

SPATIAL AND TEMPORAL DISTRIBUTION OF PROTOZOA AT CUEVA DE LOS RISCOS, QUERÉTARO, MÉXICO

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Abstract: Protozoa are important members of ecosystems, but protozoa that inhabit caves are poorly known worldwide. In this work, we present data on the record and distribution of thirteen protozoa species in four underground biotopes (water, soil, bat guano, and moss), at Cueva de Los Riscos. The samples were taken in six different months over more than a year. Protozoa species were ciliates (eight species), flagellates (three species), amoeboid (one species), and heliozoan (one species). Five of these species are reported for the first time inside cave systems anywhere, and an additional three species are new records for Mexican caves. *Colpoda* was the ciliate genera found in all cave zones sampled, and it inhabited the four biotopes together with *Vorticella*. The biotopes with the highest specific richness were the moss, sampled near the main cave entrance, and the temporary or permanent water bodies, with ten species each. The greatest number of species was observed in April 2006 (dry season). With the exception of water, all biotopes are studied for the first time.

INTRODUCTION

A great extent of Mexican territory is formed by sedimentary rocks that permit the formation of caves, but the number, location, and biodiversity of Mexican caves is only partially known. Records of subterranean protozoan taxa in Mexico are scarce, and refer only to caves in San Luis Potosí and Guerrero states (Osorio-Tafall, 1943; Hoffmann et al., 1986, p. 206–207), in which thirty species of sarcodines and ciliates have been reported.

There are records of nine protozoa species from caves in North America. In Bermuda, Hill et al. (1986) recorded *Euplotes iliffei* in subterranean anchialine habitats and Small et al. (1986) recorded *Glauconema bermudense* in marine caves. Holsinger (1966) found *Paramecium multimicronucleatum* and *Spirostomum ambiguum* in Virginia, and Barr (1968) reported *Phacus* sp., *Paramecium* sp., *Halteria* sp., *Diffflugia* sp., and *Peranema* sp. as free-living inhabitants of the Mammoth Cave system in Kentucky.

Protozoa are cosmopolitan and tolerate a wide range of physicochemical factors, including pH, temperature, oxygen concentration, and salinity. They are not randomly distributed, but live in microhabitats, small regions that may be as tiny as a few cubic centimeters, within a body of water or a moist environment such as soil, vegetation, or the bodies of plants and animals (Bamforth, 1985). They occupy many different biotopes, in the sense of Olenin and Ducrottoy (2006).

According to Hoffmann et al. (1986, chap. III), roots, leaf and animal debris, and the guano of bats provide the primary energy sources in open system caves. Indeed, guano is considered the most important biotope, but water, soil, and moss also play important roles as sustainable habitats for diverse communities of microorganisms and metazoan taxa.

The protozoan trophozoite or cyst phase enters caves in water flow or infiltration through soil, in air currents, and by troglophile fauna present in the cave (Golemansky and Bonnet, 1994) and accidental or troglaxene organisms. The aim of this work is to record and analyze protozoan spatial and temporal distribution associated with different biotopes over more than a year at Cueva de Los Riscos.

MATERIALS AND METHODS

Cueva de Los Riscos is located in Jalpan de Serra, Querétaro, Mexico, at 1122 m asl, 21°11'38"N, 99°30'50"W. It is a mixed underground system (horizontal and vertical) with a length of 440 m (Lazcano-Sahagún, 1986a, p. 32; 1986b, p. 77–79), with four zones (A–D) and four entrances (Fig. 1); a detailed cave description is in Espino del Castillo et al. (2009).

Six visits were made to Cueva de Los Riscos from November 2005 to June 2007 (Table 1). Samples, including water, soil, bat guano, and moss, were collected where available in four large zones in the cave, A, B, C, and D (Fig. 1); other areas in narrow tunnels were not sampled. Access to the cave was done without special equipment, but for biosafety we wore face masks with filters (Milter disposable 3M 8210 N95).

Water samples were collected by using sterile pipettes; guano and soil samples were obtained with sterilized metallic

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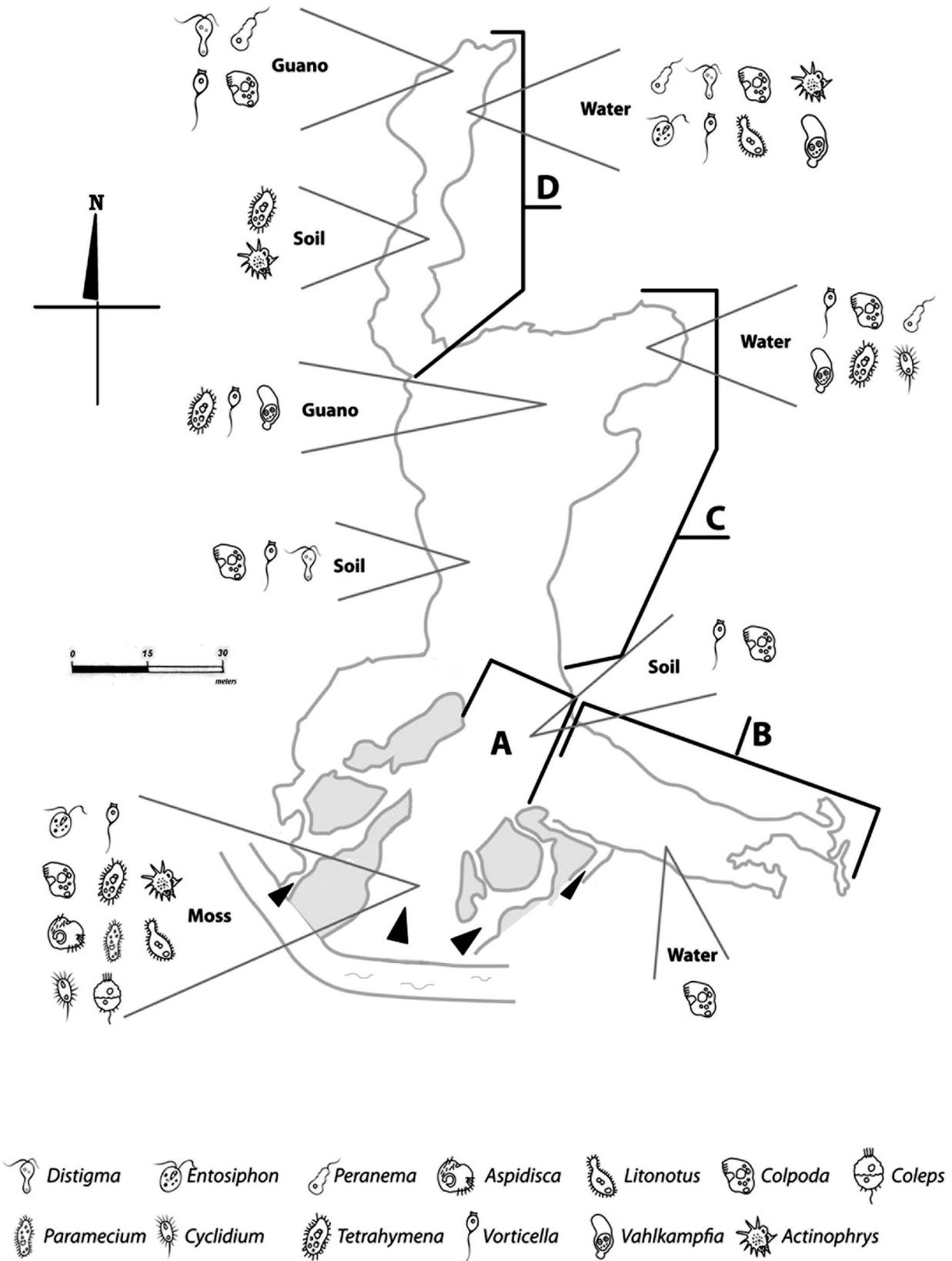


Figure 1. Cueva de Los Riscos map drawn by D. McKenzie of the Association for Mexican Cave Studies in 1966 (Lazcano Sahagún, 1986b, p. 79), modified by A. Espino del Castillo, M. Hernández, J.B. Morales-Malacara, and L. González of the Universidad Nacional Autónoma de México in March 2007, showing collecting places and species biodiversity at each biotope. Black arrow heads represent cave entrances.

Table 1. Distribution of protozoa genera recorded on each biotope during the study at Los Riscos Cave.

		Year														
		2005						2006						2007		
Rainy Season		Dry Season			Rainy Season			Dry Season			Rainy Season			Rainy Season		
November		April			June			October			March			June		
Zone	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes	Genus	Biotopes
A	NC	NC	<i>Entosiphon</i>	Moss
A	NC	NC	<i>Vorticella</i>	Moss
A	NC	NC	<i>Colpoda</i>	Moss
A	NC	NC	<i>Tetrahymena</i>	Moss
A	NC	NC	<i>Aspidisca</i>	Moss	<i>Vorticella</i>	Soil
A	NC	NC	<i>Paramecium</i>	Moss
A	NC	NC	<i>Cyclidium</i>	Moss
A	NC	NC	<i>Coleps</i>	Moss
A	NC	NC	<i>Actinophrys</i>	Moss
B	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
C	NC	NC	<i>Distigma</i>	Soil	<i>Vahlkampfia</i>	Water	<i>Vahlkampfia</i>	Guano	Water	
C	NC	NC	<i>Vorticella</i>	Soil, Water	<i>Vorticella</i>	Water	<i>Vorticella</i>	Guano	Water	
C	NC	NC	<i>Colpoda</i>	Soil	Water	<i>Tetrahymena</i>	Guano	Water	
C	NC	NC	Water	
D	<i>Peranema</i>	Water, Guano	NA	NA	NA	NA	NA	NA	...	
D	<i>Distigma</i>	Water	<i>Vorticella</i>	Water, Guano	NA	NA	NA	NA	NA	NA	...	
D	<i>Entosiphon</i>	Water	<i>Colpoda</i>	Guano	NA	NA	NA	NA	NA	NA	<i>Vahlkampfia</i>	Guano
D	<i>Vorticella</i>	Water, Guano	<i>Tetrahymena</i>	Soil	NA	NA	NA	NA	NA	NA	...	
D	<i>Colpoda</i>	Water	<i>Actinophrys</i>	Soil	NA	NA	NA	NA	NA	NA	...	
D	<i>Litonotus</i>	Water	NA	NA	NA	NA	NA	NA	...	
D	<i>Actinophrys</i>	Water	NA	NA	NA	NA	NA	NA	...	

Note: NC means no samples collected.
NA means no protozoa recorded.

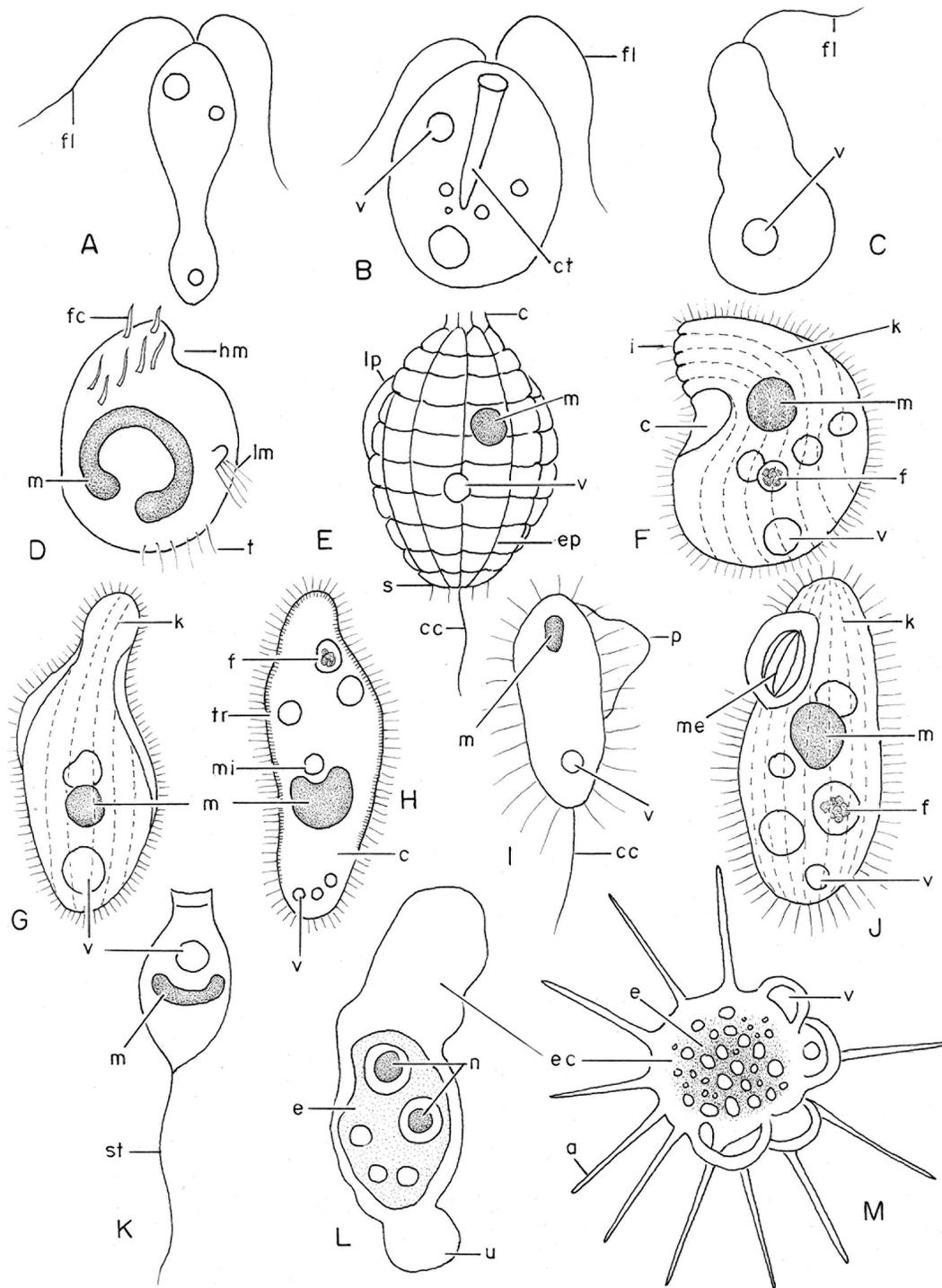


Figure 2. Protozoa species biodiversity found at Cueva de Los Riscos. Data in parentheses correspond to length and width measurements in micrometers, except for *Actinophrys sol* which corresponds to diameter. A. *Distigma* sp. (8.0 × 2.5); B. *Entosiphon* sp. (20.0 × 12.0); C. *Peranema* sp. (17.0 × 4.0); D. *Aspidisca* sp. (40.0); E. *Coleps hirtus* (40.0 × 30); F. *Colpoda* sp. (36.0 × 30.0); G. *Litonotus* sp. (80.0 × 34.0); H. *Paramecium caudatum* (200.0 × 60.0); I. *Cyclidium* sp. (20.0 × 10.0); J. *Tetrahymena pyriformis* (35.0 × 24.0); K. *Vorticella* sp. (25.0 × 20.0); L. *Vahlkampfia* sp. (30.0 × 10.0); *Actinophrys sol* (25). Abbreviations: a = axopodium; c = cytostome; cc = caudal cilium; ct = conical tubule; e = endoplasm; ec = ectoplasm; ep = ectoplasmatic plates; f = food vacuole; fc = frontoventral cirri; fl = flagellum; hm = higher adoral zone of membranelles; i = indentation; k = kinety; lm = lower adoral zone of membranelles; lp = lateral projection; m = macronucleus; me = membranelles; mi = micronucleus; n = nucleus; p = paroral membrane; s = spines; st = stalk; t = transversal cirri; tr = trichocysts; u = uroid; v = contractile vacuole.

spoons, and for moss we utilized forceps. Approximately 2 ml or 2 cc of each sample was placed in one sterilized Falcon tube containing 5 ml of nutritive sterile pea infusion (Patterson and Hedley, 1992, p.17–18). Additionally, 100 ml or 100 cc of each sample was collected in sterilized 100 ml glass jars. Moss samples were collected in plastic bags. All samples were transported in a cooler without ice, in order to maintain all samples in good condition.

All laboratory procedures started within 24 hours of collection. In all cases, to obtain polyxenic cultures (multiple species), 2 ml of each sample was added to natural infusions of straw, rice, wheat, pea, and corn (Lee et al. 1985; Jahn et al. 1979, p. 10–12) and chemical media (Chalkley, peptone, and RPMI-1640 Sigma) (Manwell, 1968, p. 559–572; Kudo, 1971, p. 848–852). The samples and cultures were maintained in the dark at 25 °C in an incubator, except for the moss samples, which were exposed to sunlight. To identify the protozoa and for a photomicrographic record, all the cultures were periodically examined by using phase-contrast, differential-interference-contrast and bright-field microscopes (Nikon Labophot-2 with Nikon Digital Sight DS-2Mv and Nikon FX-35DX incorporated camera; Zeiss Axioskop 2 plus with Zeiss AxioCam MRC system). Diagnostic characters were studied with staining and impregnation techniques such as Harris hematoxylin, Klein, protargol, and butanol-nigrosine (Borror, 1969; Kudo, 1971, p. 863; Lee et al., 1985; Silva-Neto, 2000).

We utilized the Jaccard index to estimate the degree of similarity between biotopes with regard to genera. The similarity values obtained were summarized by clustering, using the UPGMA method (program NTSYS pc. v. 2.2, Exeter Software, Setauket, New York).

RESULTS

We identified thirteen protozoa species, with three flagellates (*Entosiphon* sp., *Distigma* sp., *Peranema* sp.), one naked amoebae (*Vahlkampfia* sp.), one heliozoan (*Actinophrys sol* Ehrenberg), and eight ciliates [*Aspidisca* sp., *Litonotus* sp., *Colpoda* sp., *Coleps hirtus* (O. F. Müller), *Paramecium caudatum* Ehrenberg, *Cyclidium* sp., *Tetrahymena pyriformis* (Ehrenberg), and *Vorticella* sp.] (Table 1, Fig. 2). The protozoan distribution in relation to the cave zones and biotopes from all seasons is shown in Figure 1.

The species *Entosiphon* sp., *Distigma* sp., *Aspidisca* sp., *Litonotus* sp., and *Tetrahymena pyriformis* have not been recorded in caves anywhere in the world. The following species are new records for Mexican caves: *Actinophrys sol*, *Colpoda* sp., and *Paramecium caudatum* (Table 2).

The relative species richness among the cave biotopes, seasons, and zones, is shown in Figures 1 and 3. The most diverse biotopes were moss and water, and the soil was the least diverse (Table 1).

The greatest number of protozoan taxa was present in April 2006 (dry season), and the lowest was in October

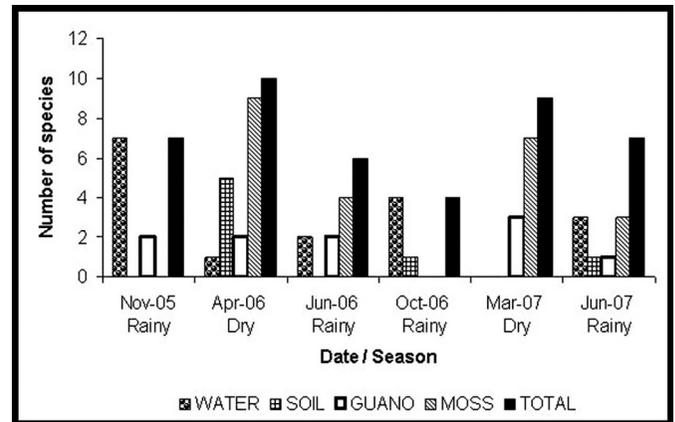


Figure 3. Number of protozoa species for each biotope and season at Cueva de Los Riscos. Note that some species appear in the totals for more than one biotope for each date.

(rainy season) of the same year, when flagellates were absent, with Zone A having the highest diversity

The genus *Colpoda* was found on all biotopes, zones, and periods. *Vorticella* sp. and *Tetrahymena pyriformis* were recorded in all biotopes and during all periods. The genus *Coleps* was recorded only in Zone A moss in April 2006.

Entosiphon was recorded in samples from five months (rainy and dry seasons), except for October 2006. The heliozoan *Actinophrys sol* was observed only in November 2005 (rainy season) and April 2006 (dry season). The amoeboid genus *Vahlkampfia* was recorded three times: October 2006 (rainy season), March 2007 (dry season), and June 2007 (rainy season).

In Zone B, we found only the genus *Colpoda*, which was observed in a draining water sample.

From the Jaccard similarity index two clusters were obtained; the first grouped the biotopes water, guano, and soil, and the second cluster included only the moss biotope. We obtained a similarity index of 0.6 between the water and guano biotopes, a similarity of 0.54 when both biotopes were compared with the soil, and a similarity of 0.38 when these three biotopes were compared with the moss.

DISCUSSION

According to Corliss (2002), the protists are cosmopolitan in overall distribution, and, in particular, most protozoa play roles mainly as phagotrophs (particulate consumers). Free-living species have a very broad distribution as planktonic or benthic forms. Free-swimming flagellates and ciliates are the most important consumers of bacteria in aquatic and terrestrial ecosystems.

Considering the functional groups of protozoa in ecosystems proposed by Pratt and Cairns (1985), the species found in the biotopes at Cueva de Los Riscos

Table 2. Genera recorded in this study, with previous cave records, if any.

Europe	Previous Records			Los Riscos Cave ^a	Record
	USA	México	References		
...	<i>Peranema</i> sp.	...	Barr, 1986	<i>Peranema</i> sp.	P
...	...	<i>Peranema</i> sp.	Hoffmann et al., 1986		
...	...	<i>Vahlkampfia</i> sp.	Hoffmann et al., 1986	<i>Vahlkampfia</i> sp.	P
<i>V. limax</i>	Golemansky and Bonnet, 1994		
<i>V. magna</i>	Golemansky and Bonnet, 1994		
<i>V. tachipodia</i>	Golemansky and Bonnet, 1994		
<i>Cyclidium oligotrichum</i>	Carey et al., 2001	<i>Cyclidium</i> sp.	P
<i>C. fuscum</i>	Carey et al., 2001		
<i>C. glaucoma</i>	Golemansky and Bonnet, 1994		
...	...	<i>Cyclidium</i> sp.	Hoffmann et al., 1986		
...			
...	...	<i>Tetrahymena</i> sp.	Hoffmann et al., 1986	<i>Tetrahymena pyriformis</i>	P
...	...	<i>Vorticella</i> sp.	Hoffmann et al., 1986	<i>Vorticella</i> sp.	P
...	...	<i>Vorticella</i> sp. cf. <i>microstoma</i>	Osorio-Tafall, 1943		
<i>Colpoda</i> sp.	Barr, 1986	<i>Colpoda</i> sp.	M
<i>C. steinii</i>	Golemansky and Bonnet, 1994		
<i>Paramecium caudatum</i>	Golemansky and Bonnet, 1994	<i>Paramecium caudatum</i>	M
...	<i>Paramecium</i> sp.	...	Barr, 1986		
...	<i>P. micromultinucleatum</i>	...	Holsinger, 1966		
...	...	<i>P. aurelia</i>	Hoffmann et al., 1986		
<i>Actinophrys sol</i>	Golemansky and Bonnet, 1994	<i>Actinophrys sol</i>	M
<i>A. vesiculata</i>	Golemansky and Bonnet, 1994		
...	...	<i>Actinophrys</i> sp.	Osorio-Tafall, 1943		
...	...	<i>Coleps</i> sp.	Hoffmann et al., 1986	<i>Coleps hirtus</i>	W
...	...	<i>Coleps</i> sp. cf. <i>hirtus</i>	Osorio-Tafall, 1943		
...	<i>Entosiphon</i> sp.	W
...	<i>Distigma</i> sp.	W
...	<i>Aspidisca</i> sp.	W
...	<i>Litonotus</i> sp.	W

^a This study.

W—New world and Mexican record for caves.

M—New Mexican record for caves.

P—Previously recorded in Mexican caves.

correspond to bactivores-detritivores (*Aspidisca* sp., *Colpoda* sp., *Coleps hirtus*, *Cyclidium* sp., *Litonotus* sp., *Paramecium caudatum*, *Tetrahymena pyriformis*, *Vorticella* sp., *Entosiphon* sp. and *Peranema* sp.), saprotrophs (*Distigma* sp.), and non-selective omnivores (*Actinophrys sol*). Foissner and Berger (1996) reported some species of *Litonotus* as predators. The main functional role of substrate-associated protozoa is the processing of dead organic matter and its associated bacterial flora (Pratt and Cairns, 1985).

Members of genera *Coleps*, *Vorticella*, *Tetrahymena* and *Paramecium* are very common in many ecosystems and have been previously reported in some cave biotopes (Table 2). Previous records of Mexican cave protozoa include species that were observed only in water samples, in contrast to our findings from multiple biotopes. As shown in Figure 1, *Vorticella* sp., a detritivorous genus, was recorded in all biotopes of the cave; therefore we consider this species as having a broad distribution.

The flagellates are typically smaller, but much more abundant than ciliates and often mixotrophic in their nutrition; they occupy both planktonic and benthic levels (Corliss, 2002). We found the genera *Entosiphon*, *Distigma*, and *Peranema* as benthic organisms.

The samples collected during October 2006 (rainy season) had the lowest diversity, with only four genera, probably because of the excessive water flow in the cave, as compared with April and May, that probably washed out protozoan populations. However, some puddles remained as propitious microhabitats for the development of protozoan communities and other organisms like small metazoans.

For the water biotope, we observed the highest species richness during November 2005 and October 2006 (rainy season). For the soil biotope we found the highest number of species during the spring months; however samples were not obtained for all collections during this period. The bat guano biotope presented only slight differences in species richness among seasons (Figure 3). April 2006 and March 2007 (dry season) had the highest number of species inhabiting the moss substrate.

We conclude for this cave system that the spring months, corresponding to the dry season, have the highest protozoan diversity.

According to Finlay et al. (1998), the same ciliate species are found wherever their preferred habitat is found. Free-living ciliates may be ubiquitous, as they are continually being distributed by effective passive dispersal; these statements can be taken into account when we analyze the protozoa recorded previously in world caves.

According to Hausman and Hülsmann (1996), some species of *Colpoda* can resist lower temperatures, which favors its establishment in several habitats. However, caves are systems where microclimate conditions are almost constant through the year in dark zones, and this environmental stability could be a dominant factor for

some protozoa species found throughout the cave, such as *Colpoda* sp. and *Vorticella* sp.

Cyst production by protozoa is sometimes just part of the life-cycle, but often is a response to unfavorable environmental factors, such as desiccation, temperature, or starvation, and is triggered in response to these conditions. Cyst formation has been documented in species of the genera *Colpoda*, *Vahlkampfia*, *Actinophrys*, and *Paramecium* (Hausmann and Hülsmann, 1996, p. 154–157), which were found at Cueva de Los Riscos, and this capability facilitates the presence of these species in different seasons inside the cave.

The differences in specific richness in the cave zones can be explained as a function of the type of biotopes present. The greatest values were obtained in Zone A (the light zone), followed by Zone D (the darkest and deepest zone), and Zone C (the twilight and dark zone). The elevated specific richness of Zone A could be explained because of the presence of moss, a biotope that favors protozoan population growth. In Zone C, we found small bodies of water formed by temporary water sources, and also permanent gour pools, which provide a more suitable habitat for protozoan communities. Water facilitates oxygen uptake, contains food resources, has surface tension for movement and dispersion, and is a medium that also facilitates reproduction; this explains the record of seven protozoa genera. In Zone D, permanently dark and where vampire bats (*Desmodus rotundus* (Geoffroy) and *Diphylla ecaudata* Spix) were present in all seasons, the predominant biotope was the guano of the hematophagus bat, which had fewer species than the moss and water substrates, but provides nutrients such as nitrogen compounds for protozoa and is, according to Hoffmann et al. (1986, chap. III), a major energy source in caves, supporting five genera of heterotrophic protozoa. Zone B (twilight zone), had the lowest specific richness, but this can be due the small number of samples collected there because of its inaccessibility.

The Jaccard index demonstrated a similarity degree of 0.6 between the water and the bat guano biotopes, which shared mainly bactivore-detritivore species. Water and guano biotopes shared four species with the soil biotope, for a 0.54 similarity index. The biotopes guano, water, and soil were distributed into the darkest areas of the cave and proved to be suitable substrates for the protozoan colonization and establishment.

Soil is a microhabitat that could be frequently exposed to variable degrees of desiccation affecting the ciliates, flagellates and amoebae. That could explain why we only found six species (three ciliates, one flagellate, and the heliozoan), as compared with other biotopes. However, a cave system usually exhibits high humidity, preventing desiccation. Nevertheless, protozoan diversity in soil was lowest, probably due to other factors, such as granulometric and physical characteristics that could prevent free movement and dispersion, among other functions.

The community assemblages of several taxa of protozoans in the same time and place could be explained by their trophic roles in relation to the type and availability of food resources. The protozoa have a wide spectrum of food requirements, and these were available in the different cave biotopes documented. Broad tolerances of the most common taxa suggest that some species should be found in nearly every natural system (Pratt and Cairns, 1985) and explain why most of the species recorded in the present work also have been reported in habitats other than caves worldwide. In respect to this particular cave ecosystem, previous data refer only to protozoa from water samples of caves. We found that the all the biotopes considered in this study were suitable for many protozoa, favoring reproduction and providing food sources, among others requirements.

CONCLUSIONS

We sampled four biotopes in Cueva de Los Riscos that were inhabited by thirteen protozoa species. Each biotope provided favorable conditions, but they harbored different species compositions and richness throughout the dry and rainy seasons. We recorded for the first time five protozoa species in caves worldwide; an additional three species are new records for Mexican caves. With these data we conclude that protozoa have a wide distribution in cave systems, but more studies are needed to complete the records in these ecosystems.

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