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Short communication

Soil microbial communities along the route of a venturous cycling trip

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ABSTRACT

The purpose of the present study is not solely scientific but arose from a rare fusion of art and science during a venturesome bicycle trip from Austria (Europe) to Laos (Asia). The artist Wolfgang Burtscher produced every-day dirt tire imprints on papers (*tripmarks*) to document his journey (www.tripmarks.at) Contributing to public awareness in this International Year of Soils 2015 we took a closer look at the dirt forming these pieces of art, namely to investigate the inherent microbial life. As such, we benefited from minute soil samples along a global transect, which permitted the classical hypothesis on microbial cosmopolitanism to be evaluated. DNA from 0.1 g soil was extracted and amplified with specific primers for fungi and actinobacteria to obtain denaturing gradient gel (DGGE) fingerprinting patterns. Spatial variables were generated by applying trend surface analysis and principal coordinates of neighbour matrices. Canonical correspondence analysis was also used to check for driving factors.

Our results show that microbial variability is primarily being influenced by environmental rather than spatial patterns. Clearly distinct communities in the soils of distant sampling sites were formed for actinobacteria but not for fungi. Furthermore, cosmopolitan phylotypes with relative abundances as high as 80.5% (fungi) and 74.0% (actinobacteria) were detected. These findings show that Eurasian soil microbial communities follow environmental rather than spatial constraints. For the artist, these results emphasize the broad diversity of microorganisms present on his tripmarks joining the facet of the beauty and variety of life with his dirty tire imprints.

1. Introduction

Since the dogma of *everything is everywhere,but the environment selects* was formulated in the early 20th century (Baas Becking, 1931; Beijerinck, 1913), several studies showed evidence of a microbial cosmopolitanism (Fenchel and Finlay, 2004; Finlay, 2002). It is supposed to be due to passive dispersal, dormant life stages and short generation times. Though, it has also been shown that there exist relationships between certain microbial groups or species and environmental parameters (Fierer and Jackson, 2006; Griffiths et al., 2011). However, modern molecular and fingerprinting techniques that permit a circumstantial depiction of community variations led to a debate about the correctness of the above mentioned statement (Green and Bohannan, 2006), claiming that the species are distributed following certain spatial patterns. However, theories and assumptions of global distribution patterns are still unclear, as sampling efforts on a global scale are high and data analyses complex. To dig deeper into the influence of space on microbial communities, space can be used either as a predictor or a co-variable in such statistical models (Dray et al., 2006), affecting ecological patterns and processes at different scales (Legendre and Legendre, 1998).

The project *tripmarks* benefited from soil samples achieved by the fusion of art, science, sports and adventure (Burtscher and Nagler, 2014; Fig. 1). We investigated the biodiversity and the spatial distribution of actinobacteria and soil dwelling fungi along a bike route from Austria to Laos to test to what extent spatial and environmental variables are shaping the microbial communities on a continental scale Bearing in mind the aforementioned dogma we expect that the environmental variables have a larger influence on the communities than the spatial ones, and such patterns will be group-specific. By investigating fungi and actinobacteria, we are able to test this hypothesis for two important soil microbial groups from different domains. While fungi were screened on domain level, actinobacteria were selected as key stone group representing soil bacteria, being one of the dominant bacterial phyla containing one of the largest genera, *Streptomyces* and playing a crucial role in organic matter decomposition (Ascher et al., 2012). In combination with the genetic fingerprinting-DGGE method, the choice of actinobacteria as targets was made because they yield less

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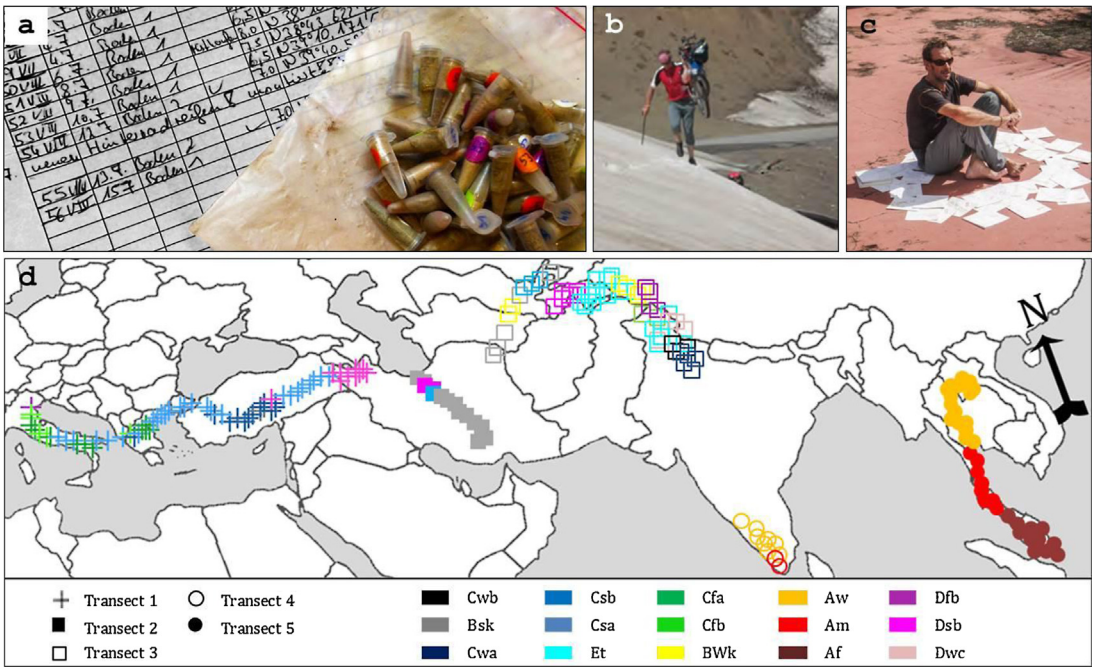

Fig. 1. The Adventure—(a) The soil samples after their long journey; (b) Wolfgang Burtscher during the trip; (c) sampling points along the *tripmarks*-route. Symbols represent different transects interrupted by flights or bus rides; colors represent climate zones according to Kottek et al. (2006). Main climates: A = equatorial, B = arid, C = warm temperate, D = snow, E = tundra. Precipitation: W = desert, f = fully humid, s = summer dry, w = winter dry, m = monsoonal. Temperature: k = cold arid, a = hot summer, b = warm summer, c = cool summer, t = tundra.

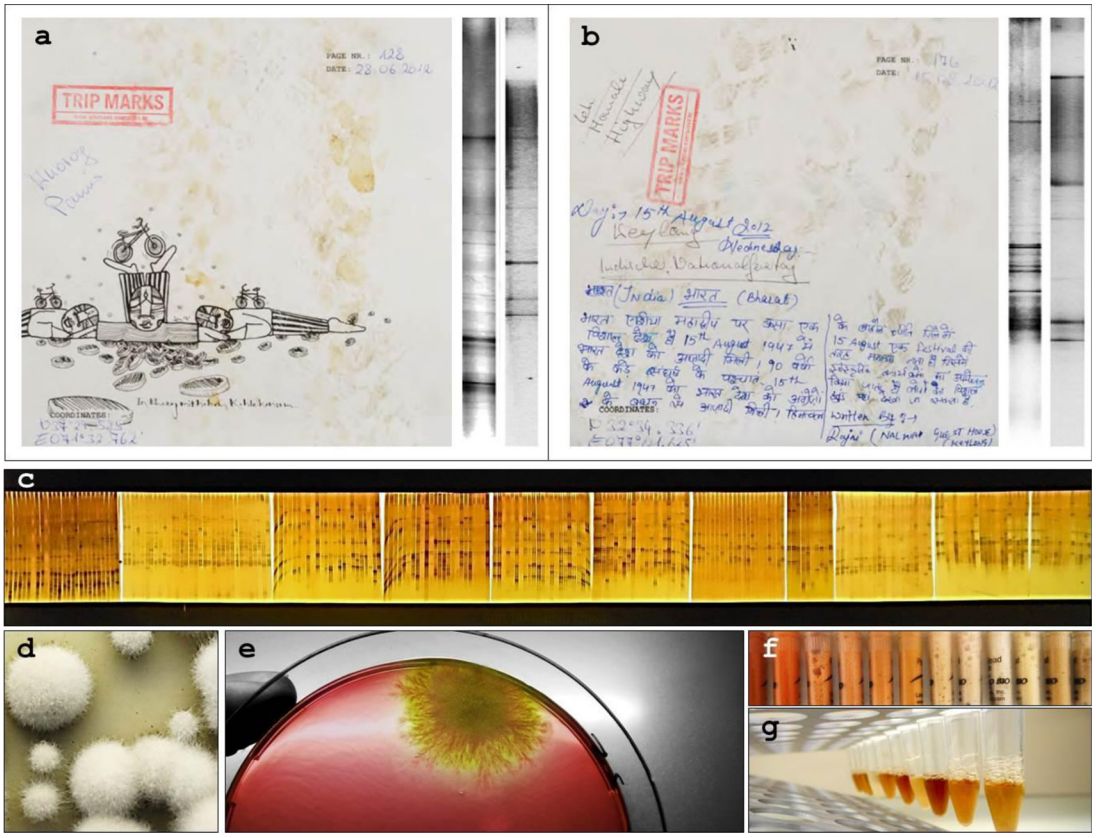

Fig. 2. The Art—(a and b) Two *tripmarks* with their corresponding DGGE lanes for fungi (left) and actinobacteria (right); (c) the DGGE's are arranged on a black light box and used for exhibitions joining art and science; (d and e) various pictures of growing microorganisms have been created and published in an art catalogue (Burtscher and Nagler, 2014); (e and f) Soil DNA extraction seen from an artistic point of view.

complex patterns with respect to community-level screening (Heuer et al., 1997; Ascher et al., 2012), allowing to better monitor changes as a function of changing edaphic and climatic factors.

To generate variables mapping large as well as medium scale spatial patterns we used the GPS-data of the sampling points to conduct a trend surface analysis (Gittins, 1968) and a principal coordinates of neighbour matrices (PCNM). Then we used the variables obtained by these methods to model spatial relationships in a canonical correspondence analysis. To date, a combination of the broader-scale spatial variables represented by polynomial functions and the finer-scale spatial variables represented by PCNM-variables has not been conducted, although the influence of both scales have been shown to be important (Legendre and Legendre, 1998).

It is important to point out that the objective of this study was not only to monitor the microbial communities along the bike route, but to somehow outreach beyond the soil scientist community in this United Nation's Year of Soils (Insam et al., 2012; United Nations, 2014). While scientific publications are neat, elaborate and often hard to follow, art is more spontaneous and subjective in its interpretation, being in this way susceptible to everyone. The goal of both disciplines is, however, to spread an idea or a finding and to motivate people to think. The fusion of art and science is therefore both, fruitful and restrictive as the scientific findings have to be easy to follow and the artwork must be done within the frame of the scientific question. Being a robust, rapid, low-cost and simple method to screen a large number of samples we chose the DGGE-approach. At the same time, these gels are beautiful and can be seen as a work of art, making it clear that dirt is alive, but that it is not the same everywhere (Fig. 2), also from a microbiological perspective.

2. Materials and methods

2.1. Study area and sampling strategy

The sampling area ranged from the city of Innsbruck, Austria to an uninhabited point of central Laos, South-East Asia. Within 11 months, from February 2012 to January 2013, a total of 197 soil samples were taken from five transects at sea levels ranging from 0 to 5330 m and from high mountainous to equatorial climate zones (Fig. 1). Soil samples were collected from the topsoil in sterile 0.2 ml PCR-tubes and they were air-dried overnight, a treatment that has been shown not to alter significantly microbial fingerprinting patterns in the soil (Fierer et al., 2003). The pH-value was determined with Litmus paper, and the total organic carbon (TOC) and nitrogen (TON) by using a CN analyser (Flash EA 1112, Thermo Electron Corporation, Germany). The C-to-N ratio was calculated after removal of inorganic C with 1 M HCl, and effervescence of CO_2 was used for a rough classification of the bedrock chemistry, distinguishing between carbonaceous and not carbonaceous.

2.2. Fingerprinting

Total DNA was extracted from 0.1 g soil using PowerSoil DNA Isolation Kit (MO Bio Laboratories Inc., Carlsbad, USA). DNA quantification of extracts was performed by µl-spectrophotometer (Picodrop TM). Amplification was performed according to Vainio and Hantula (2000) (GCFR1/F390) and Ascher et al. (2012) (243f/1401r and GC968f/UNI1401r) for fungi and actinobacteria, respectively. DGGE fingerprinting was performed following a modified protocol from Muyzer et al. (1993) and using an

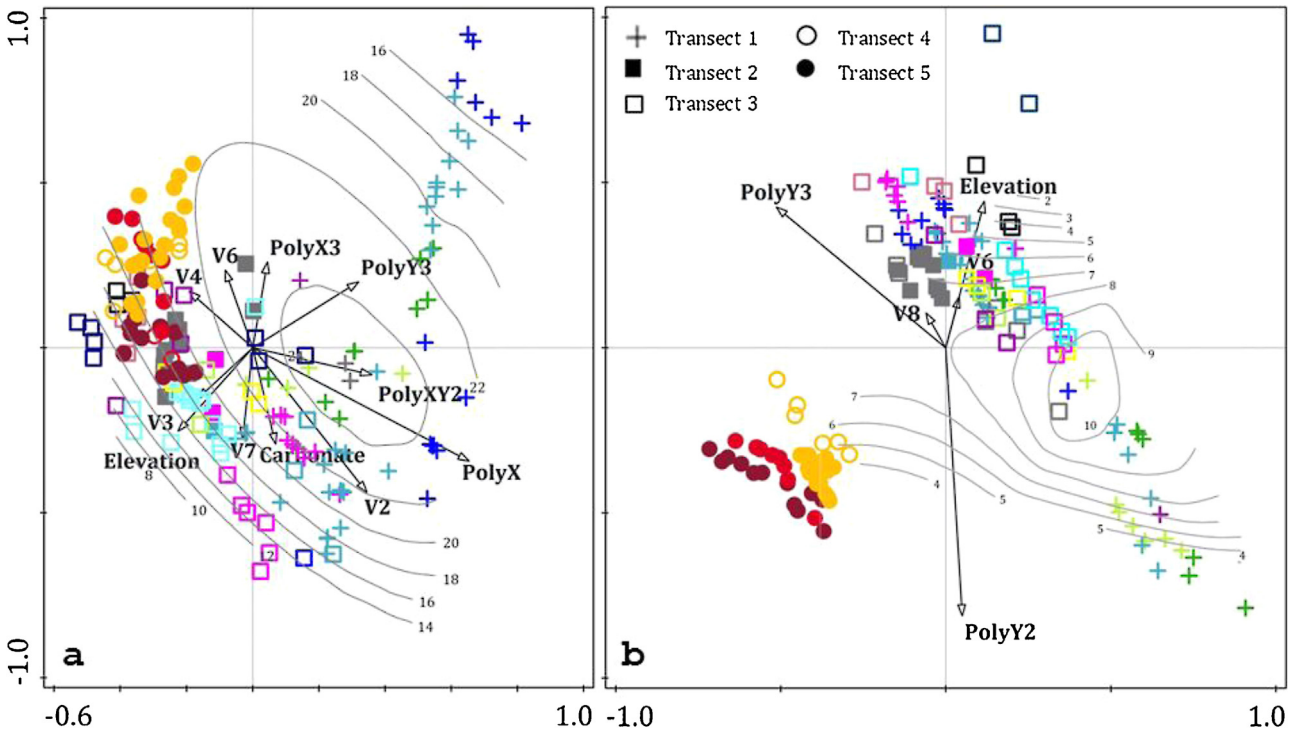

Fig. 3. The Science—Relationship between microbial communities and environmental/spatial factors according to CCA based on DGGE profiles for (a) fungi and (b) actinobacteria. Symbols indicate transects (transect 1. Austria to Iran, transect 2: Iran North to South, transect 3: Iran to India, transect 4: South India, transect 5: Singapore to Laos), colors represent the climate zones (Kottek et al., 2006) and contour lines represent the average number of different phylotypes. The arrows (vectors) affiliated with each of the axes indicate the most significant environmental variables (Elevation = altitude of the sampling point, Carbonate = Presence/absence of CO_3^{2-} in the soil) medium scale spatial patterns (V2–V7 = PCNM-derived variables) and large scale spatial patterns (PolyX–Y3 = variables obtained by trend surface analysis) and their relative effects on fungal communities. See Fig. 1 for climate, precipitation and temperature information.

IngenyphorU® buffer system (Ingeny Int., The Netherlands). Polyacrylamide gels (7%) were prepared with a denaturant gradient from 45 to 65% and a lab-specific non-molecular weight marker composed of mixed DNA of varying GC-contents used to compare the bands across multiple gels. Bands were visualized using silver nitrate (fungi; Sanguinetti et al., 1994) or SybrGreen (actinobacteria; Ascher et al., 2012) and the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany). Band detection was conducted using GelCompar II software (Applied Maths, Belgium).

2.3. Data analysis

Data analysis is divided into (I) the collection of environmental information; (II) the generation of medium and large scale spatial variables; and (III) the testing of the hypotheses through the combination of the community related data with the environmental datasets. (I) First, GPS data were transposed to .gpx data and embedded in a world climate map (Kottek et al., 2006). (II) Spatial variables were obtained at two levels: In R (R Core Team, 2015), general, large scale surface trends were determined transforming GPS-data to third-degree orthogonal polynomials ("Poly + binomial term") in a trend surface analysis (TSA). Being a global surface fitting procedure to estimate a regular grid of points on a surface from scattered observations, this method approximates the mapped data by a polynomial expansion of the coordinates of the sample points applying the least squares principle and creates variables that describe large scale spatial patterns over the whole sampled area (Legendre and Legendre, 1998). To obtain narrower spatial patterns, we determined the principal coordinates of neighbour matrices (PCNM) (Borcard and Legendre, 2002) of detrended data, selecting PCNMs modelling positive spatial correlations and labelling them from V1 to V21. This procedure (a) computes a pairwise distance matrix between the sampling locations, (b) constructs a truncated distance matrix, and (c) performs a principal coordinate analysis (PCoA) of the truncated matrix. The principal coordinates of this PCoA are the PCNM-variables. After testing for significance of the PCNM variables, most significant trend surface and PCNM variables were selected applying forward selection. (III) Finally, a canonical correspondence analysis (CCA) including all significant variables and phylotypes presence/absence data was conducted with 9999 permutations, down-weighting rare species in Canoco 5 (Šmilauer and ter Braak, 2012).

3. Results

In the 197 soil samples, a total of 125 different phylotypes were detected for fungi and 102 for actinobacteria. For fungi, 2.4% of phylotypes were found in 60–100% of all of the examined samples and 11.2% of the phylotypes occurred in 30–60% of the samples. For actinobacteria, however, only 1% of the phylotypes were globally distributed with relative abundances of 60–100%, while 98% of the phylotypes showed a relative abundance from 0 to 30%.

3.1. Canonical correspondence analysis

3.1.1. Fungi

The CCA of the fungal data displayed 41.56% of the variance of the phylotypes with respect to the environmental variables. Axes 1 and 2 accounted for 15.1 and 10.8% of the adjusted variation, respectively (Fig. 3a). Three environmental variables were found to influence the fungal communities: (I) elevation, (II) bedrock chemistry and (III) the affiliation to certain climate zones. (I) The elevation explained 1.5% of the total variation ($p = 0.001$) and influenced mostly samples from transect 3 and more precisely from the tundra, snow and cold arid climate zones in one direction and summer dry, warm temperate samples from transect 1 in the other direction. (II) The bedrock chemistry (presence or absence of carbonate) accounted for 1.7% ($p = 0.012$) of the variability, separating samples of the equatorial zones of South-East Asia without carbonate from carbonaceous samples in Northern Turkey, Iran, Uzbekistan and Tadjikistan. (III) Additionally, samples belonging to the same climate zones were clustered together, equatorial, fully humid climates (Af) and tundras (Et) having the greatest ability to explain their community compositions (2.3 and 1.9%) ($p = 0.001$), followed by equatorial, monsoonal (Am) and warm temperate climates with warm and dry summers (Csb) (1.8%) ($p = 0.002$). Equatorial, winter dry (Aw), warm temperate, fully humid with hot summers (Cfa) and snowy, fully humid climates with warm summers (Dfb) each explained 1.7% of the total variation ($p = 0.011$, 0.021 and 0.031). Furthermore, the diversity of phylotypes was found to be higher for European samples of temperate climates and lower for tundras (Et), snowy (Dsb) and warm desert climate samples (Cwa) (Fig. 3a).

Five PCNM variables represented the medium-scale spatial patterns and explained 1.4, 1.2, 1, 1% ($p = 0.001$) and 0.7% ($p = 0.025$) of the overall variation in the fungal community composition. Two variables (V2 and V6) discriminated between transects 1 and 5, while V4 separated transect 1 from transect 4, and V3 differentiated transect 1 from 3. The variable V7 isolated snow (Dsb)-samples of transect 3. Four significant polynomial variables (PolyX, Y3, X3 and XY2) explained 2.5, 1.9, 1 and 0.8% of the total variation ($p = 0.001$), where PolyX was the most important large-scale spatial pattern, separating samples from Turkey from those of Southern India and South-East Asia. PolyY3 was found to separate samples from Greece and Western Turkey from those of the Pamir Mountains in Tajikistan belonging to the tundra. PolyX3 isolated the communities of the high altitude Dsb-samples from Tadjikistan (Fig. 3). Finally, PolyXY2 discriminated warm temperate, Southern European samples of transect 1 from high altitude, cold adapted communities of Northern India.

In summary, environmental variables, medium scale and large scale spatial patterns explained 55, 20 and 25% of the variation, respectively.

3.1.2. Actinobacteria

The CCA of actinobacteria displayed 42.7% of the variance of the phylotypes, axes 1 and 2 accounted for 13.3% and 9.0% of the adjusted variation, respectively. Two environmental variables showed a significant influence on the community composition of actinobacteria: (I) elevation and (II) the affiliation to certain climate zones. (I) Elevation explained 1.7% of the total variation ($p = 0.023$), separating low lying sampling points of transects 4 and 5, as well as the samples from Italy and Albania from the higher elevation sampling points in the Pamir (transect 3) and Iranian mountains (transect 2). (II) The affiliation to the climate zones Aw (equatorial, winter dry), Af (equatorial, fully humid) and Am (equatorial, monsoonal) each explained 2.7% of the total variation ($p = 0.001$), while Cfa (warm temperate, fully humid, hot summer) and Bsk (cold arid, summer dry) explained 2 and 1.8%, respectively ($p = 0.031$ and 0.04). The diversity in the communities was higher in the samples from temperate climate zones (Cfa, Cfb and Csa) containing 8–10 phylotypes on average and lower in soils from equatorial (Aw, Af, Am) and cold arid (Bsk, Dwc) climate zones; as well as in those from the highest altitude from transect 3 with 2–8 phylotypes (Fig. 3b).

The PCNM-variables V6 and V8 explained 2 and 1.9% of the total variance ($p = 0.005$ and 0.011, respectively), in which V6 separated

Eastern Asian and European temperate samples from all the other samples and V8 isolated even more the temperate European samples from all the others (Fig. 3b).

The large-scale spatial patterns PolyY2 and PolyY3 were highly significant ($p = 0.001$), accounting for 2.7 and 2.1% of the total variation. While PolyY2 was found to separate west- and east-lying samples from those samples taken in the center of the trip, PolyY3 discriminated samples from the west (Italy, Albania and Greece) from all the samples (Fig. 3b).

In summary, environmental variables, medium scale and large scale spatial patterns explained 61, 17 and 22% of the variation, respectively.

4. Discussion

Here, we took benefit from the journey of a cycling artist and were rewarded with random soil samples along an intercontinental transect which allowed us to evaluate the influence of both environmental and spatial variables on microbial community composition. Assuming that the minimum sampling size decreases with the size of the investigated organisms due to a changed local: global taxa richness ratio (Finlay, 2002; Fenchel and Finlay, 2004), an increase in the sampling area does not involve a steep increase in microbial species. However, Woodcock et al. (2006) warned of an underestimation of this increase, and it has been estimated that one gram of soil contains 3×10^4 species g^{-1}, while one ton contains 4×10^6 (Curtis et al., 2002). This legitimates the criticism that none of the samples analysed here show the full spectrum of microbial populations. Nevertheless, we showed that some of the phylotypes were present in almost every sample, corroborating the view that microbial species are globally distributed and falling in line with several large-scale studies (Fenchel and Finlay, 2004; Prospero et al., 2005; Fierer and Jackson, 2006). The fact that cosmopolitanism is restricted to some single species could lead to the assumption that few generalists are present everywhere, while the majority of the species are present in only up to one third of the samples. An observation made by Hughes Mariny et al. (2006) who attributes these patterns to variations in range sizes of different microbial species indicating variation in extinction rates. It is also important to mention that no seasonal changes have been considered in the present work; as such, the number of cosmopolitans is expected to be even higher than determined here.

A major part of the variation remained unexplained probably due to a lack of information on other important environmental variables such as soil moisture, UV-radiation and seasonal changes. However, although our results show that the explainable variation in the community composition was also related to large- and medium-scale spatial patterns, it is important to underscore that the greater percentage was explained by the local environmental factors. Specifically, the few investigated environmental variables like elevation, pH, C and N content, climate zone and the C/N ratio explained approximately the same amount of the variation as all the significant large- and medium-scale spatial variables together.

For fungi, large-scale spatial patterns explained the biggest fraction of the variation, as shown in the CCA-plot. They were represented by four variables mostly indicating a divergence in the community composition between geographically separated samples belonging to Europe or Central-Eastern Asia. In addition, PCNM variables indicated some differences in the communities according to their transect affiliation and pointing out finer spatial patterns. The environmental factor elevation correlated negatively with the number of phylotypes, indicating that samples from higher elevations contain fewer species. Moreover, the samples containing carbonate seem to harbor different microbial communities in comparison with samples with different bedrock chemistry. All the identified spatial and environmental variables were found to be independently overlapping and influencing the species composition of each sample, thereby explaining only a relatively small amount of the overall variation. Therefore, no evidence was found for a distinct clustering based on transects or climate zones, which supports the hypothesis of an environmental selection of adequate species out of an existing pool of microorganisms (Finlay, 2002; Fenchel and Finlay, 2004). Actinobacteria, however, formed two distinct clusters; on the one hand, equatorial climate samples from Southern India and South-East Asia clustered apart from the remaining samples, being influenced by elevation, a big scale and a medium scale spatial factor. On the other hand, temperate, arid, snow and tundra samples clustered together, but they were separated according to their elevation and large-scale spatial influences. Such divergence of communities could be a sign of the selective effect of the environment causing a non-global dispersal with specialised species of actinobacteria. Summing up the explained variations of all variables allows us to test to what extent spatial and environmental variables are shaping two selected soil microbial populations and shows that the most of the variation can be explained by environmental and not spatial patterns.

The current study is looking through a small window into the microbial communities of soil along a transect and corroborates the historical statement claiming that *everything is everywhere* as we found cosmopolitan species and similar community-patterns in samples from distant places. This statement is also supported by the estimate that 10^{18}–10^{20} microorganisms are transported annually between continents (Gans et al., 2005) and over an inter-hemispheric scale (Prospero et al., 2005). Despite the fact that only a few environmental factors have been investigated in this study, the influence of spatial factors has been addressed intensively, thereby generating large- and medium-scale spatial patterns and testing the influence of both, using only the best-fitting factors. Nevertheless, their influence has not been more pronounced than the influence shown by the local environmental factors and in turn, existing biogeographical patterns seem to be formed by selective, environmental factors, *the environment selects.*

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