Associations of Yeasts with Spotted-Wing *Drosophila* (*Drosophila suzukii*; Diptera: Drosophilidae) in Cherries and Raspberries

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A rich history of investigation documents various *Drosophila*-yeast mutualisms, suggesting that *Drosophila suzukii* similarly has an association with a specific yeast species or community. To discover candidate yeast species, yeasts were isolated from larval frass, adult midguts, and fruit hosts of *D. suzukii*. Terminal restriction fragment length polymorphism (TRFLP) technology and decimal dilution plating were used to identify and determine the relative abundance of yeast species present in fruit juice samples that were either infested with *D. suzukii* or not infested. Yeasts were less abundant in uninfested than infested samples. A total of 126 independent yeast isolates were cultivated from frass, midguts, and fruit hosts of *D. suzukii*, representing 28 species of yeasts, with *Hanseniaspora uvarum* predominating. This suggests an association between *D. suzukii* and *H. uvarum* that could be utilized for pest management of the highly pestiferous *D. suzukii*.

Yeasts are believed to be an important source of nutrients for and are vectored by *Drosophila* (27, 32). *Drosophila* development is affected by the species of yeast available as food for the larvae (33). Also, microbial community composition of the larval substrate affects fitness in terms of susceptibility to parasitism (3). More diverse yeast communities appear to be preferred food sources (26), and bicultures of yeasts are generally more preferred than monocultures (33, 34), though this is not universal (27). Yeasts have been shown to affect *Drosophila* reproduction, with yeast composition in fly diet affecting egg production by orders of magnitude (8, 9, 29). In some species of *Drosophila*, yeasts are even offered as nuptial gifts in courtship (35, 36, 37). Yeast-colonized substrates are preferred oviposition sites for most fruit-breeding *Drosophila*, rather than sites where bacteria or molds predominate (22). *Drosophila buzzatii* was found to prefer feeding and ovipositing on the same species of yeast, and both *Drosophila* larvae and adults prefer to feed on particular yeast species when offered a choice of pure yeast cultures (12, 39).

In 2008, a new highly pestiferous *Drosophila* species, spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), invaded the western United States (7). *D. suzukii* is unique in that it oviposits on marketable fruit relative to over-ripe or damaged fruit, and its injury facilitates colonization by other *Drosophila* species (40). If untreated, it is capable of causing a potential 860 million dollars of revenue loss annually to blackberries, raspberries, and cherries in California, Oregon, and Washington (40). Knowledge of potential yeast associations could be used in lure development. Given that strong associations are common between yeast and *Drosophila* spp., we hypothesized that *D. suzukii* has a preferred yeast species or community that both larvae and adults can feed upon.

MATERIALS AND METHODS

Cultivation of yeasts from live *D. suzukii* larvae. Infested fruit were collected as available from September 2010 through August 2011 for larval extraction. Individual larvae were removed from infested fruit using Featherweight forceps (BioQuip, Rancho Dominguez, CA). Forceps were dipped into 100% ethanol and flamed twice in succession and allowed to cool for sterilization. The larva was surface sterilized by submergence in 70% ethanol, rinsed in sterile distilled water, and placed in the center of a

Rose Bengal chloramphenicol agar (RBCA; prepared according to the manufacturer's instructions; Oxoid, United Kingdom) plate for 30 min to 1 h depending on larval activity. Once a larva began to cross its own trail, it was removed and placed in a *Drosophila* culture bottle (177-ml stock bottles; Applied Scientific, Schrerville, IN) containing approximately 50 ml of Jazz Mix *Drosophila* medium (Applied Scientific, Schrerville, IN) with a Kimwipe delicate task wiper (Kimberly-Clark, Fullerton, CA) embedded in the media and closed with BuzzPlugs (Applied Scientific, Scherville, IN). The adult was sexed and identified upon successful eclosion. Yeasts from larvae that did not survive to emergence or were not *D. suzukii* were not included in this study. One colony of each morphological type was isolated and identified as described below, and at least two colonies per plate were chosen if the morphology appeared identical for the entire plate.

Cultivation of yeasts from live *D. suzukii* adults. Adult *D. suzukii* organisms were collected live from the field during times of peak populations in each crop (July through August 2011) either by sweep netting (cherries) or by vacuum sampling (raspberries). They were immediately returned to the laboratory (within 1 h of collection) and were first submerged in sterile distilled water and then in 70% ethanol for surface sterilization, and then they were rinsed in sterile distilled water. This prevented them from swallowing the ethanol used for surface sterilization and killing the alimentary canal yeasts. The alimentary canal was removed by gently pulling the anterior and posterior parts of the fly simultaneously with sterilized forceps. The alimentary canal was then streaked across an RBCA plate. One colony of each morphological type was isolated and identified, and at least two colonies per plate were chosen if the morphology was identical for the entire plate.

Fruit extractions. Two to four fruit of each sample (fruit variety and infestation type) were macerated until particles were less than 0.33 mm in diameter inside a Whirl-Pak sterile filter bag (Nasco, Inc., Atlanta, GA). A subsample of the fruit juice taken early in the season (when we would

TABLE 1 Summary of total yeasts isolated from all locations (n = 126) by species or pooled by genus in cases of multiple species

	No. positive for yeast isolation								
	Cherry				Raspberry				
Yeast	Adult (n = 3)	Larva (n = 13)	Uninfested fruit (n = 4)	Infested fruit (n = 3)	Adult (n = 8)	Larva (n = 21)	Uninfested fruit (n = 5)	Infested fruit (n = 5)	Total
Hanseniaspora spp.	3	13	3	3	8	19	5	8	62
Pichia spp.	1	3	0	2	1	5	1	6	19
Metschnikowia spp.	0	2	0	6	0	0	3	2	13
Cryptococcus spp.	0	0	9	0	0	1	0	1	11
Aureobasidium pullulans	0	0	3	2	0	0	0	0	5
Candida spp.	0	1	0	1	1	2	0	0	5
Sporobolomyces spp.	0	0	3	0	0	0	0	1	4
Debaryomyces spp.	0	0	0	0	0	0	2	1	3
Udeniomyces pyricola	0	0	1	0	0	0	1	0	2
Meyerozyma guilliermondii	0	0	0	0	0	0	1	0	1
Moniliella megachiliensis	0	0	0	0	0	0	0	1	1

expect the wild *Drosophila* community to be mostly *D. suzukii*) was frozen and kept at −80°C for terminal restriction fragment length polymorphism (TRFLP) yeast community analysis. Nine ml of sterile distilled water was added to one ml of the sample juice, and then serial dilutions were performed. These dilutions were plated on RBCA plates by spreading 0.1 ml of the dilution evenly across the plate. The dilution that resulted in 30 to 300 colonies per plate was used to quantify the number of CFU of yeast per gram of sample. Three to five colonies of each morphological type were isolated and identified as described below.

Infested and uninfested raspberry samples (Isabel, Maravilla, and Pacifica varieties) were taken from fields known either to have a fly population or not to have a fly population, as raspberries show no external signs of infestation. Cherries (varieties Rainier, Royal Ann, and unknown) have a characteristic oviposition mark; therefore, cherries were designated infested or uninfested by visual inspection. A subsample of uninfested fruit from each sampling date was also checked for the presence of larvae, and no larvae were found. Fruit were collected from multiple varieties of each fruit type from June to August 2011, as available. Fruit were then inspected when macerated for evidence of larvae within the fruit, and a subsample of infested fruit were used for larval extractions. Ten fruit from each sample were used to measure the Brix sugar content of the sample to account for potential variability in the ripeness of the samples.

Yeast identification. Approximately 1 μl of yeast from the final potato dextrose agar (PDA) plate was placed in 50 μl of MilliQ water and heated at 95°C for 10 min to lyse the cells. PCR amplification was performed in 50-μl reaction mixtures with 2 μl of the lysed DNA template, 25 μl 2× Promega Go *Taq* Green Master Mix (Promega, Madison, WI), and 1 mM MgCl$_2$ using the NL1 and NL4 primers at 0.2 μM reaction concentration (23) to amplify the 600-bp D1/D2 domain of the large (26S rRNA) subunit. Samples were sequenced at the UC Davis College of Biological Sciences Sequencing Facility at the University of California, Davis (http://dnaseq.ucdavis.edu/). The sequencing reaction was carried out on a 3730 capillary electrophoresis genetic analyzer with BigDye Terminator v3.1 cycle sequencing chemistry (Applied Biosystems, Foster City, CA). Electropherograms were analyzed using Sequence Scanner (v1.0; Applied Biosystems, Foster City, CA). The rRNA sequences were compared against the NCBI database using the nucleotide Basic Local Alignment Search Tool (BLASTn) program (http://blast.ncbi.nlm.nih.gov) (2) for taxonomic identification of the yeast isolates. A 98% match or higher was used to assign species name; anything less was identified solely to the genus level. Cultures were deposited in the University of California, Davis, Phaff collection (see Table S2 in the supplemental material).

TRFLP. Yeast communities from infested and uninfested cherries and raspberries were profiled using TRFLP analysis methods that were previously described (6). Briefly, DNA was extracted from the fruit juice sam-

ples (1). The internal transcribed spacer (ITS) regions (1 and 2) surrounding the 5.8S rRNA gene were amplified using HEX fluorescent-labeled ITS1 and ITS4 primers (23). Purified PCR products were then digested using the restriction enzymes HaeIII, DdeI, and HinfI. The digested DNA was submitted to the UC Davis College of Biological Science Sequencing Facility at the University of California, Davis, for quantification. Traces were visualized using Peak Scanner Software (v.1.0; Applied Biosystems, Foster City, CA), and peaks with more than 50 fluorescence units were considered true signals. Yeast species assignments were made by referencing the ITS TRFLP database for pure yeast cultures (6). As ITS1 and ITS4 are able to amplify plant 26S rRNA, cherry (*Prunus avium*) and raspberry (*Rubus ideaus*) major and minor terminal fragment profiles were predicted using SeqBuilder software (v.7.0; DNAStar) and the publicly accessible ITS rRNA sequences for the fruit. DdeI had the best diversity for our samples in terms of greatest number of operational taxonomic units (OTUs). Therefore, relative abundance for the species in our samples was determined using the height of the fluorescence peak for this restriction cut. HaeIII and HinfI were used for supporting the identification assignment.

RESULTS

D. suzukii larvae were isolated from three varieties of each fruit type for a total of 34 individuals and 46 unique frass-yeast isolations (Table 1). *Hanseniaspora uvarum* was isolated from 32 of the 34 larvae. The next most frequently isolated species were *Pichia kluyveri* and *Pichia terricola*, which were found in 5 and 3 of the larvae, respectively, for a total of 8 isolations of the genus *Pichia* (see Table S1 in the supplemental material). Yeasts were also isolated from the alimentary canal of both male and female adult *D. suzukii* flies, for a total of 11 individuals and 14 unique yeast isolations (Table 1). As was the case for the larval extractions, *H. uvarum* was the most prevalent yeast isolated from *D. suzukii* adults. The fruit juice dilutions showed some variation in yeast community composition between infested and uninfested cherry samples, with *Cryptococcus* spp. dominating in uninfested fruit relative to the *Metschnikowia* spp. in infested fruit (Table 1).

The Brix sugar content values and the yeast community profiles were similar for infested and uninfested fruit juices, although fewer yeast colonies were present and fewer were identified in the uninfested samples (Table 2). Infested cherry fruit had >10³-fold higher CFU/ml fruit juice than uninfested fruit. Interestingly, in cherries, infested fruit had no mold colonies

TABLE 2 Brix and CFU per ml fruit juice for yeasts and molds from infested and uninfested fruit juice isolations

Fruit	Date (2011)	Result					
		Brix sugar content (±SE)		Yeast CFU		Mold CFU	
		Infested	Uninfested	Infested	Uninfested	Infested	Uninfested
Cherries							
Unknown	14-Jun		16.5 ± 0.4		3.3×10^3		1.0×10^2
	14-Jun	15.0 ± 0.4	19.6 ± 1.0	3.2×10^7	1.9×10^3	0	1.0×10^3
Royal Ann	23-Jun	20.0 ± 0.6	20.0 ± 0.6	1.1×10^7	3.2×10^3	0	1.0×10^3
Rainier	12-Aug	24.0 ± 1.0	24.0 ± 1.0	4.0×10^6	$<1.0 \times 10^2$	0	0
Raspberries							
Isabel	23-Jun	10.7 ± 0.3	9.8 ± 0.1	8.0×10^6	4.6×10^2	1.0×10^3	1.0×10^3
Maravilla	23-Jun	11.5 ± 0.4	11.3 ± 0.3	6.0×10^2	3.0×10^2	1.0×10^3	1.0×10^3
	21-Jul	10.2 ± 0.2	8.8 ± 0.4	5.0×10^4	2.6×10^3	1.0×10^3	1.0×10^3
Pacifica	23-Jun	9.0 ± 0.3	10.5 ± 0.2	1.0×10^7	$>1.0 \times 10^3$	0	1.0×10^2
	21-Jul	9.8 ± 0.4	9.1 ± 0.1	4.0×10^5	2.8×10^5	1.0×10^4	1.0×10^2

(Table 2). In raspberries, yeasts and molds were present in infested and uninfested fruit, although yeasts were more prevalent in both. There was no noticeable difference in the quantity of yeast between infested and uninfested raspberries, unlike the cherry samples. However, one sample of infested Pacifica raspberry and one sample of infested Isabel raspberry did exhibit a 10^3-fold higher CFU than the uninfested samples. Of the yeast colonies isolated, *H. uvarum* comprised a similar proportion of both uninfested and infested colony isolates (see Table S1 in the supplemental material). *Cryptococcus* spp. were more prevalent in uninfested samples, with 9 isolations in uninfested and 1 in infested samples (Table 1). *Metschnikowia* spp. and *Pichia* spp. showed an opposite trend, with 3 and 1, respectively, in uninfested fruit and 8 each in infested fruit. A small portion of uninfested (3) and infested (2) samples were comprised of the yeast-like fungus *Aureobasidium pullulans*.

A total of 126 unique yeast isolations were identified in this study, for a total of 28 yeast species. *H. uvarum* was the predominant yeast and was ubiquitous across all sample types, with 59 individual isolations. *P. terricola* was the next most common, with 10 individual isolations, although it was more common in raspberry samples. This was followed closely by *P. kluyveri*, with 9

isolations. All other species were found on 5 or fewer occasions (see Table S1 in the supplemental material).

TRFLP yeast community profiles for early-season infested and uninfested fruit juices were able to detect species of yeast in three of the infested samples (Fig. 1). The infested cherry sample of unknown variety collected on 14 June 2011 had a high abundance of *P. kluyveri*, with *H. uvarum* detected at a lower abundance. The infested Royal Ann cherry sample had a low abundance of *Pichia kudriavzevii*. The only infested raspberry sample in which yeast was detected was the Pacifica variety, which had a high abundance of *H. uvarum*. The fruit's DNA was detected in all infested fruit samples, and only *P. kluyveri* in the infested cherry sample was in higher abundance than the sample's fruit DNA. No yeasts were detected in any uninfested fruit samples, and fruit DNA was prevalent (Fig. 2).

DISCUSSION

The most frequently identified yeast species from adult and larval *D. suzukii* were *H. uvarum*, *Metschnikowia pulcherrima*, *P. terricola*, and *P. kluyveri*. *H. uvarum* is considered a widespread yeast species; however, it and other apiculate yeasts are most frequently isolated from mature fruits, early stages of wine fermentation,
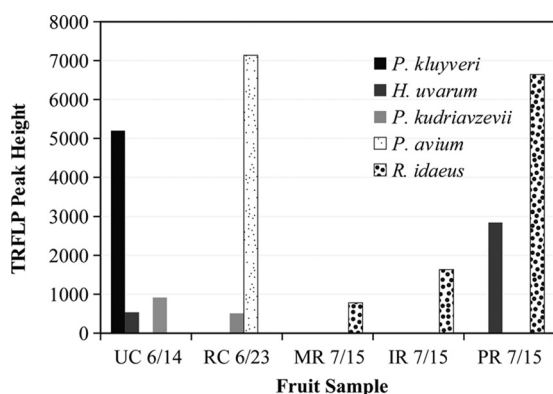

FIG 1 DdeI fragment fluorescent peak heights and identifications for infested fruit. Fruit samples were the following: UC, unknown cherry; RC, Royal Ann cherry; MR, Maravilla raspberry; IR, Isabel raspberry; and PR, Pacifica raspberry. Yeast species were *Pichia kluyveri*, *Hanseniaspora uvarum*, and *Pichia kudriavzevii*. Fruit species were *Prunus avium* and *Rubus idaeus*.
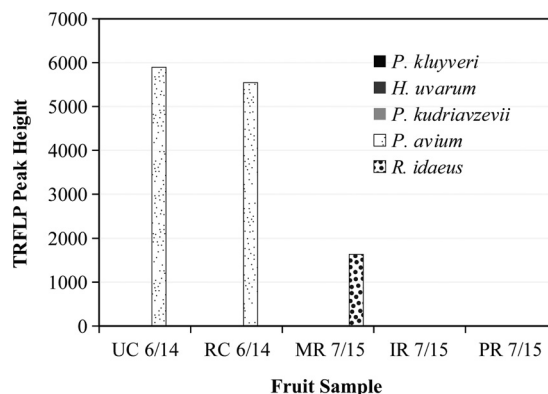

FIG 2 DdeI fragment fluorescent peak heights and identifications for uninfested fruit. Fruit samples were the following: UC, unknown cherry; RC, Royal Ann cherry; MR, Maravilla raspberry; IR, Isabel raspberry; and PR, Pacifica raspberry. Yeast species were *Pichia kluyveri*, *Hanseniaspora uvarum*, and *Pichia kudriavzevii*. Fruit species were *Prunus avium* and *Rubus idaeus*.

fermentative spoilage, and *Drosophila* (19, 20, 21, 31). *M. pulcherrima* is also often associated with feeding and breeding sites of insects, and yeasts isolated from *Drosophila* were once thought to be predominantly from the genus *Pichia* (16, 38). For both larvae and adults, the most commonly identified yeast species was *H. uvarum*, suggesting that there is an association between *D. suzukii* and *H. uvarum*.

Infested and uninfested cherry fruit juice contained a broader diversity of yeasts, although still being dominated by the fruit- and insect-associated species that were mentioned above. Ubiquitous phylloplane and soil yeasts were more common in the uninfested fruit juice isolations, with the yeast-like fungus *A. pullulans* and *Cryptococcus* spp. predominating. *A. pullulans* has been termed a plant-pathogenic fungus (11), and *Cryptococcus* spp. are considered to be generalist yeasts that are frequently isolated from the phylloplane (16). These yeasts can utilize a broad variety of carbon sources (16). Infested cherries predominately contained the yeast species *H. uvarum* and *Metschnikowia* spp., which utilize a narrower range of carbon sources (16). Uninfested and infested raspberry communities were much more similar both in composition and in quantity of yeasts in the sample, with *Pichia* spp. and *Hanseniaspora* spp. dominating. Theoretically, once a cherry has been infested with *D. suzukii* and yeast introduced into the mesocarp, larvae can travel throughout the fruit, thus yeasts can infest the entire fruit mesocarp and reach a high population. On the contrary, larval travel and, thus, yeast growth within a raspberry may be limited to the individual damaged drupelet, although larval *D. suzukii* movement in various fruits remains unknown. As fungicides are often applied to control plant-pathogenic fungi in raspberries, differences in fungicide use in organic versus conventional fields are a confounding factor. Organic fungicides have been found to reduce the overall yeast population and shift the microbial population toward the yeast fungus *A. pullulans* (10).

TRFLP analysis of yeast communities within infested versus uninfested fruit juice revealed yeast species present at detectable levels in a few of the samples. Unfortunately, the ITS primers also amplify plant DNA, which was present at a high level in fresh fruit juice and was often more abundant than yeast DNA. Detection of yeast by TRFLP analysis in fruit juice does not seem to correlate well with the culture-based methods. Samples with higher yeast CFU did not necessarily have high yeast TRFLP peaks, and *P. kudriavzevii* was not identified in any of the fruit juice yeast cultures. This could be due to sampling from the fruit dilutions or could be a limitation of the TRFLP database, which may not be able to distinguish all relevant species of *Pichia*. Despite these contradictions, the development of a primer that is more specific for yeast than plant DNA would allow TRFLP to become a useful analytical tool for yeast community comparisons.

Knowledge of *Drosophila* preference for yeast substrates has been instrumental in the development of trap attractants for *Drosophila* spp. (5). For more than 70 years, banana mash fermented by baker's yeast has been used to attract *Drosophila* spp. (24, 25, 30), and *Drosophila* spp. are common nontarget captures in *Torula* yeast-based food lures for tephritid fruit flies (18). Common synthetic volatiles for *Drosophila* spp. attraction included chemicals released by yeast fermentation of fruits, such as ethanol, acetic acid, methyl acetate, ethyl acetate, acetaldehyde, and *n*-propanol (4, 14, 15, 41). One disadvantage of using synthetic volatiles is that carbon dioxide, a fermentation product produced in live yeast baits, is not released (28).

A specific and highly attractive lure is an important part of integrated pest management strategies. Initial trap designs for monitoring *D. suzukii* utilized apple cider vinegar, grape wine, a baker's yeast and sugar water mixture, or a vinegar/wine mixture as trap bait (17, 40). A major problem with current baits is that other *Drosophila* species comprise a large part of trap captures, making it difficult to process traps, especially for non-entomologists who may have difficulties distinguishing *D. suzukii* from other *Drosophila* species. A live yeast bait with a yeast species that is specifically associated with *D. suzukii* could alleviate this problem and could be both more attractive and more selective for *D. suzukii*. The predominant yeast species associated with other temperate *Drosophila* species are not as heavily skewed to one species as we have seen with *D. suzukii* (13). Our work suggests *H. uvarum* as a species with which *D. suzukii* has a specific association, making it a good candidate species for a more attractive and selective lure. Further studies are needed to verify its long-range attractiveness to adult flies.

ACKNOWLEDGMENTS

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