**A peer-reviewed version of this preprint was published in PeerJ on 16 May 2017.**

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Brown T, Otero C, Grajales A, Rodriguez E, Rodriguez-Lanetty M. 2017. Worldwide exploration of the microbiome harbored by the cnidarian model, *Exaiptasia pallida* (Agassiz in Verrill, 1864) indicates a lack of bacterial association specificity at a lower taxonomic rank. PeerJ 5:e3235 https://doi.org/10.7717/peerj.3235

# Worldwide exploration of the microbiome harbored by the cnidarian model, *Exaiptasia pallida* indicates a lack of bacterial association specificity at a lower taxonomic rank.

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**Keywords**

Cnidaria, microbiome, host-microbe interaction, coral, sea anemone.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2338v1 | CC BY 4.0 Open Access | rec: 4 Aug 2016, publ: 4 Aug 2016

**Abstract**

Examination of host-microbe interactions in basal metazoans, such as cnidarians is of great interest from an evolutionary perspective to understand how host-microbial consortia have evolved. To address this problem, we analyzed whether the bacterial community associated with the cosmopolitan and model sea anemone *Exaiptasia pallida* shows specific patterns across worldwide populations ranging from the Caribbean Sea, and the Atlantic and Pacific oceans. By comparing sequences of the V1-V4 hypervariable regions of the bacterial 16S rRNA gene, we revealed that anemones host a complex and diverse microbial community. When examined at the phylum level, bacterial diversity and abundance associated with *E. pallida* are broadly conserved across geographic space with samples, containing largely *Proteobacteria* and *Bacteroides.* However, the species-level makeup within these phyla differs drastically across space suggesting a high-level core microbiome with local adaptation of the constituents. Indeed, no bacterial OTU was ubiquitously found in all anemones samples. We also revealed changes in the microbial community structure after rearing anemone specimens in captivity within a period of four months. These results contrast with the postulation that cnidarian hosts might actively select and maintain species-specific microbial communities that could have resulted from an intimate co-evolution process. Instead, our findings suggest that environmental settings, not host specificity seem to dictate bacterial community structure associated with this sea anemone. More than maintaining a specific composition of bacterial species some cnidarians associate with a wide range of bacterial species as long as they provide the same physiological benefits towards the maintenance of a healthy host. The examination of the previously uncharacterized bacterial community associated with the cnidarian sea anemone model *E. pallida* is the first global-scale study of its kind.



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## Introduction

Insights into the microbiome diversity of metazoan hosts have triggered a considerable interest in uncovering the regulatory principles underlying host/microbe interactions across multicellular organisms. Over the last several years, microbial symbionts living with vertebrates have been clearly shown to influence disease, physiological and developmental phenotypes in their host (Tremaroli & Backhed 2012; Blaser et al. 2013; Le Chatelier et al. 2013; Lozupone et al. 2013; Ridaura et al. 2013). In many marine invertebrates, bacteria associated with host epithelium have also been shown to play a pivotal role in host development (McFall-Ngai et al. 2013). For instance, in the bobtail squid, the bioluminescent bacteria, *Allivibrio fisheri* (Beijerinck 1889; Urbanczyk et al. 2007) are required symbionts from early host developmental stages so that a functional and healthy light organ can develop (McFall-Ngai 1994; Nyholm & McFall-Ngai 2004). Similar profound effects have been documented for more basal metazoans such as cnidarians. In the case of *Hydra viridis* (Medusozoa: Hydrozoa), induced absence of a microbial community in host polyps causes strong developmental defects and reduces asexual reproduction via budding (Rahat & Dimentman 1982). This suggests that the evolution of microbes and host interactions dates back to earlier diverging metazoan lineages (i.e. cnidarians), which has triggered an imperative interest to understand whether bacterial cores comprised of specific species have evolved in intimate association with their hosts since the early times of metazoan evolution (Bosch and Miller 2016).

Despite the simple body plans in cnidarians molecular analyses of the microbiota associated with these early-diverging organisms, predominantly corals (Anthozoa: Scleractinia), have uncovered an unprecedented bacterial diversity (Rohwer et al. 2001; Bourne & Munn 2005;



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Sunagawa et al. 2009; Rodriguez-Lanetty et al. 2013). Additionally, the species composition and structure of these microbial partnerships are complex and dynamic. The association between coral host and the consortia of these microorganisms, including bacteria, fungi, viruses and the intracellular microalgae *Symbiodinium*, has been referred to as the coral holobiont (Rohwer et al. 2001). Several studies demonstrate that certain bacterial groups associate specifically with some coral species (Rohwer et al. 2001; Morrow et al. 2012; Speck & Donachie 2012; Bayer et al. 2013; Rodriguez-Lanetty et al. 2013), implicating the effects of coevolution between coral lineages and certain bacterial strains. However, other studies have revealed that the dominant bacterial genera differ between geographically-spaced hosts of the same coral species (Klaus et al. 2007; Kvennefors et al. 2010; Littman et al. 2010) and even locally within reefs (Kvennefors et al. 2010), which suggests that environmental factors are largely responsible in shaping coral-associated microbial community diversity.

The elucidation of the mechanisms that mediate the complex interactions between microbial communities and anthozoans may be facilitated by studying a tractable model system that can be cultured and manipulated in laboratory conditions. For this purpose, the sea anemone (Actiniaria) *Exaiptasia pallida*, previously known as *Aiptasia pallida* (see Grajales & Rodriguez 2014), has been proposed as a model organism to study various aspects of the cell biology and physiology of anthozoan-*Symbiodinium* symbiosis (Weis et al. 2008) (Fig. 1). The use of this cnidarian model system has advanced our understanding of the molecular and cellular mechanism underlying anthozan/*Symbiodinium* regulation (Davy et al. 2012). Likewise, the *E. pallida* model system could benefit investigations regarding the influence of the associated microbiota on physiological, developmental, and disease-resistant host cnidarian phenotypes.



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However, we still lack baseline knowledge about the bacterial diversity and assemblages associated with this sea anemone. In the current study, we characterized the composition, structure and specificity patterns of microbial communities associated with the sea anemone *E. pallida* from worldwide populations using samples from the Caribbean Sea, and the Atlantic and the Pacific oceans. We then compared the microbial composition of natural *versus* specimens reared in the laboratory over several periods of time as means to document the effect of aquarium conditions in the composition and structure of microbial taxa.

## Material and Methods

*Sample collection, DNA extraction and V1-V4 16S rRNA pyrosequencing:* Specimens of *Exaiptasia pallida* were collected from 10 wild populations from different ocean basins worldwide (Table 1) including the Caribbean Sea; Northeastern and Western Atlantic; and Eastern, Central and Northwestern Pacific. Ethanol-preserved samples from the populations above were obtained from the invertebrate collection at the American Museum of Natural History (AMNH). Four additional groups of samples of *E. pallida* were added to the study. One group was obtained from a commercial pet store, a second group from an outdoor flow-through sea water system at the Keys Marine Laboratory (KML, Florida) and the other two were reared in the lab for different time periods: one from a six-year laboratory reared clonal population (CC7) originally obtained from a reef in the upper Florida Keys and the second from anemones more recently collected from the KML (Florida) and maintained in the lab for four months. Total DNA was extracted from the entire body of the collected sea anemone samples (3-4 per population site) using the DNeasy Plant Mini Kit DNA (Promega, Madison, WI) following the standard protocol recommended by the manufacturer.



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To assess DNA quality and lack of PCR inhibition, the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTACGACTT-3') (Jeong et al. 2014) were used to amplify a region of nearly 1500 bp following a protocol previously published from our research group (Rodriguez-Lanetty et al. 2013). Total DNA from those samples exhibiting efficient amplification of the 16S rRNA gene were then pyrosequenced at the Molecular Research LP sequencing facility (Shallowater, Texas) with the goal to examine the diversity (richness and abundance) of bacterial species associated on the surface and in the tissue of *Exaiptasia pallida*. The high throughput sequencing was done on the V1-V4 16S rRNA region (~495bp).

*Analysis of microbial community:* Barcodes, primers and short sequences (<200 bp) were removed from the raw read data using QIIME (Caporaso et al. 2010); sequences with ambiguous base calls and those with homopolymer runs exceeding 6 bp were also filtered out using QIIME. Operational taxonomic units (OTU) were defined clustering at 3% divergence (97% similarity) followed by removal of singleton sequences and chimeras (Edgar et al. 2011; Legendre & Gallagher 2001). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII [http://rdp.cme.msu.edu; (DeSantis et al. 2006)] and NCBI (www.ncbi.nlm.nih.gov).

Community similarity analysis was performed by nonmetric multidimensional scaling (nMDS) using the Bray–Curtis distance metric after Hellinger standardization (Legendre & Gallagher 2001). This analysis was conducted in the R version 3.02 package VEGAN (Core 2011; Oksanen et al. 2011). Furthermore, spatial patterns in community composition and structure were explored using hierarchical cluster analysis in PRIMER-E (Clarke & Warwick 2001). A permutation similarity profile test (SIMPROF; Clarke et al. 2008) was performed to identify clusters of



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141 samples with statistically significant internal structure ($p<0.05$).

142 **Results**

143 A high richness of bacterial species (a total of 12,585 OTU, at 3% dissimilarity cutoff) was
144 revealed to engage in association with the sea anemones across ocean basins (Supplemental
145 Table 1). The highest average bacterial OTU richness were obtained from the *E. pallida* samples
146 obtained from a commercial pet store (1671 $\pm$ 144) and the CC7 samples (1358 $\pm$ 225), which
147 were approximately four and three times higher respectively than anemones with the lowest
148 OTU richness from a natural population in Hawaii (409 $\pm$ 227).

149 Overall, the bacterial communities were dominated by *Proteobacteria* and *Bacteroides* followed
150 by the phyla *Firmicutes* and *Cyanobacteria* (Fig. 2). *Proteobacteria* dominated the communities
151 (>50%) associated with samples collected from all wild populations (Pacific and Atlantic
152 Oceans, Caribbean Sea). However, the bacterial communities associated with some anemones
153 from the Northeastern Atlantic and from an outdoor flow-through sea water system in KML
154 (Florida Keys) were dominated by *Firmicutes* (nearly 70%). Anemones collected from the KML
155 outdoor sea water system were transported and maintained in re-circulating indoor aquaria using
156 artificial seawater during a period of four months. During this short period the microbial
157 community underwent a considerable shift of bacterial phyla. *Firmicutes* decreased from ~ 70%
158 to abundances of less than 1%, and *Cyanobacteria* and *Proteobacteria* increased in relative
159 abundances, ~42% and 44% respectively. The bacterial communities associated with anemones
160 reared in the laboratory for six years (clone CC7, from an unknown Florida Keys population),
161 using Instant Ocean Water, were dominated by *Proteobacteria* (68%), with similar relative
162 abundances to those detected in many other wild populations.



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Within *Proteobacteria*, the class *Alphaproteobacteria* was most commonly dominant in anemones from the North Pacific (51%), Caribbean (53%), Atlantic (47%), four-month lab reared (58%), and six-year lab reared (78%) populations (Supplemental Fig. 1). *Gammaproteobacteria* and *Alphaproteobacteria* were equally common in the Eastern Pacific (44% and 40%) and the commercial pet store (48% and 44%) anemone populations. *Gammaproteobacteria* was found to be most common in Central Pacific anemone samples (53%). The anemones from the outdoor sea water system at the KML that were dominated by *Firmicutes* also had a very distinct group of *Proteobacteria*, represented mainly by *Deltaproteobacteria* (45%).

By examining the composition and structure of the bacterial community associated with *E. pallida* based on OTUs, we detected considerable differences among populations and geographical locations. The multivariate ordination of the bacterial communities did not exhibit clear grouping of the samples based on geographical origin (nMDS, Fig. 3). However, anemone samples reared in captivity showed less variability and each of the three captivity groups (4-month *versus* 6-year *versus* pet store) clustered in its own ordination grouping.

Hierarchical cluster analyses and similarity profile test (SIMPROF) were performed to detect bacterial community structure among the samples independent of their geographical origin. These statistical analyses revealed that very distinct microbial communities characterized most of the samples. While the 49 anemone specimens were collected from ten and four wild and captive populations respectively, SIMPROF analyses detected 32 significant bacterial assemblages (SIMPROF, $p<0.05$; Fig. 4). Out of these 32 groupings, fifteen groups were conformed by two samples and only one group was conformed by three samples. The remaining 16 samples were not clustered in any group indicating their unique bacterial assemblage. These



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clustering analyses produced similar results to the previous nMDS analysis and revealed that the associated bacterial community in wild anemones did not show geographical patterns. On the other hand, bacterial communities from captive *E. pallida* were more similar to each other. Out of 12,585 OTUs, no single bacterial OTU was shared among all anemones regardless of the geographic origin. The most prevalent OTU (OTU9148; *Vibrio tiubiashii*) was found in 75% of the samples and only 92 OTUs (less than 1% of the total discovered OTUs) were shared by one quarter of all samples (Supplemental Fig 2). Based on taxonomic classification, species within the genera *Vibrio*, *Nautella*, *Ruegeria*, *Marinobacter*, *Lentisphaera*, and *Flaviobacterium* were common representatives within the microbial community associated with *E. pallida*.

## Discussion

This study revealed the previously uncharacterized bacterial community associated with the cnidarian sea anemone model *E. pallida* at different locations throughout the Northern Hemisphere. This examination of anemone/bacterial association is the first global-scale study of its kind. The results show that a complex and diverse microbial community colonizes the anemone and varies considerably both among and within sampling locations. This indicates a lack of a bacterial core community, which is comprised of specific species that have evolved in intimate association with this cnidarian host.

When examined at the phylum level, bacterial diversity and abundance associated with the cosmopolitan sea anemone *E. pallida* are broadly conserved across geographic space with samples, containing largely *Proteobacteria* and *Bacteroides.* However, the species-level makeup within these phyla differs drastically across space suggesting a high-level core microbiome with local adaptation of the constituents. There was no a single bacterial OTU ubiquitously found in



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all anemones samples. This finding differs from the postulation, based on a study conducted in the class Hydrozoa, that cnidarian hosts actively select, regardless of environmental conditions, and maintain species-specific microbial communities (*sensu* Fraune & Bosch 2007). Our results indicate that differences in global and local environmental factors might play important roles sorting the composition of bacterial species that associates with the actiniarian *E. pallida*. This paradigm is supported by a recent study which also demonstrated that environmental parameters such as salinity, dissolved oxygen, and ammonium are key drivers in the regulation of the composition and structure of bacterial communities associated with scleractinian corals (Lee et al. 2012). Moreover, changes in the microbial community structure were revealed after rearing specimens of *E. pallida* in captivity within a period of just four months, supporting the idea that differences in aquatic environments have a strong effect on shaping associated bacterial assemblages at the species level.

Unlike hydrozoans, our findings suggest a lack of coevolution between a sister lineage within cnidaria (Anthozoa: Actiniaria) and specific bacteria. Within anthozoans, most of the studies exploring the bacterial diversity via culture-independent approaches have been done within the subclass Hexacorallia, more specifically within the order Scleractinia (i.e. stony corals). In this group a number of coral-associated microbial exploratory studies have shown that corals harbor some of the most highly diverse and abundant microbial communities in marine invertebrates after Porifera (Sunagawa et al. 2009; Mouchka et al. 2010; Bourne & Webster 2013; Rodriguez-Lanetty et al. 2013). Evidence supporting a clear co-evolution or co-diversification pattern between prokaryotes and corals is however absent. While some studies have shown species-specific patterns of bacteria/host associations (Littman et al. 2009; Sunagawa et al. 2010), recent



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studies using high throughput 16S r RNA gene sequencing have shown that microbial communities associated with scleractinian corals are not species specific (Hester et al. 2016; Meistertzheim et al. 2016; Zhang et al. 2015) and are controlled primarily by external environmental conditions rather than the coral holobiont (Pantos et al. 2015). Although there may be little support of co-evolutionary patterns, it seems that some core bacteria groups might have broadly specialized to associate with scleractinian corals regardless the host species lineage (Ainsworth et al. 2015).

Based on our findings, we propose that more than maintaining a specific species composition of bacteria, *E. pallida*, and perhaps many other anthozoans, associate with a wide range of bacterial species as long as they provide the same physiological benefits towards the maintenance of a healthy host. To certain extent this explanation is supported by the fact that at higher taxonomic level we detected more similarities across populations in the host-associated microbial structure. The particular bacterial assemblage that may engage in symbiosis with the anemone host will then depend on the existing pool of bacterial species filtered by the environmental conditions of the host habitat, provided that these bacteria belong to a preferred bacterial group with similar ecological functions. It is interesting to note that current global distribution of *E. pallida* seems the result of recent invasion events, based on the lack of host population genetic structure (Grajales & Rodríguez 2016; Thornhill et al. 2013), and yet we were able to detect a complete turnover of the bacterial community at the species level associated with this invasive host anemone across global scale. Our study highlights the importance of the interplay between host, microbial symbiont diversity (both functionally and phylogenetically) and the environment to delineate the patterns of host/microbial symbiont associations.



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**Acknowledgments**

We would like to thank Ms. Cindy Lewis for collecting sea anemones in the Florida Reef Tract and Dr. John Pringle for providing the CC7 *Exaiptasia pallida* clonal line used in this study. We are grateful to the member of the IMaGeS Lab, Mr. Daniel Merselis, Dr. Anthony Bellantuono, Ms. Katherine Dougan, Ms. Cindy Lewis, and Ms. Ellen Dow for their comments during the writing of early draft versions of this manuscript. This research was funded by NSF-IOS (1453519) grant awarded to MRL; and partially supported by the Lerner-Gray Fund for Marine Research (AMNH) and a NSF Doctoral Dissertation Improvement Grant (NSF DEB 1110754) to AG and ER.

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**Figure Legends:**

Figure 1: *Exaiptasia pallida*, the sea anemone species used in the host-associated microbial community study (Photograph taken by Tanya Brown).

Figure 2: Prevalence and distribution of bacterial phyla identified in the microbial community associated with the anemone *Exaiptasia pallida* across all natural population sites, including the lab reared populations.

Figure 3: Nonmetric multidimensional scaling (nMDS) ordination using V1-V4 16S rDNA OTUs (derived from high throughput 454 sequencing) of the microbial community associated with *Exaiptasia pallida* from all collected natural and lab reared populations. nMDS are based on Bray– Curtis dissimilarity distance after Hellinger transformation and Kruskal's stress is 0.206. Richness of OTUs per anemone sample in each population site is proportional to the size of the data sample point on the graph.

Figure 4: Hierarchical clustering dendogram of bacterial communities associated with *Exaiptasia pallida* specimens from all wild and captive populations. Solid lines indicate significant branches (SIMPROF, $p<0.05$) while dashed lines are unsupported. Colors of samples indicate geographical sampling location: Blue – Caribbean, Purple – North Atlantic, Green – Pacific, Pink – Keys Marine Lab, Yellow – Commercial Pet Store, Orange – 4 Month Captive, Red - 6 Year Clonal Captive. The numbers indicated below the sample names display the significant SIMPROF groupings.



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448 Supplemental Figure 1: Prevalence and distribution of bacterial classes within the phylum
449 Proteobacteria identified in the microbial community associated with the anemone *Exaiptasia*
450 *pallida* across all population sites.

451 Supplemental Figure 2: Frequency of V1-V2 16S rDNA OTUs shared among samples collected
452 in the study. Inset shows a close-up section of the main graph displaying the few OTUs shared
453 from 79% to 40% of the analyzed samples.

454 Table 1: Location of population sites and sampling information within site

455 Supplemental Table 1: Average number and standard deviation of OTUs found in all samples by
456 population location.

457

458



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Table 1:

| Location ID | Location | Samples (N) | Latitude | Longitude |
|---|---|---|---|---|
| Morelos | Puerto Morelos, Mexico (Caribbean) | 3 | N 20 50 18.57 | W 86 53 02.86 |
| Baja-Sur | Pichilingue, Baja California Sur, Mexico (Pacific) | 4 | N 24 15 46.46 | W 110 36 52.12 |
| Sesoko | Sesoko Island, Okinawa, Japan (Pacific) | 4 | N 26 38 10.70 | E 127 51 55.03 |
| FerryR | Ferry Reach, Bermuda (Atlantic) | 4 | N 32 22 01.51 | W 64 39 36.22 |
| Oahu | Oahu Waikiki, Hawaii, USA (Pacific) | 3 | N 21 16 40.32 | W 157 50 01.04 |
| Florida | Florida Keys National Marine Sanctuary, USA (Atlantic) | 4 | N 25 03 61.03 | W 80 25 38.02 |
| Carenera | Carenera Island, Bocas del Toro, Panama (Caribbean) | 4 | N 09 20 50.34 | W 82 15 18.67 |
| Achotines | Achotines lab, Pedasi, Panama (Pacific) | 4 | N 07 25 50.46 | W 80 11 36.24 |
| Madeira | Madeira Island, Portugal (Atlantic) | 4 | N 32 42 52.84 | W 16 45 47.85 |
| Canaria | Las Palmas Island, Gran Canaria, Spain (Atlantic) | 3 | N 28 19 09.18 | W 15 25 56.89 |
| KML | Outdoor flow-through aquariums at Key Marine Lab, Long Key, Florida, USA | 3 | N 24° 49.567 | W 80° 48.884 |
| Shortlab | Anemones from KML brought to laboratory captivity for 4 Months | 4 | N 24° 49.567 | W 80° 48.884 |
| CC7 | Clone CC7 in laboratory captivity for 6 Years | 3 | Unknown | |

| | | | |
|---|---|---|---|
| Petstore | Unknown Collection Site | 2 | Unknown |

Figure 1

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Figure 2

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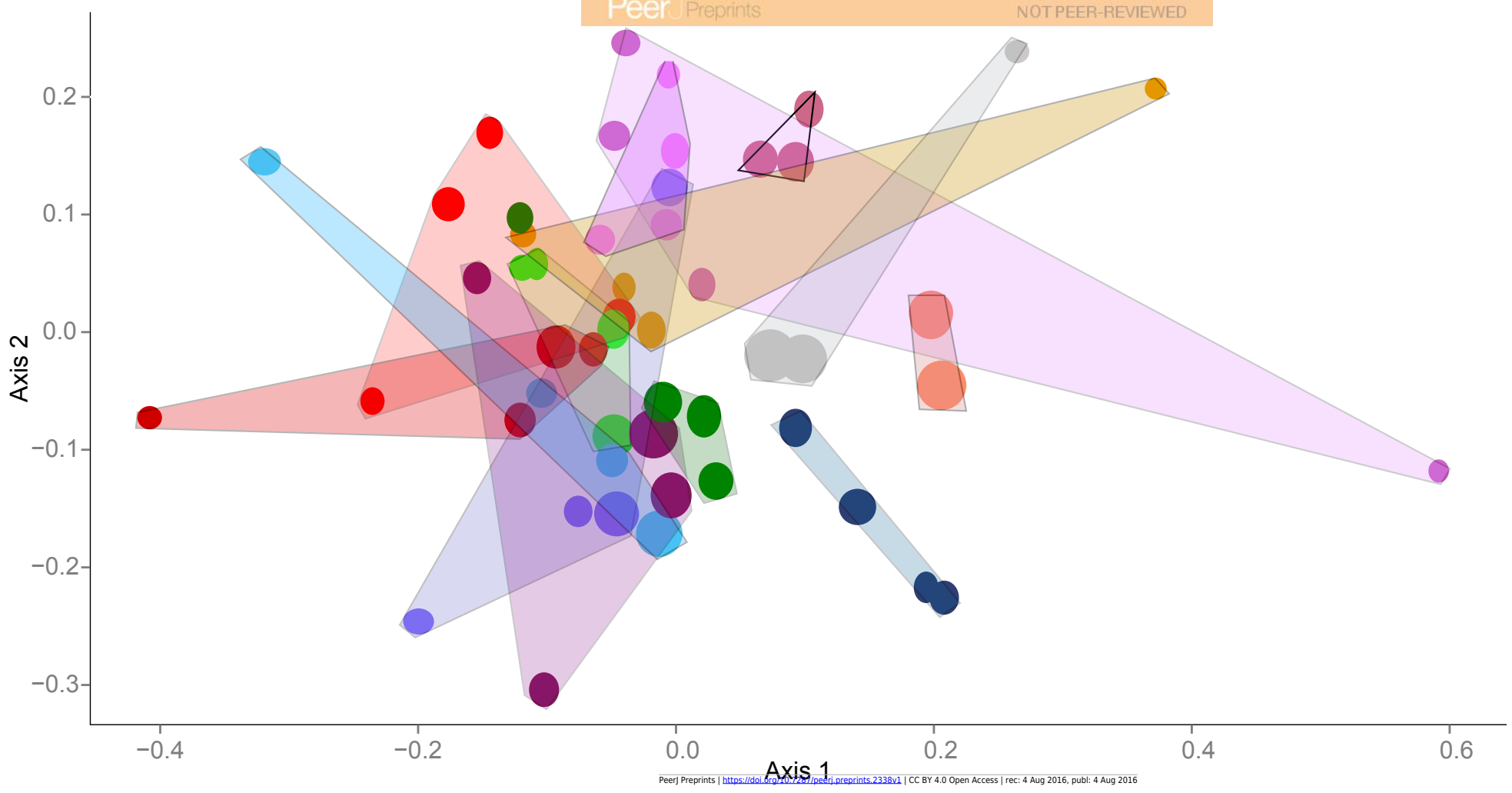


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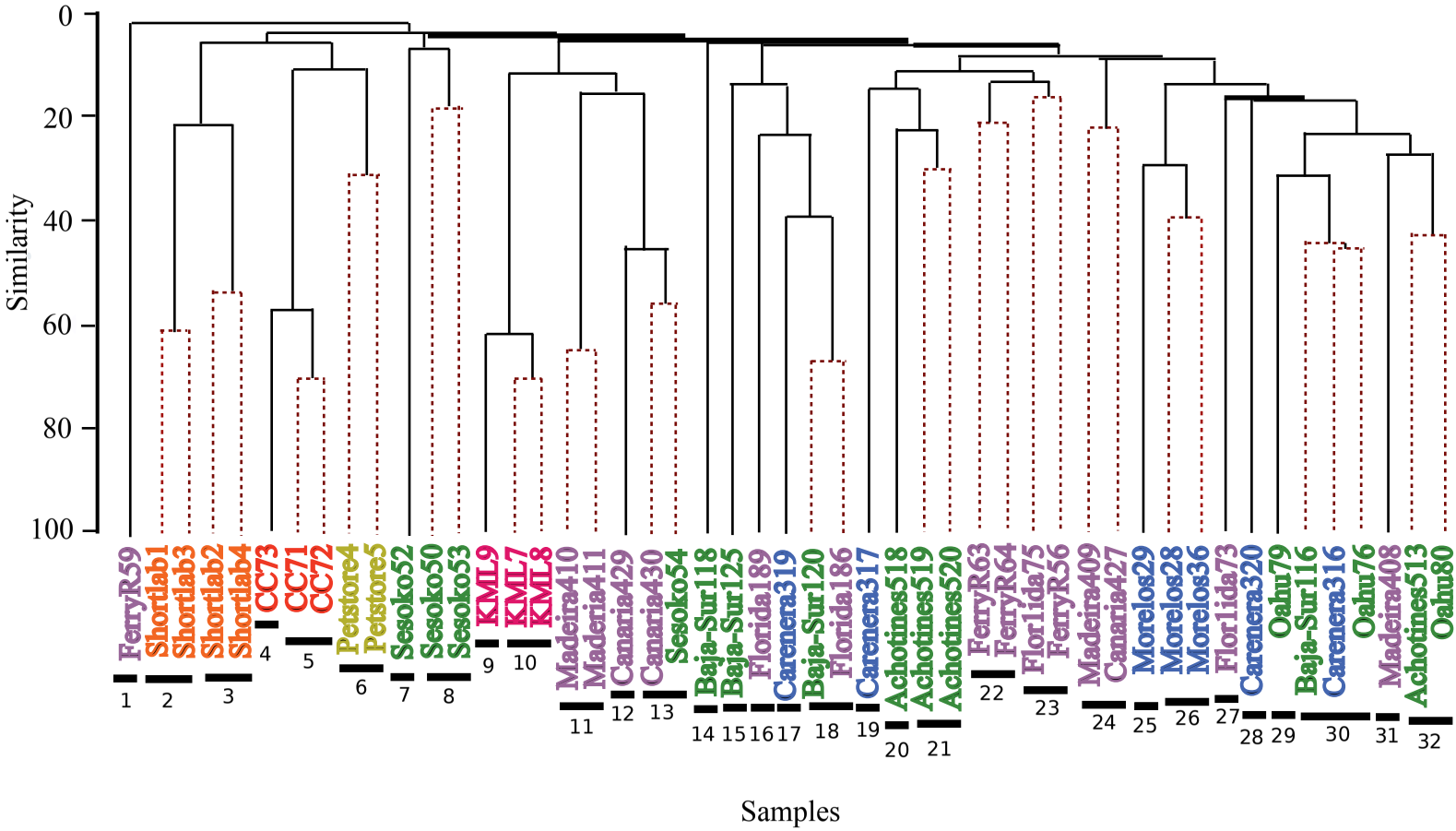


Figure 4