DISCRIMINATION OF CHROZOPHORA OBLONGIFOLIA AND RICINUS COMMUNIS FROM TAIF, KSA USING DNA BARCODING

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Abstract

The use of highly discriminative approaches for the identification and characterization of genotypes (especially with known medicinal value) is essential for plant conservation and appropriate use. Therefore, molecular characterization and phylogenetic analysis of Chrozophora oblongifolia L. and Ricinus communis L. collected from Taif were presented using ITS, ITS2, rbcLa and matK sequences. rbcLa region recorded higher percentage of the variable sites within the two species. Tajima test and Transition/Transversion bias (R) showed no molecular evolution within C. oblongifolia genome, whereas, they indicated an accelerated evolution within R. communis genome. The intraspecific divergence of ITS and ITS2 was lower than that of matK and rbcLa loci. The phylogenetic analysis proved that the rbcLa site showed a higher intraspecific difference than ITS2 in C. oblongifolia. Although, accessions of R. communis from the same country or continent showed limited genetic diversity, they did not get together and revealed intraspecific divergence. For a rapid and accurate identification of Euphorbiaceous species having medicinal importance, our analyses enforced the employment of the ITS2 mini-barcode as a universal barcode.

Key words: Chrozophora; Ricinus; ITS; ITS2; matK; rbcLa.

Introduction

DNA barcoding is an accurate taxonomic method using one or a few short, standardized DNA loci. The internal transcribed spacer (ITS) locus as a complementary marker and rbcLa + matK as core barcodes were selected as the standard barcode for the identification and characterization of plants. However, the three-barcode system encountered some difficulties in identifying other plants, especially medicinal species (Hebert et al., 2003; Hollingsworth et al., 2009). Among these selected barcoding loci and due to its high rate of successful amplification and discrimination power among all tested regions, the ITS2 site was considered as a helpful minibarc ode for projects involving a large number of environmental samples as mentioned by Chen et al., (2010) and Tang et al., (2016). Successful characterization and identification for a target species subsequently depends on a comprehensive DNA barcode database using samples from several geographic sites.

Genus Chrozophora comprises 7-8 species belonging to family Euphorbiaceae, that are generally herbs and shrubs. This genus exists in Mediterranean regions, West Africa and South Asia and possess several biological activities such as C. oblongifolia has antimicrobial and antioxidant activity. The whole plant is utilized for wounds to improve healing, other parts such as seeds and leaves are utilized as a laxative, a depurative agent and in treating skin diseases. In Saudi Arabia, the plant is also used against purifying blood and jaundice (Ramzi et al., 2011; Dipankar et al., 2011). Compared to other medicinal plants, it is clear that the genetics of C. oblongifolia has not been studied much.

On the other side, the castor bean (Ricinus communis) occurs in subtropical and tropical countries as an oilseed crop. It was thought that R. communis has originated in eastern Africa (Vavilov, 1951; Zeven & Zhukovsky, 1975).

Castor oil is an essential industrial raw material for great products such as lubricants, coatings, paints, soaps, plastics, cosmetics, and medications for skin affections (Brigham, 1993). Castor seeds contain poison ricin, ribosome-inactivating proteins (RIPs), that can be extracted and used as a bioweapon, moreover, castor oil has been sophisticated as a component of biodiesel in Brazil (Endo et al., 1987; da Silva et al., 2006). Its plants are considered invasive weeds because they spread in indiscriminate areas such as river banks, roadsides and the agricultural field fringes. R. communis is considered as self-pollinated or cross-pollinated by wind (Meinders & Jones, 1950; Brigham, 1967), with outcrossing the dominant reproduction method. Although, R. communis is an agro-economically important plant, it was clear that it possesses low genetic diversity and no geographic patterns of genetic association based on several molecular markers such as SSR, SNP, EST-SSR, AFLP, SCOT, RAPD, ISSR and TRAP that have been used in assessing its genetic diversity (Allan et al., 2008; Bajay et al., 2009; Foster et al., 2010; Kallamadi et al., 2015; Thatikunta et al., 2016; Wang et al., 2017; Simões et al., 2017). However, Agyenim-Boateng indicated that the wild samples of R. communis in Southern China were rich in the genetic variability through SRAP analysis and their clusters were closely related to regions in contrast to previous results which showed the absence of a geographically organized genetic population (Agyenim-Boateng et al., 2019). R. communis genetics still needs further molecular study through DNA barcoding approach.

Given all of the above, our objectives of this study were: 1) to assess molecular characterization for C. oblongifolia and R. communis collected from Taif using four DNA barcodes; 2) to determine the genetic variance on the basis of its ancestry; 3) to examine evolutionary rate of the two species under study.
Materials and Methods

**Plant materials:** C. oblongifolia and R. communis (family Euphorbiaceae) were collected from the highlands of Taif, KSA. They were identified according to Colleenette (1999).

**Extraction of DNA and The PCR sequencing:** Young leaves were utilized for the extracting of the genomic DNA depending on CTAB method (Doyle & Doyle, 1987). The primers for the four loci (ITS, ITS2, matK and rbcL) were introduced in Table 1. PCR products of the four DNA barcodes of C. oblongifolia and R. communis were sequenced at Macrogen Inc., South Korea. All obtained sequences were submitted to NCBI-GenBank (Their accessions are mentioned in Table 2).

**Statistics and phylogenetic trees:** Sequences of C. oblongifolia and R. communis were subjected to BLAST to retrieve the most related species of Euphorbiacea from GenBank database of NCBI. In total, loci of ITS, ITS2, matK and rbcL retrieved 178 accessions from GenBank database, 4 accessions were belonging to C. oblongifolia and 129 accessions for R. communis (Table 2), while the others were different Euphorbiaceous species. For alignment, MUSCLE method was performed (Edgar, 2004; Tamura et al., 2013). The statistics; sequence length, (\%) variable sites, Average evolutionary divergence and transition/transversion bias (R) and nucleotide exchange rates were calculated by Maximum Likelihood method (Tamura et al., 2013) utilizing MEGA6 software. 1000 bootstrap replicates have been performed through the phylogenic trees building (Saitou & Nei, 1987) using the Neighbor-Joining method.

### Table 1. List of DNA barcoding primers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer name</th>
<th>Primer sequences (5’–3’)</th>
<th>Ann. temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>AB101</td>
<td>F AC CGAATTCTAGGTCCGTTGTAAGTGGTCC</td>
<td>52°C</td>
</tr>
<tr>
<td></td>
<td>AB102</td>
<td>R TAGAATTCCCGGTTGCCTCGGGTTAC</td>
<td></td>
</tr>
<tr>
<td>ITS2</td>
<td>ITS-S2F</td>
<td>F ATGCGATACTTGGTGTAATGC</td>
<td>52°C</td>
</tr>
<tr>
<td></td>
<td>ITS4</td>
<td>R TCCCTCCGGTTATTGATATGC</td>
<td></td>
</tr>
<tr>
<td>rbcLa</td>
<td>rbcLa</td>
<td>F ATGTACCACAAACAGAGACTAAAGCG</td>
<td>52°C</td>
</tr>
<tr>
<td></td>
<td>rbcLa</td>
<td>R GTAATAATCAAGTCCACCRG</td>
<td></td>
</tr>
<tr>
<td>matK</td>
<td>matK-KIM1</td>
<td>F ACCCGATCCATCTGGAAATCTTTGTC</td>
<td>52°C</td>
</tr>
<tr>
<td></td>
<td>matK-KIM3</td>
<td>R CGTACAGTACTTTTGTGTACAGG</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. GenBank accessions numbers of C. oblongifolia and R. communis.

<table>
<thead>
<tr>
<th>Species</th>
<th>ITS</th>
<th>ITS2</th>
<th>matK</th>
<th>rbcLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrozophora</td>
<td>LC503611</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ricinus</td>
<td>LC503619</td>
<td>LC503620</td>
<td>LC503621</td>
<td>LC503622</td>
</tr>
</tbody>
</table>

**Results and Discussion**

**Chrozophora oblongifolia:** For molecular characterization of C. oblongifolia, the length of sequence, variable sites % and ratio of GC of the two candidate sites (ITS2 and rbcLa) were obtained. After BLAST, ITS2 retrieved three accessions from Saudi Arabia, whereas, rbcLa sequence retrieved only one accession from Japan.

Statistical data revealed that sequence length of rbcLa was greater than that of ITS2, whereas its GC ratio was lower than ITS2. The percentage of variable sites was higher in rbcLa region and subsequently it was more divergent than ITS2 (Table 3). ITS2 locus reported that transitions did not occur, whereas transversions ranged from 9.13 to 16.61 with Transition/Transversion bias (R) equal to 0.00 (Table 4) demonstrating very little or no molecular evolution within C. oblongifolia genome. Moreover, the relative evolutionary rate test of Tajima (Tajima, 1993) confirmed the previous result by accepting the null hypothesis of equal evolution rates between C. oblongifolia from Taif and the other related accessions collected from different regions of Saudi Arabia (P-value was higher than 0.05) (Table 5). We did not obtain sufficient data about rbcLa region because there was only one accession retrieved from the Genbank.

The genetic variation analysis of plant species and their relatives from other countries is a critical aspect of conserving biodiversity. Advanced sequencing technologies can achieve complete screening of plant biodiversity, because they discover and test a major number of molecular markers at a comparatively low price. These markers have been widely utilized in species with or without an available reference genome for genomic selection, linkage map structure and the investigation of plant genetic diversity (Verma et al., 2015; Pavan et al., 2017).

Here, ITS2 and rbcLa sequences represented the genetic divergences of C. oblongifolia and its related accessions retrieved from GenBank. The phylogentic tree of ITS2 demonstrated that all C. oblongifolia collected from KSA were grouped together (Fig. 1), whilst rbcLa tree exhibited intraspecific divergence between the two accessions from Taif and Japan (Fig. 2). The phylogentic analysis showed that the rbcLa site exhibited a higher intraspecific variance than ITS2 This reflected the benefit of using ITS2 due to its conserved secondary structure which is related to the low intraspecific variability (Keller et al., 2010).
Table 3. Statistics of DNA barcodes for *C. oblongifolia* and *R. communis*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>ITS</th>
<th>ITS2</th>
<th>matK</th>
<th>rbcLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence length</td>
<td><em>Chrozophora</em></td>
<td>-</td>
<td>323</td>
<td>-</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td><em>Ricinus</em></td>
<td>620</td>
<td>289</td>
<td>770</td>
<td>517</td>
</tr>
<tr>
<td>GC ratio</td>
<td><em>Chrozophora</em></td>
<td>-</td>
<td>63.0</td>
<td>-</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td><em>Ricinus</em></td>
<td>56.0</td>
<td>60.0</td>
<td>32.0</td>
<td>46.0</td>
</tr>
<tr>
<td>Number of related accessions</td>
<td><em>Chrozophora</em></td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Ricinus</em></td>
<td>28</td>
<td>51</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>% Variable sites</td>
<td><em>Chrozophora</em></td>
<td>-</td>
<td>0.13</td>
<td>-</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td><em>Ricinus</em></td>
<td>13.0</td>
<td>0.01</td>
<td>10.1</td>
<td>14.7</td>
</tr>
<tr>
<td>Average evolutionary divergence</td>
<td><em>Chrozophora</em></td>
<td>-</td>
<td>0.00</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td><em>Ricinus</em></td>
<td>0.01</td>
<td>0.01</td>
<td>0.12</td>
<td>0.18</td>
</tr>
<tr>
<td>(the overall mean distance)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Mean nucleotide substitution rates in the four loci for *C. oblongifolia* and *R. communis*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locus</th>
<th>Transition</th>
<th>Transversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A→G</td>
<td>G→A</td>
</tr>
<tr>
<td><em>C. oblongifolia</em></td>
<td>ITS2</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>ITS</td>
<td>17.6</td>
<td>11.3</td>
</tr>
<tr>
<td><em>R. communis</em></td>
<td>ITS2</td>
<td>41.8</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>matK</td>
<td>8.56</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>rbcLa</td>
<td>16.6</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Table 5. Tajima relative rate tests for *C. blongifolia* and *R. communis*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locus</th>
<th>Outgroup</th>
<th>Test group</th>
<th>RI</th>
<th>RD</th>
<th>RA</th>
<th>RB</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. oblongifolia</em></td>
<td>ITS2</td>
<td>Riyadh</td>
<td>Taif Makkah-Almadinah Road</td>
<td>320</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Makkah-Almadinah Road</td>
<td>Taif Riyadh</td>
<td>320</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>ITS</td>
<td>Indonesia</td>
<td>Taif India</td>
<td>367</td>
<td>0</td>
<td>59</td>
<td>0</td>
<td>59.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UK</td>
<td>Taif Switzerland</td>
<td>219</td>
<td>0</td>
<td>17</td>
<td>5</td>
<td>6.55</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KSA</td>
<td>Taif China</td>
<td>496</td>
<td>1</td>
<td>93</td>
<td>0</td>
<td>93.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>ITS2</td>
<td>KSA</td>
<td>Taif Yemen</td>
<td>283</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lebanon</td>
<td>Taif UAE</td>
<td>287</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Indonesia</td>
<td>Taif India</td>
<td>143</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0.33</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tunisia</td>
<td>Taif Egypt</td>
<td>285</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UK</td>
<td>Taif Switzerland</td>
<td>209</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Venezuela</td>
<td>Taif USA</td>
<td>276</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><em>R. communis</em></td>
<td>matK</td>
<td>India</td>
<td>Taif Yemen</td>
<td>374</td>
<td>0</td>
<td>228</td>
<td>0</td>
<td>228.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>India</td>
<td>Taif Pakistan</td>
<td>390</td>
<td>0</td>
<td>240</td>
<td>0</td>
<td>240.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>China</td>
<td>Taif Japan</td>
<td>390</td>
<td>0</td>
<td>240</td>
<td>0</td>
<td>240.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>rbcLa</td>
<td>South Africa</td>
<td>Taif East African savanna</td>
<td>388</td>
<td>0</td>
<td>240</td>
<td>0</td>
<td>240.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Venezeula</td>
<td>Taif USA</td>
<td>372</td>
<td>0</td>
<td>227</td>
<td>1</td>
<td>224.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Costa Rica</td>
<td>Taif Cuba</td>
<td>363</td>
<td>0</td>
<td>225</td>
<td>0</td>
<td>225.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

- The Tajima relative rate test was performed to test the equality of evolutionary rate of *Chrozophora* oblongifolia and *Ricinus communis* and the other related species depending on different outgroups.
- RI represents the identical regions in sequences, RD represents the divergent regions in the three sequences, RA represents the unique differences in the sequence A, RB represents the unique differences in the sequence B.
- χ² greater than 3.841 (p < 0.05) points out the acceleration in evolution, P value ( > 0.05) refuse evolution (acceptation of the null hypothesis of equal rates between lineages).
Fig. 1. Phylogenetic analysis of *C. blongifolia* and *R. communis* based on ITS2 by Neighbor-Joining method.
**Ricinus communis**: The sequence of *R. communis* genome represents an important resource for studying the genome evolution (Musshoff & Madea, 2009). *R. communis* from Taif highlands was examined to determine and compare the efficiencies of PCR and sequencing of ITS, ITS2, *matK* and *rbcLa* for molecular characterization. After BLAST, the four regions retrieved 28, 51, 28 and 22 accessions belonging to *R. communis* from various countries. The sequence length, GC ratio and variable sites % of the four candidate loci were obtained from the MUSCLE alignment results (Table 3). Statistics showed that *matK* recorded the highest sequence length (770 bp) followed by ITS (620 bp), *rbcLa* (517 bp), ITS2 (289 bp). ITS and ITS2 had the highest GC ratio. *rbcLa* region recorded higher percentage of the variable sites than the others and was subsequently more divergent than that of the other sequences.

All sequences reported that transition rates occurred more than those of transversion (Table 4) and Transition/Transversion bias (R) values demonstrated a noticeable molecular evolution within *R. communis* genome collected from Taif. The results of Tajima relative evolutionary rate for ITS, *matK* and *rbcLa* were similar to those of the Transition/Transversion method. Except ITS2, the three loci reject the null hypothesis of equal evolution rates between *R. communis* from Taif and the other related accessions retrieved from GenBank (p<0.05) indicating accelerated evolution within *R. communis* genome (Table 5). This might be due to the outcrossing process through cross-pollination by wind. Outcrossing is believed to be the “norm” in the wild plants and is usually supported by self-incompatibility (Richards, 1996).
Fig. 3. Phylogenetic analysis of *R. communis* based on ITS by Neighbor-Joining method.

For phylogenetic analysis, four parameters represented the genetic differences of *R. communis* accessions. Within accessions of *R. communis*, the intraspecific distance (overall mean distance) of ITS and ITS2 was lower than that of *matK* and *rbcLa* loci (Table 3 & Figs. 1-4). According to the construction of the phylogenetic trees of ITS and ITS2 by the neighbor-joining method, all *R. communis* accessions were joined in a monophyletic clade and the other Euphorbiaceous species were divided into three clades. Accessions belonging to the Arabian Peninsula, Africa, China and India revealed obvious intraspecific divergence. Based on the ITS2 phylogenetic tree, *R. communis* (Taif) occurred in a separate sub-clade showing little variability from the other origins (Fig. 1), whereas, it grouped with the accession from UK in ITS tree (Fig. 3). On the contrary, *R. communis* accessions were divided into two groups based on the construction of the phylogenetic trees of *rbcLa* and *matK* (Figs. 2 & 4). The first one gathered accessions retrieved from GenBank, and the other group combined *R. communis* (Taif) with accessions from USA and China. Although, accessions from the same country or continent showed limited genetic diversity, they did not get together and revealed intraspecific divergence. *R. communis* (Taif) was found in a separate sub-clade showing noticeable polymorphism from other accessions based on *rbcLa* sequence (Fig. 2). Our results based on DNA barcodes confirmed those of Allan *et al.* (2008), Foster *et al.* (2010) and others who used various molecular aspects that *R. communis* had low genetic diversity, may be due to population bottlenecks. Furthermore we detected some geographically based patterns of genetic relatedness.
Overall, characterization and phylogenetic analyses of the castor bean showed that limited genetic variation detected for nuclear genomic sequences (ITS and ITS2) was consistent with the low plastid genetic diversity (rbcLa and matK). The genetic divergence distribution of ITS was compatible to that of ITS2 for R. communis and the other Euphorbiaceous species. They were divided into separate clades revealing the suitability of ITS and ITS2 as DNA barcodes for distinguishing different species of Euphorbiaceae. Although, the ITS locus contained enough variable sites for species identification in many specimens, it could not be amplified in C. oblongifolia, this may be due to the variability of ITS and including variable insertions or deletions at this taxonomic level. The Chinese Plant BOL Group considered ITS2 to be a useful alternative to ITS for easy amplification and sequencing. Thus, the ITS2 locus might be a suitable barcode for medicinal plants due to its secondary structure (Han et al., 2013).
Unfortunately, rbcLa and matK exhibited confused authentication power for various species of Euphorbiaceae. Both rbcLa and matK showed obvious difference of C. oblongifolia and R. communis (Taif) from their related accessions. Thus, they were somewhat unsuitable genetic loci for authentication of C. oblongifolia and R. communis, because of the absence of a clear intraspecific relatedness. Kress et al., mentioned that, although rbcLa as a source for barcoding and most commonly sequenced for phylogenetic studies, it has little contribution to species level identification. Moreover, universality of matK site was demonstrated to be low in some researches which may restrict its employment as a barcode. Although, high rates of nucleotide substitutions in matK made it a suitable barcode marker for species identification, but it was difficult to amplify and sequence them regularly across varied lineages (Anon.; 2009; Kress et al., 2009).

Conclusions

Characterization of C. oblongifolia and R. communis from Taif was done depending on four specific loci (ITS, ITS2, rbcLa and matK). Based on statistical analyses and phylogenetic study using ITS2 sequence, we conclude that C. oblongifolia and R. communis were close to their related accessions from different locations. The short ITS2 site served as an effective barcode compared to the long ITS site in plant identification. Both rbcLa and matK revealed distinct divergence of C. oblongifolia and R. communis from the retrieved accessions. A combination of rbcLa and matK was recommended to be used as a barcode for Euphorbiaceous plants.

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