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Biodiversity of cactophilic microorganisms in western Argentina: community structure and species composition in the necroses of two sympatric cactus hosts

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ABSTRACT

The cactus-yeast-*Drosophila* system is a model system in evolutionary biology, and the participating saprotrophic microorganisms represent one of the most thoroughly studied microbial communities. However, much of the cactus-dominated regions of South America, home to endemic versions of this classical system, remain understudied. A combined morpho-physiological and molecular approach was employed to identify the fungal members of the cactus-yeast-*Drosophila* system in western Argentina. We identified twenty one species of saprotrophic organisms in the necroses of *Opuntia sulphurea* and *Trichocereus terscheckii* in a region of sympatry, where both cacti are exploited by cactophilic *Drosophila*. After excluding opportunistic isolates, we determined that the saprobe community of *O. sulphurea* was composed of eight species (including the first consideration of filamentous fungi as community members), whereas the community of *T. terscheckii* represented a subgroup of the former. We explain this nested pattern by considering the physiological and ecological attributes of both hosts and vectors involved.

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Introduction

Cacti are a central physiognomic element of most arid and semi-arid regions of the New World, dominating the plant communities of desert environments (Anderson, 2001). Their large population sizes, along with their capacity to store massive amounts of water and maintain high energy production efficiencies in arid climates (Ehleringer and Monson, 1993) has led to their establishment as key players in the ecological dynamics of neotropical and nearctic deserts (Fleming and Valiente-Banuet, 2002; Wolf & Martínez del Rio, 2003). In particular, cactus necroses represent extremely rich microhabitats in an otherwise hostile environment, hosting outstandingly diverse arthropod (Olson, 2000; Castrezana and Markov, 2001) and microbial communities (Foster and Fogleman, 1993; Lachance et al., 1988). Among this biodiversity that depends on the exploitation of decaying cactus tissues, rests the cactus-yeast-*Drosophila* system, a model for studies in evolutionary biology (Barker and Starmer, 1982), microbial ecology (Ganter, 2011) and chemical ecology (Fogleman and Danielson, 2001).

The system relies on three major interactors: (a) members of the family Cactaceae, both prickly pears (*Opuntia* spp.) and columnar cacti, whose necrotic cladodes and stems are used as substrata (and are therefore referred to as hosts); (b) the community of saprotrophic microorganisms that initiate and participate in the decomposing process; and (c) a guild of cactophilic species of the genus *Drosophila*, whose adult and larval stages feed on the decaying cactus tissues, also known as "rots", and the microorganisms present (Barker and Starmer, 1982; Starmer et al., 1991). The process is initiated when cacti are damaged or senescent, leading to the initial stages of decomposition which are thought to be dominated by bacteria (Lachance et al., 1988; Fogleman and Foster, 1989). The volatile compounds produced during the bacterial fermentation of the tissues attract certain species of arthropods, among which are cactophilic *Drosophila*. These feed and lay their eggs on the necrosis, inoculating in the process certain species of fungi (especially yeasts). These yeasts grow vigorously, chemically and physically modifying the substratum and serving as food for larvae while producing host specific volatile profiles which in turn are used by the flies as cues for suitable environments (Fogleman and Foster, 1989; Barker and Starmer, 1999; Fogleman and Danielson, 2001). The interaction between cactophilic *Drosophila* and yeasts has been described as a diffuse mutualism (Starmer et al., 1991), with yeasts benefiting through their use of flies as vectors that transport them to temporally bounded and spatially dispersed resources (Ganter, 2011).

The study of the cactophilic yeast community was pivotal in the early steps of microbial ecology as a discipline (Lachance and Starmer, 1998), and is therefore one of the most thoroughly studied microbial communities. The diversity of yeasts found in cactus necroses is quite limited, with relatively few endemic species dominating the habitat (Ganter, 2011). These species are nutritionally specialized (Lachance et al., 1988), and make up a community that is temporally persistent (Latham, 1998) and different from other sympatric yeast communities (Ganter et al., 1986; Ganter, 2011), supporting the existence of the cactophilic niche. Within this community, some yeasts are considered to be generalists and are widely distributed, others are restricted to certain host species or geographic regions (Starmer et al., 1991). Both community structure and species composition are qualities that depend on a myriad of factors, of which vector ecology and host chemistry are likely to be the most important (Ganter et al., 1986; Heed et al., 1976; Starmer et al., 1991). It is, therefore, expected that the biological attributes of both vectors and hosts have an impact on the structure of the cactophilic microbial community at a local scale, raising the value of research aiming at the characterization of regional versions of the classical cactus-yeast-*Drosophila* system.

One of the most successful clades of the genus *Drosophila* is the *repleta* species group (Throckmorton, 1975), which diversified in the arid and semi-arid regions of the New World due to their ability to colonize cactus necroses (Durando et al., 2000; Oliveira et al., 2012). Within this group, the *buzzatii* cluster is a monophyletic group of seven species found mainly in open, xerophytic regions of South America's "diagonal of dry formations" (Manfrin and Sene, 2006). Two of these species, *Drosophila buzzatii* and *D. koepferae*, occur in the Andean regions of western Argentina (Fontdevila et al., 1988; Ruiz and Wasserman, 1993). *D. buzzatii* uses necrotic cladodes of *Opuntia* spp. as primary hosts, whereas *D. koepferae* mainly exploits columnar cacti of the genera *Cereus* and *Trichocereus* (Fanara et al., 1999; Hasson et al., 2009; Soto et al., 2012). Nonetheless, a certain degree of overlap in host exploitation occurs in the vast areas where both species live in sympatry, with the two species emerging from both resources, despite maintaining their preference for their respective primary host (Hasson et al., 1992, 2009). In some of these regions of sympatry, two dominant species, the prickly pear *Opuntia sulphurea* and the columnar cactus *Trichocereus terscheckii* (cardón) are used as feeding and breeding substrata by both fly species (Soto et al., 2012). Although this oligophagous habit is not uncommon in the *D. repleta* group (Oliveira et al., 2012), most *Drosophila* species only exploit a single host at a given locality, as occurs for example in the cactus-yeast-*Drosophila* system of the Sonoran desert (Fogleman and Abril, 1990; Fogleman and Danielson, 2001). Since cactophilic yeasts disperse exclusively through the use of flies as vectors, both their biogeography and their realized niche are dependent on the ecology of their vectors (Ganter, 2011). Consequently, systems with highly specialized *Drosophila* species, that feed and breed on a single cactus host, develop differentiated cactus-specific yeast communities even in sympatry (Starmer and Fogleman, 1986; Ganter, 1988, 2011). Whether or not this pattern of isolated communities will develop in systems where there is a constant flow of vectors between different cactus species is still an open question.

Once the requisite of dispersion has been fulfilled, the second factor affecting yeast community structure is the presence of toxic host chemicals (Starmer et al., 1991). Columnar cacti are known to present more complex chemistries than prickly pears, being rich in allelochemicals such as triterpene glycosides, medium-chain fatty acids, alkaloids and sterol diols (Fogleman and Danielson, 2001). Several cases in which the cactus chemistry affects host use by both flies and

microorganisms have been reported (Starmer and Fogleman, 1986; Fogleman and Abril, 1990). With this respect, host-vector and host-microbe chemical interactions in the arid regions of South America may be analogous to those taking place in the Sonoran desert. So far, alkaloids isolated from *T. terscheckii* were shown to differentially affect fitness related traits in *D. buzzatii* and *D. koepferae* (Corio et al., 2013; Padró et al., submitted; Soto et al., 2014), and samples of *T. terscheckii* and *O. sulphurea* have been shown to differ in their relative concentrations of medium-chain fatty acids (Carreira et al., 2014; Padró and Soto, 2013). These two types of compounds are known to shape patterns of host use by cactophilic *Drosophila* and yeasts in the Sonoran desert (Fogleman and Danielson, 2001; Fogleman and Heed, 1989), however, their impact in the structure of other similar systems has been seldom considered.

The cactophilic yeast community has been studied in a wide range of geographic locations (see for example Barker et al., 1984; Starmer et al., 1987, 1990). However, many arid regions of South America, home to endemic species of cactophilic *Drosophila*, remain understudied. This is basically the case for species of the cluster *D. buzzatii*, a model for evolutionary studies (Manfrin and Sene, 2006), for which only preliminary notes on associated yeasts have been published (Moraes et al., 2005; Spencer et al., 1996). The aim of this study is to characterize the fungal community present in the decaying tissues of *T. terscheckii* and *O. sulphurea* in an area of sympatric occurrence, where both plants are used as hosts by *D. buzzatii* and *D. koepferae*. To this end, we characterize the saprotrophic yeast-like and microfungal community of both host species. Our goal was to establish the structure and composition of the microbe community in the cactus-yeast-*Drosophila* system in the arid regions of South America.

Materials and methods

The study was performed in the Valle Fértil Natural Reserve (30° 38′ 1″ S, 67° 27′ 59″ W, San Juan province, Argentina). The reserve is an area of high endemism situated within the Monte phytogeographic region (Cabrera, 1971), with warm and dry weather and 6–9 months of periodic droughts per year (Mirré, 1976). Vegetation is typical of a xeric environment, with scarce arboreal presence, mainly represented by the genus *Prosopis*, and a landscape otherwise dominated by shrubs of the family Zygophyllaceae and cacti (Roig-Juñent et al., 2001). Among the latter, the most frequent are columnar cacti of the genera *Trichocereus* and *Cereus* and two species of the genus *Opuntia*, *O. sulphurea* (the most abundant) and *O. cordobensis*.

Collections were made on Mar. 2012, Mar. 2013 and Apr. 2014. A total of 17 samples of decaying tissues of *O. sulphurea* and 15 of *T. terscheckii* were collected by aseptically cutting one sample of necrotic tissue per plant and placing it in individual sterile vials that were subsequently transported and stored under refrigerated conditions. Previous work in the area of study has shown that these two cactus species are the only ones to develop active necroses (Soto et al., 2012). All samples were collected within the natural reserve in a transect approximately 200 m wide and 2 km long in which both species were evenly interspersed. The number of samples taken per host meets the number empirically established by Lachance and Starmer (1998) as guaranteeing "an accurate reflection of community composition for cactus necroses".

Upon arrival at the laboratory, a portion of approximately 1–2 g of tissue from each sample was weighed and suspended in 20 ml of sterile saline solution. Suspensions were placed in an orbital shaker at 160 rpm for 48 hr at 28 °C, after which a fraction of the solution was serially diluted and plated in triplicate on glucose-peptone-yeast extract agar (GPY agar) with the addition of 100 mg l^{-1} of chloramphenicol (Lachance and Starmer, 1998) to inhibit bacterial growth. Plates were incubated at 28 °C for 72 hr, after which all colonies were examined and classified into morphotypes according to colonial and microscopical characteristics. For macroscopic characterization color, aspect and radial size of the colony were recorded. For microscopical characterization a piece of the colony was mounted on a slide and stained with Floxine B and calcofluor white M2R if necessary, and observed with a Carl Zeiss Axioskop microscope under white or UV light. For unicellular yeast-like and dimorphic isolates, budding pattern and size and shape of vegetative cells, or the characteristics of the hyphae, were recorded. For filamentous isolates, the morphology of the conidogenous cells, conidial ontogeny and conidia size and shape were recorded. Following the protocol of Lachance and Starmer (1998), for each plate the number of colonies of each morphotype was counted to estimate the number of CFU per mg of necrotic tissue, and one or two representative colonies per morphotype were then purified by restreaking them twice in new plates with the same medium.

To accomplish an accurate identification, approximately half the strains of each morphotype were randomly selected and characterized using a combined physiological and molecular approach. The D1/D2 domain of the large subunit (26S) rDNA was chosen as molecular marker given its optimal resolution at the level of species and the availability of a very complete database (Kurtzman, 2006). Each isolate was cultured in 25 ml of liquid GPY medium and placed in an orbital shaker for 48 hr at 28 °C. In the case of unicellular organisms (yeast and yeast-like taxa), 2–3 ml of the culture were centrifuged to obtain 50–100 mg of cells. In the case of filamentous fungi, a similar amount of mycelium was placed in an Eppendorf tube and ground using a plastic pestle. Afterwards, total genomic DNA was extracted using the kit ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, Irvine, California, USA) following the manufacturer's specifications. DNA samples were quantified by fluorometry using the Quant-iT DNA HS assay kit (Invitrogen, Carlsbad, California, USA). The approximately 600 bp long D1/D2 domain of the LSU rDNA was symmetrically amplified using the universal fungal primers NL-1 and NL-4 (O'Donnell, 1993) in a 50 μl reaction volume, containing: 5 μl of 10× PCR buffer (Applied Biosystems, Foster City, California, USA), 1.5 mM $MgCl_2$, 125 μM each of dATP, dCTP, dGTP, dTTP, 200 nM of each primer, 0.025 U/μl AmpliTaq Gold DNA polymerase (Applied Biosystems), and 10–20 ng of genomic DNA. PCR conditions, modified after Kurtzman and Robnett (1997), included an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. A final extension of 7 min at 72 °C completed the reaction. PCR products were purified using the

PureLink™ Quick PCR Purification Kit (Invitrogen). Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in both directions using the same primer combinations. Sequences were obtained on an ABI 3730xl DNA Analyzer, and subsequently analyzed using the software Sequence Scanner (Applied Biosystems). Consensus sequences were computed from the forward and reverse sequences using Vector NTI Advance 10 (Invitrogen) software package. Resulting gene sequences were deposited in and GenBank, and accession numbers are shown in Table 1.

Sequences were compared against databases using the basic local alignment search tool of BLAST software program from NCBI (BLAST, 2011). Isolates were assigned to a certain species using a cut-off for intraspecific polymorphisms of 1 % (*i.e.* approximately six non-contiguous differences with respect to the nearest type strain in a pairwise alignment) (Kurtzman and Robnett, 1998; Kurtzman, 2006). Highly similar sequences (those with high bit score) were obtained from the site and included in a phylogenetic analysis to confirm species identification. When possible, two reference sequences of each species were used in the analysis, including that of the type strain. When only a single reference sequence was available in the databases, sequences from closely related taxa were also included. Sequences were aligned with the multiple sequence alignment program ClustalW (Thompson et al., 1994) using standard parameters. Phylogenetic analysis was performed using maximum likelihood with software MEGA version 6 (Tamura et al., 2013), and branch support was calculated using 1 000 replicates of bootstrap (Felsenstein, 1985). Initially, all sequences were included in a single phylogenetic analysis. However, correct alignment of deeply divergent sequences could not be achieved, leading to the misplacement of several taxa or the collapse of deep branches. Therefore, and since this marker has been traditionally used for intraphylum phylogenies (Fell et al., 2000; Kurtzman and Robnett, 1997, 1998), sequences belonging to different phyla were aligned and analyzed separately, resulting in three different phylogenies. The nucleotide substitution model was chosen separately for each of these partitions using the model selection implementation of MEGA 6. In all three cases, TN93 + Γ was determined to be the model with the greatest goodness-of-fit according to the Bayesian Information Criterion (BIC).

Finally, the API C aux system (Biomerieux) was used to physiologically characterize the sequenced strains, by testing their ability to assimilate 19 different sources of carbon. In case subsequent tests were needed, they were prepared using standard methods (Lachance et al., 1988; Yarrow, 1998). This allowed us to further confirm species identification in the few cases where the rDNA sequence lacked the appropriate level of resolution, as well as to compare the physiological profile of our strains to those reported in bibliography for the respective type strains.

Since cactus rots are very rich microhabitats, the probability of isolating opportunistic organisms that are not members of the cactophilic microbial community as an ecological entity is high. However, such cases of opportunistic infections can be separated from endogenous cactophilic saprobes due to their low frequency of isolation and low within-rot

Table 1 – Molecular characterization of fungal isolates and their comparison to corresponding type strains

Species	GenBank accession number of sequenced strains	Percentage of identity with type strain (n° of nucleotidic differences)
Acremonium stromaticum	KP017410	99 % (4–5)
Candida sonorensis	KP017409	99 % (1)
Cryptococcus terrestris	KP017375, KP017376	100 % (0)
Dipodascus australiensis	KP017377, KP017378, KP017379, KP017380, KP017381, KP017382	99 % (1–4)
FIESC	KP017391	100 % (0)
FSSC	KP017414	100 % (0)
Fusarium lunatum	KP017368, KP017369, KP017370, KP017371, KP017372, KP017373, KP017374	99 % (1–3)
Fusarium oxysporum	KP017397, KP017398, KP017399	99 % (0–1)
Galactomyces candidum	KP017413	99 % (2)
Magnusiomyces capitatus	KP017400	99 % (1)
Magnusiomyces ingens	KP017403	98 % (6)
Magnusiomyces spicifer	KP017393, KP017394, KP017395, KP017396	99 % (0–4)
Peyronellaea prosopidis	KP017406	100 % (0)
Phoma opuntiae	KP017404, KP017405	99 % (1–3)
Pichia cactophila	KP017383, KP017384, KP017385, KP017386, KP017387, KP017388, KP017389, KP017390	99 % (3)
Prototheca zopfii	KP017367	99 % (2)
Rhodospiridium fluviale	KP017412	99 % (3)
Rhodotorula sp.	KP017402	97 % (13)
Sporopachydermia cereana 'australis'	KP017361, KP017362, KP017363, KP017364, KP017365, KP017366	99 % (1–3)
Tortispora phaffii	KP017401	99 % (3)
Yarrowia deformans	KP017407, KP017408	99 % (0–1)

FIESC = *Fusarium incarnatum-equiseti* species complex. FSSC = *Fusarium solani* species complex.

densities (Barker et al., 1987; Lachance et al., 1988; Starmer et al., 1991, 2006; Ganter, 2011). Five criteria were therefore defined in order to discriminate species that are autochthonous to the rotting cactus as habitat: (1) Dominance (*i.e.* is the species with the highest density for a given sample); (2) High frequency of recovery (>10 % of total samples); (3) High density (>30 UFC/mg of necrotic tissue); (4) Temporal persistence (repeated isolation from samples of different years) and (5) Association with cactus necroses in other areas of study. Species satisfying all or most of these criteria were considered as members of the cactophilic community.

Finally, species richness indices and rarefaction curves per host species were calculated using the software EstimateS (Colwell, 2013), using a sample-based incidence dataset. For other statistical enquiry, the software Statistica 7 (StatSoft Inc., 2001) was employed.

Results

An average of 242 and 289 colonies per plate were obtained for *Opuntia* and *Trichocereus* samples, respectively. After morphological inspection, 21 different morphotypes were established and a total of 105 fungal strains were isolated and purified. Approximately half of the representatives of each morphotype, adding up to 55 strains, were then randomly chosen for LSU rDNA sequencing and physiological profiling. All of these were successfully assigned to already described species using the rDNA sequence, except for an isolate from a *T. terscheckii* sample that was assigned to the genus *Rhodotorula*. This strain, here referred to as *Rhodotorula* sp., presents 13 nucleotide differences and a 97 % global similarity with the type strain of *Rhodotorula araucariae*, and probably belongs to an undescribed species (Table 1). Furthermore, only in two cases did species identification rely on physiological capabilities, given that LSU rDNA sequencing lacked the resolution needed for correct species assignment. This was the case for *Magnusiomyces spicifer*, which cannot be differentiated from *Saprochaete clavata* (Fig 1A; see also Kurtzman and Robnett, 1995), and *Rhodospiridium fluviale*, which clustered with *Sporidiobolus microsporus* as well (Fig 1B). Strains were eventually assigned to *M. spicifer* and *R. fluviale* due to their ability to assimilate D-xylose (Smith & Poot, 1998) and maltose (Fell et al., 1988, 1998), respectively.

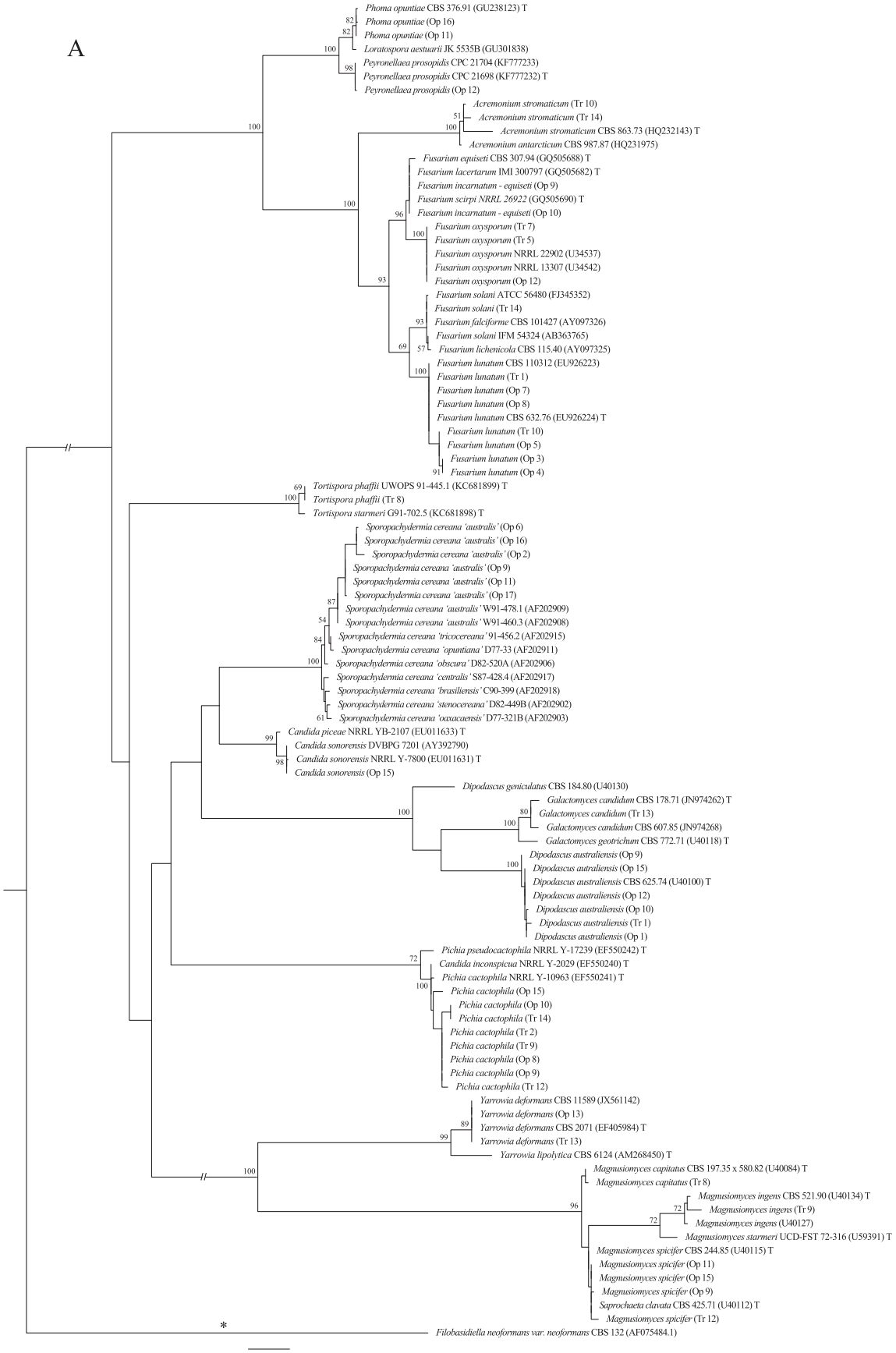
From the 21 taxa identified (Fig 1), 20 were fungi and one was a green alga of the genus *Prototheca*. Within the fungi, 17 isolates were assigned to the phylum Ascomycota and three to the Basidiomycota, a pattern of biodiversity consistent with previous surveys of the microbiota associated with rotting cacti (Lachance et al., 1988). Despite isolating a relatively high proportion of filamentous fungi, adding up to more than one third of all species, most of these were taken to represent opportunistic or accidental infections (see below for a detailed discussion), and are therefore not likely to be members of the cactophilic community. The only exception to this was *Fusarium lunatum*, which was present in more than two thirds of the sampled necroses of both *O. sulphurea* and *T. terscheckii* (Table 2), always in high densities and most of the times being the dominant organism present.

Isolates that clustered within the group identified as *F. lunatum* were the only ones to show a certain degree of phenotypic variability. These differed widely in the amount of red pigment produced, as well as in the onset of its production. Likewise, they differed in their ability to assimilate xylitol, sorbitol, N-acetyl-glucosamine and raffinose. However, the 26S rDNA sequences of these strains were very similar, and they all grouped with the sequence of the type strain of the species (Fig 1A). Otherwise, the only isolated strain that differed from the physiological profile of the species to which it was phylogenetically assigned was *Prototheca zopfii*. Despite being clearly nested within the *P. zopfii* clade (Fig 1C), strains recovered from cactus rots possessed the ability to grow on a medium containing galactose as the sole carbon source, contrary to all described species and varieties of the clade (Ueno et al., 2005). Due to this characteristic, these isolates would be assigned to *P. stagnora* in most keys of the genus (Arnold and Ahearn, 1972; Padhye et al., 1979; Pore, 1998, 1985; Ueno et al., 2005).

Eleven species of saprobes did not fulfill any of the aforementioned criteria designed to identify cactophilic microorganisms. These included *Acremonium stromaticum*, *F. oxysporum*, *Galactomyces candidum*, *Peyronellaea prosopidis*, *Phoma opuntiae*, *M. ingens*, *M. capitatus*, *R. fluviale*, *Rhodotorula* sp. and a member each of the *F. incarnatum-equiseti* (FIESC) and *F. solani* (FSSC) species complexes. All of these were only recovered from 1 to 3 samples and always at very low densities (Table 2), and were therefore not considered to be cactophilic organisms. Similarly, two species of yeasts generally considered as cactophilic, *Tortispora phaffii* and *Candida sonorensis* (Lachance and Kurtzman, 2013; Ganter, 2011), were also not considered to be active members of the community under study, given that they were recovered from only one cactus sample each.

The cactophilic community of Valle Fértil was, therefore, defined to consist of eight species of organisms: *Cryptococcus terrestris*, *Dipodascus australiensis*, *F. lunatum*, *M. spicifer*, *Pichia cactophila*, *P. zopfii*, *Sporopachydermia cereana* 'australis' and *Yarrowia deformans*. These eight species added up to 78 % of all isolates. Most of these organisms had already been described as cactophilic species (Ganter, 2011), except for *F. lunatum*, *Y. deformans* and *C. terrestris*, which represent novel members of this community. From these eight cactophilic species, *Pi. cactophila*, *F. lunatum*, *P. zopfii*, *Y. deformans*, *M. spicifer* and *Di. australiensis* were recovered from both cactus hosts, although the last two had much higher incidences in *Opuntia* samples. The remaining species, *S. cereana* and *C. terrestris*, were isolated exclusively from samples of *O. sulphurea*.

The presence of most cactophilic species was largely constant through successive samplings, with similar frequencies of isolation in samples from different years (Fig 2). The only exceptions to this were the absence of *M. spicifer* from samples of 2012, and the exclusive presence of *Y. deformans* in samples of 2014. On the contrary, almost all species considered allochthonous to cactus necroses were only found in samples corresponding to a particular year, reinforcing the hypothesis that they represent fortuitous infections. The only non-cactophilic species to be isolated from samples corresponding to successive years were *Ph. opuntiae*, an *Opuntia* endophyte, and *F. oxysporum*, an extremely common member of


A
Phoma opuntiae CBS 376.91 (GU238123) T
Phoma opuntiae (Op 16)
Phoma opuntiae (Op 11)
Loratospora aestuarii JK 5535B (GU301838)
Peyronellaea prosopidis CPC 21704 (KF777233)
Peyronellaea prosopidis CPC 21698 (KF777232) T
Peyronellaea prosopidis (Op 12)
Acremonium stromaticum (Tr 10)
Acremonium stromaticum (Tr 14)
Acremonium stromaticum CBS 863.73 (HQ232143) T
Acremonium antarcticum CBS 987.87 (HQ231975)
Fusarium equiseti CBS 307.94 (GQ505688) T
Fusarium lacertarum IMI 300797 (GQ505682) T
Fusarium incarnatum - equiseti (Op 9)
Fusarium scirpi NRRL 26922 (GQ505690) T
Fusarium incarnatum - equiseti (Op 10)
Fusarium oxysporum (Tr 7)
Fusarium oxysporum (Tr 5)
Fusarium oxysporum NRRL 22902 (U34537)
Fusarium oxysporum NRRL 13307 (U34542)
Fusarium oxysporum (Op 12)
Fusarium solani ATCC 56480 (FJ345352)
Fusarium solani (Tr 14)
Fusarium falciforme CBS 101427 (AY097326)
Fusarium solani IFM 54324 (AB363765)
Fusarium lichenicola CBS 115.40 (AY097325)
Fusarium lunatum CBS 110312 (EU926223)
Fusarium lunatum (Tr 1)
Fusarium lunatum (Op 7)
Fusarium lunatum (Op 8)
Fusarium lunatum CBS 632.76 (EU926224) T
Fusarium lunatum (Tr 10)
Fusarium lunatum (Op 5)
Fusarium lunatum (Op 3)
Fusarium lunatum (Op 4)
Tortispora phaffii UWOPS 91-445.1 (KC681899) T
Tortispora phaffii (Tr 8)
Tortispora starmeri G91-702.5 (KC681898) T
Sporopachydermia cereana 'australis' (Op 6)
Sporopachydermia cereana 'australis' (Op 16)
Sporopachydermia cereana 'australis' (Op 2)
Sporopachydermia cereana 'australis' (Op 9)
Sporopachydermia cereana 'australis' (Op 11)
Sporopachydermia cereana 'australis' (Op 17)
Sporopachydermia cereana 'australis' W91-478.1 (AF202909)
Sporopachydermia cereana 'australis' W91-460.3 (AF202908)
Sporopachydermia cereana 'tricocereana' 91-456.2 (AF202915)
Sporopachydermia cereana 'opuntiana' D77-33 (AF202911)
Sporopachydermia cereana 'obscura' D82-520A (AF202906)
Sporopachydermia cereana 'centralis' S87-428.4 (AF202917)
Sporopachydermia cereana 'brasiliensis' C90-399 (AF202918)
Sporopachydermia cereana 'stenocereana' D82-449B (AF202902)
Sporopachydermia cereana 'oaxacaensis' D77-321B (AF202903)
Candida piceae NRRL YB-2107 (EU011633) T
Candida sonorensis DVBPG 7201 (AY392790)
Candida sonorensis NRRL Y-7800 (EU011631) T
Candida sonorensis (Op 15)
Dipodascus geniculatus CBS 184.80 (U40130)
Galactomyces candidum CBS 178.71 (JN974262) T
Galactomyces candidum (Tr 13)
Galactomyces candidum CBS 607.85 (JN974268)
Galactomyces geotrichum CBS 772.71 (U40118) T
Dipodascus australiensis (Op 9)
Dipodascus autraliensis (Op 15)
Dipodascus australiensis CBS 625.74 (U40100) T
Dipodascus australiensis (Op 12)
Dipodascus australiensis (Op 10)
Dipodascus australiensis (Tr 1)
Dipodascus australiensis (Op 1)
Pichia pseudocactophila NRRL Y-17239 (EF550242) T
Candida inconspicua NRRL Y-2029 (EF550240) T
Pichia cactophila NRRL Y-10963 (EF550241) T
Pichia cactophila (Op 15)
Pichia cactophila (Op 10)
Pichia cactophila (Tr 14)
Pichia cactophila (Tr 2)
Pichia cactophila (Tr 9)
Pichia cactophila (Op 8)
Pichia cactophila (Op 9)
Pichia cactophila (Tr 12)
Yarrowia deformans CBS 11589 (JX561142)
Yarrowia deformans (Op 13)
Yarrowia deformans CBS 2071 (EF405984) T
Yarrowia deformans (Tr 13)
Yarrowia lipolytica CBS 6124 (AM268450) T
Magnusiomyces capitatus CBS 197.35 x 580.82 (U40084) T
Magnusiomyces capitatus (Tr 8)
Magnusiomyces ingens CBS 521.90 (U40134) T
Magnusiomyces ingens (Tr 9)
Magnusiomyces ingens (U40127)
Magnusiomyces starmeri UCD-FST 72-316 (U59391) T
Magnusiomyces spicifer CBS 244.85 (U40115) T
Magnusiomyces spicifer (Op 11)
Magnusiomyces spicifer (Op 15)
Magnusiomyces spicifer (Op 9)
Saprochaeta clavata CBS 425.71 (U40112) T
Magnusiomyces spicifer (Tr 12)
Filobasidiella neoformans var. neoformans CBS 132 (AF075484.1)
0.05

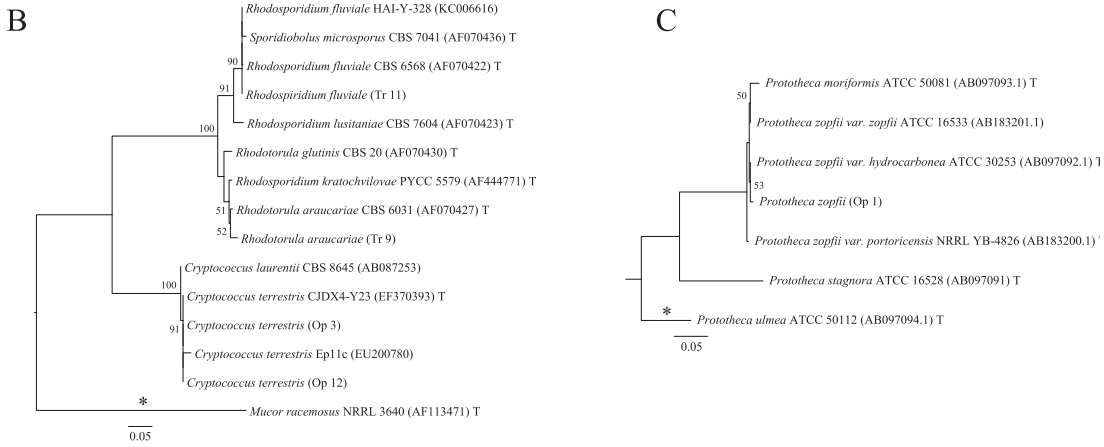


Fig 1 – Maximum likelihood phylogenetic trees built using the sequences of the D1/D2 domain of the 26S rDNA. Each tree corresponds to a different phylum: (A) Ascomycota, (B) Basidiomycota, (C) Chlorophyta. All trees were built using the TN93 + Γ substitution model and 1 000 bootstrap replications (numbers on branches). Cactus species (Op = *Opuntia sulphurea*, Tr = *Trichocereus terscheckii*) and sample number (Table 2) are annotated for all sequences obtained for this analysis (for GenBank accession numbers see Table 1). Accession numbers and strain identification codes are shown for all sequences obtained from GenBank. T = type strain. The scale bar represents the number of substitutions per nucleotide position for each tree. The out-group of each phylogeny is marked with an asterisk.

the phyllosphere that exploits niches as both phytopathogen and endophyte of an enormous array of plants (Fourie et al., 2011), including cacti (Nobel, 2002). Their repeated isolation was, therefore, expected.

Rots from both cacti showed similar levels of microbial diversity (Shannon diversity index: 2.24 vs 2.29; inverse Simpson diversity index: 7.45 vs 6.88, for *Opuntia* and *Trichocereus*, respectively), although *Opuntia* rots presented a slightly

Table 2 – Identified species of saprotrophic organisms and their pattern of occurrence in samples of both cactus hosts

Species	*Opuntia sulphurea*																*Trichocereus terscheckii*														Frequency	Mean density[a]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
F. lunatum			X	X	X		X	X	X			X	X	X		X	X	X			X	X	X	X	X		X		X	X		X	69	362.4
Pi. cactophila								X	X	X					X				X			X		X		X			X		X			31	678.5
Di. australiensis	X						X		X	X		X			X		X	X																25	100.7
S. cereana 'australis'		X				X			X		X					X	X																	19	766.0
Y. deformans													X														X			X	X	X		16	457.9
M. spicifer									X		X				X														X					13	61.4
P. zopfii		X																		X				X									X	13	56.2
C. terrestris				X				X	X			X																						13	51.8
F. oxysporum												X										X		X										9	19.3
Ph. opuntiae											X					X																		6	28.1
A. stromaticum																											X				X			6	14.3
FIESC									X	X																								6	5.6
To. phaffi																									X									3	57.9
FSSC																															X			3	26.5
G. candidum																														X				3	25.8
Pe. prosopidis												X																						3	12.4
Ca. sonorensis															X																			3	6.8
R. fluviale																												X						3	6.0
M. ingens																										X								3	4.4
M. capitatus																									X									3	2.0
Rhodotorula sp.																									X									3	0.3

The 'X' represents the presence of a microbe in a given sample. Species are ordered according to their frequency, which is calculated with respect to the total amount of samples (i.e., 32).

[a] Calculated as the mean CFU mg^{-1} of rot tissue. This was obtained by averaging the number of colonies of each species in all positive samples and correcting by dilution factors.

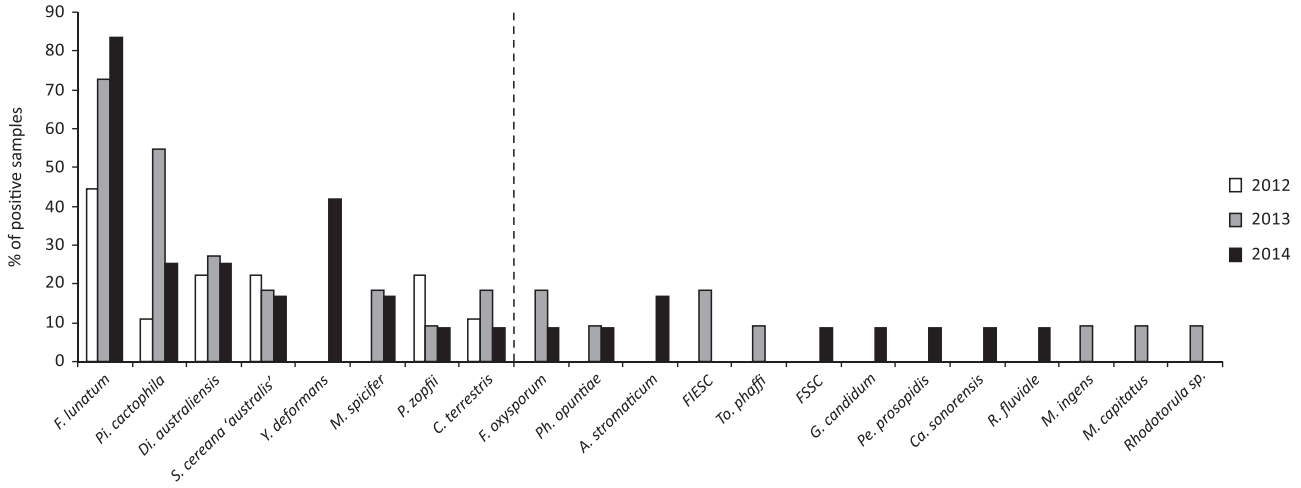


Fig 2 – Frequency of isolation of each species in the samples of three consecutive years. The dotted line separates cactophilic species (to the left) from opportunistic species (to the right). Sample size: nine for 2012, 11 for 2013 and 12 for 2014.

richer cactophilic mycobiota (*i.e.* considering only the eight species listed above), with 2.13 ± 1.13 mean cactophilic species per sample (range 1–5), compared to 1.73 ± 0.80 (range 1–3) for *Trichocereus*. Such differences were, nonetheless, not significant (one-way ANOVA, $p = 0.201$). Consistent with this poorer cactophilic diversity, *T. terscheckii* proved to be much more prone to opportunistic infections. The consequently higher incidence of species present in a single sample results in much higher values of estimated species richness for *Trichocereus* (Fig 3). Similarly, the incidence-based species richness estimators Chao2 (Chao, 1987) and ICE (Lee and Chao, 1994) wee also higher for *Trichocereus* than for *Opuntia*: 26.2 ± 10.1 vs 16.14 ± 3.91 and 33.73 vs 19.01, respectively.

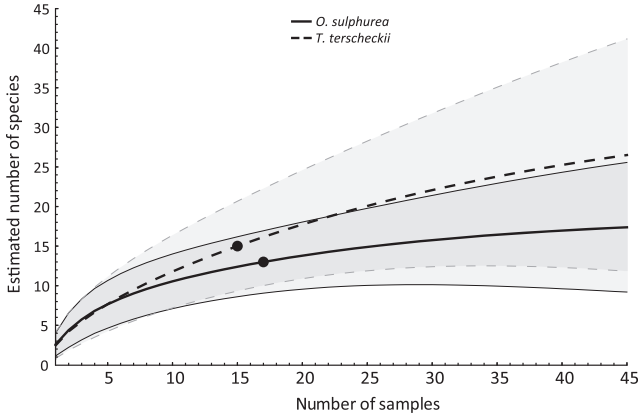


Fig 3 – Rarefaction curves for both cactus hosts, showing the estimated number of observed species with increased sampling effort. The gray shaded areas correspond to 95% CI. Curves are extrapolated using the nonparametric methods of Colwell et al. (2012), with 500 randomizations and up to three times the number of samples gathered for *T. terscheckii*. Sampling effort attained is shown with black dots.

Discussion

The cactus-yeast-*Drosophila* system has proven to be an extremely fruitful model for the development of widely diverse disciplines (Lachance and Starmer, 1998). It has also represented a very important step in our understanding of yeast biodiversity, contributing to the discovery of numerous species (Ganter et al., 2010), as well as to our understanding of microbe-host and microbe-vector interactions that shape the structure of microbial communities (Starmer, 1981; Starmer and Phaff, 1983; Ganter, 1988; Anderson et al., 2004). However, despite numerous studies aimed at characterizing local versions of the cactophilic yeast community in the southern hemisphere (Barker et al., 1984; Spencer et al., 1996; Moraes et al., 2005), few of these tried to relate species composition and diversity patterns to the peculiarities of both hosts and vectors involved in different geographical scenarios.

We recovered 21 species of saprotrophic organisms from decaying tissues of cacti in Valle Fértil, a locality situated in western Argentina. According to the criteria we established to discriminate between cactophilic and exogenous organisms, eleven species, all of which were rare and generally present at low densities, were taken to represent opportunistic or accidental infections. Moreover, these species have been associated with the exploitation of other niches, being described as phytopathogens, endophytes, soil-dwellers and fruit/tree inhabitants (*Acremonium*: Pinochet and Stover, 1980 – *Fusarium*: Fourie et al., 2011; O'Donnell et al., 2008, 2009 – *Galactomyces*: Gente et al., 2006 – *Peyronellaea*: Crous et al., 2013 – *Phoma*: Ranzoni, 1968; Suryanarayanan et al., 2009 – *Magnusiomyces*: Gadea et al., 2004; Starmer et al., 2003 – *Rhodospiridium*: Fell et al., 1988). Furthermore, the isolate considered here as *Rhodotorula* sp., a potential new member to this genus, was only recovered from one sample of *T. terscheckii* in which it was found at an extremely low density (Table 2). Despite several species of cactophilic *Rhodotorula* having been described (Ganter, 2011), the low frequency in which this isolate was

recovered does not support the claim that this species is autochthonous to cactus necroses. Likewise, two other species, *To. phaffii* and *Ca. sonorensis*, which have been considered elsewhere as cactophilic species, were recovered from only a single rot each, and in the case of the second, at a very low density. Despite the fact that these probably represent species adapted to the exploitation of rotting cacti, the evidence gathered during this study (frequency of positive rots = 0.03) does not support the hypothesis that these species play an important ecological role in the area under study. A conservative approach is favored, excluding them from the cactophilic community of Valle Fértil until more information is gathered. The fact that most of these 13 species were not recovered from samples corresponding to multiple years (Fig 2) further strengthens their exclusion from the core cactophilic community, which is characterized by temporal persistence and stability (Latham, 1998).

The saprotrophic community endemic to cactus necroses in the studied locality was, therefore, defined to consist of eight different species (Table 2). Among this biodiversity, we recovered several species that have cosmopolitan distributions and are basically generalists with respect to the host cactus that they exploit. This is the case of *P. cactophila*, *S. cereana* and *P. zopfii* (Lachance et al., 1988; Starmer et al., 2006). The first one of these species is the most common isolate of cactus necroses on a worldwide basis (Ganter, 2011), belonging to an entirely cactophilic clade (Starmer et al., 2003). Other extremely common isolates are members of the *S. cereana* complex (Ganter, 2011), which were shown to represent a cluster of closely related species that still lack formal taxonomic description (Lachance et al., 2001). From the three species of *S. cereana* reported to inhabit cactus necroses in the Argentinean territory (Lachance et al., 2001), only *S. cereana* '*australis*' was recovered in the present study. It is worth noting that this species has been isolated from columnar cacti rots in Brazil and Venezuela (Lachance et al., 2001; Rosa et al., 1994), yet it has only been recovered from *Opuntia* rots in Argentina (Lachance et al., 2001 and present study). Finally, the presence of the non-photosynthetic, yeast-like green alga *Prototheca* in necroses exploited by cactophilic *Drosophila* has been widely documented (Starmer and Heed, 1977; Starmer and Phaff, 1983; Barker et al., 1987). Many of these works have not undertaken a species-level identification of this organism (Ganter et al., 1986; Barker et al., 1987; Ganter, 1988), while others have reported the presence of the species *P. zopfii* (Starmer and Phaff, 1983; Barker et al., 1984; Starmer and Fogleman, 1986). Our phylogenetic analyses showed that the *Prototheca* strain isolated from both cactus hosts is clearly nested within the *P. zopfii* clade, clustering with the sequence of the type strain of *P. zopfii var. hydrocarbonea* (Fig 1C). However, our isolates showed the ability to grow on galactose as a sole carbon source, a character supposedly absent from the *P. zopfii* clade and, on the other hand, characteristic of *P. stagnora* (Ueno et al., 2005). Our results show that the cactophilic *Prototheca* isolated in Valle Fértil is a galactose-utilizing variety of *P. zopfii* (closely related to *P. zopfii var. hydrocarbonea*), the same conclusion at which Lachance et al. (1988) arrived for the yeast-like algae present in their areas of study.

Some of the other species that constitute the cactophilic community of Valle Fértil, such as *Di. australiensis* and *M. spicifer*, have been described as less frequent isolates, only recovered from certain host cacti or particular geographic locations (Ganter, 2011). In our survey, these two species of yeast-like fungi were recovered from both hosts, being the first time they have been isolated from a columnar cactus. However, only one *Trichocereus* rot was positive for each of these species. This difference in isolation frequency between cactus species (*Di. australiensis* was 6.2 times more likely to be isolated from *Opuntia* than from *Trichocereus*; *M. spicifer* 2.5 times), plus the fact that *Opuntia* rots are the only known habitat for *M. spicifer*, probably indicates that these organisms do not actively exploit columnar cacti.

Therefore, only three organisms are dealt here as autochthonous to cactus necroses for the first time. These are *Y. deformans*, the filamentous fungi *F. lunatum*, and the basidiomycetous yeast *C. terrestris*. The first of these species was historically considered a variety, or a synonym, of the well-studied *Y. lipolytica* (van Uden and Buckley, 1970). Only recently was the name reinstated and identified as a separate species (Bigey et al., 2003; Knutsen et al., 2007). Although there is no ecological information on this species, isolates identified as *Y. lipolytica* (or *Ca. lipolytica*) have been commonly isolated from both cacti (Barker et al., 1984) and the gut of cactophilic *Drosophila* (Shishata and Mrak, 1952), as well as other *Drosophila* associated resources in South America (Morais et al., 1995). It is possible that these isolates were in fact *Y. deformans*. On the other hand, *C. terrestris* has been recently described from isolates recovered from samples of soil in a forest in Oklahoma and the surroundings of a timber factory in Brazil (Crestani et al., 2009). Our survey is the first to indicate that this organism exploits saprotrophic niches, as well as showing its association with decaying cacti. Finally, species of the genus *Fusarium* have been traditionally described as phytopathogens, and although they certainly are among the most common causal agents of plant infections (Yli-Mattila, 2010; Fourie et al., 2011), they play a central ecological role as saprobes as well (Subramanian, 1955). *F. lunatum* has been isolated exclusively from cladodes of the genera *Opuntia* and *Gymnocalycium* (Schroers et al., 2009), and has been recently identified as one of the causal agents of the cladode spot disease in *O. ficus-indica* (Flores-Flores et al., 2013). However, our data (Table 2) demonstrate that this species is not only a phytopathogen of cacti, but it also dwells as a saprotroph in the necroses of such plants. Furthermore, this is the first time it has been isolated from columnar cacti. *F. lunatum* was recovered from more than two thirds of the sampled rots of both *O. sulphurea* and *T. terscheckii*, being the most common organism present (69 % of total samples were positive) and always attaining high densities. Beyond its mere frequency, *F. lunatum* most likely plays a crucial role in the dynamics of the system. Its aggressive form of tissue penetration (Flores-Flores et al., 2013) must affect directly the rate of liquefaction of cactus tissues, the process responsible for the generation of suitable conditions for the growth of *Drosophila* larvae (Fogleman and Danielson, 2001). Moreover, although the fermentation abilities of *F. lunatum* have never been tested, all species of the genus *Fusarium* are considered potent fermentative organisms (Ueng and Gong, 1982; Christakopoulos et al., 1989; Kurakov et al., 2011). It is, therefore, likely that this species is also central in generating the

volatile patterns that initially attract cactophilic *Drosophila* to the necrosis. Finally, the vectoring of other *Fusarium* species by *Drosophila* has also been demonstrated (Swart and Swart, 2003). Although no filamentous fungus has ever been acknowledged as being native to the cactophilic habitat, there is no good reason why yeast and yeast-like fungi should be considered as the exclusive eukaryote inhabitants of this particular environment. In a similar vein as has been expressed above, Lachance et al. (1988) proposed the inclusion of *Geotrichum* species to the cactophilic community mainly by citing their frequency in necroses, their potential as phytopathogens and their use of *Drosophila* as vectors.

Although diversity indexes rendered similar values for both hosts, the mycobiota associated with *Trichocereus* had much higher values of expected species richness, as shown by the values of Chao2 and ICE estimators. This can be attributed to the fact that these numbers (as well as the asymptotic values for the curves in Fig 3) are largely based on the probability of isolating further infrequent species. As a whole, the community of *T. terscheckii* was found to consist of fewer cactophilic species, while at the same time having a higher incidence of opportunistic and infrequent inhabitants. From the 15 species isolated from *T. terscheckii*, 60 % were found in only one sample; a value that drops to 38 % for samples of *O. sulphurea*. The consequences that this depauperate and less predictable microbiota can have for the biology of the associated *Drosophila* remains to be explored. However, despite not attaining sampling efforts that guaranteed the saturation of rarefaction curves, especially in the case of *T. terscheckii*, the cactophilic community of both resources is likely to be correctly characterized, with unobserved species representing further exogenous infections. This is validated by the repeatability of isolation of cactophilic species (Fig 2), as well as the meeting of the sampling criterion for cactus necroses established by Lachance and Starmer (1998) (it should be noted as

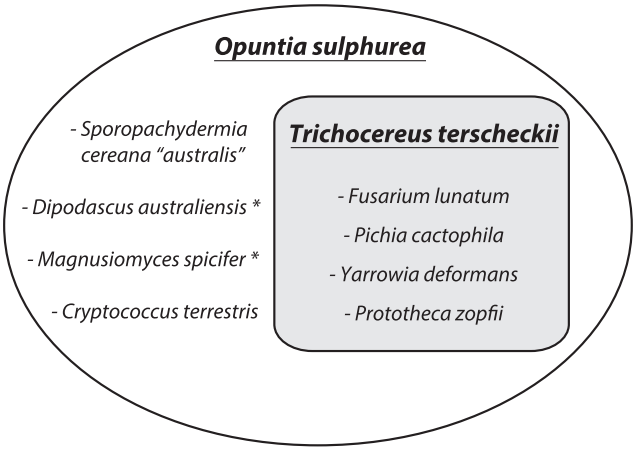


Fig 4 – Representation of the cactophilic saprotrophic community of both host plants. The community of *T. terscheckii* is completely nested within that of *O. sulphurea*. The placement of *M. spicifer* and *Di. australiensis* as exclusive inhabitants of *T. terscheckii* is highly probable yet not conclusive, and they are therefore marked with an asterisk.

well that the number of isolated species lies within the 95 % CI at saturation values for both hosts, see the black dots in Fig 3).

As already discussed, analysis of the pattern of occurrence of the eight cactophilic species in both cactus hosts under study, revealed an interesting pattern. All of the species were found in the decaying tissues of *O. sulphurea*, while only a subset of these was present in *T. terscheckii* (Fig 4). Such a pattern of nested biodiversity has not been described, to our knowledge, in any study that characterized the cactophilic communities associated with different cactus species living in sympatry. Since the presence of a microbe in a given host depends, in the first place, on the vectoring processes performed by cactophilic *Drosophila*, and secondly on the chemistry of the host cactus (Starmer et al., 1991), we propose that the particular biological characteristics of both hosts and vectors that constitute the cactus-yeast-*Drosophila* system in western Argentina are causing this peculiar pattern of nestedness. First of all, the shared use of hosts between both species of cactophilic *Drosophila* in this region is quite uncommon, and clearly differs from the characteristic specificity of other studied systems (Fellows and Heed, 1972; Heed et al., 1976). This constant flow of flies between cactus species most likely results in the homogenization of the associated mycobiotas. Secondly, the more complex chemistry of *T. terscheckii* might be involved in the selection of a limited subset of organisms capable of tolerating that particular environment. For example, *O. sulpurea* and *T. terscheckii* differ in their relative concentrations of medium-chain fatty acids (Carreira et al., 2014; Padró and Soto, 2013), compounds that are known to be inhibitors of yeast growth (Starmer, 1982; Starmer and Fogleman, 1986). Although further empirical corroboration is required, these two processes acting simultaneously may be responsible for the nested structure of cactophilic microbial diversity (Fig 4). The possibility that the absence (or low frequency) of these four saprobe species from *T. terscheckii* rots is spurious and a result of sampling bias is low, given the relatively high frequency in which, for example, *S. cereana* '*australis*' and *Di. australiensis* were found in *Opuntia* rots. This pattern is further validated by previous works, since *S. cereana* '*australis*' has been exclusively isolated from *Opuntia* samples in Argentina (Lachance et al., 2001), while *Opuntia* rots are the only known natural habitat of *M. spicifer* (de Hoog et al., 1986), as well as the most common for *Di. australiensis* (von Arx, 1997; Ganter, 2011). Furthermore, the nested pattern is even robust to whether or not *M. spicifer* and *Di. australiensis* are considered to inhabit exclusively *Opuntia* rots.

Ganter (2011) argued that the yeast's use of arthropod vectors for dispersal is the reason they managed to escape the 'everything is everywhere, the environment selects' pattern of microbial spatial distribution. The reason for this is that such a relationship results in the microorganism's biogeography resembling that of its vector. It was, therefore, expected that a system consisting of two vectors that have the ability to exploit both available hosts will result in these hosts developing highly similar microbial communities. On the other hand, several studies have pointed out that host chemistry may be more important than the activity of the vector in determining yeast community structure (Ganter et al., 1986; Starmer et al., 1980), although the covariation between host species and vector species may result in a confounding effect

on the relative importance of these two processes (Ganter et al., 1986; Starmer, 1981). The lack of strict host specificity in our site of study may effectively decouple both processes and allow for a reevaluation of their importance for determining species composition in the associated microbial communities. So far, both factors seem to play equally essential roles.

Despite the intense study that this model system has received, many of the cactus-dominated arid regions of the New World are still poorly studied. In those regions, analogous communities to that present in the Sonoran desert may provide novel insights into the ecological and evolutionary dynamics of microbe-vector and microbe-host interactions. So far, the study of the system present in western Argentina has resulted in the discovery of some unique qualities, including an important role of filamentous fungi and a nested pattern of microbial biodiversity in distinct sympatric hosts. The identification of new species of cactophilic organisms was also possible, and the necrotic tissues of cacti demonstrated they still represent a source of unknown yeast species. The characterization of the saprotrophic community of cactus necroses exploited by members of the *D. buzzatii* cluster effectively adds a new dimension to the ecology and evolutionary history of these flies, considered to be model organisms for studies in evolutionary biology.

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