November 2004

# Soil microfungi in the forest-agricultural landscape of San Luis, Costa Rica

Ellyn Yakowenko

# Soil microfungi in the forest-agricultural landscape of San Luis, Costa Rica

**Ellyn Yakowenko**

Department of Biology, Saint Mary's Honors College of Maryland

---

## ABSTRACT

Soil microfungi are known to play key roles in ecosystem function and are often the first indicators of a breakdown in stability (Volk 2001). This study compares soil microfungal abundance and diversity across a tropical human land use gradient in the San Luis valley of Costa Rica. Microfungi were cultured from soil samples collected from primary forest, secondary forest, pasture, and coffee farm sites using the Soil Dilution Plate Technique (Christensen 2001). Thirty-six soil samples yielded a total of 17,321 isolates of 41 different morphological species. Each habitat type was characterized by the dominance of a few abundant species while the rest of the community was comprised of an extensive number of 'rare' species. Differences in species richness and diversity across habitats were found to be statistically significant: secondary forest sites were found to be the most diverse (H'= 2.213, S = 33), followed by coffee farm (H'= 1.113, S = 24), pasture (H'= 0.506, S = 21), and primary forest sites (H'= 0.314, S = 26), respectively. In contrast to previous studies on microfungal diversity (Cabello and Arambarri 2002), this study determined that intermediate to high levels of soil disturbance caused by human land use actually may facilitate microfungal diversity in the tropics, instead of hindering it.

## RESUMEN

Se sabe que los microhongos de suelo juegan un papel clave en la función de los ecosistemas y a menudo son los primeros indicadores de estrés (Volk 2001). Este estudio compara la abundancia y la diversidad de los microhongos en una gradiente de uso humano en el valle de San Luis, Costa Rica. Los microhongos fueron cultivados a partir de muestras de suelo recogidas en bosques primarios, bosques secundarios, potreros y cafetales usando el Soil Dilution Plate Technique (Christensen 2001). Trienta y seis muestras de suelo produjeron 17,321 aislados de 41 especies morfológicos diferentes. Cada tipo de hábitat estuvo caracterizado por la dominancia de unas cuantas especies abundantes, mientras que el resto de la comunidad estuvo constituido de un número extenso de especies 'raras'. Las diferencias en la riqueza y diversidad de las especies entre los hábitats fueron estadísticamente significativas: los sitios de bosque secundario fueron los más diversos (H'= 2.213, S = 33), seguidos por los cafetales (H'= 1.113, S = 24), los potreros (H'= 0.506, S = 21) y los bosques primaries (H'= 0.314, S = 26), respectivamente. En contraste con estudios anteriores sobre la diversidad de microhongos (Cabello and Arambarri 2002), este encontró que las pertubaciones del suelo de nivel intermedio a alto causadas por el uso humano en efecto incrementan la diversidad de los microhongos en los trópicos, en vez de disminuirla.

## INTRODUCTION

Fungi play a major role in the functioning of global ecosystems, namely by acting as natural biocontrol agents, sources of food, mutualists, and active participants in nutrient cycling (Hawksworth 1991). They contain a vast network of tiny threads used to secrete enzymes and break down organic debris (Rossman 2002). Thus, fungi are able to

decompose complex nutrient sources, efficiently cycling leaves, wood, and other organic matter. Carbon, nitrogen, sulfur, and phosphorous are recycled back into the soil by fungi, making these essential nutrients available for plant usage. Due to the fact that they are major components of soil biomass, fungi keep the by-products derived from the decay of materials in their tissues, rather than in the atmosphere (Hawksworth 1991). These characteristics are crucial not only for primary production of nutrients but also in the long-term functioning of an ecosystem.

Considering their global importance, very little is known about this kingdom of organisms (Bills 1995). Fungi, especially microfungi suffer the fate of many of earth's 'invisible' inhabitants (Nee 2004). It is estimated that 1.5 million fungal species exist but only 5 percent are currently described, even though fungi are the second largest group of organisms in the world after insects (Hawksworth 1991). Furthermore, most of the described species are macrofungi, visible to the naked eye. Microfungi are the most diverse group of all fungi, outnumbering visible fungi 30 to 1; however, they are also the least studied and described.

Although it may be easy to overlook these microscopic organisms, microfungi are key species in monitoring forest stability and health (Hawksworth 2002). Their roles in pathogenesis and decomposition mean that they form a vital part of the links in the food web in ecological communities. They are the thread that ties the whole food web together, since they are the primary decomposers (Volk 2001). Thus, they may be the first indicators of detrimental changes in either a community's biotic or abiotic factors, which have the possibility of leading to significant changes in ecosystem functioning.

It is widely accepted that excessive land use by human populations can have serious detrimental effects on local ecosystems and this is especially true when changes in soil microfungal life are considered. As indicators of ecosystem health, soil microfungi are important monitors of the soil disturbance (Hawksworth 1991). Cabello and Arambarri (2002) found evidence to support that disturbance of soil in Argentina correlated with a decrease in soil microfungal biodiversity. They compared microfungi of disturbed forest to those of undisturbed forest and found that disturbance has an especially great impact on species richness.

This study linked modern land use to microfungal abundance and diversity in the tropics of Costa Rica. It measured the heterogeneity of microfungi at specific habitats and then broadened its focus to compare microfungal diversity and abundance over a human use gradient.

## METHODS

### Study area and collection of materials

Soil samples were collected in the San Luis valley, Costa Rica. Although most of the pristine forest of the valley has been cleared for pasture and farming, some areas of both original forest and successional forest still remain. Four distinct habitat types were sampled: primary forest, secondary (disturbed) forest, pasture, and coffee farms not using fungicides (Figure 1). Three different locations were selected for sampling for each

habitat type, yielding a total of 12 test sites. Each site had a similar elevation (approximately 800 m) and was subjected to similar weather patterns at the time of this study. At each of these 12 sites, three replicate soil samples were collected randomly from the topsoil layer. While standing at a single point, leaf litter and debris were raked, by hand, away from the soil. Soil that was in direct contact with litter was avoided. A small shovel was used to transfer between 20 to 30 grams of topsoil to a labeled plastic Ziploc bag. The shovel was washed and cleaned with water after each sample was collected to prevent cross contamination. All samples were then carried back to La Estación Biológica Monteverde for testing and processing within 48 hours.

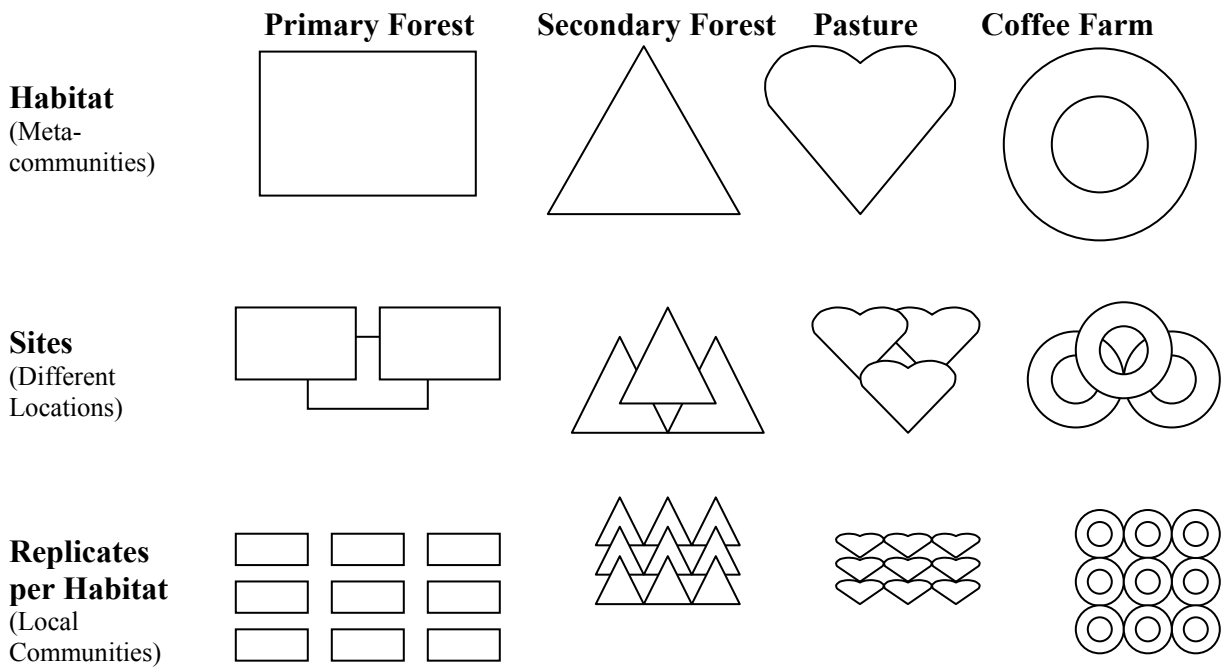


*Figure 1. Sampling protocol and experimental design. Four different habitats (meta-communities) were chosen: Primary Forest, Secondary Forest, Pasture, and Coffee Farm. Three separate sites of each habitat were located for study. At each of the three sites, three replicate samples were collected, for a total of 9 replicates per habitat or 36 replicates over all four habitat types.*

Collections were made 4-11 November 2004, during the latter half of the rainy season, a period marked with an increase in mist precipitation. All primary and secondary forest sites were located in the Ecolodge. Pasture samples were obtained from the cattle pastures of the Salazar, Ramírez, and Leitón families. Finally, non-fungicidal farmland sites were located in Finca La Bella in the coffee farms of Oldemar Leitón, Gilbert Lobo, and Álvaro Vega.

## Media

The media used for isolation and identification was a Malt Extract Agar base with rose bengal solution (0.035 g/L) to inhibit the growth of bacteria and reduce radial extension of the microfungal colonies. In order to completely prevent any form of bacterial growth, *Eritromicina MK* was added to the water used in the consequent serial dilutions (50.0 mg/L). This type of antibiotic is known to interfere with bacterial ribosome function while, at the same time, not adversely affecting the establishment of microfungal isolates. Identifications were made on the Malt Extract Agar and then isolates were transferred by hyphal tip picks and sub-cultured on nutrient agar (with 5 g/L peptone, 3 g/L meat extract, 15 g/L agar, and pH of 6.8) using the streak plating technique.

## Sample Preparation

The soil dilution plate technique (Christensen 2001) was used to isolate colonies of microfungi from the soil substrate. Five grams of soil were removed from each Ziploc bag and shaken with 125 ml of water in a watertight container. A total of two serial dilutions were made, giving final dilutions of 1:1,000, and 1:10,000. As each subsequent dilution was made, they were shaken vigorously to break up soil aggregates and ensure proper and even suspension of the substrate. Dilutions were made for each of the 36 soil samples, yielding a total of 72 dilutions. Before inoculation, each sample dilution was again shaken and the inoculate was withdrawn while the suspension was still in motion. Pipettes were sterilized with boiling water after each subsequent dilution was made to prevent contamination. Then, 1 ml of soil suspension was plated on agar plates (Cabello and Arambarri 2002). The plates were incubated at room temperature and monitored daily. Incubation time was determined by observing when colony morphology on the isolation plate was distinct and when the average colony size ranged from 20-60 isolates per plate. For this study, the incubation time was 6 days for the 1:1,000 dilution plates. Insufficient isolate numbers on a majority of the 1:10,000 dilution plates allowed for observation of microfungi, but prevented further data collection.

## Indirect Observation of Soil Microfungi

After incubation, agar plates were examined under a stereomicroscope. The number of colonies on each plate was counted. Each isolate was examined based on morphological characteristics and subsequently categorized into morphological species based on these characteristics. These ‘species’ of isolates were based on colony surface texture similarities, hyphal pigments, exudates, margin shapes, color changes in the media, and any other readily observable characteristics that made one isolate distinct from another (Azaz and Pekel 2002). The morphological species type on each plate and its correlating abundance were recorded for each replicate.

Sample populations were isolated using hyphal tip picks to nutrient agar. These isolates were incubated at room temperature for 3 days and then re-examined under the microscope to attempt to identify the microfungal species present.

### Analyses

The number of species and the number of isolates per species were recorded for each replicate using the 1:1,000 dilution agar plates. For the purpose of this study, isolate refers to each individual colony. Species abundance (n), the number of isolates of a species in a community, was determined over all three sampling levels: replicate, site, and habitat (meta-community). The Shannon-Weiner Index of Diversity (H') and evenness (E) were calculated across all three sampling levels. The Kruskall-Wallis Test was performed to compare differences in the average species richness (S), diversity (H'), evenness (E), and abundance (N), of microfungi across the 9 replicates for the four habitat types. This is a test of average local community diversity, at the level of the replicate. To compare differences in the diversity of the habitats on the level of the meta-community, H' values were contrasted using modified t-tests.

Percent abundance (number of isolates of a species divided by the total number observed in a sample) was calculated. Based on this proportion, species were ordered from the most abundant to least abundant in order to compare the relative abundance of major species occurring in more than one sample. 'Rare' species are those that were found infrequently among the isolates, and does not imply that there are few specimens of the taxon that are currently known to be in existence (Polishook et al. 1996). Finally, community composition was determined by calculating alpha (local), beta (species turnover), and gamma (regional) richness as well as the average habitat breadth over all three sampling levels.

## RESULTS

### Abundance and Diversity

A total of 17,312 isolates were recovered from the 36 soil samples, ranging from 0 to 4,244 isolates per sample. On average, the coffee farm habitat type showed the greatest abundance of microfungi, with an average of 772 isolates per sample. Pasture locations showed an average of 590 isolates per sample, secondary forest showed 502, and finally, primary forests were the least abundant, with an average of 61 isolates per sample.

The dominance-diversity distributions for all microfungi recovered from the soil samples (Figure 1) show that in the microfungal community of San Luis, only a few species were abundant (those accounting for more than 5 % of the total isolates). In fact, of all 41 species found, all but four have abundances below 1 percent. In general, samples were dominated by one morphological species, which comprised over 55 % of the total number of isolates. Most other species found were rare in the communities examined.

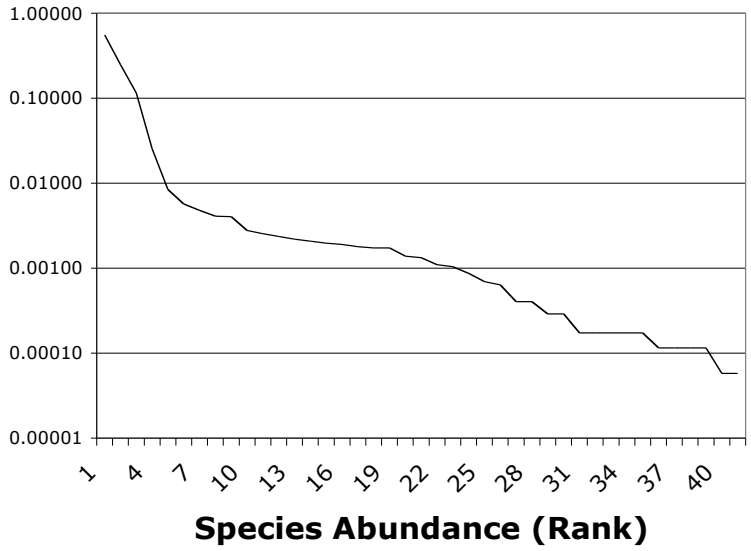


*Figure 2. Species-abundance distributions of microfungi isolated from soil samples collected from San Luis, Costa Rica. Species are ordered from the most abundant at left to the least abundant at right as a logarithm of the percentage of the total isolates of each sample.*

Figure 2 shows the dominance-diversity curves for each habitat. Again, each habitat type is characterized by a few very common species and a majority of very rare species (some of which may only have been observed one time). For example, the primary forest habitat was dominated by one species that comprised over 95 % of the total number of isolates examined. Figure 2 shows that the coffee, primary forest, and pasture habitats of San Luis all had relatively similar species dominance distributions, while the secondary forest showed a more even distribution than all of the rest. Of all habitat types, the secondary forest was the most even (E = 0.607), followed by the coffee farms (E = 0.350), pastures (E = 0.166), and primary forest (E = 0.096), respectively.

In addition to being the most even habitat type in terms of species abundance, the secondary forest was the most diverse habitat (Shannon-Weiner Diversity Index). A correlation between species evenness and diversity was observed across the four habitats. Those habitats with the highest evenness also had the highest diversity, while those with the lowest evenness had the lowest diversity. Thus, the secondary forest had the highest meta-community diversity (H' = 2.213), coffee farms followed next (H' = 1.113), then pastures (H' = 0.506), and finally the primary forest (H' = 0.314). When modified t-tests were performed to statistically compare the diversity values across habitats, all H' values were found to be statistically different (Table 1).

*Table 1. Results of the modified t-tests for comparisons between each metacommunity habitat type. All results were shown to be statistically significant*

| | t | p |
|---|---|---|
| **Primary forest to secondary forest** | 24.32 | <0.001 |
| **Primary forest to pasture** | 7.67 | <0.05 |
| **Coffee farm to primary forest** | 34.92 | <0.001 |
| **Coffee farm to secondary forest** | 27.68 | <0.001 |
| **Coffee farm to pasture** | 26.79 | <0.001 |
| **Pasture to secondary forest** | 21.83 | <0.001 |

The Kruskal-Wallis test was used to compare average local species richness, diversity, evenness, and abundance across habitats. Average local species richness was found to be statistically significant. However, average local species diversity, evenness, and abundance across habitat types were found not to be significant. Thus, while the meta-community diversity differs by habitat, the average local diversity indices do not differ.

*Table 2. Kruskal-Wallis test values comparing the average local (replicate level) species richness, diversity, evenness, and abundance. Only average local species richness was shown to be statistically significant, while diversity, evenness, and abundance were shown not to be statistically significant*

| | S | H' | E | N |
|---|---|---|---|---|
| **H** | 8.303 | 5.747 | 3.086 | 4.91 |
| **P-value** | 0.0402 | 0.1246 | 0.3786 | 0.1785 |
| **H-corrected for ties** | 8.411 | 5.755 | 3.09 | 4.913 |
| **Tied P-value** | 0.0382 | 0.1242 | 0.378 | 0.1783 |

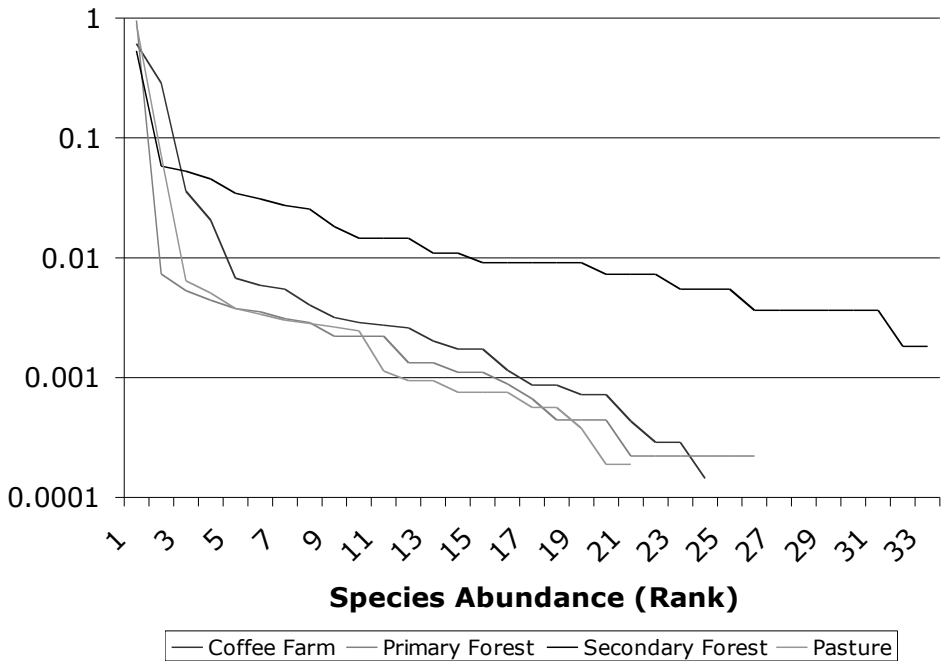


*Figure 3. Species-abundance distributions of microfungi isolated from soil samples of four habitat types of the San Luis valley, Costa Rica. Species in each habitat type (coffee farm, primary forest, secondary forest, and pasture) are ordered from most abundant at left to least abundant at right as the logarithm of the percentage of total isolates of each sample.*

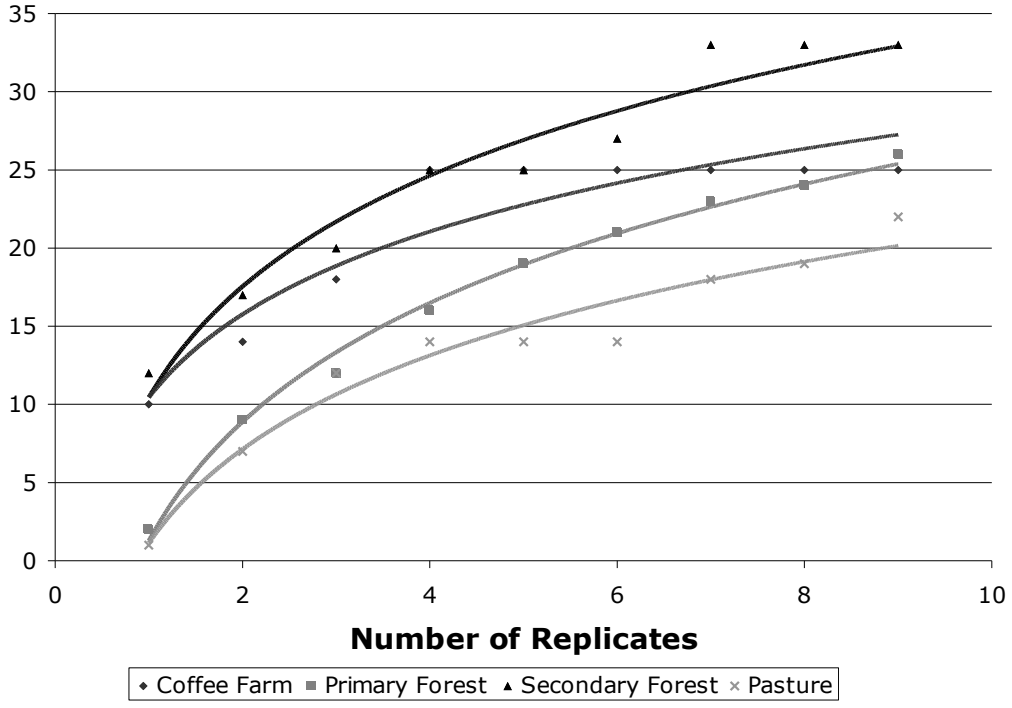


*Figure 4. Species-accumulation (sampling effort) curve for each metacommunity. Lack of asymptotes shoes that exhaustive sampling of each habitat type has not been completed.*

**Community Composition**

Community richness (local and regional), turnover, and species' habitat breadth was examined for each metacommunity type as well as for the entire San Luis valley (Table 1). Local species richness, or alpha richness, determined by the average number of species in the community, was observed to be highest in the secondary forest, followed by the coffee farms, primary forest, and pasture, respectively. In terms of gamma, or regional richness, determined by the total number of species in a community, secondary forest again showed the highest values and pasture the lowest.

*Table 3. Calculated values of species richness for each meta-community habitat sampled. Alpha values indicate local richness of the species, gamma values the regional richness, beta values the species turnover between meta-communities, and finally habitat breadth, or the amount of specificity of a species.*

| | **Alpha** | **Gamma** | **Beta** | **Habitat Breadth** |
|---|---|---|---|---|
| Coffee Farm | 16 | 24 | 0.5 | 2 |
| Primary Forest | 14 | 26 | 0.619 | 1.62 |
| Secondary Forest | 18.33 | 33 | 0.6 | 1.67 |
| Pasture | 10.67 | 21 | 0.656 | 1.52 |
| **San Luis Community** | **26** | **41** | **0.394** | **2.54** |

The pastures of the San Luis valley were found to have the highest species turnover, while coffee farms were found to have the lowest. Consequently, the habitat breadth of the pasture was found to be narrower than the other habitats and coffee farms were shown to have the widest habitat breadth.

# DISCUSSION

This study sought links between modern land use and microfungal abundance and diversity in the tropics of Costa Rica by comparing these characteristics over a human use gradient and then looking at the heterogeneity of microfungi at local and regional levels. Based on previous studies (Cabello and Arambarri 2002), it was hypothesized that locations with absolutely no human land use would exhibit the highest abundance and diversity of soil microfungi and locations constantly bombarded by human pressures would exhibit the lowest. Thus, primary forest habitats were predicted to have the highest diversity, followed by secondary forests, pastures, and finally, coffee farms were proposed to have the lowest diversity due to their highest level of land use. However, the results obtained in this experiment reject this hypothesis for the microfungal community of the San Luis valley. Secondary forest sites were actually found to be the most diverse in this study, while primary forests, although proposed to be very diverse, were found to be the least diverse of all habitats sampled. There are a few plausible explanations for these findings: (1) disturbance may actually facilitate microfungal diversity instead of hindering it, (2) extreme patchiness and heterogeneity of microfungi over local scales

may disguise major regional habitat trends, and (3) inadequate sampling may not give a good picture of the entire community.

Intermediate disturbance levels may actually promote more microfungal diversity than do either extremely high levels of disturbance or absolutely no levels of disturbance. This could explain why secondary forests are the most diverse, followed closely by the coffee farms. It may be that disturbance brings about rapid adaptation in the soil types, causing microfungal species specialization and tremendous niche diversification to prevent inter-specific competition. Also, diversity in moderate to highly disturbed soil increases the functional redundancy of the fungal community by providing a buffer to the loss of individual associates (Hawksworth 1991). Thus, the ecosystem is afforded more resilience and the loss of one species will likely not cause a crisis within the community. Primary forests with undisturbed soil have a fairly predictable, stable environment and thus may have no need to diversify to the same extent. Therefore, a few species can more easily dominate the community since it is not probable that rapid change will occur that will necessitate quick adaptation.

Species composition on a local scale also could have impacted the observed results. The San Luis microfungal community was marked by a high degree of extremely localized patchiness of both abundance and diversity. As a whole, the mycota appears to be highly varied and extensive. For example, the H' values at the level of the replicate in the coffee farms ranged from 0.067 to 2.14. Such a large diversity range among replicates was typical of each habitat type, not of just the coffee farms. In fact, such variation between replicate samples most likely caused diversity, evenness, and abundance to be found not to be significant with the Kruskal-Wallis test (Table 3). By comparing the average local richness at the level of the replicate, this test could not take into account the over-riding trends that occurred at the meta-community level. Conversely, the modified t-test contrasted the H' values by comparing the total species richness for each meta-community. Thus, a more holistic view of diversity in the meta-communities was achieved.

When looking at community composition in terms of local species richness, the replicate level of sampling was equally heterogeneous. However, some interesting trends were observed at the level of the meta-community habitat type in beta values (Table 3), which indicate the species turnover between sites. Although the pasture habitat exhibited the lowest values of local and regional richness, it showed the highest amount of species turnover. This means that the pasture species of microfungi are more habitat specific and that there are more differences between this habitat and surrounding communities. Pasture also showed the smallest habitat breadth, signifying that species found in the pasture were most often found only in the pasture and not in other habitat types. The community composition of secondary forest again proved to be the most heterogeneous, exhibiting high levels of local and regional richness. Surprisingly though, secondary forest displayed lower levels of species turnover (beta richness) than the pasture and primary forest communities. This signifies that both pasture and primary forest habitats are more specialized and habitat specific in terms of richness, even though the secondary forest species are more diverse.

Another interesting trend in community composition is observed when looking at the San Luis microfungal community as a whole and combining all four meta-

communities. Species turnover in the entire microfungal community was lower than the species turnover observed at each individual habitat type (beta = 0.394). This implies that species on a broader geographical scale are more specialized and more habitat specific. The community composition is very heterogeneous and similarity between habitats is low. Another interesting observation was in the habitat breadth of the entire San Luis valley microfungal community, which exhibited a wider habitat breadth than each individual habitat type ( = 2.54). Thus, considering the community as a whole, the San Luis microfungi tend to be very habitat specific even though species from one habitat may be found in one or more other habitat types.

This study found that while local heterogeneity is extremely varied, community differences across habitats in terms of species richness and diversity may not be as wide-ranging. The degree of substrate preference of microfungi was not as highly varied across a human land use gradient as it was across different locations within each habitat. Therefore, collectors of fungi in the San Luis valley can increase the total number of species in their samples and the certainty of their data by collecting soil samples from closer sites and adding more replicate samples. Although the dominance-diversity curves (Figures 1 and 2) are characteristic of those of microfungal isolates in tropical communities (Polishook et al. 1996), it is believed that the diversity of each habitat is even more varied. Sampling in this study was not complete, as can be seen in the lack of asymptote in the species-area curve (Figure 3). This indicates that the microfungal community is extremely diverse and it has not been exhaustively sampled. However, inadequacy in sampling explains why the community turnover is so great not only between different habitats, but also between replicate samples. The microfungal diversity is amazingly high in the San Luis community and this can and should be appreciated at all spatial scales. More testing with adequate sample sizes and closer locations is important to truly obtain a real assessment of the microfungal fauna of the San Luis valley.

## ACKNOWLEDGMENTS

First, I would like to thank my advisors, Javier Méndez and Karen Masters, for humoring me and letting me do the project that I had my heart set on, even though you thought I was absolutely crazy. Special thanks to Javier for the late nights spent in the station kitchen cooking the soil samples and sterilizing the pipettes. Karen and Alan, you are absolutely amazing for taking the time to pick up my plates in San José and for dragging them all the way back to Monteverde. Also, many thanks go out to little Emmanuel and Floribeth Leitón for acting as my assistants during my stressful collection days in San Luis and thanks to Nathan and Zaida of the EcoLodge for their guidance and assistance in trying to find some of the last remaining patches of primary forest in San Luis. Thanks to Amy Rossman, for being an absolutely fabulous mycologist and going out of her way to help me out so that I could get the information needed to get my project off of the ground. Ollie, thanks for reading over my draft and inspiring me to work with fungi by making the field experiment so fun. Finally, an EXTRA special thanks to those who actually believed in me and my ability to pull this crazy project off! I couldn't have done it without you.

## LITERATURE CITED


Azaz, A. and O. Pekel. 2002. Comparison of Soil Fungi Flora in Burnt and Unburnt Forest Soils in the Vicinity of Kargicak (Alanya, Turkey). Turkish Journal of Botany. Vol. 26, pp. 409-416.

Bills, G. 1995. Analyses of microfungal diversity from a user's perspective. Canadian Journal of Botany, Vol 73 (suppl.1): S33-S41

Cabello, M. and A. Arambarri. 2002. Diversity in soil fungi from undisturbed and disturbed *Celtis tala* and *Scutia buxifolia* forests in the eastern Buenos Aires province (Argentina). Microbiological Research. 157(2), pp. 115-25.

Christensen, M. 2001. The Dilution Plate Technique for Isolation of Soil Microfungi. http://carroll1.cc.edu/~jclausz/msamanual/diltq.html

Hawksworth, D. 2002. "Why Study Tropical Fungi?" Tropical Mycology, Vol. 2. pp. 1-10.

----------1991. "The fungal dimension of biodiversity: magnitude, significance, and conservation." Mycological Research. 95(6), pp. 641-655.

Nee, S. 2004. More than meets the eye. Nature, Vol. 429: 804-805.

Polishook, J., G. Bills, and D. Lodge. 1996. Microfungi from decaying leaves of two rain forest trees in Puerto Rico. Journal of Industrial Microbiology, Vol. 17: pp. 284-294.

Rossman, A. *Microfungi: Molds, Mildews, Rusts, and Smuts.* United States Department of Agriculture, 2002. pp.63-70.

Volk, T. 2001. Encyclopedia of Biodiversity, Vol. 3. Academic Press, NY.