El Colegio de la Frontera Sur

Los hongos *Wutz anim* (ojo de muerto) de la Reserva de la Biósfera Volcán Tacaná

TESIS
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Dedicatoria

A mi abuelita Saturnina Mérida Arrollo†

que en paz descanse

A mi esposa Alejandra Guadalupe Castillejos Fuentes

A mi hija Avril Pineda Castillejos

por su amor y apoyo incondicional

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A mi padre Jaime Pineda López

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Índice

1. Introducción

Desde 1971 la UNESCO mediante el programa el Hombre y Biósfera (MaB), otorga la categoría de “Reserva de la Biósfera” para áreas geográficas tanto terrestres como acuáticas, en las que se encuentren especies endémicas, amenazadas o en peligro de extinción, que contemplen una amplia variedad de ecosistemas y gran riqueza de organismos. Su finalidad es representar la diversidad de una región, país o del planeta, promoviendo el desarrollo humano sostenible y conservando los diferentes ecosistemas y biodiversidad (UNESCO, 2009). A partir de 2003, el área en la que se encuentra el volcán Tacaná es declarada Reserva de la Biósfera Volcán Tacaná (REBIVTA); contemplando una gran variedad de ecosistemas que representan gran parte de la riqueza faunística y florística del país (Vargas y Escobar, 2000; SEMARNAT, 2013). El sitio abarca 6,378.86 ha (CONANP, 2011) y se localiza aproximadamente a 30 Km del centro de Tapachula, se extiende hasta las zonas altas de la ciudad, los municipios de Unión Juárez y Cacahoatán hasta la frontera con Guatemala, al norponiente con la sierra madre de Chiapas y al sur con la planicie costera del Pacífico. La ruta más utilizada para llegar a la reserva es partiendo desde Tapachula para llegar a Talquián o Chiquihuites en Unión Juárez y continuar a pie por la vereda principal hacia la cima del volcán (Mendoza, 2012; SEMARNAT, 2013).

Actualmente en la REBIVTA, así como en algunos municipios fronterizos de Chiapas y el occidente de Guatemala, se localizan asentadas comunidades de la etnia *Mam*, descendientes directos de la cultura maya que se estableció en el Soconusco desde tiempos prehispánicos. En el estado existen aproximadamente 20,000 individuos hablantes de la lengua *Mam*, lo que refleja su población aún sin estimaciones exactas

(Quintana y Rosales, 2006; Hernández, 2011). Su principal actividad agrícola es la siembra del maíz; sin embargo, cultivan también otras hortalizas, café, flores, colectan hongos y crían aves de corral, así como cerdos y borregos. Sus productos generalmente se destinan para el autoconsumo, aunque una parte se comercializa en los mercados aledaños. En cuanto al uso de los hongos (macromicetos), las personas más longevas tienen el mayor conocimiento de estos, ya sea medicinal, comestible, venenoso, entre otros; y han procurado transmitirlo a las siguientes generaciones. Las colectas de hongos se realizan por veredas del volcán y en su mayoría en temporada de lluvias. Los macromicetos recolectados son principalmente de tipo agarical, así como un grupo de hongos conocidos por la etnia como *Wutz anim* que en lengua *Mam* significa “ojo de muerto” y que usan de manera comestible y medicinal. Este último grupo presenta hongos muy similares a los géneros *Lycoperdon*, *Holocotylon* y *Bovista* (Medina, Andrade y Sánchez, 2014; SEMARNAT, 2013). De manera general estos géneros lucen carpóforos de color blanco, que al desarrollarse se tornan cafés, grisáceos o amarillos-pardos y apapelados al madurar o secarse, tienen forma de pera o globo, con pseudoestípite o sin este. Sus esporas las producen en la gleba que suele tener el mismo cambio de color que el carpóforo. Para esparcir sus esporas desarrollan un ostiolo o fragmentan su cuerpo fructífero compuesto de dos capas peridiales (exo y endoperidio). Con excepción del género *Holocotylon*, se consideran cosmopolitas, se desarrollan mejor en temperaturas templadas, en lugares abiertos, boscosos o entre bambúes. Pueden crecer en conjunto o solitarios, en suelos arcillosos, arenosos, con musgos o directamente sobre el humus (Reid, 1977; Calderón, 1986; Alexopoulus, Mims y Blackwell, 1996; Moore-Landecker, 1996; Bates, Roberson y Desjardin, 2009).

Actualmente se conocen 169 especies de *Lycoperdon*, 92 de *Bovista* y 3 de *Holocotylon* (Roskov, et al., 2014).

Los registros hasta la fecha respecto de estos hongos implican su presencia en bosques caducifolios, de coníferas, en praderas semidesérticas y en vegetación predominante de pino-encino, en climas templados y hemiboreales, en países fríos (Groenlandia, Islandia, Bélgica, Rusia, etcétera) y a bajas temperaturas entre 0-10°C. Se han reportado creciendo en el suelo, sobre el humus, en alfombras de musgos, en zonas abiertas, en caminos cubiertos de hierba, sobre el pasto, entre rocas así como en suelos arenosos con hortalizas, en altitudes que varían entre 1,050-5,000 msnm (Mariaca, Luz y Castaños, 2001; Bates, Roberson y Desjardin, 2009; Larsson, Jeppson y Larsson, 2009; Jeppson, Larsson y Martín, 2012). Por su parte Calonge, Kreisel y Guzmán (2004) describieron para la Reserva Natural El Ocote, en Ocozocuatla, Chiapas, México, una nueva especie del género *Bovista* (*B. sclerocystis*). Para la REBIVTA se conocen únicamente dos estudios que han reportado la presencia de este grupo de hongos; Pérez (2006) realizó tres registros del género *Lycoperdon*, *L. perlatum, L. flavotinctum* y *Lycoperdon* sp. Mientras que Medina, Andrade y Sánchez (2014) reportaron dos especies más del mismo género, *L. umbrinum* Pers. y *L. pedicellatum* Peck. Por su parte, respecto de *Holocotylon* además del presente estudio que logró identificarlo, no se conoce antecedentes de su presencia en la región, incluso en todo el sur del país.

Por otro lado, si se quiere conocer más respecto a este grupo de macromicetos en la REBIVTA, es importante profundizar en su aislamiento y propagación micelial en medio de cultivo, ya que la información conocida es muy escasa y estos hogos son

interesantes en la región debido a su consumo y a sus propiedades hemostáticas. En relación a lo anterior, se han realizado algunos estudios como el de Bulmer (1963) quien germinó esporas de los géneros *Bovista* y *Lycoperdon*, observando en numerosas ocasiones estructuras a las que llamó cuerpos fructíferos abortivos, producidas en placas de Petri con medios de cultivo nido de pájaro, agar extracto de malta o Czapek dox modificado. Estas estructuras presentaron características muy similares a las descritas para estos géneros en la naturaleza (peridio redondo, blanco y blando) con la diferencia que no maduran ni producen esporas. Peña y Barba (1994) realizaron estudios fisiológicos con dos cepas de *Calvatia cyathiformis* y una de *Lycoperdon* sp. sin llegar a fructificación, obteniendo un mejor desarrollo micelial para esta última cepa en agar extracto de malta, a pH entre 5-6, relaciones C/N altas (10-100) y temperaturas de incubación que varían de 25 a 30°C. Para el género *Holocotylon* no existen reportes sobre su cultivo.

Es importante reconocer que los estudios micológicos que se han realizado tanto en Chiapas como en la REBIVTA son realmente muy escasos, y que existe un claro interés por el grupo de hongos denominado *Wutz anim*, tanto cultural (ya que representan una fuente de alimento, medicina o ingreso económico) así como científico debido a que se desconocen aspectos básicos de este grupo de macromicetos y su presencia en la reserva. En base a estas consideraciones, se planteó el siguiente estudio con el objetivo de determinar las especies que comprenden el grupo de hongos *Wutz anim*, sus condiciones de crecimiento y distribución en el área de estudio, así como evaluar su capacidad para desarrollarse en medio de cultivo a nivel laboratorio.

2. Artículo

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Title: The *Wutz anim* (dead-eye) mushroom of the Tacaná Volcano Biosphere Reserve

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2.1 Summary

The Tacaná Volcano Biosphere Reserve is a very important area for the Soconusco zone; it includes different ecosystems with a variety of fauna, flora and mushrooms such as the *Wutz anim* group (word of the *Mam* ethnic group). The latter are macromycetes belonging to the Agaricaceae family characterized by being edible and have haemostatic properties. This study aims to determine the species found in the reserve and their distribution, and habitat and assess their mycelial growth in a controlled environment. Through molecular analysis it was determined that the genera *Lycoperdon*, *Holocotylon* and *Bovista* are part of the mushroom group known as *Wutz anim* and that each genus develops better in different culture media. They bear fruit in the soil between 2,800 to 3,150 masl, at average temperature of 15°C and surrounded by oak pine vegetation. This study constitutes the first report of the genus *Holocotylon* in the south of Mexico.

2.2 Keywords

Gasteroid fungi, Puffballs, Agaricales, *Lycoperdon, Bovista* and *Holocotylon*

2.3 Introduction

Since 2003, the top of the Tacaná volcano is included in the Biosphere Reserve (TVBR) because its high biodiversity and presence of Mexican endemic species (Catas and De la Maza 2010; SEMARNAT 2013). In the TVBR there are human settlements among others the *Mam* ethnic group, which is direct descendant of the Mayas established in the region since prehispanic times. Their main agricultural activity is corn production, although they collect mushrooms for consumption or local commerce (Quintana and Rosales 2006). The macromycetes used are mainly of agarical type. Among the mushrooms they gathered, the group called *Wutz anim* (deadeye) takes advantage for its edible and medicinal properties (hemostatic). They identify these mushrooms mainly by its globular shape, white or brown color, soft consistency when young, when mature for the manner in which spores are expelled, through its hole at the top (ostiole) and the fruiting zone (Peña and Barba 1994; Medina et al. 2014).

For the general characteristics previously mentioned, it is assumed that these mushroom belong to the former group Gasteromycetes (currently Agaricomycetes) characterized by the structure of the hymenium, commonly known as "puffballs". These include among others, *Calvatia* spp and the genera *Bovista*, *Holocotylon* and *Lycoperdon.* The latter currently includes 169 species (Alexopoulus et al. 1996; Roskov et al. 2014), practically present worldwide in

temperate zones, in open or wooded areas, growing together or alone, in clay soils, with moss or on humus (Calderón 1986; Moore-Landecker 1996). Of the genus *Holocotylon*, only three species are known: *H. anomalum* Zeller, *H. texense* Lloyd and *H. brandegeeanum* Lloyd (Roskov et al. 2014). Growing conditions are similar to the previous genus and its distribution is reported in southeastern United States and northern Mexico (Bates et al. 2009). Meanwhile, the genus *Bovista* has 92 species (Roskov et al. 2014), cosmopolitan distributed, grows between bamboos, open places and in temperate zones (Reid 1977).

There is a clear interest in this group of mushrooms both cultural (in the *Mam* ethnic group) as well as scientific; despite studies and efforts, the fungistic knowledge both in Chiapas as in the Soconusco has been lost, and even more speaking specifically of the group called *Wutz anim*. In the TVBR, only the presence of *Lycoperdon umbrinum* Pers., *L. pedicellatum* Peck, (Medina et al. 2014) *L. perlatum* Pers., *L. flavotinctum* Bowerman and *Lycoperdon* sp. (Pérez 2006) has been reported. Also, a new *Bovista (B. sclerocystis*) species was identified in El Ocote Nature Reserve, approximately 450 km of TVBR (Calonge et al. 2004). While for the genus *Holocotylon* as mentioned above, there are no reports for southern Mexico.

On the other hand, as for the cultivation of mushroom of this group, spores germination has been reported for *Lycoperdon* and *Bovista* in Malt Extract Agar (MEA) and modified Czapeck Dox (Bulmer 1963, 1964), as well as physiological studies with *Calvatia cyathiformis* and *Lycoperdon* sp. in MEA media at temperature of 25-30°C (Peña and Barba 1994). There is no record on the cultivation of species of *Holocotylon.*

Therefore, the aim of this research was to know the genera and species that comprise the group of mushrooms *Wutz anim,* know their ecology and distribution within the TVBR and exploring their mycelial growth on solid culture.

2.4 Materials and methods

Study area and samples collection

The TVBR covers 6,378.86 ha and a maximum height of 4,100 masl. It is located southeast of Mexico in the state of Chiapas, divided by the boundary line between Mexico and Guatemala (Vargas and Escobar 2000; SEMARNAT 2013). It is bordered to the east by Guatemala, to the northwest with the mountains of the Sierra Madre de Chiapas and to the south with the Pacific coastal plain (Mendoza 2012). Three trips were conducted to collect carpophores of the group *Wutz anim*. The first two trips were conducted in the same way in the months of June and September 2013, starting to collect from the community of Chiquihuites (2,062 masl) and ending up to the point known as the "La

tranca", approximately 500 m from Papales community (2,952 msl). In the third trip conducted in May the following year, collection was finished up to the site "Bosque encantado" (3,261 masl) and in the site "El campo" (3,105 masl). The three collections were conducted along the main trail towards the summit of the volcano.

Upon collecting mushroom with morphological characteristics similar to that describing the group *Wutz anim* (globular or pear shape, smooth, blotchy, warts or spines, whitish or dark color, small size, hole at the top, absence of foot) (Medina et al. 2014), the growth substrate, temperature (ambient and substrate), height (masl), vegetation and coordinates (GPS) were registered, photograph was taken as well as substrate sample (about 20 g) to determine humidity and pH. All specimens collected were transferred individually wrapped in wax paper, to the laboratory of Tropical Mushrooms of ECOSUR for further study and conservation.

To determine the pH, 10 g of the substrate were placed in 15 ml of distilled water in a beaker. It was allowed to stand for 20 min at 26°C, filtered using clean gauze and the pH of the resulting liquid was determined by a potentiometer (Rivera 2003). For humidity, five grams of substrate were taken and placed in a crucible at 105°C for two days up to constant weight. Subsequently the formula used was Moisture% = [(initial sample weight–final sample weight)/initial sample weight] x100 (Chanatásig 2014).

Isolation of mushroom collected

The vegetative isolation or multisporic technique was used (depending of the maturity of the fungus) and the next solid media with approximately 10 mg/l of ciprofloxacin: malt agar extract, potato dextrose agar and yeast (Gaitán-Hernández et al. 2006), rose Bengal medium, water agar at pH 4.5, 5.6, 7.1 and 6.8, respectively. They were incubated at 24°C, between 15 and 30 days, with periodic revisions up to achieving their isolation.

Macro and microscopic characterization of mushroom isolated

Observations were made mainly in the MEA and PDYA media at 30 days of growth, considering the color and shape of the colony, texture and type of mycelium, color change and growth immersed in the media, exudate, edge and color on the back of the plate. Microscopic features were observed with solution of 5% KOH, at 40 and 100x objective lenses of a compound microscope considering the size, thickness and color of the hyphae as well as the presence of septa and fibulae (Sánchez et al. 2000). The mycelial color annotation was performed according to Küppers (1978).

Total nucleic acid extraction and PCR of mushroom isolated

The extraction of total nucleic acids (TNA) of the mushroom previously isolated was carried out, starting with about 0.1 g of mycelium and following the methodology proposed by Liu et al. (2000). The final extract obtained was dried at 60°C for 10 min and resuspended in 35 µl of ultrapure water. For amplification 1 µl of TNA was used, the oligos employed were ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al. 1990). The PCR reactions were done in reaction volume of 20 µl, (10x Buffer, 0.2 pmol of each oligo, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase Thermo Scientific ®). The amplification conditions used in the thermal cycler (SelectCycler, Select Bioproducts ™) were: 5 min initial denaturation at 94ºC, followed by 40 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min. Finally, a final extension of 72°C for 5 min was applied. The amplicons obtained were stored at -20°C, and then purified by Quantum Prep PCR Kleen Spin pack of *Bio-Rad*® brand according to the manufacturer's instructions. TNA integrity, PCR products as well as the purified amplicons was determined by electrophoresis in 1% agar gel.

Molecular Identification

The purified PCR products were sequenced in both directions using the Macrogen Inc ™ (Seoul, Korea) capillary sequencing method. The sequences were purified and manually edited using the Bio-Edit (Hall 1999) program and used to perform BLAST (Basic Local Alignment Search Tool) searches in the GenBank (http://www.ncbi.nlm.nih.gov/). Results showing highest percentages of identity, coverage and lower error value were selected. Subsequently, these sequences were deposited in the GenBank database, see Table 1.

Phylogenetic reconstruction was performed with the MEGA6 (Tamura et al. 2013) program, using reported and non-redundant sequences of the genera *Lycoperdon*, *Holocotylon* and *Bovista* from the NCBI database (http://www.ncbi.nlm.nihgov/) and BOLD Systems (http://www.boldsystems.org/). These sequences were aligned with those obtained experimentally, using ClustalW and Muscle tools from MEGA6 program, the alignment was manually edited. Then, the distance matrix was generated to eliminate redundant sequences (with values of 0.000). The reconstruction was performed through Maximum Likelihood method, using the Kimura 2-parameter substitution model, gamma distribution and 1000 repetitions (bootstrap) as statistical test.

Development of mycelium of mushroom isolated in culture media

Six culture media were used (Table 2): Malt extract agar (MEA), MEA complemented with Kirk (K) media (López et al. 2012), potato dextrose and yeast extract agar (PDYA) (Gaitán-Hernández et al. 2006), Melin-Norkrans culture media (MCM) (Riquelme 2011), Czapeck-Dox (Cz-D) media (Becard and Fortín 1988) and Hagem (Hg) media (Helmholz et al. 1999). Three Petri dishes per media were inoculated (repetitions) with a 30 days 0.5 cm agar mycelium fragment of the strains under study. They were incubated at 24°C and mycelial growth was recorded every third day for four weeks. The radial expansion rate (RER) was determined by the equation y=Krx+c (y=independent variable, Kr=slopes value taken as RER, x=dependent variable and c=intersection) (Riquelme 2011). With the data obtained an analysis of variance and a mean comparison test (Tukey, $p \leq 0.05$) using R statistical package (Fox 2005) was performed.

2.5 Results

Collection and Isolation of mushrooms

129 specimens (fruiting stage) were collected with characteristics similar to those previously described for the *Wutz anim* mushroom group. These were found between vegetation of mountain cloud forest (MCF), pine forest (PF), cornfield (CF), pine-oak grassland and oak forest. The majority was collected in the latter two, with 67 and 53 mushrooms, respectively. Meanwhile, altogether in the PF, CF and MCF, the least (nine in all) of these macromycetes were found. Of the mushroom collected, 26 strains were isolated in PDYA and MEA media, representing 20% of the total. The data collected from the isolated mushroom are found in Table 1.

Macro and microscopic observations of mycelia of isolated strain

81% of all cases showed a white mycelium ($N_{00}Y_{00}M_{oo}$). 50% developed a creeping type mycelium, while 54% of the strains penetrated the culture medium. The only type of edge found was irregular, 69% had cottony texture, and at the back of the Petri dish, it was observed that 85% exhibited cream color ($N_{00}Y_{20}M_{10}$). No hyphae with fibulae were observed, the hue for all hyphae evaluated was hyaline, 69% showed cenocitic hyphae while the rest presented septa. The average hyphal diameter was 1.1 µm, while 54% had characteristic thickening with range of 1-5 µm (Table 3).

Molecular Identification

The amount of TNA extracted from each isolated strain was approximately 300 mg/µl, enough for amplifying the ITS region in all the samples where expected products were obtained for oligos employed between 600 and 800 bp. The sequences obtained experimentally were compared in the NCBI database. It was found that of the 26 mushrooms isolated and characterized, two are similar to the *Lycoperdon perlatum* (ECS-20 and ECS-45) species, five to *L. ericeum* (ECS-32 ECS-33, ECS-36 ECS-38 and ECS-44) and four to *L. rupicola* (ECS-34, ECS-35 ECS-39 ECS-43), all with ≥96% identity values. Eleven were similar to *Holocotylon brandegeeanum* (ECS-19 ECS-23 ECS-24 ECS-25 ECS-26 ECS-27 ECS-28 ECS-31 ECS-37 ECS-41 and ECS-42) with ≥97% identity values. Three were similar to *Bovista graveolens* (ECS-29 ECS-30 and ECS-40) and one to *B. nigrescens* (ECS-22) with 99% identity. Coverage was 90-100% for the 26 strains. In addition, all showed 0.0 error. Therefore, it can be considered that the mushrooms comprising the referred *Wutz anim* group in the TVBR belong to the genera *Lycoperdon*, *Bovista* and *Holocotylon*.

Phylogenetic reconstruction

After comparing the 26 sequences identified between each other through distance matrices, were finally contemplated for phylogenetic analysis: six sequences with similarity by BLAST to the genus *Lycoperdon*, seven to *Holocotylon* and two to *Bovista*. In the phylogenetic tree obtained (Fig. 1) ECS-29 and ECS-40 are observed within the clade *Bovista* with 72% bootstrap values. With the exception of *L. echinatum* GBAGA10199-14, in this group all the sequences (experimental and databases) belonging to the genus *Bovista* were located, with bootstrap values between 50-94%. Most experimental sequences of the genus *Holocotylon* were arranged in a separate group (bootstrap 57 and 93%), close to the only sequence found for this genus in the database, but not included in that group. Moreover, five of the six experimental sequences similar to *Lycoperdon* through BLAST formed a separate clade supported with 95 and 76% bootstrap values. There is also the inclusion of a sequence similar to *Holocotylon* by BLAST. The ECS-45 isolate formed a group with *L. perlatum* and *Lycoperdon* sp. with 99 and 79% bootstrap values.

Distribution of the mushrooms isolated and identified in the TVBR

With the exception of the mushrooms located at 2,240 masl, 96% of the specimen corresponding to the three genus of mushroom studied that make up the *Wutz anim* group were collected in an altitudinal range that varied from 2,800 to 3,150 masl. The vegetation contemplated in the above mentioned area was of pines, oaks, as well as the site known as "El pajonal" with herbaceous shrub type vegetation surrounded by pine-oak. 92% of these macromycetes found was growing in soil with an average temperature of 14°C and substrate of 13°C. Moreover, it was observed that the samples collected at each point grew both in group or alone. The pH and average humidity of the substrate in which they were found was 5.6 and 45%, respectively (Table 1).

Mycelium development of the mushroom *Wutz anim* in culture media

The strains of the genera *Lycoperdon*, *Holocotylon* and *Bovista* presented a mycelial growth in the six media evaluated, with an average radial extension rate of 0.57±0.04, 0.66±0.06 and 0.77±0.2 mm per day respectively.

a) Genus *Lycoperdon*

With the exception of *Lycoperdon* sp. ECS-38 which had a similar statistically development in all media tested ($p=0.196$), other strains showed a variable behavior according to the media evaluated under the established culture conditions. There could not be detected a media that was most suitable for all species of this genus, although in 62.5% of cases the statistical group with the fastest growth corresponded to the MEA media. In contrast, 87.5% of the strains exhibited the slowest development in Hg media after 30 days of growth (Fig. 2).

b) Genus *Holocotylon*

Except for the ECS-23 ($p=0.162$), ECS-25 ($p=0.57$) and ECS-27 ($p=0.168$) strains which had a similar statistically development in all tested media, the other strains showed a variable behavior, based on the media assessed under the established culture conditions. No most suitable media for all the species of this genus were detected, although in 85.7% of cases, statistical groups with the fastest growth were seen in the PDYA and CzD media. In contrast, 85.7% of the strains presented slower development in the MEA media after 30 days of growth (Fig. 3).

c) Genus *Bovista*

With the exception of *Bovista* sp. ECS-30, that had a similar statistical development in all tested media (p=0.29), other strains showed a variable behavior, based on the media assessed under the established culture conditions. No most suitable media for all species of this genus were detected, while in the remaining two cases (ECS-22 and ECS-29), the statistical group with the fastest growth corresponded to the CzD, K and PDYA media. In contrast, the MCM and Hg media presented the slowest development after 30 days of growth (Fig. 4).

2.6 Discussion

Habitat records of the mushrooms studied in this work are consistent with the type of substrate (soil), temperature (10-20°C), height (2,800 to 3,150 masl) and vegetation (pine-oak) found when the mushrooms were collected during trips made along the main trail towards the summit of the volcano (129). In their research, Jeppson et al. (2012) indicate the presence of the genus *Lycoperdon* in deciduous forests in vegetation areas with temperate and hemiboreal climate, growing in soil, on humus, sandy soil and mosses carpets. In the state of Mexico, Mariaca et al. (2001) reported for the Toluca Valley and surrounding mountains, the presence of this genus in altitudes ranging from 2,500 to 5,000 masl in temperate climate and temperatures below zero up to 10°C, growing in predominant pine-oak vegetation. Mushrooms of the genus *Bovista* studied by Larsson et al. (2009) were collected in temperate countries (Greenland, Iceland, Belgium, Russia, etc) in open areas, grass-covered roads, as well as in sandy soils with vegetables. Meanwhile Bates et al. (2009) mentioned that the genus *Holocotylon* grows in the ground, on grass and between rocks, in areas of high and low elevation (1,050 to 3,500 masl), inside coniferous forest and semi-desert grasslands at low temperatures. As observed, these genera are cosmopolitan and as in the current study, these mushrooms have been reported in different altitudes, temperatures and various substrates. The lower altitudes (2,240 and 2,838 masl) recorded correspond to specimens of the genus *Holocotylon*, although this does not demonstrate a specific characteristic for that group, as they were also found at higher elevations.

In different studies that have attempted isolation from fungal spores of the genera *Lycoperdon*, *Calvatia*, *Bovista* and *Scleroderma*, there have been problems achieving mycelium development, even contamination by *Penicillium* sp. has been reported (Hoffmann 1860, Ferguson [1902 cited by Bulmer and Beneke 1961] and Cool [1912 cited by Bulmer and Beneke 1961]). Among the few attempts in the isolation or culture of this group of mushrooms, Fries (1941) was successful but reported a low percentage (0.1%). Other recent studies managing to isolate fungi of this

group are those of Bulmer and Beneke (1961, 1964), Sánchez et al. (2000) and López et al. (2012). In the present study, similar contamination problems occurred from green fungal colonies, fluffy, large and rapid growth of the genera *Trichoderma*, *Aspergillus* or *Penicillium*. Although the number of mushrooms isolated in this research represents a low percentage (about 20%) compared with the above-mentioned studies, to our knowledge it is the largest number of isolates of this type of mushroom recorded. The highest temperature observed in the field during the collections was 20°C, and on average was about 15°C, whereas incubation in this study was done at 24°C, since mycelial growth has been reported at temperatures of 25-30°C (Bulmer 1963; Peña and Barba 1994). Another point to consider is that, in Aguilar-Aguilar et al. (2011) and Vásquez-Gassibe et al. (2013) researches, some species of the genus *Lycoperdon* and *Geastrum* are considered as mycorrhizal, due to its abundance among pine trees where they have been identified in roots; while Sánchez et al. (2000) mentioned in their study that the genus *Lycoperdon* is not mycorrhizal. Despite this controversy, the cause of low number of isolates could be due to the lack of certain nutrient, association, protection or other relationship between these mushrooms and vegetation (grassland oak pine) where they were collected. In addition, the fact that the incubation temperature was different to the collection may have also affected the isolation of strains of the *Wutz anim* group.

The morphological characteristics of the fruiting body that presented the fungi collected were in general a globular form, white ($N_{00}Y_{00}M_{00}$) or brown ($N_{80}Y_{50}M_{40}$), presence of ostiole and absence of stem, similar to those described for this family by Peña and Barba (1994) and Alexopoulus et al. (1996). The characteristics of the fruiting bodies facilitated the search and collection of mushroom in the TVBR. While both macro and microscopic mycelial characteristics helped select strains for isolation and identification. Generally included mycelium with white ($N_{00}Y_{00}M_{00}$), creeping, slow growth and hyaline hyphae with thickening of up to 5 µm and absence of spores, similar to those described for this group of mushroom by Sánchez et al. (2000).

In the state of Chiapas, the presence of the genus *Lycoperdon* has been registered by Ruan-Soto and García-Santiago (2013), while for the TVBR four species are reported to date (*L. umbrinum*, *L. pedicilatum*, *L. perlatum*, *L. flavotinctum* and *Lycoperdon* sp., Pérez 2006 and Medina et al. 2014). In the present study performed in the same reserve, the genus was identified by phylogenetic reconstruction (Fig. 1) of the mushrooms that make up the *Wutz anim* group, determining that eleven strains belongs to the genus *Lycoperdon*, four to *Bovista* and eleven to *Holocotylon*. Unlike other previous studies also conducted in the TVBR, the species of mushrooms reported for this genus coincide only with *L. perlatum* (according to BLAST analysis). Meanwhile, for *Holocotylon* and *Bovista* there

are no reports even in the state. It is possible that the different species for the genus *Lycoperdon* reported in studies of Pérez (2006), Medina et al. (2014) and the current are present in the TVBR, but have not been able to be isolated, have not coincided with the time of collection or are not fully identified. Moreover, it is important to note the method of identification used in each investigation, since the physical characteristics of the species of these mushrooms are very similar and can often be confused, which could be the cause of not having any reports for the genus *Bovista* and *Holocotylon* in Chiapas. In this study, in addition to the morphological analysis, the ITS sequences obtained from isolates were analysed. While Pérez (2006) and Medina et al. (2014), they used classical morphological criteria in its identification process. Both types of identification are valid. Today molecular identification is a reliable tool to determine the species of an organism, although it has the disadvantage that many of the sequences reported in the databases, are not "cured" or confirmed (Sánchez Gómez et al. 2012). Therefore, it is important to use both types of identification to confirm the species not only of the genus of interest in this study, but for of the macromycetes in general present in the TVBR. As mentioned previously, to identify the *Wutz anim* group of mushrooms a phylogenetic reconstruction was performed, for which there were used sequences of the region ITS amplified with the oligos ITS4 and ITS5. This region was used because has proved to be amplifiable in most mushrooms tested, besides presenting a high degree of divergence between closely related mushrooms groups (Dentinger et al. 2011; Shoch et al. 2012). On the other hand, the NS3, NS8, ITS1, ITS3, ITS4, ITS5, ITS1F, LR21, LR0R and LR7 oligos have been used in other studies to amplify the complete ITS region, as well as small ribosomal subunits (SSU) and long (LSU). In phylogenetic reconstructions that included these mushrooms, there have been reported clades formed only by sequences of the same genus, the presence of sequences in clades that did not correspond, as well as clades with bootstrap values of 100% consisting solely of experimental sequences (Krüger et al. 2001, Larsson and Jeppson 2008, Bates et al. 2009, Larsson et al. 2009; Jeppson et al. 2012). Situations similar to those mentioned previously occurred in the reconstruction performed in this study, such as the formation of a clade almost exclusively by sequences of the genus *Bovista*, in addition that most experimental sequences formed clades among them without including sequences from the databases. This reflects the difference between genus and the close relationship that exists between the mushrooms of the group *Wutz anim* in the TVBR.

Moreover, one of the peculiarities of this work is that it is reported the presence of mushroom of the genus *Holocotylon*, the latter has only been reported in the US and in the north of Mexico. Suggesting an extension of the area where these fungi are located. Apart from finding macromycetes of this particular genus, phylogenetic

reconstruction reported seven different (non-redundant) sequences, suggesting that some of them may belong to different species within that group. This is important because as far as it is known, there are only three species reported for the genus in question (*H. anomalum*, *H. texense* and *H. brandegeeanum*). This opens opportunities to more studies regarding to this genus, giving rise to new species for both TVBR and for science.

There has been little research involving the cultivation of mushroom of the group of interest in this study. Yet, the results obtained by other authors are very similar, as in the work of Sánchez et al. (2000), who after four weeks of incubation obtained colonies of *L. perlatum*, of 1.5-2.5 cm in diameter in MCM. Consistent with this, in the same media in this study averaged colonies of 1.8 cm in diameter were obtained for strains of the genus *Lycoperdon* during the same growth period. In MEA media colonies of 0.8-2.6, 1.4-3.9 and 1.1-3.2 cm in diameter were recorded for the strains of the genus *Lycoperdon*, *Bovista* and *Holocotylon* respectively, during four weeks of incubation. Similar to that described by Bulmer and Beneke (1961), who tested various culture media to germinate *Calvatia gigantea* spores, stating that the MEA media is the most appropriate and that these genus needs at least four weeks of incubation to develop visible colonies. Three years later they obtained *L. curtisii*, *L. marginatum*, *L. pusillum* colonies in 2% MEA, incubated at 26°C for 2-4 weeks (Bulmer and Beneke 1964). Bulmer in that same year managed to germinate from spores in 1.5% MEA media, 24 species of puffballs including several of the genera *Lycoperdon*, *Bovista* and *Calvatia*. López et al. (2012) used among other, several strains of Lycoperdaceae family (currently Agaricaceae) to evaluate a method of conserving water and filter paper, using MEA media supplemented with Kirk to observe viability of the strains after a period of storage, found that strain of the Lycoperdaceae family developed colonies of almost 6 cm for a month of growth. In connection with this research, in the current study during the same time and in the same medium, the strains of the three genus evaluated recorded on average colonies of 2.1 cm in diameter. As observed, even though the purpose of some work was not to compare the development of these mushroom, they are grown in different media, with similar results to those obtained in this study. In addition to the evaluation conducted among media-strain interaction, it can be suggested at least one medium for every genus in which there are more possibilities of achieving greater mycelial development.

On the other hand, since a culture media that would be the most suitable was not found for the three or each genus evaluated in this study, the elements of a specific media cannot be define. Therefore, in general, some nutrients which can promote and benefit the mycelia development of mushrooms, which are present in the media used in this research are: glucose and maltose are considered the best sources of carbon for mushroom development, (Ayodele

2008); it has been reported that an increase in the concentration of these carbohydrates promotes greater mycelial growth (Torres-López et al. 2011). Meanwhile, yeast extract has been reported as the best source of nitrogen for fungal growth (Chang et al. 2006; Jo et al. 2009; Torres-López et al. 2011), in addition to being an excellent source of thiamine (Tortora et al. 2007), which plays an very important role in the development of these organisms as it contributes in the generation of energy through the breaking of carbohydrates (Kavanagh 1942; Sedlmayr et al. 1961; Chung and Tzeng 2009). Yeast extract together with that of malt are also complex substrates that contains a wide variety of proteins, fats, minerals, vitamins, sugars and salts in necessary and sufficient amounts that promote fungi mycelial growth (Torres-López et al. 2011).

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476 **2.9 Tables**

477 Table 1. Details of mushrooms collected in the Tacaná Volcano Biosphere Reserve (shown which were isolated in

478 culture media)

Strain (ECS)	Specimens[a]	Height (masl)	Coordinates (° ' '')		T (°C)		Vegetation[b]	Substrate			GenBank Accession
			North	West	Ambient	Substrate		Type[c]	pH	% Humidity	
19	1	2967	15 06 31	92 05 56.8	18	13	POG	S	6.2	45.1	KR811069
20	1	2962	15 06 56.2	92 05 55.7	15	14	POG	S	5.6	34.4	KR811070
22	1	2972	15 06 58.9	92 05 57.3	20	16	POG	S	5.9	24	KR811071
23	1	2991	15 06 57.4	92 05 57.4	19.5	12	POG	SL	5.2	57.3	KR811072
24	1	2990	15 0 657	92 05 57.2	19	17	POG	S	5.7	22.5	KR811073
25	5	2998	15 06 57.3	92 05 57.6	15	12	POG	SL	5.5	39.4	KR811074
26	1	2240	15 06 51.1	92 06 07.5	19.5	17.5	MCF	S	6.1	41.7	KR811075
27	1	2838	15 06 39.9	92 05 53.7	14	12	POG	S	5.8	39.2	KR811076
28	1	2989	15 06 58.1	92 05 57	15	13	POG	S	5.7	25.4	KR811077
29	7	3133	15 07 04.8	92 06 11.1	14	14	OF	S	5.2	49.1	KR811078
30	3	3138	15 07 04.8	92 06 11.1	13.5	14	OF	S	5.2	48.1	KR811079
31	4	3121	15 07 04.6	92 06 12.2	14.5	12	OF	S	5.5	61	KR811080
32	5	3117	15 07 05.3	92 06 12.7	13	13	OF	S	6	41.8	KR811081
33	5	3119	15 07 05.3	92 06 12.7	13	13	OF	S	5.3	47	KR811082
34	4	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811083
35	2	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811084
36	3	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811085
37	4	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811086
38	4	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811087
39	3	3105	15 07 05.4	92 06 11.3	13	14	OF	S	5.6	56.4	KR811088
40	7	3103	15 06 56.4	92 05 55.6	10.5	10.5	POG	S	5.9	34.5	KR811089
41	5	2964	15 06 56.1	92 05 55.7	10	10	POG	S	5.6	28.3	KR811090
42	5	2964	15 06 56.1	92 05 55.7	10	10	POG	S	5.6	28.3	KR811091
43	3	2978	15 06 56.3	92 05 56.0	11	10.5	POG	S	5.4	63.3	KR811092
44	3	2976	15 06 57.5	92 95 57.3	11	10	POG	S	5.5	51.6	KR811093
45	3	2976	15 06 57.5	92 95 57.3	11	10	POG	S	5.5	51.6	KR811094

479 [a]Specimens per collection point, [b]Vegetation type: POG=Pine-oak grassland, MCF=Mountain cloud forest, OF=Oak

480 forest, [c]Substrate type: S=Soil, SL=Soil and litter

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485 Table 2. Composition of culture media used

Nutriment	Culture media					
	PDYA	MEA	CzD	K	Hg	MCM
Dextrose	5 g	8 g	10 g	10 g	10 g	10 g
Yeast extract	2 g	-	1 g	-	-	-
Malt extract	-	16 g	-	16 g	3 g	3 g
Peptone	-	1 g	-	1 g	-	-
Ammonium tartrate	-	-	2 g	-	-	-
$MgSO_4 \cdot 7H_2O$	-	-	0.5 g	0.5 g	0.15 g	0.15 g
KCl	-	-	0.5 g	-	-	-
KH_2PO_4	-	-	1 g	2 g	0.25 g	0.5 g
$CaCl_2 \cdot 2H_2O$	-	-	-	0.1 g	0.05 g	-
$NH_4H_2PO_4$	-	-	-	-	0.25 g	0.25 g
$FeSO_4 \cdot 7H_2O$ (1%)	-	-	-	-	1.3 ml	1.2 ml
NaCl (1 g/100 ml)	-	-	-	-	-	2.5 ml
Thiamine	-	-	-	-	-	0.1 mg
$CaCl_2$ (1 g/100 ml)	-	-	-	-	-	6.7 ml
Mineral solution	-	-	1 ml	10 ml	-	-
Bacteriological agar	16 g	16 g	8 g	16 g	16 g	16 g
pH	5.6	4.5	5.5	5	5.4	5.8

486 MEA=Malt extract agar, PDYA=Potato dextrose and yeast extract agar, CzD=Czapeck-Dox media, MCM=Melin-
487 Norkrans culture media, K=MEA complemented with Kirk media, Hg=Hagem media. Media formulated to 1 l. To
488 PDYA infusion of potato (200 g potato/1 l distilled H_2O) was used
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503 Table 3. Mycelial isolates features

Record (ECS)	Macroscopic						Microscopic				
	Color[a]	Immersed in the media	Type[b]	Texture[c]	Irregular edge	Reverse[d]	Hyaline hyphae	Size (µm)	Thickening (µm)	Fibulae	Hyphae type[e]
19	W	-	C	C	+	C	+	1	1.5	-	C
20	W	+	C	F	+	C	+	1	-	-	C
22	C	-	C	C	+	C	+	2	-	-	S
23	C	-	C	F	+	C	+	2	5	-	C
24	W	-	C	C	+	C	+	1	-	-	C
25	W	-	P	F	+	C	+	1	5	-	S
26	C	+	P	P	+	C	+	2	4	-	S
27	W	-	C	C	+	C	+	1	3	-	S
28	W	+	C	C	+	C	+	1	5	-	S
29	W	+	A	Cw	+	B	+	2	4	-	S
30	W	-	P	Cw	+	B	+	2	3	-	S
31	W	+	C	C	+	C	+	1	-	-	C
32	W	+	C	C	+	C	+	1	-	-	S
33	W	+	F	C	+	C	+	1	2	-	S
34	W	+	F	C	+	C	+	1	2	-	S
35	W	+	F	C	+	C	+	<1	1	-	C
36	W	+	C	C	+	C	+	<1	3	-	S
37	W	+	C	C	+	C	+	<1	1	-	S
38	W	+	F	C	+	C	+	1	-	-	S
39	W	+	F	C	+	C	+	<1	-	-	S
40	C	-	C	Cw	+	B	+	1	1.5	-	S
41	W	-	F	C	+	C	+	<1	-	-	C
42	W	+	F	C	+	C	+	1	-	-	S
43	W	-	F	C	+	C	+	1	-	-	C
44	W	-	F	C	+	Y	+	1	-	-	S
45	C	-	C	P	+	C	+	2	4	-	S

504 [a]Color: W=White ($N_{00}Y_{00}M_{00}$), C=Cream ($N_{00}Y_{20}M_{10}$), [b]Type: C=Creeping, P=Plane, A=Aerial, F=Filamentous,
505 [c]Texture: C=Cottony, F=Filamentous, P=Plane, Cw=Cobweb, [d]Reverse: C=Cream ($N_{00}Y_{20}M_{10}$), B=Brown
506 ($N_{40}Y_{40}M_{20}$), Y=Yellow ($N_{00}Y_{30}M_{00}$), [e]Hyphae type: C=Cenocitic, S=Septa.

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2.10 Figure legends

Fig. 1 Consensus tree of Maximum Likelihood based on the analysis sequences of ITS mushrooms of the genera *Lycoperdon* (*), *Holocotylon* (**), and *Bovista* (***) of about 600 nucleotides. The accession numbers of the sequences appear in parentheses. Bootstrap values are expressed in percentage of 1000 replicates. Clades supported with bootstrap values of 50 or more are indicated

Fig. 2 Radial extension rate of the isolated strains of the genus *Lycoperdon* in six culture media, evaluated for four weeks. Same letters indicate no significant difference between the media of each strain, according to the Tukey test (α=0.05)

Fig. 3 Radial extension rate of the isolated strains of the genus *Holocotylon* in six culture media, evaluated for four weeks. Same letters indicate no significant difference between the media of each strain, according to the Tukey test (α=0.05)

Fig. 4 Radial extension rate of the isolated strains of the genus *Bovista* in six culture media, evaluated for four weeks. Same letters indicate no significant difference between the media of each strain, according to the Tukey test (α=0.05)

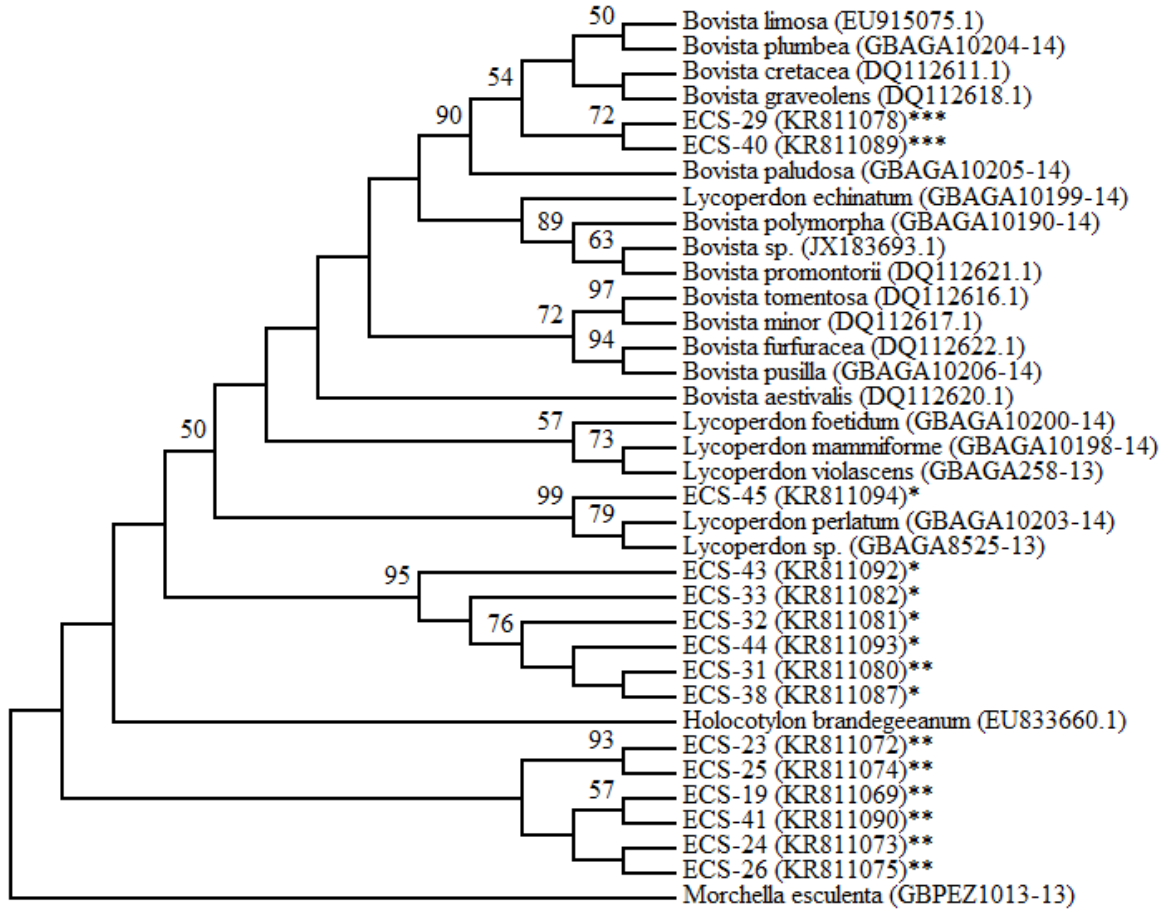
50
Bovista limosa (EU915075.1)
Bovista plumbea (GBAGA10204-14)
54
Bovista cretacea (DQ112611.1)
Bovista graveolens (DQ112618.1)
90
72
ECS-29 (KR811078)***
ECS-40 (KR811089)***
Bovista paludosa (GBAGA10205-14)
Lycoperdon echinatum (GBAGA10199-14)
89
Bovista polymorpha (GBAGA10190-14)
63
Bovista sp. (JX183693.1)
Bovista promontorii (DQ112621.1)
97
Bovista tomentosa (DQ112616.1)
72
Bovista minor (DQ112617.1)
94
Bovista furfuracea (DQ112622.1)
Bovista pusilla (GBAGA10206-14)
Bovista aestivalis (DQ112620.1)
50
57
Lycoperdon foetidum (GBAGA10200-14)
73
Lycoperdon mammiforme (GBAGA10198-14)
Lycoperdon violascens (GBAGA258-13)
99
ECS-45 (KR811094)*
79
Lycoperdon perlatum (GBAGA10203-14)
Lycoperdon sp. (GBAGA8525-13)
95
ECS-43 (KR811092)*
ECS-33 (KR811082)*
76
ECS-32 (KR811081)*
ECS-44 (KR811093)*
ECS-31 (KR811080)**
ECS-38 (KR811087)*
Holocotylon brandegeeanum (EU833660.1)
93
ECS-23 (KR811072)**
ECS-25 (KR811074)**
57
ECS-19 (KR811069)**
ECS-41 (KR811090)**
ECS-24 (KR811073)**
ECS-26 (KR811075)**
Morchella esculenta (GBPEZ1013-13)

Fig. 1

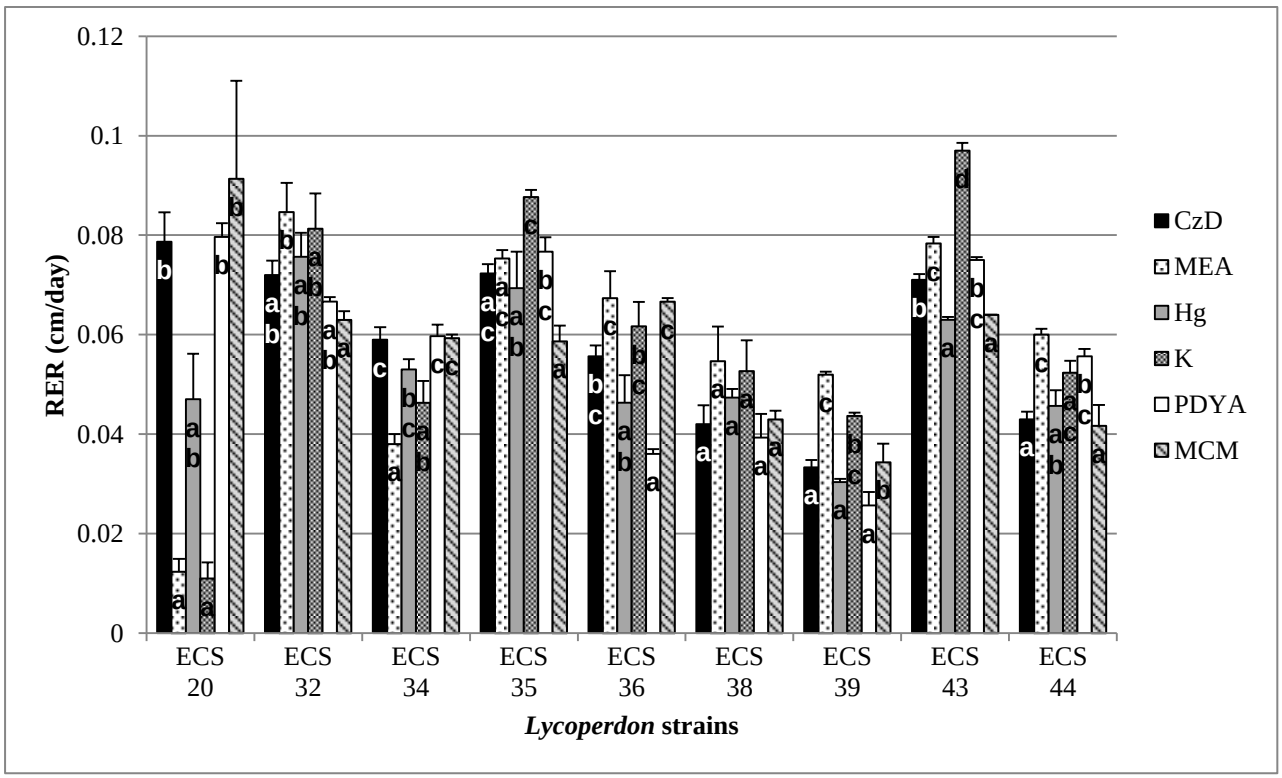
0.12
0.1
0.08
0.06
0.04
0.02
0
RER (cm/day)
ECS 20
ECS 32
ECS 34
ECS 35
ECS 36
ECS 38
ECS 39
ECS 43
ECS 44
Lycoperdon strains
CzD
MEA
Hg
K
PDYA
MCM

Fig. 2

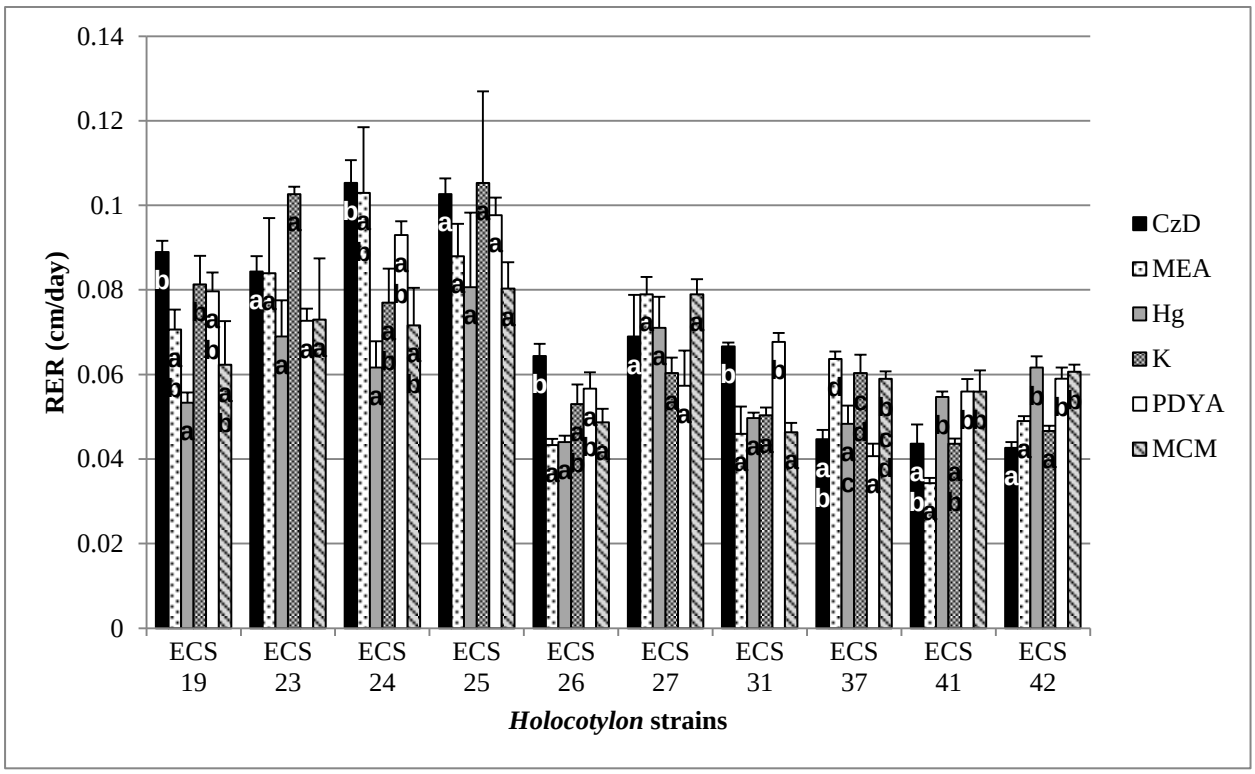
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
RER (cm/day)
ECS 19
ECS 23
ECS 24
ECS 25
ECS 26
ECS 27
ECS 31
ECS 37
ECS 41
ECS 42
Holocotylon strains
CzD
MEA
Hg
K
PDYA
MCM

Fig. 3

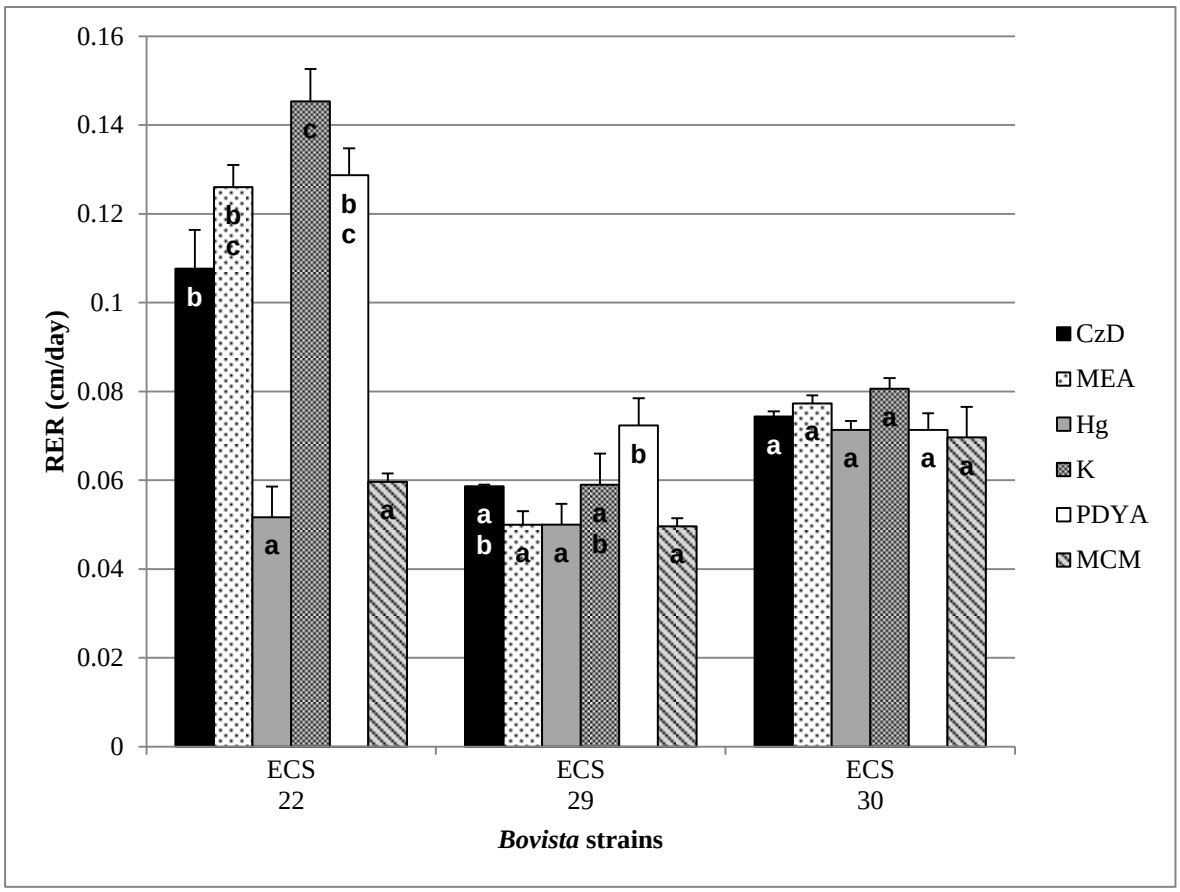
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
RER (cm/day)
b
b c
a
c
b c
a
a b
a
a
a b
b
a
a
a
a
a
a
CzD
MEA
Hg
K
PDYA
MCM
ECS 22
ECS 29
ECS 30
Bovista strains

Fig. 4

3. Conclusiones

Las especies identificadas en el presente estudio y que componen el grupo de hongos *Wutz anim* son similares según BLAST a *Lycoperdon perlatum*, *L. ericeum*, *L. rupícola*, *Bovista nigrescens*, *B. graveolens* y *Holocotylon brandegeeanum*. Sin embargo, la reconstrucción filogenética sugiere que la mayoría de las secuencias empleadas pertenecen a especies distintas a las reportadas en las bases de datos. Este estudio constituye el primer registro de *Holocotylon* en Centroamérica (sur de México) y existen posibilidades que se encuentren nuevas especies de este género en la REBIVTA.

Las condiciones de crecimiento registradas para los tres géneros en la REBIVTA comprenden temperaturas promedio de 15°C, fructificación en el suelo y desarrollo rodeado de vegetación pino encino. Su distribución contempla un rango altitudinal entre los 2800-3150 msnm abarcando la zona conocida localmente como “El pajonal”.

El desarrollo micelial de estos hongos no está ligado a un solo medio de cultivo, *Lycoperdon* creció mejor en EMA, *Holocotylon* en CzD y PDAL, mientras que *Bovista* lo hizo en CzD, PDAL y K.

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5. **Anexo 1.** Mapa de distribución de los hongos *Wutz anim* en la Reserva de la Biósfera Volcán Tacaná.