

# Biodiversity of anaerobic fungi from ruminants in republic of Macedonia

## Abstract

Anaerobic chytridiomycete fungi are found in the gastrointestinal tracts of sheep, cattle and goats, as well as in many other domesticated ruminant and nonruminant herbivores and a wide variety of wild herbivorous mammals. They are principally found associated with the fibrous plant particles of digesta and as free swimming zoospores in the fluid phase. The presence of large fungal populations in animals consuming mature pasture or diets largely composed of hay or straw together with the production of highly active fibre degrading enzymes lead to the belief that anaerobic fungi may have a significant role to play in the assimilation of fibrous feeds by ruminants. This paper is focused on anaerobic fungi because of their unusual biology and metabolism, and the biotechnological potential of their cellulases.

**Keywords:** anaerobic fungi, chytridiomycete, ruminant, herbivores, zoospores, cellulases

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## Introduction

Herbivores as well as other vertebrates are not capable of producing cellulases and/or hemicellulases. Instead, a lot of herbivores form symbiotic associations with bacteria, protozoa and fungi which produce these enzymes and therefore are capable of degrading the plant polymers. In return for the relatively constant environment and continuous flow of plant material, the microorganisms of the herbivores' digestive system provide the animal with easily usable form of carbon and energy, and ruminants with microbial proteins. Microbial eco-system of rumen is stable and at the same time dynamic.<sup>1</sup> Stability is seen in the fact that the system itself is set well, in function and bioconversion of food in volatile fatty acids. In healthy ruminants, there is no contamination of eco-system although millions of microorganisms attack the rumen every day via food, water and air. Namely, this happens because rumen-microorganisms are adapted to survive the conditions in the rumen, whereas contaminants do not survive. They are anaerobiosis, high buffer capacity and osmotic pressure. On the other hand, the eco-system is dynamic due to the change of microbial population by changing food and frequency of feeding.

The only ultra structure of anaerobic fungi, their adaptation of gastrointestinal tract of herbivores and their habitation among phylogenetically different animals teaches us that they could exist as a separate group until the time when these mammals had started diverging, at least 120 million years ago.<sup>2</sup> Although comparatively a little is known for the taxonomy of anaerobic fungi, generally it is known that they are zoospore-producing fungi and should belong to the classis of *Chytridiomycetes*. The order *Spizellomycetales* in *Chytridiomycetes* was established by Barr<sup>3</sup> with the division of *Chytridiales* in order to explain the differences in the ultra structure of zoospores. Anyway, there are a lot of similarities between the families and genera from both or dines (*Spizellomycetales* and *Chytridiales*). Six ordines are known: Neocallimastix, Piromyces, Orpinomyces, Anaeromyces, Caecomyces, and since recently Cyllamyces.<sup>4</sup> In Republic of Macedonia this is the first study to process anaerobic fungi in domestic and wild rumen animals.

## Materials and methods

### Source of organisms

Anaerobic fungi were isolated from rumen content and feces from more domestic and wild herbivorous mammals. Animals which took participation in this paper are given in Table 1. Feces was taken in several occasions around 10.00-10.30am and was picked up fresh from the ground and was taken immediately to the laboratory. Also, feces which has been old only several days was used. Only in one case was feces used, directly taken from the moufflon's large intestine. Rumen content was taken during slaughtering, directly from rumen, after which was taken to laboratory immediately.

### Medium for isolation and cultivation

Many of the cultivation techniques and media which are used in rumen-microbiology originate from Hungate<sup>5,6</sup> who studied anaerobic rumen bacteria. These techniques were modified and upgraded in order to provide media and procedures which are nowadays used constantly in the anaerobic microbiology.<sup>7</sup> With relatively few exceptions, these media and techniques are used today, together with anaerobic bags and procedures with Petri dish<sup>8,9</sup> for isolation and studying of anaerobic fungi. The basic medium which was used to isolate anaerobic fungi, was the medium 10 (M10) of.<sup>10</sup> Medium M10 is one of the numerous media used in anaerobic rumen-microbiology and for our region and climate, ideal. It contains rumen fluid and that is why it is described as complex or habitat-simulating; it is well buffered at pH from 6.5 to 6.8 with bicarbonate and/or phosphate buffers and might be solidified with 0.8-1.5% agar. The medium contains resazurin as redox indicator, micro and macrominerals, organic and/or inorganic nitrogen sources and chemically reducing agents, sodium sulfate and/or L-cystin hydrochloride.<sup>11</sup> Reducing agents and anaerobic techniques are essential during medium preparation. One or more antibacterial antibiotics, penicillin, streptomycin and chloramphenicol incorporate in the medium; this is essential during isolation of anaerobic fungi from rumen content and faecal samples.<sup>12</sup> In fact, antibiotics are necessary because of the anaerobic fungi growth which is completely inhibited in co-culture in anaerobic rumen bacteria.

In order to isolate axenic cultures from anaerobic fungi from their natural environment, free from contaminated bacteria, it is essential that repetitive subcultures are used, antibacterial antibiotics and some form of physical separation, such as cultivation of isolated colonies of agar medium.

**Table 1** Animals used in this paper

Animal	Origin of the animal (Type of animal- phylum)
Cattle( <i>Bos indicus</i> )	Z (R)
Domestic yak ( <i>Bos gruniens</i> )	Z (R)
Ankole-watusi ( <i>Bos vatusi</i> )	Z (R)
Red deer ( <i>Cervus elaphus</i> )	Z, J (R)
Fallow deer ( <i>Cervus dama</i> )	Z, J (R)
Barbary sheep ( <i>Ammotragus lervia</i> )	Z (R)
Pygmy goat ( <i>Capra nigra</i> spp.)	Z (R)
Roe deer ( <i>Capreolus capreolus</i> )	Z, J (R)
Mouflon ( <i>Ovis musimon</i> )	J (R)
Wild water buffalo ( <i>Bos bubalus</i> )	D (R)
Sheep ( <i>Ovis aries</i> )	D (R)
Goat ( <i>Capra hircus</i> )	D (R)
Chamois( <i>Rupicapra rupicapra</i> )	J (R)

Z, ZOO; J, Protected Nature Reserve Jasen; D, Domestic Animal; R, Ruminant Animal.

### Determination of morphological features of obtained isolates

In order to study the morphology and anatomy of the obtained pure isolates, cultivated in liquid medium M10, they have been additionally observed by applying the lighting and fluorescent microscopy, in different stages of their development. For that purpose, there had been a native preparation which was coloured with safranin and was directly observed. To observe some of the isolates, for which the lighting microscopy did not give enough data, fluorescent microscopy was also used where a drop of suspension was mixed with fluorochrome colour and bisbenzimidazole (5mg/l PBS) to color the nuclei. The Nucleitum fluorescent when lightened by UV light. They were determined accordingly the morphology of colonies, volume of fungal rhizoids and appearance of zoospores, with regards to.<sup>13</sup>

### Result and discussion

There are two morphological forms, monocentric (one reproductive body) and polycentric (more centres of reproduction). These forms are determined in the earliest stages of development and are invariable. In both forms, after zoospore encystation, the cyst germinates with producing germinative tube. In monocentric types, the nucleus does not enter the germinative tube. The germinative tube develops in rhizodal system with determined length. Anuclear rhizoids have double usage, grounding and absorption of nutrients. Rhizoids are of two types: typical filamentous form in *Neocallimastix* and *Piromyces*, and bulbous form in *Caecomyces*.

In polycentric types (*Orpinomyces* and *Anaeromyces*) the nucleus migrates outside the zoospore cyst in the germinative tube. The

zoospore cyst does not have a further function in the development, but the cyst wall might remain. The germinative tube elongates and ramifies in mycelium (rhizomycelium) similar to other filamentous fungi. The nucleus ramifies constantly and migrates along the individual hyphae. This form of development results in mycelium thallus with unpredictable volume with many sporangia. Unfortunately, after the continuous culture, many of the mycelium polycentric types either produce sporangia which do not differentiate in zoospores, or they do not produce sporangia anymore, and their identification becomes problematic.

In identifying chytrids, it is essential not to forget their natural morphological variation. This variation complicates the identification and classification of these organisms. Because of the temperature needs and anaerobic status of anaerobic fungi, the problem is amplified and compared to the other chytrids, growth and development of one thallus cannot be studied microscopically under normal circumstances. The differences in the medium probably contribute to more variations rather than any other factor. When the medium is too rich, as in the glucose or in filter paper, the sporangia often become abnormally big and abort. Small but mature thalli of monocentric types, except *C. communis*, are similar and only by observing of the type of discharge of zoospores and zoospores flagellation, they might differ. When anaerobic fungi are identified and classified, it is essential to make sure the sporangia are healthy and viable, and conclusions should be reached only after enough material had been observed.

In addition, there is a description of obligatory anaerobic fungi, which have been isolated during the preparation of this study. The description is based completely on the thallus morphology, as seen through the light microscope, in order to enable functional identification of genera and phyla.

#### Isolate EZ1

Endogenously developed zoosporangia, spheric with diameter of 65.71 µm; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 20 µm in diameter; main rhizoid is often coiled; rhizoids might have strongly narrowed places, rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore.

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic fungi of,<sup>13</sup> the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.

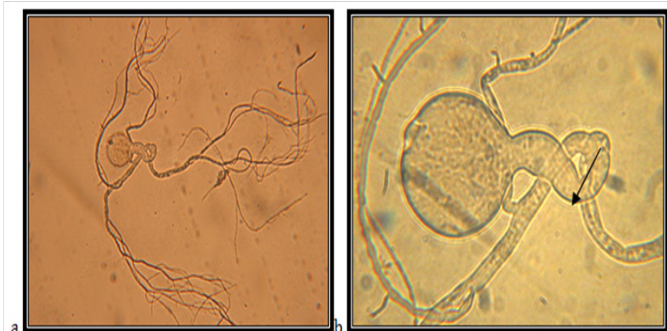
In Malaysia, *N. frontalis* has been isolated from rumen material and droppings from a buffalo, ox, sheep, goat and deer.<sup>14</sup> Also, rumen material has been isolated from oxen in New Zealand,<sup>15</sup> Canada,<sup>16</sup> Australia<sup>17</sup> and USA<sup>18</sup> and from the rumen, the saliva and droppings from sheep in Great Britain<sup>19,20</sup> (Figure 1) (Figure 2).

#### Isolate EZ2

Endogenously developed zoosporangia, spherically elongated (egg-like); 157.14 µm in diameter; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 17.14 µm in diameter, coiled; main rhizoid is often coiled; rhizoid might have strongly narrowed places; rhizoid system is spread up to 1 mm in diameter, zoospores are liberation through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores

are variable in length and shape, globular zoospores with 10-25µm in diameter, with 10 to 30 flagella, 35-50µm long.

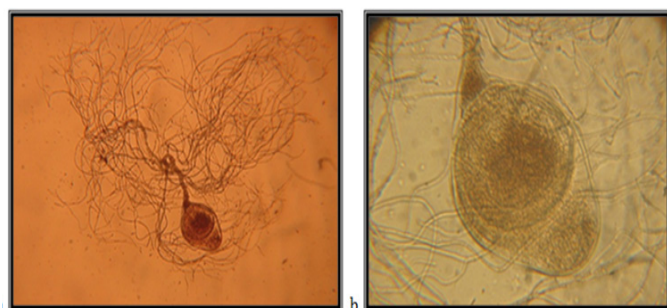
The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Neocallimastix frontalis* (Figure 3).



**Figure 1** Strain EZ1- *N. frontalis*. Monocentric thallus with one sporangium, wide neck, main rhizoid coiled (narrow).a, Magnification 10x; b, Magnification 40x.



**Figure 2** Polyflagellate zoospore of *N. frontalis*.



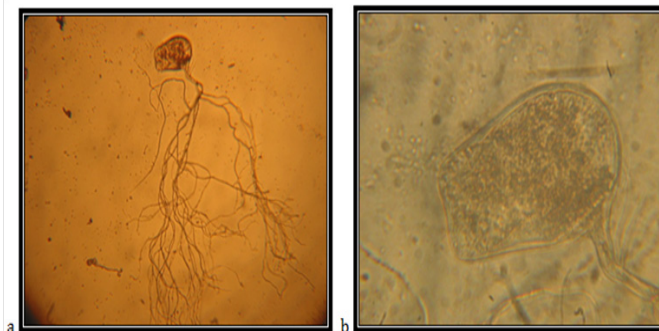
**Figure 3** Strain EZ2- *N. frontalis*. Monocentric thallus with short egg-like sporangiophore. a. Magnification 10x; b. Magnification 40x.

### Isolate EZ3

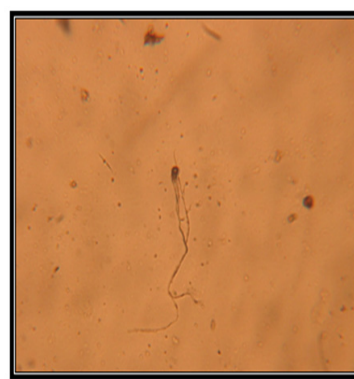
Zoosporangia, asymmetrically spheric, 161.24µm in diameter; widespread rhizoid, the neck is not narrowed or slightly narrowed, the main axis, close to the sporangium, up to 14.28µm in diameter, coiled; the main rhizoid is often coiled; rhizoid system is spread up to 600µm in diameter; zoospores are liberated through side pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape; spheric zoospores, polyflagellates.

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic

fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Neocallimastix* spp (Figure 4) (Figure 5).



**Figure 4** Strain EZ3- Monocentric thallus with zoosporangium, in the moment of liberation of zoospores. a. Magnification 10x; b. Magnification 40x.

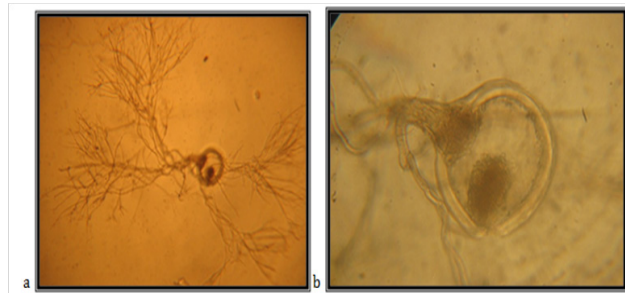


**Figure 5** *Neocallimastix* spp- polyflagellate zoospore.

### Isolate EJI

Zoosporangia, symmetrically spheric, 119.99µm in diameter; widespread rhizoid, the neck is slightly narrowed, the main axis, close to the sporangium, up to 25.71µm in diameter, coiled; the main rhizoid is often coiled; rhizoid system is spread up to 800µm in diameter; zoospores are liberated through side pore, accompanied by fast decomposition and cracking of the sporangium wall, which is clearly double-layered; zoospores are variable in length and shape; spheric zoospores, polyflagellates.

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Neocallimastix* spp (Figure 6).



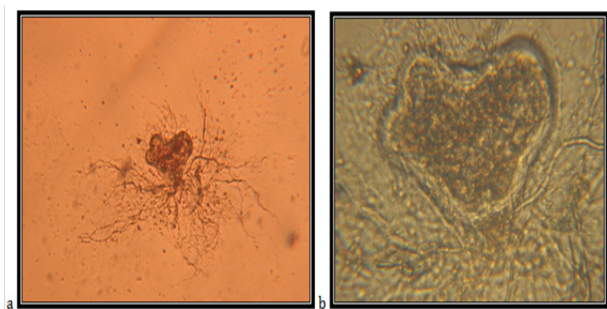
**Figure 6** Strain EJI- Monocentric thallus with zoosporangium in the moment of liberation of zoospores. a. Magnification 10x; b. Magnification 40x.



### Isolate SZ1

Monocentric sporangia, double pear-like 31\*115µm; with several papillae; the main rhizoid is filamentously spread, it elongates up to 180µm, discharge follows after the decomposition of the papillae; zoospores are ovoid, 3.1-8.0µm, uniflagellate. The isolate is taken from feces of a roe deer (*Capreolus capreolus*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces mae*.

In Malaysia, *P. mae* has been isolated from the rumen and feces of oxen, buffalos, sheep and goats, as well as from the duodenum of the sheep. Also, this type has been isolated from the rumen of Elk in Canada<sup>16</sup> and in the rumen of sheep in France<sup>21</sup> (Figure 7) (Figure 8).



**Figure 7** Strain SZ1- *Piromyces mae*, endogenous sporangium with two papillae. a. Magnification 10x; b. Magnification 40x.



**Figure 8** Uniflagellate zoospores- *P. mae*.

### Isolate SJ1

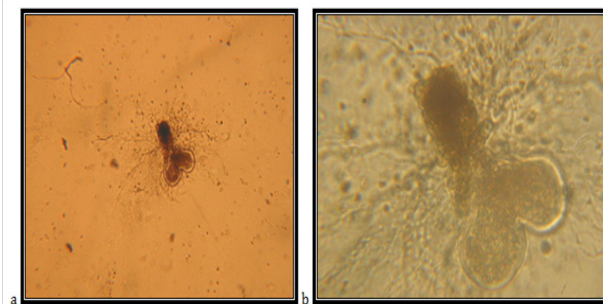
Monocentric sporangia, heart-like, 48\*89µm; with several papillae; the main rhizoid is filamentously branched, it elongates up to 330µm, discharge follows after the decomposition of one or two papillae; zoospores are spheric to ovoid, uniflagellate. The isolate is taken from feces of a roe deer (*Capreolus capreolus*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces mae* (Figure 9).

### Isolate EJ2

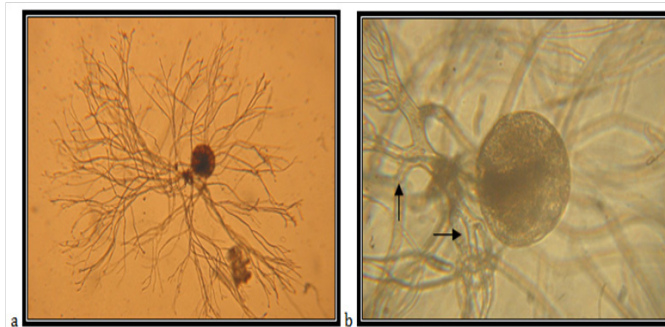
Monocentric sporangia, endogenous, with diameter of 97µm; the main rhizoid is large, 583.13µm in diameter, without constrictions or slightly constricted in the neck area, branched with constrictions on the rhizoid; zoospore examination is with decomposition of a wide apical

part of the wall; zoospores are quite variable in the shape, uniflagellate. The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces communis*.

In Malaysia, *P. communis* has been isolated from the rumen of sheep, goat, ox, water buffalo and deer, and from the duodenum of the sheep.<sup>22</sup> The coil of the main rhizoid is often found in the endogeneous forms. *Piromyces communis* is, also isolated from the rumen of sheep in Great Britain,<sup>23</sup> and France,<sup>24</sup> as well as from the rumen of oxen in Canada<sup>16</sup> (Figure 10).



**Figure 9** Strain SJ1- *Piromyces mae*, endogenous sporangium with two papillae. a. Magnification 10x; b. Magnification 40x.



**Figure 10** Strain EJ2- *Piromyces communis*, endogenous sporangium with coiled main rhizoid; some rhizoids are constricted (arrows). a. Magnification 10x; b. Magnification 40x.

### Isolate EJ3

Monocentric sporangia, endogeneous, ellipsoid with diameter of 31.42µm; rhizoids are sometimes with constrictions; the main rhizoid is usually not branched, ending in a poorly branched system; zoospores are uniflagellates. In cultures, this type is easy to differentiate from other *Piromyces* spp. because of how small the sporangium is. It has been found only in Malaysia, in the rumen of Javan rusa,<sup>25</sup> goat, sheep and in the duodenum of sheep.<sup>22</sup>

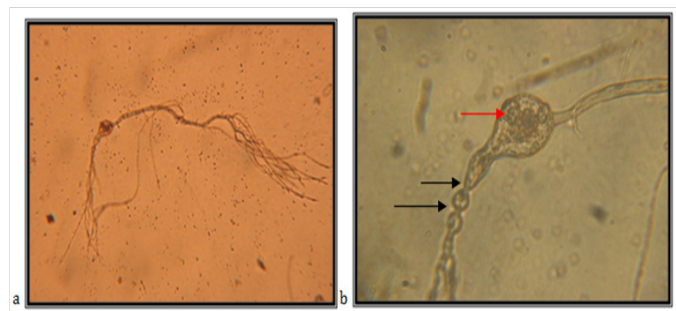
The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces minutus* (Figure 11).

### Isolate EJ4

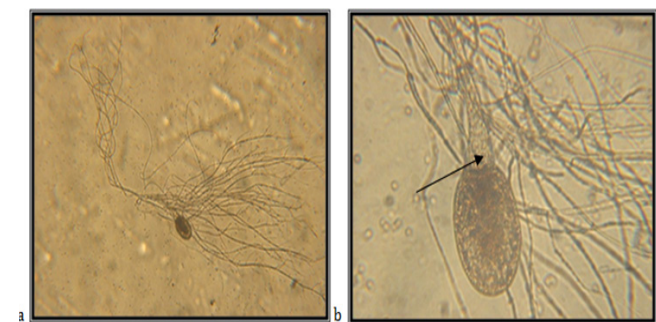
Monocentric; sporangia are elongated and ovale, 74.28\*51.42µm; main rhizoid is filamentously branched, it elongates up to 800µm; sporangium might lay on both shorter and longer sporangiophore

without constrictions; discharge of zoospore follows after the decomposition of the sporangium wall; zoospores are spheric to ovale, 2.0-11.0µm.

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces mae* (Figure 12).



**Figure 11** Strain EJ3- *Piromyces minutus*, straight and unbranched main rhizoid terminating in sparsely-branched rhizoids. Main rhizoid with constriction (black arrows). Red arrow- zoospores. a. Magnification 10x; b. Magnification 40x.



**Figure 12** Strain EJ4- *Piromyces mae*, sporangium with short sporangiophore (arrow). a. Magnification 10x; b. Magnification 40x.

### Isolate SJ2

Monocentric; sporangia are strictly endogeneous, ellipsoid or in a shape of pear, with diameter of 171µm; main rhizoid is not branched, ending in a branched system; zoospore discharge follows after a wide apical pore, occasionally two pores of a large sporangium; zoospores are uniflagellates.

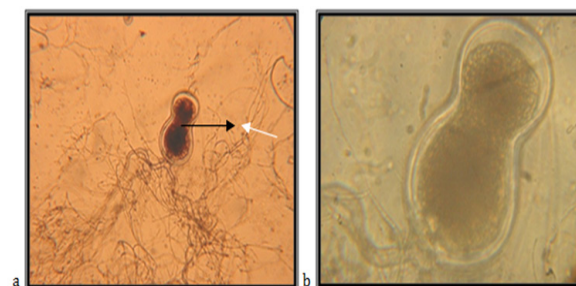
In cultures, this type is easy to differentiate from other *Piromyces* spp. because of how small the sporangium is. It has been found only in Malaysia, in the rumen of Javan rusa,<sup>25</sup> goat, sheep and in the duodenum of sheep.<sup>22</sup> The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces minutus* (Figure 13).

### Isolate Z2

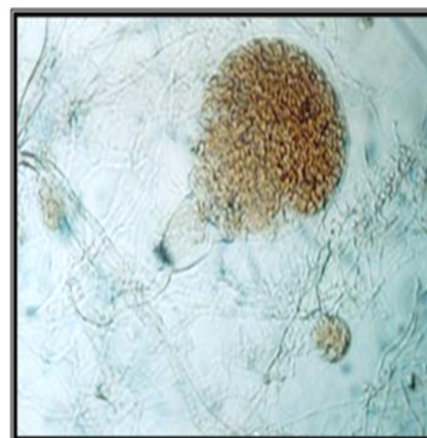
Endogenously developed zoosporangia, spheric, 83.61µm in diameter; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 32µm in diameter, coiled; main rhizoid is often coiled; rhizoid system is spread up to 1mm in diameter,

zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, spheric zoospores 7-22µm in diameter, with 7 to around 30 flagella.

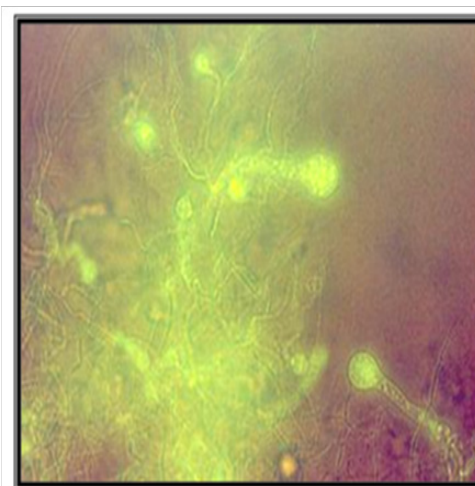
The isolate is taken from feces of a zebu (*Bos indicus*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Neocallimastix frontalis* (Figure 14) (Figure 15).



**Figure 13** Strain SJ2- *Piromyces minutus*, rhizoid system with two main rhizoids. Arrows shows two-layer wall of sporangiophore, full of spores. a. Magnification 10x; b. Magnification 40x.



**Figure 14** Strain Z2- *Neocallimastix frontalis*, exogenous sporangium with short, egg-like sporangiophore, full with spores. Magnification 900x.

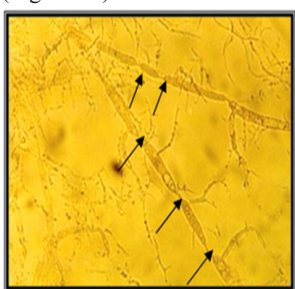


**Figure 15** Strain Z2- *Neocallimastix frontalis*, under fluorescent microscopy. Magnification 400x. Fluorescence occur in nuclei.

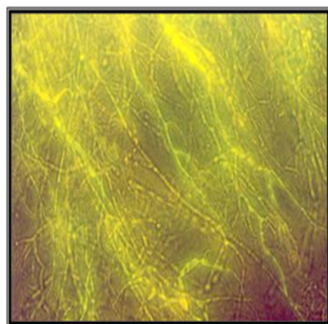


### Isolate L2

Mycelium with undetermined length is raised above the zoospore cyst in germination; polycentric. Zoospores were not found. Constrictions of the hyphas are in the shape of isthmus. Only because of the absence of zoospores, were we not able to determine for sure what genus of polycentric anaerobic fungi they were; but the presence of constrictions in shape of isthmus which are correctly positioned, means that these are polycentric fungi of genus *Anaeromyces*. The isolate is taken from the feces of llama (*Lama glama*), kept in ZOO, Skopje (Figure 16) (Figure 17).



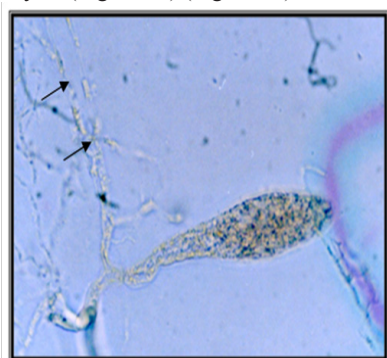
**Figure 16** Strain L2- *Anaeromyces* spp., polycentric strain. Magnification 400x. Arrows- constricted hiphas.



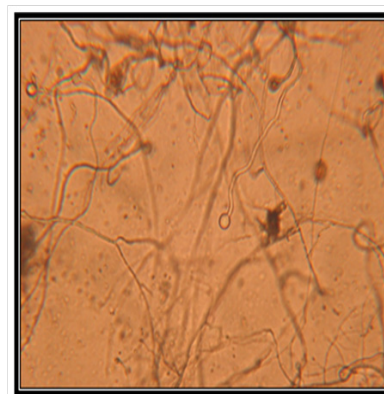
**Figure 17** Strain L2- *Anaeromyces* spp., polycentric strain under fluorescent microscopy. Magnification 400x. Fluorescence occur in nuclei.

### Isolate J2

Single longitudinal sporangia,  $28 \times 106 \mu\text{m}$ , in the shape of bobbin (with apical bud), formed on sporangiophores, uniflagellates zoospores, and hyphas-constricted. The isolate is taken from feces of a domestic yak (*Bos gruniens*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Anaeromyces elegans*. This type can be often found in the rumen of oxen and buffalos in Malaysia (Figure 18) (Figure 19).



**Figure 18** Strain J2- *Anaeromyces elegans*, polycentric strain. Magnification 450x. Arrows- constricted hiphas.

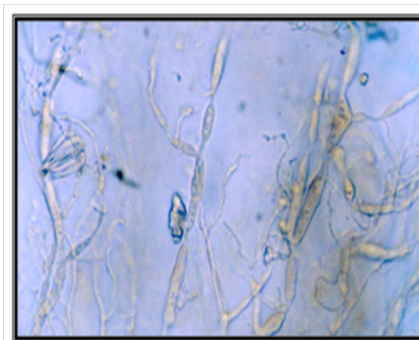


**Figure 19** Uniflagellate zoospores- strain J2. Magnification 40x.

### Isolate V4

Mycelium with undetermined length is raised above the zoospore cyst in germination; polycentric. Zoospores were not found. Constrictions of the hyphas. Only because of the absence of zoospores, were we not able to determine for sure what genus of polycentric anaerobic fungi they were.

The isolate is taken from the feces of watusi (*Bos vatusi*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of polycentric genus of anaerobic fungi *Orpinomyces* spp. or *Anaeromyces* spp (Figure 20).



**Figure 20** Strain V4- polycentric strain. Magnification 450x.

### Isolate BO3

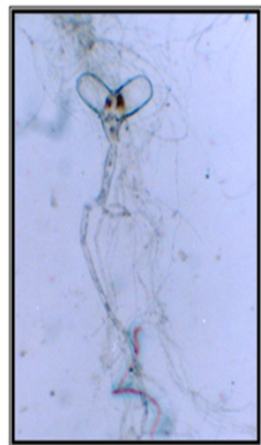
Monocentric; sporangia are double pear-like (heart-like),  $39-61 \times 89-128 \mu\text{m}$ , with several papillae; main rhizoid is filamentously branched, elongated to  $450 \mu\text{m}$ ; discharge follows after one or two papillae; spheric zoospores,  $2.0-12.5 \mu\text{m}$ , uniflagellate, rarely two to four flagella. The isolate is taken from the feces of a barbary sheep (*Ammotragus lervia*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>13</sup> the description of the isolate corresponds completely to the description of *Piromyces mae* (Figure 21).

### Isolate ESI

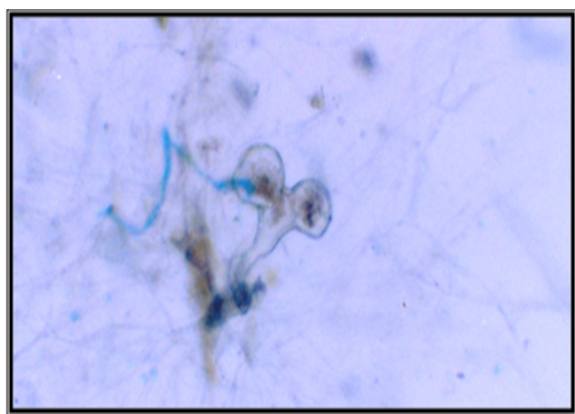
Filamentous fungus, with monocentric thallus; with double oval sporangia which are kept on one sporangium with diameter of around  $77 \mu\text{m}$ . When they are mature, the upper half of the sporangium opens and zoospores are liberated. Zoospores have a diameter of  $7.3 \mu\text{m}$ ; uniflagellate.

The isolate is taken from the feces of a red deer (*Cervus elaphus*), kept in the ZOO in Skopje. Accordingly the key to determine the

anaerobic fungi of<sup>f3</sup> the description of the isolate corresponds completely to the description of *Piromyces citronii* (Figure 22).



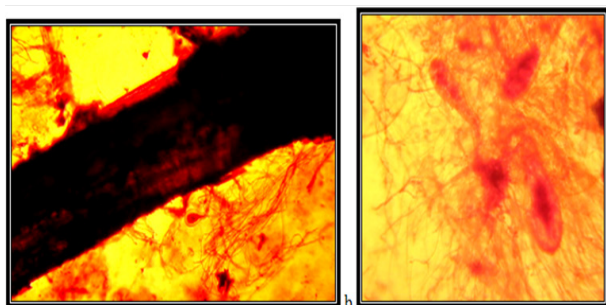
**Figure 21** Strain BO3- *Piromyces mae*, endogenous sporangium with two papillae. Magnification 450x.



**Figure 22** Strain ESI- *Piromyces citronii*, monocentric thallus. Magnification 450x.

### Isolate J1

Monocentric; sporangia are elongated; main rhizoid is filamentously branched, elongated to 450 $\mu$ m; spheric zoospores, 2.0-12.3 $\mu$ m, uniflagellate, rarely two to four flagella. The isolate is taken from the feces of a yak (*Bos gruniens*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>f3</sup> the description of the isolate corresponds completely to the description of *Piromyces mae* (Figure 23).

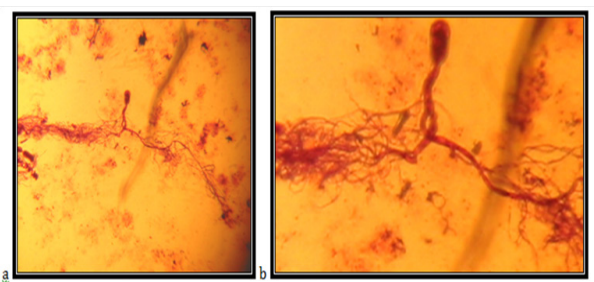


**Figure 23** Strain J1- *Piromyces mae*, monocentric strain. a. Sporangia on digested piece of straw; magnification 10x; b. Magnification 40x.

### Isolate J3

Endogenously developed zoosporangia, ovally elongated; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 18  $\mu$ m in diameter, coiled; main rhizoid is often twirled; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, 5-18 $\mu$ m in diameter, with 7 to around 30 flagella.

The isolate is taken from the feces of a yak (*Bos gruniens*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of<sup>f3</sup> the description of the isolate corresponds completely to the description of *Neocallimastix frontalis* (Figure 24).

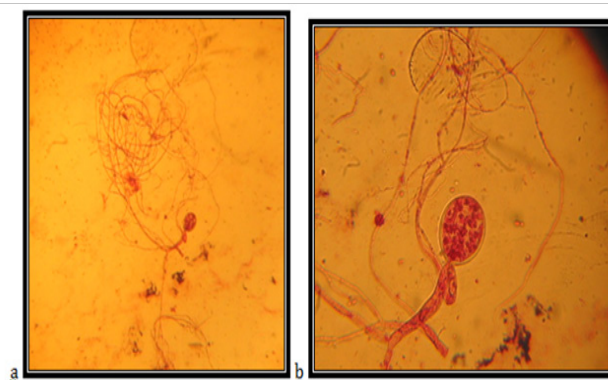


**Figure 24** Strain J3- *Neocallimastix frontalis*, sporangium on long sporangiophore, full with spores. a. Magnification 10x; b. Magnification 40x.

### Isolate MRI

Endogenously developed zoosporangia, spheric with diameter of 42 $\mu$ m; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 15 $\mu$ m in diameter, coiled; main rhizoid is often coiled; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, often spheric, with 7 to around 30 flagella.

The isolate is taken from the feces of a moufflon (*Ovis musimon*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of<sup>f3</sup> the description of the isolate corresponds completely to the description of *Neocallimastix frontalis* (Figure 25).



**Figure 25** Strain MRI- *Neocallimastix frontalis*, sporangium with long sporangiophore, with spores. a. Magnification 10x; b. Magnification 40x.

## Conclusion

The study reveals that 19 isolates of obligatory anaerobic fungi, which have been isolated during the preparation of this study. The description is based completely on the thallus morphology, as seen through the light microscope, in order to enable functional identification of genera and phyla. Among these, three isolates are identified as polycentric, and 16 are identified as monocentric. The present study gives a baseline data of biodiversity of anaerobic fungi from ruminants in Republic of Macedonia.

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None.

## Conflict of interest

The authors declare that there is no conflict of interests to declare regarding the publication of this paper.

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