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## **Saprolegniosis in goldfish, *Carassius auratus*, associated with *Saprolegnia parasitica*; molecular characterization and electron microscopy**

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**Running title:-** Saprolegniosis in goldfish (*Carassius auratus*)

### **ABSTRACT**

*Saprolegnia* strain was isolated from infected goldfish, *Carassius auratus*, fingerlings. Fish showed extensive hyphal growth on the skin, fins, gills and eyes. The isolate was molecularly identified through 18S rRNA gene and internal transcript spacer, ITS, sequencing. The isolate showed 18S rRNA gene nucleotide identity, 91.6%, and ITS homology with *Saprolegnia parasitica*. Scanning electron microscopy was applied for evaluating the pathogenicity for the retrieved isolate; which proved its high virulence accompanied with the high mortality rate reaching 100% of the infected fingerlings.

**Keywords:** Saprolegniosis, *Carassius auratus*, molecular characterization, Electron microscopy.

### **INTRODUCTION**

Ornamental fishes share in the worldwide trade by a value of about US\$ 900 million, playing a role in some countries' income and occupation (Meshgi et al., 2006). Family Cyprinidae is one of the most commercially important and commonly available ornamental fish (Sharma et al., 2011). This industry is threatened by losses due to infectious diseases,

including fungal diseases. *Saprolegnia* has been considered as one of the most destructive oomycete pathogen in fish hatcheries and larval stages specially; *S. parasitica*, which account for major losses in aquaculture (Hussein et al., 2001; Bruno & Wood, 1999; Van West, 2006; Van den berg et al., 2013). Saprolegniosis is always associated with prolonged low water temperature at early spring, late autumn and winter. Although, *Saprolegnia*-infected fish are

easily recognized by the cotton-like, white to grayish patches on the skin and gills ([Stueland et al., 2005](#)), but it is still difficult to reach the full data about the causative strain unless following both traditional and recent diagnostic tools. The lack of a robust taxonomy in the genus *Saprolegnia* (Oomycetes) is leading to the presence of incorrectly named isolates in culture collections and of an increasing number of mis-assigned named sequences in DNA databases ([Sandoval-Saierra et al., 2014](#)). In addition, accurate species delimitation is critical for most biological disciplines. A recently proposed approach to solve species delimitation (taxon diagnosis system) of difficult organisms is the definition based on molecular analyses of rRNA-encoding genes. Analysis of 18SrRNA gene and internal transcribed spacer (ITS) can be used for species identification; furthermore it provides information about new species and their genetic diversity ([Paul and Steciow 2004](#); [Steciow et al., 2007](#); [Sandoval-Saierra et al., 2014](#)). On the same hand, the ultra-structure of the early invasive stages can draw the scenario of the fungus pathogenicity through its phenotypic features including; long boat hooks on the spores and their germination rate/pattern ([Thoen et al., 2011](#)). The aim of the current investigation was to identify the causative agent associated with goldfish fingerlings mortality using both traditional and molecular methods of diagnosis. Also, scanning microscopic analysis was performed for tracing the pathogenesis during the early infection stages.

## MATERIALS AND METHODS

### Fingerlings sampling

Moribund and freshly dead Goldfish *Carassius auratus* (*C. auratus*) fingerlings were obtained from a private ornamental fish farm located at South Cairo, Egypt with history of high mortality during mid February 2014. Freshly dead fingerlings were transported as soon as possible to the wetlab of fish diseases and management, Faculty of Veterinary Medicine, Benha University, Egypt. Moribund fish were kept in glass aquaria at water temperature  $16\pm 1^{\circ}\text{C}$  for subsequent examination.

### Clinical examination

Fish samples were examined for clinical signs and postmortem according to ([Schaeperclaus, 1992](#)).

### Isolation

Isolation of *Saprolegnia* spp from infected fish was performed according to the method of [Stueland et al. \(2005\)](#), with modifications. Parts of the outer surface of the examined fish, covered with cotton wool-like fungal mats, were washed up with sterilized distilled water to get rid of solid particles trapped within fungal mats from the surrounding water and cleaned with 70% ethyl alcohol. Samples from the skin lesions were inoculated onto sterile plates of Sabouraud's dextrose agar (SDA) (Difco Lab., USA) with chloramphenicol. Culture plates were incubated at  $20^{\circ}\text{C}$  for 3–5 days with regular daily inspection for any expected fungal growths. Fungal growth was observed and identified according to [Willoughby \(1985\)](#) and [Hatai et al. \(1990\)](#). In order to stimulate the production of sexual organs, a part of the growing fungus was aseptically transferred into tube containing sterile pierced hemp seeds in sterile tap water, incubated at  $20^{\circ}\text{C}$  and was observed

periodically for up to 2 months (Johnson Jr, 1956).

### Molecular characterization

Genomic DNA was extracted from 200 µl of the isolate cells, using high pure PCR template preparation kit, catalog no. 11796828001, Roche, with modifications. The fungus cells were centrifuged at 3000 Xg for 5 min. and the supernatant was discarded. The cells were re-suspended in 200 µL PBS, 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O; 1.4 mM KH<sub>2</sub>PO<sub>4</sub> and treated with 10 µL lyticase, 0.5 mg/ml at 37°C for 30 minutes. The cell solution was incubated with 200 µL binding buffer and 40 µL proteinase K for 10 min at 72°C. The DNA was purified from crude lysate using sephadex column according to kit instruction manual. The size of the extracted DNA was checked by electrophoresis on a 0.9% agarose gel against a Lambda-HindIII digest marker (New England BioLabs, Hitchin, Hertfordshire, UK) with ethidium bromide staining.

For PCR amplification of 18S rRNA gene, the used primers were 5/-aacctgggtgatcctgccagt-3/ and 5/-tgatcctctgcaggttcacctac-3/. (Borchiellini *et al.*, 2001). The internal transcribed spacers 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 were amplified through ITS1, 5/-tccgtaggtgaacctgcgg-3/, and ITS4, 5/-tctccgcttattgatatgc-3/ primers, (White *et al.*, 1990). Reaction mixture, 50 µL, contained 10X EX taq buffer II (Mg<sup>2+</sup> plus), 0.2 µM primer, 400 µM dNTP each, 2.25 U Takara EX-Taq Polymerase (Takara, Japan) and 5–30 ng DNA template. PCR was performed with an initial denaturation step of 3 min at 95 °C. The PCR reaction continued with 30 cycles of 1 min at 95 °C, 40 s. at the annealing temperature of 55 °C, for amplification

of 18S rRNA gene and ITS, and 72 °C for 1 minute, followed by final extension at 72 °C for 10 minutes.

PCR products were confirmed by running on 1.2 % agarose gel and stained with ethidium bromide. PCR amplicons were eluted from the gel, using QIAquick Gel Extraction Kit, catalog no. 28704 Qiagen. The 18S rRNA gene and ITS amplicons were analyzed by sequencing, using capillary 3500 series genetic analyzer, Applied Biosystems.

The 18S rRNA gene and ITS sequences were checked by FASTA screening to determine their similarity to known fungus species in the DNA database. The recovered 18S rRNA gene sequence was aligned with those from DNA database, using CLUSTAL Omega,

<http://www.ebi.ac.uk/Tools/msa/clustalo/>. Construction of the consensus phylogenetic tree was done through submission of the aligned sequences, including the sequence gaps, to the Molecular Evolutionary Genetic Analyses, MEGA, software version. Bootstrap method, provided as a phylogeny test, in the MEGA software, was performed, using a number of 500 Bootstrap replications.

### Scanning Electron Microscopy (SEM)

#### Preparation of the attached secondary spores

Zoospores production and preparation were performed according to Willoughby *et al.* (1983). Briefly, hemp seeds, colonized by the fungus, were incubated in sterile tap water at 20°C for 2 days. The water surrounding the seeds contained numerous motile secondary zoospores. Zoospores

concentrations were adjusted to be  $1 \times 10^4/L$ .

Zoospore attachment was performed following **El-Feki and Refaat, (2014)** with some modifications. Two healthy goldfish of an average weight  $20.0 \pm 0.4$  g and average length  $8 \pm 0.2$  cm were collected from private aquarium shop. Pieces of skin were aseptically collected and immersed in spore suspension for 180 min., to allow zoospores attachment. Skin pieces with germinating zoospores were kept for SEM in 5 mL of 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, and stored at 4 °C for 2 days. Fixed skin samples were rinsed in 0.1M phosphate buffer, re-fixed in 2% osmium tetra-oxide for 12 hours, washed 4-5 times in distilled water for 15 min., and dehydrated in ethanol series using automatic tissue processor (Leica Microsystems, Wetzlar, Germany). The specimens were immersed in t-Butyl alcohol (2-methyl-2-propanol) three times for 10-20 min. at 4°C. The dehydrated samples were critical point dried using CO<sub>2</sub> critical point dryer (Tousimis Autosamdri-815). Dried specimens were mounted on aluminum stubs and coated with gold/palladium, using sputter coater (SPI- Module). The specimens were examined at 17KV using a scanning electron microscope, JOEL-JSM-5500 LV, Japan.

## RESULTS

### Clinical and microscopic examination

Gross examination of the freshly dead fingerlings showed extensive cotton wool-like growths on the skin, fins, gills and eyes (Fig. 1, a) , the Sabroud's dextrose agar (SDA) plate was covered with white dense mycelia growth (data not shown).

The asexual reproduction, of the current fungus isolate, was characterized by presence of branched non septated hyphae, together with masses of mature and immature sporangia (Fig. 1, b). Two months post inoculation on hemp seeds containing tap water, the sexual organs were observed forming terminal oogonia with centric oospores (Fig.1, c). Periodical examination of the culture along three months showed the presence of abundant gammae (Fig. 1, d).

### Characterization of fungal isolate, based on 18S rRNA gene and ITS sequences

The PCR amplification of 18S rRNA gene yielded 1.8 kb, while 750 bp was amplified from ITS region (Fig. 2). The sequenced ITS amplicon showed G/C ratio of 46 % (Accession no. KT807577). The resulted 18S rRNA gene sequence was registered in database under accession no. AB985402. The FASTA homology showed that the 18S rRNA gene of the current isolate had 94% nucleotide identity with that of *S. parasitica*, strain recorded in Taiwan (accession number HQ384412), and both formed monophyletic clade, but with an observable genetic distance (Fig. 3).

### Scanning electron microscope (SEM)

The fine structure of the retrieved fungus and its pathogenicity was confirmed through SEM. More than one germinating secondary spore with appressorium like structure appeared as club shape attached to the end of long germinating tube at the site of skin invasion were observed (Fig.4, A, B). The newly germinating spore showed the presence of hair like structures and presence of globular adhesive materials (pad) (Fig. 4, C) these adhesive

materials were progressive and cone shape along the germination tube (Fig. 34, D).

## DISCUSSION

*S. parasitica* is global freshwater pathogenic fungus (Van West, 2006). As an opportunistic microorganism, the host immunity is crucial in inducing infection; hence suppressed immunity enables its invasion and induction of disease condition. Depending on the history of the disease in this study; an attempt for water exchange to the aquaria, where fish was subjected to sudden decrease in the temperature and handling stress. Eissa *et al.* (2014) observed drastic increase in the plasma cortisol levels post handling and low temperature stress. It is well known that cortisol is the key hormone in fish stress response suppressing the immune system, rendering the host vulnerable to infection (Cortes *et al.*, 2013).

Secondary zoospores have been considered as the infective stage of *S. parasitica*, encysting on the host fish and forming secondary cysts that release the next generation of laterally biflagellate (Robertson *et al.*, 2009). In winter season, the temperature drop below 10 °C predispose saprolegnia attack through triggering zoospore release (Bly *et al.*, 1992). The infected fish showed cotton like growth on the skin, fins, gills and eyes with mortality reaching 100%. The nearly same results were observed in tilapia, angel fish and carp infected with saprolegnia species (Iqbal *et al.*, 2012, Eissa *et al.*, 2013 and Mortada *et al.*, 2013).

For diagnosis of saprolegnia, the steps included; examination of both of asexual and sexual mode of reproduction. The asexual reproduction was characterized by the presence of

branched non septated hyphae with sporangia containing zoospores. The obtained results were matched with those described by Seymour (1970) and Daugherty *et al.* (1998). On the other hand, the sexual organs appeared 2 months post inoculation on hemp seeds containing tap water. Coker (1923) was able to identify saprolegnia species based on the sexually produced oospores, which differ in number, size and shape from species to species. In the present study, the oogonia was embedded with centric oospores matching the findings of Seymour (1970) and Johnson Jr. *et al.* (2002), implicating the causative agent belonging to Saprolegniales. Gemmae formation is characteristic structure for *S. parasitica* identification which was observed in three months culture. Vega-Ramirez *et al.* (2013) noticed that the isolated *S. parasitica* didn't produce sexual organs and instead abundant gemmae was formed; which was persistent for long time.

The molecular evolutionary patterns of Saprolegnia are still not well understood, due in part to the lack of molecular markers suited to resolve the deep phylogeny of this genus (Lara & Belbahri, 2011). The phylogenetic relationship between the current isolate and those of the *S. parasitica* was investigated based on 18S rRNA gene and ITS regions sequencing. The nearest sequence deposited in database of our ITS regions sequence was *S. parasitica* C8 (JN400038), which recorded in isolate from China. This fungal isolate was isolate from *Pelteobagrus fulvidraco* eggs.

The homology between ITS sequence of our isolate and that of deposited in database, acc.no. JN400038 was 100 % identity. This result confirms the global distribution of this fungus

isolate, among freshwater fishes, causing fish mortality outbreak. The length of amplified 18S rRNA gene in this study was longer than those of recorded in previous studies (**Lara & Belbahri, 2011**), increasing the accuracy for identification of the current isolate. The molecular studies have supported the validity of 18 species of *Saprolegnia* and identified 11 potential new species (**De la Bastide et al., 2015**).

Although most of *saprolegnia* species are opportunistic pathogens but some strains are pathogenic and cause primary infection to both fish and eggs (**Willoughby and Pickering, 1977**). The mode of zoospore attachment is one of the virulence factors and pathogenicity indicator. Where the mechanism of fungal infection involves; adhesion of the zoospores and germination (**Dieguez-Urbeondo et al., 2007**). The phenotypic characters of the secondary zoospores of *Saprolegnia* can be used for recognizing its virulence; like hairs which is required for attachment to the host (**Beakes 1982 and Willoughby, 1994**). The electron microscope revealed the presence long hair attached spores. In addition to the adhesive materials which render the secondary cystospore resistant to detachment (**Durso et al., 1993**). Appressorium is another important structure was observed while cystospore germination. **Money et al. (2004)** had described the appressorium a swollen structure formed at the tip of a hypha or a germ tube at the point of contact with the host cell, that facilitates the penetration of the hyphae into the hard cuticle mechanically or through enzymatic activity. Finally, the presented diagnosis approach of *Saprolegnia* species might help setting the basis for a suitable identification of

species in this economically important genus and will help to better understand the emergence of pathogenicity of current isolate in the different oomycete groups.

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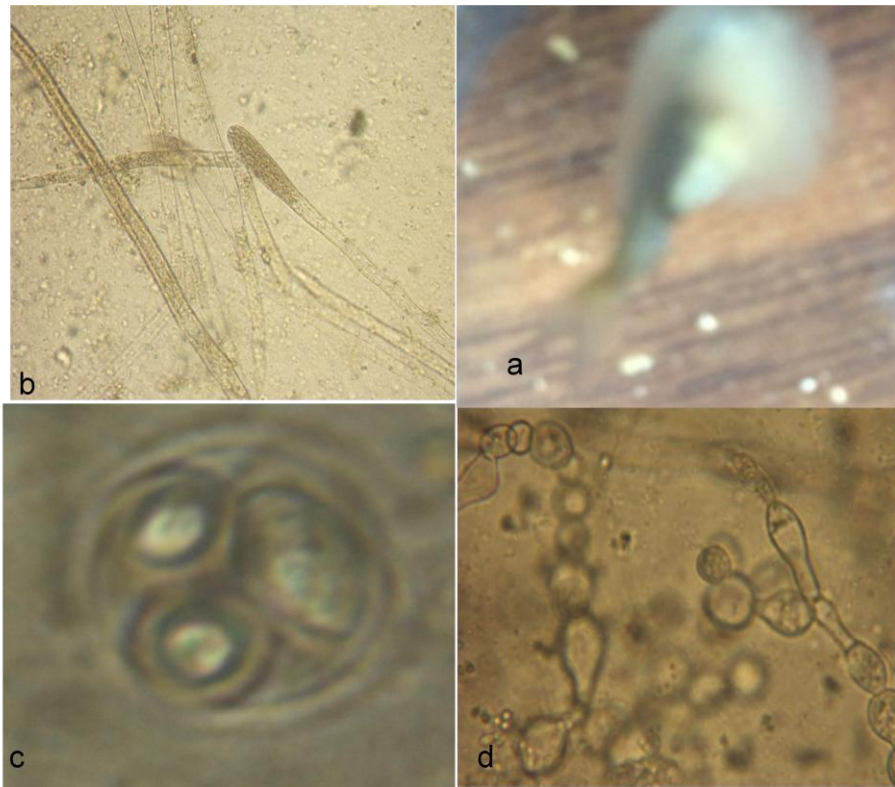
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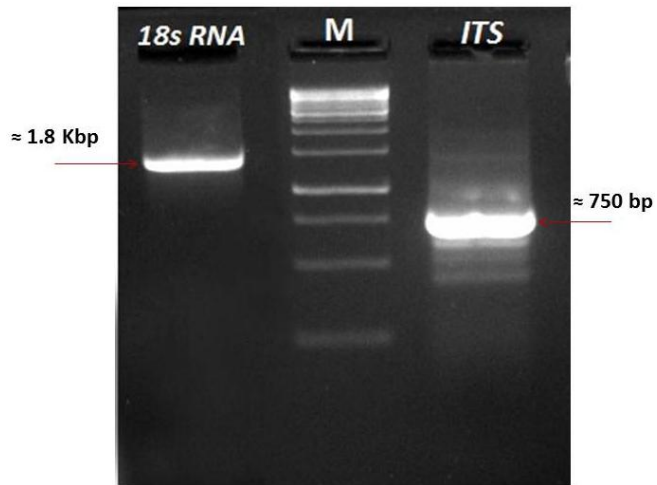
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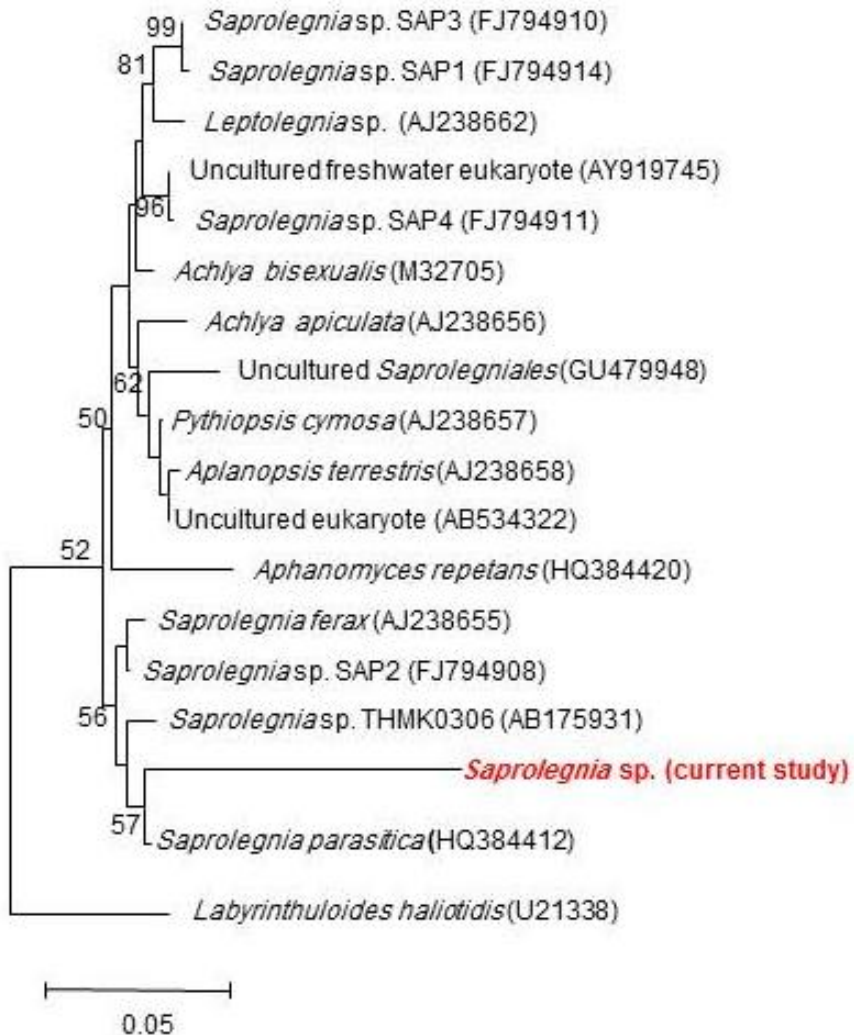
Saprolegniosis in goldfish (*Carassius auratus*) associated with *saprolegnia parasitica*; molecular characterization and electron scanning



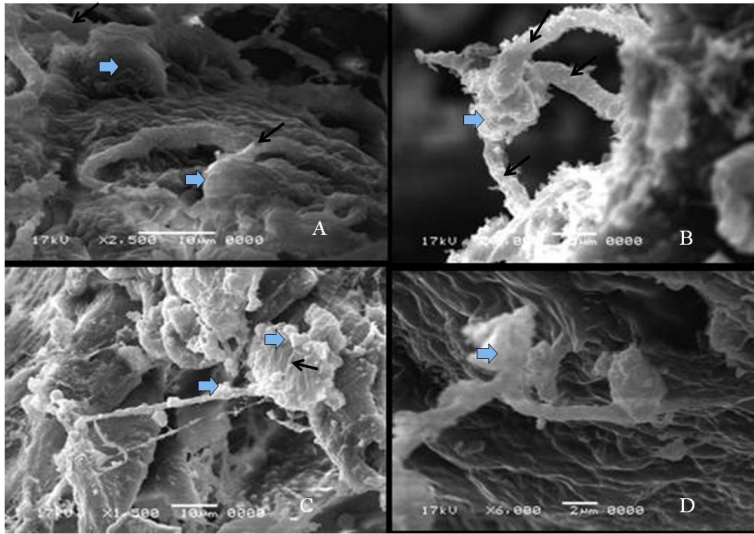
**Figure 1:** (a) goldfish fingerling warped with cotton wool like hyphal mats,(b) Asexual reproduction showing; branched nonseptated hyphae & sporangia filled with large number of spherical sporangiospores (arrow) (c) sexual reproduction showing; oogonia with centric oospores, (d) germination of oospore with formation of Gemmae.



**Figure 2: PCR products of 18S rRNA gene and ITS region; M is 1k DNA ladder**



**Figure 3:** Phylogenetic tree of 18S rRNA sequence of the retrieved saprolegnia isolate (accession number in gene bank HQ384412)



**Figure 4:** Scanning electron microscope; A,B) a newly germinating secondary zoospores of *S. parasitica* with germinating tube (black arrows) and appersoria (blue arrows) C) secondary zoospores of *S. parasitica* with globular adhesive materials around the zoospore (blue arrows) with hair like tuft on the secondary cyst (black arrow) D) deep inclusion of the emerging tubes into the skin, note the accumulation of the adhesive materials around the germinating tube (arrow).



## مرض السابرووليجنيوسيس فى اسماك الجولد. دراسات جزيئية ومسح الالكترونى

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فى هذه الدراسة تم عزل فطر السابرووليجنيا باراسيتكا والمتسببة فى نفوق اسماك الجولد فى منتصف شهر فبراير مع وجود اعراض من نموات قطنية على الجلد والزعانف والخياشيم والعين كما لو كانت الاسماك ملفوفة بالقطن وقد تم تصنيف هذه العترة من خلال التكاثر الجينسى والاجنسى لها وايضا من خلال التتابع الجينى ل18S rRNA و ITS والذى اظهر تشابه بنسبه 100% مع السابرووليجنيا باراسيتكا. بالاضافة الى ذلك فقد تم عمل مسح الكترونى لتقييم ضراوة العترة المعزولة والتي اثبتت شدة ضراوته حيث وصلت نسبة النفوق الى 100%.