Nectar-living yeasts of a tropical host-plant community: diversity and effects on community-wide floral nectar traits

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We characterize the diversity of nectar-living yeasts and measure the associations between yeasts and nectariferous plants and the effect of yeasts on nectar traits in a tropical environment. Using a series of hierarchically nested sampling units, we extracted nectar from an assemblage of host plants representative of the diversity of life forms, flower shapes, and pollinator types occurring in the tropical area of Yucatan-Mexico. Yeasts were isolated from single nectar samples and DNA-identified, yeast cell density in nectar was estimated, and sugar composition and concentration were quantified using HPLC. In contrast to previous studies from temperate region, diversity of nectar-living yeasts in the plant community was characterized by a relatively high number of equally-common species with low dominance. Analyses predict highly diverse nectar yeast communities in a relatively narrow range of tropical vegetation. Significant associations between specific yeast species and host plants were also detected and the impact of the interaction between yeasts and host plants on the effect of yeast cell density on nectar sugars was evident. This study provides an overall picture of the diversity of nectar-living yeasts in tropical host plants and suggests that a key factor affecting community-wide patterns of nectar traits is not nectar chemistry, but rather the kind of yeasts in interaction with host plants associated to flower visitors.

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13 **ABSTRACT**

14 We characterize the diversity of nectar-living yeasts and measure the associations between yeasts

15 and nectariferous plants and the effect of yeasts on nectar traits in a tropical environment. Using

16 a series of hierarchically nested sampling units, we extracted nectar from an assemblage of host

17 plants representative of the diversity of life forms, flower shapes, and pollinator types occurring

18 in the tropical area of Yucatan-Mexico. Yeasts were isolated from single nectar samples and

19 DNA-identified, yeast cell density in nectar was estimated, and sugar composition and

20 concentration were quantified using HPLC. In contrast to previous studies from temperate

21 region, diversity of nectar-living yeasts in the plant community was characterized by a relatively

22 high number of equally-common species with low dominance. Analyses predict highly diverse

23 nectar yeast communities in a relatively narrow range of tropical vegetation. Significant

24 associations between specific yeast species and host plants were also detected and the impact of

25 the interaction between yeasts and host plants on the effect of yeast cell density on nectar sugars

26 was evident. This study provides an overall picture of the diversity of nectar-living yeasts in

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INTRODUCTION

Floral nectars are sugar-rich environments that frequently harbor distinctive microbial communities. Studies on microbial diversity conducted, among other, by Brysch-Herzberg (2004), Pozo, Herrera & Bazaga (2011), Álvarez-Pérez & Herrera (2013), Jacquemyn et al. (2013) and Mittelbach et al. (2015) have revealed that floral nectar is frequently colonized by specialized sugar-consumer yeasts in the Ascomycota and Basidiomycota, along with several bacterial groups. However, most of the studies addressing questions about nectar-living microorganisms have been conducted in temperate areas, resulting in a still-poor knowledge of nectar microbial diversity in tropical habitats. Only three preliminary assessments of the frequency of microbial-cell density in floral nectars in several tropical environments have been conducted so far (Herrera et al., 2009; Canto & Herrera, 2012; Belisle et al., 2014). Altogether, these earlier studies showed that the incidence of microorganisms in tropical nectars was higher than in temperate areas, and provided a glimpse of the high diversity harbored in tropical host plant communities. Diversity assessments in tropical nectars are still necessary to obtain a more complete view of microbial distribution linked to nectars across different environments and latitudes. Other aspect of the impact of nectar-microbial diversity is that microorganisms can account for a significant fraction of community-wide variance of nectar traits, since presence of yeast cells alters nectar sugar composition and concentration (the microbial imprint; Canto & Herrera, 2012). So far, the evidence indicates that differential yeast effect on nectars is associated with characteristics inherent to plant (type of nectar) and pollinator type. For example, pollinators are the main source of inocula for the initial establishment of microbial communities in nectars as they introduce their mouthparts into the nectaries in their search for nectar rewards (Canto et al., 2008). The initial assemblage of microorganisms colonizing a flower will therefore largely depend on the type of pollinator visiting host plants (Belisle, Peay & Fukami, 2012; de Vega & Herrera, 2013; Mittelbach et al., 2015). However, after initial colonization, the order of yeast species arrival to nectar and nectar features will strongly influence the growth of arriving microorganisms and allow some species thriving but not others, with the consequence that the resulting microbial community consists of a cluster of phylogenetically related species (Herrera et al., 2010, Peay, Belisle & Fukami, 2012; Vannette & Fukami, 2014). It has been therefore hypothesized that, in a given community of nectariferous plants, nonrandom plant-

60 microorganism associations can produce a mosaic of different qualities of floral nectars at the

61 community level with potential effects on plant-pollinator interactions (Canto & Herrera 2012).

62 To characterize diversity of nectar-living microorganisms in a tropical environment and to gain

63 insights on factors driving community-wide variance in nectar traits, we analyze in this paper the

64 assemblage of yeast and yeast-like species (collectively termed 'yeasts' hereafter) occurring in

65 floral nectars of tropical environments of the Yucatan Peninsula, Mexico. By isolating and

66 identifying culturable yeasts from the floral nectar from many animal-pollinated plants species

67 and individuals , quantifying their population densities in nectar, and estimating nectar sugar

68 concentration and composition in the host plant nectar, we will specifically assess (1) how

69 diverse is the community of nectar-living yeasts in a tropical environment, (2) the existence of

70 predictable associations between nectar yeasts and host plants, and (3) the differential impact of

71 yeasts on nectar sugar composition associated with different host plants. Yeast diversity is

72 discussed in relation to the different nectars structuring the plant community sampled and the

73 role of different host plants and types of yeasts as sources of associations between plants and

74 yeasts, all of which will ultimately influence the plant-pollinator interactions. Our results predicts

75 the existence of a relatively high diverse assemblage of nectar-living yeasts showing significant

76 correspondence with the diversity of their host plants, as well as a significant impact of the

77 interaction between yeasts and host plants in the effect that yeasts exert on floral nectars.

78 **MATERIALS AND METHODS**

79 *Study area*

80 Field sampling was conducted from September 2008 to November 2009 at 28 localities from an

81 area of tropical vegetation (approx. 430 km^2), located between Chuburna and Dzilam de Bravo

82 towns and the Cuxtal Ecological Reserve in north-western Yucatan, Mexico. The study area

83 includes coastal dune and adjacent dry forest environments, which elevation ranges between 1-

84 10 m. Climate is semi-arid at the coastal dune strip and subtropical at the dry forest, with a mean

85 temperature of 26 °C in both types and annual rainfall of 370 mm and 1077 mm, respectively.

86 The vegetation is a low, open scrub dominated by xerophytes, halophyte herbs, thorny bushes,

87 palms and 1-3 m treelets growing on sandy, nutrient-poor soils in the dune strip. The dry forest is

88 made up of cacti, thorny shrubs and deciduous medium-height trees (3-8 m tall) growing on

89 limestone bedrock soil with a thick litter layer (Chan-Vermont, Rico-Gray & Flores, 2002; Canto

90 & Herrera, 2012). Permission to collect in natural areas of Yucatan was granted by Secretaría del

91 Medio Ambiente y Recursos Naturales, Delegación Yucatán-Subsecretaría de Gestion para la

92 Protección Ambiental: Dirección General de Vida Silvestre (oficio 00837/09).

93 *Sampling method*

94 To provide an overall picture of the diversity of nectar-living yeasts in floral nectars of the

95 studied area, nectar samples were obtained from an assemblage of 18 host plant species

96 belonging to 14 genera and 10 botanical families (Table 1), representing the diversity of life

97 forms, flower shapes, pollinator types and taxonomic categories occurring in the area. Plant

98 species were individually sampled in their respective flowering peak, trying to include as

99 possible main all flowering periods occurring during the year. At each locality a single plant

100 species was sampled (usually only one plant species was flowering in each place at the time of

101 nectar collection), except for species from coastal dune environments where nectar collection

102 was done at several sites and times. We adopted a five-tiered series of hierarchically nested

103 sampling units for nectar collection, namely nectar samples or drops (Drop), individual plants

104 (Individual), plant species (Species), plant genus (Genus), and botanical family (Family).

105 Individual plants for nectar collection were chosen at random from the assemblage of individuals

106 growing at the respective locality and the criteria used for collecting nectar samples in each

107 individual plant was that flowers were approximately the same age, not wilting, but already open

108 at the time of collection in the field. This allowed that flowers were exposed to prior pollinator

109 visitation and the nectar therefore susceptible to have been colonized by yeasts. Three single

110 nectar samples (drops) were extracted from each flower using sterile microcapillary tubes with

111 calibrated scale of volume (Drummond®). The volume of nectar drops ranged from < 0.50 to 1

112 µL. Flowers used in the sampling were fully open at the time of nectar collection. Three to six

113 flowers were individually sampled from each plant and 6-10 individual plants were surveyed by

114 plant species. Of the three of nectar drops obtained from each flower, one was used for DNA-

115 based identification of yeasts, another for quantification of yeast cell density and the other to

116 estimate the sugar composition and concentration, using in each case methods described below

117 (see Appendix for further details in the numbers of nectar drops used in each method).

118 *Yeast isolation and DNA-identification*

119   The respective nectar drops were individually streaked onto YM agar plates (1.0 % glucose, 0.5

120   % peptone, 0.3 % malt extract, 0.3 % yeast extract, 2.0 % agar) with 0.01 % chloramphenicol,

121   and incubated at 25 °C until microbial colonies were detectable (2-20 days). From 439 nectar

122   drops plated, a total of 158 colonies (approximately 1-5 colonies grew per agar plate) were

123   resulted. Colonies were observed at 10x-40x magnification under a microscope (Olympus CX31)

124   and phenotypically different colonies were purified by streak-plating. A single clone (an entire

125   colony) of each purified morphotype was used for species identification. As much as possible, all

126   colonies produced by nectar drop were DNA-sequenced. The large-subunit (26S) ribosomal

127   DNA gene (D1/D2 region) was two-way sequenced for each clone using the primer combination

128   NL1-NL4, according to Kurtzman & Robnett (1998) and Lachance et al. (1999). Raw sequences

129   were edited, assembled and consensus sequences were obtained using Geneious Pro 8.1.7

130   bioinformatics software (Biomatters Ltd, Auckland, New Zealand). Nucleotide collection

131   databases at GenBank were queried with the Basic Local Alignment Search Tool (BLAST;

132   Altschul et al., 1997) to look for named yeast species with DNA sequences matching those

133   obtained for the isolates. All sequences queried yielded significant correlations with named yeast

134   accessions in GenBank databases, generally at 98-100 % of sequence coverage and identity.

135   Resulting DNA-species and the associated sampling information (Drop, Individual, Species,

136   Genus and Botanical family) was used in the analyses of yeast diversity. The yeasts studied are

137   maintained in the Centro de Investigación Cientifica de Yucatan (CICY) and their corresponding

138   DNA sequences have been deposited in GeneBank under the accessions listed in Table 1.

139   *Cell counts and nectar sugar composition and concentration*

140   The density of yeast cells was estimated in the respective nectar drop using a Neubauer chamber

141   and standard cell count procedures (Herrera et al., 2009). To do this, the initial volume of nectar

142   drops was measured with calibrated micropipettes (Dafni 1992), then each nectar sample was

143   diluted using a 0.5 % lactophenol cotton blue solution to obtain a final volume up to 1.5-6 times

144   more than the initial volume. Each diluted sample was loaded on the counting chamber and

145   examined under a microscope. Cells were counted in each of 16 quadrants of the counting

146   chamber and cell density was calculated using the formula: cells per μL = average number of

147   cells counted in the quadrants multiplied by the dilution factor and for the fixed volume of the

148   chamber.

149   The sugar composition and concentration of nectar was measured following procedures

150   described by Herrera et al. (2006) and Canto et al. (2011) and using ion-exchange high-

151   performance liquid chromatography (HPLC). Samples of nectar selected were individually

152   blotted onto a 10 mm x 12 mm sterile Whatman 3MM paper wick and immediately after

153   absorption, were placed into sterile envelopes and stored at 25-26 °C in silica gel. For analytical

154   procedure, nectar-containing wicks were individually placed into Eppendorf tubes and 1 mL of

155   HPLC-grade water was added to each one. Each diluted sample was filtered using a 0.4 µm

156   polyvinylidenedifluoride (PVDF) filter and 5 µL of that solution injected into a Dionex DX 500

157   HPLC system (Dionex, Sunnyvale, CA, USA). The HPLC system was equipped with an effluent

158   degas module, a GP 40 gradient pump, a CarboPac PA10 (4 mm x 50 mm) guard column and a

159   CarboPac PA10 (4 mm x 250 mm) analytical column. It also had an ED40 electrochemical

160   detector for pulsed amperometric detection in integrated amperometric mode, with the normal

161   preloaded wave form for sugar detection (Dionex Corp., 1994). The column was eluted (flow

162   rate 1 mL min^{-1}) isocratically with 40 mM NaOH (50 % solution; J.T. Baker, Deventer, The

163   Netherlands) and kept at 24 °C during analysis. The concentrations of sucrose, glucose and

164   fructose in each nectar sample (g of solute per 100 mL solution) were calculated by integrating

165   the area under the corresponding chromatogram peaks, then using linear regression models fitted

166   to the data of standard sugar-solutions, and then calculating the expected values of concentration

167   corresponding with the integrated area of each type of sugar contained in the analyzed samples.

168   Two independent HPLC measurements were done on each diluted sample, and replicate results

169   averaged for the analyses.

170   *Data analysis*

171   In order to characterize the species diversity of nectar yeasts and to compare diversity estimates

172   across the different hierarchical sampling levels considered (i.e., Drop, Individual, Species,

173   Genus, Family), the analytical framework suggested by Chao et al. (2014) was performed with

174   the R package iNEXT (Hsieh, Ma & Chao, 2016). This method generalizes the sample-size-

175   based approach of Colwell et al. (2012) and the coverage-based approach of Chao & Jost (2012)

176   to produce and expand rarefaction-extrapolation curves of species based on Hill numbers (Hill,

177   1973). Hill numbers are a mathematically unified family of diversity indices, differing among

178   themselves only by an exponent q. These indices provide a suitable framework for measuring

179   diversity because (1) they are expressed in units of effective numbers of species, (2) by using

180   algebraic transformation, they are easily associated to key diversity indexes such as Shannon

181   entropy and Gini-Simpson index, and (3) their estimations can be effectively generalized to

182   incorporate hierarchical levels of diversity in a species assemblage (Chao et al., 2014). For each

183   sampling level (Drop, Individual, Species, Genus, and Family), an incidence matrix was built up

184   by recording the presence-absence across sampling units of each of the 158 DNA-species

185   identified. The first three Hill numbers (Hill, 1973), which are associated to estimators of species

186   richness and species dominance, were calculated for each level and their corresponding

187   rarefaction and extrapolation curves were constructed. The first Hill number ($q = 0$) used in the

188   analysis estimates the expected yeast-species richness (number of species) in the assemblage of

189   nectar host plants. The second Hill number ($q = 1$) is the exponential of Shannon entropy index

190   and estimates yeast diversity with respect to equally-common species and species richness

191   (Shannon diversity). The third Hill number ($q = 2$) is the inverse Simpson concentration index

192   and measures the dominance of yeast species in the species assemblage (Simpson diversity); see

193   Hill (1973) for further details of Hill numbers. To compare among hierarchical sampling levels,

194   rarefaction and extrapolation sample-size-based were produced for each level to provide

195   asymptotic estimators of diversity based on Hill numbers with their respective 95 % confidence

196   intervals constructed by a bootstrap method (Chao et al., 2014). One potential issue in our

197   sampling is that it included many different plant species each with a relatively low replication.

198   To account for this as far as possible, firstly, all yeast species that occurred only once were

199   excluded from the analysis, as they were likely to be allochthonous; secondly, an analysis of

200   sampling completeness was conducted to estimate the sample size needed to achieve that the

201   proportion of undetected autochthonous species remains unchanged even when the sample size

202   increases (Chao & Jost, 2012). To this end, a sample completeness curve was constructed by

203   combining the sample-size-based and the coverage-based estimations. Extrapolations were

204   extended up to double the initial sample size (i.e., 122 nectar samples) for all sampling levels,

205   which allow us to make predictions about the yeast diversity that can be detected in each

206   sampling level using a similar sampling effort. The number of nectar samples examined in each

207   level was 122, 54, 17, 13, 10 for Drop, Individual, Species, Genus, and Family, respectively.

208   Correspondence analysis was conducted using the R package ca (Greenacre, Nemadic &

209   Friendly, 2016) to obtain a statistical and graphical visualization of associations between nectar-

210 living yeasts and host plants. This analysis is a geometric technique for displaying the rows and

211 the columns of a contingency table as points in a low-dimensional space such that the positions

212 of the row and column points are consistent with their associations in the table. The analysis

213 produces correspondence-dimensions based on the profiles (relative frequency of yeast taxa

214 corresponding with the respective host plant), weighted average of profiles (centroid of the space

215 representation), chi-square Euclidean-distances (proximity between points), and the total inertia

216 (total contribution of yeast taxa and host plant to the between-taxa correspondence). For yeasts

217 and host plants data, contingency tables were produced using yeast species as column variables

218 and plant species as row variables. All singletons were excluded from the analysis. The first

219 three dimensions obtained from the analysis were plotted to generate biplots representing

220 correspondence between yeast and host plant taxa.

221 A power model with two categorical factors (Zahn, 2010) was proposed to test both, the

222 association between the logarithm (+1) of yeast cell density and the logarithm of nectar sugars

223 concentration in nectar samples and, the contribution of different types of yeasts and host plants

224 after taking out the variance due to yeast cell density. This variable was set as causal variable and

225 the sugar concentration as response variable. Different groups of yeasts (Yeast) and host plants

226 species (Plant) were treated as co-factors in the model. Sample sizes in a number of

227 combinations between yeast and host plants are less than five and yeast groups do not occur

228 across all host plants, therefore in order to get a robust analysis, the Yeast was classified into five

229 groups. Groups of yeast were *Metschnikowia*, *Papilotrema*, *Ustilago*, Other yeasts, and Without

230 yeasts. The *Metschnikowia* group included to the close related *Metschnikowia ipomoeae*, *M.*

231 *koreensis*, *M. lochheadii*, and *Metschnikowia* sp. Similarly, The *Papilotrema* included to

232 *Cryptococcus laurentii var. laurentii*, *P. nemorosus*, and *P. rajasthanensis*, and The *Ustilago*

233 group included *Ustilago sparsa* and *Ustilago* sp. The yeast species with very small sample size

234 were included in the named Other yeasts group and the Without yeasts group, contained all cases

235 that did not have yeast cells in nectar and not produce any microbial colony when plated. Given

236 that data are structured as an incomplete design, an interaction term (Yeast x Plant) was added to

237 test multiplicative effects of yeasts and host plants, rather than additive effects. A Type III

238 approach for unbalanced data was used to calculate the sums of squares. The Akaike Information

239 Criterion (AIC) was applied to measure the goodness of fit of the power model taking into

240 account the number of parameters included and to find the best model that fits the data with the

241   minimum number of parameters. Power regressions were calculated separately for sucrose,

242   glucose, and fructose. In four cases, nectar samples produced more than one yeast species. In

243   each of those cases, yeast identity assigned in the analysis was selected at random drawing from

244   yeast species co-occurring in the respective case. Analyses were done with R software (R

245   Development Core Team, 2016).

246   **RESULTS**

247   *Yeast diversity*

248   A total of 39 species of yeasts were identified, comprising 48 % Ascomycota and 52 %

249   Basidiomycota (Table 1). The number of colonies produced by nectar drop is reported in the raw

250   data file, the number of nectar drops by host plant species is reported in the Appendix. There was

251   practically a single yeast species per nectar drop in all cases and only in four nectar samples

252   occurred two or three different yeast species (see raw data). The most frequent ascomycetous

253   yeasts were *Metschnikowia koreensis* (n = 13), *M. lochheadii* (n = 11), and *Kurtzmaniella*

254   *cleridarum* (n = 12), and for basidiomycetous yeasts were *Ustilago* species (n = 14)

255   *Cryptococcus laurentii var. laurentii* (n = 12), and *Sympodiomycopsis paphiopedili* (n = 8).

256   Analysis of diversity predicts that the overall species richness of yeasts in the sampled nectar

257   community (Hill number $q = 0$) was between 25-34 species, which was in the same order of

258   magnitude as the number of equally-common species ($q = 1$, 22-34 species) or dominant species

259   ($q = 2$, 19-33 species). Rarefaction and extrapolation curves were consistent in showing that

260   there remained a number of unrecorded yeasts at Genus and Family sampling levels of the plant

261   community surveyed. None of the three diversity estimates used reached an asymptote at those

262   levels in the sampling hierarchy. At the Species level, species richness reached an asymptote at

263   sample size doubling initial sampling effort, i.e., n = 17. Analyses also showed that the number

264   of species harbored at Drop and Plant levels was nearly complete sampled since the three

265   estimators of diversity reached an asymptote at approximately 100 and 50 sampling units,

266   respectively. The maximum predicted species values were 25 for species richness ($q = 0$), and 22

267   for equally-common species ($q = 1$) and 19 for dominant species ($q = 2$). At all levels, estimation

268   of the species richness is roughly comparable to the dominance.

269   Rarefaction and extrapolation curves also allow us to make two predictions of Hill numbers for

270   equally-common species ($q = 1$) and dominance ($q = 2$) of yeasts in the host plant community. In

271   the first scenario, Drop and Individual sampling categories for nectar collection reach an

272   asymptote and harbor relatively low yeast diversity. In the second, Species, Genus and Family

273   categories do not reach an asymptote; even when extrapolations double initial sample size and

274   them keep relatively high, unrecorded yeast diversity. These last categories have the highest

275   predicted diversity of yeasts (Fig. 1, $q = 0$, $q = 1$, $q = 2$). Completeness curves show that sample

276   completeness was nearly achieved at the Drop and Individual levels (1 and 0.99 respectively)

277   with the current sample size. At Species level sampled completeness was close to one (0.89) and

278   at higher-order levels, the maximum sample completeness was 0.76 and 0.65 for Genus and

279   Family respectively (Fig. 1, sampling completeness).

280   *Yeast-plant association*

281   Correspondence analysis revealed a significant number of associations between yeasts and host

282   plants (Fig. 2). The most extreme correspondence was observed between *K. cleridarum* with

283   *Opuntia dillenii*. The other closest associations were *Starmerella* sp. and *Metschnikowia*

284   *ipomoeae* corresponding to the host plant *Ipomoea crinicalyx*, *Clavispora lusitaniae* with *Agave*

285   *angustifolia*, and *M. koreensis* with *Tecoma stans*. Looser associations were *Candida*

286   *sorbosivorans* with *Passiflora foetida*, *Metschnikowia* sp. with *Lonchocarpus longistylus*,

287   *Sporidiobolus ruineniae* with *Merremia dissecta*, *Papilotrema flavescens* with *Bravaisia*

288   *berlandieriana*, and *Kwoniella mangrovensis* with *Operculina pinnatifida*. The weakest

289   associations were observed between two groups of species, including *Saitozyma flava*, *Ustilago*

290   *sparsa*, and *Ustilago* sp. with *Ipomoea hederifolia* and *Ipomoea triloba*, and *Vishniacozyma*

291   *taibaiensis*, and *Naganishia liquefaciens* with *Piscidia piscipula* (Fig. 2).

292   *Yeast effects on nectar sugars*

293   Nectar samples containing yeasts have lower average concentration of sucrose, glucose, and

294   fructose than which nectar samples not containing yeasts, irrespectively of the yeast species and

295   host plant (Table 2). In general, significant regressions were found between yeast-cell density

296   and nectar sugar concentration. The concentrations of sucrose, glucose and fructose decreased

297   with increasing density of yeast cells (Fig. 3). Different yeasts groups (Yeast) showed no

298   different impacts on the concentration of nectar sugars (Table 3). In contrast, different plant

299   species (Plant) tend to show different impacts on the concentration of nectar sugars after taking

300   out the overlap variance of yeast cell density (Table 3). For instance, nectar samples of *P. foetida*

301   had the highest concentration of glucose (8.4 g x 100 mL^{-1}) and fructose (8.8 g x 100 mL^{-1}) while

302   samples of *O. pinnatifida* showed consistently the lowest concentration in both sugars (0.1 and

303   0.2 g x 100 mL^{-1} for glucose and fructose respectively). For sucrose concentration, nectar

304   samples of *L. longistylus* showed the highest concentration (17.5 g x 100 mL^{-1}) and samples of

305   *Gymnopodium floribundum* the lowest (0.06 g x 100 mL^{-1}). However, the AIC reveals that the

306   multiplicative impact of the interaction between Yeast and Plant was more important to the

307   regression model than the additive effect of each factor (Table 3). The best power model that fits

308   the data is one that includes the yeast cell density as predictor of nectar sugar concentration and a

309   multiplicative effect of the interaction between yeasts and host plant species (Table 3). To

310   illustrate the interaction between Yeast and Plant factors and its impact on nectar sugar

311   concentration along with the overlap effect of yeast cell density, scatter plots for representative

312   yeast species and their respective host plant species are showed (Fig. 4).

313   **DISCUSSION**

314   No other studies on nectar-living yeasts have been conducted in tropical nectariferous plants so

315   far, excepting Herrera et al. (2009) and Canto & Herrera (2012), where frequency of yeasts in

316   floral nectar samples was assessed in three regions, two at Southern Spain and one in Southern

317   Mexico. Diversity of nectar yeasts, however, was not explicitly addressed in these two previous

318   studies, although their results suggest differences between temperate and tropical regions.

319   Another similar study was conducted by Mittelbach et al. (2015) in a subtropical environment of

320   the Canary Islands. We will first discuss diversity patterns found in the present study and then

321   compare them with previous findings. Then we will discuss on the association between yeast

322   species and host plants and on the implications of differential yeast effects on nectar sugars.

323   *Yeast diversity*

324   Our results indicate that the assemblage of yeasts in the plant community surveyed was made up

325   of a relatively high number of species at the highest sampling levels (plant genera and botanical

326   families), along with a substantial number of equally-common species and relatively low species

327   dominance at all sampling levels considered. Low dominance in the assemblage is pointing a

328   bias inherent in the diversity estimates due to under-sampling at higher sampling levels and

329   oversampling at lower levels (Chao & Jost, 2015). This tropical plant community harbored a

330   higher diversity of nectar yeasts than our sampling design was able to detect. While the expected

331   yeast diversity at the drop- and individual-based levels was acceptably estimated with the sample

332   size set in this study, the analysis predicts that diversity remarkably increased at higher levels in

333   the sampling hierarchy. Reducing the number of nectar drops replicates per plant, as well as the

334   number of individual plants per species, while increasing the number of plant species by genera

335   and families, will probably achieve a more encompassing picture of diversity of nectar-living

336   yeasts in the tropical plant assemblage studied.

337   One frequent diversity patterns of animal and plant diversity is the latitudinal gradient of species

338   richness (Pianka, 1966; Hillebrand, 2004). Although latitudinal clines in species richness are

339   discernible in several groups of marine bacterioplankton and phytoplankton microorganisms (e.g.

340   Fuhrman et al., 2008; Schattenhofer et al., 2009; Barton et al., 2010), these clines for microbial

341   diversity have been lees studied, in particular for diversity associated to tropical floral nectars.

342   Although more studies are necessary, our results along with those of the other studies reveal a

343   possible tendency of lower latitudes to support more nectar-living yeast species than which

344   higher latitudes. For example, in temperate communities, Herzberg, Fischer & Titze (2002)

345   studied microfungal diversity in the nectars of native plants of Germany, reporting a species

346   richness of 20 yeasts in a total of 25 different plant species. Pozo, Herrera & Bazaga (2011)

347   found 12 yeast taxa in 24 plant species in Southern Spain, later Álvarez-Pérez & Herrera (2013)

348   in a large plant assemblage from Southern Spain found 20 yeasts in nectar of 30 plant species.

349   Most recently, Mittelbach et al. (2015) reported nectar fungal diversity from a subtropical plant

350   community in the Canary Islands. A total of 34 yeasts species were found in 8 native plant

351   species. Belisle et al. (2014) reported 38 microfungi species, associated to mouthparts of 21

352   hummingbirds and 6 bats species of Costa Rica. In this work, corresponding to a tropical

353   environment, 18 nectariferous plants were surveyed, finding a total of 39 yeast taxa. Yeast

354   species richness seems therefore to steadily decrease from tropical community of Yucatan and

355   subtropical community in the Canary Islands to temperate plant communities of Southern Spain

356   and Germany.

357   *Yeast-plant associations*

358   The diversity of nectar-living yeasts in our sample was also shaped by associations between

359   yeast, host plants and flower visitors. This pattern creates a mosaic of nectar-environments at

360   community level where habitat features are filters that influence the probability that the taxa with

361   their specified traits are able to join and persist as members of a local community (Soininen,

362   2012; Hillebrand & Blenckner, 2002). According to our results and previous evidence (e.g.,

363   Lachance et al., 2001; Lachance et al., 2008; Lachance et al., 2016), two kinds of non-exclusive

364   filters may have an effect on nectar-yeast interactions. First, floral nectar may act as a yeast

365   community filter owing to its physicochemical and nutritional factors such as availability of

366   nutrients, water activity and presence of yeast limiting/enhancing solutes, which altogether can

367   lead to physiological specialization in nectar-living yeasts (Lievens et al., 2015). Our results

368   show the existence of frequent yeast and host plant correspondences, which is compatible with

369   the existence of nectar filters that 'sieve' yeasts arriving to nectar and drive yeast distribution

370   across host plants. However, experimental evidence culturing yeasts under different nectar

371   environments are necessary in order to test the existence of this kind of filter. Second, flower

372   visitors can be also seen as ecological filter as they show particular associations with yeasts.

373   Different plant species have different pollinators that can transport different yeast species to

374   floral nectars. In a preliminary nectar-yeast assessment from South African plants, de Vega,

375   Herrera & Johnson (2009) observed that differences among plant species in yeast incidence were

376   related to variations in pollinator types. Mittelbach et al. (2015) also found that differences in

377   pollinator types partly explained variation in nectar yeast composition between the Canary

378   Islands plants. Pollinators of plants sampled for this study included solitary bees, stingless bees,

379   hummingbirds, beetles, and bats. It thus seems reasonable to postulate that these different groups

380   will carry different yeast species, and the closest yeast-plant correspondences are also resulted

381   from particular flower visitors carrying particular yeasts to flowers. For example,

382   correspondence between *K. cleridarum* and the cactus *O. dilleni* is explained by the association

383   of this yeast with beetles of genus *Carpophilus*, which arrive to cactus flowers to feed on nectar

384   and pollen and release yeast cells to this environment (Lachance & Starmer, 2008).

385   Correspondence showed by *Starmerella* sp. and *M. ipomoeae* with *I. crinicalyx* denote that the

386   flower visitors are bees and nitidulid beetles (Rosa et al., 2003; Lachance et al., 2001). The

387   strong association of *M. ipomoeae* and *M. lochheadii* with *Ipomoea* species is resulted from the

388   association of these yeasts with *Conotelus* beetles (Lachance et al., 2001). In contrast, looser

389   yeast-plant correspondences involved mostly basidiomicetous yeasts (except *C. sorbosivorans*)

390   that have been isolated in non-flower, non-nectar substrates and probably arrive to nectar through

391   accidental contamination or air dispersal (Lachance et al., 2001; Valério, Gadanho & Sampaio,

392   2002; Fell & Tallman, 1980; Yang et al., 2010). Additionally, plant-yeast species

393   correspondences mostly involved ascomycetous yeasts. In fact, ascomycetous yeasts showing

394   correspondence with plants all belong to the same class Saccharomycetes (subphylum

395   Saccharomycotina), while basidiomycetous taxa isolated from nectar belong to several classes

396   such as Tremellomycetes, Ustilaginomycetes, Microbotryomycetes, and Hyphomycetes

397   (subphyla Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina).

398   *Yeast effects on nectar*

399   Our results show that the overall effect of yeast cell density on nectar sugars generally involves

400   changes in the composition of nectar sugars that denote not only a chemical signature of yeast

401   metabolism, but also a nectar quality-impoverishment, since sugar concentration decreases with

402   increasing yeast cell density. This phenomenon has been previously reported by Herrera, García

403   & Pérez (2008) and de Vega & Herrera (2013). By reducing the nutritional value of nectar,

404   foraging behavior of pollinators is affected and nectar-living yeasts become a factor that drives

405   plant-pollinator interactions (Herrera, Pozo & Medrano, 2013; see also Vannette, Gauthier &

406   Fukami, 2013; Good et al., 2014; Schaeffer & Irwin, 2014). Although more data from additional

407   tropical communities are needed, it is reasonable to expect that nectar-living yeasts will have

408   ecologically significant implications in plant-pollinator interactions at the community level

409   because of their effects on community-wide floral nectar traits and foraging behavior of flower

410   visitors. Results in this study also show that nectar alteration by yeasts is not a rare phenomenon

411   in the community of host plants surveyed and probably, is more frequent in tropical plant

412   communities than currently acknowledged.

413   Yeast cell density and the interaction between different yeast groups and host plants account for

414   the majority of the variance observed in nectar sugars concentration in this study. Although

415   different yeast groups were found to have no different impacts on nectar traits, their interaction

416   with host plants impacted on nectar sugars concentration. One explanation is that initial sugar

417   concentration of nectar depends on the variance inherent to plant species in their nectar secretion.

418 Nectar-living yeasts can match or mismatch with traits of initial nectar (e.g. because of
419 physiologic requirements of yeasts), therefore, different types of yeasts will differ in their ability
420 to grow in different nectars of different plants (Herrera, Pozo & Bazaga, 2014). Moreover floral
421 nectars frequently contain plant metabolites that prevent for yeast degradation of nectar (Adler,
422 2000; Thornburg et al., 2003; Herrera et al., 2010; Heil, 2011; Nepi, 2012). The final result is
423 that some types (or species) of yeast will occur in some specific host plants but will not occur in
424 others. That pattern was observed across host plants in this study. For example, *Metschnikowia*
425 group occur in *I. crinicalyx*, *O. pinnatifida* and *T. stant* but it did not occur in the rest of the host
426 plants. Similarly, *Papilotrema* group occurred only in *I. hederifolia*, *M. aegyptia*, *M. dissecta* and
427 *O. pinnatifida*, and *Ustilago* group occurred only in *I. hederifolia*, *I. nil*, *I. triloba*, and *M.*
428 *dissecta*.

429 The observed diversity of nectar-living yeasts in the assemblage of host plants surveyed most
430 likely represent only a small portion of actual species numbers occurring in floral nectar in the
431 area, suggesting that tropical communities of nectars harbor an impressive, so far undiscovered
432 diversity of yeast taxa associated to flower-nectar environments. The diversity of this type of
433 yeasts is not only characterized by an important number of equally-common species with low
434 dominance, but also by significant species correspondences between yeasts and nectariferous
435 plants. Finally, the impact that the interaction between differente types of yeasts and
436 nectariferous plants exert on nectar sugars observed in this study, suggest for the existence of a
437 nectar filtering process that sieves the initial assemblage of yeast species arriving to nectar from
438 pollinators mouthparts, thus opening the opportunity to yeast specialization.

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Figure 1

Diversity of tropical nectar-living yeasts

Diversity of nectar-living yeasts at the different hierarchically nested sampling levels used in nectar collection: nectar drops (Drop), individual plants (Individual), plant species (Species), plant genus (Genus), and botanical family (Family). Plots show (A) species richness (Hill number for $q = 0$), (B) equally-abundant species ($q = 1$), (C) dominance ($q = 2$), and (D) sample completeness curve. Diversity curves were constructed using rarefied (solid lines) and extrapolated nectar samples (dashed lines) with sample-size-based (left panels) estimations. Each curve was extrapolated up to double the base reference sample size. Observed reference size for each category curve is denoted by a different symbol. The 95 % confidence intervals (color-shaded regions) were obtained by a bootstrap method based on 200 replications.

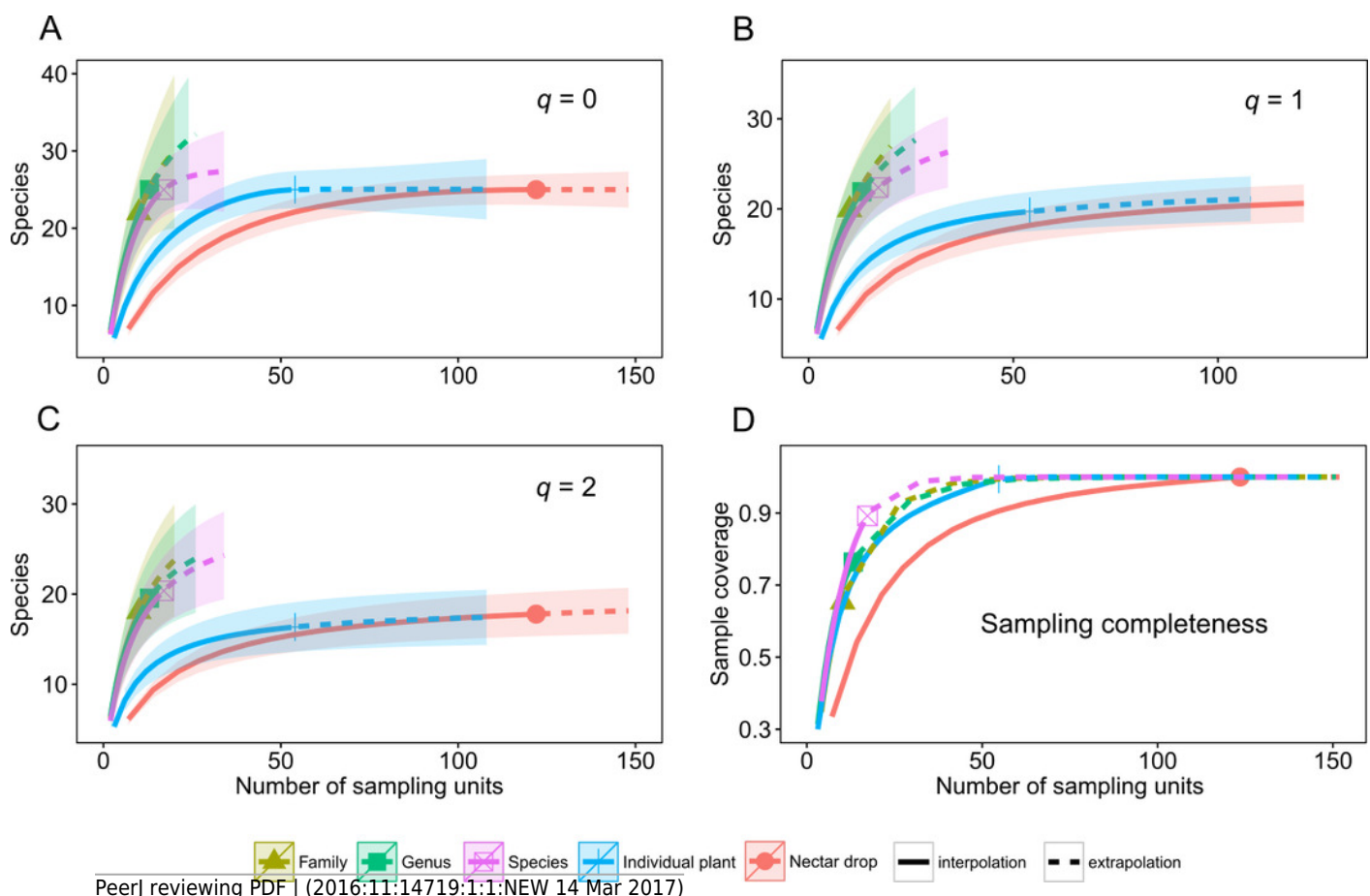



Figure 2

Correspondence analysis for nectar-living yeasts

Correspondence analysis for yeasts isolated from floral nectar samples. Plots show the associations between yeasts and host plants. Yeasts are depicted by filled triangles and letters and Host plants by filled circles and numbers. Distances among points depict the similarity between members of the same yeast group or of the same plant group. Whiting dashed-line ellipses indicate significant ($p < 0.05$) correspondences between yeasts and host plants. Percentage of contribution of each dimension to total variation is shown in parenthesis in the respective dimension. The points depicting the extreme correspondence of *Kurtzmaniella cleridarum* with *Opuntia dillenii* were extracted from the graphic analysis so that the remained correspondences are better observed. Yeasts: (a) *Starmerella* sp., (b) *Wickerhamiella occidentalis*, (c) *Candida sorbosivorans*, (e) *Clavispora lusitaniae*, (f) *Metschnikowia ipomoeae*, (g) *Metschnikowia lochheadii* (h) *Metschnikowia* sp., (i) *Metschnikowia koreensis*, (k) *Vishniacozyma taibaiensis*, (l) *Saitozyma flava* (m) *Cryptococcus* sp.1, (n) *Cryptococcus laurentii* var. *laurentii*, (o) *Papilotrema flavescens*, (p) *Cryptococcus* sp.2, (q) *Naganishia liquefaciens*, (r) *Hannaella siamensis*, (s) *Kwoniella mangrovensis*, (t) *Rhodotorula paludigena*, (u) *Sporidiobolus ruineniae*, (v) *Sympodiomycopsis paphiopedili*, (w) *Ustilago sparsa*, (x) *Ustilago* sp. Host plants: (1) *Agave angustifolia*, (2) *Bravaisia berlandieriana*, (3) *Gymnopodium floribundum*, (4) *Ipomoea crinicalyx*, (5) *Ipomoea hederifolia*, (6) *Ipomoea nil*, (7) *Ipomoea triloba*, (8) *Lonchocarpus longistylus*, (9) *Merremia dissecta*, (10) *Operculina pinnatifida*, (12) *Passiflora foetida*, (13) *Piscidia piscipula*, (14) *Tecoma stans*.

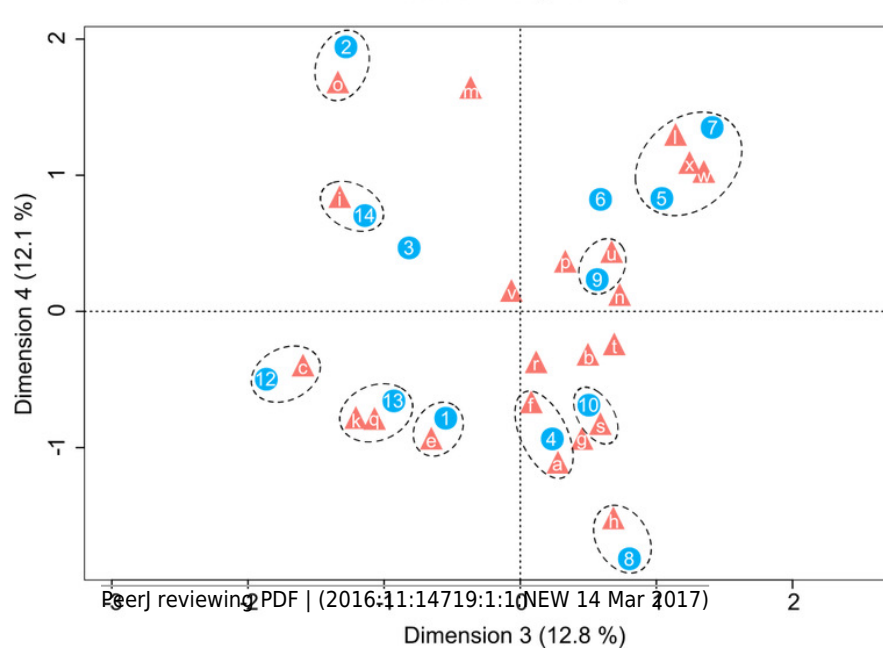
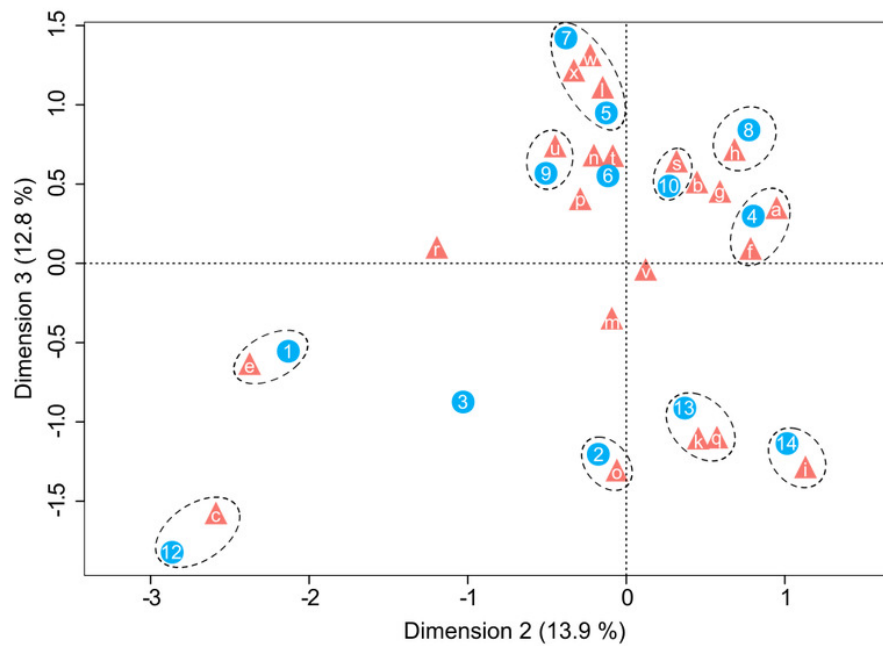
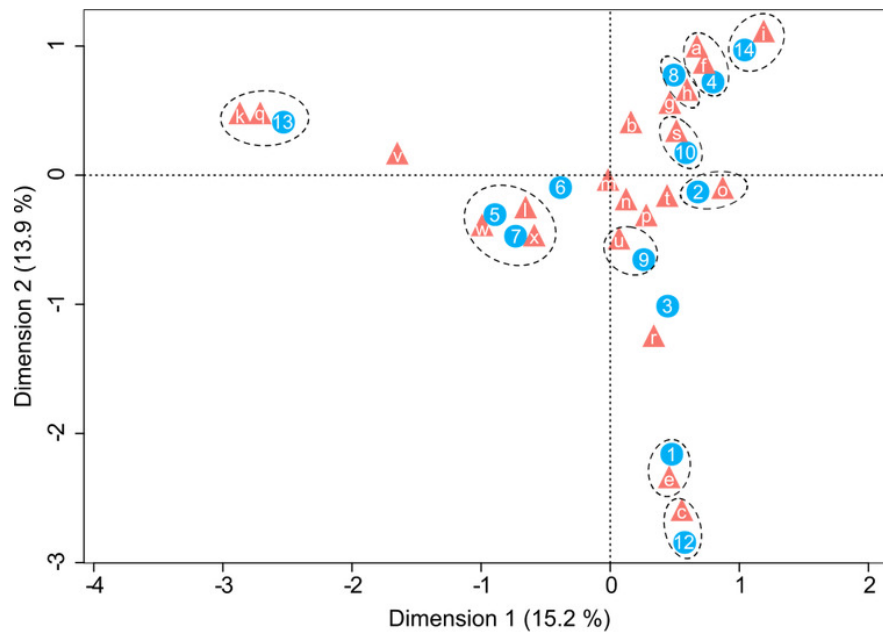



Figure 3

The relationship between nectar sugars and yeast cell density.

Overall power regression between yeast cell density and the concentration of three nectar sugars: (A) sucrose, (B) glucose and (C) fructose). Models are shown in each panel along with their statistical significance. The percentages of variance of each sugar explained by each model (adjusted R^2) are 10 %, 30 %, and 36 % for sucrose, glucose, and fructose, respectively.

A

$y = 2.0x^{-0.1}$, $p < 0.0001$

B

$y = 1.7x^{-0.1}$, $p < 0.0001$

C

$y = 1.9x^{-0.1}$, $p < 0.0001$

log(cell_density + 1)

nectar samples without yeasts   nectar samples with yeasts

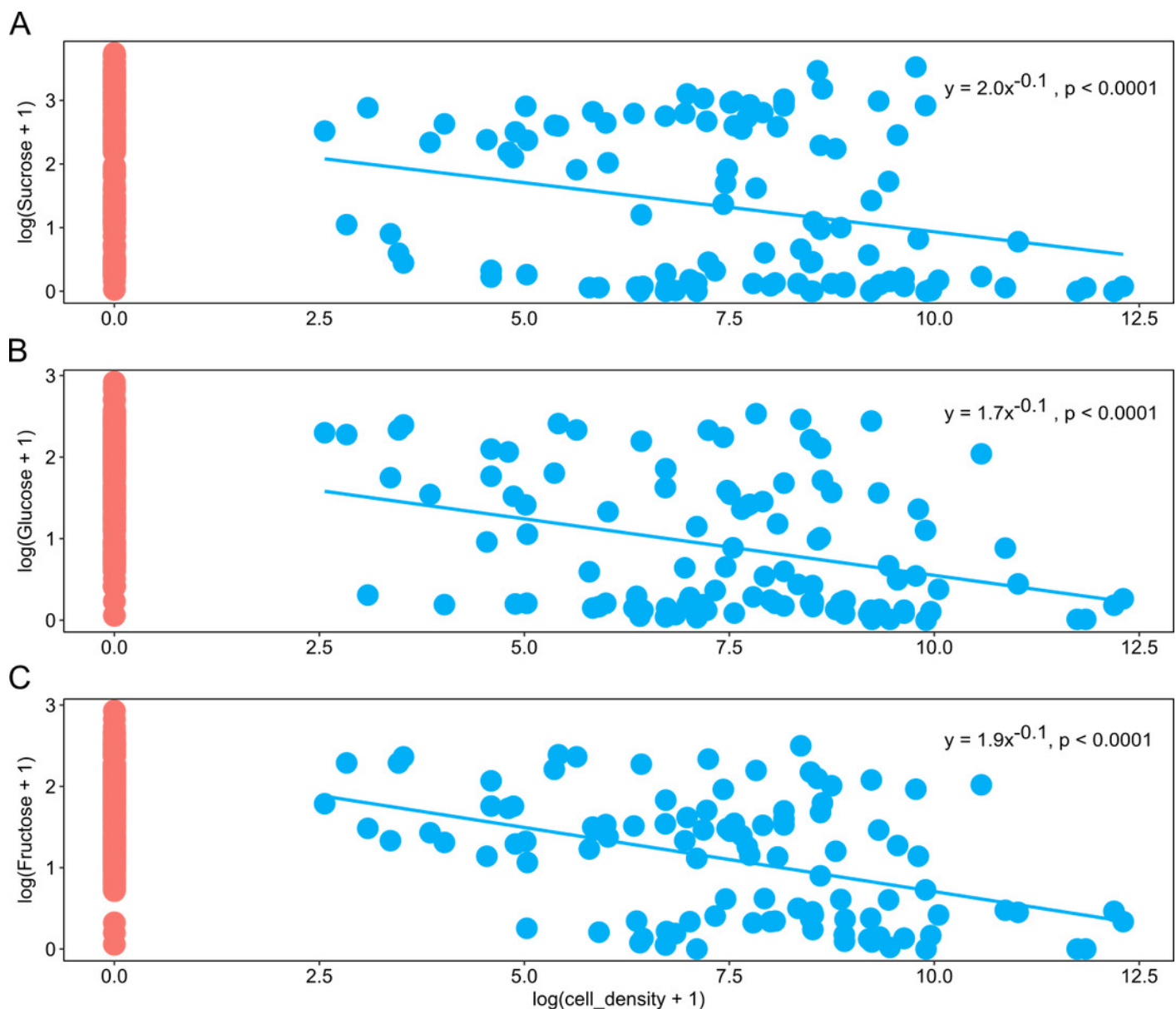
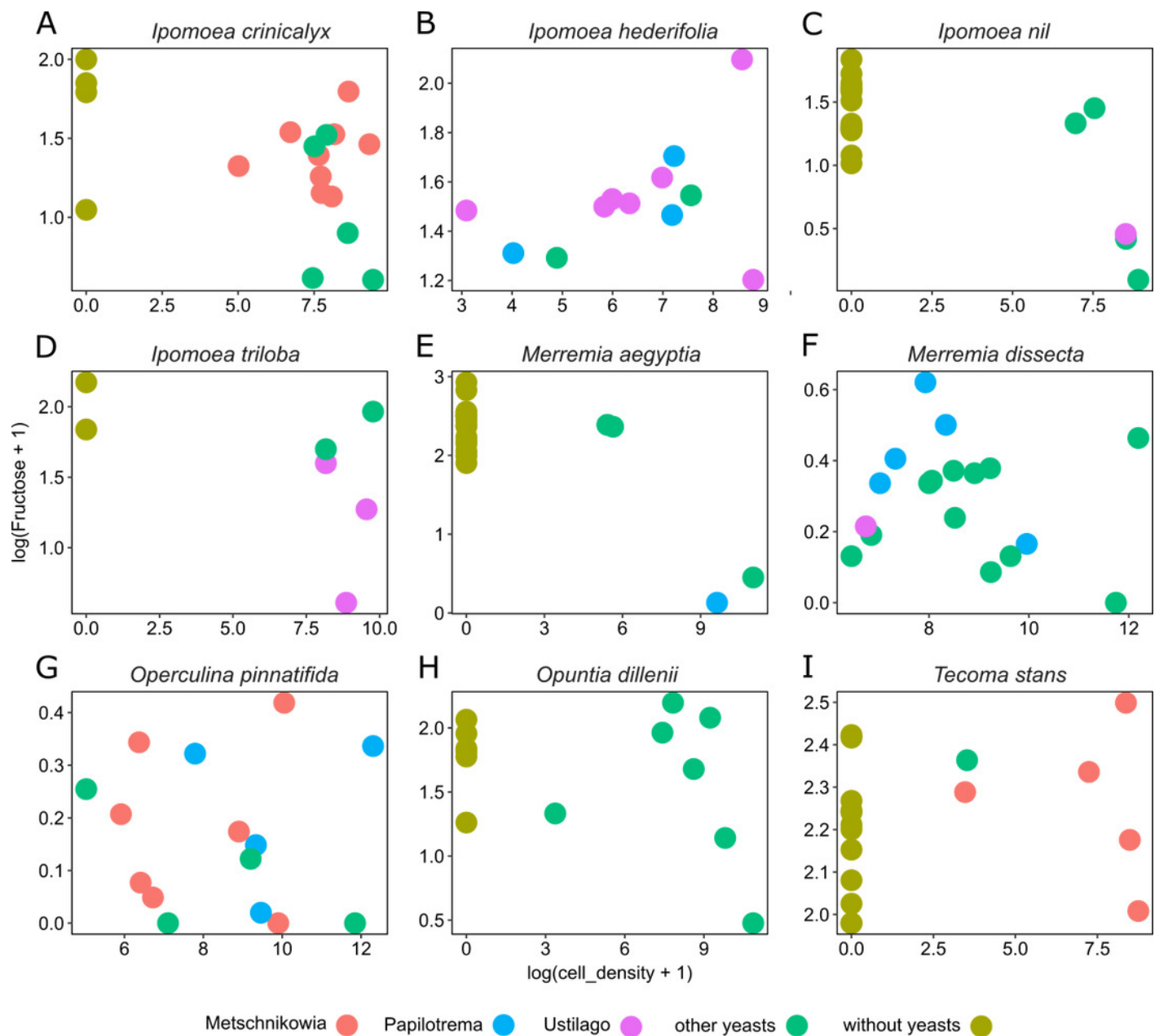



Figure 4

Differential effects of nectar-living yeasts.

The effect of the interaction of different types of yeasts and host plants on the relationship between yeast cell density and the concentration of sugar in nectar samples. Data are shown only for fructose concentration in nectar of (A) *Ipomoea crinicalyx*, (B) *Ipomoea hederifolia*, (C) *Ipomoea nil*, (D) *Ipomoea triloba*, (E) *Merremia aegyptia*, (F) *Merremia dissecta*, (G) *Operculina pinnatifida*, (H) *Opuntia dillenii*, and (I) *Tecoma stans*. Not all yeasts are distributed in all host plants, some yeasts seem to be specific to some plants.

Nectar-living yeasts obtained from floral nectar of tropical plants

Yeast species isolated from floral nectar in a tropical community of host-plants. Plant species, their respective botanical family, as well number of nectar samples or drops collected by plant species and individuals are reported along with frequency of each yeast species in nectar samples and accession numbers.

1 **Table 1.** Yeast species isolated from floral nectar in a tropical community of host-plants. Plant species,

2 their respective botanical family, as well number of nectar samples or drops collected by plant species and

3 individuals are reported along with frequency of each yeast species in nectar samples and accession

4 numbers.

Plant species (Botanical family)	Designation	n	Accession numbers	
			CICY	NCBI
Agave angustifolia (Agavaceae)	*Candida sorbosivorans*	1	CICYRN019	KX908033
	Clavispora lusitaniae	4	CICYRN016	KX908034
	Hannaella siamensis	3	CICYRN007	KX908035
Bravaisia berlandieriana (Acanthaceae)	*Papilotrema flavescens*	3	CICYRN004	KX908036
	Cryptococcus sp.1	1	CICYRN011	KX908037
Cordia sebestena (Boraginaceae)	*Candida apicola*	1	CICYRN065	KX908038
Gossypium hirsutum (Malvaceae)	*Candida versatilis*	1	CICYRN061	KX908039
	Vishniacozyma taibaiensis	1	CICYRN053	KX908040
	Starmerella bombicola	1	CICYRN055	KX908041
	Sympodiomycopsis paphiopedili	1	CICYRN063	KX908042
Gymnopodium floribundum (Polygonaceae)	*Candida sorbosivorans*	1	CICYRN041	KX908043
	Cryptococcus laurentii var. *laurentii*	1	CICYRN040	KX908044
	Papilotrema flavescens	1	CICYRN039	KX908045
Ipomoea crinicalyx (Convolvulaceae)	*Candida etchellsii*	1	CICYRN313	KX908046
	Candida powellii	1	CICYRN303	KX908047
	Metschnikowia ipomoeae	2	CICYRN320	KX908048
	Metschnikowia lochheadii	8	CICYRN304	KX908049
	Metschnikowia sp.	1	CICYRN310	KX908050
	Starmerella sp.	2	CICYRN337	KX908051
	Wickerhamiella occidentalis	1	CICYRN341	KX908052
Ipomoea hederifolia (Convolvulaceae)	*Cryptococcus laurentii* var. *laurentii*	3	CICYRN225	KX908053
	Hannaella sinensis	1	CICYRN264	KX908054
	Pseudozyma sp.	1	CICYRN249	KX908055
	Sympodiomycopsis paphiopedili	2	CICYRN325	KX908056
	Ustilago sp.	3	CICYRN228	KX908057
	Ustilago sparsa	6	CICYRN256	KX908058
Ipomoea nil (Convolvulaceae)	*Cryptococcus* sp.1	1	CICYRN217	KX908059
	Saitozyma flava	1	CICYRN207	KX908060
	Sporidiobolus ruineniae	1	CICYRN201	KX908061
	Sympodiomycopsis paphiopedili	1	CICYRN218	KX908062
	Ustilago sp.	1	CICYRN180	KX908063
	Wickerhamiella occidentalis	1	CICYRN182	KX908064
Ipomoea triloba	*Saitozyma flava*	1	CICYRN280	KX908065

(Convolvulaceae)	*Ustilago* sp.	3	CICYRN277	KX908066
	Pseudozyma sp.	1	CICYRN286	KX908067
Lonchocarpus longistylus (Fabaceae)	*Metschnikowia* sp.	3	CICYRN002	KX908068
Malvaviscus arboreus (Malvaceae)	*Candida versatilis*	1	CICYRN058	KX908069
Merremia aegyptia (Convolvulaceae)	*Aureobasidium* sp.	1	CICYRN221	KX908070
	Papilotrema nemorosus	1	CICYRN208	KX908071
	Priceomyces melissophilus	1	CICYRN210	KX908072
	Sympodiomycopsis paphiopedili	1	CICYRN209	KX908073
Merremia dissecta (Convolvulaceae)	*Cryptococcus laurentii* var. *laurentii*	4	CICYRN105	KX908074
	Cryptococcus sp.2	6	CICYRN166	KX908075
	Cryptococcus sp.3	1	CICYRN179	KX908076
	Hannaella siamensis	3	CICYRN107	KX908077
	Papilotrema rajasthanensis	1	CICYRN169	KX908078
	Rhodotorula paludigena	1	CICYRN188	KX908079
	Sporidiobolus ruineniae	4	CICYRN109	KX908080
	Ustilago sp.	1	CICYRN177	KX908081
Operculina pinnatifida (Convolvulaceae)	*Candida parazyma*	1	CICYRN165	KX908082
	Cryptococcus laurentii var. *laurentii*	4	CICYRN132	KX908083
	Hannaella siamensis	1	CICYRN134	KX908084
	Kwoniella mangrovensis	2	CICYRN127	KX908085
	Metschnikowia ipomoeae	4	CICYRN161	KX908086
	Metschnikowia lachancei	1	CICYRN155	KX908087
	Metschnikowia lochheadii	3	CICYRN144	KX908088
	Metschnikowia sp.	2	CICYRN150	KX908089
	Rhodotorula paludigena	1	CICYRN185	KX908090
	Wickerhamiella occidentalis	1	CICYRN137	KX908091
Opuntia dillenii (Cactaceae)	*Kurtzmaniella cleridarum*	12	CICYRN094	KX908092
	Candida etchellsii	1	CICYRN080	KX908093
Passiflora foetida (Passifloraceae)	*Candida bombi*	1	CICYRN051	KX908094
	Candida sorbosivorans	3	CICYRN014	KX908095
Piscidia piscipula (Fabaceae)	*Vishniacozyma taibaiensis*	3	CICYRN042	KX908096
	Naganishia liquefaciens	6	CICYRN046	KX908097
	Sympodiomycopsis paphiopedili	3	CICYRN048	KX908098
Tecoma stans (Bignoniaceae)	*Metschnikowia koreensis*	13	CICYRN036	KX908099
	Metschnikowia ipomoeae	3	CICYRN027	KX908100
	Cryptococcus sp.2	1	CICYRN024	KX908101

Nectar samples with yeasts and nectar samples without yeasts

Comparisons of average (± SD) concentrations of the three nectar sugars between nectar samples containing yeast cells and nectar samples without cells. T-tests and statistical significance are shown for each sugar.

1   **Table 2.** Comparisons of average (± SD) concentrations of the three nectar sugars between nectar samples containing yeast cells and nectar
2   samples without cells. T-tests and statistical significance are shown for each sugar.

Nectar sugars	Nectar samples (g of solute per 100 mL solution)				
	With yeasts	Without yeasts	t	d.f	p
Sucrose	6.3 ± 7.8	10.6 ± 11.1	4.48	215	< 0.0001
Glucose	2.5 ± 3.2	5.5 ± 3.8	7.95	196	< 0.0001
Fructose	2.9 ± 2.9	5.6 ± 3.5	8.81	172	< 0.0001

Power model analysis for the effect of yeast cell density, different yeast types and host plants

Statistical significance for power models of the effects of yeast cell density on the concentration of the three nectar sugars. The Akaike Information Criterion (AIC) values are shown for each variable/factor included in models. The lower AIC, the better the power model fits to the data . F-values were calculated based on Type III sum of square approach.

1 **Table 3.** Statistical significance for power models of the effects of yeast cell density on the concentration of the three nectar sugars. The Akaike
2 Information Criterion (AIC) values are shown for each variable/factor included in models. The lower AIC, the better the power model
3 fits to the data. F-values were calculated based on Type III sum of square approach.

	Sucrose				Glucose				Fructose			
	F	d.f	P-value	AIC	F	d.f	P-value	AIC	F	d.f	P-value	AIC
Yeast cell density	5.88	1, 73	0.0177	-120.79	7.85	1, 73	0.0064	-197.73	4.48	1, 73	0.0375	-186.11
Yeast	1.19	3, 73	0.3170	-126.77	0.43	3, 73	0.7298	-206.26	0.84	3, 73	0.4950	-190.26
Plant	26.90	17, 73	< 0.0001	-126.77	21.44	17, 73	< 0.0001	-206.26	15.85	17, 73	< 0.0001	-190.26
Yeast x Plant	2.57	8, 73	0.0155	-117.15	2.81	8, 73	0.0089	-194.57	2.64	8, 73	0.0133	-180.08
Total AIC				-126.77			-197.73	-206.26				-190.26