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Measuring Species Richness Based on Microbial Community Fingerprints: the Emperor Has No Clothes

Danovaro and colleagues (3) recently compared microbial community diversity and richness estimates obtained using automated ribosomal intergenic spacer analysis (ARISA) with those obtained using analysis of terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes. While community fingerprinting methods such as ARISA and T-RFLP are useful for comparative analyses, they are not useful ways to assess the richness or diversity metrics of complex communities (4). Such methods are inherently limited by their detection threshold or, more precisely, by their dynamic range (5). Simply put, the number of peaks detected in either T-RFLP or ARISA assays will grossly underestimate the actual richness of any community with a long-tailed rank abundance distribution. Microbial communities, including aquatic and terrestrial bacterial assemblages (1, 2, 8, 10, 11), are generally found to approximate long-tailed distributions, such as lognormal, power law, or log-Laplace distributions (7). When taken in conjunction with differences in the contents of microbial communities, this property makes counting peaks in fingerprint patterns an unproductive exercise.

Danovaro and colleagues also conclude "that ARISA is more accurate than T-RFLP analysis on the 16S rRNA gene for estimating the biodiversity of aquatic bacterial assemblages." The basis of the authors' conclusion is that ARISA profiles contain more peaks than T-RFLP profiles generated from the same community sample. We assert that neither method has been shown to be more or less accurate based on the data presented in the paper. ARISA and T-RFLP analysis provide different levels of resolution. This leads to differences in the number of peaks and in the evenness of detected phylotypes but not to an increase in accuracy so far as estimating microbial diversity. Moreover, while the two methods partition the diversity in the community differently, neither method yields distinguishable categories that correspond to named taxonomic levels, such as species or genera. Consequently, neither method can be considered better from a taxonomic perspective.

While this letter is proximally in response to Danovaro and colleagues' paper, it is also intended to address a wider swath of the microbial ecology literature (see, for example, reference 6), in which authors fail to meaningfully discuss how the exclusion of rare taxa by the detection limits of such assays affects the interpretation of their data (9). For reasons we do not understand, investigators seem reluctant to acknowledge the limitations of such methods. So we will say what we all already know: the emperor has no clothes (http://www.andersen.sdu.dk/vaerk/hersholt/TheEmperorsNewClothes_e.html); current microbial community fingerprinting methods cannot provide reliable diversity indices.

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Authors' Reply

Estimating prokaryote diversity in natural ecosystems is a priority in current ecological research (17, 22). Spatial and temporal patterns of microbial diversity are obscure, especially in aquatic systems (8). The study of microbial biogeography is still in its infancy (10, 15), and even less is known of the relationship between microbial diversity and ecosystem functioning (19). In aquatic systems, for instance, and particularly in marine ecosystems, which cover more than 70% of the Earth's surface and more than 95% of the biosphere, simple questions are not only without answers but often even without hypotheses. What is the total number of microbial species on Earth? How many bacterial species can live in a liter of marine water (or kg of sediment)? Is there a latitudinal gradient of prokaryotic diversity? Is microbial diversity coupled with metazoan diversity, or do the two display idiosyncratic relationships? All these unanswered key questions provide evidence of the delay in microbial ecology research, especially when large spatial scales are considered. The development of accurate, rapid, and universally adopted methods for the determination of prokaryote diversity is, therefore, not only auspicated but needed to construct solid and consistent data sets, enabling the development of large spatial and temporal studies.

Recently, Danovaro et al. (5), comparing two fingerprinting

techniques (terminal restriction fragment length polymorphism [T-RFLP] analysis and automated ribosomal intergenic spacer analysis [ARISA]) using several marine samples, provided evidence that ARISA is more effective than T-RFLP analysis in measuring bacterial diversity. ARISA estimates of bacterial species richness were always significantly higher than (sometimes double) those obtained using T-RFLP analysis, as a result of a higher taxonomic resolution. In the preceding letter, Bent et al. stressed that bacterial species richness, estimated using fingerprinting techniques (such as T-RFLP analysis and ARISA), does not reflect the actual prokaryote diversity, as these methods capture only the dominant members of complex assemblages and lose the rare taxa. The problem posed by Bent et al. is crucial in current microbial research and repeatedly recognized by several authors (3, 14, 20, 21), and all fingerprinting methods, including denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism analysis, length heterogeneity PCR, and others, could be subjected to similar criticisms. However, the point is, what is the question we pose? If the question is determining the absolute number of bacterial species, Bent et al. are absolutely right and our efforts should be addressed to other techniques allowing the accurate identification of the species present in the sample. But if the question is comparing levels of biodiversity in habitats and ecosystems or looking at the impacts of different kinds of stimuli on biodiversity, then a comparative approach based on a suitable fingerprinting technique could be very useful. This holds true in particular when large numbers of samples are collected, as in the case of the research carried out in the field of microbial ecology. In addition, it could be noted that, at present, no single method is completely devoid of a potential bias. Despite the well-recognized pitfalls of PCR-based rRNA analysis (32), the profiling of bacterial rhizosphere and bulk communities with denaturing gradient gels has proved to be a powerful method allowing a cultivation-independent analysis of large numbers of rhizosphere and bulk soil samples (27, 28, 29). Wieland et al. (33) showed that, although TGGE patterns can represent biased sequence frequency distributions, they are useful for monitoring the variation of complex communities in response to a variety of experimental conditions with the large sets of samples needed for monitoring natural soil assemblages.

Bent et al. criticized the fingerprinting techniques for their inability to identify accurately bacterial taxa to the species level. It is evident that a fingerprinting technique cannot provide a complete inventory of all bacterial taxa present in a sample. However, the fact that these methods are now largely utilized worldwide is creating a large data set, thus enabling comparisons among samples (3). In addition, despite their relatively low sensitivity toward less abundant microbial taxa, fingerprinting techniques have been shown to produce results consistent with those obtained using more-sophisticated and time-consuming approaches (such as cloning and sequencing [6, 12]). This, indeed, makes fingerprinting analyses still a highly suitable tool for testing scientific hypotheses which require the analysis of a large number of natural samples. Any method allowing a more accurate identification of the number of bacterial taxa is potentially more useful for testing scientific hypotheses or for investigating bacterial assemblages. From this perspective, the higher performance of ARISA than of T-RFLP analysis has been remarked (5).

The importance of the taxonomic resolution (taxonomic sufficiency) in ecological studies has been posed several times in other disciplines (13, 25). Although the debate is still open, it can be concluded that in most cases, changes detected in biodiversity analysis at the species level are evident (and sometimes more evident) also at a lower taxonomic resolution (e.g., the genus or family). We think that all scientists might agree that it would be better to always have the highest taxonomic resolution (i.e., a detailed species list) in any ecological research, but this is often impossible due to the high number of samples or to a limitation in time or facilities. To a certain extent, it could be concluded that the higher the spatial scale of the investigation, the lower the resolution power of the methodologies employed.

In this regard, the criticism of Bent et al. toward T-RFLP analysis and ARISA for their inability to provide clearly distinguishable taxonomic categories are not fully justified. The 16S rRNA gene is recognized to be insufficient to define phylogenetic relationships among closely related species (4, 7, 11, 23, 31). Conversely, the use of the internal transcribed spacer (ITS) region (a more varied molecular marker) has been shown to enable discrimination to the species level and even within species (9, 11, 18, 31, 34). This genetic marker is being rapidly and largely utilized, and only in a very few cases has it failed in discriminating to the species level (7). Moreover, different ITS sequences (i.e., different ARISA peaks) have been shown to identify bacteria with distinct ecological roles (16, 26), and this property might result in being extremely useful for estimating the functional diversity of ecologically relevant taxa within a complex assemblage. This can represent only the first step toward a more detailed analysis of the physiological and metabolic properties of bacterial species and thus toward the integration of taxonomy and a determination of function.

Bent et al. concluded that most authors do not discuss adequately the biases induced by the exclusion of "rare" taxa from fingerprinting-based diversity estimates. We agree that identification of rare taxa is a priority in current ecological research, including microbiological research (1, 30). The abundances and identities of rare bacterial taxa can be extremely relevant, for instance, in testing processes of ecological facilitation. Moreover, certain ecological processes can be tested only if a large number of taxa are present due to the increased connectedness of species and functional groups. The identification of rare bacterial species in mixed assemblages is now possible only using sophisticated methods (24), including the recently developed "parallel tag sequencing strategy" (30). The development of the use of these or other techniques, enabling a more accurate identification of bacterial species, is clearly recommended, but their application in large-scale studies will require further research and technological efforts.

We do not agree that scientists around the world are performing "unproductive exercises" when they measure bacterial species richness using fingerprinting techniques. We believe that this is a matter of questions asked and of tools. Fingerprinting techniques are helping us to elucidate important yet undescribed characteristics of natural prokaryotic assemblages in the fields of ecology, biogeography, and applied microbiology. Nonetheless, we believe that it could be a shared opinion that further technological and scientific developments are needed to enhance our ability to investigate microbial diversity. Fingerprinting techniques are like prêt-a-porter clothes: dressing prêt-a-porter is maybe not as glamorous as being dressed by a top stylist, but it does not leave you naked.

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