment, 10 plants were inoculated with groups of 5 mealybugs and another 10 plants with groups of 20 individuals. Therefore, each accession number (i.e., virus source tissue) was used as source material for 20 insect-group inoculation events. After a 24-h inoculation access period (IAP), mealybugs were removed from the test plants with a fine brush. These plants were then treated with an insecticide. The test plants were maintained in a greenhouse and sprayed with insecticide and fungicide until tested for viruses. We pruned all plants periodically to avoid overgrowth. The plants in this experiment were inoculated by mealybugs in October 2007 and then went through the winter in a light-supplemented greenhouse. Petiole samples were harvested in April and August 2008 for reverse-transcription polymerase chain reaction (RT-PCR)-based detection of grapevine viruses (described below).

Following results from the first experiment, all nontransmissible virus accessions were tested again; a similar protocol was used to screen these virus sources. In this experiment, the number of mealybugs per group was increased and longer AAP and IAP were used to maximize transmission rates. Fifty mealybugs were transferred to each of 10 healthy test plants for an AAP and IAP of 48 h each. Test plant maintenance and pesticide spraying were the same as described above. The plants from this

TABLE 1. Summary of transmission experiments performed with two mealybug species, *Planococcus ficus* and *Pseudococcus longispinus*

Source plant[a]	Viruses in source plants[b]	Virus detected[c]	Transmission rate[d]		
			5 insects	20 insects	50 insects
Planococcus ficus					
LR100	GLRaV-5	None	0/10	0/10	0/10
LR102	GLRaV-1, -2, -5, RSP, GVB	GLRaV-1, -5	0/10	0/10	5/10, 4/10
LR106	GLRaV-4	GLRaV-4	0/10	1/10	–
LR109	GLRaV-2, -3, RSP, GFkV	GLRaV-3	6/10	7/10	–
LR110	GLRaV-2, -4, GFkV	None	0/10	0/10	0/10
LR118	GLRaV-9	GLRaV-9	0/10	2/10	–
LR135	GLRaV-4, GVA	GVA	0/10	0/10	5/10
LV92-02	GLRaV-1, -2, -7, GFkV, GVB	GLRaV-3	4/10	8/10	–
LV92-04	GLRaV-2, -4, -5, RSP, GFkV, GVB	GLRaV-3	2/10	4/10	–
LV93-09	GLRaV-1, -5, GVA, GFkV	None	0/10	0/10	0/10
Pseudococcus longispinus					
LR100	GLRaV-5	None	–	0/74	–
LR118	GLRaV-9	GLRaV-9	–	13/74	–

[a] Source plant material was from a virus collection at the University of California, Davis.

[b] Abbreviations: GLRaV, *Grapevine leafroll-associated virus*; GVA, *Grapevine virus A*; GVB, *Grapevine virus B*; RSP, *Grapevine rupestris stem pitting-associated virus*; GFkV, *Grapevine fleck virus*. Plants were not tested for RSP and GFkV.

[c] Virus detected in test plants. Infection of viruses in the test plants was assayed by reverse-transcription polymerase chain reaction.

[d] Transmission rate is based on the proportion of plants inoculated with GLRaV by groups of 5, 20, or 50 individual mealybugs. Number of positive plants/number of test plants for groups of 5, 20, and 50 mealybugs used as vectors during the inoculation access period. Ten to twenty mealybugs were used for the *Pseudococcus longispinus* trials.

trial were inoculated by mealybugs in September 2008 and then went through the winter in a light-supplemented greenhouse. Petiole samples were harvested in April 2009 for virus detection. In both trials, mealybug transmission from healthy grapevines to healthy test plants and test plants with no mealybug inoculations were used as controls, ensuring that our mealybug colonies were not naturally infected and that virus spread had not occurred within the greenhouse during experimental periods.

Screening for *Pseudococcus longispinus*-transmissible viruses. Two sources of *Pseudococcus longispinus* were used for this experiment: one from a vineyard in San Luis Obispo, CA and the other from a natural infestation in a greenhouse on the UC Davis campus. Colonies were established with single adult females; mealybugs were maintained on sprouted potato tubers (*Solanum tuberosum*). GLRaV sources were also obtained from a virus collection at UC Davis (9). Virus accessions LR100 and LR118, which are infected with GLRaV-5 and GLRaV-9, respectively, were used as sources. The vines were rooted and transplanted to 4-liter pots in a greenhouse until they were ≈1.5 m tall. *Pseudococcus longispinus* individuals (mixed-life stages) were allowed a 2-week AAP on virus source plants. One-node rooted cuttings of healthy grapevines (cv. Cabernet Franc) were used as test plants. After a 2-week AAP, leaves of the mealybug-infected source plants were cut into sections and arranged on healthy test plants to allow insects to crawl off as the leaf dried. In all, ≈10 to 20 mealybugs were observed feeding on each test plant; a total of 74 plants was infested with potentially viruliferous insects. After a 2-week IAP, the test plants were sprayed with an insecticide to kill all mealybugs. *Pseudococcus longispinus* individuals from healthy grapevines and test plants with no mealybug inoculation were used as controls. Test plant maintenance and pesticide spraying were the same as described above. All plants were assayed at 9 months postinoculation using RT-PCR (see below for methods).

Virus detection. All samples were sent to the Whitter Laboratory in the Department of Veterinary Medicine at UC Davis for total RNA extraction by the ABI 9600 automated RNA extraction system. Before testing the samples for grapevine viruses, a housekeeping control (NADH dehydrogenase subunit 5 gene) was used to evaluate the quality of extracted RNA (22). If samples did not pass this housekeeping test (i.e., NADH amplicon was not amplified), we again extracted RNA from those plants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All plants were tested for nine grapevine viruses by RT-PCR as previously described (26). Briefly, viruses screened in this project included GLRaV-1, -2, -3, -4, -5, -7, and -9, *Grapevine virus* A (GVA; genus *Vitivirus*), and *Grapevine virus* B (GVB; genus *Vitivirus*). The enzymes and reagents used for RT-PCR were obtained from Invitrogen Life Technologies (Carlsbad, CA). The sequences for primers used for RT-PCR have been published (26). The RT reaction was performed at 52°C for 30 min, followed by a PCR activation step at 94°C for 2 min; amplification of 35 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min; and a final extension step at 72°C for 7 min. Amplification products were analyzed by electrophoresis on 1.5% agarose gels and visualized on a UV transilluminator following ethidium bromide staining.

RESULTS

Virus transmission by *Planococcus ficus*. The results of virus transmission experiments by *Planococcus ficus* are summarized in Table 1. As expected, increasing the number of vectors during the IAP increased transmission efficiency. GLRaV-1, -3, -4, -5, and -9 were transmitted by *Planococcus ficus*; GLRaV-4 and -9 had not been shown to be vector-transmitted before. GLRaVs from both single-infection accession numbers LR106 and LR118 (GLRaV-4 and GLRaV-9, respectively) were successfully transmitted to healthy test vines (Table 1). Although accession number LR109 was infected with multiple grapevine viruses, only GLRaV-3 was transmitted from source vines to test plants. Accession numbers LV92-02 and LV92-04 were infected with multiple grapevine viruses but not with GLRaV-3; however, the test plants were positive for GLRaV-3. The reason for this inconsistency is not clear. It might be due to in-greenhouse mealybug transmission, contaminated mealybug colonies, or contaminated test plants. However, all of our greenhouse controls were negative for all viruses tested. In addition, other transmission tests resulted only in the transmission of viruses present in their respective source plants. Therefore, we believe these source plants were infected with GLRaV-3 but virus detection failed, possibly due to a population level within plants that was too low for standard RT-PCR detection. Accession numbers LR102, LR110, LR135, and LV93-09 were also infected with multiple grapevine viruses but none of these viruses was transmitted from source vines to test plants with 5 or 20 *Planococcus ficus* individuals per plant (Table 1). When the number of vectors was increased to 50 individuals per plant, *Planococcus ficus* transmitted GLRaV-1 and -5 from LR102 to healthy test plants, and one test plant was infected with both viruses (Table 1). In addition, *Planococcus ficus* also transmitted GVA from LR135 to test plants (Table 1). Engelbrecht and Kasdorf (8) showed that GVA was dependent on GLRaV-3 for its transmission by *Planococcus ficus* but we found that *Planococcus ficus* transmitted GVA independently of GLRaV-3. However, LR135 was co-infected with GLRaV-4, and we do not know whether GLRaV-4 plays any role in GVA transmission. In summary, *Planococcus ficus* transmitted GLRaV-1, -3, -4, -5, and -9, and GVA.

Virus transmission by *Pseudococcus longispinus*. The results of virus transmission by *Pseudococcus longispinus* are summarized in Table 1. Nine months after inoculation, none of the inoculated plants tested positive for GLRaV-5 and 18% of the inoculated plants tested positive for GLRaV-9. As above, all controls were negative for grape viruses tested.

DISCUSSION

Traditionally, studies aimed at the identification of vectors of plant pathogens focus on a few insect species and strains of a specific pathogen. These simple vector–pathogen combinations are desirable because the research focus is often on an important vector or pathogen. In addition, due to evolutionary constraints of such systems, one insect is often more commonly found associated with a specific disease and the most likely vector of its respective etiological agent (23). However, it is important to consider that extensive within-vector and -virus species variation in transmission efficiency has been documented for insect-borne plant viruses (2,12,33). On the other hand, for complex systems such as grapevine leafroll disease, which is caused by several viruses transmitted by many insect species, screening all possible individual combinations is technically challenging and labor intensive (10). Here, an alternative approach for the identification of new vector–virus relationships that resulted in pathogen transmission to plants was employed by screening a large number of viruses in multiply infected plants. We showed that GLRaV-4 and GLRaV-9 are vector transmitted and mealybug-borne, which represent new findings, with *Planococcus ficus* transmitting both GLRaV-4 and -9 and *Pseudococcus longispinus* transmitting GLRaV-9. Mealybug transmission of GVA was previously demonstrated (8). These results suggest that this approach is helpful in determining if viruses are vector-borne, and which of a complex of viruses may be transmitted by a specific insect species. Constraints of this approach include the fact that multiple infections must be available for studies and little information is generated in addition to the identification of new virus–vector associations.

To visually evaluate the degree of mealybug–GLRaV specificity, we reconstructed phylogenies for vector-transmitted GLRaVs

and its vector species (Supplementary Material). GLRaVs in the genus *Ampelovirus* form two groups (Figs. 1 and 2), one composed of GLRaV-1 and -3 and the other with all other species, as previously reported (20). Despite the limited number of taxa used in the mealybug phylogenetic tree, species were grouped similarly to a more thorough analysis of this insect family (15). Importantly, overlaying experimental transmission data on these trees highlighted a few aspects of virus–vector interactions in this system. First, research seems biased toward GLRaV-3, potentially because it has been shown to be the most important GLRaV in many grape-growing regions. In this analysis, all mealybug species transmitted GLRaV-3. In addition, there seems to be no evidence of virus–vector specificity or co-evolution between virus and vector (crossed, not parallel lines connecting taxa in both trees). That is best evidenced by *Planococcus ficus*, which transmits five GLRaVs. Finally, a few mealybug species tested did not transmit GLRaV-1 (Supplementary Table 1); however, it is not clear whether the lack of transmission was due to virus–vector specificity or other factors, such as the use of nontransmissible strains.

Many biological factors determine the successful vector transmission of plant viruses. A given insect species proven to be a vector of a given virus does not always transmit the virus. Because of the nature of genetic variations among populations, different biotypes of vector species and different isolates of a given virus affect the results of transmission tests (16,24). The virion concentration in diets has been shown to influence the transmission efficiency of a closterovirus (25). Genetic variation among insect or virus populations and low virus population within source tissues may explain why some mealybug-transmissible GLRaVs were not transmitted in our tests. In addition, the within-plant distribution of GLRaVs in infected grapevines is uneven (19), likely introducing variability in transmission studies. Together, any of these factors could explain why, for example, GLRaV-4 from only one virus source was transmitted when it was present in other four sources. Furthermore, transmission of some semipersistent viruses has been shown to depend on helper viruses (24). For example, *Parsnip yellow fleck virus* is dependent on *Anthriscus yellows virus* for its aphid transmission (7) and *Rice tungro badnavirus* is dependent on *Rice tungro waikavirus* for its leafhopper transmission (17). Although there is no evidence that the transmission of ampeloviruses is dependent on helper viruses, this may have been a factor in this study because most source plants used were infected with multiple viruses.

Nault (23) proposed that plant viruses in the same genus share vectors; for the family *Closteroviridae*, Karasev (18) suggested that three genera exist within the family and each genus is transmitted by a specific family of insects. The literature review and phylogenetic analysis conducted here confirms the proposals made by both authors (see Supplementary Material). All viruses in the genera *Closterovirus*, *Crinivirus*, and *Ampelovirus* are transmitted only by aphids, whiteflies, and mealybugs, respectively (Fig. 2). The phylogenetic placement of the aphid-borne

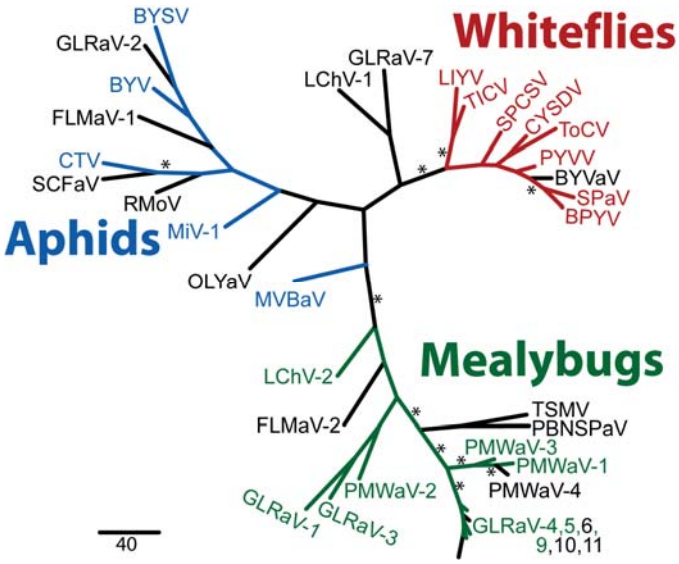

Fig. 2. Maximum parsimony tree of virus species in the family *Closteroviridae*; asterisks indicate >70% branch support. Colored branches indicate experimental evidence for virus transmission by a specific family of insect vectors. Blue = aphids, *Aphididae*; red = whiteflies, *Aleyrodidae*; and green = mealybugs, *Pseudococcidae*. Vectors that have not been identified for taxa are in black. Supplementary Material provides more detail.

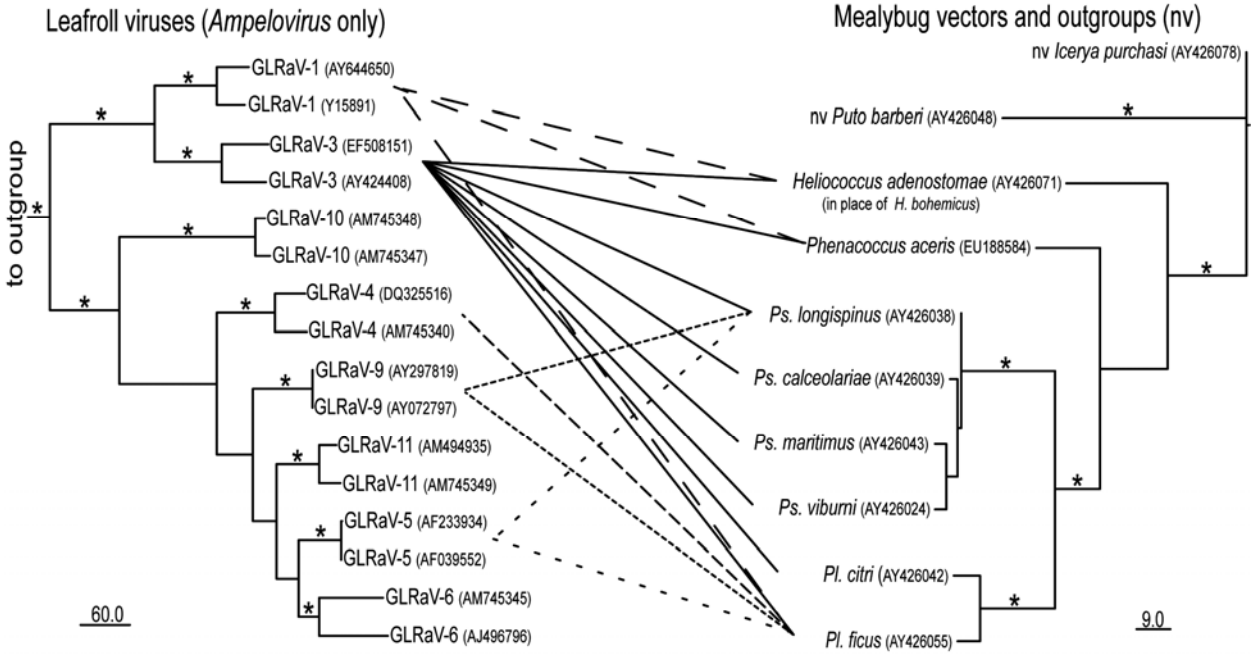

Fig. 1. Maximum parsimony trees of grapevine ampeloviruses (left) and all mealybug species shown to transmit grapevine leafroll-associated viruses; asterisks indicate >70% branch support. Lines indicate which virus species were transmitted by each mealybug vector; nv = nonvector species. Supplementary material provides more detail.

Mint vein banding-associated virus within the family *Closteroviridae* is interesting, and is discussed in further detail in relation to its aphid transmission by Tzanetakis et al. (32). The transmission of GLRaV-1 and -3 by soft scales (*Hemiptera, Coccidae*), a sister taxon of mealybugs, was not addressed in our study (1,30). Among the eight *Ampelovirus* spp. (taxonomically accepted, in addition to proposed GLRaV-10 and -11) causing grapevine leafroll disease, five have now been shown to be vector-transmitted (4,8,10,28,30, and this work). There are no reports on GLRaV-6, -10, and -11 transmission; however, it is expected that these are also mealybug-borne. These viruses were not included in this study. GLRaV-2 and -7 were not transmitted in our trials despite multiple assays, suggesting that these viruses are not mealybug-borne. These results are consistent with the phylogenetic placement of both species (21) and confirm previous reports in the case of GLRaV-2 (8).

The data obtained in our study suggest a general lack of transmission specificity between mealybugs and GLRaVs; however, this observation is supported by a limited number of data points, and more research on this topic is necessary. Due to the fact that no comparative transmission studies have been performed for any GLRaVs, there is no information available regarding the relative efficiency with which these viruses are transmitted or what factors affect competence. Furthermore, existing data on transmission biology of GLRaVs are strongly biased toward GLRaV-3, limiting meaningful comparisons in this respect. Future work should also explore the specificity of *Ampelovirus* transmission by soft scales. Finally, all closteroviruses for which studies have explored multiple aspects of transmission biology were transmitted in a semipersistent manner (21,24). Limited information is available for GLRaVs in that regard but work conducted support that hypothesis (4,31). Although vector transmission is essential to pathogen spread and GLRaVs are of growing economic importance worldwide, this disease complex still lacks fundamental knowledge regarding virus–mealybug interactions required for the development of disease management practices.

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