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Molecular phylogenetic analysis of wild Tiger's milk mushroom (*Lignosus rhinocerus*) collected from Pahang, Malaysia and its nutritional value and toxic metal content

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Abstract

Tiger's Milk mushroom has been used for medicinal purposes by local aborigines to treat asthma, breast cancer, cough, fever and food poisoning. Molecular phylogenetic analysis utilizing RNA polymerase II, second largest subunit (RPB2) gene, identified the wild Tiger's Milk mushrooms collected from the state of Pahang in Malaysia for this study as *Lignosus rhinocerus* in the order Polyporales. The tuber, stipe and pileus of this mushroom were analyzed for their basic nutritional composition (fat, protein, and carbohydrate) and toxic metal content profile (Cadmium, Lead and Mercury). The moisture content of these mushroom parts varied from 32.22% (pileus) – 46.31% (stipe). The dry matter of the mushrooms contained 2.76% (stipe) – 6.60% (pileus) proteins, 0.21% (pileus) – 0.30% (tuber) fat, 1.76% (stipe) – 4.38% (tuber) ash and 38.47% (stipe) – 56.30% (pileus) carbohydrates. The toxic metal content of the mushroom samples ranged from 0.03–0.12 mg/kg for Cd, 0.80–1.94 mg/kg for Pb and 0.05–0.10 mg/kg for Hg. The present study demonstrated that *L. rhinocerus* is a potential source of food due to its high carbohydrate content. In addition, the trace levels of toxic metals in this mushroom are within the safe level for consumption.

Introduction

Tiger's Milk mushroom (*Lignosus* spp.) are native to tropical parts of the world and has been utilized as traditional medicine to treat asthma, breast cancer, cough, fever and food poisoning (Lee *et al.*, 2012). *Lignosus* spp. belong to the Polyporaceae family in the phylum Basidiomycota and comprise of six species; *L. dimiticus*, *L. ekombitii*, *L. goetzii*, *L. rhinocerus*, *L. sacer* and *L. hainanensis* (Ryvarden and Johnson, 1980; Núñez and Ryvarden, 2001; Douanla-Meli and Langer, 2003; Cui *et al.*, 2010). Tiger's Milk mushroom has three distinct parts: cap (pileus), stem (stipe), and tuber (sclerotium). They are polypores as the stem is centrally joined to the brownish woody cap that grows from a tuber in soil in humid environment. The irregularly shaped tuber has leathery flesh consisting of whitish mycelia (Chroma meter values are $L^* = 65.73$, $a^* = 3.76$, $b^* = 10.79$). In Malaysia, *Lignosus rhinocerus* is the most popular and specifically sought after medicinal mushroom by the *Semai aborigine* upon request by urban middlemen consisting mostly of local herbalists (Lee *et al.*, 2009).

In a recent report, the phylogenetic of wild Tiger's Milk mushroom collected from Perak was studied using the RNA polymerase II second largest subunit (RPB2) gene (Sotome *et al.*, 2008). The RPB2 gene is an excellent tool for phylogenetic studies (Sun *et al.*, 2008) and this gene, which encodes the second large subunit of RNA Polymerase II, is highly conserved among eukaryotes and the Archae group (Matheny *et al.*, 2007). It encodes a protein with a modest rate of evolutionary change and its polypeptide sequence has been used for phylogenetic studies in green plants and Ascomycetes (Malkus *et al.*, 2006). In addition, Reeb *et al.* (2004) also highlighted the importance of protein-coding genes in resolving deep phylogenetic relationships.

Currently, local herbalists are collecting Tiger's Milk mushroom from the remote regions of the states of Pahang and Perak in Malaysia to accommodate the growing demand. Although this mushroom has been widely used and consumed as traditional medicine, information on its nutritional properties and toxic metals contents are scarce. Hence, this study is

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essential to mushroom growers and enthusiasts to understand the nutritional values and toxic metals content of wild Tiger's Milk mushroom. Raised awareness of the nutritional content and safety of this mushroom will promote the cultivation and consumption of this mushroom.

Materials and Methods

Mushroom materials

Matured Tiger's Milk mushrooms were collected from forest in Kuala Lipis, Pahang, Malaysia. Fresh Tiger's Milk mushrooms were divided into tubers, pilei and stipes and these parts weighed between 350–500 g. The separated tubers, pilei and stipes were then washed several times, blotted dry, sliced, oven-dried at 60°C, powdered in a blender and kept in airtight containers at 4°C prior to proximate and toxic metal analysis. DNA was extracted from the tuber using NucleoSpin® Plant II (Macherey-Nagel, GERMAN). A hundred milligrams of mycelial cells were washed using ethanol. Three hundred micro liter of PL1 buffer was added into the sample and homogenized followed by incubation for 10 min at 65°C. Then, 100 µL of chloroform was added followed by centrifugation for 15 min at 20,000 x g. The top aqueous layer was pipetted into a new 1.5 ml tube. The lysate then filtered using NucleoSpin® Filter and centrifuged at 11 000 x g for 2 min. DNA binding condition was adjusted by adding 450 µL of PC buffer after removing NucleoSpin® Filter. NucleoSpin® Plant II Column was placed in collection tube and 700 µL of sample was centrifuged at 11 000 x g for 1 min. Then, 400 µL of PW1 buffer was added into the column and centrifuged for 1 min at 110,000 x g. Flow through was discarded and 700 µL of PW2 buffer was added into column and centrifuged for 1 min at 110,000 x g. Flow through was again discarded and 200 µL of PW2 buffer was added into column and centrifuged for 2 min at 110,000 x g to remove wash buffer and dry the silica membrane completely. Next, NucleoSpin® Plant II Column was placed into a new tube. Fifty micro liter of PE buffer was pipetted onto the membrane and incubated for 1 min at 65°C and centrifuged for 1 min at 110,000 x g to elute the DNA. Further elution of DNA was performed by adding another 50 µL of PE buffer onto the membrane and incubated for 1 min at 65°C and centrifuged for 1 min at 110,000 x g.

DNA amplification and sequencing

The forward primer (LRRPB2C-F: 5' AGA CCC ACAAGGAGTTCA A 3') and reverse primer (LRRPB2C-R: 5' TGAGGTTGT CCC AGTTGT AT 3') were designed based on known RPB2 sequences in GenBank using Primer Premier (version 5.0) software. The protocols for polymerase chain reaction (PCR) were 1 cycle of 95ºC for 15 min followed by 35 cycles of 94ºC for 1 min, 53ºC for 1 min, 72ºC for 25 sec and 1 cycle of 72ºC for 10 min. PCR products were then purified using Wizard®SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA). Gel slice containing targeted amplicon was dissolved by adding 10 µL of Membrane Binding Solution per 10 mg of gel slice and incubated at 50-65°C until gel slice is completely dissolved. Then, SV Minicolumn was placed in collection tube and dissolved gel mixture was transferred to the Minicolumn assembly and incubated for 1 min at room temperature. Next, the Minicolumn assembly was centrifuged at 16000 x g for 1 min and flow through was discarded. Afterward, Minicolumn was reinserted into the collection tube and 700 µL of Membrane Wash Solution was added and centrifuged at 16000 x g for 1 min. Then, flow through was again discarded. Next, collection tube was emptied and recentrifuged for 1 min to remove residual ethanol. Subsequently, Minicolumn was transferred to new tube and added with 50 µL of Nuclease-Free Water to the center of the column. Minicolumn was incubated for 1 min followed by centrifuged at 16000 x g for 1 min. Then, Minicolumn was discarded and DNA was stored at -20°C and sent to sequence commercially at 1st BASE Pte Ltd (SINGAPORE).

Phylogenetic analysis

Multiple orthologous RPB2 sequences were retrieved using BLASTx (Altschul *et al.*,1990) with a minimum match cut-off value of $E \leq 10^{-107}$. A total of 10 unique taxa containing 215 characters were selected for further analyses. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) followed by visual inspection and manual adjustment. Phylogenetic analyses were conducted using MEGA version 5 (Tamura *et al*., 2011). Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method from distance matrixes calculated by the *Jones Taylor-Thornton* model. The robustness of the trees was evaluated by bootstrap analysis of 1000 random iterations. All trees were then viewed and edited using TREEVIEW 1.6.6 (Page, 1996) with *Termitomyces* sp. and *Tricholoma subareum* being rooted as outgroups.

Proximate and toxic metal analysis

Proximate composition which included percentage moisture, fat, crude protein, fibre, carbohydrate and ash was determined according

to the standard method of the Association of Official Analytical Chemists (AOAC, 2003). All proximate components were analyzed in triplicate and reported as mean on percentage of dry weight basis. Determination of moisture: Moisture content was determined by oven drying method. An amount of 1.5 g sample was accurately weighed in a clean, dried crucible (W_1). The crucible and its contents were placed in an oven at 100-105°C for 6-12 hr until a constant weight was obtained. Then the crucible and its contents were placed in desiccator for 30 min to cool and weighed again (W_2). The moisture percentage was calculated by the following formula:

$$\text{Moisture percentage} = \frac{W_1 - W_2 \times 100}{\text{Wt. of sample}}$$

Where
W_1 = Initial weight of crucible + Sample
W_2 = Final weight of crucible + Sample
Note: Moisture free samples were used for further analysis.

Determination of ash: For the determination of ash, clean empty crucible was placed in a muffle furnace at 600°C for an hour, cooled in desiccator and then weight of empty crucible was noted (W_1). One gram of moisture free sample was weighed in the crucible (W_2) and samples were ignited over a burner with the aid of blowpipe, until charred. Then, the crucible was placed in a muffle furnace at 550°C for 2-4 hr. The crucible was then cooled and weighed (W_3) and the percentage of ash was calculated by the following formula:

$$\text{Ash percentage} = \frac{\text{Difference in Wt. of Ash} \times 100}{\text{Wt. of sample}}$$

Difference in wt. of Ash= $W_3 - W_1$

Determination of crude protein: Protein in the sample was determined by Kjeldahl method. Dried samples (1.0 g) were placed in digestion flasks. Fifteen milliliters of concentrated sulphuric acid was added together with 8 g of digestion mixture of potassium sulphate and copper sulphate i.e. K_2SO_4 : $CuSO_4$ (8: 1). The flask was swirled to mix the contents thoroughly then heated to start digestion until the mixture become clear (blue green in color). The digest was cooled and transferred into 100 ml volumetric flask and volume was made up to mark by the addition of distilled water. Distillation of the digest was performed in Markam Still Distillation Apparatus (Khalil and Manan, 1990). Ten milliliters of digest was introduced in the distillation tube and 10 ml of 0.5 N NaOH was gradually added through the similar approach. Distillation was continued for at least 10 min and NH3 produced was collected as NH_4OH in a conical flask containing 20 ml of 4% boric acid solution with a few drops of modified methyl red indicator. During distillation yellowish color appears due to NH_4OH. The distillate was then titrated against standard 0.1N HCl solution till the appearance of pink color. A blank was also run through all steps as above. Percentage of crude protein content of the sample was calculated by using the following formula:

$$\text{Percentage of crude Protein} = 6.25^{*} \times \%N \quad (^{*}\text{. Correction factor})$$

$$\text{N percentage} = \frac{(S-B) \times N \times 0.014 \times D \times 100}{\text{Wt. of the sample} \times V}$$

Where
S = Sample titration reading
B = Blank titration reading
N = Normality of HCl
D = Dilution of sample after digestion
V = Volume taken for distillation
0.014 = Milli equivalent weight of Nitrogen

Determination of crude fat: Crude fat was determined by ether extract method using soxhlet apparatus. Approximately 1 g of moisture free sample was wrapped in filter paper, placed in fat free thimble and then introduced in the extraction tube. The receiving beaker was weighed, cleaned, dried and filled with petroleum ether and fitted into the apparatus. Next, extract was transferred into clean glass for washing and then placed in an oven at 105°C for 2 hr and cooled in a desiccator. The percentage of crude fat was determined by using the following formula:

$$\text{Crude fat percentage} = \frac{\text{Wt. of ether extract} \times 100}{\text{Wt. of sample}}$$

Determination of crude fibre: Sample was weighed (0.2 g) (W_0) and transferred to porous crucible. Then the crucible was placed into Dosi-Fibre unit and the valve kept in "OFF" position. Then, 150 ml of preheated H_2SO_4 solution and some drops of foam-suppresser were added to each column before heating at 90% power. When it started boiling, the power was reduced to 30% and left for 30 min. Valves were opened for drainage of acid and rinsed with distilled water thrice to completely ensure the removal of acid from sample. The same procedure was used for alkali digestion by using KOH instead of H_2SO_4. Afterward, samples were dried in an oven

at 150°C for 1 h and allowed to cool in a desiccator and weighed (W_1). Then, the samples in crucibles were placed in a muffle furnace at 55°C for 4 hrs, allowed to cool in a desiccator and weighed again (W_2). Calculations were done using the formula:

$$\text{Crude fibre percentage} = \frac{W_1\text{-}W_2 \times 100}{W_0}$$

Determination of carbohydrate components: Soluble sugar was extracted three times from 2 g samples with 80% ethanol using soxhlet extractor and refluxed for 2 hr as described by Bainbridge *et al.* (1996). Reducing sugar was determined from the ethanolic extract by the ferricyanide method (AOAC, 2003). Two mililiters of extract was added into 8.0 ml of the ferricyanide reagent and the absorbance was read at 380 nm using glucose as standard. The total sugar content of the samples was determined by hydrolyzing 25 ml of the sugar extracted above in 100 ml beaker using 5 ml concentrated hydrochloric acid as described earlier (Bainbridge *et al.*, 1996). Total sugar was then determined on 2 ml of the hydrolysate using ferricyanide method. The starch content of the samples was determined on 200 mg residue of the ethanolic extract obtained above by refluxing the residue with 0.7M HCl for 2.5 h. The acid hydrolysate was neutralised, made up to volume in 500 ml standard flask with distilled water and then filtered through a Whatman No. 541 filter paper. The starch in the original sample was then determined as reducing sugar, using the ferricyanide method described above. The reducing sugar was then converted to starch content using the AOAC (2003) equation.

Determination of toxic metals: Dried mushroom samples (500 mg) were introduced into the digestion vessels together with 3 ml nitric acid and 5 ml hydrogen peroxide. After 40 min of digestion, the vessels were cooled to room temperature for 30 min. The clear solution volume was made up to 50 ml for each sample using deionized water. Then, samples were submitted to atomic absorption spectrophotometer (AAS) (GBC906A, Australia) to determine the content of Cd and Pb. Additionally, total mercury in samples of mushroom was determined using Advanced Mercury Analyzer (AMA 254) without the need for chemical pretreatment of the sample. Determination of heavy metal concentrations in mushrooms and their substrate were performed using the method of calibration curve according to the absorber concentration (Wagner, 1998; Sperling and Welz, 1999).

Results and Discussion

The phylogenetic relationship of the collected wild Tiger's Milk mushroom was studied by comparing amino acid sequences of RPB2 gene of this mushroom with previously published orthologous sequences from other fungi species. The generated NJ tree (Figure 1) indicated that the sample collected from Pahang (GenBank accession number: JN088735) is closely related to the *L. rhinocerus* (GenBank accession number: AB368132) sample collected from Perak, suggesting that these two mushroom samples are geographical similar (99% bootstrap confidence support). The topology of the NJ tree generated from these sequences supported previously described taxonomic relationships associating the *Lignosus* sp., which is more closely related to the *Polyporus* sp. than to those in a more distinct cluster such as *Ganoderma* sp. (Sotome *et al.*, 2008). Some members of the Polyporales such as *Ganoderma lucidum*, *Grifola frondosa* and *Trametes versicolor* are commercially cultivated and marketed for their potential as traditional and complementary medicine.

The proximate compositions of *L. rhinocerus* are presented in Table 1. The results of the proximate analysis of *L. rhinocerus* show that the mushroom is rich in protein, fat, carbohydrate, ash and moisture. This agrees with Moore and Chi (2005) that mushrooms has high nutritional attributes and potential applications in food industries. Crude protein content of *L. rhinocerus* (Table 1) was highest in the pileus (6.60%), followed by tuber (3.04%) and stipe (2.76%). These findings seem to be consistent with Vetter and Rimoczi (1993), which reported that pileus of *Pleurotus ostreatus* contained the greatest protein concentration. However, the distribution of proteins within a fruiting body and changes in protein content during the development of a fruiting body still remain unclear.

Carbohydrate content (pileus =56.30% and tuber =51.30%) and moisture content (pileus =32.22%, stipe =46.31% and tuber =39.82%) in *L. rhinocerus* were higher compared to *Ganoderma* spp. (Aremu *et al*., 2009) and *Pleurotus tuber-regium* (Ezeibekwe *et al*., 2009). The high moisture content of *L.rhinocerus* indicates that this mushroom is highly perishable (Table 1). Hence, great care must be taken in their handling and presentation as high moisture contents promote microbial growth. Fat content of *L. rhinocerus* was generally low, ranging from 0.21% in pileus, 0.27% in stipe and 0.46% in tuber (Table 1). It is encouraging to compare these figures with

Table 1. Nutrient profile (g/100 g dry matter, mean ± SD[a]) of *Lignosus rhinocerus*

	Tuber	Pileus	Stipe
Carbohydrate	51.30±0.10	56.30±0.26	38.47±0.49
Moisture	39.82±2.22	32.22±5.54	46.31±14.00
Protein	3.04±0.59	6.60±1.38	2.76±0.60
Fat	0.46±0.32	0.21±0.09	0.27±0.07
Ash	4.38±2.71	1.77±0.28	1.76±0.93

Table 2. Concentrations of toxic metals content in wild *Lignosus rhinocerus* (mg/kg) (mean ± SD[a])

Element	Tuber	Pileus	Stipe
Mercury (Hg)	0.05±0.03	0.10±0.08	0.07±0.06
Cadmium (Cd)	0.06±0.03	0.12±0.10	0.03±0.01
Lead (Pb)	1.03±0.01	1.94±0.03	0.80±0.29

[a]Mean ± SD (n = 5)

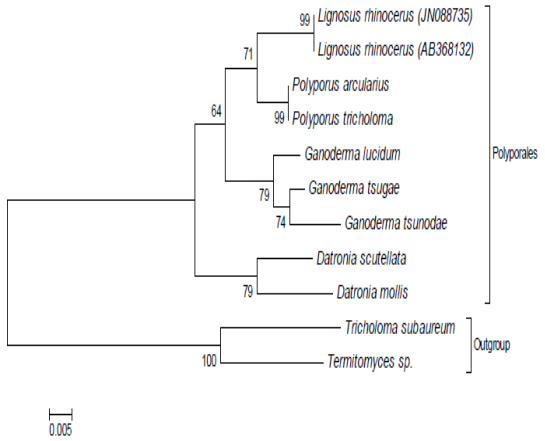

Figure1. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method from distance matrixes calculated by the *Jones Taylor-Thornton* model.

those of Aletor (1995) for various species of edible mushrooms and that of Ogundana and Fagade (1982) for *P. tuber-regium*. This finding, while preliminary, suggests that *L. rhinocerus* could be suitable in weight restriction diets because it contain high carbohydrate with low fat content.

When the nutritional value of mushrooms is evaluated, perhaps the most important factor is their dry matter/moisture content, which directly affects the nutrient content of mushrooms (Mattila *et al.*, 2002). Fresh mushrooms contain 5–15% dry matter (Kurzman, 1997) while ash content of mushrooms is usually 5–12% of dry matter (Kalac, 2009). Present ash values of *L. rhinocerus* (1.76 – 4.38%) are lower compared to *Russula delica* (5.61%), *Cantharellus cibarius* (9.44%), *P. tuber-regium* (7.25%), *Auricularia auricula* (5.84%) and *Ganoderma* spp. (7.8%) (Ouzouni *et al*., 2007; Aremu *et al.*, 2009; Ezeibekwe *et al*., 2009). Suggesting *L. rhinocerus* contain low mineral content. This variability is dependent on the mushroom species and other parameters such as environmental temperature, relative humidity during growth and proportional amount of metabolic water that may be produced or utilized during storage (Crisan and Sands, 1978).

Wild mushroom consumption has increased in recent years due to their delicate flavour and texture as well as their high content of trace minerals (Ouzouniet *et al*., 2007). Therefore, it is necessary to investigate the metal content in wild species, given the fact that many of them are known to accumulate high levels of heavy metals such as cadmium, mercury, lead and copper (Kalac and Svoboda, 2000). Thus, it is crucial to investigate the bioaccumulation of toxic metals (Hg, Cd and Pb) in wild *L. rhinocerus* that grow in the soil.

Mercury (Hg) concentrations in tuber, stipe and pileus of wild *L. rhinocerus* were between 0.05 – 0.10 mg/kg, cadmium (Cd) concentrations between 0.03–0.12 mg/kg and lead concentrations between 0.80 –1.94 mg/kg (Table 2). Generally, mushrooms can be very rich in cadmium (Demirbas, 2001). Cadmium is a by-product of the production of zinc (Zn) and lead (Pb), and the pyrometallurgical production of zinc is the most important anthropogenic source in the environment. Other major sources are fossil fuel combustion and waste incineration. Cadmium is known as a principal toxic element, since it inhibits many life processes (Vetter, 1993) and cadmium accumulation in fungi has been demonstrated by Schmitt and Meisch (1985). Current cadmium levels in *L. rhinocerus* are lower than other previously studied mushrooms (Demirbas, 2001; Kalac and Svoboda, 2000; Rudawska and Leski, 2005; Sesli and Tuzen, 1999).

Many studies support the connection between mercury levels and the concentration of humic matter (Demirbas, 2001). The mean mercury level in macrofungi surpasses, by two orders of magnitude, that of green plants (green plants: 0.015 ppm; macrofungi: 1–1.5 ppm) and varies according to the type of fungi. Current mercury levels in *L. rhinocerus* are lower than terrestrial saprophytic species and mycorrhizal except for the concentration of *Lepista nuda* stalk (0.17 ± 0.08 to 0.72 ± 0.20 µg/g) (Melgar and García, 2009). Although the mechanism of mercury accumulation is not clearly understood, Melgar and García (2009) suggested the elements of genotype, environment and soils could be factors underlying the accumulation of mercury in wild mushrooms. On the other hand, heavy metal concentrations in the mushroom are hardly affected by pH or organic matter content of the soil (Sesli and Tuzen, 1999; Demirbas, 2002).

Lead is especially toxic to the growing brain and can affect the behavioral development of the young, even at low concentrations (Demirbas, 2001). The comparison of current lead levels (0.84 mg/kg) in the

stipe of *L. rhinocerus* to 60 species of common edible mushrooms in Italy exhibited higher lead levels in *L. rhinocerus* compared to *Amanita ovoidea* (0.57 mg/kg), *Amanita rubescens* (0.70 mg/kg), *Boletus aestivalis* (0.74 mg/kg), *Boletus appendiculatus* (0.64 mg/kg), *Boletus erythropus* (0.74 mg/kg), *Clitocybe geotropa* (0.68 mg/kg), *Cortinarius praestans* (0.70 mg/kg), *Coprinus comatus* (0.80 mg/kg), *Hygrophorus penarius* (0.83 mg/kg), *Morchella esculenta* (0.74 mg/kg), *Xerocomus chrysenteron* (0.54 mg/kg), and *Xerocomus dryophilus* (0.69 mg/kg) (Cocchi *et al.*, 2006). Current result suggested the Lead content in *L. rhinocerus* is in safe range (< 2.0 mg/kg) when compared to European Union Regulation for maximum levels of permissible contaminants in foodstuff especially mushroom (Sembratowicz and Rusinek-Prystupa, 2012).

Conclusion

Present results demonstrated that the wild Tiger's Milk mushrooms collected from the state of Pahang in Malaysia for this study were *Lignosus rhinocerus*. It contained high levels of carbohydrates, which indicate this mushroom as a potential energy source. In addition, due to their low toxic metals content, it is suggested that wild *L. rhinocerus* are safe for consumption. Current findings add substantially to our understanding of nutritional and toxic metal contents of wild *L. rhinocerus*.

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