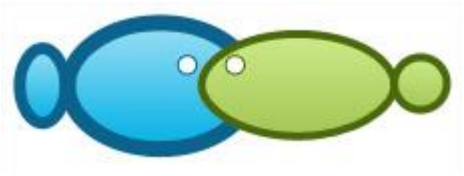

Isolation, identification, and characterization of *Ankistrodesmus falcatus* from Lake Chini, Pahang, Malaysia

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Abstract. This study was designed to isolate pure species of *Ankistrodesmus falcatus* from Lake Chini Pahang in Malaysia and confirm the identity/characterize the microalgae molecularly. To achieve this, *Ankistrodesmus* sp. was isolated from the water samples taken from the lake by centrifugation and agar plate streaking. Phenotypically, *Ankistrodesmus* sp. is a unicellular needle-like shape green alga which has both ends gradually tapering in a fusiform manner. The mean size of the microalgae was 35µm in length and 2.5µm in diameter. Using molecular characterization of the 18S rDNA, the identity of the algae was confirmed to be *Ankistrodesmus falcatus* with 98% similarity with the top sequenced microalgae of the same species on the NCBI (2019) website. Although the water quality of the Lake varied compared to standard, it seems not to have affected the growth of the microalgae species negatively. Studies on the optimization of the mass production protocol for this important microalgae species are recommended.
Key Words: 18S rDNA, *Ankistrodesmus* sp., freshwater lake, microalgae, water quality.

Introduction. The current interest in microalgae research is borne out of its potential for biofuel production, alternative nutritional supplement, and use for animal feeding. Considering the current challenges with energy demands and deterioration of the environment with fossil fuel, microalgae represent an environmentally friendly, economically viable yet renewable source of alternative energy for the modern age (Rodolfi et al 2009). This is because of its high lipid content (50-80%), biodegradable and non-toxic nature of the macroalgae which does not possess many competitive uses as compared to other higher photosynthetic plants (Moazami et al 2011; Schenk et al 2008). Also, low yields, slow growth rate, high land/water requirements, rainforest deforestation and loss of native biodiversity/ecosystem are part of the major drawbacks of the use of crop-based plants for biofuel compared to microalgae (Sheehan et al 1998; Puppan 2002; Foley et al 2007; Sharma et al 2012).

Microalgae can also be used in the rearing of aquatic organisms as their microscopic size, high levels of sterols, essential fatty acids and proteins make them appropriate for larvae fish and filter-feeding fishes (García et al 2012; Sharifah et al 2016; Khatoon et al 2017). Many studies have also demonstrated the potential of some microalgae in the treatment of bacterial diseases such as *Vibrio* (Austin et al 1992;

Naviner et al 1999). A good example of these microalgae is the *Ankistrodesmus* sp. which not only serves as an excellent source of biofuel (Sheehan et al 1998; Griffiths & Harrison 2009) but also as nutrition alternatives and disease control measures against *Streptococcus agalactiae* in fish (Sharifah et al 2016).

With the promising prospect of the microalgae industry, much research has been conducted into isolation, characterization as well as mass production of different microalgae species (Khatoon et al 2017). However, germplasm resources development is becoming a prominent problem inhibiting the growth of the industry (Doan et al 2011; Zhang et al 2014). Therefore, screening and isolation of indigenous microalgae species with desirable attributes is urgently needed. The simple morphology of many unicellular microalgae causes taxonomical misidentification due to morphological similarities (Shakeel et al 2018). Hence, in the bit to identify and characterize promising algae species accurately, morphological identification must be accompanied by molecular characterization. This study aims at screening and isolating indigenous microalgae *Ankistrodesmus falcatus* from Lake Chini Pahang in Malaysia using centrifugation and agar plate streaking. The identity of the microalgae was also confirmed and characterized through the 18S rDNA sequence analysis.

Material and Method. The algal sample for this study was collected from eight stations selected at Lake Chini, Malaysia, namely: Gumum, Pulau Balai, Jerankang, Gunting Teratai, Mempitih, Kenawar, Serodong, and Melai. The study took place between April 2018 and August 2019. The lake's geographical coordinates are 3.43°N, 102.92°E, and is located about 250 km from the country's capital (i.e., Kuala Lumpur as seen in Figure 1). It is only 100 km from Kuantan and flows into the Pahang River via the Chini River (Shuhaimi & Lim 2006).

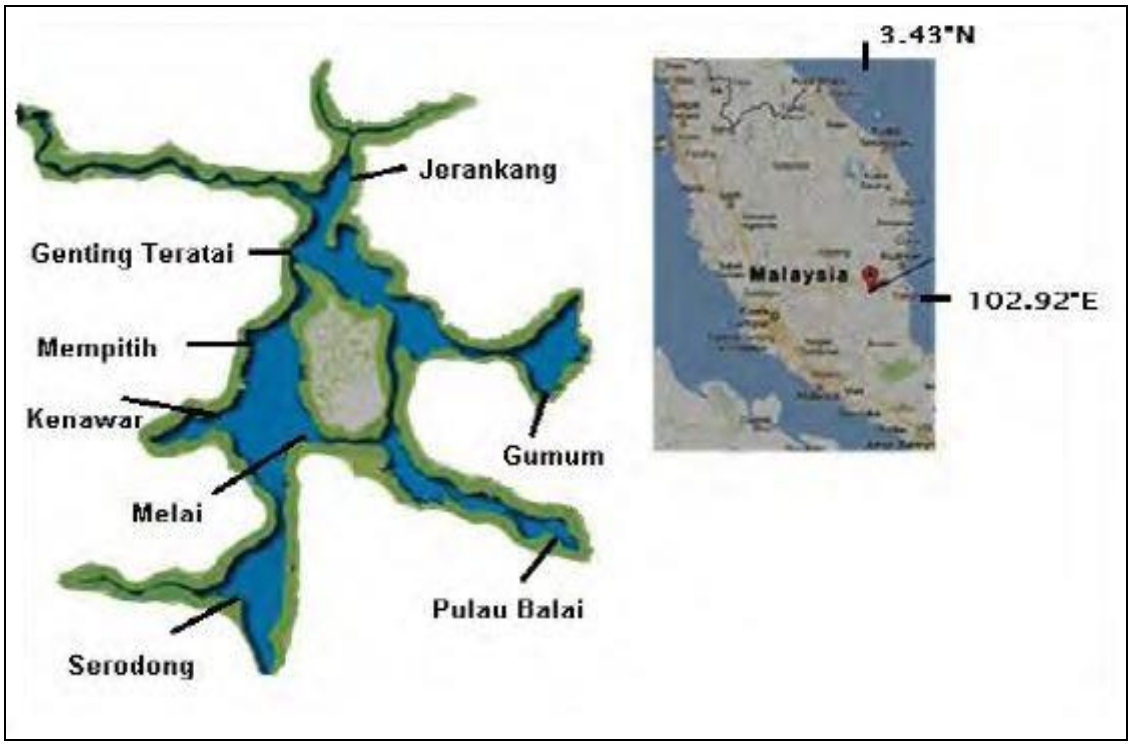

Figure 1. Sampling stations at the Chini, Pahang Lake used for this study (Source: Shuhaimi & Lim 2006).

The sample collection of microalgae was done using different sizes of plankton net (i.e., 20, 25, 50, and 100 µm) to target certain sizes of microalgae. The samples were stored in an icebox (4°C) and transported to the laboratory for further analysis. Water quality was also analyzed during sampling at the lake for each station. Temperature, dissolved oxygen (DO), and pH were recorded in-situ using Hanna's digital multi-parameter water checker (Model HL 98126). A water sample was also collected and taken to the laboratory

for the determination of ammonia (NH_3), nitrite (NO_2), nitrate (NO_3), and phosphate (PO_4) following standard protocol by APHA (2005). Summary statistics of the water quality were obtained using Minitab 14 software for Windows. The result was then presented in a table.

Centrifugation and agar plate streaking techniques (Phang & Chu 1999) were used to obtain the pure culture of *Ankistrodesmus* sp. In brief, the microalgae were harvested and washed four times by centrifugation in 5000rpm for 5 minutes (each time by discarding supernatant and resuspension). Sterile agar plates were then prepared by mixing 1.5g of bacteriological agar powder with 100ml of pre-sterile Bold's Basal Medium (BBM) medium. Upon complete dissolution of the agar (through heating and stirring, they were poured into 3 to 5 sterilized Petri dishes (100 × 15mm diameter) and cooled down to room temperature. With the aid of a sterilized pipette, suspension of the initial stock was dropped into each petri dish and spread over the agar using a sterile platinum hook. The Petri dishes were placed in an upside-down position and tightened up with a para-film tape as it was incubated under laboratory conditions for 7days. Samples of colonies were taken on slides and observed under a compound microscope (40x magnification) to confirm the purity of culture and photographs were taken with an electron microscope (1400x magnification). Mixed culture was sub-cultured (streaked) to obtain the pure culture intended. Single colonies from agar plates after successive sub-culturing were up scaled using a 50 ml test tube containing 10ml liquid growth medium. Sub-culturing of the obtained pure stock was done every 2 weeks to maintain healthy and good germplasm stocks for future studies.

Identification of the green microalgae was done by consulting the standard monographs key by Edward and David (2010), while the species was confirmed using the 18S rDNA sequence analysis. Harvested algae (by centrifugation) for DNA analysis were in the exponential growth phase. The cells were grounded to a fine powder using liquid nitrogen. The DNA extraction was done following the manufacturer manual of a commercial Plant Genomic DNA kit. The extracted DNA was observed in a 1% agarose gel electrophoresis and used for PCR amplification. PCR amplification was performed using a real-time PCR system (Biometra Model-II T gradient thermoblock, serial#: 2706187, Germany). Amplification reactions used the following eukaryotic primers pair NS1 5'-GTAGTCATATGCTTGTCTC-3' and 18L 5'-CACCTACGGAAACCTTGTTACGACTT-3' (Hamby & Zimmer 1988; White et al 1990; Remias et al 2012; Uetake et al 2014).

The PCR condition for 18S rRNA was done using the protocol described by Kullen et al (2000). In brief, initial denaturation was 3 min at 94°C, followed by 34 cycles of 30 sec denaturation at 94°C, annealing was done at 60°C for 30 sec, and extension at 72°C for 30 sec, a final extension of 72°C for 5 min and was held in 4°C until use. The PCR product was purified using the Qiagen kit following the manufacturer's label. The purified PCR product and primers were sent for sequencing at the 1st Base Laboratories Sdn. Bhd., Malaysia. 18S rDNA sequences obtained from the isolated microalgae were compared with the GenBank database using BLAST. Phylogenetic trees were constructed using the neighbour-joining method using MEGA version 6 (Wu et al 2015; Okomoda 2018; Okomoda et al 2019). The confidence of the branching patterns was assessed by 1000 bootstrap replicates in the neighbour-joining analysis.

Results and Discussion. According to Adakole et al (2003), near neutral or slightly alkaline pH are indicators of unpolluted lakes. Similarly, a pH of 6-8 is considered able to support the normal biological activities of aquatic animals in a natural water body (EEC 1980). The pH of Lake Chini was within these recommended levels (Table 1). The slightly acidic nature of the freshwater lake may be due to the huge quantity of deteriorating materials within the lake (although not quantified but observable during sampling). This observation is like the earlier findings of Ahmad et al (2011) on the same lake. However, an alkaline pH was reported by Njenga (2004) for Lake Kolleru in India. Although the mean dissolved oxygen of Lake Chini was slightly lower (4.6 mg/l) than the recommended optimum requirement for aquaculture activities (Boyd 1982), this was not thought to have affected the targeted microalgae. Depletion of dissolved oxygen in water encourages the microbial reduction of nitrate to nitrite and sulphate to sulphide, giving

rise to severe problems (Adakole et al 2003). However, the values of these toxic compounds were very low as observed in the current study compared to the levels that can affect the aquatic organism as reported by Ridha and Cruz (2001), Akinwole (2005), and Somervilla et al (2014). The temperature was also optimum in comparison with what is obtainable in the tropics.

Table 1

Water quality of water samples collected from different stations in Lake Chini, Pahang

Station	*Temperature*	*DO*	*pH*	*NH_3*	*NO_3*	*NO_2*	*PO_4*
Gumun	29.06	4.72	7.09	0.42	0.01	0.004	0.25
Pulau Balai	30.17	6.64	6.08	0.01	0.01	0.011	0.18
Jerankang	28.77	3.04	6.12	0.03	0.01	0.001	0.06
Gunting Teratai	29.85	4.21	6.08	0.01	0.01	0.007	0.06
Mempitih	30.46	5.09	6.12	0.02	0.01	0.005	0.04
Kenawar	29.47	3.64	5.7	0.04	0.01	0.007	0.08
Serodong	30.72	4.42	6.01	0.03	0.01	0.008	0.03
Malai	31.61	4.95	6.19	0.02	0.01	0.006	0.02
Mean	30.01±0.32	4.58±0.38	6.17±0.14	0.07±0.05	0.01±0.0	0.006±0.001	0.09±0.03

Ankistrodesmus sp. is known to be very common in eutrophic lakes and slow-flowing rivers; however, some species are adapted to acid waters (Bellinger & Sigee 2010). It is, therefore, no surprise that they were successfully isolated in the current study. Isolation of microalgae by traditional means is well established in the earlier works of Beyerinck (1890) and Miquel (1892). Two different techniques, centrifugation, and streaking were used to isolate and purify the *Ankistrodesmus* sp. For purification of the free-living planktonic algae, centrifugation serves to concentrate the quarry relative to the contaminants and thus, increases the chances for success in later stages of purification.

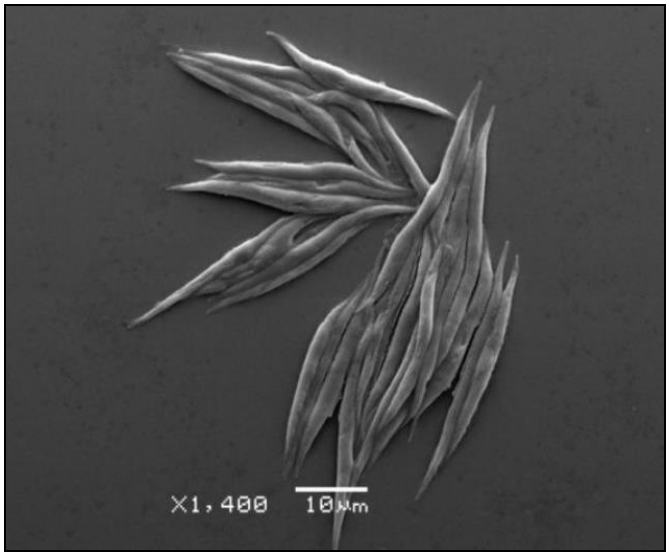

Figure 2. Isolated pure culture of *Ankistrodesmus falcatus* under electron microscope scanning (Source: original figure).

This has been proven extremely effective in the separation of algal cells or its colonies from each other and from bacteria or detritus (Reardon et al 1979). However, to achieve this, a uniform density of centrifugation, timing, and speed is very critical to success. Centrifugation is continued until the heavier or barely sedimented cells form a supernatant which is then replaced by a fresh sterile medium and the process is repeated as many times as possible (Hoshaw & Rosowski 1973). This method has also been successfully used by Pringsheim (1946), Phang & Chu (1999) for the isolation and purification of other microalgae species.

The morphological characteristics of *Ankistrodesmus* sp. was matching with the reference given in Bellinger and Sigee (2010). However, as opined earlier, the simple morphology of many unicellular green algae always leads to taxonomical difficulties. Duong et al (2012) and Wu et al (2015) had stated that morphological data are largely unreliable for the identification of green microalgae to species level. According to Talukdar et al (2012), the cells of *Ankistrodesmus* sp. are fusiform, solitary, and clustered without a colonial sheath. The chloroplast is parietal, pyrenoids are absent and they vary in sizes ranging between 30 to 36µm long and 2.5 to 4.5µm in diameter. The microscopic description of Smith (1933) and of Fritsch and West (1927) of the *A. falcatus* is said to be a unicellular uninucleate needle-like shape green alga cell with gradually tapering ends. All this description fits perfectly with what was observed for the microalgae in this study as shown in Figure 2 coupled with the observation of mean size of 35µm in length and 2.5µm in diameter. However, other microalgae such as *Selenastrum* sp. have a very close resemblance to the *Ankistrodesmus* sp. with the only difference being that the first is strongly curved compared to the latter (Komarkova-Legnerava 1969). This form of species discrimination and identification is very subjective, unreliable, and misleading. Hence, to get rid of such taxonomic confusion which leads to probable failure in identifying the species correctly, molecular characterization is inevitable.

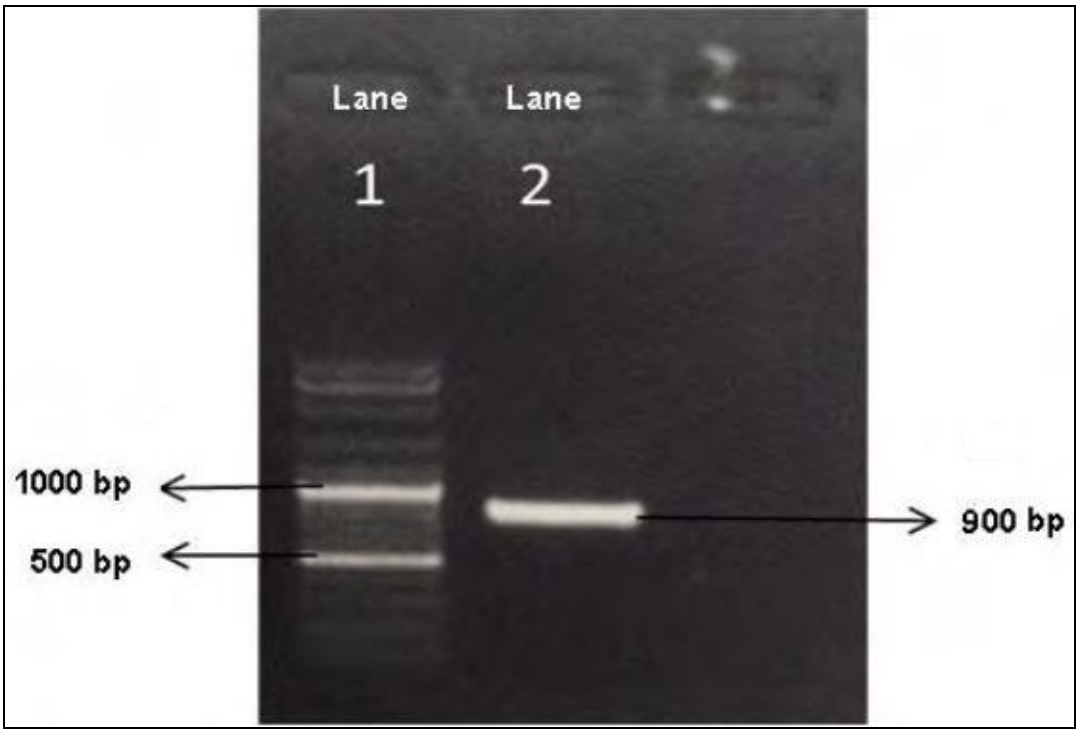

Figure 3. Singe band obtained after purification of the PCR product. Lane 1: 100bp ladder (Multi, EzWay); Lane 2: Purified product (Source: original figure).

Table 2
Gene sequence blast of *Ankistrodesmus falcatus* with retrieved sequences from GenBank

Species	*Voucher*	*Accession No.*	*Similarity (%)*
Ankistrodesmus falcatus	NFW1	KC852902	98
Podohedriella falcata		JN630515	94
Ankistrodesmus sp.	NDem 8 6/18 T-6d	AY846373	94
Ankistrodesmus fusiformis	SAG 2005	X97352	94
Ankistrodesmus sp.	Mary 8/18 T-2w	AY846372	94
Ankistrodesmus sp.	KMMCC 23	JQ315545	94

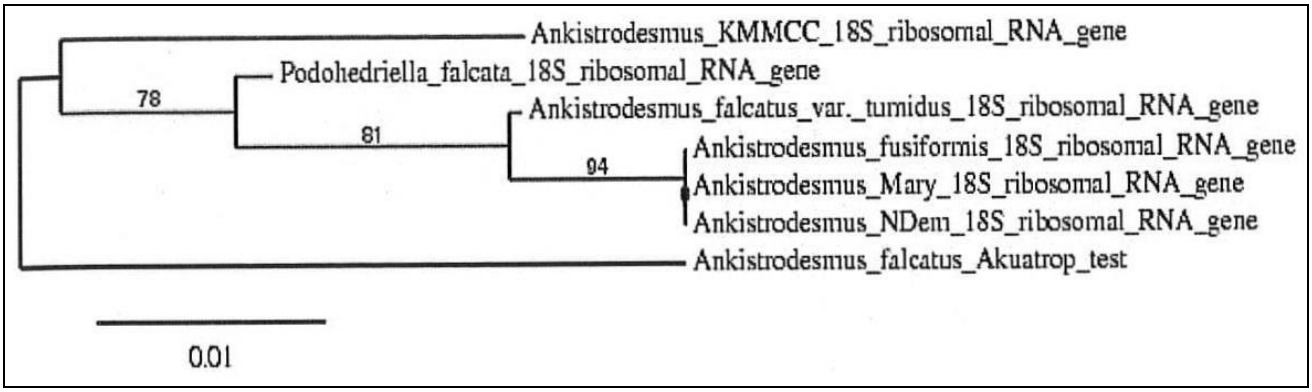

Figure 4. Divergence similarity with the top sequences ('*Ankistrodesmus falcatus* AKUATROP test' indicates the sequence from the current study).

Molecular data provided results that are more reliable and provide possible new insights into the phylogenetic relationship of different algae (Soylu et al 2012). In this study, PCR amplification and purification obtained a single band at a near 900bp mark (Figure 3), of which 975bp sequence was obtained. The species isolated was thereafter identified to be *Ankistrodesmus falcatus* with a maximum identity of 98% similarity observed with GenBank accession number KCB852902 in NCBI (2019) website (Table 2). Top sequences were aligned to see the divergence of evolution which indicated that 78% similarity was seen (Figure 4). *Podohedriella falcate* as a synonym of *A. falcatus* was observed with 97% maximum identity. Much more of an unlikely similarity was shown between *A. falcatus* and *A. fusiformis* which may probably need some review into their sequence and morphological characteristics to avoid any ambiguity in identification. In-depth, molecular analysis of the species can help clarify this taxonomic puzzle. A similar approach has been done by several researchers in the identification of other algae species (Krienitz et al 2001; Fawley et al 2004; Fawley et al 2006).

Conclusions. The current study observed that the water quality parameters of the Lake varied significantly when compared to specified standard. However, that does not seem to negatively affect the growth of the microalgae species *Ankistrodesmus falcatus* in this current study. Following the successful isolation of this indigenous macroalgae from the lake, future studies can focus on the optimization of the mass production techniques for the algae for different alternative uses.

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