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Application Note

DNA Barcode *ITS* Effectively Distinguishes the Medicinal Plant *Boerhavia diffusa* from Its Adulterants

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Abstract

Boerhavia diffusa (*B. diffusa)*, also known as Punarnava, is an indigenous plant in India and an important component in traditional Indian medicine. The accurate identification and collection of this medicinal herb is vital to enhance the drug's efficacy and biosafety. In this study, a DNA barcoding technique has been applied to identify and distinguish *B. diffusa* from its closely-related species. The phylogenetic analysis was carried out for the four species of *Boerhavia* using barcode candidates including nuclear ribosomal DNA regions *ITS, ITS1*, *ITS2* and the chloroplast plastid gene *psbA-trnH.* Sequence alignment revealed 26% polymorphic sites in *ITS*, 30% in *ITS1*, 16% in *ITS2* and 6% in *psbA-trnH*, respectively. Additionally, a phylogenetic tree was constructed for 15 species using *ITS* sequences which clearly distinguished *B. diffusa* from the other species. The *ITS1* demonstrates a higher transition/transversion ratio, percentage of variation and pairwise distance which differentiate *B. diffusa* from other species of *Boerhavia.* Our study revealed that *ITS* and *ITS1* could be used as potential candidate regions for identifying *B. diffusa* and for authenticating its herbal products.

Keywords: Adulterant; *Boerhavia diffusa*; *ITS*; DNA barcoding; Punarnava

Introduction

Boerhavia is one of the highly polymorphic genus in Nyctaginaceae family [1]. About 40 species are distributed in tropical, subtropical and temperate regions. Among these, 6 species are reported in India and *Boerhavia diffusa (B. diffusa)* is indigenous [2]. *B. diffusa* is described as Punarnava by an Indian system of medicine, Ayurveda [3]. Roots and whole plants of *B. diffusa* are used in the Ayurvedic and Unani systems of medicine in Arabian countries [4] and many tribal communities in India still use it for the treatment of jaundice and various other liver disorders. It has anti-inflammatory, diuretic, fibrinolytic, anti-convulsant properties [5–8] and also used as carminatives [9–10]. The two pharmaceutically important alkaloids, Punarnavine-1 and Punarnavine-2, belonging to the group of quinolizidine were separated from *B. diffusa* [11–12].

B. diffusa is known to be extensively adulterated with other species like *Boerhavia erecta*, *Boerhavia repanda*, *Boerhavia coccinea* and *Boerhavia verticillata*. *B. diffusa* have taxonomical conflicts with *B. coccinea*, *Boerhavia repens*, *Boerhavia tetranda* and *Boerhavia albiflora*, making it difficult to distinguish from one another [13–14]. The species *B. verticillata* display similar morphological features and phytochemical properties with *B. diffusa*, but they differ by their habits [15]. Determination of plant specimens by DNA barcodes will be an effective, reliable and simple pharmacognostic tool to resolve the confusion in morphological identification. Due to different rates of evolution, nuclear ribosomal internal transcribed spacer (*ITS*) regions have become the routine marker in evolutionary studies at different taxonomic levels [16,17]. There is a report using the chloroplast intergenic spacer *psbA-trnH* for identifying

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the *Dendrobium* species of Chinese pharmacopoeia and *psbA-trnH* is recommended as an ideal DNA barcode candidate [18]. Recently the sequence variations are used to develop specific markers for the identification and authentication of drugs and herbal formulations [19].

The objective of the present study is to evaluate an ideal barcode candidate for distinguishing and authenticating the species *B. diffusa* from its common adulterants.

Results

Genomic DNA was isolated from the species of *B. diffusa*, *B. repanda*, *B. erecta* and *B. verticillata* and used for PCR amplification of the *ITS* and *psbA-trnH*. The obtained sequences were submitted to GenBank. The size and accession number for the gene *ITS* and *psbA-trnH* is shown in Table S1. Additionally, *ITS* sequences from 11 species of *Boerhavia* were taken from the GenBank (Table S2) and used for sequence alignments.

Multiple sequence alignment and pairwise alignment analysis were performed for nuclear *ITS* and chloroplast *psbA-trnH* (Figure S1). The *ITS* region consists of *ITS1, 5.8S rDNA* and *ITS2*. The ribosomal sites of *5.8S rRNA* and *28S rRNA* are highly conserved. The regions *ITS1* and *ITS2* were compared by multiple sequence alignment, where *ITS1* showed more variation than *ITS2*. Phylogenetic analysis using *ITS1* and *ITS2* indicated *B. diffusa* and *B. erecta* in the same clade while *B. verticillata* and *B. diffusa* was shown in the same clade when using *psbA-trnH* region for phylogenetic analysis (**Figure 1**). The tree also constructed using *ITS* region clearly distinguished the morphologically similar species *B. diffusa* from the 14 other species of *Boerhavia* as shown in the **Figure 2**.

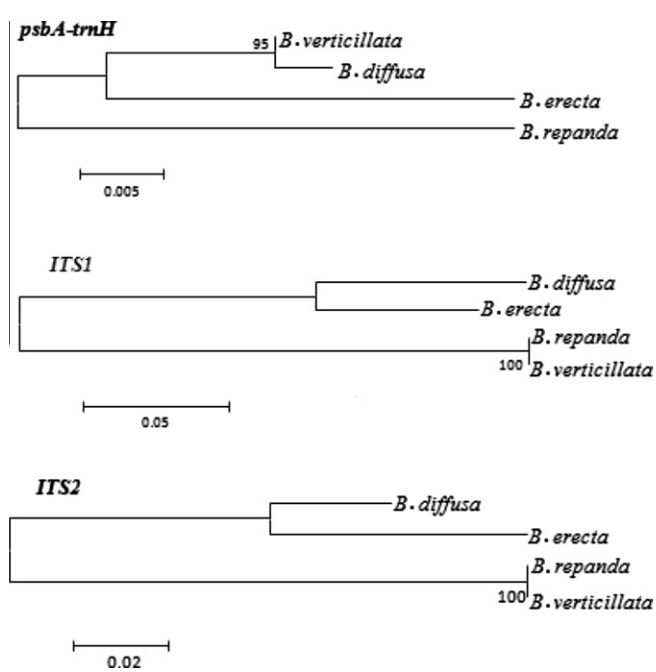

Figure 1 Phylogenetic trees of the four *Boerhavia* species constructed using *ITS1, ITS2* and *psbA-trnH*
Phylogenetic trees were constructed by Minimum Evolution method using *ITS1, ITS2* and *psbA-trnH*, respectively, for the four species of *Boerhavia*, including *B. diffusa*, *B. erecta*, *B. repanda* and *B. verticillata*.

We further analyzed the nucleotide variations of *ITS* and *psbA-trnH* between different species. Percentage of variation shown in **Figure 3** indicated that *ITS* demonstrated higher inter-specific divergence. The Wilcoxson rank test indicated significant variation between the species for *ITS1* when compared to *ITS2* and *psbA-trnH*. BLAST 1 and distance method also indicated that *ITS1* showed higher identification percentage at species level (**Table 1**).

Discussion

Recent molecular methods like DNA barcoding have been extensively used for species identification, diversity, forensic medicine and ecological studies [20–21]. It also plays an important role in the identification of traditional medicinal herbs. *ITS2* has been effectively used in differentiating morphologically similar species like *Swartzia grandifolia* and *Swartzia longicarpa* and also in solving the controversial species *Caranga rosea* and *Caranga sinica* of the family Fabaceae [22]. Medicinal plant species like the family Polygonaceae [23] and the genus *Dendrobium* [24] have been identified using *ITS2* region. In addition, *ITS1* was used to demonstrate that species of *Amomum villosum* belongs to the family Zingiberaceae [25].

In our study, multiple sequence alignment of *ITS1* and *ITS2* from four *Boerhavia* species showed that *B. diffusa* had a unique basepair variation, which can distinguish it from the other three species, despite the fact that they share many morphological similarities. On the other hand, although *psbA-trnH* distinguishes some species of Polygonaceae [18], less sequence variation in *psbA-trnH* was revealed among the four species of *Boerhavia.* This result is consistent with a previous report that *psbA-trnH* does not show any variation for closely-related Cycad species [26]. Hence, *ITS1* may be a better barcode region for distin-

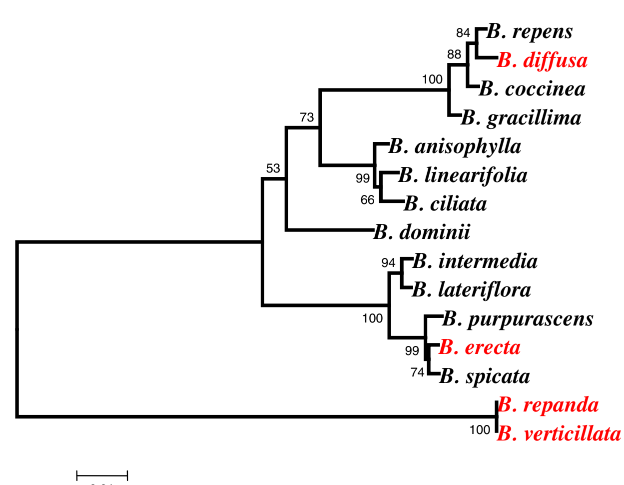

Figure 2 Phylogenetic tree of the 15 *Boerhavia* species constructed using *ITS*
Phylogenetic tree was constructed by Minimum Evolution method for the 15 species of *Boerhavia* using *ITS* region.