UNIVERSIDADE DE LISBOA
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A STUDY OF NUCLEO-CYTOPLASMIC LARGE DNA VIRUSES: DETECTION AND CHARACTERIZATION OF VIRUSES IN ENVIRONMENTAL AMOEBA

Mariana Costa Fugas

DISSERTAÇÃO
MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

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Dissertação orientada pela Prof. Dra. Maria Filomena Caeiro (Centro de Estudos do Ambiente e do Mar – Universidade de Aveiro / Faculdade de Ciências – Universidade de Lisboa)

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Abstract

Amoeba associated microorganisms (ARMs) are bacteria or viruses that share a symbiotic relationship with amoebas. Many ARMs are associated with human diseases and it has been reported the acquisition of resistance inside their host. These facts highlight the importance of finding and characterizing ARMs in a public health's perspective.

In the present work, amoebas from environmental samples have been isolated and identified by optical microscopy and molecular methods. Amoeba associated viruses, especially from the NCLDV group, were also subjected to a PCR screening. From the 12 collected samples, it was possible to isolate 17 amoebas, of which 11 were identified by molecular methods. No viruses were detected but in 2 of these amoebas, a bacterium was identified which shares an 89% sequence identity with the *Marivirga tractuosa species*.

If amoebas allow the replication of a *Mimivirus*, which belongs to the NCLDV group, would they be able to replicate iridoviruses that belong to the same group? An *Acanthamoeba castellanii* cell line was experimentally inoculated with three *Ranavirus*: FV3 (Frog Virus 3), Ma3B and BoA (respectively, a *Triturus marmoratus* and a *Triturus boscai* isolate). The PCR test suggested an association between *A. castellanii* cells with the Ma3B and BoA viruses. VLPs (Virus-like particles) were visualized by electron microscopy in the Ma3B inoculated amoebas.

Ranavirus from several *Triturus* species are currently being characterized and the sequences of their *Major Capsid Protein* and *DNA polymerase* genes are identical. Could the sequencing of a less conserved gene like the *Flap endonuclease*, allow the distinction? The sequencing and analysis of the Flap gene was able to group viruses FV3 and LMO (*Lacerta monticula* virus) as they present an equal gene sequence. The three *Triturus marmoratus* isolates presented an equal Flap gene amongst them and were also grouped. Between the two groups, this gene sequence has a difference of 8 nucleotides, confirming the separation of the FV3 and LMO viruses from the other iridoviruses tested.

Key words: NCLDVs; Amoeba; *Acanthamoeba castellanii*; Iridovirus; Flap endonuclease; Environment;

Resumo

As amibas são microrganismos ubíquos que podem apresentar uma de duas formas: a forma locomotiva denominada trofozoóide e a forma dormente ou cisto. Estas amibas de vida-livre (AVL) predam sobretudo bactérias, algas, leveduras e outros protistas. Por vezes, os mesmos organismos de que se alimentam tornam-se seus parceiros endossimbiontes, vivendo no interior da amiba sem que sejam digeridos. A estes microrganismos chamamos de MRAs (microrganismos resistentes a amibas) e entre eles encontram-se bactérias do género *Legionella* como a espécie *L. pneumophila* que causa a doença do Legionário ou a Febre de Pontiac, eucariotas como a espécie *Cryptococcus neoformans* e até mesmo vírus como é o caso do *Mimivirus*, que apresenta um dos maiores genomas encontrados até hoje.

Algumas espécies de AVLs causam doenças humanas como é o caso da *Naegleria fowleri*, *Balamuthia mandrillaris* e algumas espécies do género *Acanthamoeba*. Podem causar infecções do sistema nervoso central e no caso das últimas, podem também causar uma infecção ocular denominada queratite amibiana. Além desta capacidade de causar doença, as AVLs são também hospedeiras de muitos MRAs que podem eles próprios causar outras doenças. Sabe-se também que estes MRAs parecem ganhar resistências enquanto permanecem no interior destes hospedeiros. Isto é explicado pelo facto das amibas abrigarem muitos microrganismos diferentes, o que leva a que a pool genética seja enorme e a possibilidade destes MRAs de evoluírem para potenciais patogénios aumente. É assim de extrema importância a procura de MRAs dentro de amibas como um ponto fulcral na prevenção em saúde pública.

Os vírus encontrados até hoje associados a amibas constituem um grupo recente composto pelo mimivirus, mamavirus, marseillevirus e lausannevirus. Todos foram associados a espécies de *Acanthamoeba* sendo que os dois primeiros compõem a família *Mimiviridae* e os dois últimos a família *Marseilleviridae*. O mimivirus foi o primeiro vírus a ser encontrado e o seu nome deriva da capacidade de imitar (do inglês *mimicking*) uma bactéria, apresentando um tamanho de 750 nm, muito superior a qualquer vírus até então descoberto. Foi assim cunhado o termo vírus gigante sendo que o seu genoma é também um dos maiores genomas virais estudados, composto por 1180 quilo pares de base (qpb). Alguns estudos sugerem que o mimivirus pode ser internalizado por macrófagos humanos e murinos e que a sua entrada é mediada por um processo de fagocitose, pelo que este poderá ter como alvo células fagocíticas humanas. Já foi também sugerido que o mimivirus consegue causar pneumonia em ratos, em condições experimentais, e que poderá ser uma causa para pneumonias adquiridas tanto na comunidade como em ambientes hospitalares. Percebe-se assim, a importância de detectar MRAs e em especial, vírus associados a

amibas que poderão vir a explicar a causa de doenças humanas para as quais ainda não foi associado um agente responsável.

O presente trabalho pretende então isolar e identificar amibas de amostras ambientais e encontrar vírus associados a estas, especialmente do grupo dos NCLDVs, do qual fazem parte os *Mimivirus*. Para isto, foram aplicados métodos de isolamento de amibas em Placas de Petri com agar feitas com três meios diferentes: H_2O, meio de Page e água-do-mar artificial a 75%. As amibas foram observadas e caracterizadas por microscopia óptica e foi utilizado também um protocolo de PCR directo para identificar as mesmas por métodos moleculares. Para detectar os vírus, procedeu-se também a um protocolo de PCR directo e a microscopia electrónica de transmissão. Os primers utilizados têm por alvo as regiões genómicas da polimerase de DNA (primers dos LDVs – Large DNA vírus – e dos adenovírus), da MCP (*Major Capsid Protein* – dos *Ranavirus* (*Iridoviridae*)) e das regiões L396 e R596 do genoma do mimivirus, que codificam, respectivamente, uma helicase e uma tiol oxidoreductase.

Para além dos objectivos acima referidos, coloca-se também a questão de que se as amibas permitem a replicação de *Mimivirus*, permitirão também a replicação de outros NCLDVs, como é o caso dos iridovírus? Para responder a esta questão, uma linha celular de *Acanthamoeba castellanii* foi inoculada com três ranavirus: FV3 (Frog Virus 3), Ma3B e BoA (isolados, respectivamente, de um *Triturus marmoratus* e de um *Triturus boscai*).

Pensa-se também que a utilização de genes conservados, como é o caso dos genes da polimerase de DNA ou da MCP, para distinguir entre vírus semelhantes por vezes não são suficientes. Vários isolados virais de tritões (*Triturus marmoratus* e *Triturus boscai*) estão a ser de momento caracterizados e sabe-se que têm sequências semelhantes nas regiões genómicas da polimerase de DNA e da MCP. Poderá o gene menos conservado da putativa Flap endonuclease permitir a distinção entre estes iridovírus? Foram desenhados *primers* para este gene utilizando as sequências do gene da Flap endonuclease dos vírus FV3 e CMTR (*Common midwife toad ranavirus*) – com os números de acesso NC_005946.1 (101656-102747) e JQ231222.1 (15499-16593), respectivamente. Estas sequências foram alinhadas no programa ClustalX e analisadas no programa BioEdit. A sequência do FV3 foi submetida ao programa PrimerBlast e o par de *primers* mais adequado foi seleccionado e comparado com o alinhamento feito previamente. Escolheram-se as bases degeneradas e os *primers* foram nomeados de F-Flap e R-Flap, *primers forward* e *reverse*, respectivamente. Os produtos de PCR obtidos com estes *primers* foram depois sequenciados e analisados por ferramentas bioinformáticas tais como os programas BioEdit, ClustalX, FinchTV e blastn.

Os principais resultados reportam o isolamento de 17 amibas de entre 12 amostras ambientais diferentes. Destas amibas, 11 foram identificadas por microscopia óptica e por métodos moleculares. Não foi detectado nenhum vírus mas em duas destas amibas foi detectada por PCR uma bactéria cuja sequência de DNA apresenta uma identidade de 89% com a espécie *Marivirga tractuosa*, caracterizada como sendo resistente a certos antibióticos.

No ensaio de infecção *in vitro* de células *A. castellanii* com ranavirus, o teste de PCR sugere algum tipo de associação entre os iridovírus Ma3B e BoA e as células inoculadas. Por microscopia electrónica, podem ser observadas VLPs (*Virus-like particles*) nas amibas infectadas com o vírus Ma3B. Fica por esclarecer a natureza destas VLPs e que tipo de associação apresenta o vírus BoA com as amibas.

A sequenciação e análise do gene da Flap endonuclease permitiu agrupar os vírus FV3 e LMO (isolado de uma *Lacerta monticula*). Os três isolados de *Triturus marmoratus* (Ma3A, Ma3B e Ma3P) apresentam sequências idênticas e constituem outro grupo. Entre os dois grupos, a sequência deste gene difere em 8 nucleótidos confirmando a separação dos vírus FV3 e LMO dos outros iridovírus testados.

Palavras-chave: NCLDVs; Amiba; *Acanthamoeba castellanii*; Iridovírus; Flap endonuclease; Ambiente.

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1. Introduction

Free living amoebas (FLA) are phagocytic protists that can be present in numerous environments[1]. They can appear in one of two forms: a locomotive form in which they feed and multiply termed trophozoite and a dormant form, named cyst[2]. They mainly prey on bacteria, fungi, algae and other protozoa[1] but there is evidence that they can phagocyte particles with sizes higher than 0.5 µm and do not need to recognize specific markers to ingest them[3].

Amongst the many FLA species that can be found in nature, the ones associated with human diseases are as few as one species from the *Naegleria* genus (*N. fowleri*), one from the *Balamuthia* genus (*B. mandrillaris*) and several species of *Acanthamoeba*. They can all cause fatal central nervous system (CNS) infections and in the case of the latter species, they can also cause cutaneous lesions and a sight-threatening infection of the cornea termed Amoebic Keratitis[4].

Along with the ability to cause disease in humans, FLA species can also harbour intracellular pathogenic bacteria such as *Legionella pneumophila* and other species from the same genus, which cause Legionnaires' disease and Pontiac fever[4,5]. It has also been discovered that other pathogenic bacteria including *Mycobacterium avium*, *Escherichia coli* O157:H7 and *Vibrio cholerae* can survive and reproduce within FLA species, *in vitro*[4]. They have been associated with eukaryotes, namely with *Cryptococcus neoformans*, which creates a symbiotic relationship with FLA species, and have also been associated with viruses[3]. These viruses comprise a recent and unique group composed by the Mimivirus, Mamavirus, Marseillevirus and most recently the Lausannevirus, all of which have been related to *Acanthamoeba* species[6-9].

In 1992, in the quest to find a pneumonia causing agent, Timothy Rowbotham, the officer in charge of Britain's Public Health Laboratory Service, discovered a microorganism that resembled a small Gram-positive coccus. The apparent bacteria was obtained from the water of a cooling tower in Bradford (England) and it multiplied within the amoeba *Acanthamoeba polyphaga*[10] but it could not be cultured in agar plates and the molecular identification using the universal 16S rRNA bacterial primers also failed. Moreover, it could not pass through a 0.22 µm pore filter, the standard experimental procedure to separate bacterial cells from viruses and so it was named "Bradfordcoccus". In 2003, the mistake was corrected when this sample was brought to the University of Marseille (France) and Didier Raoult and his team revealed the ultrastructure of the found microorganism by electron microscopy. Several bodies were observed within the infected amoeba which appeared to have regular icosahedral forms, similar to the iridoviruses capsid. The new found virus was then termed

Acanthamoeba polyphaga mimivirus (APMV) for its amoebal host and mimicking ability to disguise itself as bacteria[11].

In the years that followed, the APMV virus was thoroughly studied, for its biology and molecular characteristics. The virus capsid has a diameter of approximately 0.5 µm however it is covered by numerous well compacted fibers that increase its particle size to 0.75 µm, hence the term giant virus. APMV is a double stranded DNA virus with a genome size of 1180 kbp (kilo base pairs), which make it one of the largest viral genomes and even larger than the genomes of several bacteria[11]. However, the most remarkable characteristic of this viral genome is that it includes numerous genes which were thought to belong only to cellular organisms such as: amino-acyl transfer RNA synthetases, translation elongation factors (EF-Tu) and tRNAs as well as components of DNA repair pathways. This intricate gene assembly poses several questions about the established frontier between viruses and parasitic cellular organisms[12].

Five years after the correct identification of APMV, a new strain of this virus was discovered and termed Mamavirus since when observed by transmission electron microscopy (TEM) it appeared larger than the Mimivirus. The Mamavirus was isolated from the water of a cooling tower in Les Halles (Paris, France) and as with the Mimivirus, the Mamavirus induces the formation of a giant viral factory and has a multi-layered membrane covered by several fibers[13]. Its particle size only reaches 0.65 µm but its genome is indeed larger than the genome of the Mimivirus with 1200 kbp[14,15]. Alongside with this discovery, La Scola *et al* [13] observed smaller particles, with 50 nm in size and an icosahedral form which were present both in the viral factories and the cytoplasm of the infected cells. They named this new smaller virus Sputnik for its association with the Mamavirus. When inoculated into *Acanthamoeba castellanii* cells, Sputnik did not replicate by itself, but rather only when either the Mamavirus or the Mimivirus was co-infecting the amoebal cells[13,14]. It was found that Sputnik was indeed infecting the Mamavirus because not only it led to the formation of some abnormal Mamavirus particles but it also decreased the amoebal cell lysis. The Mimivirus is also able to be infected by Sputnik but in this case, the number of amoebal cells co-infected is lower. From these characteristics, Sputnik was considered to be more than a satellite virus, as it is truly a parasite of other viruses and so it was named a virophage[14].

In 2009, it was discovered another giant virus termed Marseillevirus which was isolated in the same manner as the Mamavirus, using metal plaques in a cooling tower to create a biofilm which was then homogenized into sterile water and filtered. The filters were then shaken in Page's amoebal saline (PAS) and each suspension was inoculated onto *A. polyphaga* microplates. After roughly 19 weeks, cell lysis could be observed and the virus was detected by TEM. The viral particles had an icosahedral form and a diameter of about

250 nm covered by fibers with globular ends. Marseillevirus has a genome of about 368 kbp composed by a circular double-stranded DNA molecule[8].

Regarding Sputnik, it was recently put to the test whether other giant viruses could be affected by this virophage and the results are consistently referring to a negative effect on both the Mimivirus and the Mamavirus but with the Marseillevirus the case is a tad bit more complicated. If this virus was present, Sputnik did not enter the amoebal cell but it could still delay the replication cycle of the Marseillevirus. If there were viral factories (VFs) already present, the replication cycle occurred as if Sputnik was not added. But if Sputnik was present before the infection of the Marseillevirus, the latter was still internalized but was unable to replicate. These conclusions point to a target of this virophage at an early stage of infection, but which one still remains unclear[14].

In 2010, with the discovery of the *Cafeteria roenbersensis* virus (CroV), a giant virus that infects marine flagellate present in the zooplankton, came the discovery of another virophage termed Mavirus[16] proving that Sputnik was not an isolated case.

Lausannevirus is the most recent addition to this group of giant viruses associated with *Acanthamoeba* species. It was discovered in 2011 after the co-cultivation of *A. castellanii* cells with samples from the water of the Seine River (France). Eight hours post infection it was observed icosahedral particles of about 200 nm in several large cytoplasmic vacuoles of the infected amoebas. A few VFs could also be observed, in certain amoebal cells, and the particles had no fibers surrounding the viral capsid. Its 346 kbp genome can have one of two possible conformations: a circular molecule or a linear molecule with terminal repeats. After the genome analysis, it was determined that its gene sequence is 89% similar to that of the Marseillevirus[9].

Since 2008, twenty one new *Acanthamoeba* growing giant viruses have been isolated by La Scola and his team, including the Mamavirus and the Marseillevirus, which clearly confirm the relative abundance of this kind of viruses in nature[17].

The Nucleo-Cytoplasmic Large DNA Virus clade or NCLDV is a group composed, as the name suggests, of large DNA viruses whose replicative cycle is carried exclusively in the cytoplasm of the host cell or it starts in the nucleus but it is completed in the cytoplasm. The relative independence of the NCLDVs from the host cells is consistent with the fact that all these viruses encode several conserved proteins which perform most of the key life-cycle processes such as for instance, DNA polymerases, DNA helicases and ATPase pumps for DNA packaging. Amongst these viruses we can find the members of the *Mimiviridae* family (the Mimivirus and the Mamavirus), the *Marseilleviridae family* (the Marseillevirus and the Lausannevirus), as well as others like the only member of the *Asfarviridae* family, the ASFV (African Swine Fever Virus), members of the genus *Ascovirus, Poxvirus* such as Vaccinia

virus, *Iridoviruses* like FV3 (Frog Virus 3) and *Phycodnaviruses*. The analysis of the genomes of several members of the NCLDVs has led then to the establishment of an ancestral NCLDV virus which is predicted to have coded in its genome proteins such as Topoisomerase II, RNA polymerase, the Capping enzyme and the G5R-like Flap endonuclease, among others[16,18]. These proteins are then coded by conserved genes that can be targets in the search for new NCLDVs.

It has been recently proposed a new viral order termed *Megavirales*, which is based on several criteria to group both NCLDV viruses and other large viruses that, for genetic reasons, were not included in the NCLDVs group, such as the elements of the *Herpesviridae* and *Polydnaviridae* families[16].

With respect to their ability to phagocyte very large particles and harbour many different microorganisms, amoebas in general are considered to be a melting pot. They can have symbiotic relationships with bacteria, eukaryotes and viruses which create the perfect opportunity for these intracellular organisms to exchange and evolve numerous genes between them, as well as with the amoeba itself. It has been reported that the ability of these amoeba resistant microorganisms (ARMs) to multiply within this phagocytic host can prepare them to survive and multiply within human macrophages[3]. It has indeed been reported that human and murine macrophages can phagocyte the Mimivirus and that its entry is mediated by a phagocytosis pathway, suggesting that human phagocytic cells can be a target for this giant virus[19]. In addition, it has been strongly suggested that the Mimivirus can cause pneumonia under experimental conditions in mice and many studies support the possibility of the Mimivirus to be a human pathogen and a possible cause for both community and hospital acquired pneumonia[11].

It is then fairly clear why it is of the outmost importance to study these amoebal associated giant viruses, particularly when one considers the extreme genetic promiscuity between several microorganisms within the amoebal host which lead to a very diverse gene pool and hence to numerous possibilities of new and improved human pathogens.

2. Objectives

Considering the importance of studying amoeba and associated microorganisms, the present work had two main objectives:

- The isolation of FLA species derived from environmental samples;
- Optical microscopy and molecular identification of the isolated amoebas;
- The search for viruses within these amoebas, with special emphasis in NCLDV viruses.

The viruses were detected by PCR using specific primers to amplify: the DNA polymerase gene region of large DNA viruses[20] and Adenovirus[20]; regions L396 and R596 of the mimivirus genome, which code for an helicase and a thiol oxidoreductase[21], respectively; and the *Major Capsid Protein* gene of *Ranavirus* (*Iridoviridae*)[22].

TEM and optical microscopy have also been applied for a better characterization of the isolated amoebas and viruses.

The other objectives of this work aimed to answer two leading questions:

- If *Acanthamoeba* species can replicate *Mimivirus*, which belong to the NCLDV group, would they be able to replicate iridoviruses *in vitro*?

To address this question, *A. castellanii* cells were inoculated with FV3 and two isolates of *Ranavirus*: Ma3B[23] and BoA[23] (isolated from a *Triturus marmoratus* and a *Triturus boscai*, respectively).

- Is it possible to distinguish between closely related *Ranavirus* using a less conserved gene than those frequently applied, and could it be used routinely in the lab?

The *MCP* and *DNA polymerase* genes are mostly conserved among several iridoviruses from the *Ranavirus* genus, such as FV3, LMO (*Lacerta monticula* iridovirus)[24], Ma3A[23], Ma3B[23], Ma3P[23] (iridoviruses isolated from three different *Triturus marmoratus*) and BoA[23], increasing the difficulty in separating them as unique viruses. The *G5R-like Flap endonuclease* gene is present in the ancestral NCLDV virus but it is only found in poxviruses, iridoviruses, EHV (*Emiliania huxleyi* virus) and the mimivirus[18], which possibly make it a core gene that is less prone to be maintained unaltered in the genome but it is still conserved enough to allow the design of specific primers. Therefore, a PCR protocol using newly designed Flap primers was applied.

3. Methodology

3.1 Cell Culture

3.1.1 Animal cells

Vero cells were cultured in CO_2 independent medium (Gibco®) supplemented with 1X Glutamax (Gibco®) and 1X Gentamicin (Gibco®) plus 10% Fetal Bovine Serum (FBS - Gibco®) in T flasks (Nunc®). At confluence, cultures were washed twice with 1X PBS-A (Gibco®), incubated with Trypsin (Gibco®) at 37ºC until detachment and distributed in 24 or 12 well Nunc® plates. These plates were then incubated at 37ºC and monitored frequently.

3.1.2 Amoeba cells

Acanthamoeba castellanii cells were maintained in culture using the Peptone Yeast extract Glucose (PYG) complete medium[25] (see Annex 1) plus a mixture of antibiotics and antimycotic (Anti-Anti® - Gibco®) 2X concentrated. When the cultures had too many debris, the amoebal cells were recovered by a centrifuging step (300g, 5 minutes), the supernatant discarded and the cells ressuspended in new PYG complete medium plus 2X Anti-Anti (Gibco®), maintaining the cultures in the same T flasks (Orange Scientific®). To make fresh cultures with fewer cysts, the same method was applied and the amoebal cells were distributed over several T flasks.

For longer maintenance of this cell line, the amoebal cells were recovered by centrifuging at 300g for 5 minutes, the supernatant discarded, and the cells ressuspended carefully in Freezing medium (see Annex 1). *A. castellanii* cells were then distributed in 2 mL tubes and these were placed in a Cryo 1ºC freezing container (Nalgene®) at -80ºC.

3.2 Isolation of free living amoeba

Several samples of water were collected in clean bottles (approximately 500 mL) from six locations described in Table 1 as AW1, AW2, Granjal, Ferragudo, LO1 and LO2 (for the specific locations see Annex 2). All samples had sediments which were further recovered by a centrifuging step (1000g, 30 min). All samples were inoculated in three different 1.5% non-nutrient agar (Fagron®) media: distilled H_2O, Page amoeba saline (PAS)[26] and artificial sea water (ASW) at 75% (see Annex 1). These Petri dishes were overlaid with 100µL of an autoclaved *E. coli* K12 culture in the shape of a plus-sign, and 50µL of the sediments were inoculated at the centre. They were kept right sided at room temperature, sealed with parafilm M® and protected from direct light[27].

From the Tagus River region (around the Passeio do Tejo area – see Annex 2), six samples were obtained (see Table 1) on a low tide. One sample was obtained directly

beneath a small bridge (E1 Ponte), another in the surrounding area of the said bridge (E1b), and the last two samples were collected close to shore and depending on the presence (E3) or absence (E2) of vegetation. The two water samples, LTW (Left Tagus Water) and RTW (Right Tagus Water), were collected next to a nearby floating dock, respectively at the left and right of the same dock. The first four samples were obtained through usage of an acrylic cylinder which recovered a small portion of sludge that was processed so that the amoebal cells derived from these samples came from 1 cm below the surface. As with the previous samples, they were all inoculated in triplicates consisting of approximately 1 cm^2 for the sludge samples, or 50µL for the water samples at the centre of the autoclaved *E. coli* plus-sign. These Petri dishes were also kept at room temperature, right sided, sealed with parafilm M® and protected from direct light[27].

Table 1 - Samples obtained for the isolation of free living amoeba.

Collection Date	Designation	Region	Coordinates (D.D.)
September 2011	Alqueva Water 1 (AW1)	Alqueva Lake	38.265183, -7.3881
September 2011	Alqueva Water 2 (AW2)	Alqueva Lake	38.377733, -7.37155
October 2011	Granjal (G)	Dão River	40.414083, -8.090717
March 2012	Ferragudo (Fer)	Portimão Bay	37.11035, -8.519833
March 2012	Lagoa Odivelas 1 (LO1)	Odivelas Lake	38.2026, -8.110633
March 2012	Lagoa Odivelas 2 (LO2)	Odivelas Lake	38.1908, -8.118233
April 2012	E1 Ponte	Tagus River	38.781939, -9.090930
April 2012	E1b	Tagus River	38.781835, -9.090984
April 2012	Left Tagus Water (LTW)	Tagus River	38.783570, -9.089755
April 2012	Right Tagus Water (RTW)	Tagus River	38.783461, -9.089760
April 2012	E2	Tagus River	38.781897, -9.091204
April 2012	E3	Tagus River	38.781948, -9.091128

D.D. – Decimal Degrees

The Petri dishes were observed frequently with a Zeiss® microscope at a magnification of 100X and when amoebal cells were detected, they were cut from the agar with surgery sterile knifes (Braun®) and placed in a new Petri dish with the same conditions from where they were cut.

3.3 Virus production and titration

For the amoeba infection assay, FV3 virus was produced in Vero cells with a multiplicity of infection of < 0.01. In a T75 flask (Nunc®): the medium of a sub confluent culture (approximately 10^7 cells) was discarded and the cells were inoculated with 3 mL of a 10^3 pfu/mL viral suspension. The incubation step occurred in the rocker for 30 minutes, at room temperature. It was then added 12 mL of supplemented CO_2 independent medium (Gibco®)

supplemented with 2% FBS (Gibco®) and the T flask (Nunc®) placed at 30ºC in an incubator. The virus was collected in a 15mL Falcon tube (Orange Scientific®) 6 days after infection and centrifuged at 4000g for 5 minutes. The supernatant was removed to a new tube and termed extracellular virus. The remaining pellet was ressuspended in 3 mL of 1X PBS-A (Gibco®) and placed for 2h at -20ºC. These cells were then rapidly thawed at 37ºC and centrifuged at 4000g for 5 minutes. The new supernatant was collected to a Falcon tube (Orange Scientific®) and labelled intracellular virus.

For the titration procedure, 100 µL of several dilutions of both intra and extracellular virus, namely from 10^{-1} to 10^{-3}, were inoculated. First, the medium of a sub confluent 24 well Nunc® plate, containing approximately $5X10^{6}$ Vero cells, was discarded. The cells were then inoculated with the virus and incubated for 30 minutes in the rocker at room temperature. It was then added 400 µL of titration medium (CO_2 independent medium (Gibco®) supplemented with 2% FBS (Gibco®) plus 2% Sephadex® G-50 (Pharmacia®)) and the plate incubated at 30ºC. After 13 days, the infection was halted by introducing a few drops of a 10% formaldehyde solution in each well and the plate placed in the rocker for 1h at room temperature. The supernatants were then discarded and a few drops of a 10% Giemsa (Sigma®) solution were added to each well. The staining lasted for 15 minutes after which the excess dye was washed with water and the Nunc® plate observed to count the number of viral plaques.

3.4 Amoeba Infection Assay

The assay was initiated with a fresh culture of *A. castellanii* cells where the adherent cells were inoculated with 10 µL of a 10^{7} pfu/mL viral solution while the ressuspended ones were inoculated with 50 µL of the same solution. For the adherent amoeba, a negative control T Flask (Nunc®) was made without virus inoculation, and the other T flasks (Nunc®) were inoculated with FV3, BoA[23] and Ma3B[23] viruses, respectively. After the inoculation, 2 mL of PYG complete medium plus 2X Anti-Anti (Gibco®) was added to the cells. The ressuspended *A. castellanii* cells were combined in the same Falcon tube (Orange Scientific®) and centrifuged at 750g for 5 minutes. The supernatant was discarded and the pellet was ressuspended in 2 mL of PYG complete medium with 2X Anti-Anti (Gibco®). The cells were divided into two Falcon tubes (Orange Scientific®), one as a negative control while the other was inoculated with FV3. The tubes were kept in the rocker for 1h before the cells were distributed onto another T flask and another 1 mL of PYG complete medium plus 2X Anti-Anti (Gibco®) was added. All T flasks were kept at room temperature and protected from direct light.

3.5 DNA extraction techniques

For the DNA extraction, amoebas were recovered by washing around the area left by the cut agar in the Petri dish with 1 mL of PAS. The sample was then recovered and centrifuged at 300g for 5 minutes. The supernatant was discarded and 500 µL of PAS were added. The samples were then centrifuged at 7200g for 5 minutes and the supernatant discarded, adding 200 µL of PAS to the remaining pellet[27].

The DNA extraction was achieved using the EasySpin® Genomic DNA Minipreps Bacteria Kit (Citomed®) and the Jet Quick® Blood and Cell Culture DNA Spin Kit (Genomed®). Both kits shared the same first steps: adding RNase A (Albet®) to the 200 µL sample at a final concentration of 0.4 mg/mL and incubate for 5 minutes at 37ºC followed by an addition of Proteinase K (Eurobio®) at a final concentration of 2 mg/mL and incubation for 10 minutes at 60ºC. The remaining protocol was followed accordingly to the product's manufacturer.

3.6 Polymerase Chain Reaction (PCR)

3.6.1 Primer design – Flap primers

Flap primers were designed based on the sequences of the FV3 and CMTR (Common midwife toad ranavirus) Flap genes. FV3 Flap is termed RAD2 and its accession number is NC_005946.1 (101656-102747). CMTR Flap can be accessed by the number JQ231222.1 (15499-16593). The two sequences were aligned in Clustal X®[28] and the alignment was analysed in BioEdit®[29]. FV3 Flap sequence was submitted to PrimerBlast® (National Library of Medicine) that generated a few possible primer pairs. The most suitable pair was selected and compared with the previous alignment where the base wobbles were chosen and the final primers designed. They were named F-Flap and R-Flap, forward and reverse primers, respectively (see Table 4 in Annex 3).

3.6.2 PCR

The PCRs were prepared using the kit My Taq® Red Mix (Bioline®) and the set-up of the reactions were made accordingly to the instructions except for the reaction volume which was 5 µL for the viral screening PCRs, 12,5 µL for the amoeba identification PCRs or 25 µL for the amplification of the Flap gene. All reactions were performed in a T personal® (Biometra®) cycler and the DNA originated from a DNA extraction method as per the method described above except in Direct PCR reactions. The PCR products were resolved on 1% agarose gels using TBE 1X concentrated and stained with ethidium bromide (5 µg/mL).

For the viral screening, the applied primers tested the presence of *Mimivirus* (primers F396/R396 and F596/R596[21]), large DNA virus (primers HV/Cons[20]), *Adenovirus* (primers

Adeno/Cons[20]) and *Ranavirus* (primers OLT1/OLT2R – that correspond to primers 4 and 5 in Table 2 of Mao *et al* (1997)[22] – see Table 4 in Annex 3). All reactions were submitted to the same cycling conditions: initial denaturation at 95ºC for 3 minutes followed by 40 cycles of 95ºC for 30 s, 40ºC for 30s and 72ºC for 1 min. A final extension was performed at 72ºC for 5 minutes before the samples were kept at 4ºC.

Amplification of the 18S rRNA region, for the amoeba identification PCRs, was accomplished with the EUKA and EUKB[30] primers. When the latter pair failed to produce a result, primers III and IX[31] were applied to amplify the ITS genomic region between the 18S and 28S rRNA genes. In both cases the cycling conditions used were as follow: initial denaturation of 95ºC for 3 minutes and 35 cycles of 95ºC for 30 s, 58ºC for 30 s and 72ºC for 1 minute. This was followed by a final extension of 72ºC for 5 minutes before going to 4ºC.

For the Flap gene amplification, primers F-Flap and R-Flap were used and the DNA provided referred to six different viruses already isolated: FV3, LMO[24], BoA[23], Ma3A[23], Ma3B[23] and Ma3P[23]. The cycling conditions were the same as with the amoeba identification PCRs described above.

3.6.2.1 Direct PCR – Isolated Amoeba Samples

For both viral screening and amoeba identification, another PCR was made termed Direct PCR and it was prepared with samples concentrated in a Dilution Buffer® provided by the Phire® Plant Direct PCR Kit (Finnzymes®). The samples, in this case, were obtained by cutting a small square of agar from the amoeba Petri dishes and ressuspending it in 1 mL of PAS, pipetting several times and submitting it to a 5 second vortex. The PAS was then recovered to a new microtube and centrifuged at 300g for 5 minutes. The supernatant was discarded and the pellet was ressuspended in 10 µL of Dilution Buffer® (Finnzymes®), followed by a quick vortex. The samples were left at room temperature for 30 minutes and submitted to a spin down. In Direct PCR reactions, 1 µL of this DNA was used, applying the same enzyme and protocol as described previously. All reactions were submitted to the same cycling conditions as the other corresponding PCRs.

3.6.2.2 Direct PCR – Amoeba Infection Assay

For the Amoeba Infection Assay, the presence of the inoculated viruses was tested using the Direct PCR method. 20 µL of all cultures from the adherent *A. castellanni* cells were collected and centrifuged at 300g for 5 minutes. The supernatant was recovered and 1 µL was ressuspended in 10 µL of Dilution Buffer® (Finnzymes®). These solutions were further diluted (1:10) in nuclease free water (Cleaver Scientific®) and termed “Extracellular Virus”. The remaining pellet was ressuspended in 100 µL of PAS and centrifuged at 300g for 5

minutes. The supernatant was discarded and the washing step repeated. The final pellet was ressuspended in 10 µL of PAS and 1 µL of this solution was diluted in 10 µL of Dilution Buffer® (Finnzymes®). As with the other samples, these solutions were also further diluted (1:10) in nuclease free water (Cleaver Scientific®) and termed "Intracellular Virus" samples. 1 µL of both Extracellular and Intracellular Virus samples were used in Direct PCR reactions using the same enzyme kit and protocol as before. All samples were amplified in duplicates using primers EUKA and EUKB for one reaction and primers OLT1 and OLT2R for another (see Table 4 in Annex 3). The cycling conditions for the first reaction were the same as for the amoeba identification PCRs. The second reaction was submitted to the cycling conditions described above for viral screening PCRs.

3.7 Sequencing

All PCR products were purified with the Purelink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®) following the gel purification procedure as described in the product's manual. For sequencing, all samples were sent to a commercial lab (StabVida®, Lisbon, Portugal). The retrieved sequences were then analysed using the FinchTV® (Geospiza®) program for the chromatogram analysis, ClustalX®[28] for the sequence alignment, BioEdit®[29] for sequence and alignment analysis and blastn® (National Library of Medicine, USA) for database search.

3.8 Optical Microscopy

All amoebal preparations were performed by cutting a square of the agar Petri dishes and placing it in a microtube containing 100 µL of PAS. The agar was ressuspended several times and the remaining medium was recovered and spread onto a microscope slide. The amoebas were then observed in two different microscopes: Motic® TYPE 101M and Leica® DM LB. It was possible to take images using the Moticam® 5.0 camera and the Motic® Images Plus 2.0 program for the first microscope, and Leica® DFC320 camera and Leica® Application Suite program for the last. All images were taken in bright field or phase contrast when appropriate and processed in Microsoft® Picture Manager (Microsoft®) and Paint® v6.1 (Microsoft®). The scale bars were introduced with ImageJ® 1.45S (National Institute of Health, USA).

3.9 Electron Microscopy

The electron micrographs were obtained and kindly provided by Dr António Pedro Alves de Matos and all the procedures were completed in Curry Cabral Hospital by his team.

4. Results and Discussion

4.1 Isolation and identification of free living amoeba

Although it was possible to identify, by molecular methods, the majority of the isolated amoebas, it is important to point out that this was only possible due to the Direct PCR method. Two different DNA extraction protocols were applied (see mark 3.5 in Methodology) but it was not possible to obtain DNA in order to identify the isolated amoebas (data not shown). This could be due to the established idea that the amoebal DNA is not easy to extract[32] and since the kit methods applied focused on bacterial or animal cell DNA extraction protocols, this result is somewhat to be expected. It was recently described a method to better extract DNA from protists[33] but the resources required for such a procedure were not fully available in the lab and for a quicker way to get the essential DNA the kit methods were applied.

Since the DNA could not be extracted with these methods a new and quicker way to identify the isolated amoebas was in order. The solution was to develop and apply a Direct PCR protocol in which the amoebas were recovered by a centrifuging step and ressuspended in a Dilution Buffer from a commercial kit (Finnzymes®) which released the amoebal DNA most likely due to a detergent agent and/or enzymes that degrade the amoebal membranes. With this protocol it was then possible to obtain PCR products which were purified and further sequenced. The EUKA and EUKB primers were applied to amplify the eukaryotic 18S rRNA gene[30] and when this pair failed to produce a positive result, primers III and IX were applied to amplify the genomic region between the 18S and 28S rRNA gene region, that include *internal transcribed spacer* (ITS) regions[31], hence the term ITS primers. This was the case of amoebas AW1 and AW2.

From the 12 samples collected (see Table 1) it was possible to obtain 17 amoebas which were observed by optic microscopy and the images obtained are reunited in Figures 1, 2 and 3. Table 2 reunites the DNA sequencing results of the isolated amoebas.

When in Petri dishes, all amoebas seemed to have different morphologies since the agar distorts any observation, hence all duplicate samples (amoebas with a small letter next to the name of the collected sample in Table 2) were maintained in separate cultures. Only after the optic microscopy analysis was it possible to visualize the true form of the isolated amoebas and group some of them as possibly belonging to the same genus.

Amoebas AW1 and GH had morphologically similar trophozoites, as seen in Figures 1A and 1C. It is also possible to group amoebas AW2 and GP since they display a similar floating morphology (see Figures 1B and 1D). After DNA sequencing analysis, amoebas AW1, GH and GP were identified as *Vannella* sp. with sequence identities over 87% and

relatively low E values. Amoeba GP has the highest E value in this group mainly because the DNA sequence retrieved is smaller than the other amoebal DNA sequences and its overall quality is also diminished (see Annex 4). Amoebas AW2 were indeed identified as *Vannella simplex* with a 99% sequence identity and the lowest E value in this group (see Table 2) although its DNA sequence is almost as small as the amoeba GP (see Annex 4). These results point to a high probability that all four amoebas belong to the *Vannella* genus which is clearly supported by the similar morphologies observed by optic microscopy.

Fer amoebas had a unique and distinctive morphology (see Figure 1E), especially due to their small size but were unable to be later identified because of a sample contamination.

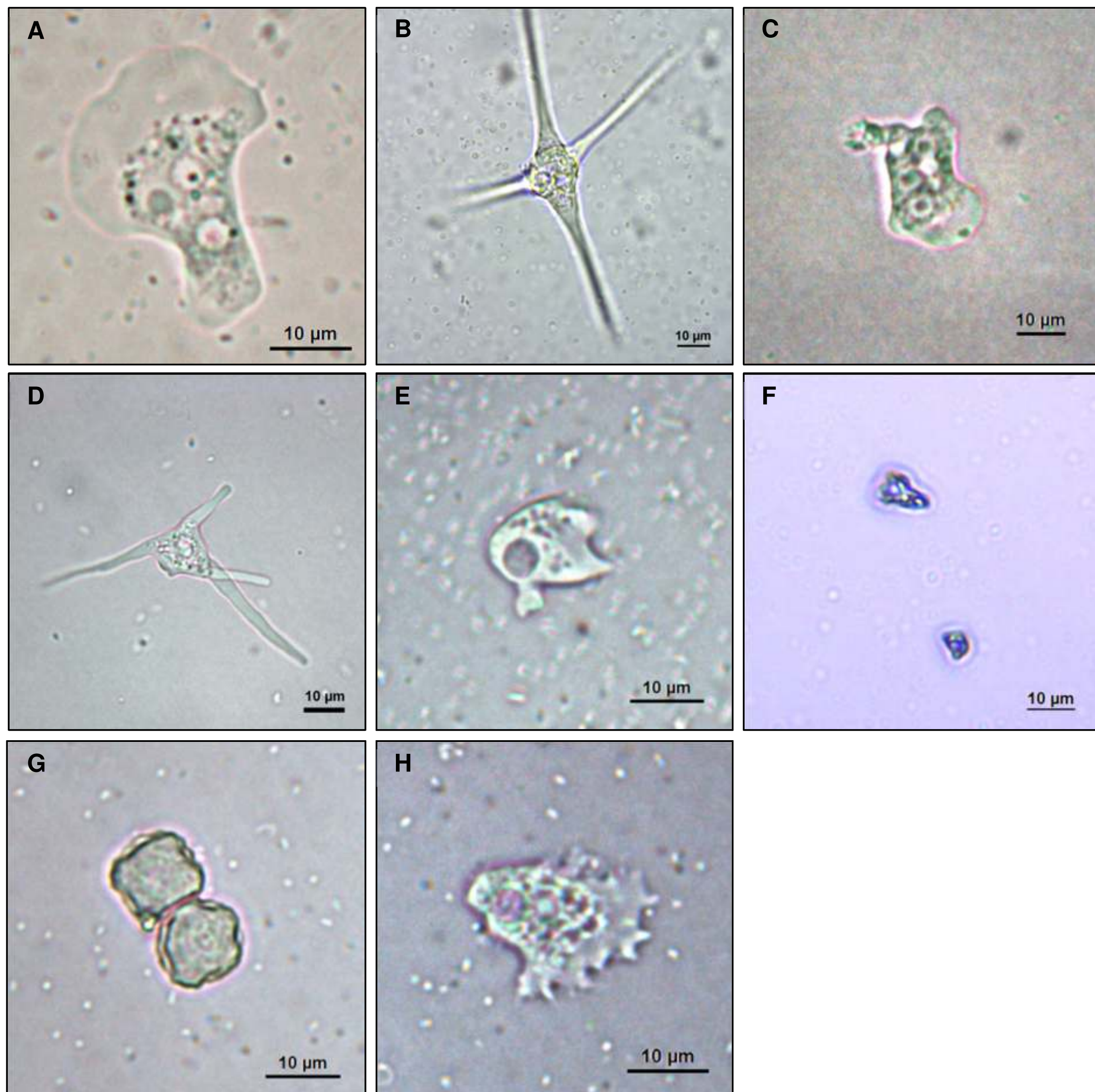

Figure 1 – Free living amoeba isolated from AW1, AW2, Granjal, Ferragudo, LO1 and LO2 collected samples. All images were obtained in bright field. The amoebas were termed after the sample from where they were isolated: **A** - AW1 trophozoite; **B** – AW2 trophozoite; **C** – GH trophozoite; **D** – GP trophozoite; **E** – Fer trophozoite; **F** – LO1 trophozoite; **G** – LO2 cyst; **H** – LO2 trophozoite.

This was also the case for amoebas LO1, as it was possible to observe very small amoebal-like particles (see Figure 1F) but the DNA sequencing results revealed a contamination as well.

Amoebas LO2 were assumed to belong to the *Acanthamoeba* genus due to the similarities of their cystic form (see Figure 1G) to the *A. castellanni* cell cysts that were already available in the lab. It was also possible to group these amoebas with amoebas 2, 3, 4, 5 and 6 of the Tagus River region (Figures 2B, 2D, 2F, and 2H), because they also display similar *Acanthamoeba* cyst forms. Although in Figure 2G it can only be observed its trophozoite form, amoeba 5 also displayed the typical *Acanthamoeba* cyst (data not shown),

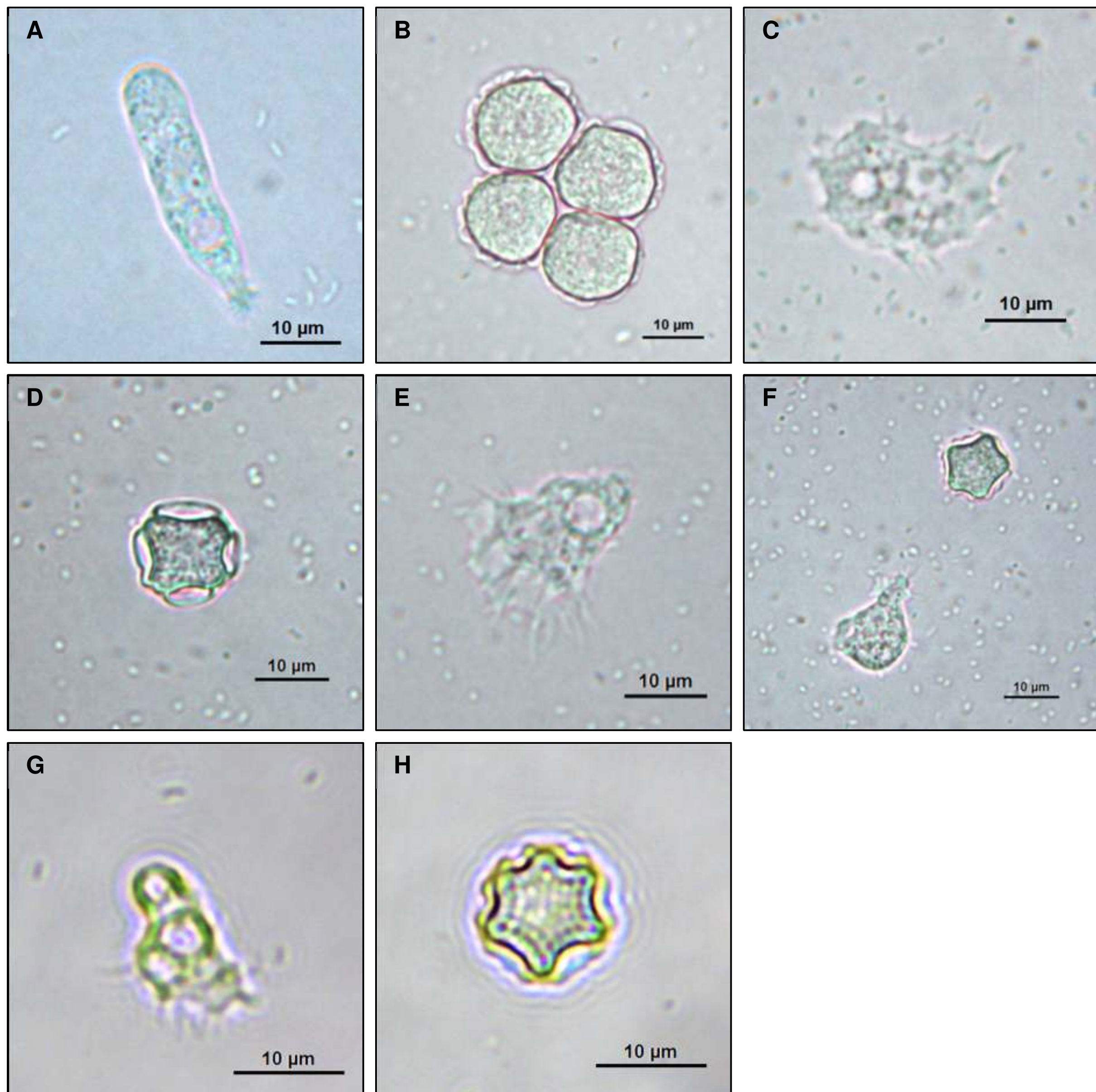

Figure 2 – **Free living amoeba isolated from the Tagus River region.** All images were obtained in bright field. The amoebas were named by numbers corresponding to a specific sample and media in which they grew (see Table 2): **A** – 1 trophozoite; **B** – 2 cyst; **C** – 2 trophozoite; **D** – 3 cyst; **E** – 3 trophozoite; **F** – 4 trophozoite and cyst; **G** – 5 trophozoite; **H** – 6 cyst.

hence the grouping referred above. Regarding their trophozoite forms, all amoebas within this group had similarities (see Figures 2C, 2E and 2G) but it was not as easy to compare between them as through the cystic form. From the DNA sequencing analysis, it was than possible to point amoeba 2 as an *Acanthamoeba lenticulata* with a 99% sequence identity and a low E value, amoeba 5 belongs to the same genus with a 97% sequence identity and a slightly lower E value, and amoeba 6 is an *Acanthamoeba rhysodes* with the lowest E value and a sequence identity of 97% (see Table 2). The higher E value in this group belongs to the amoeba 2 DNA sequence possibly due to its smaller size (see Annex 4). These results confirm the grouping of amoebas 2, 5 and 6 which do belong to the *Acanthamoeba* genus. Unfortunately, amoebas LO2, 3 and 4 could not be identified through DNA sequencing because their purified PCR products had a concentration lower than the minimal required for such a procedure. As amoebas 2, 5 and 6 were indeed proved to belong to this genus and amoebas LO2, 3 and 4 do share a highly similar morphology with the first, it is then plausible to admit that these three amoebas could also be members of the *Acanthamoeba* genus.

Amoeba 1 had a very distinctive way of locomotion, moving forward as propelled by the uroid rear region. As seen in Figure 2A, they had an elongated morphology unlike any of the other amoebas. The DNA sequencing than proved they do in fact belong to another genus

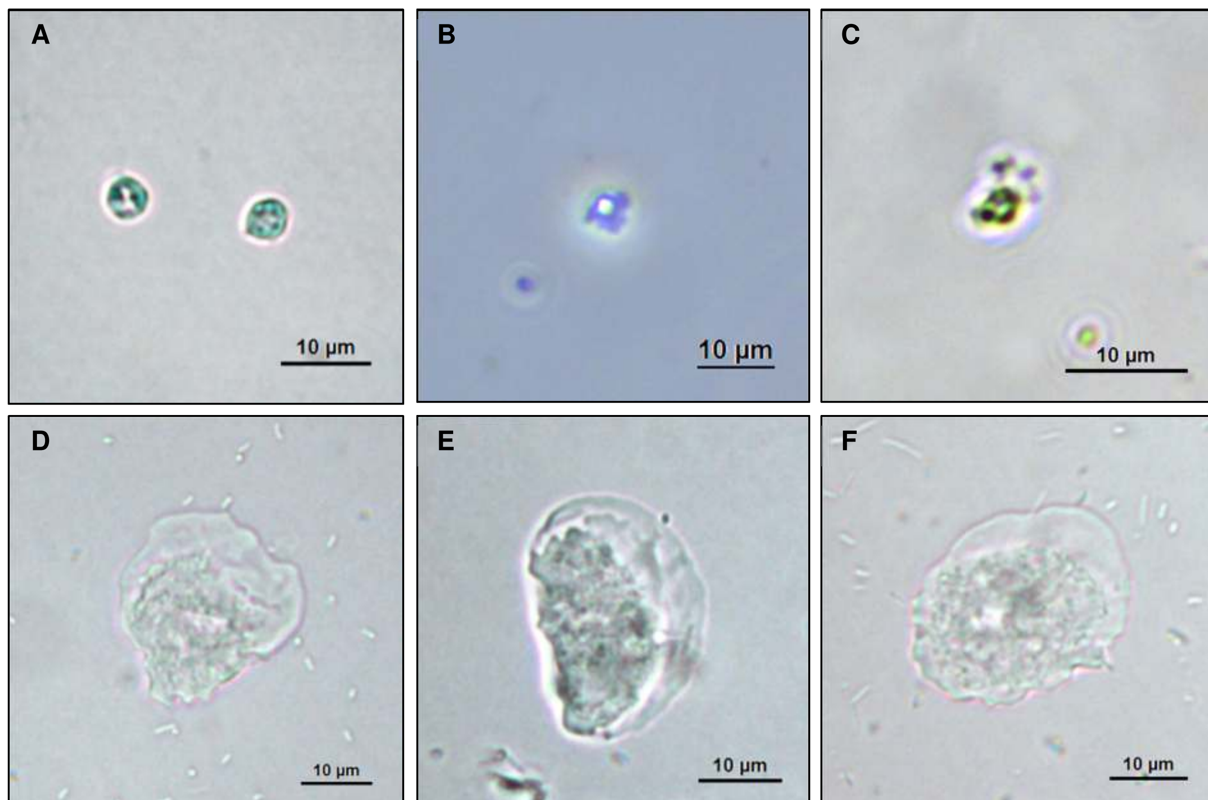

Figure 3 – **Free living amoeba isolated from the Tagus River region.** All images were obtained in bright field except for **B** which was obtained in phase contrast. The amoebas were named by numbers corresponding to a specific sample and media in which they grew (see Table 2): **A** – 7 cyst; **B** – 7 trophozoite; **C** – 7 trophozoite; **D** – 8 trophozoite; **E** – 9 trophozoite; **F** – 10 trophozoite.

and more accurately to the *Saccamoeba limax* species with a 99% sequence similarity and one of the lowest E value (see Table 2).

Amoebas 7 were incredible small and very hard to observe but in Figure 3A it is possible to see what could be their cystic form. In Figure 3B and 3C it can be observed the trophozoite form, in phase contrast and in bright field, respectively. The first image clearly demonstrates that this could be in fact an amoebal-like living cell and the second scarcely displays its general form. It was not possible to identify these amoebas by molecular methods possibly due to their small size and hence the aggravated difficulty in recovering several amoeba to reunite enough template DNA for the PCR reactions.

Finally, it was also possible to group amoebas 8, 9 and 10 to the same genus derived from their remarkable similar trophozoite form, as it can be seen in Figures 3D, 3E and 3F. They did not display a cystic form and were quite different from any of the other amoebas. The DNA sequencing revealed that they in fact belong to the *Platyamoeba oblongata* species with sequence identities higher than 84% and low E values (see Table 2). Amoeba 10 has an E value quite different from amoebas 8 and 9 due to a smaller DNA sequence obtained (see Annex 4). Despite this fact, the maximal identity is very similar amongst the three amoebas and the optical microscopy visibly confirms that they to belong to the same genus.

Table 2 – Amoebas isolated from the collected samples and blast results regarding their sequenced 18S rRNA gene or ITS genomic region. The grey lines indicate the samples that could not be analysed by DNA sequencing.

Sample	Agar medium	Amoeba	Blast results	Query coverage	Maximal Identity	E value
AW1	H_2O	AW1	*Vannella* sp.	57%	88%	6e-29
AW2	PAS	AW2	*Vannella simplex*	92%	99%	1e-31
Granjal	H_2O	GH	*Vannella* sp.	87%	89%	3e-37
Granjal	PAS	GP	*Vannella* sp.	96%	87%	8e-12
Ferragudo	PAS	Fer				
LO1	PAS	LO1				
LO2	PAS	LO2				
E1b	PAS	1	*Saccamoeba limax*	92%	99%	6e-112
LTW	PAS	2	*Acanthamoeba lenticulata*	100%	99%	7e-91
E1 Ponte	H_2O	3				
E1b(a)	H_2O	4				
E1b(b)	H_2O	5	*Acanthamoeba* sp.	68%	97%	7e-107
E1 Ponte	75% ASW	6	*Acanthamoeba rhysodes*	86%	97%	8e-177
E2(a)	75% ASW	7				
E2(b)	75% ASW	8	*Platyamoeba oblongata*	96%	87%	8e-93
E2(c)	75% ASW	9	*Platyamoeba oblongata*	93%	89%	3e-98
E2(e1)	75% ASW	10	*Platyamoeba oblongata*	83%	84%	5e-12

Only 11 out of 17 isolated amoebas have been identified by molecular methods. This has occurred mainly because the amoebas were not established in axenic cultures and the agar Petri dish allows their growth only to a certain extent. The amoebal recovery in this kind of medium is also impaired and ultimately, decreases the DNA concentration available for the PCR reactions.

All amoebal sequences that led to the identification of the isolated amoebas are, without exception, smaller than the PCR product obtained for each one (see Annex 4). This could be explained by the fact that although the Direct PCR method was an improvement comparing to the PCR reactions applying purified template DNA, it does not provide high quantities of PCR products, hence the difficulty in having high concentrations of DNA after the purification step and ultimately impairing the sequencing procedures. This may be explained by the presence of possible PCR inhibitors in the Direct PCR reactions, since although the DNA has been released into the medium, other amoebal particles are still present in the sample, even after the last spin down before removing 1 µL for the PCR reaction.

4.2 Virus screening

To assess the presence of NCLDVs in the isolated amoebas, five different PCR protocols were applied using the primers HV and Cons for the DNA polymerase gene of large DNA virus (LDV), primers Adeno and Cons for the same region of *adenoviruses*, primers OLT1 and OLT2R for the MCP gene of *Ranavirus*, and primers for the L396 and R596 genome regions of *Mimivirus*. As with the amoeba identification PCRs, this screening was only

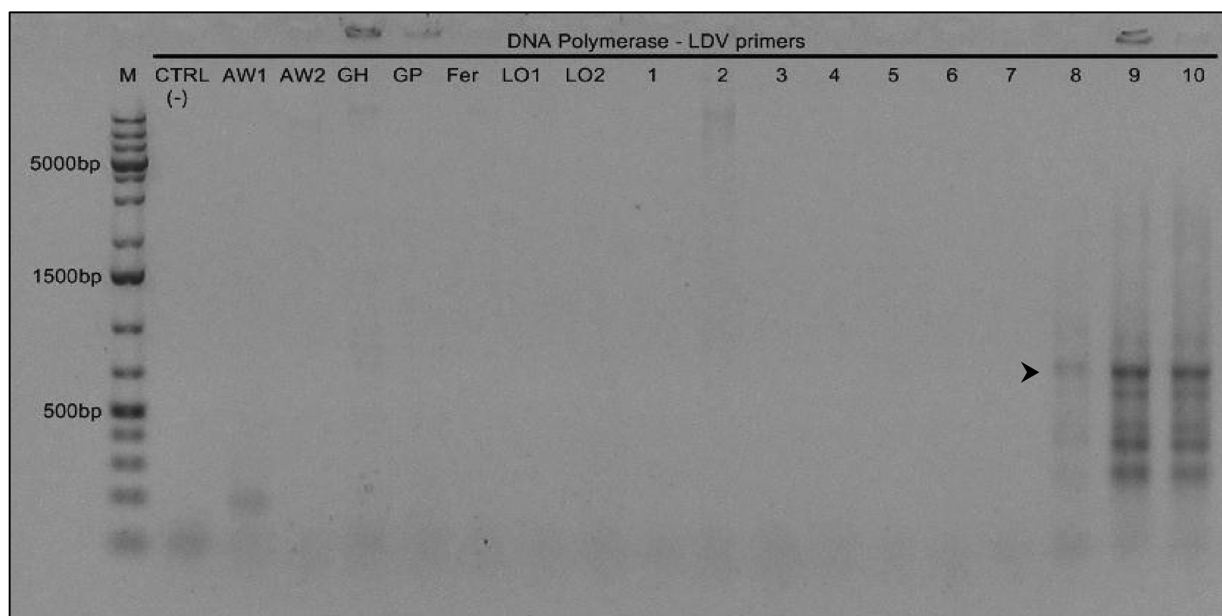

Figure 4 – Photograph of a 1% agarose gel with the PCR products obtained when using the large DNA virus primers. The arrow (➤) displays the PCR products that were further analysed by DNA sequencing. LDV – Large DNA virus; M – Marker; CTRL (-) – Negative Control; AW1, AW2, GH, GP, Fer, LO1, LO2 and 1-10 – Amoebas isolated from the respective samples.

possible due to the Direct PCR method. The PCR protocols whose template was DNA recovered by an extraction method failed to produce any positive result (data not shown).

In Figure 4 it is possible to observe an expected PCR product of about 700bp in samples 8, 9 and 10, indicated by the arrow. This could point to the presence of a large DNA virus in these three amoebas. The presence of more than one PCR product in each sample is also to be expected since the annealing temperature used in this protocol was fairly low (40ºC) and the authors of these primers had already reported the occurrence of multiple bands when using these primers with culture samples[20]. The DNA sequencing of these PCR products revealed a contamination for both amoebas 9 and 10. Sample 8 had a weaker PCR band so there was not enough DNA after the purification step for this PCR product to be sequenced.

The results for the PCR protocol that uses the Adeno primers can be seen in Figure 5. The samples with a positive result were the same as with the primers for the large DNA viruses, but unlike the latter, these results reveal a PCR product of about 350bp although the expected band should have around 1200bp. Since the authors for these primers also report the occurrence of partial sequences when using the *Adenovirus* primers[20], these products were also investigated by DNA sequencing. The sequencing results revealed that these

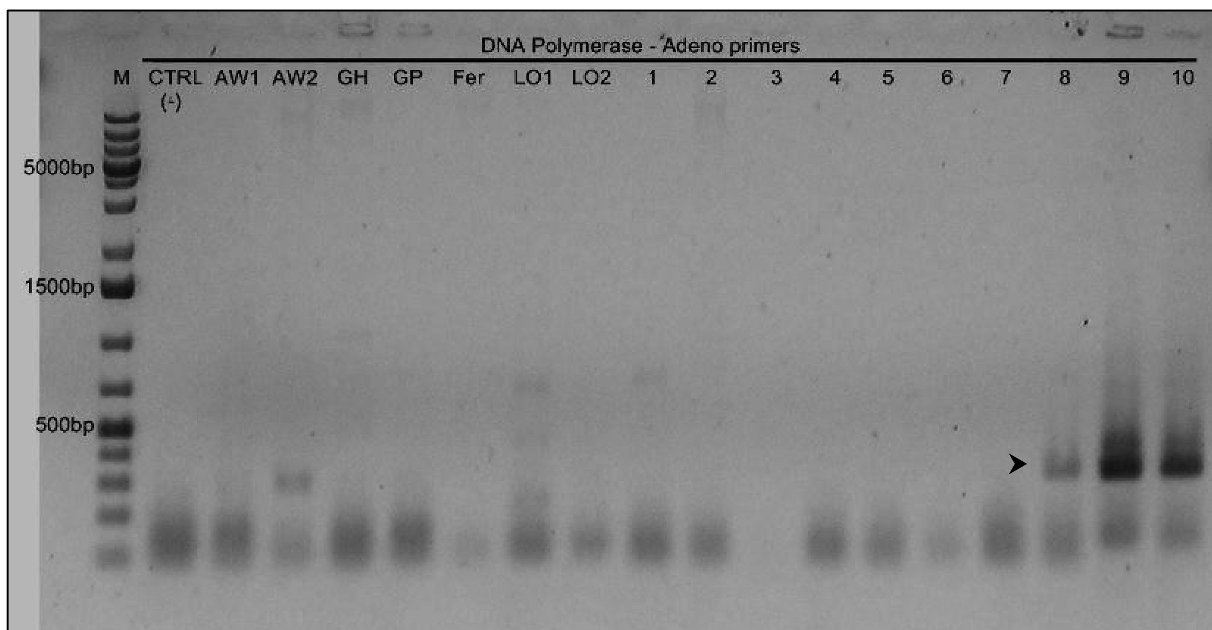

Figure 5 – Photograph of a 1% agarose gel with the PCR products obtained when using the Adenovirus primers. The arrow (➤) displays the PCR products that were further analysed by DNA sequencing. M – Marker; CTRL (-) – Negative Control; AW1, AW2, GH, GP, Fer, LO1, LO2 and 1-10 – Amoebas isolated from the respective samples.

products were in fact the PCR amplification of bacterial DNA which most likely happened due to the low annealing temperature used (40ºC) and the fact that these primers have base wobbles which potentially allow the detection of more DNA polymerases than the adenoviral

DNA polymerase (for the obtained sequences see Annex 5). Although this result was far from expected, this is still a curious finding, since the bacterium detected in sample 9 and 10 has 89% sequence identity to the *Marivirga tractuosa* species (see Table 3) which is reported as an antibiotic resistant bacterium, namely to gentamicin, kanamycin and streptomycin[34]. Although this is important for a public health concern, it is relevant to point out that this bacterium is usually found only in marine environments[34] (as confirmed by the fact that samples 9 and 10 are indeed derived from a marine water sample). No studies so far have reported an amoebal host for this bacterium, so this result could indicate the very first *M. tractuosa* amoebal association.

Table 3 – Blast results for the PCR products of the Adenovirus screening. Only the forward sequences were considered.

Sample	Primer	Blast results	Query coverage	Maximal Identity	E value
9 Adeno	Forward (Adeno)	*Marivirga tractuosa*	90%	89%	6e-44
10 Adeno	Forward (Adeno)	*Marivirga tractuosa*	90%	89%	3e-41

As seen in the micrograph of Figure 6, amoeba 10 does present a particle that can resemble a transverse cut of this bacterium (indicated by the arrow) and has an approximate size of about 0.5 µm, which is the diameter described for *M. tractuosa*[34]. The image then supports the detection of this unexpected ARM by applying the Adenovirus primers and that perhaps could not be detected by the application of specific bacterial primers. This result highlights the broadly applicable characteristics of these wobble base primers.

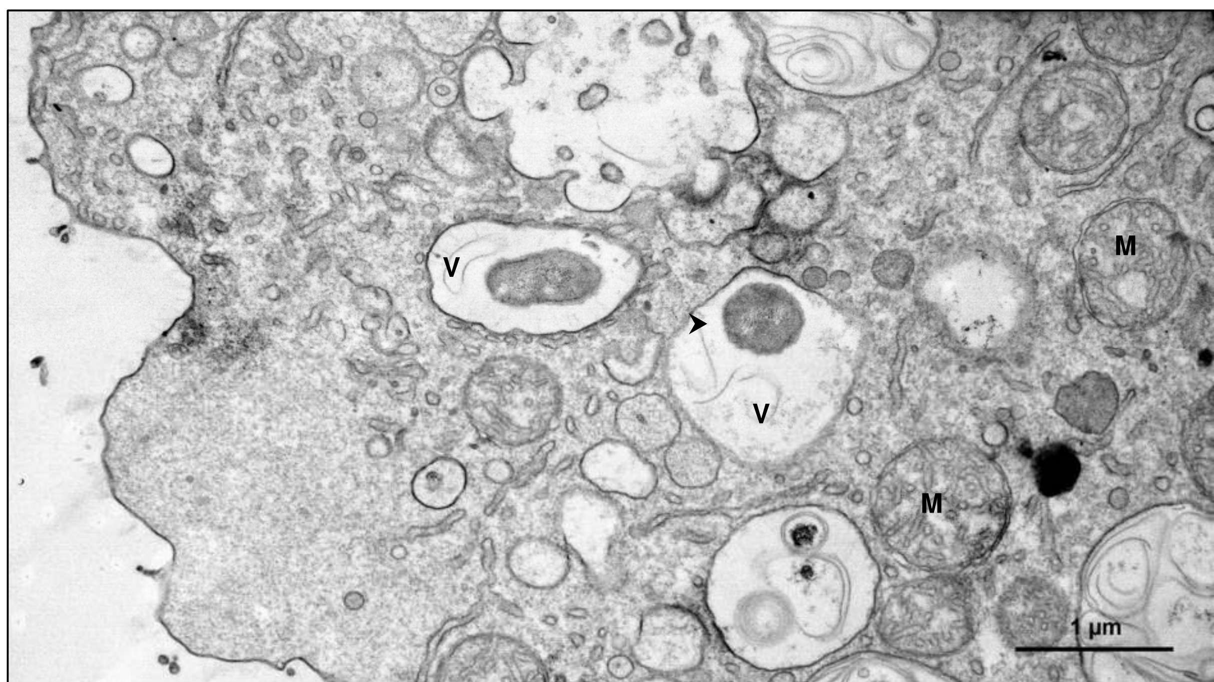

Figure 6 – Micrograph of the trophozoite form of amoeba 10. The arrow (➤) points to a possible *Marivirga tractuosa* specimen. V – Vacuole; M – Mitochondria.

When applying the primers for the MCP gene of iridoviruses, PCR products with the expected band size of about 500bp were detected in samples AW1 and LO1, as seen in Figure 7. However, the DNA concentration of these PCR products was very low and after the purification step there was not enough DNA for the sequencing procedure. As such, perhaps the expected 500bp bands were not specific to the MCP gene and rather a non-specific

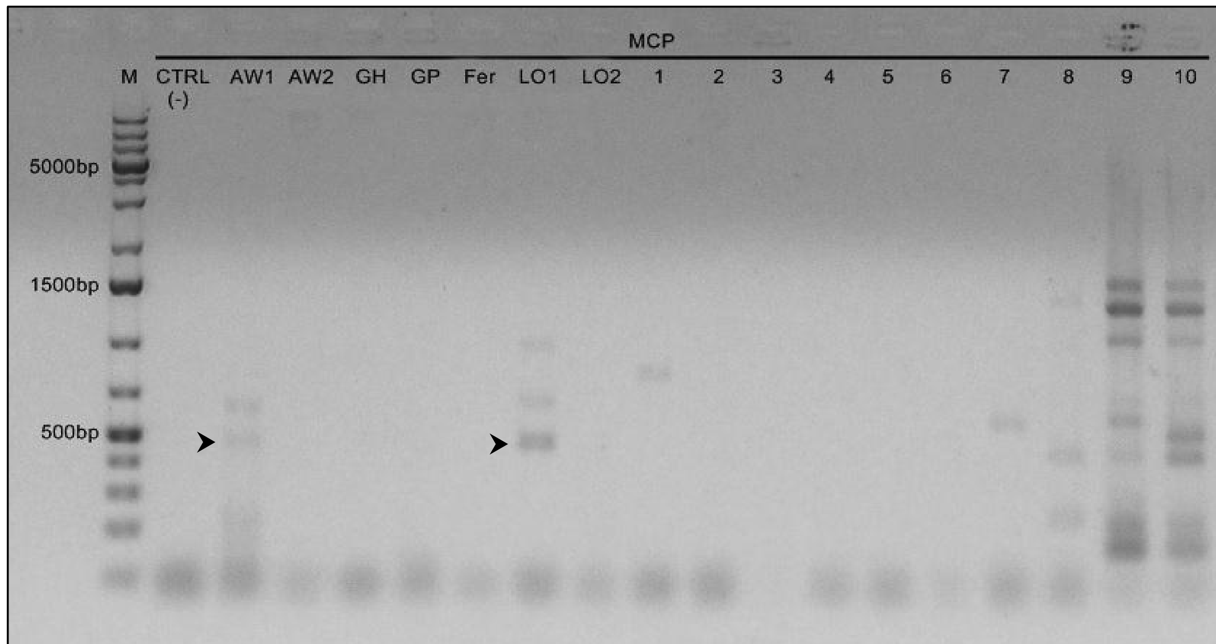

Figure 7 – Photograph of a 1% agarose gel with the PCR products obtained when using the MCP primers. The arrows (➤) displays the PCR products that were further analysed as a possible viral amplification. M – Marker; CTRL (-) – Negative Control; AW1, AW2, GH, GP, Fer, LO1, LO2 and 1-10 – Amoebas isolated from the respective samples.

product which was amplified only due to the low stringency of these PCR reactions.

The application of the primers for both the L396 and R596 mimiviral genomic regions led to no specific PCR products, although some DNA amplification can be seen on Figures 8 and 9. These bands occur most likely because the annealing temperature is only 40ºC, allowing even some samples to have strong PCR bands. These strong products have, however, lower sizes than the expected 1560bp for the L396 region and as so were not further analysed by DNA sequencing. For the R596 region, the non-specific bands usually have a size higher than the expected 879bp and were not sequenced as well.

The PCR results for virus screening were generally successful, however they can be improved mainly because the tested amoebas were derived from old samples that should have been tested as soon as they were collected and as such, probably lost their ARMs over time, which limited the possibility of detection by the PCR method. This has been described by several authors including R. Gast *et al*[27] which reported the loss of *Legionella pneumophila* specimens from the collected amoebas after one year in culture[27]. The

amoebas that tested positive for the bacteria *M. tractuosa* were derived from one of the most recent samples, which could explain why they were still detected by PCR.

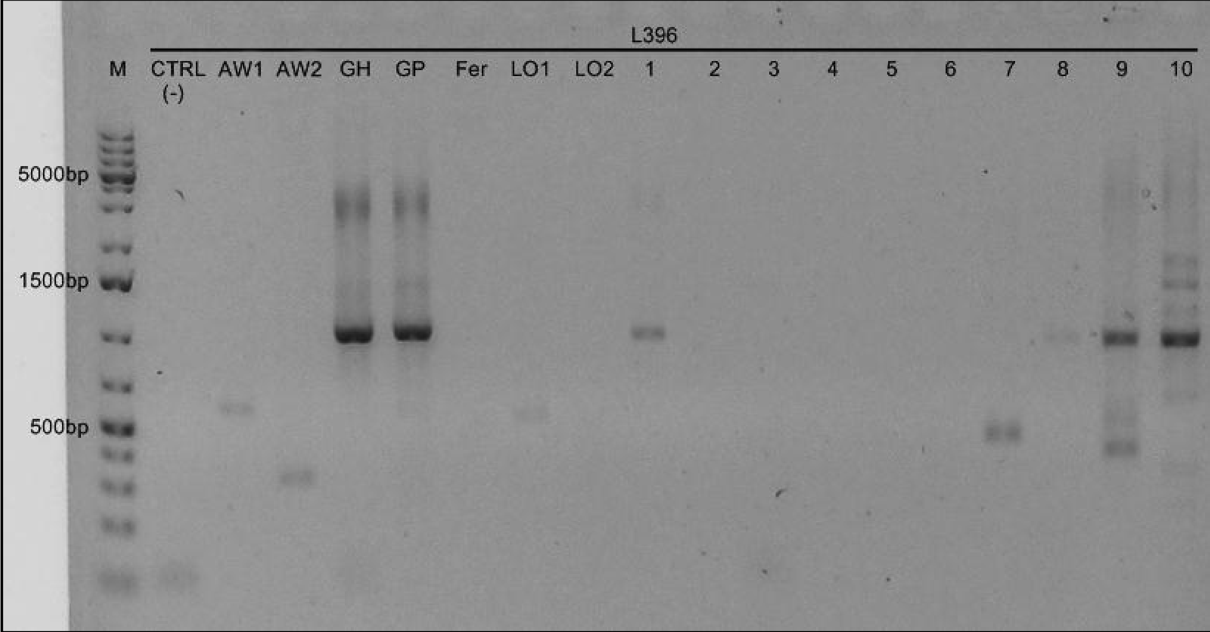

Figure 8 – Photograph of a 1% agarose gel with the PCR products obtained when using the L396 primers for Mimivirus. M – Marker; CTRL (-) – Negative Control; AW1, AW2, GH, GP, Fer, LO1, LO2 and 1-10 – Amoebas isolated from the respective samples.

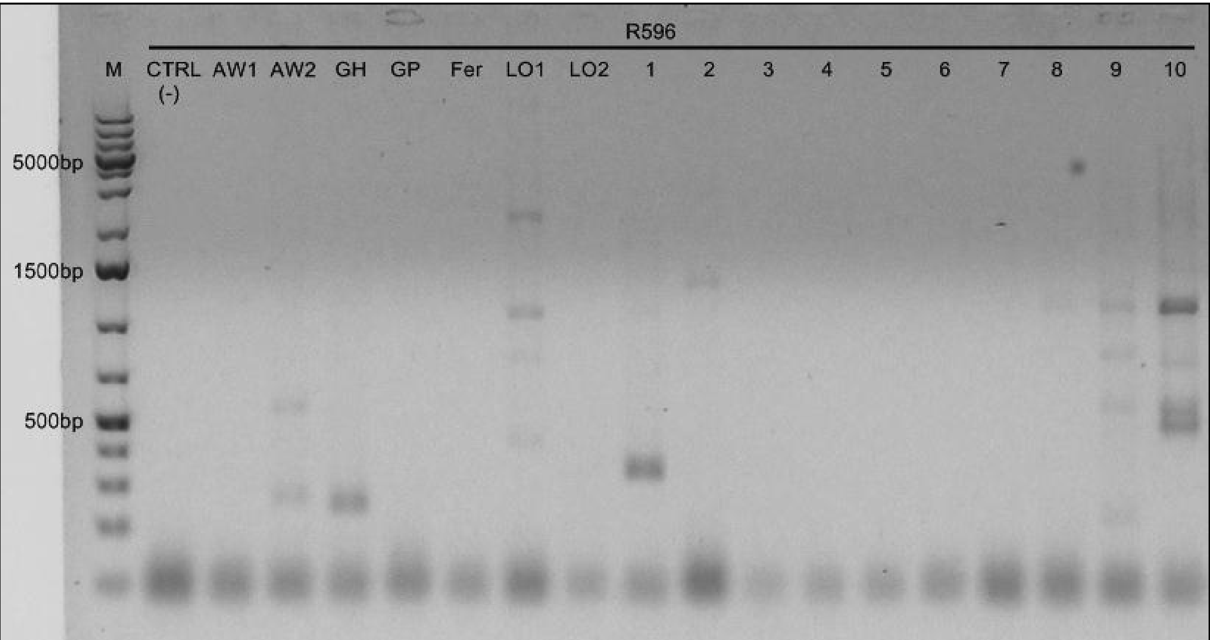

Figure 9 – Photograph of a 1% agarose gel with the PCR products obtained when using the R596 primers for Mimivirus. M – Marker; CTRL (-) – Negative Control; AW1, AW2, GH, GP, Fer, LO1, LO2 and 1-10 – Amoebas isolated from the respective samples.

No virus-like particles (VLPs) were detected by TEM in either of the isolated amoebas possibly because the TEM samples were also not as fresh as required. The amoebas should

have been processed as soon as possible for the TEM procedures as well and due to its characteristics, a method to rapidly increase the number of amoebas should have been applied in order to increase the probability of finding ARMs. These amoeba enrichment methods were indeed applied as with each cut from one Petri dish to another, the amoebas multiplied and adapted to the laboratory conditions, but unfortunately this was a time consuming method that decreased the possibility of finding ARMs in the isolated amoebas.

4.3 Amoeba Infection Assay

First and foremost, it is important to mention that the viruses that were inoculated in this assay were not the same as the ones that were produced due to the fact that the viral titration indicated a very low number of pfu/mL which aggravated the probabilities of any amoebal infection. As so, the amoebas were inoculated with other three batches of viruses previously produced and titrated, namely, the FV3, BoA[23] and Ma3B[23] viruses.

The infected amoebas were observed weekly and after 4 weeks it was possible to notice some variations in the cultures as all the *A. castellanni* cells presented a cystic form except the FV3 infected adherent amoebas. This culture displayed a great number of trophozoites and therefore it was thought that the FV3 virus had an infectious effect over this cell line that inhibited the formation of cysts which is a characteristic of these amoebas after so long in culture, at room temperature. The other adherent cells, however, had a fairly normal evolution in culture and although the virus did not seem to have replicated, it could have still been internalized before the cystic formation. As such, all amoebas were sampled to be tested by PCR except the ones that had been infected in suspension which were unable to be observed as there were too many amoebas in the culture flask.

The Amoeba Infection Assay samples were tested in two separate PCR protocols, one using the EUK primers and the other using the MCP primers. The use of the first primers tested the presence of DNA in the Direct PCR samples, proving that the template was available for the PCR reactions and the second pair tested the presence of the inoculated virus.

As it can be seen in Figure 10, when applying the EUK primers, all samples had positive results except the B sample which corresponds to the Extracellular Virus sample of *A. castellanni* infected with FV3. This may have happen because it is the same sample that after 4 weeks still had present numerous trophozoites which over time could have been compromised and their DNA exposed and degraded, as suspected by numerous debris in the culture medium. The cystic form is much more robust and increases the protection of the genomic DNA, which allowed the amoeba DNA amplification of the other samples. In the sample of *A. castellanni* infected with FV3, after the centrifugation step, the number of intact

trophozoites in the supernatant was diminished and so the DNA concentration was insufficient, impairing the PCR detection of amoebal DNA. None the less, these results still indicated that the Direct PCR method was successful and so there was DNA present in the samples that tested positive when applying the EUK primers. (The positive control for this reaction referred to a sample of *A. castellanii* cells ressuspended in Dilution Buffer which was used to test the viability of the PCR reaction.)

The results for the PCR reactions using the MCP primers can also be seen in Figure 10

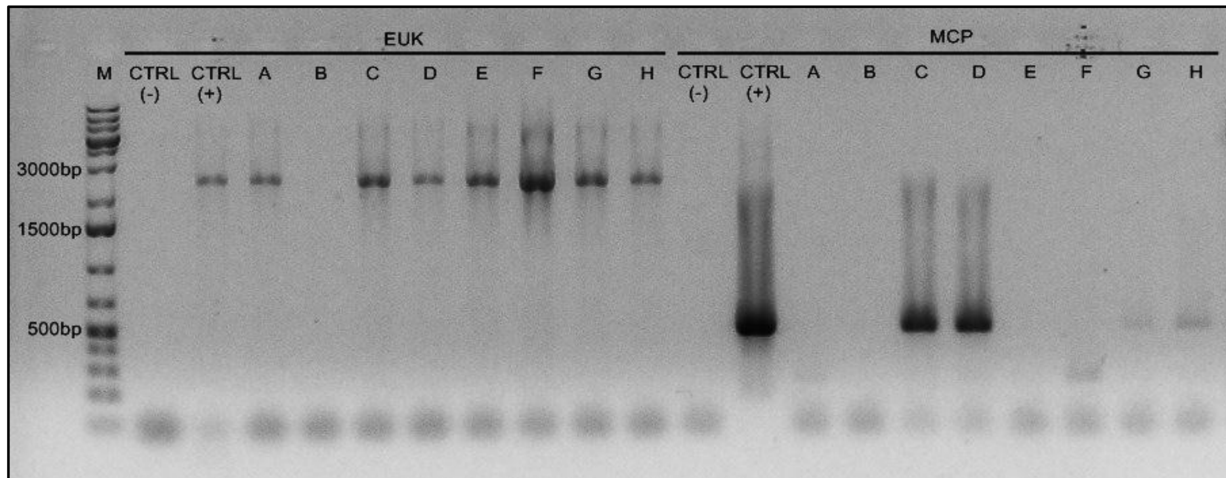

Figure 10 – Photograph of a 1% agarose gel with the PCR products obtained for the Amoeba Infection Assay samples. The results of the EUK primers PCRs can be seen at the left of the image and the ones for the MCP primers PCRs can be seen at the right. M – Marker; CTRL (-) – Negative Control; CTRL (+) – Positive Control; A – D (Extracellular virus samples): A – Test Control, B – *A. castellanii cells* + FV3 virus, C – *A. castellanii* cells + BoA virus, D – *A. castellanii* cells + Ma3B virus; E – H (Intracellular virus samples): E – Test Control, F – *A. castellanii cells* + FV3 virus, G – *A. castellanii* cells + BoA virus, H – *A. castellanii* cells + Ma3B virus.

and as expected, sample B did not have any amplification possibly for the reasons described above but neither did samples A and E. The result of the latter is consistent with the fact that samples A and E are the assay's test control (*A. castellanii* cells) and since no virus was inoculated, no PCR amplification was to occur.

Samples C and D reveal a PCR product quite concentrated which is indicative that intact viral DNA was collected from the supernatants (Extracellular Virus samples) of both BoA and MA3B inoculated cultures. This could have occurred because of one of two reasons: first, if the virus did replicate in amoebal cells, the number of virus particles increased in the culture medium or the inoculated virus could have remained intact in the exterior of these cells and the positive result is derived from the original viral particles.

Sample F did not have a positive result which could be explained by the fact that the trophozoites could have not allowed the entry of FV3 but were still inhibited to form cysts and hence no intracellular viral DNA could be detected, although there were positive results with

the EUK primers for this sample. (The template of the positive control for this PCR reaction was DNA extracted from FV3).

The most striking result is, however, the fact that the Intracellular samples G and H also had a slight amplification. This could indicate that although the amoebas were washed twice before the ressuspension in dilution buffer, viral DNA was still associated with the remaining pellet. The remaining question was whether the viral DNA came from viruses that were somehow still present on the surface of said cells, or was it actually recovered from viral particles that had entered the amoebal cells before they formed cysts?

To answer the burning question, the cultures were sampled and processed for TEM but unfortunately results could not be obtained for all inoculated cultures. As seen in Figure 11, there are VLPs present in an *A. castellanii* cyst inoculated with the Ma3B virus. These particles have an icosahedral form, much like the iridovirus capsids, and a size of about 300nm which is consistent with the size of the inoculated virus. These VLPs appear to be devoid of DNA as the interior is characterized by a white colour but this image suggests that the virus was indeed internalized. Whether the virus lost its DNA while on the interior of the amoebal cell and did not replicate or these VLPs are the result of an abnormal viral replication, remains to be enlightened.

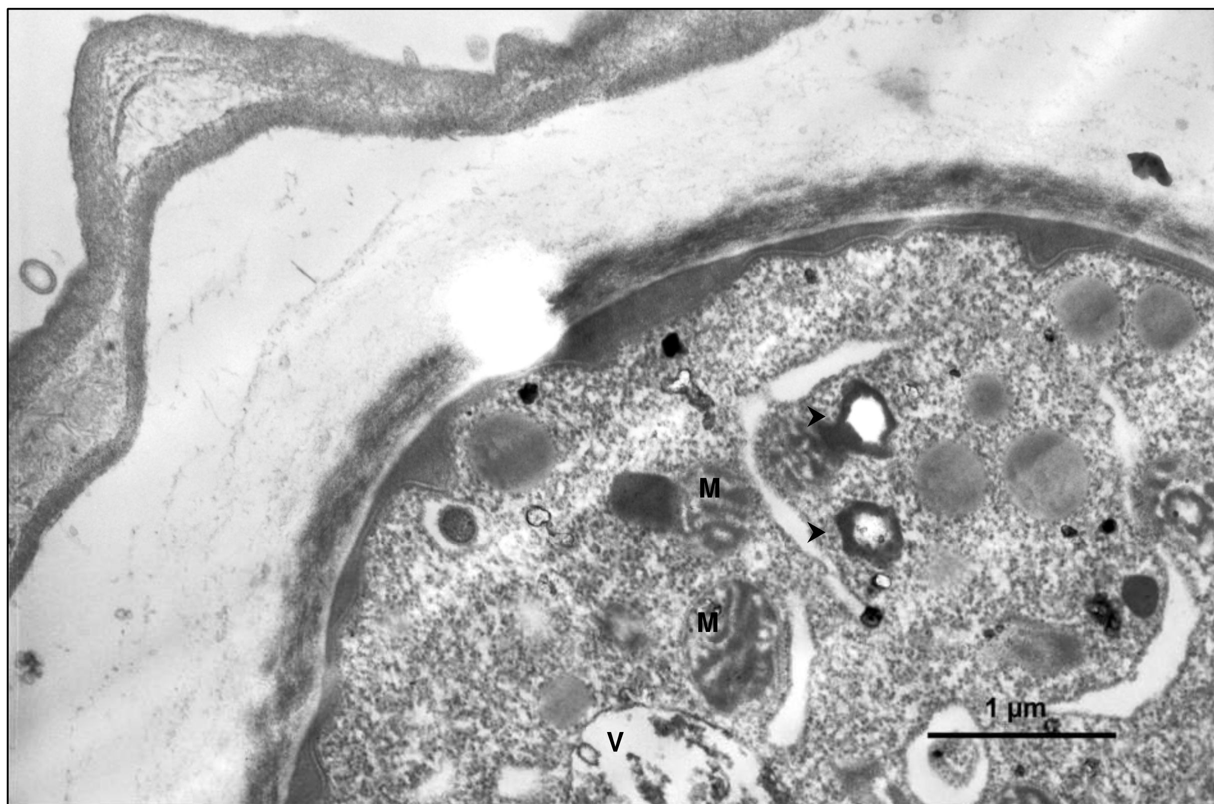

Figure 11 – Micrograph of an *A. castellanii* cyst infected with the Ma3B virus. The arrows (➤) point to possible VLPs. V – Vacuole; M – Mitochondria.

4.4 Flap endonuclease

As seen in Figure 12, the Flap primers successfully amplified the endonuclease gene in all six virus tested, which is revealed by the strong expected band size of about 570bp. The primers were designed with base wobbles to allow the amplification of this less conserved gene in several related iridoviruses but it appears that this also allows the occurrence of some non-specific DNA amplification. This can probably be avoided if the extracted DNA is obtained from concentrated extracellular virus samples, without any contamination of cellular DNA.

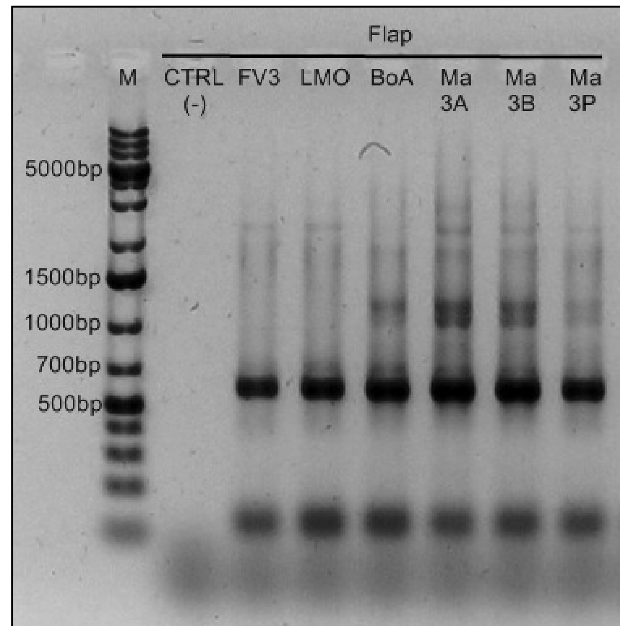

Figure 12 – Photograph of a 1% agarose gel with the PCR products obtained for the *Flap endonuclease* gene. M – Marker; CTRL (-) – Negative Control; FV3 – Frog Virus 3; LMO – *Lacerta monticula* iridovirus; BoA – *Triturus boscai* iridovirus; Ma3A, Ma3B and Ma3P – *Triturus* m*armoratus* isolates.

The PCR products were sent for sequencing in both directions (forward and reverse) and the resulting DNA sequences were aligned to give the consensus sequences reunited in Table 7 in Annex 6. The only exception was the BoA virus because the forward sequence had poor quality and did not allow a good alignment with the reverse sequence and as so, there is no consensus sequence for this virus.

To obtain a good result that allowed the correct analysis of this gene sequence, the BoA sequence was discarded and the resulting alignment with the remaining five consensus sequences can be seen in Figure 13. According with previous studies[23,24], FV3 and LMO viruses are identical because the nucleotide alignment seen in Figure 13 match in both viruses. As such, it is plausible to say that in respect to the *MCP*, *DNA polymerase* and *Flap endonuclease* partial sequences, these viruses are genetically identical. Regarding Ma3A, Ma3B and Ma3P viruses, the alignment indicates that they have an equal Flap gene partial sequence but it differs from the FV3 virus, which is the type species of this genus. This result indicates that the *Triturus marmoratus* isolates are in fact one and the same, but can be classified as a separate species from FV3 and LMO viruses.

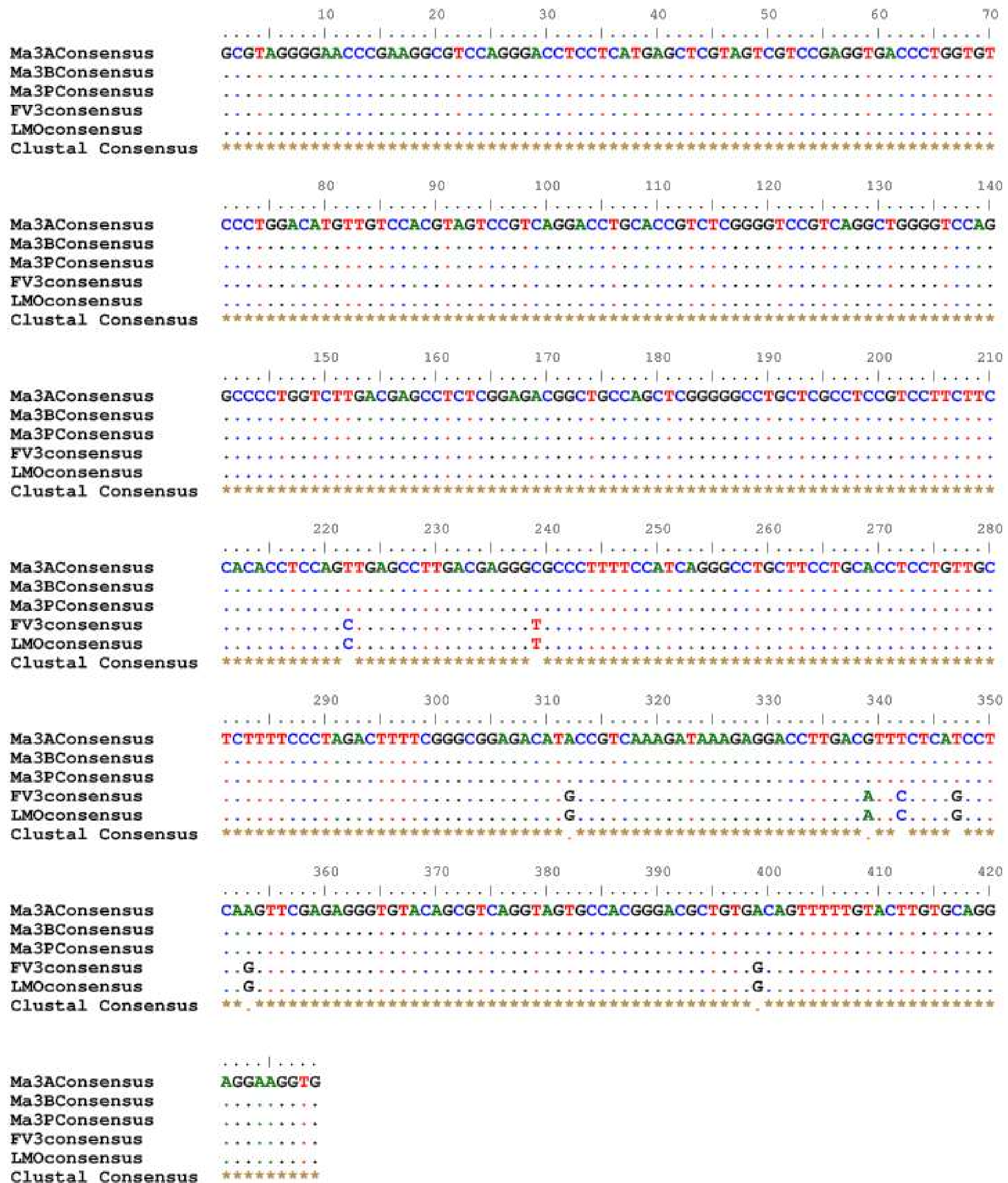

Figure 13 – Graphic image of the alignment considering the consensus sequences of a partial region of the *Flap endonuclease* gene of FV3, LMO[25], Ma3A[24], Ma3B[24], Ma3P[24] viruses. The alignment was cropped to the most conserved region between the considered sequences. The dots indicate the same nucleotide for all DNA sequences and the bold letters indicate the ones that differ between them.

5. Conclusions

A Direct PCR protocol was applied that allowed the success of the identification of the isolated amoebas as well as the virus screening. It is a highly recommended method if the work requires a simpler and faster way of detecting a specific organism which was the case for the isolated amoebas in the present study. The Direct PCR method does present some advantages especially because it comprises faster, simpler and more practical procedures. It was not, however, fully developed and improvements in the collection and processing of the samples are in order. To resolve these issues, a new way of recovering the desired amoebas from the agar must be found as well as a better and faster way of adapting such amoebas to an axenic culture medium as the PYG complete medium.

To successfully accomplish the detection of amoebal viruses, the samples must be processed as soon as they are collected so that no ARMs are lost. A filtration procedure could also be applied, especially to separate the amoebas from the other microorganisms present in environmental samples which would lead to a better observation of said amoebas. Alongside with these, the remaining of the samples could also be inoculated in the *A. castellanii* cell line similarly to what was described by La Scola and his team for the isolation of the Mamavirus[13]. This could allow the detection of possible viruses without an accurate detection of their natural host but if both procedures are concluded in parallel, perhaps there would be an increase of the probabilities of detecting a virus in amoebas and the production and characterization of said virus.

Overall, the virus screening PCRs fulfilled their role as the main procedure to detect possible NCLDVs, because it is a wide range technique, requires simple PCR procedures and can be modulated to allow the detection of various virus families by simply applying the respective primers. The application of degenerate primers appears to increase the number of ARMs that can be detected, especially when the annealing temperatures are fairly low. This could be an advantage when the search for ARMs is not limited to a specific virus family or even to a specific microorganism. Perhaps in the future, the search for viruses in amoebas can be widen to include other viruses than those that have already been associated with them (like the mimivirus) or that are related to the already found amoeba associated viruses (like the NCLDV based families).

The Amoeba Infection Assay PCR results have indicated an association with the inoculated viruses Ma3B and BoA. The TEM results have highlighted the presence of VLPs in the Ma3B inoculated amoebas but posed more questions regarding the nature of such VLPs. Further

studies are required to assess if these viruses really enter the *A. castellanii* cells and whether they can replicate in these amoebal cells. To address these questions the assay should be reformulated specially by increasing the number of viral particles inoculated in order to increase the probability of infection. Also, the amoebas should be monitored and sampled daily and observed by TEM microscopy preferably before they turn into cysts, which due to their nature, prejudices the TEM procedures. The answer to these questions is of special interest if one considers the fact that although it is known that the iridoviruses are present in several environments, their natural reservoir is still unidentified.

With the use of the Flap endonuclease primers it was possible to distinguish the tested iridoviruses. The LMO virus was grouped with the type species of the *Ranavirus* genus, the FV3 virus, concluding that they have a genetically identical Flap gene. The *T. marmoratus* iridoviruses could also be grouped with a genetically identical Flap gene but a difference of eight nucleotides separates them from the FV3 group. The BoA iridovirus remains to be characterized in this gene. A new DNA extraction from this virus could improve the PCR amplification and hence the DNA sequencing of this endonuclease gene and help to sort it into one of these groups or to create a new group with a different Flap gene. In conclusion, this gene has proved to be a good genetic target allowing the distinction between closely related iridoviruses and the Flap primers could be considered as a routinely useful tool.

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7. Annex 1

PYG complete medium[26]

100 g proteose peptone (Difco®)

10 g yeast-extract (HIMEDIA®)

4.9 g $MgSO_4 \cdot 7H_2O$ (Fluka®)

5 g sodium citrate • $2H_2O$ (Panreac®)

0.1 g $Fe(NH4)_2(SO4)_2 \cdot 6H_2O$ (Merck®)

1.7 g KH_2PO_4 (Fluka®)

1.97 g $Na_2HPO_4 \cdot 7H_2O$ (Merck ®)

45 g glucose (Difco®)

0.295 g $CaCl_2$ (Merck®)

in 5 L of deionized water

Freezing medium

20 % Fetal Bovine Serum (Gibco®)

10 % Dimethyl sulfoxide (DMSO)

70 % PYG complete medium

PAS (Page amoebal saline)[27]

120 mg NaCl (AppliChem®)

4 mg $MgSO_4 \cdot 7H_2O$ (Fluka®)

4 mg $CaCl_2 \cdot 2H_2O$ (Merck®)

142 mg Na_2HPO_4 (Merck®)

136 mg KH_2PO_4 (May and Backer Ltd®)

in 1 L of deionized water

Artificial Sea Water (ASW) (kindly provided by the Oceanography Institute (IO), Lisboa, Portugal)

24.540 g NaCl

4.090 g Na_2SO_4

0.7 g KCl

0.2 g $NaHCO_3$

0.1 g KBr

0.003 g H_3BO_3

0.003 g NaF

11.1 g $MgCl_2 \cdot 6H_2O$

1.540 g $CaCl_2 \cdot 2H_2O$

0.017 g $SrCl_2 \cdot 6H_2O$

in 1 L of deionized water

8. Annex 2

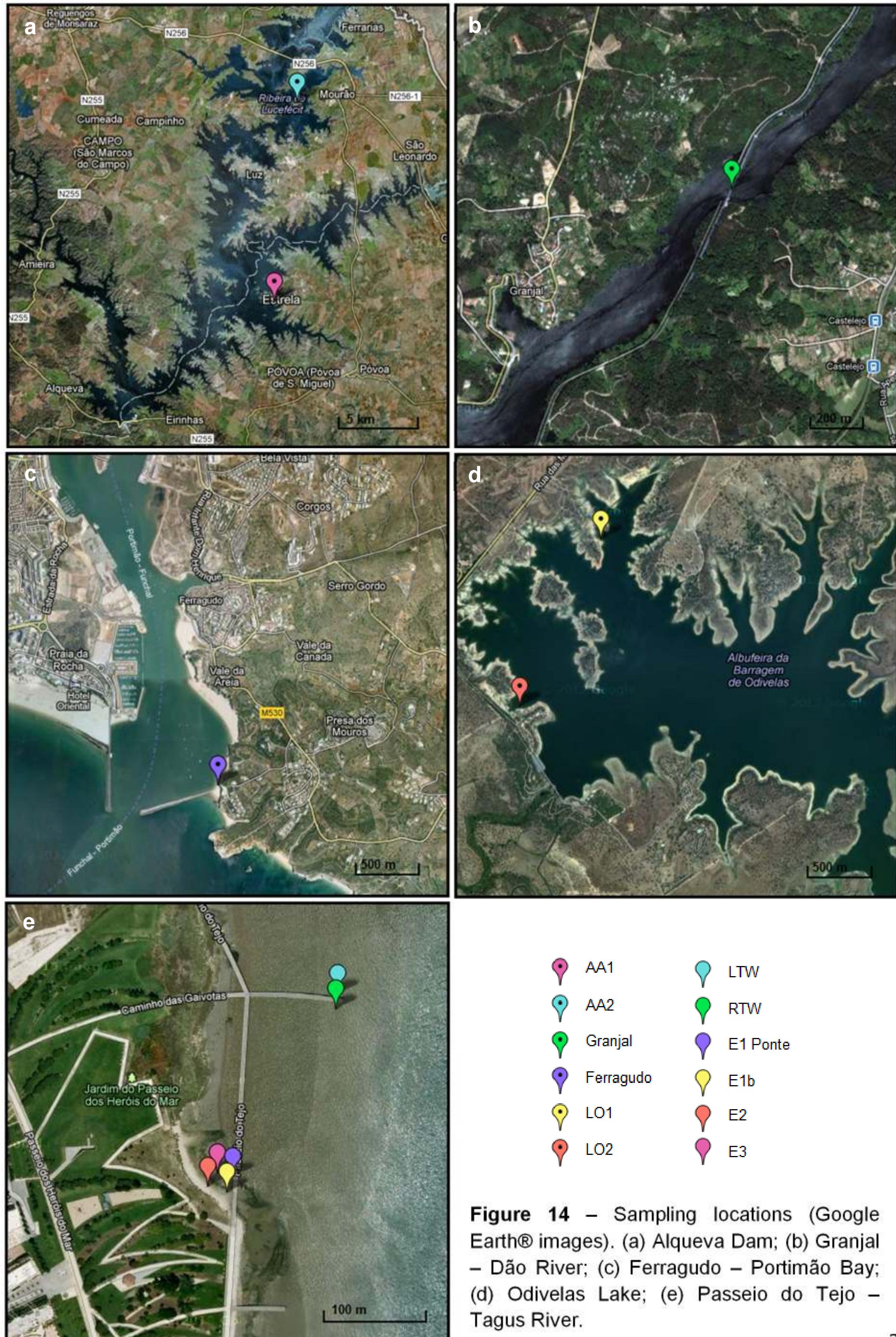

Figure 14 – Sampling locations (Google Earth® images). (a) Alqueva Dam; (b) Granjal – Dão River; (c) Ferragudo – Portimão Bay; (d) Odivelas Lake; (e) Passeio do Tejo – Tagus River.

9. Annex 3

Table 4 – Primers used in the PCR reactions.

Target	Genomic region	Primer name	Primer sequence	Primer size	Tm	Tanneal	Expected product size
Mimivirus	*L396*	F396	TTA ATC ATC TTC CAA AAA ATT TAA TTC	27bp	47.9ºC	45ºC	1560bp
		R396	ATG GCG AAC AAT ATT AAA ACT AAA A	25bp	49.9ºC		
	R596	F596	ATG TCG TTA TCA AAA CAA GTA GTT CC	26bp	53.6ºC	49ºC	879bp
		R596	CTA ATT TTC AAT ATA GTG CGT AGA TTC TA	29bp	51.5ºC		
Large DNA viruses (LDVs)	*DNA polymerase*	HV	cggaattctaGAYTTYGCNWSNYTNTAYCC3	30bp	60.94ºC	58ºC	400-700bp
		Cons	cccgaattcagatcTCNGTRTCNCCRTA	28bp	61.39ºC		
Adenovirus	*DNA polymerase*	Adeno	gggaattctaGAYATHTGYGGNATGTAYGC	30bp	60.71ºC	58ºC	1200bp
		Cons	cccgaattcagatcTCNGTRTCNCCRTA	28bp	61.39ºC		
Ranavirus	*MCP*	OLT1	GAC TTG GCC ACT TAT GAC	18bp	42ºC	36ºC	500bp
		OLT2R	GTC TCT GGA GAA GAA GAA T	19bp	39ºC		
rRNA genomic region	*ITS*	III	CAC ACC GCC CGT CGC TMC KAC CGA TTG	27bp	67.9ºC	58ºC	1500bp-2500bp
		IX	WGA CTC CTT GGT CCG TGT TTC AAG AC	26bp	60.5ºC		
rRNA genomic region	*rRNA 18S*	EukA	AACCTG GTT GAT CCT GCC AGT	21bp	64ºC	61ºC	1600 - 2000bp
		EukB	TGATCC TTC TGC AGG TTC ACC TAC	24bp	72ºC		
Flap endonuclease gene	*Flap*	F-Flap	KYC MTG GRS GCS TYC GSK WT	20bp	59.82ºC	57ºC	567bp
		R-Flap	SKY CTY RGG AGC YAY SSW ST	20bp	59.75ºC		

bp - base pair | Tm – Melting temperature | Tanneal – Annealing temperature

10. Annex 4

Table 5 – DNA sequences obtained for the identification of the isolated amoebas. All sequences were acquired with the forward primer.

Amoeba	Primer	DNA sequence	Sequence size (bp)	PCR product size (bp)
AW1	III	NNNNTATGTGATGGGAGGACTGGCCCTCTTGTTTTTCAGGAGAGGAAAGGAAACTATTGAATGTTTATATTTAAAGGAAGCAAANTTCGT AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCTAAATAAACGATCTTTTCCCTTCANTTCCCCCCTTCATTCNCCTAATCCAAAATT CCNCGAAATGGNCAATCCTNTTTAT	201	2300
AW2	III	NNNNNNNNNNNNCAANNNCATCCTCGGACTGAAGTAGTTATCTAGCAGCAATGTTAGATGCTATTTTGAGAAGTTGATTAAACCTTATCA TCTAGAGGAAG	89	1500
GH	EUKA	NNNNNNNNNANNCCTGCAAGTGCGAGTGATTCGGTGCATCCATCAGTAAAATGAGGGAAGGCGCTCAGGATGACTGCAAAAGGCTCAT TAAAACAGTTATTGGAGCGGGATGGTCAGAATACGACAACAAAGGTAAGTNTTCNCCACCT	140	1700
GP	EUKA	NNNNNNNNNNNNNNNNGCANNTGCGAGTGATTCGGTGCATCCATCAGTAAAATGAGGGAAGGCGCTCANGATGACTGNN	63	1700
1	EUKA	NNNNNNNNNNTANATNNNCCNTGCACGTCTAAGGATAAATATTTTTTAACCGTAAACCTGCGGATGGCTCATTAAATCAGTTACAATCTA CTCTATTGTATAAATTACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCAAAAAATTTGAACTCGCAAGGGAACAAATGTATTTATT AAGGAGCAAAATCAATACAGACCATTCCAAACTCTAGATTATACTTTAACCGGTATTTTTTAGGGGGTTN	242	1900
2	EUKA	NNNNNTGTCTCAGATTAGCCATGCATGTCTAAGTATAAGCTTGTTTTATACGGCGAGACTGCGGATGGCTCATTAAATCAGTTATAGTTT ATTTGATGGTCTCTTGACACGGCGCAAGCCGTATCATTTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCGTAAAATCTCGAG CGCGGGGGGTGGTCTTGGGGTG	197	2500
5	EUKA	NNNNNTCNNCGTNNNNNCATTNNGCNNTAGCATGTCTAAGTATAAGCTTGTTTATACGGCGAGACTGCGGATGGCTCATTAAATCAATT ATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTACCTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCG CGGGGGGCGGGGCTTCACGGCTCTGTTCTCGCATGCGCAGAGGGATGTATTTCTTAGGTTAAAAACCTGCGTAGAAAGAAATGGCTTT TTACTCGTGGTAACTCGACTTCTCCCGTTCAGACCGCACTACCACCCGTAATTAAGGGAAAGCCTCGAACCGCCATTTGAATGTTTTTTC TGAAT	358	2500
6	EUKA	GNGNNGNACGNGNNTANCANTACTCCATGCATGTCTAAGTATAAGCTTGTTTATACGGCGAGACTGCGGATGGCTCATTAAATCAGTTA TAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTACCTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGC GGGGGATGGGGCTTCACGGCTCTGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACCAGCGTAACCAGCAATGGCTACA ATCTCCTGGTGATTCATAGTAACTCTTTCGGATCGCATTCATGTCCTCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCCTATT ACTTTCGATGGTAGGATAGAGGCCTACATTTACCGTAACTGGCAATTAATCATTAAGTTTCAACTCCCGATATGTATCCAC	439	2500
8	EUKA	NNNNNNTATTTAGACTAGCCATGCAATGAAGTATAAATCGCTTAATACTGATGAAACTGTGAACGGCTCATTAAATCAGTTATAGTTTATT TGATGGTATTGGAATTTATTCCAACTACATGGATAACTGTAGTAACCTAGAGCTAATACATGCGTAACAATTCAACTTTTATGGAAGAATT GGCACTTATTAGATATCAAACCAATATATCTTCGGATATTTACTGGTGAATCATAATAACTGAACAGACCGAATTGCCTTTGTGCACGCGA GGTTTCATTCGAATTTCGGCGCGCTCATCTACACGTTAATATN	310	2000
9	EUKA	NNNNNACTATTTAGACTAGCCATGCAATGAAGTATAAATCGCTTAATACTGATGAAACTGTGAACGGCTCATTAAATCAGTTATAGTTTAT TTGATGGTATTGGAATTTATTCCAACTACATGGATAACTGTAGTAACCTAGAGCTAATACATGCGTAACAATTCAACTTTTATGGAAGAAT TGGCACTTATTAAATATCAAACCAATATATCTTCGGATATTTACTGGTGAATCATAATAACTGAACACATCCCATTGCCTTTGTGCAGGCG ACGTTTCATTCAAATTTCCGCCCTATCNACAANATGTCACTAAAAAN	315	2000
10	EUKA	NNNNNNANNAAANTNNTTNANACTAAGCCATGCAATGAAGTATAAATCGCTTACTACTGATGAAACTGTAAACGGCGCATTAATCATAGT AANGGTGTTATTAGATGG	102	2000

11. Annex 5

Table 6 – DNA sequences of the PCR products obtained with the Adenovirus primers. All sequences were acquired with the forward primer.

Amoeba	Primer	DNA sequence	Sequence size (bp)	PCR product size (bp)
9	Adeno	NNNNNNNNNNNNNCGAGANTNTATCGCTCTTACAATGGCGGCACGACTATGGAAATTGTACCCAACCTGCCCATTTCTCAGTTCTA CCATATCAGCATTGATGATAACGAACCTTACAATATTTATGGAGGCCTTCAGGACAACGGCTCATGGTACGGCCCCTCCTCCTCCCC AGGTGGAGTTGAAGCCAGAGATTGGAACTCTATAGGTTACGGAGATGGTTTCAGGGTACTTAAACATCCGACAAAAAACATCATTTA CTCTGATATGCAAGGTGCTCAGAATGTTGGGAGACACCCAGCCCTNAACNCNCGGGGA	305	350
10	Adeno	NNNNNCNNNNNNNNNNGAGANTNTATCGCTCTTACATGGCGGCACGACTATGGAAATTGTACCCAACCTGCCCATTTCTCAGTTCTA CCATATCAGCATTGATGATAACGAACCTTACAATATTTATGGAGGCCTTCAGGACAACGGCTCATGGTACGGCCCCTCCTCCTCCCC AGGTGGAGTTGAAGCCAGAGATTGGAACTCTATAGGTTACGGAGATGGTTTCAGGGTACTTAAACATCCGACAAAAAAGATCGTTTC CTCTGAAATGCAAGGGGCTCATAATGTGGGGANCTCACGGGACGACNCCCTTCGN	300	350

12. Annex 6

Table 7 – Consensus DNA sequences obtained for the *Flap endonuclease* gene of the tested iridoviruses. All sequences refer to consensus sequences except the BoA DNA sequence.

Virus	*Flap endonuclease* gene DNA sequence	Sequence size (bp)
FV3	GGGGCGTCTGCGTAGGGGAACCCGAAGGCGTCCAGGGACCTCCTCATGAGCTCGTAGTCGTCCGAGGTGACCCTGGTGTCCCTGGACATGTTGTCCACGTAGTCCG TCAGGACCTGCACCGTCTCGGGGTCCGTCAGGCTGGGGTCCAGGCCCCTGGTCTTGACGAGCCTCTCGGAGACGGCTGCCAGCTCGGGGGCCTGCTCGCCTCCGT CCTTCTTCCACACCTCCAGCTGAGCCTTGACGAGGGTGCCCTTTTCCATCAGGGCCTGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGGCGGAGACA TGCCGTCAAAGATAAAGAGGACCTTGACATTCCTCAGCCTCAGGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGCTGTGGCAGTTTTTGTACTTGTGC AGGAGGAAGGTG	438
LMO	GCGTAGGGGAACCCGAAGGCGTCCAGGGACCTCCTCATGAGCTCGTAGTCGTCCGAGGTGACCCTGGTGTCCCTGGACATGTTGTCCACGTAGTCCGTCAGGACCT GCACCGTCTCGGGGTCCGTCAGGCTGGGGTCCAGGCCCCTGGTCTTGACGAGCCTCTCGGAGACGGCTGCCAGCTCGGGGGCCTGCTCGCCTCCGTCCTTCTTCC ACACCTCCAGCTGAGCCTTGACGAGGGTGCCCTTTTCCATCAGGGCCTGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGGCGGAGACATGCCGTCAA AGATAAAGAGGACCTTGACATTCCTCAGCCTCAGGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGCTGTGGCAGTTTTTGTACTTGTGCAGGAGGAAG GTGCCGTCCACGGCGAT	443
Ma3A	TGTTGGGGGGGTTTGCGTAGGGGAACCCGAAGGCGTCCAGGGACCTCCTCATGAGCTCGTAGTCGTCCGAGGTGACCCTGGTGTCCCTGGACATGTTGTCCACGTA GTCCGTCAGGACCTGCACCGTCTCGGGGTCCGTCAGGCTGGGGTCCAGGCCCCTGGTCTTGACGAGCCTCTCGGAGACGGCTGCCAGCTCGGGGGCCTGCTCGCC TCCGTCCTTCTTCCACACCTCCAGTTGAGCCTTGACGAGGGCGCCCTTTTCCATCAGGGCCTGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGGCGG AGACATACCGTCAAAGATAAAGAGGACCTTGACGTTTCTCATCCTCAAGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGCTGTGACAGTTTTTGTACTT GTGCAGGAGGAAGGTG	443
Ma3B	CGTGTGGGGGGGGTATGCGTAGGGGAACCCGAAGGCGTCCAGGGACCTCCTCATGAGCTCGTAGTCGTCCGAGGTGACCCTGGTGTCCCTGGACATGTTGTCCAC GTAGTCCGTCAGGACCTGCACCGTCTCGGGGTCCGTCAGGCTGGGGTCCAGGCCCCTGGTCTTGACGAGCCTCTCGGAGACGGCTGCCAGCTCGGGGGCCTGCTC GCCTCCGTCCTTCTTCCACACCTCCAGTTGAGCCTTGACGAGGGCGCCCTTTTCCATCAGGGCCTGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGG CGGAGACATACCGTCAAAGATAAAGAGGACCTTGACGTTTCTCATCCTCAAGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGCTGTGACAGTTTTTGTA CTTGTGCAGGAGGAAGGTGCCGTCCACGGC	456
Ma3P	GCGTAGGGGAACCCGAAGGCGTCCAGGGACCTCCTCATGAGCTCGTAGTCGTCCGAGGTGACCCTGGTGTCCCTGGACATGTTGTCCACGTAGTCCGTCAGGACCT GCACCGTCTCGGGGTCCGTCAGGCTGGGGTCCAGGCCCCTGGTCTTGACGAGCCTCTCGGAGACGGCTGCCAGCTCGGGGGCCTGCTCGCCTCCGTCCTTCTTCC ACACCTCCAGTTGAGCCTTGACGAGGGCGCCCTTTTCCATCAGGGCCTGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGGCGGAGACATACCGTCAA AGATAAAGAGGACCTTGACGTTTCTCATCCTCAAGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGCTGTGACAGTTTTTGTACTTGTGCAGGAGGAAG GTG	429
BoA*	TGTTGTCATGGGGGCGTTCGGGATGCCCATGTGAACCAGCCTCACACAGCACAGCTAGGCCTAGTTGGGGGCGTCTGGGTAGGGGAACCCGAAGGCGTCCAGGGA CCTCCTCATGAGCTAGTAGTAGTCCGAGGTGACCGTGGTGTCCCTGGACATGTTGTCCATGTAGTCCGTCAGGACCTGCACCGTCTTGGGGTCCGTCAGGCTGGGGT CCAGGCCCCTGGTCTTGACGAGCCTATGGGAGACGGCTGCCAGATCGGGGGCTTGCTTGCCTCAGTCATTTTTCCACACCTCCAGTTGAGCCTTGACGAGGGCGCC CTTTTCCATCAGGGCATGCTTCCTGCACCTCCTGTTGCTCTTTTCCCTAGACTTTTCGGGAGGAGACATACCGTCAAAGATAAAGAGGACCTTGACGTTTCTCATCCTC AAGTTCGAGAGGGTGTACAGCGTCAGGTAGTGCCACGGGACGGTGTGACAGTTTTTGTACTTGTGCAGGAGGAAGGTGCGTCCACGGCGATGTCATGCCGCCATCT GACNNNNANNNNNNNN	536

* This sequence refers to the reverse complement sequence obtained with the reverse primer R-Flap.