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SHORT COMMUNICATION

Evaluation of species limits of *Hortonia* by DNA barcoding

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Abstract: A study was carried out to determine the species limits of the endemic genus *Hortonia* by DNA barcoding with a view to establishing conclusive molecular evidence regarding the speciation of *Hortonia* in Sri Lanka. Total DNA was extracted from all three species and the purity of the extracted DNA was confirmed. Internal transcribed spacer (ITS) region and the *trnH-psbA* region were first amplified by the polymerase chain reaction (PCR) with specific primers and ligated to a pBlueScript vector followed by plasmid purification. The purified plasmids containing the DNA of interest were subjected to sequencing. Sequence homology of ITS and *trnH-psbA* from all three species were compared using MacVector software. Between *H. ovalifolia* and *H. floribunda,* the ITS region showed a 2.37 % sequence divergence and the *trnH-psbA* region showed a 1.5 % sequence divergence. Between *H. ovalifolia* and *H. angustifolia,* the ITS region and *trnH-psbA* region showed a 3.36 % and 1.89 % sequence divergences, respectively. The percentage sequence divergence between ITS and *trnH-psbA* regions of *H. floribunda* and *H. angustifolia* were 3.36 % and 2.65 %, respectively. The high sequence divergence values clearly indicate that the genus *Hortonia* has three different species. Considering the percentage sequence divergence values, *H. ovalifolia* and *H. floribunda* are more closely related to each other than to *H. angustifolia*.

Keywords: Barcoding, *Hortonia*, ITS, sequence divergence, *trnH-psbA*.

INTRODUCTION

Despite its relatively small size, Sri Lanka possesses a high level of biodiversity because of its topographic and climatic heterogeneity as well as its coastal influence. A noteworthy feature of Sri Lanka's biodiversity is the remarkable high proportion of endemic species among its flora and fauna. In the island's flora, the highest species diversity is recorded among the flowering plants. The endemic plant species diversity comprises 927 or 28 % of the flowering plants, of which 60 % are found in the lowland Wet Zone (Gunatilleke *et al*., 2008).

Sri Lanka is currently experiencing a tremendous increase in novel descriptions of its endemic diversity. *Hortonia* is a genus endemic to Sri Lanka, belonging to the family Monimiaceae Juss., which had possible origins in Gondwanaland about 100 −120 million years ago (Jayasekara, 1997). *Hortonia* is considered as a surviving genus of the ancestral plants from which the Monimiaceae alliance has evolved. Plants of this family are trees or shrubs and rarely climbers, and it comprises about 39 genera, where 440 species are widely spread in the tropical and subtropical regions of America (Leitao *et al*., 2000). This family occurs in Sri Lanka, Oceania, Polynesia, Australia, Malaysia, Madagascar and South America (Leitao *et al*., 2000). It is very rare in Africa and not reported from India.

Hortonia has had several taxonomic treatments: Wight (1853) considered three species (*H. angustifolia, H. ovalifolia, H. acuminata*); Hooker and Thomson (1855) described only one species, namely, *H. floribunda* while keeping *angustifolia* and *acuminata* as varieties; Thwaites (1864) too considered *H. floribunda* and placed *angustifolia*, *ovalifolia* and *acuminata* as varieties; a subsequent study described *H. floribunda* with *ovalifolia* as a variety and *H. angustifolia* with *angustifolia* and *acuminata* as varieties (Trimen, 1895). Later, *Hortonia* was considered as one species during a study on the phylogenetic affinities of the Monimiaceae based on cpDNA gene and spacer sequences. These data were

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the basis of Renner's assertion that *H. ovalifolia* and *H. angustifolia* were identical with *H. floribunda* (Renner, 1998).

The most recent revision of the family, however, recognizes three species of *Hortonia*; *H. ovalifolia, H. floribunda* and *H. angustifolia* (Dassanayake, 1996). Leaf morphology has been used as the main character to key the three species. According to this revision, leaves elliptic, rounded at base and apex belong to *H. ovalifolia*, leaves lanceolate to narrowly ovate and major veins arched belong to *H. floribunda* and leaves narrowly lanceolate to narrowly elliptic with major veins parallel to margin belong to *H. angustifolia*. In terms of the conservation status, *H. ovalifolia* is endangered while *H. angustifolia* is critically endangered (IUCN, 2007), and according to our studies, *H. floribunda* also has become increasingly difficult to locate (*unpublished data*). A study was undertaken to investigate the phytochemistry of all three species, based on the near identity of TLC patterns and the mosquitolarvicidal activity of the leaf extracts of the three species. Previously, the authors have reported the isolation of several new butenolides with mosquitolarvicidal activity from the leaves of all three species (Ratnayake *et al*, 2001, 2008a, 2008b). It was concluded from these studies that the three species were phytochemically very similar. In another attempt Yakandawala and Rubasinghe (2003) re-evaluated the species limits of *Hortonia* using a large number of morphological characters and concluded the presence of three species*, H. ovalifolia, H. floribunda* and *H. angustifolia.* With a view to establishing conclusive molecular evidence regarding the speciation of *Hortonia* in Sri Lanka, the work described herein aimed to differentiate the species using DNA barcoding.

The surge in the application of molecular biology information to answer systematic and evolutionary questions has resulted in significant contributions to both plant and animal systematics. Among the different approaches used in molecular systematics, DNA sequencing has become one of the most widely used, particularly for species identification and discrimination among closely related species. DNA barcoding is a technique in which species identification is performed by using DNA sequences from a small fragment of the genome, the DNA barcode.

METHODS AND MATERIALS

Leaf samples of *H. angustifolia*, *H. floribunda* and *H. ovalifolia* were collected from Kanneliya, Hakgala Botanic Gardens and the Adams Peak area in September 2009. The plants were identified at the National Herbarium of Royal Botanic Gardens, Peradeniya where voucher specimens are kept and the leaf samples were kept at – 20 ^{o}C until use. Total DNA was extracted using a recommended procedure (Keb *et al*., 2002). Purity of the extracted DNA was confirmed by spectrophotometry. DNA from all three species were subjected to polymerase chain reaction (PCR) using high fidelity KOD plus DNA polymerase (Toyobo, Osaka, Japan) and universal primers for the internal transcribed spacer (ITS) region and the *trnH-psbA* region. The primer sequences were as follows: ITS5af (5'-CCTTATCATTTAG AGGAAGGAG-3') (Stanford *et al*., 2000), ITS4r (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990): trnHf (5'-CGCGCATGGTGGATTCACAATCC-3') and psbA3f (5'-GTTATGCATGAACGTAATGCTC-3') (Sang *et al*., 1997). The PCR conditions for both ITS region and *trnH-psbA* regions were 3 min at 94 ^{o}C for heating, followed by 30 cycles of 30 s at 94 ^{o}C for denaturing, 30 s at 49 ^{o}C for annealing and 1 min at 68 ^{o}C for extension.

Amplified ITS and *trnH-psbA* regions were purified using a GeneClean kit and phosphorylated using T_4 polynucleotide kinase. Phosphorylated PCR products were ligated to a pBlueScript vector previously digested with *EcoR* V. The ligated vector was transformed to *E.coli* strain JM109 and the cells were grown at 37 ^{o}C in Luria Bertani (LB) medium containing ampicillin for 12−15 h. Cells were harvested by centrifugation, followed by plasmid purification and the purified plasmids containing the DNA of interest were subjected to sequencing using a 3100 sequencer (Applied Biosystems). Sequence homology of ITS and *trnH-psbA* from all three species were compared using MacVector software.

RESULTS

The sequence lengths of ITS and *trnH-psbA* regions of all three species were 802 and 527 base pairs, respectively. Between *H. ovalifolia* and *H. floribunda,* the ITS region showed a sequence difference in 19 base pairs accounting for 2.37 % sequence divergence and the *trnH-psbA* region showed a sequence difference in 8 base pairs accounting for 1.5 % sequence divergence. Between *H. ovalifolia* and *H. angustifolia,* the ITS region showed a sequence divergence in 27 base pairs accounting for 3.36 % sequence divergence and the *trnH-psbA* region showed a sequence difference in 10 base pairs accounting for 1.89 % sequence divergence. Moreover, the percentage

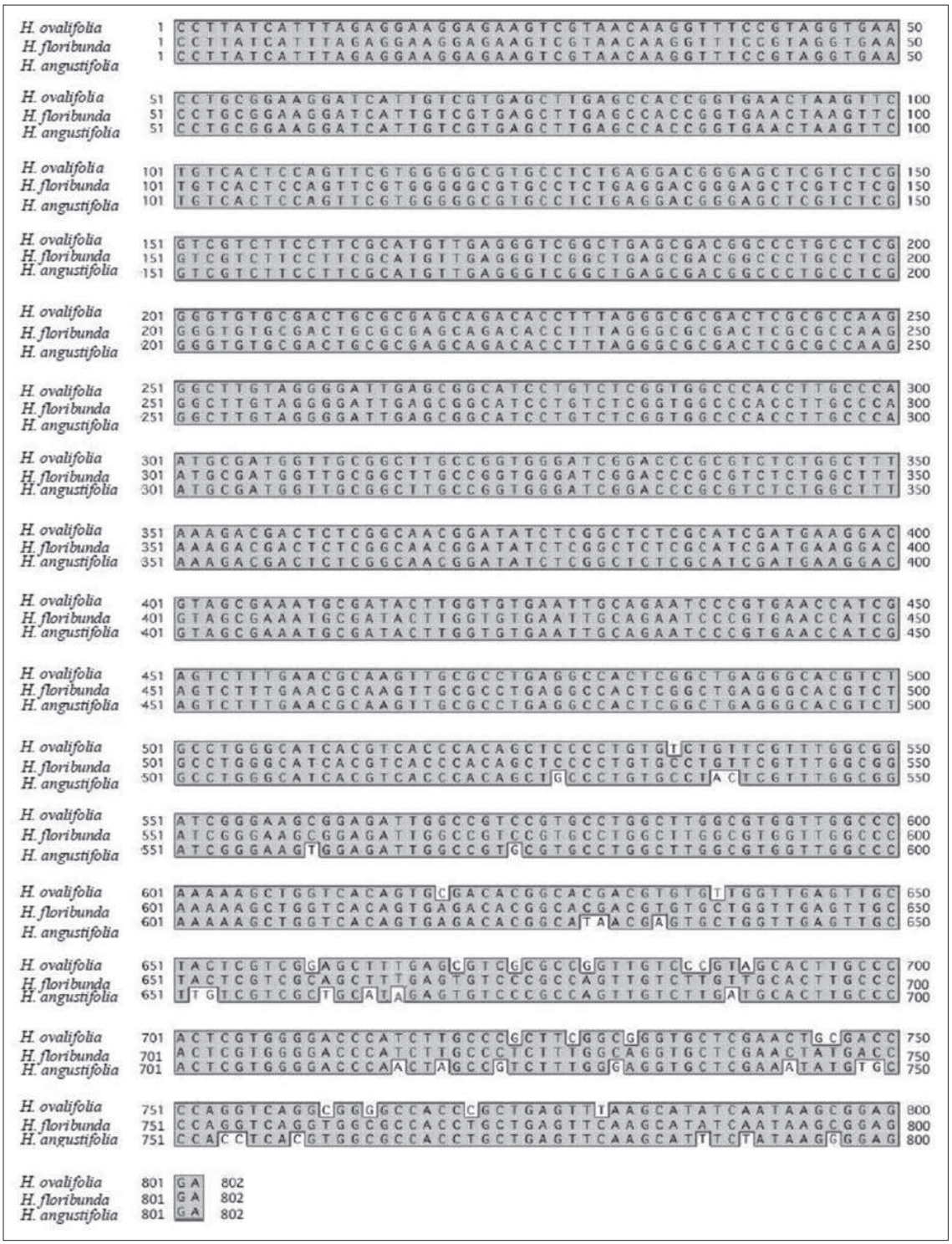

Figure 1: Comparison of nucleotide sequences of ITS region. Nucleotide sequence alignment was performed using Mac Vector software.

sequence divergence between ITS and *trnH-psbA* regions of *H. floribunda* and *H. angustifolia* were 3.36 % and 2.65 %, respectively (Figures 1, 2 and Table 1). There was no divergence at all in both pairs of loci when sequences from different individuals of the same species were compared.

Table 1: Percentage sequence divergence between the three species of the genus *Hortonia*

Species	Percentage sequence divergence	
	ITS region	*trnH-psbA* region
H. ovalifolia vs H. floribunda	2.37	1.50
H. ovalifolia vs H. angustifolia	3.36	1.89
H. floribunda vs H. angustifolia	3.36	2.65

DISCUSSION

Methods to identify species using short orthologous DNA sequences have been initiated to facilitate biodiversity studies. For plant molecular systematic investigations at the species level, the ITS region of nuclear ribosomal cistron (18S-5.8S-26S) is the most commonly sequenced locus (Alvarez & Wendel, 2003). With the exception of ferns, the ITS region has shown broad utility across photosynthetic eukaryotes and fungi. In plant phylogenetic studies the ITS region is widely employed for species level discrimination. A recent study proposed that the *trnH-psbA* intergenic spacer plastid region that has good priming sites, and a shorter length with interspecific variation would be suitable as a universal barcode for land plants (Kress *et al*., 2005). Both ITS and *trnH-psbA* regions therefore have the potential to discriminate among closely related plant species.

Figure 2: Comparison of nucleotide sequences of trnH-psbA region. Nucleotide sequence alignment was performed using Mac Vector software

Intraspecific variation in both these DNA barcodes is known to be extremely low, compared with interspecific variation. Notwithstanding phytochemical similarities, the high sequence divergence values clearly indicate that the genus *Hortonia* has three different species because a 0.5 % sequence divergence is sufficient to discriminate among plant species (Kress *et al*., 2005). Considering the percentage sequence divergence values, *H. ovalifolia* and *H. floribunda* are more closely related to each other than to *H. angustifolia*. This corroborates with the recent work on re-evaluation of the species limits of *Hortonia* by empirical methods (Yakandawela & Rubasinghe, 2003).

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