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 assemblages 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 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 sampled plant community was characterized by a relatively high number of equally-common species with low dominance. Analyses predict highly diverse nectar yeast communities in the relatively narrow range of tropical vegetation. Significant associations between specific yeast species and specific host-plants were also detected and the impact of yeasts on nectar of different host-plants was related to yeast diversity and host-plant associations with flower visitors. 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 associated to flower visitors.

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2   community-wide floral nectar traits

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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 assemblages and the effect of yeasts on nectar traits in a tropical

16   environment. Using a series of hierarchically nested sampling units, we extracted nectar from an

17   assemblage of host-plants representative of the diversity of life forms, flower shapes, and

18   pollinator types occurring in the tropical area of Yucatan-Mexico. Yeasts were isolated from

19   single nectar samples and DNA-identified, yeast-cell density was estimated, and sugar

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

21   temperate region, diversity of nectar-living yeasts in the sampled plant community was

22   characterized by a relatively high number of equally-common species with low dominance.

23   Analyses predict highly diverse nectar yeast communities in the relatively narrow range of

24   tropical vegetation. Significant associations between specific yeast species and specific host-

25   plants were also detected and the impact of yeasts on nectar of different host-plants was related

26   to yeast diversity and host-plant associations with flower visitors. This study provides an overall

27   picture of the diversity of nectar-living yeasts in tropical host-plants and suggests that a key

28   factor affecting community-wide patterns of nectar traits is not nectar chemistry, but rather the

29   kind of yeasts associated to flower visitors.

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). After initial colonization, however, 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). It has been therefore hypothesized that, in a given community of nectariferous plants, nonrandom plant-microorganism associations can produce a mosaic of different qualities of floral nectars at the community level with potential effects on plant-pollinator interactions (Canto & Herrera 2012).

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

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

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

64 the floral nectar of tropical plant environments of the Yucatan Peninsula, Mexico. By isolating

65 and identifying culturable yeasts from the floral nectar from many species and individuals of

66 animal-pollinated plants, quantifying their population densities in nectar, and estimating nectar

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

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

69 predictable associations between nectar yeasts and host-plants; and (3) the differential impact on

70 nectar composition of yeast species associated with different host-plants. Yeast diversity is

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

72 role of different host-plants and yeasts as sources of associations between plants and nectar-

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

74 predicts the existence of a relatively high diverse assemblage of nectar-living yeasts showing

75 significant correspondence with the diversity of their host-plants, as well as significant

76 differences between yeasts and host-plants in the effect that exert on floral nectars.

77 **MATERIALS AND METHODS**

78 *Study area and species sampled*

79 Field sampling was conducted from September 2008 to November 2009 in 28 sites located in a

80 relatively narrow area of tropical vegetation (approx. 430 km^2) in northwest Yucatan Peninsula,

81 Mexico. This area includes a coastal dune portion, between Chuburna and Dzilam de Bravo

82 towns, and the adjacent dry forest, between the Dzibichaltun Mayan Ruins and the Cuxtal

83 Ecological Reserve. Elevation ranges between 1-10 m and climate is semi-arid at the coastal

84 dune strip and subtropical at the dry forest, with a mean temperature of 26 °C in both types and

85 annual rainfall of 370 mm and 1077 mm, respectively. The vegetation is a low, open scrub

86 dominated by xerophytes, halophyte herbs, thorny bushes, palms and 1-3 m treelets growing on

87 sandy, nutrient-poor soils in the dune strip. The dry forest is made up of cacti, thorny shrubs and

88 deciduous medium-height trees (3-8 m tall) growing on limestone bedrock soil with a thick litter

89 layer (Chan-Vermont, Rico-Gray & Flores, 2002; Canto & Herrera, 2012).

90 *Sampling method*

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

92 studied area, nectar samples were obtained from an assemblage of 24 host-plant species

93 belonging to 17 genera and 14 botanical families, representing the diversity of life forms, flower

94 shapes, pollinator types and taxonomic categories occurring in the area (see Canto & Herrera

95 2012 for a complete list of species sampled). We adopted a five-tiered series of hierarchically

96 nested sampling units for nectar collection, namely nectar drops (Drop), individual plants

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

98 criteria used for collecting nectar samples was that flowers were already open at the time of

99 collection in the field, thus exposed to prior pollinator visitation and the nectar therefore

100 susceptible to have been colonized by yeasts. Three nectar drops were obtained from each

101 flower, and 3-6 flowers were sampled from each of 6-10 individuals per surveyed plant species.

102 Each sample was extracted with sterile calibrated micropipettes and its volume was calculated

103 following methods used by Herrera et al. (2009). Of the three nectar drops obtained from each

104 flower, one was used for DNA-based identification of yeasts, another for quantification of yeast

105 density and the other to estimate sugar concentration and composition, using in each case the

106 methods described below.

107 *Isolation and identification*

108 Nectar drops were individually streaked onto YM agar plates (1.0 % glucose, 0.5 % peptone, 0.3

109 % malt extract, 0.3 % yeast extract, 2.0 % agar) with 0.01 % chloramphenicol, and incubated at

110 25 °C until microbial colonies were detectable (2-20 days). Colonies were observed at 10x - 40x

111 magnification and phenotypically different colonies were purified by streak-plating. From 532

112 nectar drops collected, a total of 158 isolates were obtained from the resulting yeast colonies.

113 The large-subunit (26S) ribosomal DNA gene (D1/D2 region) was two-way sequenced for each

114 isolate using the primer combination NL1-NL4, according to Kurtzman & Robnett (1998) and

115 Lachance et al. (1999). Raw sequences for each isolate were edited, assembled and consensus

116 sequences were obtained using Geneious Pro 8.1.7 bioinformatics software (Biomatters Ltd,

117 Auckland, New Zealand). Nucleotide collection databases at GenBank were queried with the

118 Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997) to look for named yeast

119 species with DNA sequences matching those obtained for the isolates. All sequences queried

120   yielded significant alignments with named yeast accessions in GenBank databases, generally at

121   98–100 % of sequence coverage and identity. Resulting DNA-species and the associated

122   sampling information (nectar drop, individual plant, host-species, genus and botanical family)

123   was used in the analyses of yeast diversity. The strains studied are maintained in the Centro de

124   Investigación Cientifica de Yucatan (CICY) and their corresponding DNA sequences have been

125   deposited in GeneBank under the accessions listed in Table 1 along with their designation,

126   frequency and respective host plants (botanical family).

127   *Cell counts and nectar sugar composition and concentration*

128   The density of yeast cells was estimated in each nectar drop using a Neubauer chamber and

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

130   drops was measured with calibrated micropipettes. Each nectar sample was diluted using a 0.5 %

131   lactophenol cotton blue solution to obtain a final volume up to 1.5 – 6 times more than the initial

132   volume. Diluted samples were loaded on the counting chamber and examined under a

133   microscope (Olympus CX31) at 10x - 40 x magnifications. Cells were counted in each of 16

134   quadrants of the counting chamber and cell density was calculated using the formula: cells per μl

135   = average number of cells counting in the quadrants multiplied by the dilution factor and for the

136   fixed volume of the chamber.

137   The composition and concentration of nectar was measured following procedures described by

138   Herrera et al. (2006) and Canto et al. (2011) and using ion-exchange high-performance liquid

139   chromatography (HPLC). Nectar samples were blotted onto a 10 x 12 mm sterile Whatman

140   3MM paper wick and immediately after absorption, were placed into sterile envelopes and stored

141   at 25-26 °C in silica gel. For analytical procedure, nectar-containing wicks were individually

142   placed into Eppendorf tubes and 1mL of HPLC-grade water was added to each one. For each

143   sample, 5 μL of solution was filtered using a 0.4 μm polyvinylidenedifluoride (PVDF) filter and

144   injected into a Dionex DX 500 HPLC system (Dionex, Sunnyvale, CA, USA). The HPLC system

145   was equipped with an effluent degas module, a GP 40 gradient pump, a CarboPac PA10

146   (4mm×50mm) guard column and a CarboPac PA10 (4mm×250mm) analytical column. It also

147   had an ED 40 electrochemical detector for pulsed amperometric detection in integrated

148   amperometric mode, with the normal preloaded wave form for sugar detection (Dionex Corp.,

149   1994). The column was eluted (flow rate 1 mL min^{-1}) isocratically with 40mM NaOH (50%

150   solution; J.T. Baker, Deventer, The Netherlands) and kept at 24 °C during analysis. The

151   concentrations of sucrose, glucose and fructose in each nectar sample (g of solute per 100 mL

152   solution) were calculated by integrating the area under the corresponding chromatogram peaks,

153   then using linear regression models fitted to the data of standard sugar-solutions, and then

154   calculating the expected values of concentration corresponding with the integrated area of each

155   type of sugar contained in the analyzed samples. Two independent HPLC measurements were

156   done on each sample, and replicate results averaged for the analyses.

157   *Data analysis*

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

159   across the different hierarchical sampling levels considered (i.e., drop, individual, species, genus,

160   family), the analytical framework suggested by Chao et al. (2014) was performed with the R

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

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

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

164   1973). Hill numbers are a mathematically unified family of diversity indices, differing among

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

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

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

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

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

170   sampling level (drop, individual, species, genus, and family), an incidence matrix was built up by

171   recording the presence-absence across sampling units of each of the 158 DNA-species identified.

172   The first three Hill numbers (Hill, 1973), which are associated to estimators of species richness

173   and species dominance, were calculated for each level and their corresponding rarefaction and

174   extrapolation curves were constructed. The first Hill number ($q = 0$) used in the analysis

175   estimates the expected yeast-species richness (number of species) in the assemblage of nectar

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

177   estimates yeast diversity with respect to equally-common species and species richness (Shannon

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

179   measures the dominance of yeast species in the species assemblage (Simpson diversity); see Hill

180   (1973) for further details of Hill numbers. To compare among hierarchical sampling levels,

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

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

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

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

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

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

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

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

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

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

191   extended up to double the initial sample size for all sampling levels, which allow us to make

192   predictions about the yeast diversity that can be detected in each sampling level using a similar

193   sampling effort.

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

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

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

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

198   of the row and column points are consistent with their associations in the table. For yeasts and

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

200   plant species as row variables. All singletons were excluded from the analysis. The analysis

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

202   corresponding with the respective host-plants), weighted average of profiles (centroid of the

203   space representation), chi-square Euclidean-distances (proximity between points), and the total

204   inertia (total contribution of yeast taxa and host-plant to correspondence precision). The first

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

206   correspondence between yeast taxa and host-plants.

207   To identify possible associations between yeasts and plant species in terms of yeast effects on

208   nectar sugars, regression methods were applied using R software (R Development Core Team,

209   2016). Yeast-cell density (Log_{10} transformed) was used as predictor variable and sugar

210 concentration in nectar samples was used as response variable. To identify differences in nectar

211 concentration due to the intrinsic traits of yeasts and plants species, yeast species (Yeast) and

212 plant species (Plant) were treated as factors. Three interaction factors were added to test

213 multiplicative effects of the interactions between the Yeast and Plant, between yeast-cell-density

214 (Cell density) and Yeast, and between Cell density and Plant factors. Linear regressions were

215 calculated separately for sucrose, glucose, and fructose to predict particular changes in sugar

216 concentrations based on the effect of yeast-cell density on nectar (Canto & Herrera, 2012).

217 **RESULTS**

218 *Yeast diversity*

219 A total of 39 species of yeasts were identified, comprising 48 % ascomycetous and 52 %

220 basidiomycetous species (Table 1). The most frequent ascomycetous yeasts were *Metschnikowia*

221 *koreensis* (n = 13), *Metschnikowia ipomoeae* (n = 12), and *Kurtzmaniella cleridarum* (n = 12),

222 and for basidiomycetous yeasts were *Ustilago* species (n = 14) *Cryptococcus laurentii* (n = 12),

223 and *Sympodiomycopsis paphiopedili* (n = 8). Analysis of diversity predicts that the overall

224 species richness of yeasts in the sampled nectar community (Hill number $q = 0$) was between 25-

225 34 species, which was in the same order of magnitude as the number of equally-common species

226 ($q = 1$, 22-34 species) or dominant species ($q = 2$, 19-33 species). Rarefaction and extrapolation

227 curves were consistent in showing that there remained a number of unrecorded yeasts at Genus

228 and Family sampling levels of the plant community surveyed. None of the three diversity

229 estimates used reached an asymptote at these levels in the sampling hierarchy. At the Species

230 level, species richness reached an asymptote at sample size doubling initial sampling effort, i.e.,

231 n = 34. In contrast, species diversity estimates obtained with Hill numbers $q = 1$ and $q = 2$ did

232 reach an asymptote at that level. Analyses also showed that the number of species harbored at

233 Drop and Plant levels was nearly complete sampled. At those levels, the three estimators of

234 diversity reached an asymptote at approximately 100 and 50 sampling units, respectively. The

235 maximum predicted values were 25 yeast species for species richness Hill number $q = 0$), and 22

236 and 19 species for estimates of equally-common ($q = 1$) and dominant ($q = 2$) species,

237 respectively. Again, estimated species richness was roughly comparable to higher-order diversity

238 estimates incorporating information on dominance.

239   Rarefaction and extrapolation curves also allow to identify two predictions of Hill numbers for

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

241   one scenario, Drop and Individual sampling categories for nectar collection reach an asymptote

242   and harbor a relatively low yeast diversity. In other scenario, Species, Genus and Family

243   categories do not reach an asymptote, even when extrapolations double initial sample size. Those

244   categories have the highest predicted diversity of yeasts (Fig. 1, $q = 0$, $q = 1$, $q = 2$).

245   Completeness curves show that sample completeness was nearly achieved at the Drop and

246   Individual levels (1 and 0.99 respectively) with the current sample size in the survey. At Species

247   level sampled completeness was close to one (0.89) and at higher-order diversity estimates, the

248   maximum sample completeness was 0.76 and 0.65 for Genus and Family respectively (Fig. 1,

249   sampling completeness).

250   *Yeast-plant association*

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

252   plants (Fig. 2). The most extreme correspondence was observed between *Kurtzmaniella*

253   *cleridarum* with *Opuntia dillenii*. The other closest associations include *Starmerella* sp. and

254   *Metschnikowia ipomoeae* corresponding to the host-plant *Ipomoea crinicalyx*; *Clavispora*

255   *lusitaniae* with *Agave angustifolia* and *M. koreensis* with *Tecoma stans*. Looser associations

256   include *Candida sorbosivorans* with *Passiflora foetida*, *Metschnikowia* sp. with *Lonchocarpus*

257   *longistylus*, *Sporidiobolus ruineniae* with *Merremia dissecta*, *Papilotrema flavescens* with

258   *Bravaisia berlandieriana*, and *Kwoniella mangrovensis* with *Operculina pinnatifida*. The

259   weakest associations were observed between two groups of species, including *Saitozyma flava*,

260   *Ustilago sparsa*, and *Ustilago* sp. with *Ipomoea hederifolia* and *Ipomoea triloba*, and

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

262   *Yeast effects on nectar sugars*

263   Significant regressions were found for the overall effect of yeast-cell density on nectar sugars. In

264   general, concentrations of sucrose, glucose and fructose decreased with increasing density of

265   yeast cells (sucrose: $R^2 = 0.85$, $F_{1, 180} = 47.84$, p < 0.0001; glucose: $R^2 = 0.70$, $F_{1, 180} = 96.12$, p $<$

266   0.0001; and fructose: $R^2 = 0.67$, $F_{1, 180} = 93.20$, p < 0.0001). The analysis also showed that the

267   effect of yeasts on sugars was influenced by inherent differences due to yeast species (Yeast) and

268   plant species (Plant) (Table 2). Significant Plant x Yeast and Plant x Cell density interactions

269   were observed for sucrose concentration but not for glucose and fructose concentrations. The

270   Yeast x Cell density interaction effect on concentration was not significant for any sugar (Table

271   2). To illustrate how the effect of yeast cells density on sugar concentration in nectar can be

272   modified by Yeast and Plant factors, separate regressions for four representative yeasts species

273   were plotted. Figure 3 shows that the overall negative effect of yeast cells density on nectar

274   sugars did not remain consistent across yeast and plant species. For example, *M. ipomoeae*

275   showed no effect on concentration of any nectar sugars of *I. crinicalyx*, while *Cry. laurentii* had

276   negative effect on concentration of sucrose and fructose in *M. dissecta* and *O. pinnatifida,* but no

277   statistically significant effect on the nectar of *I. hederifolia*. Moreover, *Metschnikowia lochheadii*

278   showed overall negative effects on sugar concentrations in *O. pinnatifida* while *Ustilago sparsa*

279   had not significant effect on any nectar sugars.

280   **DISCUSSION**

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

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

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

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

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

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

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

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

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

290   *Yeast diversity*

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

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

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

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

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

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

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

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

299 size set in this study, the analysis predicted that diversity remarkably increased at higher levels in

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

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

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

303 yeasts in the tropical plant assemblage studied.

304 In temperate communities, Herzberg, Fischer & Titze (2002) studied microfungal diversity in the

305 nectars of native plants of Germany, reporting a species richness of 20 yeasts in a total of 25

306 different plant species. Pozo, Herrera & Bazaga (2011) found 12 yeast taxa in 24 plant species in

307 Southern Spain, later Álvarez-Pérez & Herrera (2013) in a large plant assemblage from Southern

308 Spain found 20 yeasts in nectar of 30 plant species. Most recently, Mittelbach et al. (2015)

309 reported nectar fungal diversity from a subtropical plant community in the Canary Islands. A

310 total of 34 yeasts species were found in 8 native plant species. Belisle et al. (2014) reported 38

311 microfungi species, associated to mouthparts of 21 hummingbirds and 6 bats species of Costa

312 Rica. In this work, corresponding to a tropical environment, 18 nectariferous plants were

313 surveyed, finding a total of 41 yeast taxa. Yeast species richness seems therefore to steadily

314 decrease from tropical community of Yucatan and subtropical community in the Canary Islands

315 to temperate plant communities of Southern Spain and Germany. One frequent diversity patterns

316 of animal and plant diversity is the latitudinal gradient of species richness (Pianka, 1966;

317 Hillebrand, 2004). The well-known latitudinal gradient of species richness is discernible in

318 several groups of marine bacterioplankton and phytoplankton microorganisms (e.g. Fuhrman et

319 al., 2008; Schattenhofer et al., 2009; Barton et al., 2010). Although more studies are necessary,

320 our results altogether with those of the studies mentioned above reveal a possible tendency of

321 lower latitudes to support more nectar yeast species than higher latitudes.

322 *Yeast-plant associations*

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

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

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

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

327 2012; Hillebrand & Blenckner, 2002). According to our results and previous evidence (e.g.,

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

357 2002; Fell & Tallman, 1980; Yang et al., 2010). Additionally, plant-yeast species

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

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

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

361   such as Tremellomycetes, Ustilaginomycetes, Microbotryomycetes, and Hyphomycetes

362   (subphyla Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina).

363   *Yeast effects on nectar*

364   Our results confirm that the overall effect of yeasts (ascomycetous and basidiomycetous taxa) on

365   nectar generally involves changes in the composition of nectar sugars that denote not only a

366   chemical signature of yeast metabolism, but also a nectar quality-impoverishment, since sugar

367   concentration decreases with increasing yeast-cell density. This phenomenon has been

368   previously reported by Herrera, García & Pérez (2008) and de Vega & Herrera (2013). By

369   reducing the nutritional value of nectar, foraging behavior of pollinators is affected and nectar-

370   living yeasts become a factor that drives plant-pollinator interactions (Herrera, Pozo & Medrano,

371   2013; see also Vannette, Gauthier & Fukami, 2012; Good et al., 2014). Although more data from

372   additional tropical communities are needed, it is reasonable to expect that nectar-living yeasts

373   will have ecologically significant implications in plant-pollinator interactions at the community

374   level because of their effects on community-wide floral nectar traits and foraging behavior of

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

376   phenomenon in the community of host-plants surveyed and probably, is more frequent in tropical

377   plant communities than currently acknowledged.

378   Different yeast species were found to have different impacts on nectar traits. One possible

379   explanation is that initial conditions in nectar can match or mismatch with traits of arriving yeast

380   (e.g. physiologic requirements of yeasts). Floral nectars frequently contain plant metabolites

381   (Adler, 2000; Thornburg et al., 2003) or high sugar concentrations that prevent for yeast

382   degradation of nectar (Herrera et al., 2010; Heil, 2011; Nepi, 2012); and nectar-living yeasts

383   differ in their relative ability to grow in different nectars of different plants (Herrera, Pozo &

384   Bazaga, 2014). In this study the close-related yeasts, *M. ipomoeae* and *M. lochheadii* (Lachance,

385   2011) and the far-related basidiomicetous yeasts *Cry. laurentii* and *Ustilago* sp., exemplify the

386   diversity of yeast effects across nectar types resulting from different yeast species matching and

387   mismatching with different floral nectars. The four yeasts partly overlapped each other across

388 different types of nectars, occur in the same plant community and showed different effects, from

389 negative to no effects, on the same type of nectar.

390 The observed diversity of nectar-living yeasts in the assemblage of host-plants surveyed most

391 likely represent only a small portion of actual species numbers occurring in floral nectar in the

392 area, suggesting that tropical communities of nectars harbor an impressive, so far undiscovered

393 diversity of yeast taxa associated to flower-nectar environments. The diversity of this type of

394 yeasts is not only characterized by an important number of equally-common species with low

395 dominance, but also by significant species correspondences between yeasts and nectariferous

396 plants. Finally, the different effects that yeasts exert on floral nectars observed in this study,

397 provide evidences for the existence of a nectar filtering process that sieves the initial assemblage

398 of yeast species arriving to nectar from pollinators mouthparts, thus opening the opportunity to

399 yeast specialization.

400 **FUNDING**

401 This work was supported by the Consejo Nacional de Ciencia y Tecnología through CB-2007-01

402 program [grant number 80031], the Ministerio de Educación y Ciencia [grant number CGL2010-

403 15964] and the Junta de Andalucía [grant number P09-RNM-4517].

404 **ACKNOWLEDGEMENTS**

405 Permission to collect in natural areas of Yucatan was granted by Secretaría del Medio Ambiente

406 y Recursos Naturales, Delegación Yucatán-Subsecretaría de Gestion para la Protección

407 Ambiental: Dirección General de Vida Silvestre (oficio 00837/09). We thank Pilar Bazaga and

408 Esmeralda López for assistance with DNA sequencing of yeasts; Atzelby López, Blanca Lizama,

409 Cesar Canché and Raymundo González for assistance in the field and laboratory; Marina García

410 for chemical analyses; Paulino Simá, Filogonio May and Alfredo Dorantes for host-plant

411 identification.

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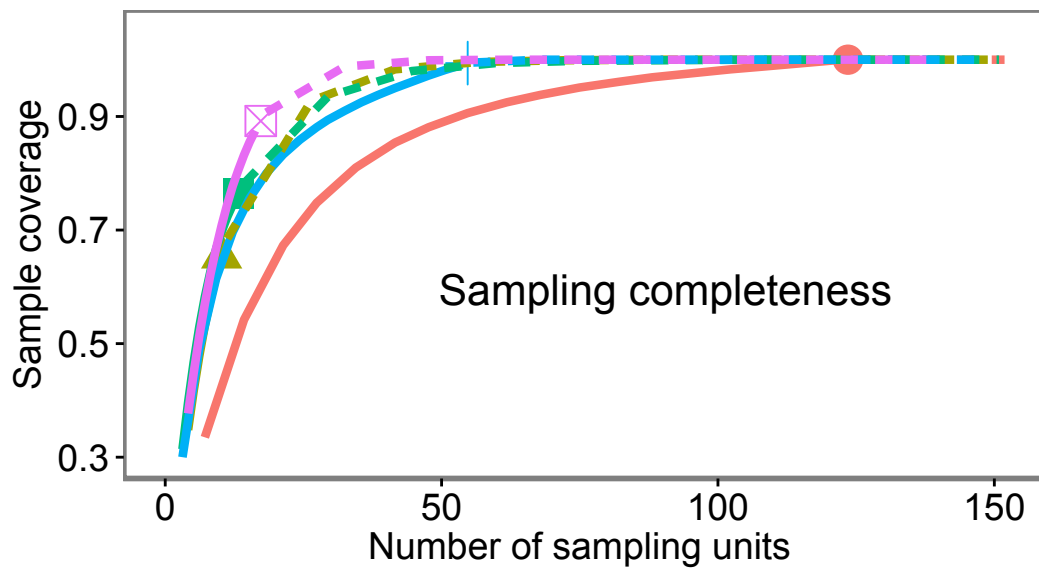
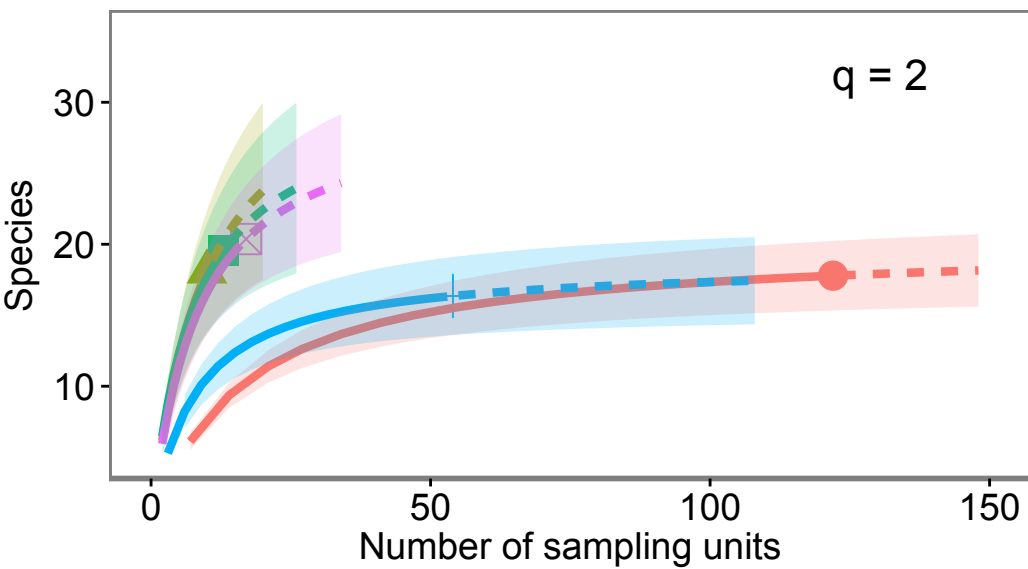
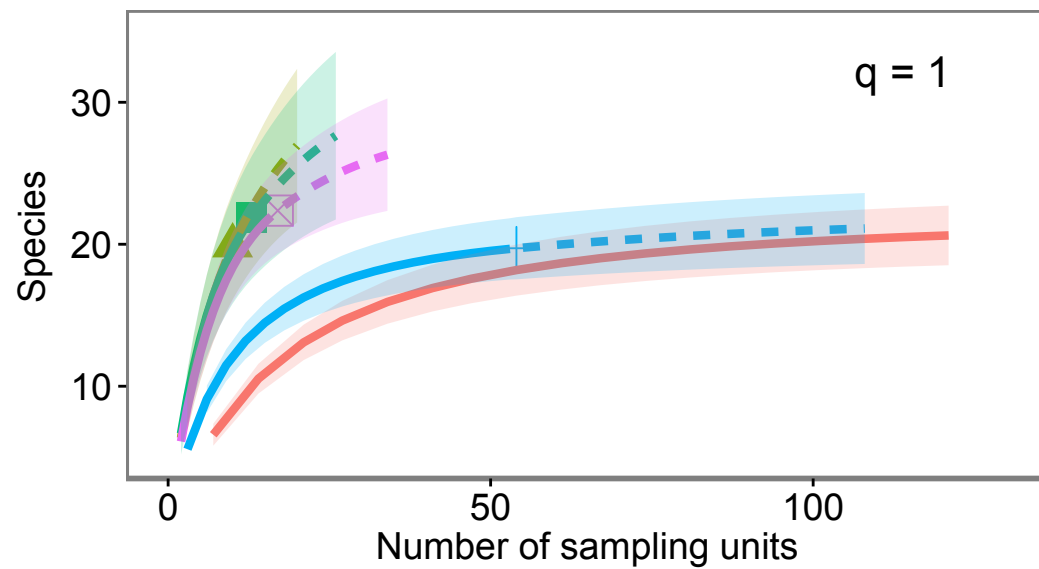
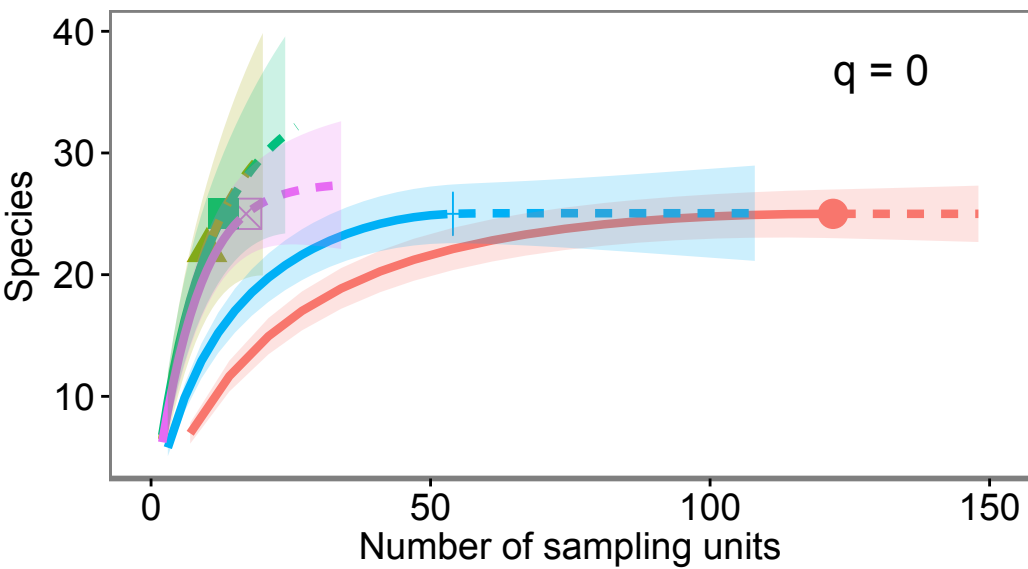
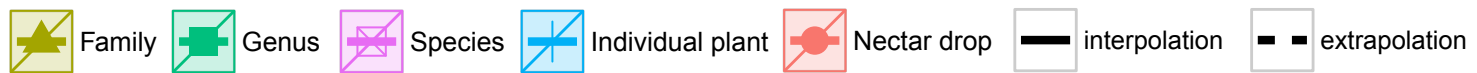
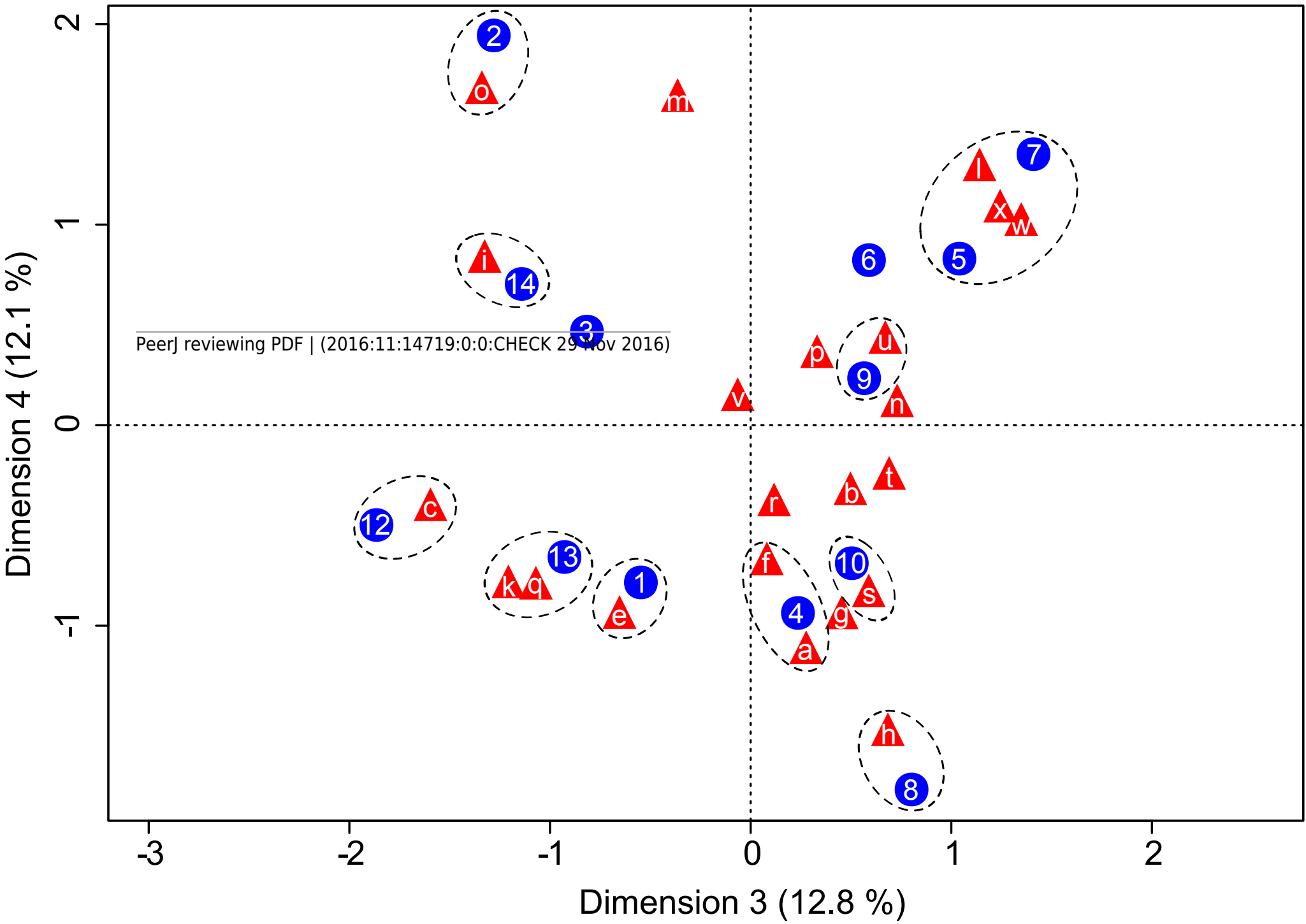
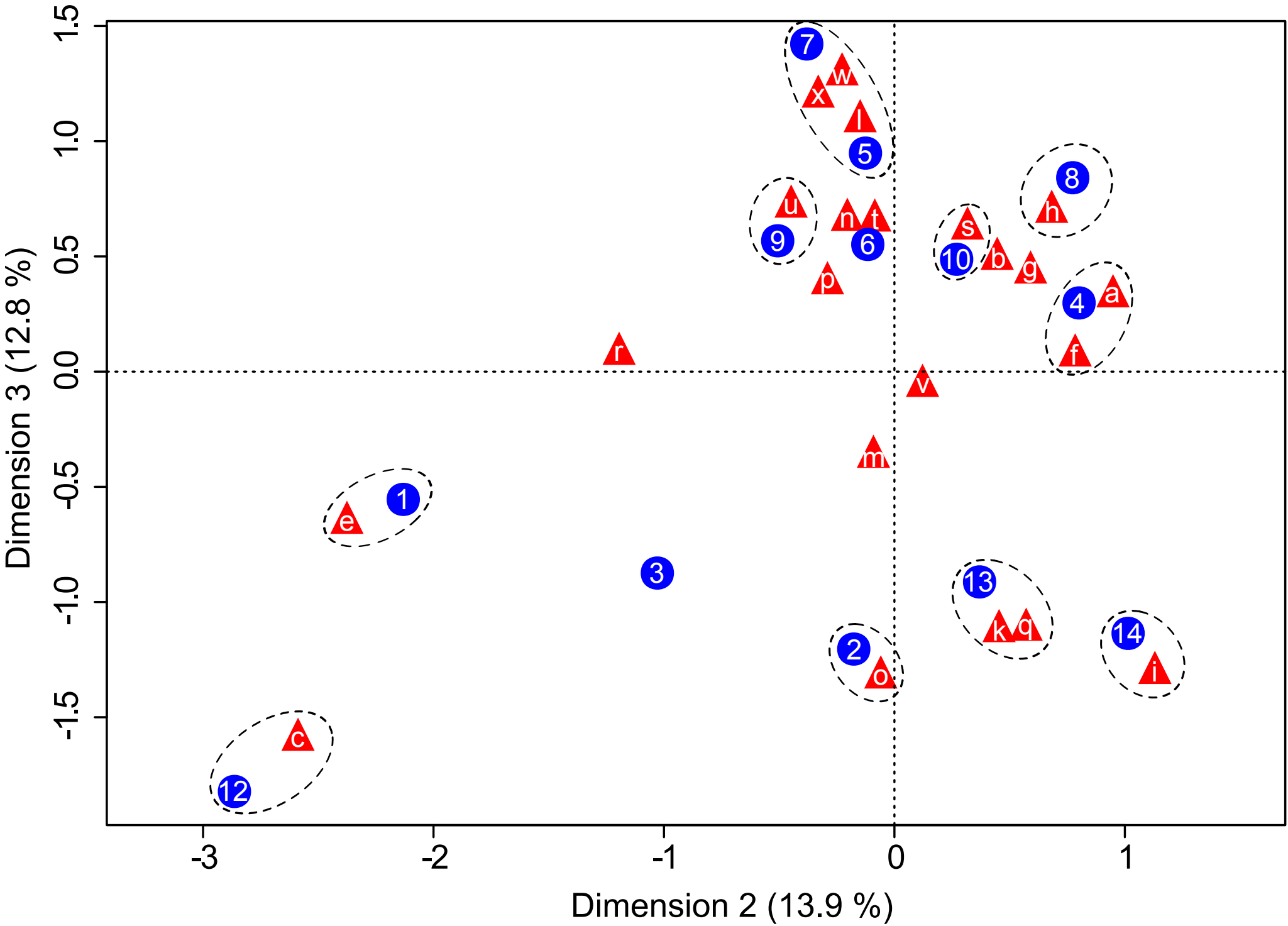
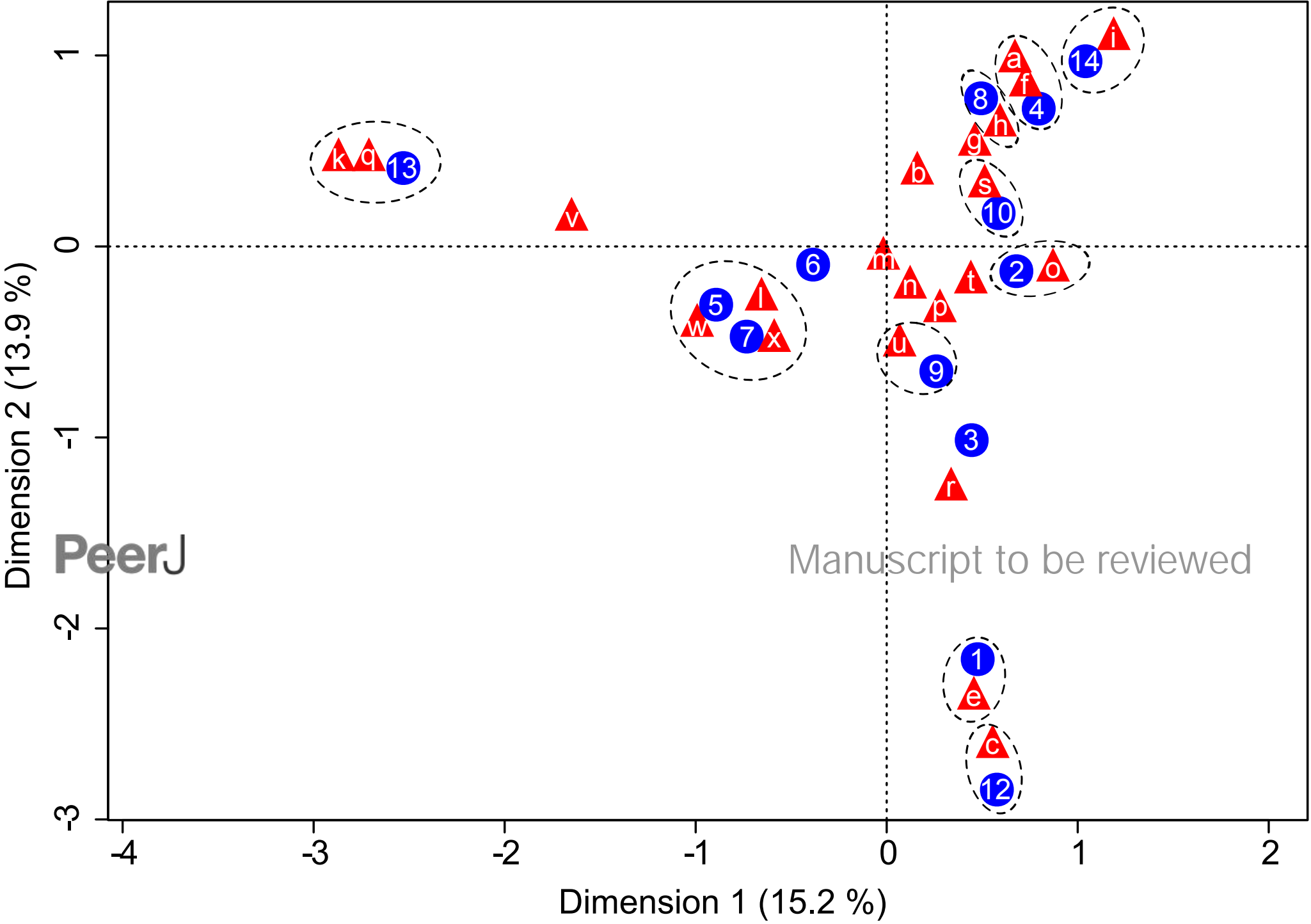
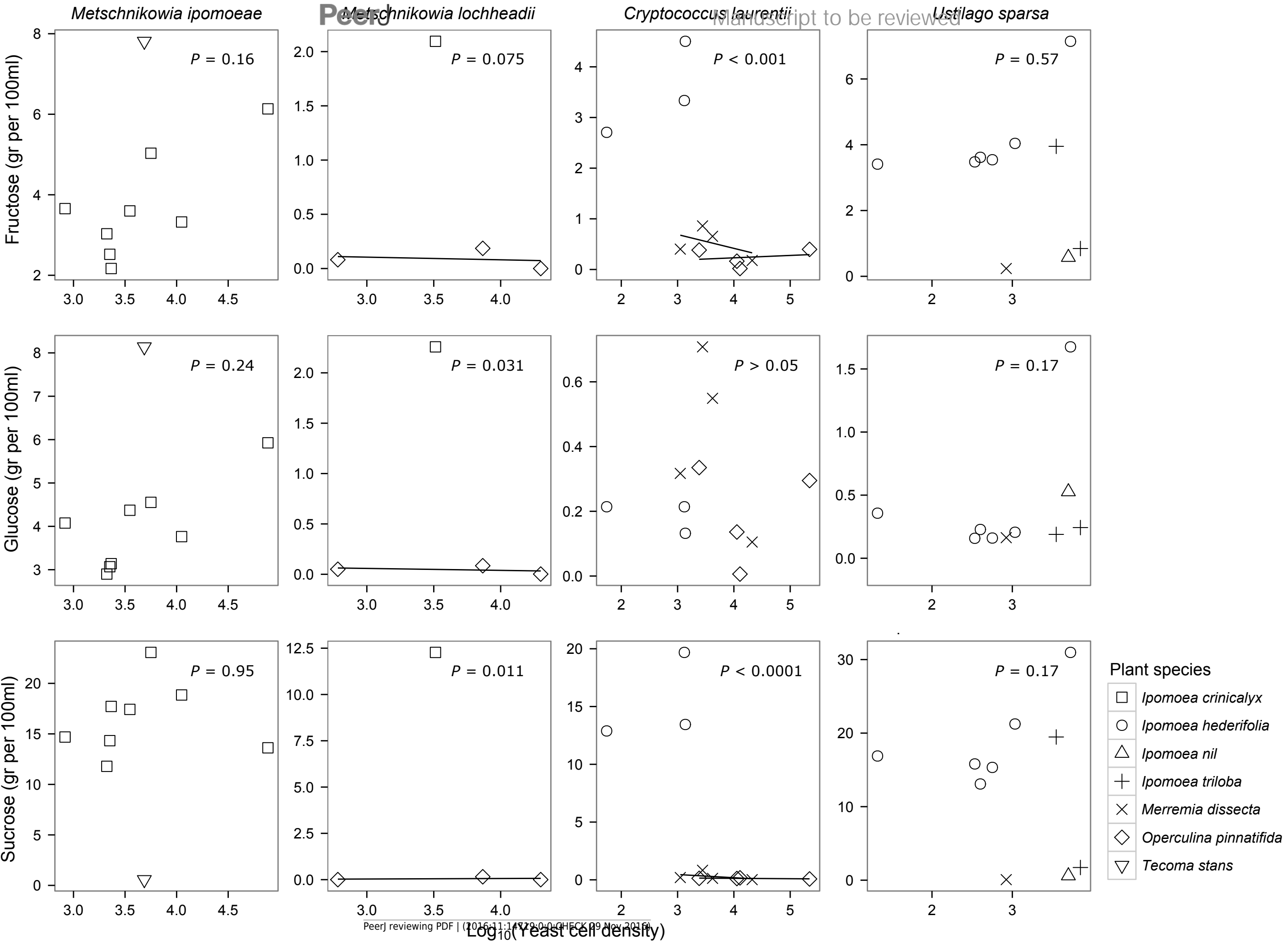
Diversity of nectar-living yeast

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 species richness (Hill number for $q = 0$), diversity in terms of equally-abundant species (Hill numbers for $q = 1$ and $q = 2$), and 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

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 member of the same yeast group or of the same plant group. Whiting dashed-line ellipses are the significant 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*.

Differential effects of nectar-living yeasts

Differential effects of four representative yeasts species (yeast cell density) on nectar sugars of floral nectar of their respective host plants. Statistical significance is shown for the p-values in each panel.

Yeasts isolated from floral nectar of tropical plants

Yeast species isolated from floral nectar in a tropical community of host-plants. Frequency of each yeast species in nectar samples is showed along with accession numbers.

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

2 each yeast species in nectar samples is showed along with accession numbers.

Host-plant (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	8	CICYRN320	KX908048
	Metschnikowia lochheadii	2	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 (Convolvulaceae)	*Saitozyma flava*	1	CICYRN280	KX908065
	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

Regression analyses for nectar-living yeasts and nectar sugars.

Regression analyses for the effects of yeast cell density (log-transformed), yeast and host plant factors, and interaction terms on nectar sugar composition.

1 **Table 2.** Regression analyses for the effects of yeast cell density (log-transformed), yeast and host plant factors, and interaction terms on nectar
2 sugar composition.

	Sucrose			Glucose			Fructose		
	F	d.f	P-value	F	d.f	P-value	F	d.f	P-value
Cell density	47.84	1, 180	< 0.0001	96.12	1, 180	< 0.0001	93.20	1, 180	< 0.0001
Yeast	4.80	35, 180	< 0.0001	2.75	35, 180	< 0.0001	2.17	35, 180	0.0005
Plant	41.86	17, 180	< 0.0001	13.03	17, 180	< 0.0001	11.56	17, 180	< 0.0001
Yeast x Plant	2.39	25, 180	< 0.0001	0.22	25, 180	1	0.36	25, 180	0.99
Cell density x Yeast	0.50	12, 180	0.90	0.41	12, 180	0.96	0.24	12, 180	0.99
Cell density x Plant	2.98	3, 180	0.0329	0.003	3, 180	0.99	0.41	3, 180	0.74