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Can the location of the isolation laboratory affect the generation of myxomycete data using moist chambers? An experiment in the Neotropics

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Abstract

The moist chamber technique, as it is used in myxomycete research, is an extremely useful method to optimize project resources. However, as it occurs with any laboratory-based protocol, the usefulness of this technique should be evaluated using contextual elements, most of which, are lacking in the literature. The present study evaluated the results obtained using the moist chamber technique with the same substrate material, synchronously, in two different laboratories in Costa Rica. Using climate data obtained at both isolation localities and microclimate data obtained directly from the moist chambers, an analysis of differences in results was attempted. Even though variations in results are intrinsic to biological surveys, the results presented herein suggested that some recorded differences could have been linked to the air humidity and the temperature of the moist chamber culture. Even though the two laboratories were only 30 km apart, an average difference of 8.5% in the values of the diversity indices was observed. Also, between 15% and 19% of the species recorded in the total dataset were only observed in one laboratory. In this manner, the present study is useful to keep in mind that myxomycete results using the moist chamber technique, can be extremely influenced by variables that are usually not considered in the general application of the protocol. Perhaps it is time to work on an optimized version of such useful technique.

Key words – Costa Rica – moist chamber culture technique – Neotropics – protocols – slime molds

Introduction

The moist chamber culture technique (Gilbert & Martin 1933) has been used extensively, for nearly a century, to generate biodiversity and ecological data sets for myxomycete research (Alexopoulos 1964, Wrigley de Basanta & Estrada-Torres 2017). As a widely accepted method to document myxomycete occurrence, moist chamber cultures have repeatedly been used to complement the results obtained from field-based surveys (Stephenson 1985). A morphologically based myxomycete species inventory generated for a given locality would be considered inadequate, by modern standards, without the use of the moist chamber culture technique to bolster the records

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obtained from successive field surveys conducted across a location's variable seasons (Novozhilov et al. 2019).

The moist chamber culture technique provides researchers several advantages which have contributed to its sustained use for developing myxomycete inventories and its increasing popularity for testing ecological hypotheses. The technique is simple, inexpensive, easily adaptable to the supplies available to researchers, and effective at inducing several species of myxomycetes to produce fruiting bodies; as such, a standardized protocol could easily be universally implemented. The technique, through use of a dissecting microscope, enables researchers to document species that produce minute fruiting bodies which are almost never documented directly in the field. Furthermore, since the collections develop at a later time (in the lab), the impact of the irregular and ephemeral nature of fruiting body formation in natural settings is minimized, thus permitting increased flexibility in scheduling site visits. Collectively, these benefits compensate the monetary investment associated with the logistics of field trips by letting researchers develop concentrated sampling efforts that focus on target material, specific ecological conditions, or understudied localities.

The myxomycete life cycle (Alexopoulos 1964) produces large numbers of different types of dispersal propagules and resistant stages which likely contribute to the high rate of success typically experienced with the moist chamber culture technique (Schnittler & Stephenson 2000, Rollins & Stephenson 2016). However, as is the case with any method used to generate primary information on microscopic organisms, the moist chamber technique has many limitations such as competing organisms (e.g. fungi and insects), potential to favour non heterothallic isolates, and the time commitment required to observe cultures over a period of months (see Wrigley de Basanta & Estrada-Torres 2017). Species that produce larger fruitifications and require longer developmental times rarely (if ever) develop in moist chamber cultures. Furthermore, the environmental parameters in the culture likely fail to emulate natural conditions and could allow propagules that are present, but would have never developed under natural conditions, to be represented in the data set.

The moist chamber culture technique essentially creates a biological microcosm with parameters that could be heavily dependent on an unquantifiable number of unknown conditions and thus represents a type of chaotic complex (see the observations about commitment to forming fruiting bodies in agar culture from Seifriz & Russell 1936). In this manner, any of the elements of the system (most which are unknown, undocumented, and unquantifiable) could potentially modify the outcome (e.g. the number and type of fruiting bodies observed) by modulating the biotic and abiotic dynamics within the culture. Among these elements, the dimensions and volume of the chamber; the origin, type and history of the material used for isolation; the mass relationships between substrate, water and the paper used to retain humidity and the environmental conditions of experimentation could substantially impact the results obtained.

Despite these known challenges, there has been little in the way of comprehensive studies aimed at quantifying the magnitude of the impacts of the potential pitfalls associated with utilizing the moist chamber culture technique. Researchers know that taxonomic data obtained from moist chamber cultures are skewed by favouring some forms (see Alexopoulos 1964, Stephenson et al. 2004, Rollins & Stephenson 2016). Several species, which can be present at a locality, rarely produce fruiting bodies in moist chambers and would likely never be documented in the absence of comprehensive field surveys. Since moist chambers are typically incubated for a period of two to four months (the variability among researchers and projects is appreciable) some of the data (i.e. species producing fruiting bodies) could be the result of cross-contamination or airborne propagules inoculating the cultures in the lab during the study. The problem with these issues is that they are difficult to control and trying to do so could be counterproductive to the process of data generation, which is a priority for documentation.

In a similar manner, the environmental conditions (e.g. temperature, humidity, air flow rate, amount/intensity/duration of incoming solar radiation, barometric pressure etc.) associated with the laboratory (where the cultures are incubated) have the potential to impact the data (e.g. the species that develop) obtained from the moist chamber culture technique. As such, the results obtained from moist chamber cultures could potentially be somewhat skewed as a result of the location and

associated parameters of the laboratory setting (see Blackwell & Gilbertson 1984 for results at different temperatures). Despite these possibilities this situation has been poorly studied even though these conditions could be relatively easy to quantify, modify, and regulate. Quantifying and characterizing the magnitude of these potential impacts is necessary to validate the usefulness and reproducibility of the moist chamber culture technique as well as evaluating the feasibility of comparing and contrasting such data sets across locations. Currently, empirical data of this nature are lacking. As such, the present study represents a simple experiment intended to evaluate the impacts of the macro- and microenvironmental parameters of both the laboratory location and the moist chamber culture on the myxomycete data obtained using this technique. For general biodiversity monitoring purposes, where easy-to-implement methods are necessary, the evaluation of these aspects is necessary in order to develop a standardized protocol appropriate for generating reliable data that can be compared and contrasted across localities and used to test ecological hypotheses.

Materials & Methods

The present study was carried out in Costa Rica during 2017. For this investigation, two different Lower Premontane Wet Forest (PWF) localities on opposite flanks of the Turrialba Volcano (3420 m) and one Upper Premontane Moist Forest (PMF) locality on the easternmost section of the Central Valley were selected (Fig. 1). Myxomycetes in these forest types are very well studied in Costa Rica and bioclimatic differences among them are based on higher precipitation in wet (~3000 mm rain/year) than in moist forests (~1500 mm rain/year). The former type occurs in the Caribbean slope at intermediate elevations (400-800 m asl) and dominant trees are in the Fabaceae, Lauraceae and Moraceae families; whereas the latter occurs in the higher elevations of Costa Rica's Central Valley (1000-1400 m asl) and is dominated by *Cupressus lusitanica*. As such, the localities for substrate collection corresponded with the Centro Ambiental Manú in the northern Caribbean region (abbreviated as PWF-NC, ~550 m, 10.157249/-83.781667), the Finca Experimental Interdisciplinaria de Modelos Agroecológicos in the southern Caribbean section (PWF-SC, ~625 m, 9.865648/-83.636507), and the Mirador de Orosi, a public park, (PMF-CV, ~1270 m, 9.817658/-83.858607), in the Central Valley.

At each locality, 60 samples of twigs (TW) and 60 samples of ground litter (GL) were collected on June 25, 2017 for a total of 360 samples from all three sites. As conceptualized herein, twigs were decomposing pieces of branches or plant shoots on the ground with diameters less than 1.5 cm and ground litter referred to decomposing plant leaves on the forest floor. All samples were individually collected and wrapped in paper bags, and no mixing of samples took place. In this manner, half of the material from each locality and substrate type (i.e., 30 samples of twigs from a single site) was immediately taken to a laboratory located near the PWF-SC site (Turrialba Laboratory), whereas the other half was taken to a laboratory located near the PWF-NC (Manú Laboratory). The two laboratories were linearly distanced by only 30 km, but due to the presence of the Turrialba Volcano (3340 m asl) between them (see Fig 1B), they were located in two slightly different climatic regions of the Costa Rican Caribbean.

In each laboratory, all 180 corresponding samples were used to make moist chamber cultures in the manner explained by Stephenson & Stempen (1994) using approximately the same amount of material for each of them. For control purposes, all petri dishes used in both laboratories (100x15 mm) were new and sterile, taken from the same box, and lined with filter paper of the same kind and brand. All moist chamber cultures were set up on June 26 and pH values were determined after one day using a Hach SensION MM110 portable pH meter. The moist chambers were placed on a lab bench and incubated at room temperature for 12 weeks, in areas with light intensity up to 1200 lux (~200 lux average), and checked only three times on July 24, August 21, and September 18, for standardization of recording times. The present study originated from the need to standardize the MC methodology, and for that, it required a standard system of recording the object of study. Hence, the three checking periods are certainly sufficient to the answer the objective of the present investigation. After all, whatever effect the checking periods would have, it will be true for all set-ups. The frequency of

checking was also established that way to ensure capturing species as they developed and before fungi would set in so that they could be identified properly and in a standard manner. All myxomycete records represent fruiting bodies that developed in culture and were identified using the morphological species concept and the scientific names according to the nomenclature of Lado (2005–2020). Some material was collected and deposited in the Myxogastrid Repository (INII) of the University of Costa Rica.

The temperature, atmospheric humidity, and barometric pressure of each laboratory was recorded for the duration of the study using both Onset HOBO U12-012 automatic loggers and Ambient Weather WS1173A microstations. The associated precipitation of each locality was also recorded using Onset RG3-M pluviometers. Additionally, the temperature and humidity within the moist chamber cultures were recorded three times during the period of study on a subset of 36 moist chambers (20% of total batch) per laboratory, prior to scanning the cultures, using an Etekcity Lasergrip 1080 infrared thermometer and a GoerTek TK-W moisture meter.

The myxomycete species presence data, obtained from the moist chamber cultures, were used to calculate Shannon´s and the Simpson's (1-D) Indices of Diversity as well as the Taxonomic Diversity Index (TDI = number of species/number of genera) for each laboratory. In myxomycete studies, both diversity indices have been used as metrics of biological diversity and were calculated herein for intra-study purposes and across-studies comparisons. The TDI is a metric that allows researchers understand intrageneric diversity, which is useful to address ecological effects such as resource utilization and niche dynamics. Additionally, the number of positive moist chambers (i.e., those showing evidence of myxomycete activity in the form of fruiting bodies or plasmodia), the number of species, and the number of records were calculated for each laboratory. Furthermore, the number and identity of species developing exclusively at one laboratory were also calculated. Collectively these biological estimators were used to compare the results obtained between the two laboratory locations.

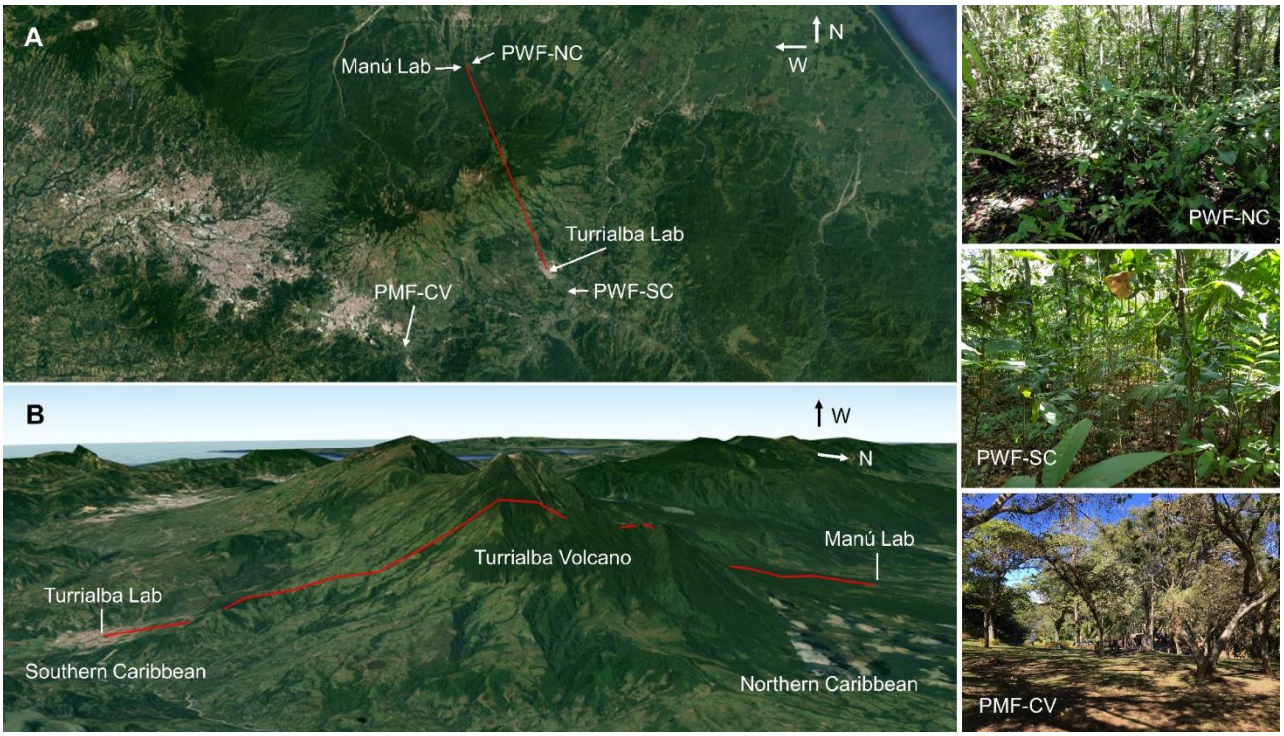

Fig. 1 – The geographical characteristics and general aspect of the three localities from where substrate samples were collected in relation to the Turrialba Volcano and the position of the two laboratories. A Aerial view illustrating the linear distance between the two laboratories (red line) and the relative position of the Central Valley (Metropolitan Area) and the Caribbean Ocean. B Side view, looking west, of the same as in A. For abbreviations see Materials & Methods.

The data for all macro- and microenvironmental parameters were evaluated to see if they reasonably approximated a normal distribution by using the Shapiro Wilk test (see Gerald 2018). Statistical hypothesis testing of the differences for these parameters between laboratory locations was accomplished using standard independent t-tests. The same test was used to evaluate differences for both the Shannon´s and the Simpson's Indices of Diversity between the laboratory locations. The recommendation of Schroeder & Jenkins (2018) was followed and both the Jaccard (presence-absence) and the Bray-Curtis (abundance) similarity indices were calculated (1 = same dataset, 0 = completely dissimilar) to test for differences in results between the two laboratory locations. These calculations were completed for both laboratories at the first and the third check-ups using the software PAST, V4.01 (Hammer et al. 2001). As the study did not seek to look at the timing of species development, only the first and last checkups were compared to see any differences between the two results.

Results

A total of 268 myxomycete records representing 26 species in 10 genera were observed when the data were pooled from the two laboratory locations. From this, 151 records representing 22 species in 9 genera were observed in the Manú Laboratory and 117 records representing 21 species in 10 genera were recorded in the Turrialba Laboratory. The percentage of moist chamber cultures with myxomycete activity was the same for both laboratories, but the Shannon's and Simpson´s Indices of Diversity differed between the two laboratories (Table 1). The calculated Jaccard Index between the two laboratories was 0.65 and the Bray-Curtis Index was 0.74. With the exception of moist chamber humidity and accumulated precipitation, both the overall climatic conditions and the moist chamber microclimatic conditions were different between the two laboratories.

Table 1 Summary statistics – mean (standard deviation) – for the macro/microenvironmental parameters and biological estimators for the Manú and Turrialba laboratory locations. The probability values (p values) associated with the respective t-tests are shown with "*" indicating a statistically significant difference ($\alpha = 0.05$) between the two laboratories

Parameter	Laboratory		p value
	Manú	Turrialba	
Microenvironmental			
Moist chamber pH, pH scale	7.2 (0.3)	7.1 (0.2)	p = 0.02*
Moist chamber temperature, °C	22.3 (1.1)	28.6 (0.9)	p < 0.0001*
Moist chamber humidity, %	79.8 (9.9)	83.3 (16.4)	p = 0.19
Macroenvironmental			
Air temperature, °C	22.6 (2.3)	24.9 (3.9)	p < 0.0001*
Air humidity, %	87.6 (4.1)	51.7 (18.7)	p < 0.0001*
Atmospheric pressure, millibars	955.0 (0.9)	943.1 (2.2)	p < 0.0001*
Accumulated precipitation, mm	669.8 (7.9/day)	606.2 (7.2/day)	p = 0.96
Biological			
Shannon's Index of Diversity	2.62	2.32	p = 0.007*
Simpson's Index of Diversity	0.90	0.84	p = 0.004*
Taxonomic Diversity Index	2.01	1.96	n/a
Number of examined moist chambers	180	180	n/a
Percentage of positive moist chambers	76 (137/180)	76 (137/180)	n/a
Number of species	22	21	n/a
Number of records	151	117	n/a
Number of unique species	5	4	n/a

Both laboratories yielded a similar number of unique species (Table 2). *Arcyria afroalpina*, *Comatricha elegans*, *C. nigra*, *Didymium clavus* and *Physarum cinereum* developed only at the Manú

Laboratory, whereas *Clastoderma debaryanum*, *D. nigripes*, *Perichaena depressa* and *Ph. superbum* developed only at the Turrialba Laboratory. Of those species, *D. nigripes* was only recorded during the first checkup, whereas *P. depressa*, *Ph. cinereum* and *Ph. superbum* were only recorded during the last checkup. No species unique to a single laboratory location were recorded during the second checkup, and most species only recorded during the first checkup were found in the Turrialba Laboratory. For the Manú Laboratory, the calculated Jaccard Index between the first and the third checkup was 0.59 and the Bray-Curtis Index was 0.68. For the Turrialba Laboratory, these values were 0.54 and 0.55, respectively.

Most shared species between laboratories were recorded at a similar percentage distribution (average of 52% of observations in Manú vs. 48% in the Turrialba) with the exception of *D. difforme*, *D. squamulosum* and *Lamproderma scintillans*, with unbalanced ratios of observation. In all these cases, the number of records in the Manú Laboratory exceeded 75% of the total number of observations. Interestingly, most shared species were slightly more frequent in the same laboratory. Only *Ph. compressum*, *Ph. didermoides* and *Ph. pusillum* were slightly more frequent in the Turrialba Laboratory.

Three more species were recorded on twigs than on ground litter. The PMF-CV locality yielded 16 species, whereas both of the lower elevation localities yielded 19 species each. As expected, some species were only recorded in one substrate or in one collecting locality. Notable examples were *Clastoderma debaryanum*, *C. tenerrima*, *Ph. superbum* and *Stemonitis fusca*, only recorded on twigs; or *A. afroalpina*, *D. clavus*, *Ph. didermoides* and *Ph. galbeum*, only recorded on ground litter. Similarly, *Cribraria violacea*, *P. corticalis* and *Ph. didermoides* were only recorded in material from PWF-SC, *D. nigripes* and *Ph. galbeum* only found in PWF-NC, and *P. depressa* was only recorded in PMF-CV.

The number of remaining plasmodia in the moist chambers was higher in the Turrialba Laboratory than in the Manú Laboratory, with a 6 to 4 ratio (49 to 33). For substrates, the observed relation of remaining plasmodia was 7 to 3 (55 to 27) for ground litter over twigs. For substrate origin, the relation was similar among all locations, with PWF-SC showing a smaller number of remaining plasmodia than the others. Except for *P. depressa* (2 records) and *D. nigripes* (1 record), all other unique species associated with one experimental laboratory, were recorded on material from more than one locality.

Discussion

All of the species recorded in the present study are common findings in Costa Rica for the respective forest types (see Rojas et al. 2018). As such, no extraordinary records were made herein. This is important to note because for a well-studied country like Costa Rica, with 242 species of myxomycetes recorded, this is exactly what would be expected using the moist chamber culture technique with typical substrates like ground litter and twigs. In this sense, the usefulness of the moist chamber technique for reliable data generation should be highlighted since the results obtained in the present study are, to some extent, representative of the substrate and forest types studied (see Alexopoulos 1953 as well for comments on the technique in temperate areas).

However, there were differences in the results obtained from the moist chamber cultures when the data from the two laboratories were compared. The ecological variables calculated with the records were all higher in the Manú Laboratory than in the Turrialba one, and differences were observed (average of 8.5% higher in Manú) in the indices of diversity. Also, taxonomic differences in the makeup of the datasets were clearly observed in both the Jaccard (0.65) and Bray-Curtis (0.74) indices as well, since they showed that similarities accounted for two thirds or three quarters of the presence-absence and abundance data, respectively. These values indicated that differences in the results obtained herein, even though they may look minor, were large enough (one third or one quarter) to be quantified.

Interestingly, such differences in dataset makeup were also observed between the comparisons made within each laboratory, when the first and the last check-ups were compared. The observed differences between the laboratories could represent the natural variability associated with the moist

chambers (see Härkönen 1981, Schnittler & Stephenson 2000 for context on this variability), with one laboratory setting favouring some species to form sporocarps while the other did not. For instance, at the end of the study period, the moist chambers in the Turrialba Laboratory showed about 33% more remaining plasmodia than those in the Manú Laboratory. Since these remaining plasmodia, as well as most common species, were independent of the substrate origin, results showed than within each laboratory, moist chambers were subjected to similar conditions. In simple words, differences in the results could have been driven by the interaction of laboratory conditions and the particularities (i.e. response to stimuli) of the different species. Similarly, to address future studies, it would be worthwhile to study the frequency of recording myxomycetes in the moist chambers as well.

Table 2 The number of myxomycete records arranged alphabetically by species name. For abbreviations see Materials & Methods

Species	Laboratory		Substrate		Original locality of the material			Records
	Manú	Turrialba	GL	TW	PWF-SC	PWF-NC	PMF-CV	
Arcyria afroalpina	4		4			2	2	4
Arcyria cinerea	29	33	15	47	28	17	17	62
Clastoderma debaryanum		3		3		2	1	3
Comatricha elegans	2			2		1	1	2
Comatricha nigra	4		1	3	2	1	1	4
Comatricha pulchella	9	6	4	11	1	7	7	15
Comatricha tenerrima	4	4		8	7	1		8
Cribraria violacea	1	1		2	2			2
Didymium bahiense	21	14	13	22	12	14	9	35
Didymium clavus	4		4		1	3		4
Didymium difforme	4	1	4	1	3	2		5
Didymium nigripes		1		1		1		1
Didymium squamulosum	11	3	13	1	5	4	5	14
Echinostelium minutum	2	1	2	1	1	1	1	3
Lamproderma scintillans	6	2	4	4		6	2	8
Perichaena chrysosperma	18	13	18	13	23		8	31
Perichaena corticalis	4	4	7	1	8			8
Perichaena depressa		2	1	1			2	2
Perichaena minor	1	1	1	1	1	1		2
Physarum cinereum	2		1	1	1		1	2
Physarum compressum	12	15	23	4	7	1	19	27
Physarum didermoides	1	2	3		3			3
Physarum galbeum	1	1	2			2		2
Physarum pusillum	1	2	2	1	1		2	3
Physarum superbum		2		2	1	1		2
Stemonitis fusca	10	6		16	1	11	4	16
Non identified plasmodia	33	49	55	27	21	33	28	82
Total number of records	151	117	122	146	108	78	82	268

Such observation is not striking and is largely logical beyond the limitations of this study. However, the tacit implication herein is that the moist chamber culture technique, as a microcosm used to obtain myxomycete presence-based data, could be highly susceptible to the external conditions exerted by the laboratory. The data presented in this study, showed that between 15% and 19% of the species recorded in the total dataset were only observed in one laboratory. Based on the observation that most of the unique species in the Turrialba Laboratory were only recorded during one of the check-ups, it seems that such potential influence of the external conditions is very finely directed on the myxomycetes inside the microcosm (i.e. Blackwell & Gilbertson 1984).

In the present investigation, objectively, differences could have been driven by several unknown factors. However, the recorded differences in both the location climate and the microclimate of the moist chambers, which could have also affected results, cannot be ruled out as

significant drivers. Of those variables, air humidity and moist chamber temperature seemed to have been important parameters. The former is particularly interesting because, as observed in the results, the Turrialba Laboratory had dryer air, but no differences were observed in moist chamber humidity. These results likely imply that moist chambers in this laboratory dried more rapidly and water had to be added more often to keep adequate moisture levels. The latter was not different between laboratories simply because it was one of the most controlled variables in the study, but the frequency of water addition could have differentially impacted the development of certain myxomycete species favouring one over the other (see Härkönen & Ukkola 2000 for a similar conclusion). Similarly, the higher microcosm temperature in the Turrialba Laboratory likely accelerated the water loss process, also increasing the frequency with which water had to be added to maintain moisture levels.

Using these arguments, it seems that *Arcyria afroalpina, Comatricha elegans*, *C. nigra*, *Didymium clavus* and *Physarum cinereum* were favoured by the steadier microclimate in the Manú Laboratory (see the standard deviation values for variables at both laboratories), whereas the presence of *Clastoderma debaryanum*, *D. nigripes*, *Perichaena depressa* and *Ph. superbum* was facilitated by the frequent fluctuations present in the Turrialba Laboratory. Similarly, species such as *D. difforme*, *D. squamulosum* and *Lamproderma scintillans* may have simply performed better in a steadier climate but were able to cope with variability in conditions. pH could have been a factor of some importance, but the differences recorded herein were so small that it seemed unlikely. The higher capacity of twigs relative to ground litter to desiccate at a slower rate along with a stronger myxomycete specificity for that substrate than for leaves on the ground (see Stephenson et al. 2008), could have played a role in the lower number of remaining plasmodia at the end of the experiment.

The similarity of the species recovered from the current study to what would be expected for the sampled forest types and substrates (based on an extensive body of literature and the experience of the first author) indicate that the results from the current study fell within expected floristic parameters (see Härkönen & Koponen 1978 or Stephenson et al. 2008 for substrate considerations). As a floristic data generator, the moist chamber technique appears to be adequate (Härkönen & Ukkola 2000, Schnittler & Stephenson 2000). However, such observation is independent of both the completeness and robustness of data acquisition, which are necessary to compare and contrast data sets across localities and studies. For practical purposes, the latter two could be greatly improved if the moist chamber technique is understood as a dynamic chaotic-like system that relies heavily on external conditions. For the purpose of testing ecological hypotheses, standardizing the moist chamber culture technique (i.e. using standard temperature and humidity levels) would be ideal. Furthermore, simply accounting for external effects would help contextualize the significance of the results and associated patterns (see Levy et al. 2014). As observed in the present study, some species of myxomycetes seem to form fruiting bodies more frequently with either steadier or more fluctuating internal conditions. Such considerations would increase the reproducibility of the moist chamber technique for purposes other than the generation of biodiversity-level data.

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