Microb Ecol (2011) 62:361–373
DOI 10.1007/s00248-011-9843-6

FUNGAL MICROBIOLOGY

# Microhabitat and Climatic Preferences of Protosteloid Amoebae in a Region with a Mediterranean Climate

**María Aguilar · Frederick W. Spiegel · Carlos Lado**

Received: 16 November 2010 / Accepted: 2 March 2011 / Published online: 22 March 2011


**Abstract** The role of microhabitat and climate variation in structuring protosteloid amoebae communities has been investigated for the first time in the Mediterranean Basin, a biodiversity hotspot for plants and animals and the largest of the world's five areas with a Mediterranean climate. Abundance data were obtained from natural substrates collected in 13 localities from central Spain, and a total of 1,504 colonies and 18 species were recorded. For this new area, it has been carried out an optimization of the culturing effort based on rarefaction analyses, thus making possible to adapt the protocol to the objectives in future research. Canonical correspondence analysis and generalized linear models showed that microhabitat type was the most important factor for differentiating the niches of the species studied, but climatic variables, especially minimum temperature of the coldest month, precipitation seasonality, and temperature range, had secondary but also important effects. Bark inhabitants tend to be more abundant in localities with high temperature range and low annual precipitation. Aerial litter was the microhabitat with the highest species richness, abundance, and evenness. Species typical of this microhabitat are more abundant when there is high precipitation, low temperature of the warmest month, and low minimum temperature of the coldest month.

M. Aguilar (✉) · C. Lado
Departamento de Micología, Real Jardín Botánico, CSIC,
Plaza de Murillo 2,
28014 Madrid, Spain
e-mail: aguilar@rjb.csic.es

F. W. Spiegel
Department of Biological Sciences, University of Arkansas,
Fayetteville, AR 72701, USA

## Introduction

Protosteloid amoebae, formerly called protostelids, are a diverse group of slime molds in the eukaryotic supergroup Amoebozoa [1, 31]. They produce simple, stalked fruiting bodies, known as sporocarps [23, 31, 33, 34]. The sporocarps always consist of a single acellular stalk and one to a few spores, but there is also a trophic stage that varies from uninucleate amoeboid and/or amoeboflagellate cells to multinucleate reticulate plasmodia [23, 31, 34]. All known protosteloid species are heterotrophic microorganisms and act as predators on terrestrial decomposers such as bacteria, yeasts, and spores of filamentous fungi [36]. Despite their morphological similarities and common lifestyle, recent molecular data suggest that they may have polyphyletic origins within the Amoebozoa [10, 31]. They can occur on many different microhabitats, such as dead aerial plant parts, bark, leaf litter, and soil [23, 36].

The ecology of this group has not been studied until recently [34], with most works focused on comparisons of species assemblages from different microhabitats at a local scale. A microhabitat is a small, localized habitat within a larger ecosystem, having conditions that sustain a limited range of organisms that form a distinct community. At different latitudes, species appear in samples from different microhabitats, and their relative abundance changes [21]. Though it has been pointed out that elevation and latitude could cause changes in species composition in a given microhabitat [21], the underlying influences of climatic factors have not been disentangled. Several studies have been made throughout the world in temperate areas [2, 3, 5, 15, 17, 18, 29, 30, 39], tropical regions [16, 19, 21, 24, 37], polar regions [20, 35], and aquatic environments [14, 40], but studies of protosteloid amoebae communities at a large scale

have not been made, due in part to the lack of comparable datasets.

In spite of all efforts, there are still many gaps in our knowledge of the distribution of protosteloid amoebae. No studies have taken place in a region with a Mediterranean climate, characterized by hot dry summers that contrast with cyclonic rains in winter [41]. There are five areas in the world with this kind of climate, all of them biodiversity hotspots for plants and animals [4] and located in the Mediterranean Basin, California (USA), parts of central Chile, the Cape region of South Africa, and areas in the south and southwest of Australia. For this study, we have selected the central area of Spain, in the Mediterranean Basin, to check if this area also harbors a high diversity of these organisms, and to provide an analysis of the diversity and ecology of protosteloid amoebae in this kind of climate. Spain has previously proved to be an excellent location for other groups of slime molds, such as dictyostelids [26] and myxomycetes [12, 13].

The objective of this paper is to report the differences in species composition and relative abundance of protosteloid amoebae between microhabitats, especially with respect to evaluating the influence of different climatic factors on these parameters. As these organisms have never been studied in localities with a Mediterranean climate and previous information about their ecology is limited, the sampling method has been emphasised, not only to test and find the optimum effort needed for the particularities of these areas but also to provide a more quantitative, statistical method that will allow comparison between different ecosystems in further studies. This optimization can be used in the future for designing new experiments in similar areas, adapting the effort to the objectives of the research.

## Material and Methods

### Sampling and Culturing

This study is based on material collected during two sampling efforts in 2006 and 2007 in two areas of central Spain (Fig. 1). Samples were collected in a total of 13 localities (Table 1), all georeferenced with a Garmin GPS 16, datum WGS 84, located in well-conserved areas between 40–41° N and 2–5° W in a range of altitude between 500 and 900 m and with different vegetation types. The first sampling (localities 1–7) took place in a region called "Alcarria." It extends principally over the province of Guadalajara but also enters Cuenca and Madrid. The second sampling (localities 8–13) comprised different locations in the west of the province of Madrid and the provinces of Toledo and Avila, in a natural region called

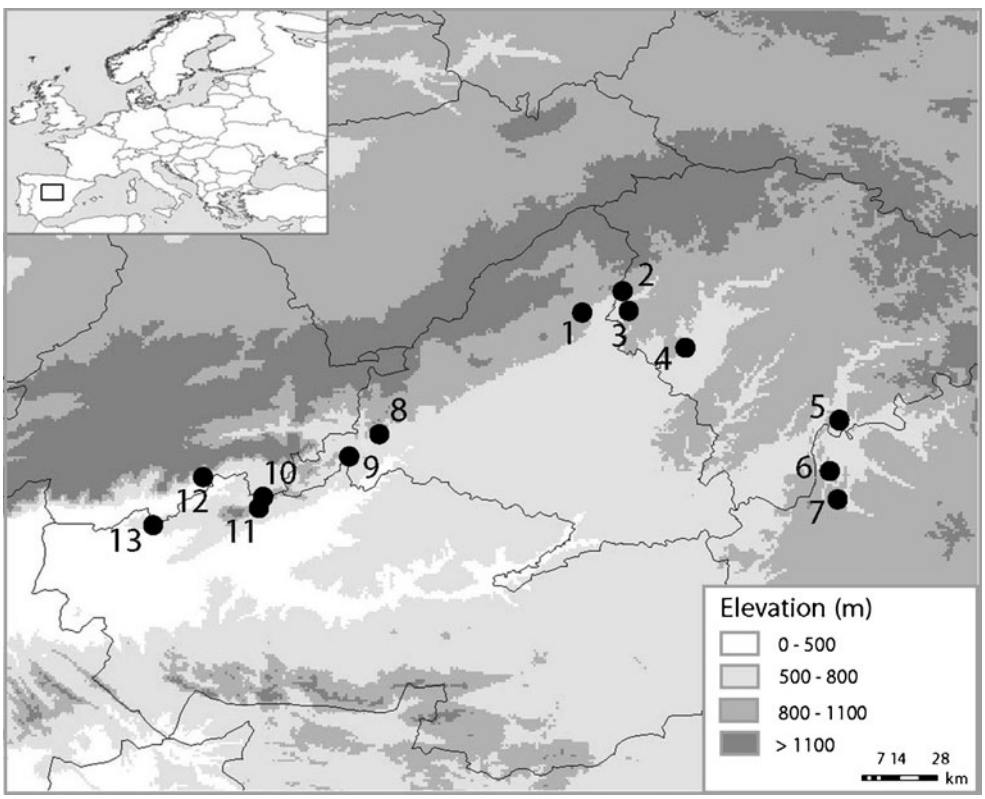


**Figure 1** Studied localities. *Black circles* show the location of the 13 localities sampled (see Table 1)

**Table 1** Sampled localities and their characteristics

| | Coordinates | Elevation (m) | Date | Description | Sample codes |
|---|---|---|---|---|---|
| Loc. 1 | 40°48′45″ N, 03°35′16″ W | 815±5 | 26 October 2006 | Mediterranean shrubland, with *Quercus* and Labiatae | M06-29–M06-38 |
| Loc. 2 | 40°53′09″ N, 03°27′19″ W | 868±3 | 26 October 2006 | Mediterranean shrubland, with *Cistus* spp. | M06-39–M06-44 |
| Loc. 3 | 40°49′32″ N, 03°26′07″ W | 880±7 | 26 October 2006 | Mediterranean shrubland, with Labiatae | GU06-01–GU06-06 |
| Loc. 4 | 40°41′56″ N, 03°14′39″ W | 805±4 | 26 October 2006 | Mediterranean shrubland, with *Quercus* sp. | GU06-07–GU06-10 |
| Loc. 5 | 40°27′36″ N, 02°43′54″ W | 765±4 | 26 October 2006 | Mediterranean shrubland, with *Quercus* sp. and Labiatae | GU06-11–GU06-16 |
| Loc. 6 | 40°17′24″ N, 02°45′47″ W | 670±5 | 26 October 2006 | Grassland in a hill, with Gramineae and Compositae | CU06-01–CU06-04 |
| Loc. 7 | 40°12′06″ N, 02′44′22″ W | 800±4 | 26 October 2006 | Mediterranean shrubland, with Labiatae | CU06-05–CU06-08 |
| Loc. 8 | 40°25′03″ N, 04′15′50″ W | 787±4 | 19 February 2007 | Mediterranean forest, with *Quercus ilex* | M07-01–M07-10 |
| Loc. 9 | 40°20′25″ N, 04′21′42″ W | 770±6 | 19 February 2007 | Mediterranean forest, with *Pinus* sp. | M07-11–M07-20 |
| Loc. 10 | 40°12′38″ N, 04′38′40″ W | 640±9 | 19 February 2007 | Mediterranean forest, with *Quercus ilex* | AV07-01–AV07-10 |
| Loc. 11 | 40°10′14″ N, 04′39′40″ W | 710±4 | 19 February 2007 | Mediterranean forest, with *Quercus ilex* | TO07-01–TO07-10 |
| Loc. 12 | 40°16′14″ N, 04′50′42″ W | 680±13 | 19 February 2007 | Mediterranean forest, with *Quercus pyrenaica* and *Pinus* sp. | AV07-11–AV07-20 |
| Loc. 13 | 40°06′49″ N, 05′00′43″ W | 530±6 | 19 February 2007 | Mediterranean forest, with *Quercus ilex* | TO07-11–TO07-20 |

"La Vera." The climate in the two selected areas is Mediterranean continental, with long, dry, and warm summers and long cold winters. Springs and autumns are mild, humid, and short. The typical vegetation of these areas mainly consists of Mediterranean forests, most of them dominated by *Quercus ilex* or *Quercus faginea*. Due to historical agricultural activities, many of the original forests have disappeared, giving rise to ecosystems in different successional stages in which shrublands predominate. These shrublands are partially determined by the soil type, being Labiatae (*Rosmarinus*, *Thymus*, *Lavandula*, *Salvia*…) the dominant vegetational components in limestones and Cistaceae (*Cistus* spp.) and Leguminosae (*Retama*) in siliceous substrates.

A total of 100 samples (44 of ground litter, 44 of aerial litter, and 12 of bark) were collected. At each site, we intended to collect ten samples from three different microhabitats and different plant species. The objective was to obtain four samples of ground litter (the layer of twigs, leaves, and other plant debris extending over the soil surface), four samples of aerial litter (assemblage of dead but still attached parts of standing plants), and two samples of bark of living plants per locality. However, this was not always possible due to the absence of appropriate plant tissues. Collections of samples were placed in separate paper bags and air-dried in the laboratory of Real Jardín Botánico. These samples were stored there with the codes shown in Table 1.

Primary isolation plates were prepared between October 2006 and June 2007, using a modification of the technique described in [23] (see also [15] and [36]). The material was cut into small (ca. 1.5–2 cm) pieces with sterile scissors. Thirty-two pieces from each sample were plated out in eight lines of four pieces forming a circle on a 9-cm Petri dish (Fig. 2) with

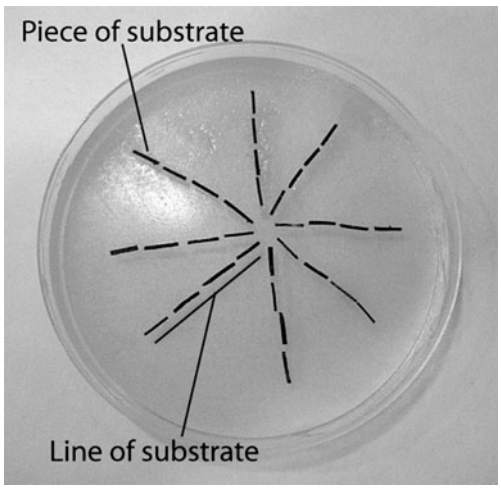


**Figure 2** A primary isolation plate with thirty-two pieces of substrate, that were plated out in eight lines of four pieces forming a circle on a 9-cm Petri dish

a weakly nutrient medium (wMY—0.002 g malt extract, 0.002 g yeast extract, 0.75 g $K_2HPO_4$, 15 g agar/L of distilled water). The material was moistened by pipetting on a few drops sterile water per line. Three plates per sample were prepared, yielding a total of 300 plates (2,400 lines and 9,600 pieces of substrate). The plates were incubated at 21°C and were surveyed for protosteloid amoebae in the second week of culture.

Species were identified on the basis of fruiting body morphology under the light microscope using both unpublished [36] and original descriptions. Nomenclature used herein follows [23] and [11]. Colonies of protosteloid amoebae were counted in each line of substrate from each plate. A colony is defined as an individual fruiting body or a patch of fruiting bodies that is separated from the nearest fruiting body of the same species by at least one field of view under a ×10 objective on a compound microscope (i.e., approximately 2.0 mm) [15]. Colony size was not taken into account for abundance measures. Photomicrographs were taken with a Nikon Eclipse 80i compound microscope using bright field optics and a Nikon Digital Sight DS-5M digital camera.

## Data Analysis

Species richness was calculated after considering only one randomly selected plate per sample, two randomly selected plates per sample, and finally all the plates cultured. This process was repeated 100 times with different random ordinations of the plates using the program R 2.6.2 [25]. The plot of the species richness vs. the number of plates per sample was subjected to a non-linear regression analysis with the program CurveExpert 1.3 [8], using as a saturation formula the Michaelis–Menten equation:

$$f(x) \sim y = Ax/(B + x)$$

In each case, the parameters $A$ and $B$ from the Michaelis–Menten formula, the standard error, and the coefficient of correlation were estimated. For a better evaluation of the results, the conditions necessary for obtaining an 80% and a 90% of the estimated maximum number of species were also calculated. Similarly, the cumulative species richness was also measured using 100 permutations with different numbers of lines of substrate per plate and per sample, using CurveExpert 1.3 to calculate the parameters $A$ and $B$.

To evaluate the extent to which the survey was exhaustive and estimate the actual number of species, two methods were used—rarefaction and a nonparametric estimator. Both methods were used for studying all the samples together and samples from the three different microhabitats separately. For the first method [27, 28], the sequence of samples was randomly permutated 100 times, and the cumulative number of species was calculated for each permutation using R 2.6.2. The plot of the mean cumulative number of species vs. the number of samples was subjected to a regression analysis with the program CurveExpert 1.3, using the Michaelis–Menten equation. The number of species was also estimated using the abundance-based coverage estimator (ACE) [6, 7], with the program Spade [32] and a cutoff point=10.

Significance of the differences in abundance between ground litter and aerial litter was tested with a chi-square test for each species, using as expected frequency the average number of colonies between the two microhabitats. When the obtained $p$ value was 0.5 or less in the chi-square test, the species were considered as having equal preferences for aerial litter and ground litter.

On the basis of their relative abundances, the species have been classified in the abundance classes described in [21]: abundant >10% of total colonies, common >5%, occasional >1%, and rare <1%. Abundance classes' boundaries were kept to be consistent with [21] and facilitate future work. Though they are informal, they provide a good tool for a quick and easy comparison of relative abundance of species between studies. A canonical correspondence analysis (CCA) was performed using abundant, common, and occasional species as dependent variables and annual mean temperature, annual precipitation, precipitation of the wettest month, precipitation of the

**Table 2** Values of the climatic variables in each locality obtained from EDIT geoplatform

| | *T* | *P* | PW | PD | PS | MTW | mTC | TR |
|---|---|---|---|---|---|---|---|---|
| Loc. 1 | 12.1 | 465 | 57 | 13 | 32 | 28.8 | −0.1 | 28.9 |
| Loc. 2 | 12.8 | 431 | 54 | 12 | 33 | 29.4 | 0.6 | 28.8 |
| Loc. 3 | 12 | 463 | 56 | 14 | 31 | 28.8 | −0.1 | 28.8 |
| Loc. 4 | 12.5 | 435 | 54 | 12 | 33 | 29.3 | 0.3 | 29 |
| Loc. 5 | 12.8 | 429 | 51 | 14 | 30 | 30.2 | −0.1 | 30.1 |
| Loc. 6 | 12.7 | 445 | 52 | 14 | 30 | 30.5 | −0.1 | 30.6 |
| Loc. 7 | 12.8 | 446 | 52 | 13 | 31 | 30.7 | −0.1 | 30.8 |
| Loc. 8 | 13.3 | 397 | 47 | 11 | 33 | 30.5 | 0.7 | 29.8 |
| Loc. 9 | 12.4 | 411 | 52 | 12 | 34 | 29.8 | −0.1 | 29.9 |
| Loc. 10 | 13.9 | 373 | 46 | 9 | 35 | 31.7 | 1.1 | 30.6 |
| Loc. 11 | 13.2 | 391 | 49 | 10 | 36 | 31.1 | 0.4 | 30.7 |
| Loc. 12 | 13.8 | 371 | 46 | 9 | 36 | 31.5 | 11 | 30.4 |
| Loc. 13 | 14.4 | 375 | 44 | 7 | 37 | 32.3 | 1.5 | 30.8 |

*T* annual mean temperature in degree Celsius, *P* annual precipitation in millimeters, *PW* precipitation of the wettest month in millimeters, *PD* precipitation of the driest month in millimeters, *PS* precipitation seasonality (coefficient of variation), *MTW* maximum temperature of the warmest month in degree Celsius, *mTC* minimum temperatures of the coldest month in degree Celsius, *TR* temperature range in degree Celsius

driest month, precipitation seasonality, maximum temperature of the warmest month, minimum temperature of the coldest month, temperature range, and microhabitat type as independent variables (Table 2), with R 2.6.2 and the vegan package [22]. Environmental data were obtained as raster layers from EDIT Geoplatform [9], and values for each sampling point were extracted using ArcGis from ESRI. Species were scaled proportional to eigenvalues, sites were unscaled (weighted dispersion equal on all dimensions), and permutation tests were carried out. For a better interpretation of the results, the correlation between all pairs of climatic variables was studied using regression analyses in R 2.6.2.

For each species, the probability distribution with the best fit was selected using various nonparametric statistics (maximum likelihood fitting, Kolmogorov–Smirnov test, chi-square test), and significance of the former climatic factors together with microhabitat type was tested using generalized linear models (GLM) in R 2.6.2. Only abundant, common, and occasional species were analyzed. Rare species were not considered in these analyses because there is not enough information about them to obtain reliable results.

## Results

### Optimization

The results of the optimization of the culture method are presented in Fig. 3 and Table 3.

The effect of culturing a different number of lines of substrate per sample has been studied using two different methods. The first one (Fig. 3b) studies the effect of always using three plates per sample, varying the number of lines that are cultured in each plate. Thus, this method takes into account in which plate the different substrate lines were initially cultured. The second method (Fig. 3c) varies the number of lines per sample selecting them randomly. Comparing the values in the table for obtaining an 80% of the species shown in Table 3, the number of lines per sample necessary (3.7) is less than three times the number of lines per plate (1.56 x 3 = 4.68). The same thing happens when observing the values in the 90% column, being the number of lines per sample (8.33) again less that three times the number of lines per plate (10.53). This effect shows that there are random differences between culture conditions that could cause that, using the same quantity of substrate, potentially more species can be obtained if the sample is divided and cultured in different plates.

The number of plates and the number of lines per plate have to be selected depending on the objectives of the study. If the main objective is finding as many species as possible, or isolating a specific rare organism, the culturing methodology should be adjusted for obtaining the maximum number of species with a reasonable effort. In this case, it is recommended to use two plates per sample to take advantage of random effects between plates. The use of three plates per sample produced an increment only of 2% (one species) with respect to the use of two plates per sample. For recovering at

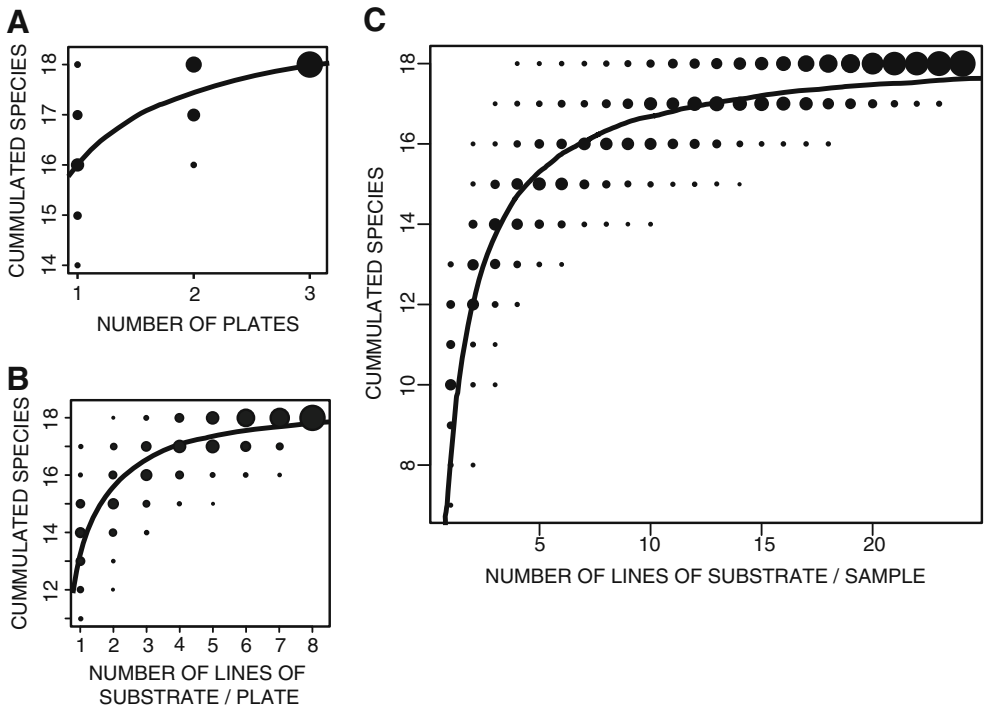


**Figure 3** Optimization analyses. **a** Cumulative species vs. number of plates per sample. **b** Cumulative species vs. number of lines of substrate per plate, using three plates per sample. **c** Cumulative species vs. number of lines per sample. These values are the means of 100 runs. The *solid line* shows the results of regression analysis using a saturation function $y = Ax/(B + x)$, where $A$ is the maximum number of species to be expected and $B$ is the number of elements needed to reach half the number of species to be expected

**Table 3** Results of the rarefaction analyses of the number of plates and the number of lines of substrate

| | *A* | *B* | s.e. | c.c. | 80% | 90% |
|---|---|---|---|---|---|---|
| No of plates/sample | 19.07 | 0.18 | 0.71 | 0.87 | 0.73 | 1.65 |
| No of lines of substrate/plate | 18.67 | 0.39 | 0.86 | 0.86 | 1.56 | 3.51 |
| No of lines of substrate/sample | 18.32 | 0.92 | 0.91 | 0.88 | 3.7 | 8.33 |

The cumulative species richness was measured using 100 permutations with different numbers of plates per sample, lines of substrate per plate, and lines of substrate per sample. Results were subjected to regression analyses using the Michaelis–Menten equation $f(x) \sim y = Ax/(B+x)$ as saturation formula

*A*, *B* parameters from Michaelis–Menten formula, *s.e.* standard error, *c.c.* correlation coefficient, *80%* number of plates or lines of substrate necessary for obtaining an 80% of the species, *90%* number of plates or lines of substrate necessary for obtaining a 90% of the species

least a 90% of the species, it is necessary to plate four lines of substrate per plate (eight in total).

If the goal of the study is characterizing the ecological preferences of a species or a group of species, it is necessary to obtain a sufficient number of occurrences of the organisms of interest. In this case, rare species are not good targets if the number of samples is limited because they are strongly affected by random errors. Using only one plate per sample made possible to recover an 85% of the species, that is, all of them except two of the rare species. Similarly, the results of changing the number of lines of substrate showed that 80% of the species are obtained using only four lines of substrate per sample. In conclusion, for an ecological study of the abundant, common, and occasional species, it is sufficient to use one plate per sample and four lines of substrate.

## Ecology

Protosteloid amoebae fruited in 95 of the 100 samples collected. The percentage of cultures positive for protosteloid amoebae (PCP=number of primary isolation plates (PIP) positive for protosteloid amoebae×100/total number of PIP) was 84%. After observing three plates per sample, a total of 1,504 colonies were found (Table 4), from which 18 species were identified. The mean number of species occurring per sample was 4.24 (range 0–13).

All 18 observed species (Figs. 4 and 5) were recovered from the aerial litter microhabitat while only 15 of the species were found in ground litter samples and 11 in samples from bark. The number of colonies was also higher in aerial litter (904 [20.5/sample]) than in ground litter (551 [12.5/sample]) and bark (49 [4.1/sample]).

The number of species estimated with rarefaction and ACE are very close to the number of species recorded from the samples. Similar results were obtained using both methods, so it can be reliably concluded that we have recovered more than 90% of the total species that would have been found with much more effort employing the same methodology (Table 5; Fig. 6) The survey was exhaustive, especially for aerial litter and ground litter (more than 85–90% of the species). This is not the case for bark for which only a 70% of the estimated number of species was found. The evenness of communities can be compared by examining the steepness of the rarefaction curves (Fig. 6b). The curve is steeped in aerial litter than in ground litter and bark, indicating a more even distribution of species among samples in aerial litter.

The most commonly encountered and abundant species in this study (Table 4) were *Protostelium mycophaga* (34% of the total colonies), *Schizoplasmodiopsis pseudoendospora* (19%), *Tychosporium acutostipes* (11%), and *Schizoplasmodiopsis amoeboidea* (10%). *Cavostelium apophysatum* (9%), and *Nematostelium gracile* (6%) were common species, while *Nematostelium ovatum* (1%), *Protosporangium articulatum* (2%), *Protostelium nocturnum* (2%), *Schizoplasmodiopsis vulgare* (2%), and *Soliformovum irregulare* (1%) were occasional species. The remaining seven species were rare.

Two rare species, *Echinosteliopsis oligospora* and *Protostelium okumukumu*, were recovered only from aerial litter samples. The rare protosteloid myxomycete, *Echinostelium bisporum*, was found on both aerial litter and bark but not on ground litter. *N. ovatum*, *P. nocturnum*, *Protostelium pyriformis*, *Schizoplasmodium cavostelioides*, and *Soliformovum irregularis* were recovered from both aerial and ground litter samples, but not from bark. The remaining species were found in all three of the microhabitats that were studied.

The abundance of each species was significantly different in aerial and in ground litter microhabitats in most cases; only *C. apophysatum*, *S. amoeboidea*, and *Schizoplasmodium cavostelioides* had a $p<0.5$ in the chi-square test (Table 4) and showed no preference for aerial litter or ground litter. *N. ovatum* and *S. pseudoendospora* were significantly more abundant in the ground litter microhabitat, but the remaining species were significantly more common in aerial litter. Rarefaction analysis showed that a high percentage of the species predicted in bark were not found. As this incomplete sampling may also affect abundance data, chi-square tests including bark were not performed due to the small number of samples collected from this microhabitat.

**Table 4** Number of colonies per species and microhabitat, absolute, and relative abundance

| | A | | G | | B | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Absolute | Relative | Absolute | Relative | Absolute | Relative | Absolute | Relative |
| *Cavostelium apophysatum** (C) | 65 | 1.48 | 67 | 1.52 | 8 | 0.67 | 140 | 1.4 |
| *Echinosteliopsis oligospora* (R) | 2 | 0.05 | 0 | 0 | 0 | 0 | 2 | 0.02 |
| *Echinostelium bisporum* (R) | 3 | 0.07 | 0 | 0 | 1 | 0.08 | 4 | 0.04 |
| *Endostelium zonatum* (R) | 8 | 0.18 | 2 | 0.05 | 2 | 0.17 | 12 | 0.12 |
| *Nematostelium gracile* (C) | 43 | 0.98 | 38 | 0.86 | 4 | 0.33 | 85 | 0.85 |
| *Nematostelium ovatum* (O) | 8 | 0.18 | 11 | 0.25 | 0 | 0 | 19 | 0.19 |
| *Protosporangium articulatum* (O) | 31 | 0.7 | 4 | 0.09 | 2 | 0.17 | 37 | 0.37 |
| *Protostelium arachisporum* (R) | 2 | 0.05 | 1 | 0.02 | 1 | 0.08 | 4 | 0.04 |
| *Protostelium mycophaga* (A) | 422 | 9.59 | 83 | 1.89 | 2 | 0.17 | 507 | 7.07 |
| *Protostelium nocturnum* (O) | 21 | 0.48 | 4 | 0.09 | 0 | 0 | 25 | 0.25 |
| *Protostelium okumukumu* (R) | 1 | 0.02 | 0 | 0 | 0 | 0 | 1 | 0.01 |
| *Protostelium pyriforme* (R) | 10 | 0.23 | 1 | 0.02 | 0 | 0 | 11 | 0.11 |
| *Schizoplasmodiopsis amoeboidea** (A) | 73 | 1.66 | 75 | 1.7 | 6 | 0.5 | 154 | 1.54 |
| *Schizoplasmodiopsis pseudoendospora* (A) | 78 | 1.77 | 182 | 4.14 | 21 | 1.75 | 281 | 2.81 |
| *Schizoplasmodiopsis vulgare* (O) | 22 | 0.5 | 9 | 0.2 | 1 | 0.08 | 32 | 0.32 |
| *Schizoplasmodium cavostelioides*** (R) | 1 | 0.02 | 1 | 0.02 | 0 | 0 | 2 | 0.02 |
| *Soliformovum irregulare* (O) | 18 | 0.41 | 4 | 0.09 | 0 | 0 | 22 | 0.22 |
| *Tychosporium acutostipes* (A) | 96 | 2.18 | 69 | 1.57 | 1 | 0.08 | 166 | 1.66 |
| Total | 904 | 20.55 | 551 | 12.52 | 49 | 4.08 | 1504 | 15.04 |

*A* aerial litter, *G* ground litter, *B* bark, (*A*) abundant, (*C*) common, (*O*) occasional, (*R*) rare

*$p<0.05$ (no significant differences between A and G; chi-square test); **$p<0.01$ (no significant differences between A and G; chi-square test)

The results of the correlation analysis between the climatic variables are shown in Fig. 7a. The variables annual mean temperature, precipitation of the wettest month, and maximum temperature of the wettest month are very highly correlated ($r^2>0.9$) so their individual effects on the species in the studied area cannot be easily distinguished.

The CCA (Fig. 7b) had a total inertia of 1.400, a constrained inertia of 0.223 (proportion 15.92%), and an unconstrained inertia of 1.177 (84.07%). Permutation tests were carried out; the test for the axes was significant ($p=0.005$), and the test for the independent variables showed that aerial litter microhabitat ($p=0.010$), annual mean temperature ($p=0.005$), maximum temperature of the warmest month ($p=0.030$), and minimum temperature of the coldest month ($p=0.015$) had significant effects. The variables that were more important for differentiating the niches of the studied species were the microhabitats, but it is interesting to observe that the climatic variables, especially minimum temperature of the coldest month, precipitation seasonality, and temperature range, have secondary but also important effects. The species that typically inhabit bark tend to be more abundant when there is a high temperature range. On the other hand, the species that have a clear preference for the aerial litter microhabitat have preference for higher values of precipitation, precipitation of the wettest month, and precipitation of the driest month. Species like *C. apophysatum*, *N. gracile*, and *S. amoeboidea* together or *N. ovatum* and *S. vulgare* have similar niches and appear more frequently together. *T. acutostipes* and *S. pseudoendospora* tend to appear in localities with higher temperatures and higher minimum temperatures of the coldest month and not in aerial litter. *C. apophysatum*, *N. gracile*, and *S. amoeboidea* have certain affinity for bark of living plants and high temperature range. *S. irregulare* and *P. nocturnum* show preference for localities with higher precipitations and lower temperatures, but *P. nocturnum* needs higher values of the minimum temperature of the coldest month.

The GLMs (Table 6) found significant contributions of at least one of the studied variables in all species but *N. ovatum*. The factors with more influence were minimum temperatures of the coldest month, which had negative effects for eight of the species (*C. apophysatum*, *N. gracile*, *P. nocturnum*, *S. amoeboidea*, *S. irregulare*, *S. pseudoendospora*, *S. vulgare*, and *T. acutostipes*) and temperature range having negative effects for four of the species (*P. nocturnum*, *S. amoeboidea*, *S. pseudoendospora*, and *T. acutostipes*). For *S. pseudoendospora* and *S. amoeboidea*, maximum temperature of the warmest month has a positive effect.

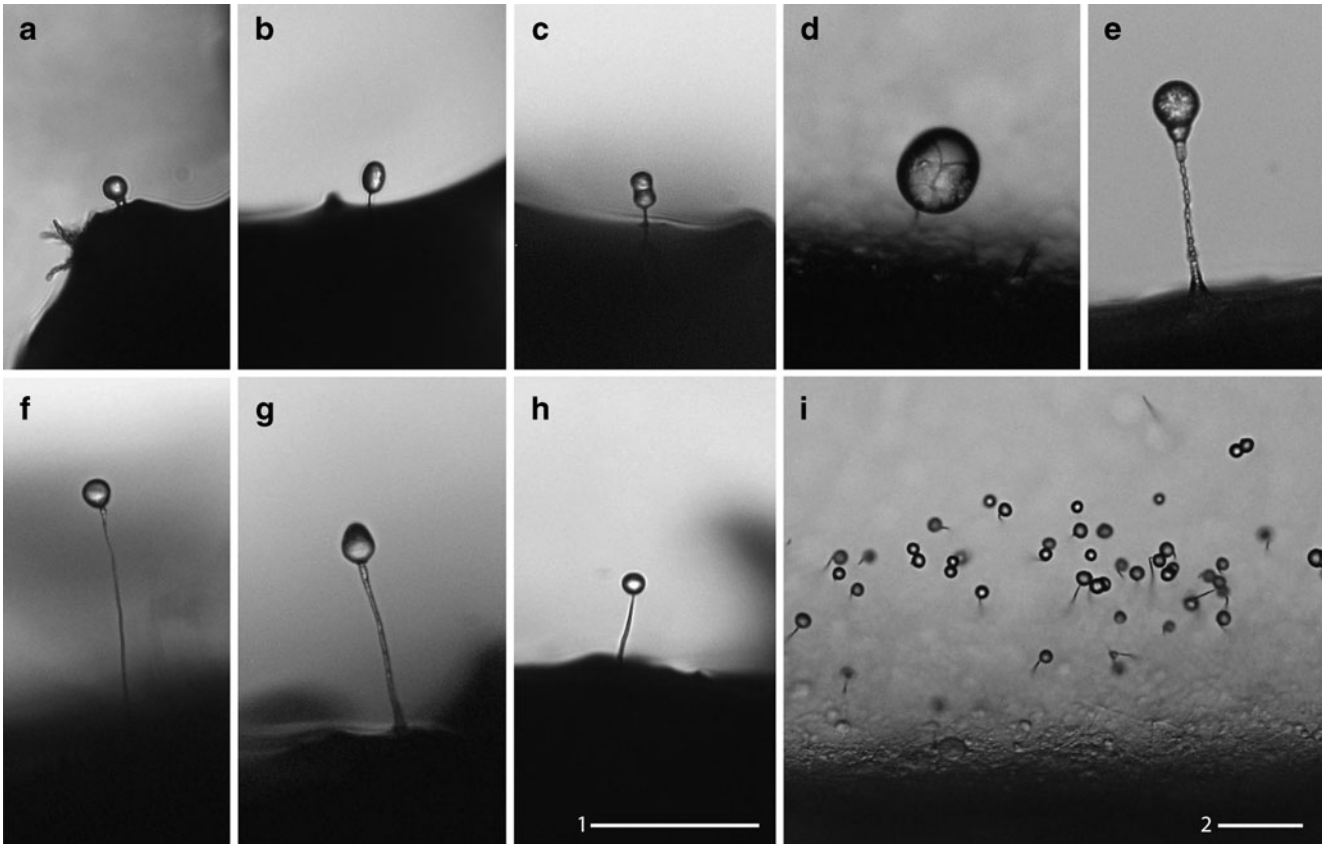


**Figure 4** Fruiting bodies of **a** *C. apophysatum*; **b** *E. bisporum* hydrated and **c** dried; **d** *E. oligospora*; **e** *E. zonatum*; **f** *N. gracile*; **g** *N. ovatum*; **h**, **i** *T. acutostipes*. *Bars* 50 μm; *1* for **a**–**h**, and *2* for **i**

## Discussion

The methods employed in this paper provide quantitative data and explore for the first time the influence of different climatic variables over protosteloid species in a relatively small area with a Mediterranean climate. The colony-counting method has the advantage of providing a more quantitative approach that makes possible the use of abundance measures and

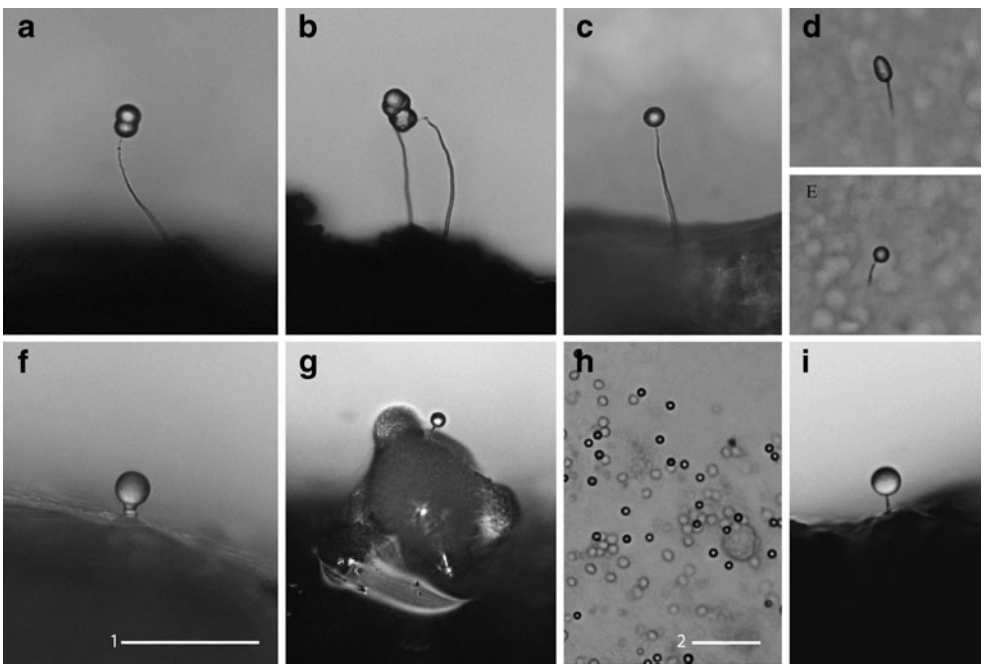


**Figure 5** Fruiting bodies of **a**, **b** *P. articulatum*, **c** *P. mycophaga*, **d** *P. arachisporum*, **e** *P. nocturnum*, **f** *S. cavostelioides*, **g** *S. pseudoendospora* fruiting on myxobacteria and **h** in group, **i** *S. amoeboidea*. *Bars* 50 μm; *1* for **a**–**g**, **i** and *2* for **h**

**Table 5** Estimates of the total species richness using the abundance-based coverage estimator and rarefaction using the Michaelis–Menten equation as saturation formula

| | Species recovered from samples | ACE | | | Rarefaction | | |
|---|---|---|---|---|---|---|---|
| | | Estimate | s.e. | 95% confidence interval | Estimate | s.e. | c.c. |
| A | 18 | 19.6 | 2.2 | 18.2, 29.9 | 18.6 | 0.3 | 0.99 |
| G | 15 | 17.8 | 3.4 | 15.4, 33.0 | 16.5 | 0.5 | 0.98 |
| B | 11 | 15.5 | 4.5 | 11.9, 34.3 | 15.4 | 0.1 | 0.99 |
| Total | 18 | 18.4 | 0.8 | 18.0, 22.9 | 18.6 | 0.3 | 0.99 |

*ACE* abundance-based coverage estimator, *A* aerial litter, *G* ground litter, *B* bark, *s.e.* standard error, *c.c.* correlation coefficient

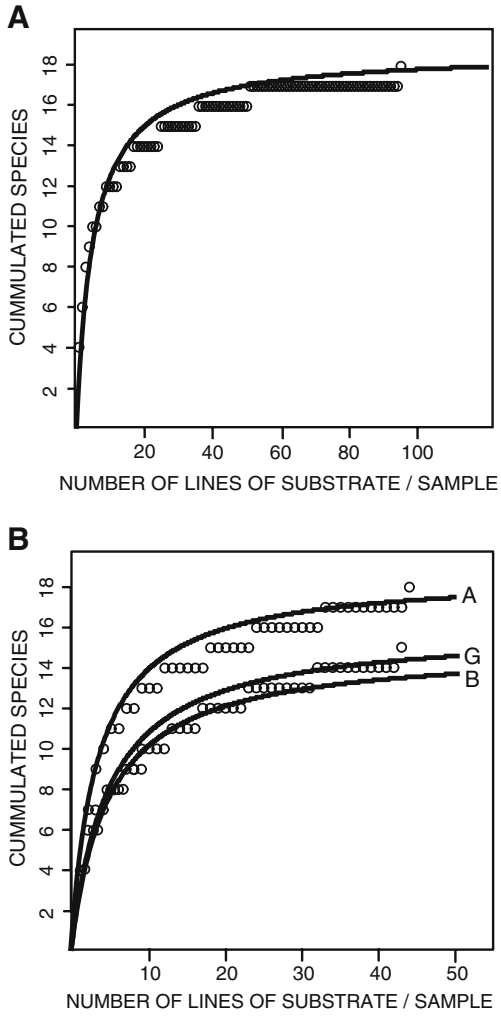


**Figure 6** Analysis of the randomly permutated sequence of all samples studied versus cumulative species numbers (*open circles*). These values are the means of 100 runs. The *solid line* shows the results of regression analysis using a saturation function $y = Ax/(B + x)$, where $A$ is the maximum number of species to be expected and $B$ is the number of samples needed to reach half the number of species to be expected. **a** Results for all the samples. **b** Results for the different microhabitats

statistics. As this study is more exhaustive than usual and samples were plated three times, it was an opportunity to emphasize the culturing methodology and optimize the effort needed for this new area. These improvements can be used in the future to generate comparable data sets for the large-scale studies that are necessary for a better understanding of the ecology of these species.

The value of counting colonies vs. counting individual fruiting bodies is that it is an easier, quicker method to carry out and that it is not affected by patchiness of food abundance and allows the use of statistics to test the significance of the observed results. Its main disadvantage could be its subjective component that may have the consequence that results obtained by different observers are not completely comparable. If the method is strictly followed, this source of error can be highly reduced because any inherent errors in the assumption of what a colony is are constant for all samples and all observers. Moreover, this method has the assumption that every colony is originated from an individual propagule that was present on the substrate, but some possible built-in errors are that (1) distinct patches of fruiting that are closer than 2 mm to each other might be separate colonies, also (2) colonies on opposite sides of a piece of substrate may be continuous across the piece of substrate but cannot be seen due to the opacity of the substrate, and (3) two or more colonies could grow together before the first observation could be made.

Another important question is to what extent our results reflect what is actually present in the field. Culture conditions in the lab are different from natural conditions, and this can affect the way propagules germinate and fruit. It is also not well-known if wMY medium has selective effects on the protosteloid organisms and if other enrichments would enable different species to grow. Observed colonies are the result of species that were present in the original samples—at least as propagules—and that were able to germinate, survive, and fruit under culture conditions. In previous studies [17], similar patterns were found on native substrates and on previously sterilized, standardized substrates that were placed in the field and colonized by spores. This suggests that the

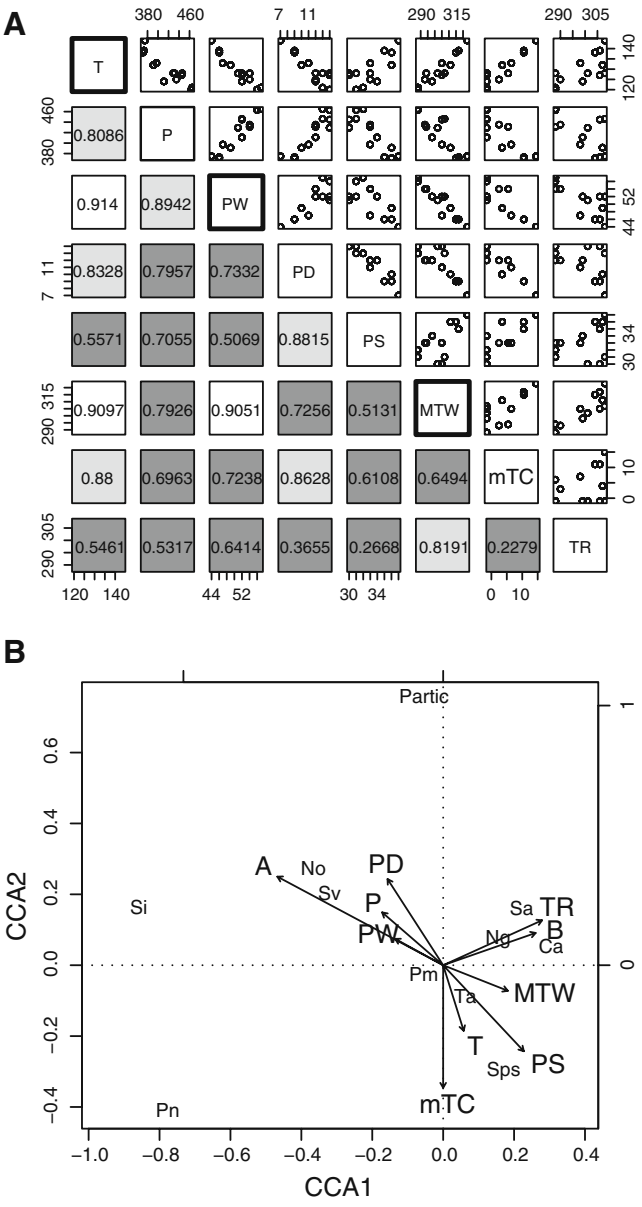


**Figure 7** **a** Correlation analyses of all pairs of climatic variables. Each variable name is shown in the intersection between the *row* and the *column* that represent its results. Results from variable pairs are presented in the intersections between *rows* and *columns* of different variables. Plots of all pairs are presented on the *right upper corner*, and their corresponding squared correlation coefficients ($r^2$) are in the *lower left corner*. Values of $r^2$ close to 1 and points forming a *line in the plot* indicate high correlation between a pairs of variables. A group of highly correlated variables is *highlighted*. *White*: $r^2>0.9$, *light gray*: $r^2>0.8$, *dark gray*: $r^2<0.8$. **b** Canonical correspondence analysis using abundant, common, and occasional species as dependent variables and climatic and microhabitat variables as independent variables. Each species point in the diagram is at the centroid (weighted average) of the site points in which it occurs, environmental variables are represented by *arrows* that run from the origin to the weights that each variable has in the linear combinations that form the axes *Ca C. apophysatum*, *Ng N. gracile*, *No N. ovatum*, *Partic P. articulatum*, *Pm P. mycophaga*, *Pn P. nocturnum*, *Sa S. amoeboidea*, *Sps S. pseudoendospora*, *Sv S. vulgare*, *Si S. irregulare*, *Ta T. acutostipes*, *P* annual precipitation, *PD* precipitation of the driest month, *PS* precipitation seasonality, *PW* precipitation of the wettest month, *T* mean annual temperature, *MTW* maximum temperature of the warmest month, *mTC* minimum temperature of the coldest month, *TR* temperature range, *A* aerial litter, *B* bark

protosteloid amoebae that are observed in culture are probably, for the most part, the ones that are actively growing and dispersing. The main drawback of using cultures is that there may be differences in the success of propagules from different species in culture, making inter-species comparisons very difficult. Problems with cultures especially affect culture-based quantitative approaches like the colony-counting method because they will underestimate the number of propagules present in the samples and be biased toward the species that germinate and fruit better in culture conditions. This problem will not be solved until quantitative environmental molecular techniques are developed for protosteloid amoebae.

Previous studies about seasonality in protosteloid amoebae [17] show evidence of the existence of cyclic changes in assemblages of protosteloid amoebae where more colonies occurred in samples collected in warmer months and fewer colonies occurred in colder months. What is seen in plates probably reflects a snapshot of what is happening in nature during some period of time just prior to the collection of the samples. If any turnover during seasonal cycles or over longer periods is occurring, it would be missed in present study. To reduce the effect of seasonal differences and get a comparable dataset, samples for this study were collected in October and February, when precipitation and temperature are close to the annual averages. This way it is possible to avoid the direct effect of summer drought and extremely cold temperatures and thus reduce the effect of cyclic changes.

On the basis of the results obtained, it is possible to adapt the culture method to each particular case, depending on the objectives of the research. If the objective is to find as many species as possible or a particular rare species, then using more than one plate per sample is highly recommended. To achieve the ecological objectives of this paper, culturing each sample only once would have provided the best fit between effort and results because only the rarer species would have been missed. Rare species are found in such small numbers that they are strongly affected by random errors, and it is very difficult to use statistics to obtain reliable conclusions about their ecological preferences. For this reason, the ecology of rare species should be studied with a more sensitive method, or with a sampling design specifically oriented which could provide enough raw data to obtain statistically reliable results and minimize errors.

Results presented herein are consistent with previous studies carried out in other temperate areas ([2, 3, 15, 17, 18, 30, 39], see also [21]), and a high percentage of positive samples and number of species per sample were obtained. These studies used different methods and sampling strategies,

**Table 6** Results of the generalized linear models for the abundant, common, and occasional species

| | Probability distribution | Significant variables in GLM |
|---|---|---|
| *C. apophysatum* | Poisson | (−)PD, (−)mTC** |
| *N. gracile* | Quasi-Poisson | (+)P, (+)PS, (−)mTC |
| *N. ovatum* | Negative binomial | |
| *P. articulatum* | Negative binomial | (−)G* |
| *P. mycophaga* | Poisson | (−)B |
| *P. nocturnum* | Negative binomial | (−)mTC, (−)TR, (−)G* |
| *S. amoeboidea* | Poisson | (+)MTW, (−)mTC*, (−)TR, (−)B* |
| *S. irregulare* | Negative binomial | (−)mTC, (−)G |
| *S. pseudoendospora* | Poisson | (+)MTW, (−)mTC*, (−)TR, (−)B* |
| *S. vulgare* | Negative binomial | (−)mTC |
| *T. acutostipes* | Poisson | (−)mTC*, (−)TR, (−)B* |

The probability distribution of data and the significant variables are shown. No indication: $p<0.05$

*GLM* generalized linear models, *P* annual precipitation, *PD* precipitation of the driest month, *PS* precipitation seasonality, *MTW* maximum temperature of the warmest month, *mTC* minimum temperature of the coldest month, *TR* temperature range, *G* ground litter, *B* bark, *(+)* positive effect, *(−)* negative effect

No indication: $p<0.05$, *$p<0.01$; **$p<0.001$

so results have to be compared with caution. In most cases [2, 3, 30, 39], protosteloid amoebae were recorded as presence data on natural substrates. Other approaches were the use of abundance data from standardized substrates [17, 18], or presence data from standardized substrates [15]. The closest area with temperate climate formerly studied, the Somiedo Biosphere Reserve in the northern part of Spain [2], showed a higher species richness. It is remarkable that protosteloid amoebae have a lower species richness in a study area comprised in the Mediterranean region, a biodiversity hotspot for other groups of organisms, in spite of the fact that this study has been more exhaustive. Nonetheless, as the sampling methods were different in each study, observed tendencies should be taken with caution. In order to confirm these results and study their causes in more detail, it would be necessary to perform a new study, including localities from both temperate and Mediterranean regions using the same quantitative method. In all other temperate areas studied, the number of species obtained was lower, except in the Mountains of Northwest Arkansas, USA [5] and Great Smoky Mountains National Park, USA [30]. In the tropics and high-latitude areas that have been previously surveyed, species richness was also lower than in this study, being higher only in forests from Malawi and Kenya [21] and Puerto Rico and Hawaii [38].

It has been previously observed that it is possible to find more differences in assemblages of species from different microhabitats in the same locality, than when comparing samples from the same microhabitat collected in different localities [18, 34]. However, species composition and relative abundance also vary in each microhabitat at different latitudes [21]. The influence of various climatic factors over species found in this study area has been studied using CCA, an ordination method that considers all species together to find the ecological variables that maximize the differences between their niches, and GLM, a parametric method that studies each species individually to find out its requirements. In the CCA, the microhabitat variables were the most important for differentiating the niches of the studied species, and the climatic variables had a secondary but also important effect, but all the variables studied only explain a 15.92% of variation in the data. The incorporation of other sources of information like biotic interactions, pH, concentration of nutrients, and controlling the effect of covariates may improve the quality of future models.

Aerial litter was the microhabitat in which more species were found, and it had the highest abundance of protosteloid amoebae, a result that was obtained in most works carried out in similar latitudes [2, 18, 39]. It was also the microhabitat with the highest evenness, suggesting that species living in this microhabitat may tolerate wider ranges of climate change or that this microhabitat is less heterogeneous than others. According to CCA, aerial litter microhabitat has significant effects on niche segregation, and the species with a clear preference for this microhabitat tend to be more abundant in localities with higher precipitation, lower temperatures of the warmest month, and they usually can tolerate lower values of minimum temperature of the coldest month. This result is also consistent with results obtained in studies made in high latitudes [20, 35]. In this kind of habitats, temperatures are low and precipitation is usually high, and most protosteloid species found are those typical of aerial litter in temperate areas.

Results from the CCA also show that the species that are typical bark inhabitants tend to be more abundant if there is a high temperature range and low annual precipitation. Bark species are usually more abundant in arid grasslands and desert ecosystems, where precipitations are low and there is a high contrast of temperatures, but in this kind of habitats are found fewer protosteloid amoebae common on dead aerial plant parts [34]. In the rarefaction analysis, this microhabitat's curve was less steep than the others, indicating that bark species were less evenly distributed in the samples.

Results obtained with GLM gave further information about the individual preferences of the species and the influence of the climatic factors studied. The problem is that the area studied is too small to have a wide sampling of the environmental conditions that the species can tolerate, so these tendencies cannot be reliably extrapolated out of this area. All species but *P. articulatum* and *P. mycophaga* show preference for localities with lower minimum temperatures of the coldest month. This variable was also significant in the CCA, and it seems to have a very important effect on protosteloid species. *N. gracile*, a species usually more common in tropical latitudes, seems to prefer higher annual precipitation and precipitation seasonality. For *S. pseudoendospora* and *S. amoeboidea*, high maximum temperature of the warmest month has a positive effect.

When comparing relative abundances of protosteloid amoebae obtained in other studies carried out in temperate areas, some differences arise, but most results are concordant with those in this study. However, comparisons between studies made so far are merely informal observations that can be used as a starting point for further work. Two abundant species in this study, *P. mycophaga* and *S. pseudoendospora*, were also abundant in all other studies in temperate areas and usually abundant or common in tropics and high latitudes. They are expected to be a major part of any biota of protosteloid amoebae [21]. *S. amoeboidea*, abundant in present study, was abundant in the Ozark Mountains of Northwest Arkansas, USA [5], in the Somiedo Biosphere Reserve [2], and in one study from tropical areas [21]. It is a widespread species but its abundance varies from locality to locality without a clear pattern. *T. acutostipes*, a species usually more abundant in temperate localities than in the tropics, was also abundant in [30] and common in the Somiedo Biosphere Reserve [2]. It is remarkable that *C. apophysatum* was a common species here. This species is usually rare or occasional in temperate areas, but it is a common or abundant species in tropical areas [21]. In the Somiedo Biosphere Reserve [2], it was an occasional species. Another interesting anomaly is that *S. irregulare* is an occasional species here. It is an abundant species in most studies in temperate areas [21], except in [29] where it is occasional. *P. articulatum*, which was not recovered from samples from Somiedo, is moderately abundant here. This species is more commonly encountered in drier habitats worldwide and has been traditionally considered a bark inhabiting species [36]. It is interesting that, here, this species was found in microhabitats other than bark, especially in aerial litter. It is also remarkable that *N. gracile*, usually a species with preference for ground litter [36], shows more preference for aerial litter in this study area. However, results about *N. gracile* may not be completely reliable because this species cannot be distinguished from *Ceratiomyxella tahitiensis* on the basis of fruiting body morphology, so it is likely we observed both of those species and one might have more preference for aerial litter than the other.

Our present results and our earlier results from Somiedo [2] confirm the excellence of Spain as study area for protosteloid amoebae. The qualitative differences in the occurrence of protosteloid amoebae in the two studies lead us to believe that comparison of their communities in the different ecoregions of Spain may prove to be useful for understanding the biogeography of these organisms in general. Just as the Mediterranean climate seems to be rich in other mycetozoans [12, 13, 26], it is rich in protosteloid amoebae. Thus, the Mediterranean climatic region of Spain can be used as a baseline for comparison with the protosteloid amoebal communities of other Mediterranean regions of the world. The use of these quantitative methods can serve as a blueprint for other studies to test and compare relative abundances of protosteloid species between areas and microhabitats, and the optimization of the sampling method that has been carried out can help to increase the effectiveness of ecological studies in this interesting bioregion. Using these methods, it will be possible to understand the influence of environmental factors on this group and compare its pattern to both those of other microorganisms and of multicellular organisms. The study of microhabitat conditions and their relationship with major climatic factors is a stepping stone for understanding both small- and large-scale distribution of this kind of organisms.

**Acknowledgments** We wish to thank Eva García Carvajal for helping in field work, Fátima Durán for technician work, Diana Wrigley de Basanta for reviewing the style of the text, and Lora Lindley Shadwick, John Shadwick, Matt Brown, George Ndiritu, and Carlos Rojas for all their kind suggestions. This work has been supported by the Research Project (CGL2005-00320/BOS and CGL2008-00720/BOS) of the Ministry of Science and Innovation of Spain and the National Science Foundation grants DEB-0316284 (PBI) and DEB-0329102 (PEET).

## References

1. Adl SM, Simpson AGB, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq

S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn LD, Mann DG, McCourt RM, Mendoza L, Moestrup Ø, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MFJR (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52:399–451
2. Aguilar M, Lado C, Spiegel FW (2007) Protostelids from deciduous forests: first data from southwestern Europe. Mycol Res 111(7):863–872
3. Best SC, Spiegel FW (1984) Protostelids and other simple mycetozoans of Hueston Woods State Park and Nature Preserve. In: Willeke GB (ed) Hueston Woods State Park and Nature Preserve, proceedings of a symposium, April 16–18. Miami University, Oxford, pp 116–121
4. Biodiversity Hotspots Conservation International (2007) http://www.biodiversityhotspots.org
5. Brown MW, Spiegel FW (2008) Assessment of protostelid diversity in Ozark Plateau oak-hickory forests in south central USA. In: Abstracts from 2007 MSA meeting at LSU, Baton Rouge, Louisiana. Inoculum vol 59, p 9
6. Chao A, Lee S-M (1992) Estimating the number of classes via sample coverage. J Am Stat Assoc 87:210–217
7. Chao A, Ma M-C, Yang MCK (1993) Stopping rules and estimation for recapture debugging with unequal failure rates. Biometrika 80:193–201
8. CurveExpert curve fitting software (2008) http://curveexpert.webhop.net/
9. EDIT geoplatform (2007) http://edit.csic.es
10. Fiore-Donno AM, Nikolaev SI, Nelson M, Pawlowski J, Cavalier-Smith T, Baldauf SL (2010) Deep phylogeny and evolution of slime molds (Mycetozoa). Protist 161(1):55–70
11. Lado C (2005–2010) An online nomenclatural information system of Eumycetozoa. http://www.nomen.eumycetozoa.com (consulted 2008)
12. Lado C (1993) Bases corológicas de Flora Micológica Ibérica, números 376–692. Cuad Trab Flora Micol Ibér 7:1–305
13. Lado C, Pando F (1997) Myxomycetes, I. Ceratiomyxales, Echinosteliales, Liceales, Trichiales. In: Flora Mycologica Ibérica, vol. 2. Real Jardín Botánico, CSIC & J. Cramer in der Gebrüder Borntraeger Verlagsbuchhandlung, Madrid
14. Lindley LA, Stephenson SL, Spiegel FW (2007) Protostelids and myxomycetes isolated from aquatic habitats. Mycologia 99(4):504–509
15. Moore DL, Spiegel FW (1995) A new technique for sampling protostelids. Mycologia 87(3):414–418
16. Moore DL, Spiegel FW (2000) Microhabitat distribution of protostelids in tropical forests of the Caribbean National Forest, Puerto Rico. Mycologia 92(4):616–625
17. Moore DL, Spiegel FW (2000) The effect of season on protostelid communities. Mycologia 92(4):599–608
18. Moore DL, Spiegel FW (2000) Microhabitat distribution of protostelids in temperate habitats in northwestern Arkansas. Can J Bot 78:985–994
19. Moore DL, Stephenson SL (2003) Microhabitat distribution of protostelids in a Tropical Wet Forest in Costa Rica. Mycologia 95 (1):11–18
20. Moore DL, Stephenson S, Laursen G, Woodgate W (2000) Protostelids from boreal forest and tundra ecosystems in Alaska. Mycologia 92(3):390–393
21. Ndiritu GG, Stephenson SL, Spiegel FW (2009) First records and microhabitat assessment of protostelids in the Aberdare region, central Kenya. J Eukaryot Microbiol 56(2):148–158
22. Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Stevens MHH, Wagner H (2008) vegan: Community Ecology Package. R package version 1.13-1. http://vegan.r-forge.r-project.org/
23. Olive LS (1975) Chapter 2: Protostelia (Protostelids). In: The Mycetozoans. Academic, New York
24. Powers DM, Stephenson SL (2006) Protostelids from tropical forests, woodlands and deserts in Australia. Mycologia 98(2):218–222
25. R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. ISBN 3-900051-07-0, http://www.R-project.org
26. Romeralo M, Lado C (2006) Dictyostelids from Mediterranean forests of the south of Europe. Mycol Prog 5:231–241
27. Schnittler M (2001) Ecology of myxomycetes of a winter-cold desert in western Kazakhstan. Mycologia 93(4):653–669
28. Schnittler M, Stephenson SL (2000) Myxomycete biodiversity in four different forest types in Costa Rica. Mycologia 92(4):626–637
29. Shadwick J, Stephenson S (2004) First records of protostelids from northern India. Fungal Divers 16:141–145
30. Shadwick JDL, Stephenson SL, Spiegel FW (2009) Distribution and ecology of protostelids in Great Smoky Mountains National Park. Mycologia 101(3):320–328
31. Shadwick LL, Spiegel FW, Shadwick JDL, Brown MW, Silberman JD (2009) Eumycetozoa = Amoebozoa?: SSUrDNA phylogeny of protosteloid slime molds and its significance for the Amoebozoan supergroup. PLoS ONE 4(8):1–13
32. SPADE software (2008) http://140.114.36.3:8080/NTHUStat/UserInfo.jsp
33. Spiegel FW (1986) Phylum plasmodial slime molds class Protostelida. In: Margulis et al (eds) Handbook of Protoctista. Jones and Barlett, Boston
34. Spiegel FW, Stephenson SL, Keller HW, Moore DL, Cavender JC (2004) Sampling the biodiversity of mycetozoans. In: Foster (ed) Biodiversity of fungi. Academic, New York
35. Spiegel FW, Stephenson S (2000) Protostelids of Macquarie Island. Mycologia 92(5):849–852
36. Spiegel FW, Shadwick JD, Lindley-Settlemyre L, Brown MW, Ndiritu G (2007) A beginner's guide to identifying the protostelids. http://slimemold.uark.edu/pdfs/Handbook1_3rd.pdf
37. Stephenson SL, Landolt JC, Moore DL (1999) Protostelids, dictyostelids, and myxomycetes in the litter microhabitat of the Luquillo Experimental Forest, Puerto Rico. Mycol Res 103:209–214
38. Stephenson SL, Schnittler M, Lado C, Estrada-Torres A, Wrigley de Basanta D, Landolt JC, Novozhilov YK, Clarck J, Moore DL, Spiegel FW (2004) Syst Geogr Plants 74:87–108
39. Tesmer J, Rulik B, Spiegel F, Shadwick J, Schnittler M (2005) Protostelids from German Beech forests. Mycol Prog 4(4):267–271
40. Tesmer J, Schnittler M (2009) Aquatic protostelids—a study from northeastern Germany. Fungal Ecol 2(3):140–144
41. Walter H (1984) Vegetation of the earth in relation to climate and the eco-physiological conditions. The English Universities Press, London