Species identification of *Rhododendron* (Ericaceae) using the chloroplast deoxyribonucleic acid *PsbA-trnH* genetic marker

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**ABSTRACT**

**Background:** *Rhododendron* is a group of famous landscape plants with high medicinal value. However, there is no simple or universal manner to discriminate the various species of this group. Deoxyribonucleic acid (DNA) barcoding technique is a new biological tool that can accurately and objectively identify species by using short and standard DNA regions. **Objective:** To choose a suitable DNA marker to authenticate the *Rhododendron* species. **Materials and Methods:** Four candidate DNA barcodes (*rbcL*, *matK*, *psbA-trnH*, and ITS2 intergenic spacer) were tested on 68 samples of 38 species. **Results:** The *psbA-trnH* candidate barcode yielded 86.8% sequencing efficiency. The highest interspecific divergence was provided by the *psbA-trnH* intergenic spacer, based on six parameters, and the Wilcoxon signed rank tests. Although there was not a clear barcoding gap, the Wilcoxon Two sample tests indicated that the interspecific divergence of the *psbA-trnH* intergenic spacer was significantly higher than the relevant intraspecific variation. The *psbA-trnH* DNA barcode possessed the highest species identification efficiency at 100% by the BLAST1 method. The present results showed that the *psbA-trnH* intergenic spacer was the most promising one of the four markers for barcoding the *Rhododendron* species. To further evaluate the ability of the *psbA-trnH* marker, to discriminate the closely related species, the samples were expanded to 94 samples of 53 species in the genus, and the rate of successful identification was 93.6%. The *psbA-trnH* region would be useful even for unidentified samples, as it could significantly narrow their possible taxa to a small area. **Conclusion:** The *psbA-trnH* intergenic region is a valuable DNA marker for identifying the Rhododendron species.

**Key words:** Deoxyribonucleic acid barcoding, *psbA-trnH*, *Rhododendron*, species identification

**INTRODUCTION**

*Rhododendron* is a very large genus in Ericaceae, with about 1000 known species in the world and more than 500 species in China.[¹,²] Most species within this genus are widely cultivated in the temperate and sub-temperate regions as ornamentals.[³] Some *Rhododendron* species have been used in traditional Chinese medicine for treatment of various diseases. For example, the stems and leaves of *Rhododendron simii* Planch and *Rhododendron anthopogonoides* Maxim have traditionally been used as folk medicines to treat chronic bronchitis.[⁴] Meanwhile, the fruit, flower, and root of *Rhododendron molle* (Blum) G. Don relieve joint pains and have a remarkable therapeutic effect on rheumatoid arthritis (RA).[⁵] However, some species with no medicinal value are easily confused with the medicinal plants because of the similar morphological characteristics. Furthermore, in China, more than sixty species in this genus are poisonous, and some toxic medicinal plants can cause severe poisoning if they are confused with others.[⁴-⁶] Therefore, it is extremely important to accurately identify the *Rhododendron* species.

As the genus was established by Linnaeus, various methods have been constantly used to revise its classification system on the basis of morphology, cytology, chemotaxonomy, and molecular taxonomy. The contemporary classifications of *Rhododendron* are based on the seminal publication of
Sleumer. Later, some researchers have conducted more morphological studies in infrageneric groupings and those studied have been integrated into the classification of Chamberlain et al. Nowadays, this taxonomic system is generally accepted by Rhododendron specialists. However, the Rhododendron genus still has some problems at various systematic classification levels and there is no simple or universal manner to discriminate the various species within the genus.

The DNA barcoding, based on a short DNA sequence to identify species has been proposed as a rapid, accurate, and convenient taxonomic tool. The Consortium for the Barcode of Life (CBOL) Plant Working Group recommended the \textit{rbcL} + \textit{matK} combination as a barcode sequence in the plant kingdom, and they also suggested that ITS (ITS2) and \textit{psbA-trnH} were good candidates for plant DNA barcoding, because of their fast evolution rates. Chen et al. found that the ITS2 region possessed many advantages compared to the plastid loci, including the \textit{rbcL} and \textit{matK} regions. They also recommended that \textit{psbA-trnH} could be a complementary barcode to ITS2 for a broad series of plants. One of the problems for plant DNA barcoding was that the previous studies were mainly carried out on a large scale and rarely on a specific genus, with many closely related species, so some studies suggested that species identification using standard DNA sequences should be carried out within a narrow taxon (such as the genus). In this study, we tried to assess the suitability of four potential DNA regions (\textit{psbA-trnH}, \textit{matK}, \textit{rbcL} and ITS2) as a DNA barcode, to identify species of \textit{Rhododendron} across 68 samples belonging to 38 species. One of the challenges for any DNA barcode was its ability in discriminating closely related species (i.e., sister-species). Furthermore, to evaluate the ability of the \textit{psbA-trnH} region, the tested data were expanded to 94 samples belonging to 53 species, including 37 samples of 20 species within Subgenus \textit{Hymenanthes} and 24 samples of 13 species within Subgenus \textit{Tsutsusi} based on Chamberlain’s classification system.

**RESULTS**

In order to be useful, a DNA barcode sequence must be easily PCR amplified with universal reaction conditions and primers, and then successfully sequenced. In our pilot study, we tested the efficiency of sequencing, by employing 68 samples, and the results showed that \textit{rbcL}, \textit{psbA-trnH}, and ITS2 candidate barcodes yielded 89.7, 86.8, and 50% success rates of sequencing, respectively. However, the efficiency of the \textit{matK} region was very poor, hence \textit{matK} was not included in the subsequent experiments. The sequence lengths, GC contents of the three regions based on the results of the CodonCode Aligner and Clustal W alignment are presented in Table 3.

An ideal DNA barcode should own significant interspecific
Table 1: The collection sites and GenBank accession of 68 samples of the Rhododendron genus

| Species name                          | Subgenus (Chamberlain et al.) | Collection sites | Voucher number | GenBank accession |
|---------------------------------------|-------------------------------|------------------|----------------|------------------|
| Rhododendron championae               | Azaleastrum                   | Jiangxi          | LS0508MT01     | ITS2             |
|                                       |                               |                  |                | psbA-trnH        |
|                                       |                               |                  |                | rbcL             |
| Rhododendron championae               | Azaleastrum                   | Yunnan           | LS0508MT02     | HQ7070044        |
| Rhododendron latoucheae               | Azaleastrum                   | Jiangxi          | LS0529MT01     | HQ706992         |
| Rhododendron moumainense              | Azaleastrum                   | Jiangxi          | LS0541MT02     | HQ707004         |
| Rhododendron stamineum, var. stamineum| Azaleastrum                   | Jiangxi          | LS0558MT01     | HQ707025         |
| Rhododendron stamineum var. stamineum | Azaleastrum                   | Yunnan           | LS0558MT02     | HQ707026         |
| Rhododendron vialii                   | Azaleastrum                   | Jiangxi          | LS0562MT01     | HQ707031         |
| Rhododendron vialii                   | Azaleastrum                   | Yunnan           | LS0562MT02     | HQ707032         |
| Rhododendron agastum.                 | Hymenanthes                   | Jiangxi          | LS0503MT01     | HQ706949         |
| Rhododendron agastum.                 | Hymenanthes                   | Yunnan           | LS0503MT02     | HQ706949         |
| Rhododendron auriculatum              | Hymenanthes                   | Jiangxi          | LS0505MT01     | HQ706950         |
| Rhododendron auriculatum              | Hymenanthes                   | Yunnan           | LS0505MT02     | HQ706956         |
| Rhododendron chihsinianum             | Hymenanthes                   | Jiangxi          | LS0510MT01     | HQ707045         |
| Rhododendron chihsinianum             | Hymenanthes                   | Yunnan           | LS0510MT02     | HQ706995         |
| Rhododendron decorum                  | Hymenanthes                   | Jiangxi          | LS0511MT01     | HQ706959         |
| Rhododendron delavayi.                | Hymenanthes                   | Jiangxi          | LS0512MT01     | HQ707047         |
| Rhododendron delavayi.                | Hymenanthes                   | Yunnan           | LS0512MT02     | HQ706961         |
| Rhododendron fortunei                 | Hymenanthes                   | Jiangxi          | LS0518MT01     | HQ706969         |
| Rhododendron fortunei                 | Hymenanthes                   | Yunnan           | LS0518MT02     | HQ706970         |
| Rhododendron glanduliferum            | Hymenanthes                   | Jiangxi          | LS0520MT01     | HQ706967         |
| Rhododendron glanduliferum            | Hymenanthes                   | Yunnan           | LS0520MT02     | HQ706976         |
| Rhododendron hemsleyanum              | Hymenanthes                   | Jiangxi          | LS0522MT01     | HQ706972         |
| Rhododendron hemsleyanum              | Hymenanthes                   | Yunnan           | LS0522MT02     | HQ706980         |
| Rhododendron jinggangshanicum         | Hymenanthes                   | Jiangxi          | LS0527MT01     | HQ706989         |
| Rhododendron jinggangshanicum         | Hymenanthes                   | Yunnan           | LS0527MT02     | HQ706989         |
| Rhododendron leptopeplum              | Hymenanthes                   | Jiangxi          | LS0530MT01     | HQ706993         |
| Rhododendron pachyphyllum             | Hymenanthes                   | Jiangxi          | LS0547MT01     | HQ707064         |
| Rhododendron similum                   | Hymenanthes                   | Jiangxi          | LS0556MT01     | HQ707070         |
| Rhododendron similum                   | Hymenanthes                   | Yunnan           | LS0556MT02     | HQ707071         |
| Rhododendron vernicosum               | Hymenanthes                   | Jiangxi          | LS0561MT01     | HQ707073         |
| Rhododendron vernicosum               | Hymenanthes                   | Yunnan           | LS0561MT02     | HQ707074         |
| Rhododendron williamsonianum          | Hymenanthes                   | Jiangxi          | LS0564MT01     | HQ707039         |
| Rhododendron williamsonianum          | Hymenanthes                   | Yunnan           | LS0564MT02     | HQ706942         |
| Rhododendron zhangjiajiense           | Hymenanthes                   | Jiangxi          | LS0566MT01     | HQ707075         |
| Rhododendron zhangjiajiense           | Hymenanthes                   | Yunnan           | LS0566MT02     | HQ707040         |
| Rhododendron molle                    | Pentanthera                   | Jiangxi          | LS0567MT01     | HQ707062         |
| Rhododendron molle                    | Pentanthera                   | Yunnan           | LS0567MT02     | HQ707063         |
| Rhododendron molle                    | Pentanthera                   | Guangdong        | LS0567MT03     | HQ707002         |
| Rhododendron mekongense               | Rhododendron                  | Jiangxi          | LS0506MT01     | HQ706996         |
| Rhododendron mekongense               | Rhododendron                  | Jiangxi          | LS0506MT02     | HQ706997         |
| Rhododendron edgeworthii              | Rhododendron                  | Jiangxi          | LS0515MT01     | HQ706962         |
| Rhododendron edgeworthii              | Rhododendron                  | Yunnan           | LS0515MT02     | HQ706963         |
| Rhododendron excellens                 | Rhododendron                  | Jiangxi          | LS0516MT01     | HQ707048         |
| Rhododendron excellens                 | Rhododendron                  | Yunnan           | LS0516MT02     | HQ707049         |
| Rhododendron kiangsiensi              | Rhododendron                  | Jiangxi          | LS0528MT01     | HQ707059         |
| Rhododendron kiangsiensi              | Rhododendron                  | Yunnan           | LS0528MT02     | HQ707060         |
| Rhododendron micranthum               | Rhododendron                  | Jiangxi          | LS0536MT01     | HQ706998         |
| Rhododendron micranthum               | Rhododendron                  | Yunnan           | LS0536MT02     | HQ706999         |
| Rhododendron rubiginosum               | Rhododendron                  | Jiangxi          | LS0554MT01     | HQ707017         |
| Rhododendron farrerae                  | Tsutsusi                      | Jiangxi          | LS0517MT01     | HQ706967         |
| Rhododendron farrerae                  | Tsutsusi                      | Yunnan           | LS0517MT02     | HQ706968         |
| Rhododendron huanense                  | Tsutsusi                      | Jiangxi          | LS0523MT01     | HQ707054         |
| Rhododendron huanense                  | Tsutsusi                      | Yunnan           | LS0523MT02     | HQ706978         |
| Rhododendron hypoblematosum            | Tsutsusi                      | Jiangxi          | LS0524MT01     | HQ707056         |
### Table 1: Contd...

| Species name                          | Subgenus (Chamberlain et al.) | Collection sites | Voucher number | GenBank accession |
|---------------------------------------|-------------------------------|------------------|----------------|-------------------|
| Rhododendron hypoblematosum           | Tsutsusi yunnan               | LS0524MT02       | HQ706981       | HQ706912          |
| Rhododendron indicum                  | Tsutsusi Jiangxi              | LS0525MT01       | HQ706982       |                   |
| Rhododendron indicum                  | Tsutsusi yunnan               | LS0525MT02       | HQ706983       |                   |
| Rhododendron mucronatum               | Tsutsusi Jiangxi              | LS0542MT02       | HQ707005       | HQ706922          |
| Rhododendron obtusum                  | Tsutsusi Jiangxi              | LS0544MT01       | HQ707006       | HQ706923          |
| Rhododendron obtusum                  | Tsutsusi yunnan               | LS0544MT02       | HQ707007       | HQ706924          |
| Rhododendron oldhamii                 | Tsutsusi Jiangxi              | LS0545MT01       | HQ707008       | HQ706925          |
| Rhododendron oldhamii                 | Tsutsusi yunnan               | LS0545MT02       | HQ707009       | HQ706926          |
| Rhododendron strigosum                | Tsutsusi Jiangxi              | LS0548MT02       | HQ707027       | HQ706928          |
| Rhododendron pulchrum                 | Tsutsusi Jiangxi              | LS0549MT01       | HQ707065       | HQ706929          |
| Rhododendron pulchrum                 | Tsutsusi yunnan               | LS0549MT02       | HQ707066       | HQ706930          |
| Rhododendron rhuyuenense              | Tsutsusi Jiangxi              | LS0551MT01       | HQ707014       | HQ706931          |
| Rhododendron rhuyuenense              | Tsutsusi Yunnan               | LS0551MT02       | HQ707015       | HQ706932          |

### Table 2: The collection sites and GenBank accession of expanded samples of the *Rhododendron* genus

| Samples name                          | Subgenus (Chamberlain et al.) | Sampling location | Voucher number | psbA-trnH |
|---------------------------------------|-------------------------------|-------------------|----------------|-----------|
| Rhododendron aganniphum               | Hymenanthes                  | Xizang            | LS0502MT01     | HQ706948  |
| Rhododendron annae                    | Hymenanthes                  | Jiangxi           | LS0504MT01     | HQ706951  |
| Rhododendron annae                    | Hymenanthes                  | Yunnan            | LS0504MT02     | HQ706952  |
| Rhododendron annae                    | Hymenanthes                  | Guangdong         | LS0504MT03     | HQ706953  |
| Rhododendron anthosphaerum            | Hymenanthes                  | Guangdong         | LS0593MT01     | HQ706954  |
| Rhododendron delavayi                 | Hymenanthes                  | Guangdong         | LS0512MT01     | HQ706960  |
| Rhododendron fortunei                 | Hymenanthes                  | Guangdong         | LS0518MT03     | HQ706971  |
| Rhododendron fortunei                 | Hymenanthes                  | Guangdong         | LS0518MT04     | HQ706972  |
| Rhododendron habrotichicum            | Hymenanthes                  | Jiangxi           | LS0597MT01     | HQ706977  |
| Rhododendron irroratum                | Hymenanthes                  | Jiangxi           | LS0526MT01     | HQ706986  |
| Rhododendron irroratum                | Hymenanthes                  | Yunnan            | LS0526MT02     | HQ706987  |
| Rhododendron irroratum                | Hymenanthes                  | Guangdong         | LS0526MT03     | HQ706988  |
| Rhododendron aberconwayi              | Hymenanthes                  | Jiangxi           | LS0514MT01     | HQ707023  |
| Rhododendron aberconwayi              | Hymenanthes                  | Jiangxi           | LS0514MT02     | HQ707024  |
| Rhododendron vernicosum               | Hymenanthes                  | Yunnan            | LS0561MT02     | HQ707030  |
| Rhododendron wardii                   | Hymenanthes                  | Xizang            | LS0531MT01     | HQ707037  |
| Rhododendron wardii                   | Hymenanthes                  | Xizang            | LS0531MT02     | HQ707038  |
| Rhododendron molle                    | Pentanthera                  | Guangdong         | LS0567MT04     | HQ707003  |
| Rhododendron schlippenbachii          | Pentanthera                  | Jiangxi           | LS0586MT01     | HQ707019  |
| Rhododendron ciliatum                 | Rhododendron                 | Jiangxi           | LS0507MT01     | HQ706958  |
| Rhododendron excellens                | Rhododendron                 | Guangdong         | LS0516MT03     | HQ706966  |
| Rhododendron billiflorum              | Rhododendron                 | Yunnan            | LS0588MT01     | HQ706994  |
| Rhododendron sargentianum             | Rhododendron                 | Jiangxi           | LS0513MT01     | HQ707018  |
| Rhododendron taronense                | Rhododendron                 | Jiangxi           | LS0519MT01     | HQ707028  |
| Rhododendron virgatum                 | Rhododendron                 | Jiangxi           | LS0563MT01     | HQ707033  |
| Rhododendron virgatum                 | Rhododendron                 | Yunnan            | LS0563MT02     | HQ707034  |
| Rhododendron mariesii                 | Tsutsusi                     | Jiangxi           | LS0535MT01     | HQ706995  |
| Rhododendron pulchrum                 | Tsutsusi                     | Yunnan            | LS0549MT03     | HQ707013  |
| Rhododendron indicum                  | Tsutsusi                     | Jiangxi           | LS0525MT03     | HQ706984  |
| Rhododendron indicum                  | Tsutsusi                     | Yunnan            | LS0525MT04     | HQ706985  |
| Rhododendron simii                    | Tsutsusi                     | Jiangxi           | LS0583MT01     | HQ707022  |
| Rhododendron fragrans                 | yunnan                       | LS0509MT01       | HQ706973       |
| Rhododendron fragrans                 | yunnan                       | LS0509MT02       | HQ706974       |
| Rhododendron wanxia                   | yunnan                       | LS0521MT01       | HQ707035       |
| Rhododendron wanxia                   | yunnan                       | LS0521MT02       | HQ707036       |
variation in DNA sequences, with a comparatively small variation between individuals, within a single species.\textsuperscript{17,21,22} Therefore, six metrics were employed to characterize interspecific versus intraspecific variation. Through comparison of interspecific genetic distances among congeneric species for three candidate barcodes, the chloroplast non-coding region \textit{psbA-trnH} exhibited the highest interspecific divergence with all three metrics, followed by ITS2, while \textit{rbcL} provided the lowest [Table 4]. Moreover, the Wilcoxon signed rank tests confirmed that \textit{psbA-trnH} provided the highest interspecific divergence between the congeneric species [Table 5]. We also found that \textit{rbcL} showed the lowest level of intraspecific variation with all three parameters, followed by ITS2, while \textit{psbA-trnH} provided the highest [Table 4]. The Wilcoxon signed rank tests showed that \textit{rbcL} has the lowest variation between conspecific individuals, whereas, \textit{psbA-trnH} showed the highest [Table 6].

The DNA barcode should exhibit a ‘barcoding gap’ between interspecific and intraspecific divergences.\textsuperscript{17,18} Although there was no clear gap in the histogram between intraspecific variation and interspecific divergence in the distributions of the three loci (\textit{rbcL}, TS2, \textit{psbA-trnH} intergenic spacer) [Figure 1], the Wilcoxon two sample tests indicated that for the three loci the distribution of interspecific divergences were higher than those of the corresponding intraspecific variations, with high significance [Table 7].

The BLAST1 method was used to test the applicability of different regions, for species identification.\textsuperscript{18} The results indicated that the \textit{psbA-trnH} intergenic spacer possessed the highest species identification efficiency at 100%, followed by \textit{rbcL} at 59%, then ITS2 at 41.2% [Table 8]. To further evaluate the ability of the \textit{psbA-trnH} region to identify the \textit{Rhododendron} species with more closely related species in a wider range, 94 samples were tested. The rate of correct identification was 93.6% [Table 8], with six failed samples [Table 9].

### DISCUSSION

In the present research, the feasibility of four potential DNA regions (\textit{psbA-trnH}, \textit{matK}, \textit{rbcL}, ITS2) as a DNA barcode of the \textit{Rhododendron} species was concretely tested. The \textit{rbcL} sequence showed advantages of higher efficiency of PCR amplification and sequencing [Table 3]. However, the variation of the sequence in the species level was insufficient to discriminate the \textit{Rhododendron} species, and the identification efficiency was only 59% [Table 8]. The \textit{matK} showed lower sequencing efficiency and its successful identification rate of 131 samples from the GenBank database was 43.8%. At the Third International

### Table 3: Success rate of sequencing, Length range, GC content

| Markers   | \textit{psbA-trnH} | \textit{rbcL} | ITS2 |
|-----------|-------------------|---------------|------|
| Number of samples / n | 68                | 68            | 68   |
| Success of Sequencing / n | 59               | 61            | 34   |
| Success rate of sequencing / % | 86.8             | 89.7          | 50   |
| Length range / bp | 450 – 493         | 666           | 245 – 249 |
| GC content / % | 32.1             | 44            | 59.3 |

### Table 4: Inter- and intraspecific genetic divergences of three candidate barcodes

| Markers   | ITS2 (34) | \textit{psbA-trnH} (59) | \textit{rbcL} (61) |
|-----------|-----------|-------------------------|-------------------|
| All interspecific distance | 0.0312 ± 0.0165 | 0.0728 ± 0.0237 | 0.0103 ± 0.0112 |
| Theta prime | 0.0293 ± 0.0109 | 0.0729 ± 0.0119 | 0.0101 ± 0.0103 |
| Minimum interspecific distance | 0.0079 ± 0.0123 | 0.0230 ± 0.0181 | 0.0045 ± 0.0110 |
| All intraspecific distance | 0.0043 ± 0.0136 | 0.0150 ± 0.0100 | 0.0002 ± 0.0007 |
| Theta | 0.0043 ± 0.0136 | 0.0151 ± 0.0105 | 0.0002 ± 0.0007 |
| Coalescent depth | 0.0043 ± 0.0136 | 0.0153 ± 0.0105 | 0.0002 ± 0.0007 |

Numbers in parentheses mean the whole sequenced samples of three candidate barcodes [Table 3].

### Table 5: Wilcoxon signed rank test for interspecific variations

| W+  | W-  | Inter relative ranks | Result |
|-----|-----|----------------------|--------|
| \textit{rbcL} | ITS2 | W+ = 4665.0, W- = 30846 | ITS2 >> \textit{rbcL} |
|\textit{psbA-trnH} | ITS2 | W+ = 35833, W- = 482.0 | \textit{psbA-trnH} >> ITS2 |
| \textit{rbcL} | \textit{psbA-trnH} | W+ = 160.0, W- = 36155 | \textit{psbA-trnH} >> \textit{rbcL} |

### Table 6: Wilcoxon signed rank test for intraspecific variations

| W+  | W-  | Inter relative ranks | Result |
|-----|-----|----------------------|--------|
| \textit{ITS2} | \textit{rbcL} | W+ = 1, W- = 0 | \textit{ITS2} = \textit{rbcL} |
|\textit{psbA-trnH} | \textit{ITS2} | W+ = 21.0, W- = 7.0 | \textit{ITS2} = \textit{psbA-trnH} |
| \textit{rbcL} | \textit{psbA-trnH} | W+ = 0, W- = 28 | \textit{psbA-trnH} >> \textit{rbcL} |
Barcoding Conference, the Plant Working Group of the Consortium for the Barcode of Life recommended the two-locus combination of *rbcL* + *matK* for plant barcoding.[11] The two proposed regions were the most useful barcodes and provided a universal framework for land plants at and above the generic levels.[13] However, they showed a lower resolution rate to identify the species within a rapid evolutionary genus such as *Rhododendron*. In the meantime, many researchers have proposed the use of ITS2 as a suitable marker for taxonomic classification.[11,21,24] However, in our study, the success rate of sequencing with ITS2 was only 50% [Table 3], and the identification efficiency was only 41.2% [Table 8]. Above all, the results indicated that *matK, rbcL*, and ITS2 were not suitable as barcodes for the identification of the *Rhododendron* species.

The *psbA-trnH* intergenic spacer is among the most variable regions in the angiosperm chloroplast genome. It is a popular tool for plant population genetics and species level phylogenetics and has been proposed to be suitable for the DNA barcoding studies.[25,26] *Rhododendron* is a rapidly evolutionary genus within the angiosperms in

### Table 7: Wilcoxon two-sample tests for distribution of intra- versus interspecific divergences

| Marker          | No. of Interspecific distances | No. of Intraspecific distances | Wilcoxon W | P value       |
|-----------------|--------------------------------|--------------------------------|------------|--------------|
| ITS2            | 549                            | 12                             | 795        | 8.5172 × 10^{-3} |
| *psbA-trnH*     | 1687                           | 24                             | 910        | 3.4910 × 10^{-7}  |
| *rbcL*          | 1803                           | 27                             | 30         | 6.5088 × 10^{-4}  |

### Table 8: Identification efficiency for potential deoxyribonucleic acid barcodes loci using the BLAST1 method

| Marker          | Number of samples (n) | Correct identification samples (n) | Correct identification efficiency (%) | Ambiguous identification samples (n) | Ambiguous identification efficiency (%) |
|-----------------|-----------------------|-----------------------------------|---------------------------------------|---------------------------------------|------------------------------------------|
| ITS2            | 34                    | 14                                | 41.2                                  | 20                                    | 58.8                                     |
| *psbA-trnH*     | 59                    | 59                                | 100                                   | 0                                     | 0                                        |
| *rbcL*          | 61                    | 36                                | 59.0                                  | 25                                    | 41.0                                     |
| *psbA-trnH*     | 94                    | 88                                | 93.6                                  | 6                                     | 6.4                                      |

### Table 9: Unsuccessful identification sample pairs in BLAST1 based on Chamberlain’s classification system

| Pairs | Samples name        | Voucher number | Subgenus   | Section   | Subsection |
|-------|---------------------|----------------|------------|-----------|------------|
| 1     | *Rhododendron irroratum* | LS0526MT01    | Hymenanthes | Ponticum | Irrorata  |
| 2     | *Rhododendron anna*   | LS0504MT01    | Hymenanthes | Ponticum | Irrorata  |
| 3     | *Rhododendron aberconwayi* | LS0526MT02   | Hymenanthes | Ponticum | Irrorata  |
| 4     | *Rhododendron excellens* | LS0516MT01    | Rhododendron | Rhododendron | Maddenia |
| 5     | *Rhododendron virgatum* | LS0563MT01    | Rhododendron | Rhododendron | Virgata  |
recent years with many closely related species and there are many artificial and natural hybrids.[23] The psbA-trnH region is one of the most variable non-coding regions of the plastid genome in the angiosperms, because of the highest percentages of variable sites.[28-30]

Moreover this variation indicated that this intergenic spacer could offer high levels of species discrimination.[27,28] In our study, first we found that the average length of the psbA-trnH intergenic spacer was rather short at 450 – 493 base pairs. The psbA-trnH sequences were relatively easy to be amplified using one pair of universal primers. Second, examination of the genetic divergences using six parameters and statistical tests confirmed that the psbA-trnH intergenic spacer possessed high interspecific divergence. Analyses of the DNA barcoding gap and the Wilcoxon twosample tests supported the notion that the mean interspecific divergence of the psbA-trnH intergenic spacer was significantly higher than its mean intraspecific variation. Third, according to the BLAST1 method, the identification accuracy using the psbA-trnH intergenic spacer was 100%, and it could identify all the species that could be identified by ITS2 or rbcL. Therefore, it was quite clear that among the four sequences, psbA-trnH was the most promising one for barcoding the species within the rapid evolutionary genus.

One of the challenges for any DNA barcode is its utility in discriminating closely related species.[13,18] In this study, to further evaluate the ability of the psbA-trnH region, to identify the closely related species in a wider range, the samples were expanded to 94 samples belonging to 53 species. The result showed that the psbA-trnH region steadily kept a higher identification efficiency. Furthermore, we specifically tested the identification ability of psbA-trnH in two subgenera, and it showed that the success rate of identification was 100% for 24 samples belonging to 13 species from Subgenus Tsutsusi and 89.2% for 37 samples of 20 species from Subgenus Hymenanthes. Therefore, psbA-trnH was confirmed as a useful marker for differentiating closely related species within Rhododendron.

Meanwhile, we noted that there were three pairs of samples which could not be accurately identified [Table 9]. The first and second pairs from three species, Rhododendron annae, Rhododendron irroratum, and Rhododendron aberconwayi, belonged to the same subsection IRRORATA, as they shared exceedingly similar morphological characteristics of the corolla shape, leaves shape, glabrous petiole, and pedicel.[3] The third pair, R. excellens and R. virgatum, was classified by Chamberlain as the same subgenus Rhododendron and the same section Rhododendron, because of their similar morphological characters: Both of them have termina

inflorescence buds, vary rarely axillary from lower leaves and the whole plant of them is densely covered with peltate scales.[1,2] The failure of psbA-trnH in discriminating these species indicated that some morphologically similar species had no sufficient interspecific variation in the psbA-trnH region. In spite of this, the psbA-trnH region would still be significant for those unidentified samples as it could narrow their possible taxa to a small area, one subgenus, one section, or even to one subsection [Table 9].

CONCLUSION

The psbA-trnH intergenic region is a potential DNA barcoding sequence for identifying the Rhododendron species. Furthermore, it would still be useful, even for those unidentified species, because it could significantly narrow the possible taxa to a small area.

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