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Isolation and Characterisation of Ascomycetes Isolated from *Eurycoma longifolia* Jack and Malay Traditional Vegetables

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ABSTRACT

Plants are the most common host for fungal communities. However, vegetables and herbs traditionally consumed by the Malay community have not been thoroughly investigated for their association with fungi. The main objective of the present study is to identify the Ascomycetes fungi associated with *Eurycoma longifolia* Jack and vegetables traditionally consumed by the Malay community. In the present study, we isolated and identified 34 isolates of the Ascomycetes fungi obtained from five traditional vegetables (*Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica*) and *Eurycoma longifolia* Jack. The isolates are identified as eight species, which are *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Fusarium oxysporum, Fusarium proliferatum, Fusarium solani, Penicillium paraherquei* and *Trichoderma asperellum*. *Aspergillus* and *Fusarium* are dominant among the isolated fungi. This report provides additional information on the diversity of fungi isolated from traditional vegetables and *Eurycoma longifolia* based on the Internal Transcribed Spacer (ITS) sequence analysis.

Keywords: Traditional vegetables, *Aspergillus* species, *Fusarium* species, *Penicillium* species and *Trichoderma* species

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INTRODUCTION

Numerous studies have been conducted on fungi and their ecological advantages, biomedical properties, natural products and taxonomic relationship (Radu & Kqueen, 2002; Guo, Wang, Sun, & Tang, 2008; Aly, Debbab, Kjer, & Proksch, 2010; Nath,

Raghunatha, & Joshi, 2012; Madhusudan, Barathi, & Prakash, 2015). Malaysia is rich in rainforest land teeming with thousands of plants with potential medicinal value such as traditional vegetables and *Eurycoma longifolia* Jack, which are the most consumed by Malaysians. Traditional vegetables (locally known as *ulam*) were reported in previous studies to contain many benefits in terms of their biomedical properties (Kumar & Sagar, 2007; Guo et al., 2008, p.136; Shukri, Alan, & Noorzuraini, 2011; Fatimah, Norazian, & Rashidi, 2012). *Eurycoma longifolia*, which belongs to the Simaroubaceae family, is a woody plant locally known as *Tongkat Ali*, that has pharmacological and medicinal value in treating various diseases.

Commonly, traditional vegetables can be eaten raw or boiled, usually as part of a side dish served with rice. It can be served as a salad and is very popular among Malaysians and visiting foreigners because of its flavour and savoury taste. Previous studies have recorded that there are more than 100 plant species from various plant families that are consumed as *ulam* in Malaysia (Hussain, Anwar, Sherazi, & Przybylski, 2008). *Ulam* is also applied in Malaysia as a medicinal remedy for controlling blood pressure and improving blood glucose concentrations among diabetes patients (Abas, Ozpinar, Kutay, Kahraman, & Eseceli, 2005). Five *ulam* were chosen in the present study, namely *Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica* as the study samples. Selection of the *ulam* was due to availability, popularity as a local favourite and frequency of use in Malaysian cuisine.

There is very limited information on the diversity of fungi isolated from herbs and vegetables, especially in tropical area. Realising the importance of this research to the nation and the world, a study of the diversity of fungi associated with these plants was conducted. The objectives of this study were to isolate the Ascomycetes fungi from *Eurycoma longifolia* Jack and the traditional vegetables and to identify the fungal isolates based on the Internal Transcribed Spacer (ITS) region sequence analysis. The findings of this study provide basic information on fungal diversity that can be used as reference in aetiology, disease control studies and understanding the role of fungi in their hosts.

MATERIALS AND METHOD

Fungal Isolation, Purification and Preservation

Thirty-four fungal isolates were isolated from *Eurycoma longifolia* Jack and five traditional vegetables commoly consumed in Malaysia (*Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica*). Three 5 × 5 mm^2 pieces from the margins of leaf tissue of each plant were taken and surface sterilised in 1% sodium hypochlorite solution by dipping for 3 min and then rinsed three times with sterilised distilled water. After the blot was dried, the pieces of tissue were placed on the surface

of a Potato Dextrose Agar (PDA) plate and incubated for 4 days. Single hyphal tip isolation was conducted to obtain pure fungal colonies. All contaminated isolates were sub-cultured on water agar (WA). The cultures were incubated for 24 hours, and then by using a dissecting microscope, a single hyphal tip of fungal growth was transferred to a PDA plate. The culture plates were incubated at 25°C for 5-7 days to allow the colony to grow. Conidial suspension of the fungi was preserved in 25% glycerol complete medium with xylose (CMX), which was then kept at -80°C for further use.

Morphological Characterisation

The fungal cultures were transferred to water agar (WA) for single conidial isolation using the conidial suspension method. All the purified isolates were tentatively identified based on morphological characteristics that were emphasised on growth rate, pigmentation, size and the shape of micro- and macroconidia, presence of chlamydospore and type of conidiophore (Raper & Fennell, 1965; Pitt, 1979; Leslie & Summerell, 2006).

DNA Extraction and Amplification of ITS Region

All the isolates were cultured on PDA and incubated for 7 days. DNA was extracted from the young fungal mycelia of the culture growing on the PDA agar plate using the UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the user's manual instructions. A standard Polymerase Chain Reaction (PCR) protocol was used to amplify the ITS gene region. The PCR reactions of the ITS region were carried out in a thermal cycler with the primer ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and the primer ITS4 (5'TCCTCCGCTTATTGATATGC-3') (White, Bruns, Lee, & Taylor, 1990). Amplification reactions were performed in a 20 µl reaction volume containing 1x Green GoTaq Buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM primer ITS1, 0.5 mM primer ITS4, 0.1U *Taq* Polymerase and a 20-ng DNA template. The ITS was amplified in a thermal cycler (Biometra TProfessional) with the following cycling protocol: initial denaturation at 95°C for 30 s; followed by 35 cycles of 95°C for 10 s, 59°C for 15 s and 72°C for 30 s, and a final extension at 72°C for 5 min.

The PCR products with an expected length of 600-650 bp were examined by gel electrophoresis in 2% agarose gel and stained with FloroSafe DNA (BIO-5170-1 ml) (Axil Scientific Pte Ltd, Singapore). The amplicons were viewed under an UV transluminator. All the PCR products were given good quality of amplicons; the DNA was amplified to 50 µl in volume for sequencing.

ITS Sequencing and Phylogenetic Analysis

The amplification product for the ITS of each isolate was purified with QIAquick® Gel Extraction Kit according to the

manufacturer's instructions. The ITS gene fragment was sent for DNA sequencing using the ABI3730XL sequencer completed by MyTACG Bioscience Company, Selangor, MY. These sequences were BLAST against GenBank (http://blast.ncbi.nlm.nih.gov/). BLASTn was used to run a search for maximum identity and the number of bases of aligned sequence. Sequence alignments and analysis were performed using the Mega 6.06 (Molecular Evolutionary Genetics Analysis 6.06; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The maximum-likelihood phylogenetic tree was constructed using the bootstrap (1000 replicates) method.

Seven ITS sequences of the Ascomycetes fungi, *Aspergillus flavus* (JX292092), *Aspergillus fumigatus* (AY214446.1), *Aspergillus niger* (JX535496.1), *Fusarium oxysporum* (KM246753), *Fusarium proliferatum* (AF291061.1), *Fusarium solani* (JN9830007) and *Penicillium paraherquei* (NR111052.1) obtained from the NCBI GenBank dataset were used. The ITS sequence of the Ustilaginomycetes fungi, *Ustilago maydis* (AJ235275.1), was used as the outgroup reference in the phylogenetic analysis.

RESULTS AND DISCUSSION

A total of 34 isolates of fungi from five traditional vegetables and one herb, namely *Oenanthe javanica* (4 isolates), *Cosmos caudatus* (8 isolates), *Persicaria odorata* (5 isolates), *Psophocarpus tetragonolobus* (4 isolates), *Cantella asiatica* (4 isolates) and *Eurycoma longifolia* (9 isolates), were evaluated in this study according to their morphological characteristics (Table 1) and ITS sequences. All the isolated fungi can be considered endophytic as all the samples collected in this study were free of disease symptoms. Endophytic fungi are microorganisms that live in the plant tissue of the host plant and build colonies without causing any harm to the host plant. They represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medicinal, agricultural and industrial uses (Radu & Kqueen, 2002; Guo et al., 2008, p. 136; Aly et al., 2010, p. 2; Nath et al., 2012, p. 8; Madhusudan et al., 2015, p. 111). However, they are a poorly investigated group of microorganisms. Metabolites emitted by endophytes are recognised as a versatile source of antimicrobial and antioxidant agents. Thus, the fungal endophytes of plants need to be explored and studied to describe their distribution, morphology and diversity.

Variations were observed in the cultures in colour, appearance, margins, texture and sectors of the colonies (Table 1; Figure 1). Based on morphological characteristics, the most important colony features of *Fusarium* species are a white to yellow or pale violet colour of abundant mycelia and white tinged with yellow or purple pigmentation in agar. The colonies of the *Aspergillus* species on the PDA were observed to have a cottony and powdery texture and they exhibited a dark green (*A. fumigatus* and *A. flavus*) or black (*A. niger*) pigmentation. The colonies of *Penicillium*

paraherquei were observed to be greyish green and have a cottony texture on the PDA, whereas *Trichoderma asperellum* displayed a cottony and powdery texture with white to light green pigmentation that became dark green with age.

Table 1
List of microfungi isolates used in this study and their morphological characteristics

Isolate no.	Host (Scientific name)	Location	Species	Pigmentation	Colony features
B2468 C2473	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang	*A. fumigatus*	Grey with green to dark green, becomes black with age	Cottony and powdery
B2478	*Cosmos caudatus*	Puchong, Selangor			
B2479 B2480	*Oenanthe javanica*	Semenyih, Selangor			
C2483	*Cosmos caudatus*	Cameron Highland, Pahang			
C2471	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang	*A. flavus*	Yellowish, becomes green with age	Powdery
B2474 B2475	*Cosmos caudatus*	Puchong, Selangor			
B2482	*Oenanthe javanica*	Semenyih, Selangor			
B2469 B2484	*Persicaria odorata*	Kajang, Selangor	*A. niger*	White, becomes black or dark brown with age	Cottony and powdery
C2470 C2472	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang			
B2476 B2477	*Cosmos caudatus*	Puchong, Selangor			
B2481	*Oenanthe javanica*	Semenyih, Selangor			
A223, A225 A229, A242	*Eurycoma longifolia*	Grik, Perak	*F. solani*	White, becomes pale yellow with age	White sparse mycelia in concentric rings
B2486	*Centella asiatica*	Puchong, Selangor			
B475, B480 B481, B483 B484	*Eurycoma longifolia*	Ayer Hitam Forest Reserve, Selangor			
B2485	*Centella asiatica*	Puchong, Selangor	*F. oxysporum*	White, becomes yellow with age	Cottony and fluffy
B2487	*Centella asiatica*	Puchong, Selangor	*F. proliferatum*	White, becomes purple with age	Cottony
B2488	*Cosmos caudatus*	Puchong, Selangor	*Penicillium paraherquei*	Greyish green	Cottony and powdery
B2489	*Persicaria odorata*				
B2490	*Persicaria odorata*	Kajang, Selangor	*Trichoderma asperellum*	White to light green, becomes dark green with age	Cottony and powdery
B2491 B2492	*Centella asiatica*	Puchong, Selangor			

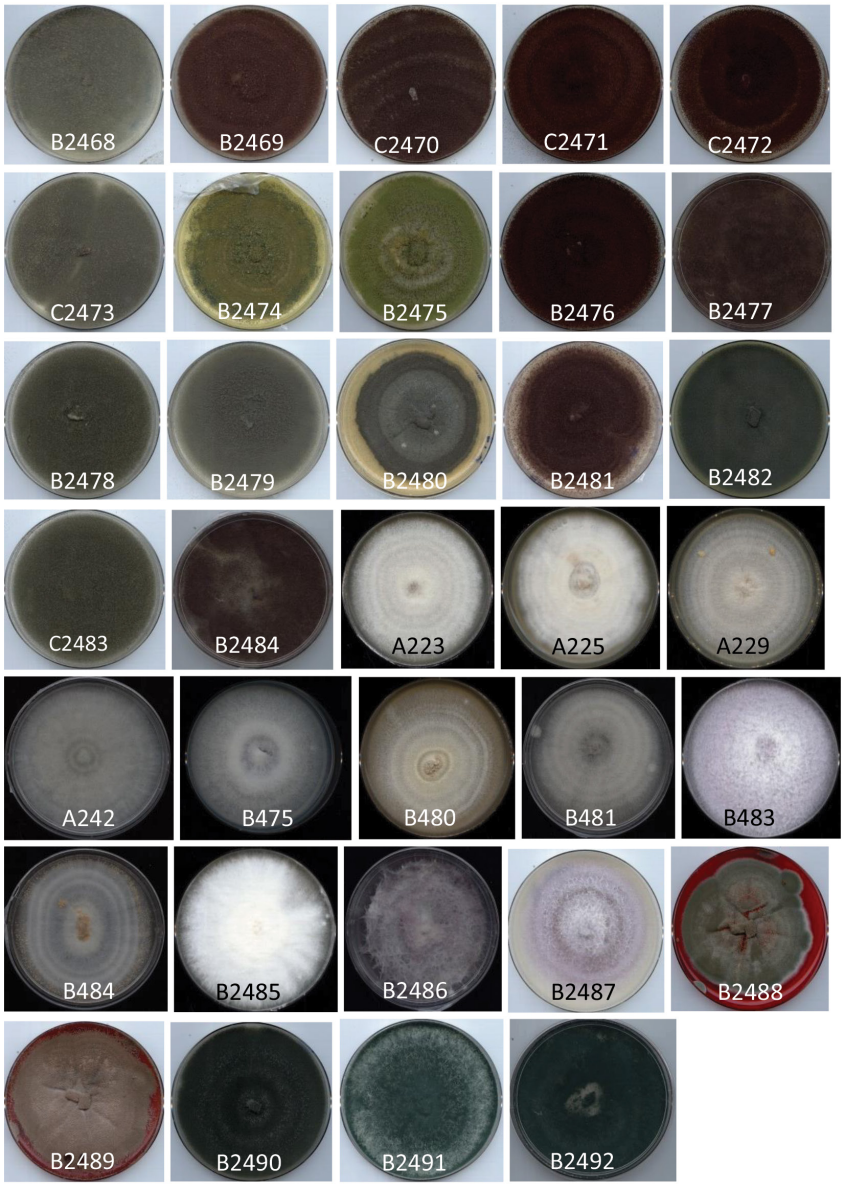

Figure 1. Colony features of *A. flavus (*isolates C2471, AB2474, B2475, B2482), *A. fumigatus* (isolates B2468, C2473, B2478, B2479, B2480, C2483), *A. niger* (isolates B2469, C2470, C2472, B2476, B2477, B2481, B2484), *F. solani* (isolates A223, A225, A229, A242, B475, B480, B481, B483, B484, B2486), *F. oxysporum* (isolate B2485), *F. proliferatum* (isolate B2487), *P. paraherquei* (isolates B2488 and B2489) and *T. asperellum* (isolates B2490, B2491 and B2492)

The *Aspergillus* and *Fusarium* species were observed to have the highest number of fungal isolates. According to the BLASTn analysis in the NCBI database, four isolates (C2471, B2474, B2475 and B2482) were successfully identified as *A. flavus,* six isolates (B2479, B2468, B2480, C2483, C2473 and B2478) as *A. fumigatus* and seven isolates (C2472, B2481, B2476, B2469, B2477, C2470 and B2484) as *A. niger* in a range of 97-99% maximum sequence identity. Twelve isolates were identified as the *Fusarium* species, mainly as *F. oxysporum* (B2485), *F. proliferatum* (B2487) and *F. solani* (A223, A225, A229, A242, B475, B480, B481, B483, B484 and B2486) with 98-99% maximum sequence identity. Isolates B2488 and B2489 were subjected to a BLAST analysis as *P. paraherquei* with 99% sequence similarity, while the remaining isolates (2490, B2491 and B2492) were identified as *T. asperellum* with 99% sequence similarity.

A study conducted by Radu and Kqueen (2002) reported on endophytes that were isolated from different types of medicinal plant including *Cantella asiatica* and *Eurycoma longifolia* Jack. In addition, Nur Ain Izzati and Wan Hasmida (2011) reported that a total number of 40 microfungi were isolated from traditional vegetables, namely *O. javanica, C. caudatus, P. odorata, P. tetragonolobus* and *C. asiatica*, that were only morphologically identified into four genera such as *Aspergillus, Fusarium, Penicillium* and *Trichoderma* species.

A phylogenetic tree was constructed using the neighbour-joining method of the MEGA Version 6.06 software with 1000 bootstrap replications. Nine additional sequences obtained from the NCBI GenBank databases were used as references including for the outgroup. Generally, the phylogenetic tree consisted of four genera, mainly *Aspergillus, Fusarium, Penicillium* and *Trichoderma.* Results from the phylogenetic tree (Figure 2) based on ITS region revealed that all the 34 endophytic fungi isolates belonging to the Ascomycete family could be divided into four terminal clades. *Aspergillus* sp. was the dominating group of fungi represented by four isolates of *A. flavus,* six isolates of *A. fumigatus* and seven isolates of *A. niger* in a 97-99% maximum sequence identity. Twelve isolates were identified as the *Fusarium* species, with *F. oxysporum*, *F. proliferatum* and *F. solani* showing a 98-99% maximum sequence identity. Isolates of *P. paraherquei* and the remaining three isolates identified as *T. asperellum* showed a 99% sequence identity.

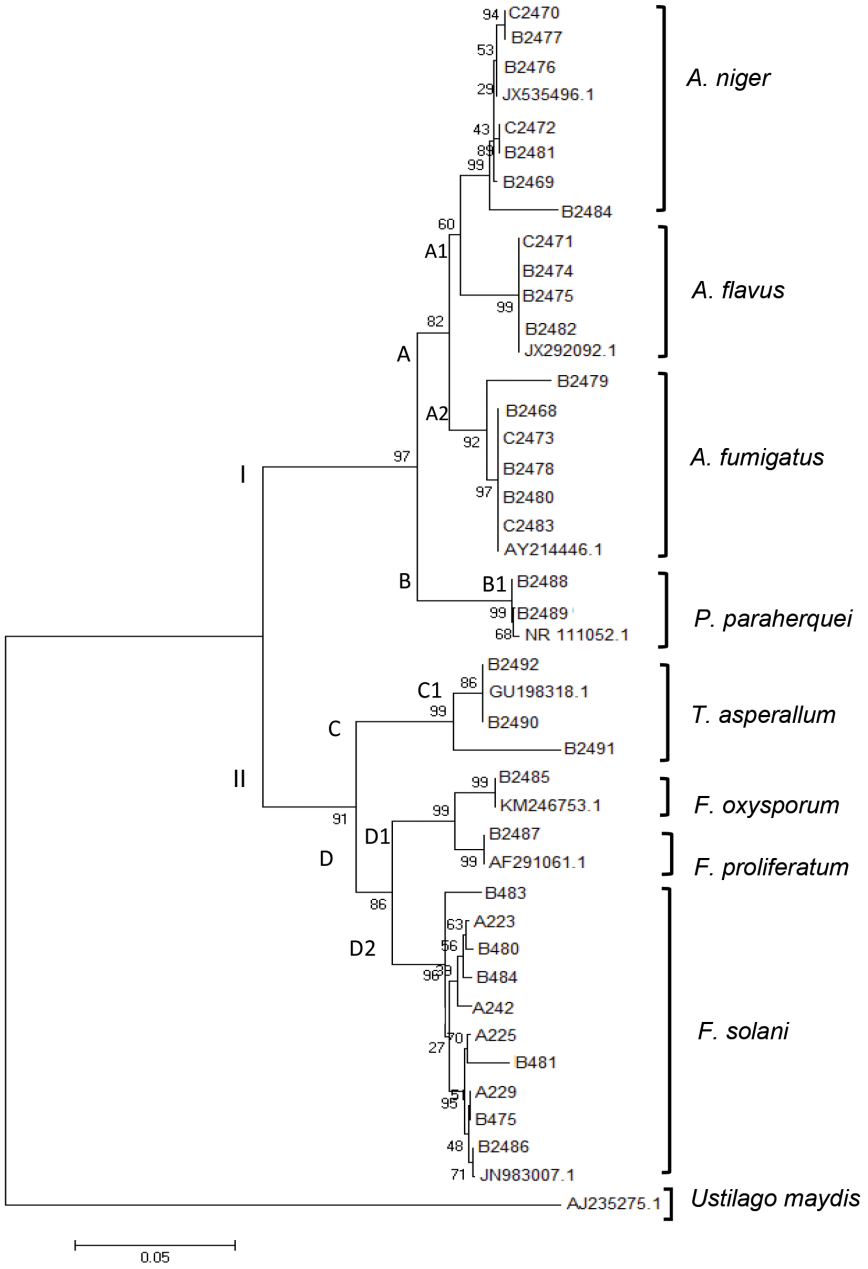

Figure 2. The evolution history was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 43 nucleotide sequences. *Ustilago maydis* (AJ235275) was taken as the outgroup.

The phylogenetic tree grouped the isolates into two major clades (Clade I and Clade II) with the outgroup (*U. maydis*) clade (Figure 2). Clade I was grouped into two clades (Clade A and Clade B) that consisted of 19 isolates, while Clade II was also grouped into two clades (Clade C and Clade D), which consisted of 15 isolates. Clade A1 consisted of 10 isolates of *A. niger* and *A. flavus*, which was the cluster formed supported by a 60% bootstrap value with two reference isolates identified as JX535496.1 (*A. niger)* and JX292092.1 (*A. flavus*). Clade A1 was branched into two small clusters that comprised 11 isolates classified into two species containing: i) *A. niger* (B2469, C2470, C2472, B2476, B2477, B2481 and B2484 isolated from *Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) supported with a 99% bootstrap value; and ii) *A. flavus* (C2471, B2474, B2475 and B2482 isolated from *Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) with a 99% bootstrap value. On the other hand, isolates of B2468, C2473, B2478, B2479, B2480 and C2483 (isolated from *Persicaria odorata, Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) were closely related to *A. fumigatus* (AY214446.1) with a 92% bootstrap value clustered in Clade A2. This proved that the ITS region of some strains was highly conserved. This is highly supported by previous studies stating that ITS region is a highly conserved region (Sumida et al., 2004; Maryam & Ehsan, 2015). Based on the phylogenetic tree, all the species were clearly placed into separate clades.

CONCLUSION

In this study, 34 fungal isolates were grouped under eight different species, *Aspergillus flavus, A. fumigatus, A. niger, Fusarium oxysporum, F. proliferatum, F. solani, Penicillium paraherquei* and *Trichoderma asperellum*. All the isolates were identified based on morphological characteristics and ITS sequence analyses. *Aspergillus* sp. was the dominant genus with 17 isolates, while two isolates of *P. paraherquei* and three isolates of *T. asperellum* were also successfully identified. These findings can be used as reference for future study on the diversity of microfungi isolated from herbs as well as the role of fungi in herbs.

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