"Año del Centenario de la promulgación de la Constitución Política de los Estados Unidos Mexicanos"

March 5th, 2017
Professor Michael Singer
Academic Editor, PeerJ

Dear Editor,

We appreciate the time and efforts of the reviewers, as well their generous comments on the manuscript. As detailed below, we have addressed all concerns and suggestions pointed out by the reviewers and we hope you find this version acceptable for publication in PeerJ.

Sincerely yours,

Azucena Canto
Researcher of the Centro de Investigacion Cientifica de Yucatan (CICY)

On behalf of all authors

Responses to reviews

To help you follow the changes made to the manuscript, our responses to suggestions are given below as sentences in blue following the corresponding passages of original reviews. Line numbers refer to the revised version.

Editor's Comments

MAJOR REVISIONS

The reviewers and I agree that your manuscript reports on an important and interesting topic. However, there is disparity between the reviewers about how well your manuscript accomplishes its goals. My own opinion is a compromise between those of the reviewers. While I think you have an opportunity to greatly improve the manuscript by addressing the comments of the reviewers, I am sympathetic to the critique of reviewer 1 that the methodology should be much more transparent so that readers can truly understand how much merit to place in your results. I also share the skepticism of reviewer 1 about whether the regression on nectar sugar concentration is truly meaningful. The statistical results do not match the figures, and the very small number of data points makes the analysis suspect. Please address this issue very clearly. You will either need to convince skeptical reviewers about the validity of that analysis, or use an alternative analysis that lacks the problems of the regression analysis. For example, you might try classifying sugar concentrations as high or low categories and analyzing this binomial variable. In addition to these large issues, please address each of the comments of the reviewers and check the language as closely as you can for grammatical mistakes, of which there are many.

We have re-edited/corrected thoroughly the methodology to clarify the field sampling and the lab treatment of nectar samples and yeasts obtained.

We have corrected figures and statistical analysis to make them congruent. We have also re-classified the causal factors to increase sample size and to improve validity of results.

We are agree with your suggestion and have conducted new analysis using a power model regression with two factors (Yeast and Plant). We have also classified yeast species to increase sample size in each level combination and at the same time maintaining the natural relationships between response variable (nectar sugar concentration) and causal variables (yeast cell density, different types of yeast and host plants). We used the Type III approach to calculate the sums of squares for unbalanced data and incomplete sampling design. The Akaike Information Criterion (AIC) was also applied to measure the goodness of fit of the power model taking into account the number of parameters included and to find the best model that fits the data with the minimum number of parameters. This new analysis lacks the problems of the regression analysis conducted in the early version. The previous figure was replaced by two new figures.

We have corrected all grammatical mistakes.

This new version has one figure and two tables more than the previous version in order to clarify the concerns of reviewers in regard to the regression analysis and sampling design.

Reviewer 1 (Anonymous)

The following details are missing from the methods section:

1. What is a nectar drop? (Line 100) How are three obtained from each flower? Were they pooled, then split or each obtained and treated separately?

A drop is defined in this study as each nectar sample extracted from a single flower; its volume depends on the volume capacity of the capillary tube used to extract nectar. Each nectar sample by flower was individually lab processed. We have re-edited the Sampling method section to clarify how nectar samples or drops were obtained from each flower and how those samples were treated (Lines 102-121).

2. Samples were extracted in 1 mL of water, but only 5 uL of that was filtered and injected? How was this small of a sample filtered? Perhaps a larger volume was filtered but only 5 ul was injected?

Each diluted sample was filtered, and then five uL of that solution was injected. We have re-edited the phrases to clarify this point (Lines 164-166).

3. How many samples per species were used? Please include as a table.

We have added an Appendix as a supplemental file with the information of the number of samples used (agar plated) by plant species in each of the three lab methods used in this study and also with the number of individual plants surveyed by plant species.

4. How can yeast species be included in the model on sugar concentrations?

Yeast species were added in the model as categorical variable. This point has been clarified in the Data analysis section (Lns. 240-241).

Were there ever multiple yeast species isolated from the same sample? This would be expected in at least a few samples. How did the authors deal with this type of sample in the regression analysis?

Only in four cases more than one yeast species were isolated from the same nectar sample. To deal with this, yeast identity in each case was assigned at random drawing from yeast species co-occurring in the respective case. Changes were made to clarify this point in the Data analysis section (Lns 258-261).

5. How many samples/species plated?

Information of the number of nectar samples (drops) were agar plated has been added in the Appendix.

How many colonies sequenced per species? How many colonies sequenced per morphotype? Was there a 1:1 correlation between consensus ID and morphotype?

Almost all purified yeast colonies and morphotypes produced by nectar samples were DNA-sequenced. The correlation between ID and morphotype ranged 98 and 100 % in all cases. We highlighted this point in the Yeast isolation and DNA-identification section (Lns 139-141).

Experimental design

The study is a survey. Sampling details are not fully described, as outlined above. For example, how were flowers chosen? Were all samples taken at one site or across multiple sites? Further, it is unclear if the plant species were sampled at the same time of the year, or peak flowering etc. or if they are comparable.

Agree. We have corrected those omissions by adding clarifications in the Sampling method (Lns. 102-114).

Validity of the findings

1) In the current study, diversity patterns are reported at the level of nectar drop, individual plant, species and genus. However, it is unclear how many samples were examined at each level, and if sampling was equal across time, space, flowering age etc. among plants. The number of samples per plant is reported in Canto and Herrera 2012, but is not repeated here (even in the supplement). Even if the number of samples taken per plant or flower were reported, we would also need to know how many colonies per sample were sequenced to interpret results.

Agree. Changes were done in the Sampling method to include further details of conditions of nectar collection (Lns. 98-122). The number of samples per plant is reported in the Appendix and also is was mentioned in the Results (Lns. 265-268). The information of the number of colonies DNA-sequenced per nectar drop was added in the raw data.

Finally, rarefaction is reported at the level of the nectar drop. However, individual drops were not re-sampled. Instead, samples from all plant species were used as replicates. Interpreting what this analysis means biologically is unclear. Is this the diversity that is estimated to occur at the level of the individual nectar drop averaged across plant species?

Maybe we miss the point in this last question. Changes were done in the Sampling method (Lns. 107-110; 114-119) and the Data analysis (Lns. 116; 193-196; 218-220) to point out that several independent nectar drops were collected from each individual plant and therefore we were able to analyze the diversity at the level of nectar drops using rarefaction. If after to read again the changes made in the two sections, the question remains unanswered, would you make the question again?

2) The authors seem to assume that there is only a single yeast species per flower/nectar drop, but do not present any evidence for this claim.

Agree. We have added the information of the number of yeast species by nectar drop in the raw data file as evidence for the claim that in the majority of nectar samples only one yeast species was produced. Also changes were made in the Yeast isolation and DNA-identification section to clarify this point (Lns. 130-132; 266-268).

Further, the sampling method used--sequencing from isolated colonies--would only be able to examine this if many or most of the colonies on the plate were sequenced (it is unclear if they are) or if cell morphotype was also used (and validated) as a method to determine species per sample.

Agree. Changes were made in the Yeast isolation and DNA-identification section to emphasize that almost all yeast colonies produced by nectar drops were sequenced (Lns. 130-132).

3) The assumption above (single yeast species per nectar drop) was used in the analyses, including the regressions were performed linking yeast density within a sample based on the yeast species IDed from that sample. As stated before, no justification for this assumption is given.

A number of corrections have been added to point out that in all cases only one yeast species was observed, except for four cases. Changes in the Data analysis (Lns. 258-260) were made to justify the assumption and give evidence of it.

In addition, this regression also assumes that the species found in the nectar is the one that is responsible for any metabolic breakdown of sugars. However, pollinators vector many microbial taxa, including yeasts and bacteria, and extinction is also possible in this system, so the assumption although likely, is not tested that the identified species is also responsible for sugar metabolism.

We are agree in that pollinators carry to flowers more than yeasts. Bacteria may also responsible for nectar sugar changes. We have mentioned in text that not only yeast are occurring in floral nectar but also bacteria. Our findings are still robust even if bacteria were occurring in nectar samples. The evidence so far, point out that both groups accounted for the majority of the post-secretion nectar features. Although our study is focus on the nectar-living yeasts rather than bacteria groups, we have clearly mentioned in text the works that have study bacteria effects on nectar (Lns. 36-37; 432-434).

4) Figure 3 reports the p-values for a regression between yeast density and sugar concentration in the nectar sample. The p-values included here are somewhat suspect. For example, what looks like a positive correlation (Figure 3a) is not significant, while a panel with 4 points that do not form any sort of a line has a p-value of 0.031. The statistics on this figure are should be checked. In addition, a regression should not even be run with only 4 points, as is done in three of the panels. Finally, there are two fit lines but only one statistic reported for Cryptococcus laurentii. Is the fit line for both species together or one of the fit lines or the model as a whole? This entire analysis is questionable for reasons outlined in 2-4 above.

We have conducted a different analysis in this new version. A power model with two categorical factors is proposed to test the association between yeast cell density and nectar sugar features. This type of analysis also allows to identify the contribution of different types of yeasts and host plants after taking out the variance due to yeast cell density. We have classified the variable yeast species into five categorical levels and used the Type III approach to calculate the square sums when data are unbalanced and the sampling design is incomplete. Results were different from the previous manuscript version but they contribute to reach robust conclusions and it is statistically correct. We really appreciate this correction, the new analysis reveals the true nature of our data. The figure has been replaced by two new figures: one figure shows the overall relationship between yeast cell density and sugar concentration (Fig. 3); the other figure shows the

multiplicative effect of different types of yeasts and host plants on nectar sugar concentration after taking out the effect of yeast cell density (Fig. 4).

5) Are the authors confident that isolated yeasts rather than other microorganisms or variation in floral traits etc. are responsible for change in nectar sugars? What about organisms that may not be culturable given the current methods?

Agree. We believe these question are worthy to take into account in our future investigations on nectars.

Comments for the Author

The paper is generally well-written and increased study of tropical nectar yeast communities is definitely welcome. This paper overlaps substantially with a previously published paper by these authors (Canto and Herrera 2012) and uses most of the same data. This would be acceptable if the analyses were justified. However, the new analyses in this paper are not valid given how the data were collected and rely on assumptions that the authors do not address as outlined above (single yeast species per nectar sample and that yeast communities are fully sampled). It is unclear if enough work has been done to merit an additional publication from these data.

Canto & Herrera (2014) and this study share similar sampling area and sampling time. However, analyzes were conducted using a data set that includes more yeast species and different assemblage of host plants (but many plant species are the same that which in the previous work). Data of this study was taken specifically to analyze the yeast diversity, the correspondence between groups of yeasts and plants, and the differential effects in regard to different yeasts and hosts. We think that the validity of our study is not lost because it shares the same sampling area and time that which the previous work. Additionally, we have done a different analysis to test the contribution of yeasts and host plants after taking out the variance of yeast cell density. The analysis allows testing hypothesis in unbalanced data and incomplete sampling design.

Reviewer 2 (Anonymous)

Basic reporting

Lns 54-57: While the host nectar environment can certainly impact what species are capable of colonizing, your sentence suggests that microbial colonization order can also be important in determining community composition, as well as variation in nectar features. Perhaps worthwhile to edit this sentence to make this point more clear, and cite Peay et al. 2012 (Proc Royal) and Vannette & Fukami 2014 (Ecology Letters).

Done. We have edited the sentence and cites were added (now Lns 57-61).

Ln 135: Change "counting" to "counted"

Done (now Lns. 156)

Ln 137: Edit sentence to state "The SUGAR composition and

concentration…."

Done (now Ln. 158).

General comment regarding units; please be consistent in either including a space or not between a number and units reported throughout the manuscript. In other words, 1mL vs. 1 mL

Agree. We have unified the format to report values and units throughout the text.

Ln 271: Change "cells" to "cell"

Done (now Ln. 333).

Ln 272: Change "yeasts" to "yeast"

Done (Ln. 334).

Ln 273: Change "cells" to "cell"

Done (Ln. 334).

Lns 304-321: I think this paragraph as a whole could be cleaned up a little.

Instead of jumping into a laundry list of results from other studies on findings of yeast diversity, perhaps prepare the reader with a better topic sentence. I think your statements on there being latitudinal clines in species richness could be moved to the front of this paragraph, and perhaps highlight that these clines for microbial diversity have been less studied (but see examples from marine systems). Then perhaps jump into your discussion of prior findings from temperate systems vs. your own. Just a suggestion.

The advice has been followed closely by editing lines 362 to 369.

Lns 370-371: To be thorough, perhaps cite Schaeffer & Irwin 2014 (Ecology) which also looked at nectar yeast impacts on plant-pollinator interactions

Agree. Done.

Check format for Jacquemyn reference (journal title)

Done.

Check format for Pianka reference (report full journal title)

Done.

Figure 1 caption: Missing period at end of last sentence. Also, please make

the following edits:

"...between MEMBERS of the same yeast…."

"Whiting dashed-ellipses INDICATE significant correspondence between

yeasts and host plants."

All these corrections were done.

Table 1 caption: Edit to state "...yeast species in nectar is REPORTED

along with.."

Done.

Figures: In terms of overall presentation of a manuscript and results, I think it looks best if color schemes are consistent across figures. For instance, a couple figures have grey axes lines, while the other has black. Different color schemes between Fig. 1 and 2 also clash for me. Perhaps use the same blue and red pastels for Fig 2 as in 1? Just a suggestion, which should be easy to implement in ggplot2.

We have done all changes suggested to figures; they look better.

Experimental design

The intent of their study is well-defined, and methods employed are sound. They are clearly defined, and build off of extensive experience and prior work on related questioning in other systems. I also commend them on their use of Hill numbers and Chao's framework for addressing diversity in this study.

Thank you.

Validity of the findings

Lns 312-313: Please double-check your reporting on yeast species richness and how many plant species were sampled. In the Methods/Results you state that 39 yeast species from 24 plant species were identified. In the Discussion here however, these numbers do not match up (41 yeast species from 18 plant species).

Done. We have double-check our numbers and corrected this typeset error.