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To cite this article: Shekinah Shamini Victor and Khomaizon A.K Pahirulzaman 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **596** 012092

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240th ECS Meeting ORLANDO, FL

Orange County Convention Center **Oct 10-14, 2021**



Abstract submission due: April 9

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Molecular Identification of Fungi Isolated from Infected Redclaw Crayfish, *Cherax quadricarinatus*

Shekinah Shamini Victor & Khomaizon A.K Pahirulzaman*

Faculty of AgroBased Industry, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan

E-mail: khomaizon@umk.edu.my

Abstract. In Malaysia, the redclaw crayfish, *Cherax quadricarinatus* is widely known as freshwater lobster due to its lobster-like appearance and habitat. Even though the exact year in which the redclaw species was introduced into the country is unknown, commercial scale culturing activity of this species has been in record since 2003 in the southern part of Peninsular Malaysia. Crayfish plague is a water mould caused by an oomycete *Aphanomyces astaci* which contributes for high mortality rate in indigenous crayfish species throughout Europe. However, the effects of the plague can vary among species, regions and local populations. Traditionally, the crayfish plague agent was seen to have only the freshwater crayfish as hosts. The identity of the pathogen is only assumption and not confirmed by research because of difficulties in cultivation and ambiguous morphological characteristics. In this study, we demonstrate the use of ITS86F and ITS4 primer pair to amplify the ITS2 region in the isolated fungi from infected *C. quadricarinatus*. The primer aligned with sequences from a range of fungal species within the Ascomycota and Basidiomycota clades. A total of 14 fungi were isolated from the infected *C. quadricarinatus*. With the constructed neighbour-joining phylogenetic tree, 7 of the ITS2 sequences were identified to most closely related to 9 fungal species within the Ascomycota clade, 5 sequences in Basidiomycota clade and 2 sequences were closely related to endophyte culture collection. Results show fungi other than *A. astaci*, could cause crayfish plague. These isolated fungi might be linked to the *A. astaci*. Genetic analysis provides easy identification of the large majority of fungi at the species level, regardless when little DNA is available. Moreover, there are very low quantities of the pathogen DNA in the sample that are detectable by some of the molecular methods.

1. Introduction

The redclaw crayfish, *Cherax quadricarinatus* is a tropical freshwater crayfish from the family *Parastacidae* [1]. The native habitat of the *C. quadricarinatus* is the river catchments in northern Australia and south eastern Papua New Guinea [2]. It was successfully farmed in Australia in 1985 [3]. The growth and development of *C. quadricarinatus* is direct, in which, it has no larval stage and grows fast. *C. quadricarinatus* able to withstand wide range of temperature, pH scale and dissolved oxygen concentrations [2]. It inhabits warm, hard and slightly alkaline waters from pH 7.0 to 8.5. It has high growth rate where the total body length can reach up to 250 mm and its wet weight can reach up to 600 g in 9 months. *C. quadricarinatus* exhibits a certain degree of phenotypic variability throughout its indigenous range and has the ability to survive and reproduce under diverse biotic and abiotic conditions from tropical to temperate zones.



Economically, *C. quadricarinatus* is exploited as a significant aquaculture and ornamental trade species [2]. In Malaysia, the estimated production from *C. quadricarinatus* farming activities in southern Peninsular Malaysia totals 12 tonnes in a year. Commercial culturing by private entrepreneurs has increased since 2009 to provide hotel and restaurant chains that has crayfish menu. The culture farm suppliers are from Australia and Indonesia. The *C. quadricarinatus* can easily adapt to the local environmental conditions as the conditions are favourable to the growth and survival of the species. There are three locations in Malaysia with wild populations of *C. quadricarinatus*, namely Parit Sulong in Johor, and Rajang Stream and Similajau River in Sarawak. Their population is highly influenced by human factors, intentionally and unintentionally [3].

The *C. quadricarinatus* aquaculture industry has a wide prospect due to its positive aquaculture attributes. Its hardy, gregarious, high growth rate and flexible diet trait increases its popularity in the aquaculture market. No larval stage in the development of *C. quadricarinatus*, thus it does not require expensive hatcheries for larval rearing and makes the production of juvenile stage easier [4]. These make *C. quadricarinatus* has high market value for both ornamental and aquaculture purposes in Malaysia. The oomycete *Aphanomyces astaci* causes crayfish plague and is responsible for high mortality rate in indigenous crayfish species throughout Europe. However, the effects of the plague can vary among species, regions and local populations [5]. Crayfish may host other filamentous organisms such as parasitic and saprobiotic oomycete and fungi. The variety of microbiota colonising healthy crayfish should be more intensively studied. Hence, this study aims to detect other fungi strains that cause crayfish plague-like infections in *C. quadricarinatus*. The findings of this study will provide information that can allow the designing and establishment of specific treatment and prevention of the outbreak and spread of various fungi and crayfish plague in general.

2. Material and Methodology

2.1. Breeding of Infected Redclaw Crayfish

This study was carried out in 2 batches. The crayfish tank was set up with three quarter de-chlorinated water equipped with suitable substrates such as PVC pipes and rocks. Thirty *C. quadricarinatus* were sampled from Chew Thean Yeang Aquatic and Pet shop and KW Aquatic Supplies in Penang, Malaysia and bred in 5 tanks (3 crayfish for each tank). The *C. quadricarinatus* were fed with commercial pellet and growth was observed until the crayfish showed signs of infection. The water in the tanks is changed once a week.

2.2. Fungal Isolation

Fungal isolation was carried out once the *C. quadricarinatus* showed signs of infection such as brownish-red melanisation on abdomen and sluggish movement. Sterilised cotton buds were moistened with sterile distilled water and used to swab 4 different parts of the crayfish which are chelipeds, abdomen, head and body. The cotton swab were streaked on potato dextrose agar (PDA) media and incubated at 25 °C in the fungal growth incubator with daily observation. The tips of the growing hyphae were repeatedly transferred onto fresh PDA media to eliminate possible bacterial contamination and produce single-strain culture per media. The morphological characteristics of the isolated fungi were recorded based on the color of aerial mycelium, mycelia texture, topography, and the reverse color of the isolates, prior to DNA extraction.

2.3. DNA Extraction

DNA from the isolated fungi were extracted using cetyl trimethylammonium bromide (CTAB) method from Doyle & Doyle, 1990. The DNA pellet was dissolved in 30 µL of sterilised distilled water and kept at -20 °C for future use. The quantity and quality of the DNA was measured using DeNovix DS-11 spectrophotometer, USA. A 260/280 ratio of 1.8 is considered 'pure' DNA.

2.4. Polymerase Chain Reaction (PCR) amplification

PCR amplification on the extracted DNA was carried out using primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') which targeted the ITS2 region between the 5.8S and 28S rRNA. Primers were synthesized by MyTACG Bioscience Enterprise, Malaysia. PCR amplification mixtures contained 20 ng of fungi DNA template in a final volume of 25 μ L reaction, following the MyTaq™ Red DNA Polymerase PCR amplification kit protocol. PCR amplification was carried out with pre-denaturation at 95 °C for 2 minutes, followed by 45 cycles at 95 °C for 30 seconds, 51 °C for 30 seconds, 72 °C for 30 seconds, with a final extension process at 72 °C for 5 minutes. The PCR product was electrophoresed on 1% agarose gel in 1x TAE buffer for 20 minutes with 100V voltage, and then viewed using AlphaImager HP System (Santa Clara, USA).

2.5. DNA Sequencing Analysis

PCR products were purified using QIAquick PCR Purification Kit following the manufacturer's protocol. 10 μ L of purified DNA was sequenced by My TACG Bioscience Enterprise, Malaysia. The fungi were identified based on Basic Local Alignment Search Tool (BLAST) analyses to find the closely related taxa from the sequences available in GenBank NCBI. These sequences were further aligned with the reference sequences acquired from GenBank using ClustalW program. A neighbour-joining tree was constructed to portray the sequence similarity and phylogenetic relationship of the isolated fungi strains.

3. Results and Discussion

3.1. Fungal Isolated from Infected *C. quadricarinatus*

The fungal isolation was carried out once signs of infection were observed, such as brownish-red melanisation, whitening of abdomen (Figure 1) and sluggish movement. The non-specific melanisation is a result from the crayfish defence system against infection. Some other signs of infection include whitening of the musculature of the ventral abdomen. At terminal stages of infection, the crayfish may appear sluggish and lack of limbs [6]. Even though *C. quadricarinatus* is a species of easy husbandry, it is still susceptible to infectious diseases. Characteristically, crayfish in particular only possess mechanism of innate immunity, a non-specific system that has no immunological memory [7]. Hence, the immune response occur at the same extent each time the host encounter a foreign material. It used a short term defense mechanisms such as phagocytosis, melanisation or clotting mechanism to defend themselves from infection [6].

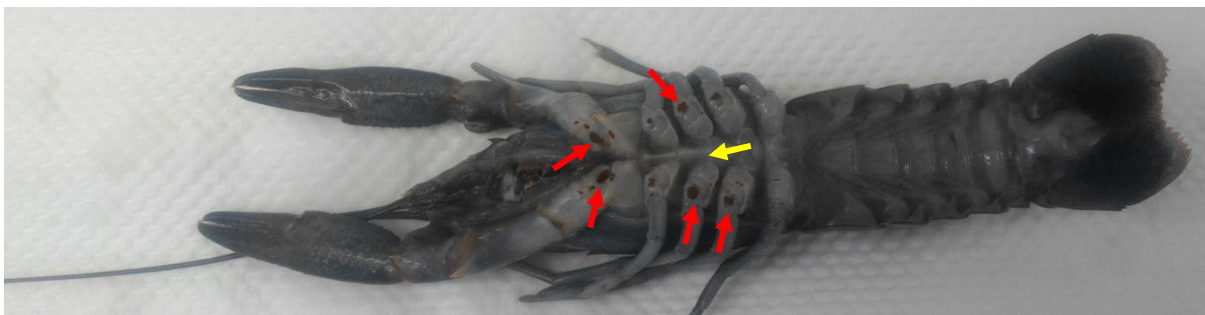


Figure 1. Infected *C. quadricarinatus* showed signs of brownish-red melanisation (red arrow) and whitening of abdomen (yellow arrow).

A total of 15 morphologically different fungi (Figure 2) were isolated from 5 parts of the infected *C. quadricarinatus*. The isolated fungi were mostly filamentous and whitish in colour with cottony texture (Table 1) and isolated from *C. quadricarinatus* cheliped. Only F13a were isolated from all 5 parts of the *C. quadricarinatus*. The other fungi isolates were isolated from either abdomen, body or cheliped. Fungi habitats are quite diverse and widely distributed on earth with enormous environmental and medical importance. Some fungi form a parasitic or symbiotic relationship with plant and animal, with cell walls built of polysaccharides and chitin. It can be easily distinguished from all other living organisms because of the principles modes of vegetative growth and nutrient intake. Fungi produced zoospores in order to survive by swimming and attach to another host, which later, it protrude a hyphae through the cuticle at the original entry site of the host. Under lack of nutrition, fungi produce spores and later forming a flagellum that will leave the host and search for another crayfish [6].

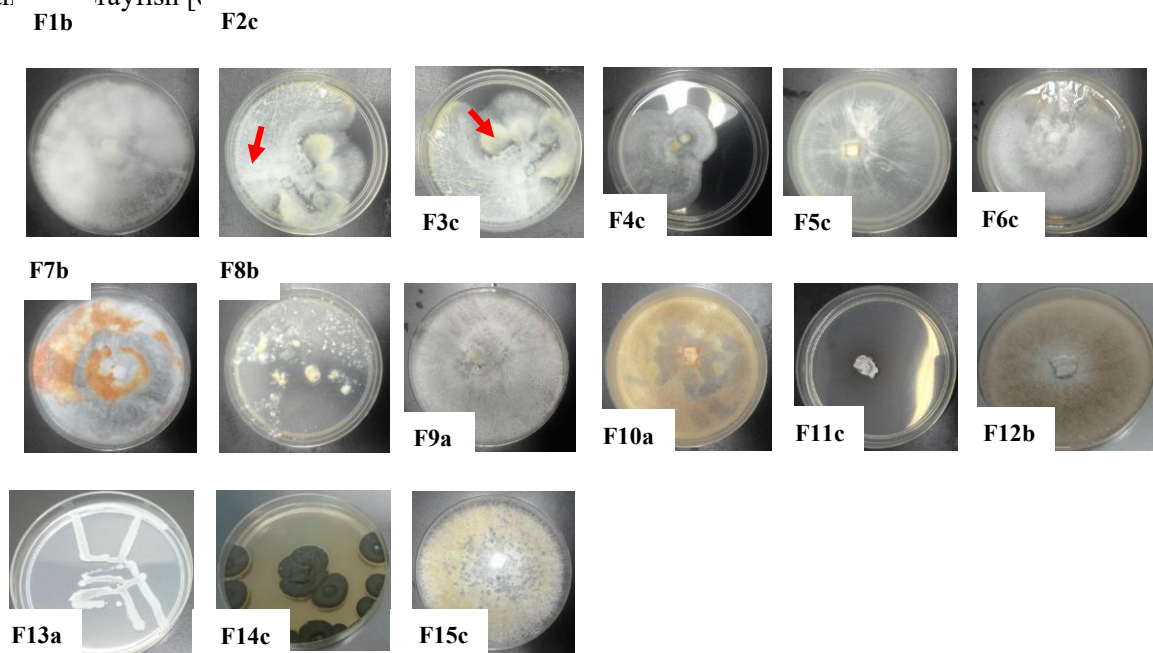


Figure 2. Fifteen fungi strains isolated from different parts of the infected *C. quadricarinatus* (a: abdomen, b: body, c: cheliped, h: head). Red arrows show the 2 morphologically similar fungi isolated on the same plate.

Table 1. Morphological characteristics of fungal isolated from infected *C. quadricarinatus*.

Fungal Isolate	Colour of aerial mycelia	Macroscopic Observation		
		Texture of mycelia	Topography	Reverse colour
F1b	Greyish - white	Cottony	Filamentous, convex, filiform,	Brownish - white
F2c	Yellowish – white	Cottony	Filamentous, raised, filiform, undulate	Dark green
F3c	Yellowish – white	Cottony	Filamentous, raised, filiform, undulate	Dark green

F4c	Yellowish - white	Cottony	Filamentous, raised, filiform,	Dark green
F5c	Greyish - white	Cottony	Filamentous, flat, entire	Greyish - white
F6c	Greyish - white	Cottony	Filamentous, flat, filiform	Greyish - white
F7b	Rusty brown and white	Cottony	Filamentous, raised, filiform	Brownish- white
F8b	Yellowish – white with pale yellow fruitbodies	Cottony	Irregular, umbonate, entire	Yellowish -white
F9a	Greyish - white	Cottony	Filamentous, raised, filiform	Grey
F10a	Rusty brown	Cottony	Filamentous, flat, entire	Brown
F11c	Brownish - white	Granular	Irregular, flat, lobate	Brownish - white
F12b	Brownish - white	Cottony	Circular, raised, entire	Dark brown
F13a	Yellowish - White	Velvety	Irregular, flat	Yellow
F14c	Dark green	Velvety	Circular, raised, convex	Blackish - green
F15c	Brownish – white with green beads	Cottony	Regular, flat, filiform	Dark brown

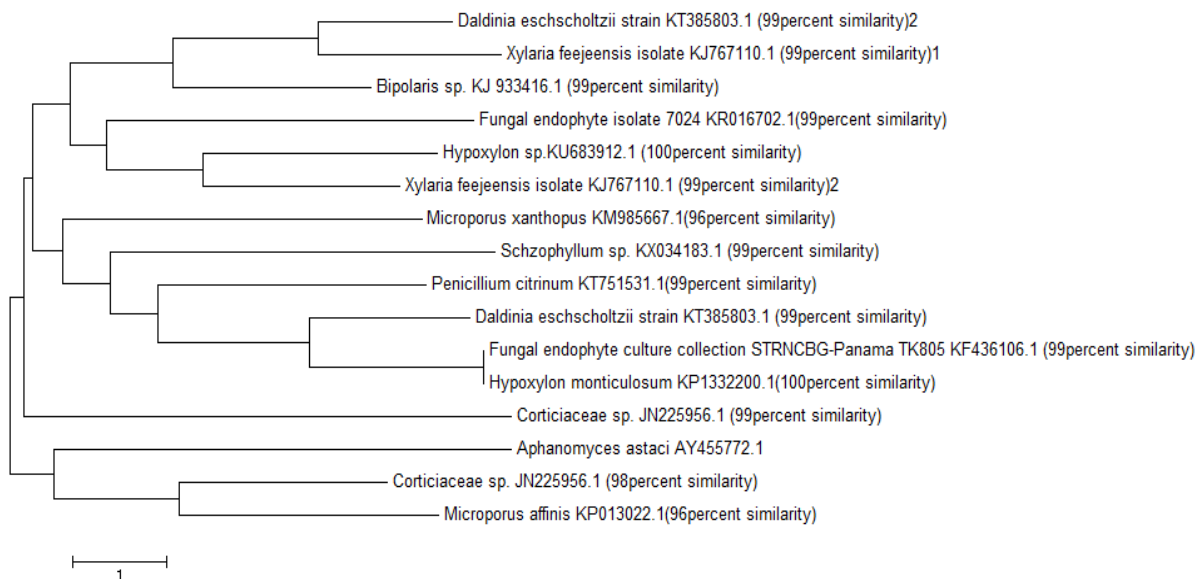
3.2. DNA Sequencing Analyses

Molecular techniques is the most powerful tools in identification, diversification, and phylogeny studies of microorganisms. In estimating the species evolutionary relationships and taxonomy identification in fungi, the internal transcribed spacer (ITS) is widely used among DNA markers [9]. The ITS rRNA region is composed of two hypervariable regions (ITS1 and ITS2) with the highly conserved 5.8S rRNA gene between them. It has been confirmed that the ITS region is applicable as a fungal barcode, and able to identify successfully a broad range of fungi (approximately 70%) [10]. In this study, primers ITS86F and ITS4, which are fungal specific [11] were used to amplify the fungal DNA. The primers were successfully amplified all 15 DNA extracted from isolated fungi, with intense bands on gel ranges approximately at 256 to 333 bp, respectively (data not shown). The percentage of similarity shown is the similarity of the sequenced fungal isolate to the DNA sequence in the GenBank database. These sequences are specific to closely related compared taxa. The ITS2 fungal isolates show 96-100% similarity to the nucleotides in the GenBank database. From the ITS2 sequences, Ascomycota had the highest representation, and some sequences are aligned with fungi from the Basidiomycota (Table 2). Seven of the ITS2 sequences were most closely related to 6 fungal species within the Ascomycota clade and all 6 sequences uniquely aligned with 5 fungal species in Basidiomycota clade. The remaining 2 sequences were closely related to endophyte culture collection. This indicates that the Ascomycota may have been more dominant in the samples.

The neighbour-joining phylogenetic tree was constructed to compare the genetic distance between the fungal that were isolated in this study, with the crayfish-plague fungi, *A. astaci* (Figure 3). The *A. astaci*, an oomycete fungus that caused crayfish plague, is extremely virulent which caused 100% death to the infected fish that lead to massive stock losses. The infected animals may not show any clinical signs at an earlier stage of infection until it reaches the terminal stage [6]. In this study, we managed to isolate 15 fungi isolates that might be linked to *A. astaci*. More detailed morphological characteristics and data on possible sequence variations are needed to identify the genetic variation between the isolated strains. Most of the isolated fungi were closely related to a wood-decaying fungi species. Yet, there is no evidence that these wood-decaying fungi could cause infection to the crayfish.

Table 2. Closest match in the GenBank database to nucleotide sequences from the ITS2 fungal isolates.

Fungi Isolate	Clades	Accession Number	Closest Match	Homology (%)	Sequence length (bp)
F1b		KX034183.1	Schizophyllum sp.	99	333
F2c	Basidiomycota	KM985667.1	<i>Microporus xanthopus</i>	96	308
F3c		KT751531.1	<i>Penicillium citrinum</i>	99	260
F4c	Ascomycota	KP1332200.1	<i>Hypoxyylon monticulosum</i> isolate 67	100	256
F5c	Unknown	KF436106.1	Fungal endophyte culture collection STRNCBG-Panama TK805	99	258
F6c	Basidiomycota	KP013022.1	<i>Microporus affinis</i>	96	305
F7b	Unknown	KR016702.1	Fungal endophyte isolate 7024	99	282
F8b	Basidiomycota	JN225956.1	Corticiaceae sp.	99	290
F9a	Ascomycota	KT385803.1	<i>Daldinia eschscholtzii</i>	99	259
F10a	Basidiomycota	JN225956.1	Corticiaceae sp	98	276
F11c	Ascomycota	KJ767110.1	<i>Xylaria feejeensis</i> isolate	99	268
F12b	Ascomycota	KU683912.1	<i>Hypoxyylon</i> sp.	100	264
F13a	Ascomycota	KT385803.1	<i>Daldinia eschscholtzii</i> strain	99	267
F14c	Ascomycota	KJ933416.1	<i>Bipolaris</i> sp.	99	257
F15c	Ascomycota	KJ767110.1	<i>Xylaria feejeensis</i> isolate	99	260

**Figure 3.** Neighbour-joining phylogenetic tree based on ITS2 sequences showing relationship between isolated fungal strain and *A. astaci*.

Conclusion

This study demonstrates that through genetic evidence, different forms of fungi that are not *A. astaci* were isolated from infected redclaw crayfish. Neighbour-joining phylogenetic tree shows the relation between the isolated fungi with *A. astaci*. The use of ITS primers with high sequence variability provide easy identification of the large majority of fungi at the species level, regardless when little DNA is available.

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