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RAPID DISCRIMINATION BETWEEN SIX *RUSSULA* (RUSSULALES, AGARICOMYCETES) SPECIES USING HIGH RESOLUTION MELTING ANALYSIS

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Abstract

The genus *Russula* is a group of edible and/or medicinal fungi with high economic and scientific value. However, some species of this genus are poisonous. In the market, this genus has been commercialized throughout various markets and commonly sold in processed forms such as dried material, making it almost impossible to accurately identify the original species. Recently, the evidence of hepatotoxic effects linked to use of *Russula* were reported and is now seriously concern. There is a need to find an approach that could help with species identification to ensure the safety use of this genus. In this research work, High Resolution Melting (HRM) assay, targeting a fragment of the Internal Transcribed Spacer 2 (ITS2) gene, was developed to authenticate six common species of *Russula*. The results showed that ITS2 regions were successful in the species-specific identification. Therefore, ITS2 DNA loci coupled with HRM method can be applied as routine tests for the verification of *Russula* species to protect consumers from health risks due to the toxic adulterants.

Russula, one of the two major groups of Russulaceae, with more than 770 species occurring mostly in temperature forests (Kirk *et al*. 2008). Some species of this genus have been harvested, consumed, and exported as an exotic food and/or medicine for many years. Modern pharmacological research has demonstrated that these species exhibit multiple therapeutic activities, including antitumour, antihypertensive, antiviral and immunomodulatory activities (Nandi *et al*. 2014). However, some species of this genus are poisonous (Saviuc and Danel 2006). Evidence of hepatotoxic effects linked to use of *Russula* were reported and is now seriously concern (Chen *et al*. 2014). In southern China, several species of this genus like *Russula abietina*, *R. alutacea*, *R. azurea*, *R. emetica*, *R. exalbicans*, *R. griseocarnosa*, *R. olivacea*, *R. Paludosa*, *R. rosea*, *R. sanguinea*, *R. sardonia*, *R. vesca*, *R. xerampelina*, etc. are commonly called ‘‘Red Mushroom’’ by the local residents (Yang and Piepenbring 2004, Li *et al*. 2010). Due to the similarity in morphologies and nomenclature, it is almost impossible for consumer to know exactly which ‘‘Red Mushroom’’ products they are buying, so this could be a potential health risk to the end users. There is a need to find an approach that could help with species identification to ensure the safety use of these fungi.

The ITS2 region of the nuclear ribosomal DNA is the formal DNA barcoding region for molecular identification of fungi due to its high degree of interspecific variability, conserved primer sites and multicopy nature in the genome (Schoch *et al.* 2012). Thus far, the ITS2 region has been shown to be able to discriminate a wide range of eukaryotes, including animals, plants and fungi (Coleman 2007, Miao *et al*. 2008, Keller *et al*. 2009, Samaga *et al*. 2014). In recent years, the rapidity and effectiveness of DNA barcoding have been advanced by HRM analysis.

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HRM has been widely used in medical research to detect cancer gene mutations and characterize clinical pathogens (Akiyoshi *et al*. 2013, Lin *et al*. 2014). This method also has been effectively used in the agricultural industry to identify and classify poultry pathogens and to assess the purity of commercial food products (Madesis *et al*. 2012, Sakaridis *et al*. 2013). So far, its application has never been exerted as a tool for species identification of fungi. Here, we report on the establishment and application of HRM analysis for reliably discriminating six *Russula* species through the use of a nuclear ITS2 gene region.

An experiment was conducted with five commonly used edible and/or medicinal *Russula* species (*R. azurea*, *R. griseocarnosa*, *R. rosea*, *R. sanguinea* and *R. xerampelina*) and one medicinal and poisonous *Russula* species (*R. emetica*). Fresh specimens of these species were collected from a local market in Yulin city, Guangxi province of China. All the samples were desiccated in silica gel. The mushroom materials were ground with liquid nitrogen, and 50 mg of fine powder was then used for DNA extraction with the plant genomic DNA extraction kit (Tiangen Biotech Co., China) following the manufacturer's instruction. PCR and HRM amplifications were performed according to Sun *et al*. (2016). The Rotor-Gene Q MDx proprietary software (version 2.3.1) was used to genotype the different species. In particular, the negative derivative of fluorescence (F) over temperature (T) (dF/dT), the normalized original curve depicting the decreasing fluorescence versus the increasing temperature, and difference curves were evaluated. In addition, a two-step procedure was used to assess the similarity of unknown HRM curves in reference to prior studies (Li *et al* 2016, Song *et al* 2016). Finally, each species was set as the "genotype" (reference species), and the average HRM genotype confidence percentages (GCPs; the value assigned to each species being compared with the genotype, with value of 100 indicating an exact match) for the replicates (disregarding the most outlying replicate) were calculated.

The melting characteristics of ITS2 amplicons from all voucher samples were assessed by plotting two different curves (Figs 1 - 2). As shown in Fig. 1, the barcode marker ITS2 was specific to these species with different melting profiles, making them easily distinguishable. Improved visualization and separation of variant melting curves from each species were demonstrated in the difference plot melt curves (Fig. 2). By assigning the *R. emetica* as the reference genotype, revealed part of the curve sitting outside the 90% confidence interval (CI) curve, all samples were successfully genotyped. Finally, with the mean *R. emetica* curve as the baseline, suggesting that all the examined species via the HRM curves are indeed different (Fig. 2).

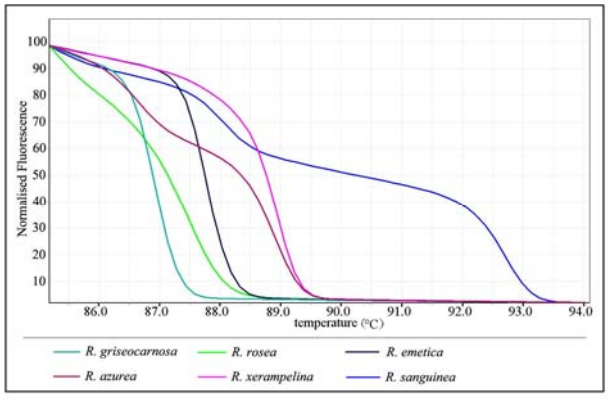

Fig. 1. The normalized melting curve graph of ITS2 region of six *Russula* species. Fluorescence is plotted against degrees Celsius (°C).

Russula fungi are an essential element in human diet and medicinal product, both directly and indirectly. However, incidents of poisoning in recent years in China have led to concerns over the toxicity of this species. A reliable identification of *Russula* species, as well as their origin and traceability, are key elements in the fields of food and medicine safety. DNA barcoding coupled with HRM technology is effective in certifying both origin and traceability of raw materials, and to detect adulterations (e.g. by mixing products from different taxa) occurring in the industrial food and medicine chains. The advantage of this method is that its performance is strongly in fluenced by the molecular variability of the organisms, and a high level of resolution is achieved when an organism has low intraspecific polymorphism, making it well distinguishable from closely related taxa (Casiraghi *et al*. 2010). An additional advantage is this method uses the melting curve changes caused by the release of an intercalating DNA dye from a DNA duplex denatured by increasing temperature, it does not require electrophoretic separation or additional handling because the whole analysis is conducted in a closed tube system.

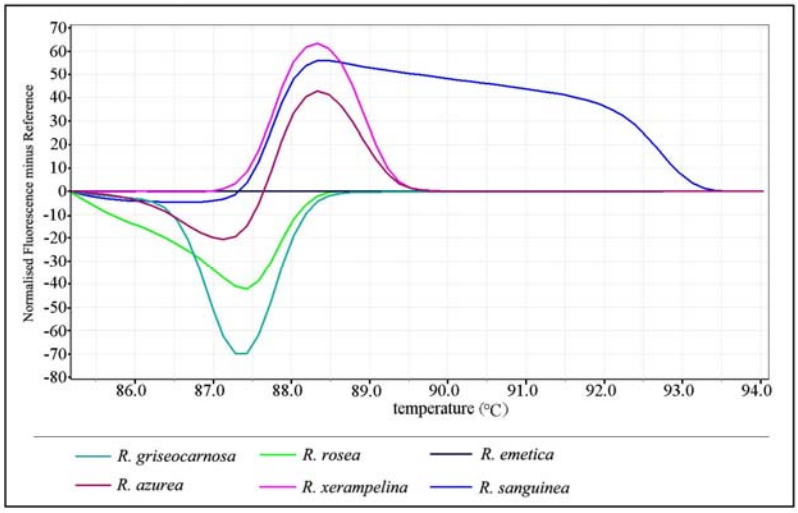

Fig. 2. Representative profiles of the melting curves (difference plot curves) of ITS2 amplicons for six *Russula* species using *R. emetica* as reference genotype.

In conclusion, HRM analysis was proven to be a fast and accurate closed tube post PCR method which permited the identification of *Russula* species via the use of ITS2 regions. This method was not only useful for identification of *Russula* fungi vouchers, but can also be used for species discrimination, authentication, and detection of adulteration in samples lacking diagnostic morphological characters.

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