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The upper respiratory tract as a microbial source for pulmonary infections in Cystic Fibrosis: Parallels from Island Biogeography

Katrine L. Whiteson[1], Barbara Bailey[2], Megan Bergkessel[3], Douglas Conrad[4], Laurence Delhaes[5], Ben Felts[6], J. Kirk Harris[7], Ryan Hunter[8], Yan Wei Lim[1], Heather Maughan[9], Robert Quinn[1], Peter Salamon[6], James Sullivan[10], Brandie D. Wagner[11], and Paul B. Rainey[12]

[1]Department of Biology, San Diego State University; [2]Department of Statistics, San Diego State University, [3]Department of Geological and Planetary Sciences, California Institute of Technology; [4]Department of Medicine, UC San Diego, [5]Department of Medicine, Pasteur Institute of Lille; [6]Department of Mathematics, San Diego State University; [7]Department of Pediatrics, University of Colorado Denver, [8]Department of Microbiology, University of Minnesota Medical School; [9]Ronin Institute; [10]Vertex Pharmaceuticals; [11]Biostatistics and Informatics, University of Colorado Denver; [12]New Zealand Institute for Advanced Study, Massey University and Max Planck Institute for Evolutionary Biology.

Corresponding Author:
Katrine L. Whiteson
San Diego State University - Biology
5500 Campinile Dr. San Diego California 92182
United States
T: 949-241-734
katrinewhiteson@gmail.com

"When we try to pick out anything by itself, we find it hitched to everything else in the Universe."
-- John Muir

Abstract

A continuously mixed series of microbial communities inhabits various points of the respiratory tract, with community composition dictated by distance from colonization sources, colonization rates, and extinction rates. Ecology and evolution theory developed in the context of biogeography is relevant to clinical microbiology and could reframe the interpretation of recent studies comparing communities from lung explant samples, sputum samples and oropharyngeal swabs. We propose an Island Biogeography model of the microbial communities inhabiting different niches in human airways. Island biogeography as applied to communities separated by time and space is a useful parallel for exploring microbial colonization of healthy and diseased lungs, with the potential to inform our understanding of microbial community dynamics and the relevance of microbes detected in different sample types. In this perspective, we focus on the inter---mixed microbial communities inhabiting different regions of the airways of patients with cystic fibrosis.

Introduction

Individual humans, and their organs and tissues, can be considered islands. Like the islands of the Galapagos, humans constitute spatially structured environments that offer microbes abundant ecological opportunity (Figure 1). The Human Microbiome Project (http://www.hmpdacc.org/) has shown that different organs and systems within the human body are inhabited by different species assemblages (1). Just as the islands of an archipelago are exposed to a reservoir of potential emigrants from the mainland, individual human organ systems are exposed to potential migrants from the surrounding milieu. Irrespective of specific habitat details, community assembly and stability is driven by dispersal rates and priority effects, with the order and timing of arrival influencing the fate of particular species (2, 3).

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The diversity of species inhabiting the islands of an archipelago depends on how close each island is to a mainland (from which colonizing species are derived), and the rate that species go extinct (Figure 2). The theory of island biogeography, born in the 1960s (4), includes islands of many different sorts: islands as mountain tops, lakes, trees, and in fact any set of niches separated by time, space or an environmental barrier. While studies of island biogeography initially focused on ecology, there has long been awareness that patterns of species diversity cannot be fully understood outside the evolutionary processes that fuel diversification (5). Indeed, incorporation of evolutionary thinking into ecology has underpinned advances in understanding adaptive radiation, speciation, and opportunities for ecosystem restoration.

In extreme cases, for example following a forest fire, the process of community assembly is given special prominence (6). However the same processes that lead to re---establishment of a forest on barren land may also govern the formation of host---associated microbial communities. Community assembly has important implications for health and disease (2, 3, 7). For example, the capacity for a pathogen to establish itself in a given niche is likely to depend upon the presence (or absence) of competing microbes (8---12), the timing of arrival and the history of colonization events. Establishment will also be affected by host factors including the immune system, the availability and quality of nutrients (their spatial and temporal distribution), and the physical structure and properties of tissues and surfaces.

Island Culture: distinct communities intermingle in the oropharynx---airway---lung ecosystem. As islands that provide ecological opportunity to microbes, the unique environment of the lung in diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disorder (COPD) and other inflammatory respiratory conditions are of special interest. Microbes immigrating to the lung come from various sources including air, water and food, but also from the oral cavity and other nearby islands such as the sinuses and even the GI tract. In the model proposed here, the oral cavity might be considered as the mainland, or the largest, most diverse proximal island, and various niches in the respiratory tract as islands (Figure 2). Like modern ecologists who find exceptions to the original Island Biogeography theory of the 1960s, it is necessary to acknowledge the influence of factors beyond the size and remoteness of island reservoirs on community diversity (as indicated by species richness) (13). In CF patients, the immune system, interspecies interactions and antibiotic pressure exert profound additional influences on microbial community structure.

Microbes traditionally inhabiting the oral cavity that are also capable of colonizing the lower airways, are likely to be an important source of *Attack* community microbes in lungs, as presented in the Climax---Attack---Model (CAM) (14). CAM postulates that there are two major microbial or viral communities inhabiting the CF lung: a well---adapted, persistent *Climax* community and an *Attack* community comprised of more virulent and transient microbes and viruses. While the oral cavity hosts a large diversity of microbes, the most abundant species found deep in explanted lung samples from patients with end---stage disease are usually from a small group of known CF pathogens, including *Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Staphylococcus aureus,* and *Burkholderia cenocepacia* (15). Furthermore, well---studied clinical isolates of *P. aeruginosa* often

harbor a subset of common mutations that reveal their adaptation to the CF lung environment (16---18). Recent evidence suggests a similar trend in *Rothia mucilaginosa*, a bacterium traditionally considered to be part of the oral microbial community (19).

Oral microbes that do not normally inhabit the lungs are likely to significantly impact lung pathophysiology through the interconnected oropharynx---airway---lung ecosystem. Even if those oral microbes remain in their endemic location (the oral cavity), they may still impact populations in the lung by producing metabolites that passively travel to the lungs (20---22), or by stimulating systemic host responses that could directly impact the deep airway microbial community. Additionally, given the potential for these oral microbes to emigrate to the lungs across the continuum of the respiratory system, these same microbes may be important competitors and thus regulators of lung---invading microbes. Bacteriophage and other agents of gene exchange may also transfer DNA encoded traits, *e.g.*, antibiotic resistance (23, 24). Microbes within the oral cavity, and their migration from that source, are therefore likely to be important contributors to lung disease. It is critical that all such sources are identified and studied so that their connection with the CF lung can be understood.

Given the potential for mainland microbes to directly and indirectly affect lung communities, we argue that to understand the evolution of polymicrobial lung communities, and their related patho---physiologies, ecological dynamics must be acknowledged both within resident lung microbial communities along with those in neighboring niches.

Some have argued that oral commensals detected in sputum samples used to interrogate lung microbial communities represent contamination from the oral cavity (15, 25, 26). However, the degree to which oral microbes may emigrate and persist in lower airways is likely to vary from one patient to the next, depending upon the presence of specific microbes as well as a variety of other environmental and host factors. Thus, tracking all airway microbes is important because oral microbes need not frequently form resident communities in the lower airways to profoundly influence polymicrobial community physiology.

A bridge to the lung: migration across the trachea. The physical continuum of the trachea connects the oral cavity and the lungs. Indeed, lung samples from healthy humans are not sterile, and they occasionally contain microbes traditionally associated with the oral cavity (27---30). The density of bacteria in the oral cavity is orders of magnitude greater than the healthy lung (27). The composition and even the existence of microbial communities in healthy human lungs is an active area of research. Whether the upper and lower respiratory tracts of healthy people contain “tourists” which are quickly expelled, or whether there exist resident microbial communities is an important question (27, 29, 31---33). Either way, differences in commensal and pathogenic microbial load in the airways of healthy humans and those affected by various respiratory conditions are likely to be affected by the rate at which new types enter the system and the rate at which they fail to colonize, or go extinct.

Healthy and diseased lungs are equally accessible to microbial migrants – they both contain a bridge across the trachea, and significantly, migration may not always result in persistent colonization.

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Healthy lungs have coordinated mucociliary clearance (34) which forces microbes out, and this limits the time for adaptation and establishment. This means the extinction rates of microbes that reach the lower airways are much higher in healthy lungs; impaired mucociliary clearance will reduce extinction rates and lead to larger numbers of species colonizing the lower airways (Figure 2). Emigration of oral microbes to the lower airways due to aspiration is important in a number of respiratory conditions, where a large fraction of infections are thought to have oral microbial etiology (35---39). Respiratory conditions associated with decreased extinction of microbes that reach the lower airways include smoking (31), vaccination and probiotics (40, 41), respiratory virus infection (42---44), pneumonia (36), HIV (45, 46), COPD (30, 47---49) and CF (50). In addition, improvements in oral hygiene practices in hospitals have been shown to prevent pneumonia (51). In newly transplanted lungs of CF patients, strains of bacteria found in the sinuses are later found in the newly transplanted lungs, again suggesting that microbes in the sinuses have a route to the lower respiratory tract (52).

In CF, the epithelia lining the airways are covered by abnormally dense mucus, trapping the cilia and rendering them nonfunctional (53). In addition, lung---specific immune responses are impaired in CF, including dysfunctional alveolar macrophages and autophagy (54---56). Together these defects allow the commonly observed opportunists, such as *P. aeruginosa* and *B. cenocepacia*, to persist in CF airways. Most patients harbor unique and persistent microbial communities, suggesting differences from patient to patient in exposure to microbial immigrants, or selective pressures, or both. While the well---known opportunistic pathogens are clearly important, they do not exist in isolation; interactions with other community members have important implications (11, 57---59). Indeed, there are increasing examples of infections driven by an altered polymicrobial community, rather than a single pathogen, and these will likely influence how clinicians diagnose and treat infections (59, 60). Pneumonia, COPD, CF, chronic sinusitis, periodontitis and otitis media are examples of infections where a commensal from one human site can emigrate to become a pathogen in another context (36, 51, 61---66). Like drifting seeds from a proximal island, exposure and survival rates of microbes from nearby habitats stands to profoundly influence microbial community composition and interactions.

Spoiled sputum? As sampling of the lungs of a living human generally requires passage through the oral cavity, the question of how frequently and how deeply oral microbes penetrate into the lower airways is unresolved. Invasive lower airway sampling methods such as bronchoalveolar lavage (BAL) enable careful sampling of a specific region of the lung with reduced potential for contamination, but these methods cannot be performed on a regular basis because of negative impacts on patients. Sputum and BAL samples are also limited in the extent to which they provide insight into spatial distribution of microbes within the airways. Currently, when typical CF pathogens are not found in CF sputum cultures, clinical microbiology labs report them as culture negative (67), essentially disregarding oral microbes as contaminants without clinical relevance. Given the interconnected topography of the oral cavity and the airways, it would not be surprising to learn that some microbial groups are found in both the oral and lung environments. Direct evidence that organisms considered oral contaminants are present in the large airway exists from early culture studies based on trans---tracheal aspirates where mixing with oral microbiota is avoided (68). In addition, next---generation sequencing has often detected surprisingly high relative abundances of species such as *Streptococcus* spp., *R. mucilaginosa*, or *Gemella* spp. in large volumes of purulent sputum (mls to tens of mls) (11,

19, 69). Overall, while there are limitations associated with sputum samples, the fact that they can be obtained by non---invasive means, and that they show patterns of diversity similar to lower and upper airways (8, 15, 16, 34), suggest it is sensible to obtain as much information from sputum samples as possible.

To directly assess lung microbial communities, multiple studies have examined the microbes present in explant lung samples. These samples carry their own caveat -- they are most often obtained from patients with end---stage disease, which have been shown to contain significant regional differences in community composition, and severely reduced diversity (30, 70, 71). In an attempt to reconcile the different caveats of sputum and explant lung samples in CF, a recent study compared throat, sputum and explant lung samples (average of all lobar bronchi) from the same CF patients, close to the time of transplant surgery (15). This study concluded that next---generation sequencing of DNA in sputum samples inaccurately represents airway microbial communities, and that oral microbes detected by this method should be regarded as contaminants.

We present a complementary analysis of the Goddard et al. 2012 data. Whereas Goddard et al present the relative abundance of taxa as bar plots, we took the relative abundance data from their supplementary material and visualize an unsupervised random forest in a multidimensional scaling plot. The barplots in Goddard et al. and our analysis both show that the overall microbial community composition of sputum samples closely resembles that of the explanted lung samples in half of the six samples tested (72) (Figure 3). In the other three patients, additional microbes traditionally considered oral commensals were found in sputum samples and were not found in the explanted lung samples. However, for all samples, the dominant microbe found in sputum was the same as the dominant microbe found in the explant lung samples. The lack of additional commensal microbes in three of the sputum samples may reflect antibiotic treatment and disease state rather than a lack of oral contamination. We therefore favor the interpretation that microbes considered as contaminants might be important components of CF lung ecology. We list some issues that warrant consideration:

First, the careful study of explanted lungs presented in Goddard et al. provides valuable information about the end---stage disease associated microbial community DNA found in CF explanted lungs. However, end---stage disease communities are not representative of microbial community composition and activity during earlier stages. One of the most consistent signatures of CF airway microbial community evolution from longitudinal sputum sampling is the increase in antibiotic resistant pathogen load as disease state becomes more severe (15, 50, 73---75). Furthermore, by the time a lung is surgically removed, many of the airways have undergone bronchiectasis and become clogged, no longer exchanging air with upper airways. Substantially reduced microbial diversity is expected in explant samples, and is very different from the microbial community composition in the airways of younger CF patients with less severe disease (76). Community interactions involving the more diverse repertoire of microbes found in younger, earlier---stage patients are integral to the evolution of the community towards a less diverse, more antibiotic resistant state found at the end---stage of respiratory diseases, and should be studied in greater detail.

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Second, sputum may come into contact with oral microbes independent of the collection of the sputum sample. Sputum may accumulate in areas between the throat and the lobar bronchi in some patients, and oral microbes may inhabit these sites. Half of the six sputum samples reported in Goddard et al 2012 had a low abundance of oral microbes. This dearth of microbes may have been due to long---term antibiotic use and acute antibiotic treatment following an exacerbation (70, 73, 75, 77). The other three sputum samples did contain a greater abundance of oral microbiota compared to the explant samples, matching microbes identified in throat swabs. The presence of oral microbes could be significant to disease progression.

Third, the authors note that they made no attempt to separate intact microbes from the surrounding material, neither in the sputum samples nor in the explanted lung samples. This is relevant because *P. aeruginosa* is known to rely on large amounts of extracellular DNA from coordinated cell death for biofilm formation (78). This DNA could accumulate over time and exaggerate the apparent abundance of *Pseudomonas* relative to other microbes, especially in a context like the lower airways of patients with severe disease, where little disruption of the microbial communities would take place (79). In many explanted lungs, the authors did in fact detect some microbes typically considered oral commensals (such as *Streptococcus spp.*), but at much lower abundances than in sputum. If the relative abundance of *Pseudomonas* was exaggerated by the sampling method, then the low but non---zero relative abundances of oral microbes could be much higher than originally considered by the authors. However it is important to note that high abundance does not equal ecosystem importance, as some oral microbes may be able to influence lung microbial communities even at very low abundances (64, 65).

Moving forward: understanding colonization and migration events in respiratory infections. Further study is required to understand the dynamics of airway microbial communities that are relevant to disease. Human airways are exposed to microbes from many sources. While the persistent microbial community of the adjacent oral cavity is likely to be a dominant source, additional sources include nearby humans (other archipelagos, in Figure 1), animals, the home, water, food and other environmental reservoirs capable of creating airborne particulate matter. By analogy to the island biogeography example, the oral cavity can be thought of as a mainland species reservoir; the communities that become established in these locations can have a profound effect on the prospective colonizers of more remote islands further down the chain. Understanding where reservoirs of relevant microbes exist could help avoid or delay colonization, inform therapeutic strategies and potentially improve clinical outcomes.

Fortunately, modern 'omics profiling --- next generation sequencing, proteomic, and metabolomics measurements --- now allows the identities and activities of both chronic microbial communities and drifting microbial seeds to be identified with unprecedented depth and breadth. New technologies and decreasing costs allow for measurement of taxonomic identities, genome content, and transcriptomic, proteomic, and metabolomic activity of microbes in sputum samples. Insights into how movement and changes in activity of lung and oral microbes may precipitate exacerbations and other pathophysiological changes should be possible. The next major challenge is designing careful studies so that the large amounts of new data can give a clear picture of the spatial and temporal dynamics of these communities, and lead to understanding of how these dynamics impact clinical

outcomes. The biological question should dictate the most appropriate sampling approach. Standardization of sample collection and processing protocols is essential to producing data that can be compared, regardless of sample type.

The island biogeography analogy can be used to develop criteria for better understanding the relationships among traditionally "oral" and traditionally "CF pathogen" microbes. There may be cases where sputum samples are contaminated, and there may also be cases where "oral" microbes become established deeper in the CF lung. Perhaps these cases could be distinguished by deep longitudinal sampling. Evidence of oral microbes becoming established in the lung could include 1) persistence over time; 2) stable abundance and 3) genetic adaptation. These three lines of evidence are all predicted by island biogeography and evolutionary theory. Additional methods may also decipher the contributions of all airway microbes to the community dynamics. So far, little change in measurable density or composition of microbiota has been observed preceding CF disease flares known as pulmonary exacerbations (73, 77, 80). However, breath gas analysis and metabolite analysis of sputum samples may capture a valuable snapshot of metabolic activity that reflects the physiology of both the human host and microbes (20, 22, 81). Transcriptomic and proteomic analyses may also give deeper insight not only into the taxonomy of microbes present, but also their activities. Finally, new imaging techniques may eventually allow a non---invasive window into the spatial distribution of sputum accumulation within the airways of patients (82).

In conclusion, understanding polymicrobial community dynamics in health and disease is a compelling challenge in 21st century medicine, with consequences for infection diagnosis and treatment. Applying Island Biogeography theory to microbial communities inhabiting niches in the human airways informs our interpretation of the role of microbes found in different human sample types. When microbes that are typical of the oral cavity are found in sputum or BAL samples from CF patients, they should be considered potential immigrants, rather than contaminants. Regardless of whether microbial seeds originate from the mainland oral cavity or an adjacent island in the airways, they have the capacity to alter the dynamics of the polymicrobial community that impacts patient's quality of life.

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Figure Legends

Figure 1. Parallels between island biogeography and polymicrobial lung colonization. A) In Island Biogeography theory, the mainland is the greatest source of species diversity, with individual island species composition depending on the distance from the mainland. B.) Human airway microbial colonization is likely to display a similar dependence on the distance from the mainland (largely the oral cavity, shown in yellow, which is the richest and most diverse source of microbes with proximity to the lung). C.) Other people, along with the air, water and other environments, are also important sources of microbes which can immigrate to the islands in the human airways, and influence the polymicrobial community

Figure 2. Classic Island Biogeography. The richness of species depends on the Colonization rate (left Y---axis) and the Extinction rate (right Y---axis), adapted from (4). Migration through the trachea offers colonization opportunity to microbes from multiple sources, and impaired mucociliary clearance decreases the extinction rate. Gray circle (1) represents a small distant island (i.e. the lung) with few species, while gray circle (2) identifies the mainland or a large proximal island with high species diversity, such as the oral cavity. Diversity is comprised of both the number of species and their distribution, or evenness, and can be indicated by different measures of species richness and frequency. The number of species, or species richness, is an indicator of diversity. The term diversity is used throughout this perspective as informed by the species richness, which can be predicted in the Island Biogeography model.

Figure 3 Multidimensional scaling (MDS) of an unsupervised Random Forest comparing the relative abundance of taxa derived from 16S sequencing of lung explant samples (red) with sputum samples (blue) and oropharyngeal swabs (green) from six CF patients (see legend), data from (15), analysis conducted in R with the package Random Forest (72). Shared community composition leads to clustering of sputum and lung samples in most cases, while some sputum and throat samples cluster together.

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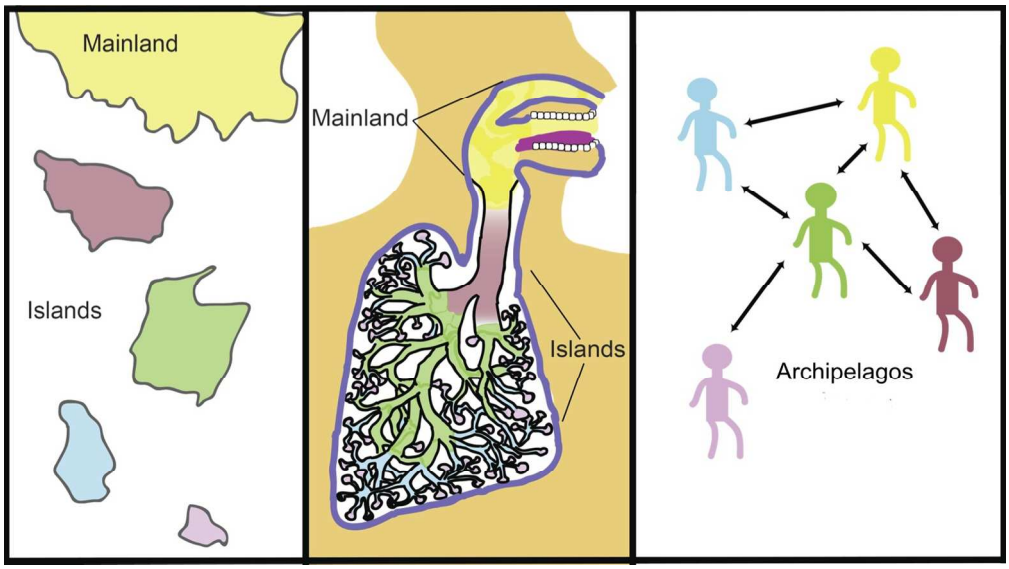

Figure 1. Parallels between island biogeography and polymicrobial lung colonization. A) In Island Biogeography theory, the mainland is the greatest source of species diversity, with individual island species composition depending on the distance from the mainland. B.) Human airway microbial colonization is likely to display a similar dependence on the distance from the mainland (largely the oral cavity, shown in yellow, which is the richest and most diverse source of microbes with proximity to the lung). C.) Other people, along with the air, water and other environments, are also important sources of microbes which can immigrate to the islands in the human airways, and influence the polymicrobial community
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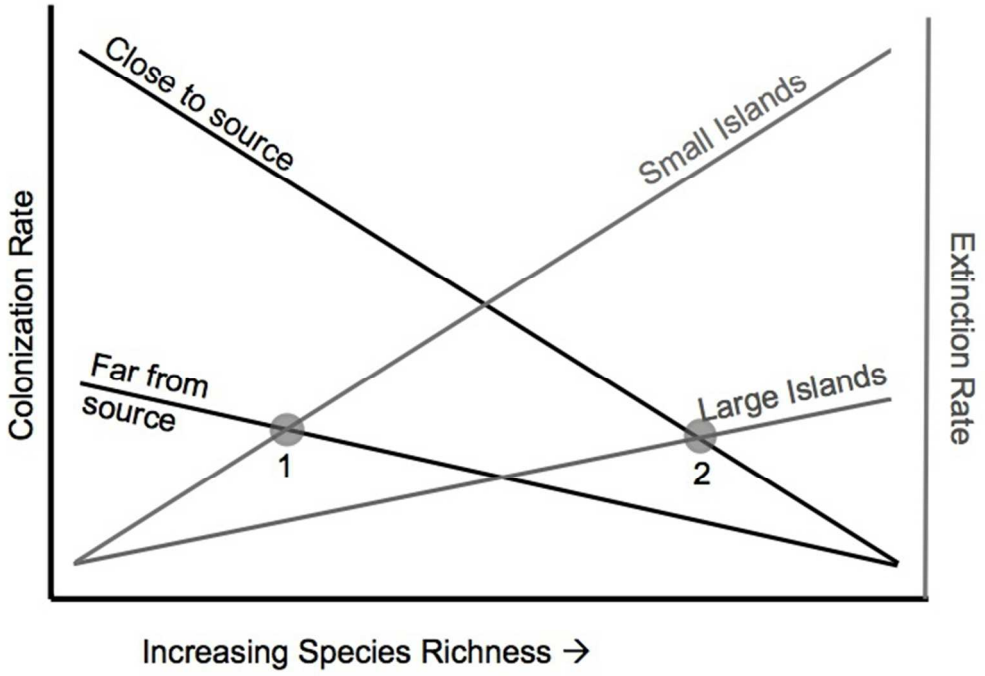

Figure 2. Classic Island Biogeography. The richness of species depends on the Colonization rate (left Y-axis) and the Extinction rate (right Y-axis), adapted from (4). Migration through the trachea offers colonization opportunity to microbes from multiple sources, and impaired mucociliary clearance decreases the extinction rate. Gray circle (1) represents a small distant island (i.e. the lung) with few species, while gray circle (2) identifies the mainland or a large proximal island with high species diversity, such as the oral cavity. Diversity is comprised of both the number of species and their distribution, or evenness, and can be indicated by different measures of species richness and frequency. The number of species, or species richness, is an indicator of diversity. The term diversity is used throughout this perspective as informed by the species richness, which can be predicted in the Island Biogeography model.
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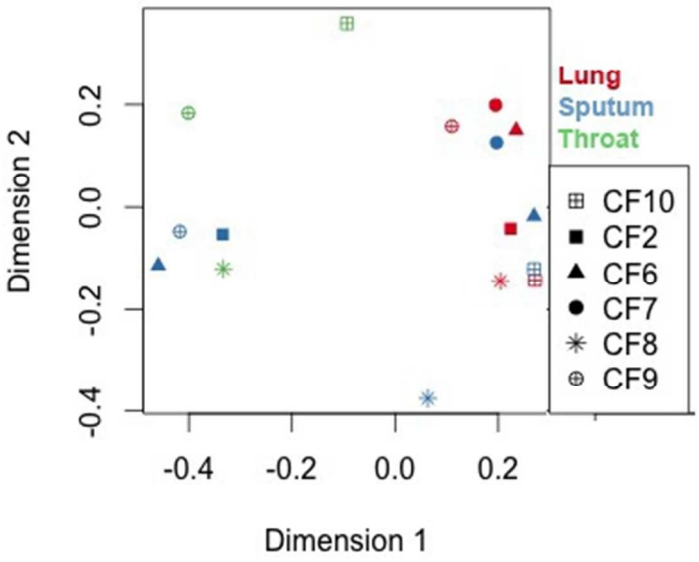

Figure 3 Multidimensional scaling (MDS) of an unsupervised Random Forest comparing the relative abundance of taxa derived from 16S sequencing of lung explant samples (red) with sputum samples (blue) and oropharyngeal swabs (green) from six CF patients (see legend), data from (15), analysis conducted in R with the package Random Forest (72). Shared community composition leads to clustering of sputum and lung samples in most cases, while some sputum and throat samples cluster together.
190x139mm (72 x 72 DPI)