Loss of chloroplast trnL_UAA intron in two species of Hedysarum (Fabaceae): evolutionary implications

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Abstract
Previous studies have indicated that in all land plants examined to date, the chloroplast gene trnL_UAA is interrupted by a single group I intron ranging from 250 to over 1400 bp. The parasitic Epifagus virginiana has lost, however, the entire gene. We report that the intron is missing from the chloroplast genome of two arctic species of the legume genus Hedysarum (H. alpinum, H. boreale). DNA sequencing of the trnL gene and trnL-trnF intergenic spacer (trnL-F), as well as portion of trnF exon in these species confirms the absence of trnL intron and shows that it has been deleted from the gene precisely along established exon/intron splicing sites. Phylogenetic analysis of trnL-F sequence data revealed that they are closely related species. This indicates that the intron was lost from the chloroplast genome before the divergence of the two Hedysarum species. It is concluded that this rare genomic structural mutation may have occurred once during the evolution of land plants.

Keywords: Chloroplast DNA; Fabaceae; Hedysarum; Structural mutation; trnL_UAA intron loss

INTRODUCTION
Based on the conserved characteristics of the primary sequence and the predicted secondary structure, chloroplast introns are classified as either group I, group II, or group III introns. A basic set of chloroplast introns, consisting of one group I intron and ca 20 group II introns, is a common feature of embryophytes (land plants) and their closest algal relatives-members of the Charophyta (Schmitz-Linneweber and Barkan, 2007). Almost all land plants and charophyte algae have a single group I intron in the trnL_UAA gene (Shaw et al., 2005). This intron is the most ancient intron that is considered to have been present in the common ancestor of cyanobacteria and acquired by plastids via vertical transmission (Asakura and Barkan 2007; Simon et al., 2003; Xu et al., 1990). The frequent loss of the intron in red algae and some of their secondary plastid derivatives as well as among green algae was already documented (Simon et al., 2003). The trnL_UAA gene is located between the tandemly arranged tRNA genes trnT_UGU and trnF_GAA in the large single copy region of the chloroplast genome in land plants (Taberlet et al., 2007; Sugiura, 1996; Taberlet et al., 1991). The trnL intron ranges from ca 250 to over 1400 bp in land plants (Shaw et al., 2005). Sequences of this trnL intron are usually coamplified with the trnL-trnF intergenic spacer and together these two fragments (hereafter trnL-F) have become the most popular and widely used noncoding cpDNA markers (Kazemi et al., 2009; Shaw et al., 2005). This intron shows sequence conservation in the regions flanking the trnL exons, while the central part is variable (Hao et al., 2009; Quandt and Stech, 2005; Bakker et al., 2000). It has a specific secondary structure and several highly conserved motifs that are found among all group I introns (Won and Renner, 2005; Shaw et al., 2005 and references therein).

Here, we report loss of the intron in two arctic species of the legume genus Hedysarum. Prior to this
report, the loss of this intron (actually whole trnL gene) in chloroplast of land plants has only been reported in the parasitic plant *Epifagus virginiana* (Wolfe et al., 1992).

**MATERIALS AND METHODS**

**Taxon sampling:** In the framework of a phylogenetic analysis of the tribe Hedysareae and allies using both nrDNA ITS and trnL-F sequences for over 70 taxa (Amirahmadi et al. unpubl. data), a subset of eight species including, *Alhagi persarum*, *Ebenus stellata*, *Hedysarum alpinum* (two accessions), *H. boreale* (two accessions), *H. aculeolatum*, *H. hedysaroides*, *Sulla pallida* and *Onobrychis pulchella* were chosen in the present work. *Astragalus curvipes* was included here as a positive control, containing the intron previously reported (Kazemi et al., 2009; Table 1).

<table>
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<tr>
<th>Species</th>
<th>Voucher accession</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
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<td><em>Astragalus curvipes</em> Traut.</td>
<td>Iran: Gorgan, Golestan National Park, Maassoumi 47553 (TARI)</td>
<td>AB485938a</td>
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<td><em>Alhagi persarum</em> Boiss. &amp; Buhse</td>
<td>Iran: Rudbar, Kazempour Osaloo 2008-1 (TMUPC)</td>
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<td><em>Ebenus stellata</em> Boiss.</td>
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<td>Canada: Saskatchewan, Prince Albert, Doppelbaur 15788 (MSB)</td>
<td>AB558515</td>
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<td><em>Hedysarum hedysaroides</em> (L.) Schinz &amp; Thell.</td>
<td>USSR: Magadan, Bilibinsky, Zaslavskaja 6184 (TARI)</td>
<td>AB558517</td>
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<td><em>Sulla pallida</em> (Desf.) Choi &amp; Ohashi (=<em>Hedysarum pallidum</em> Desf.)</td>
<td>Morocco: Oudja, Taourirt, Podlech 97026 (MSB)</td>
<td>AB558518</td>
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<td><em>Onobrychis pulchella</em> Miller</td>
<td>Iran: Khorasan, Kalate-Naderi, Ghaframan et al. 27318 (TUH)</td>
<td>AB558519</td>
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</table>

Abbreviations used in voucher information:

TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran; TMUPC, Tarbiat Modares University Plant Collection, Tehran, Iran; TUH, Tehran University Herbarium, Tehran, Iran; MSB, Herbarium of Ludwig-Maximilians-Universität, München, Germany. With the exception of TMUPC, herbarium acronyms are according to Holmgren and Holmgren (2008).

*trnL-F* sequence for *Astragalus curvipes* was retrieved from GenBank.

**DNA isolation, amplification and sequencing:** Total genomic DNA was isolated from fresh or herbarium materials using the modified cetyl trimethylammonium bromide (CTAB) procedure of Doyle and Doyle (1987). For each genomic DNA, the *trnL* intron (if present) and portions of its flanking exons were ampli-

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**Figure 1.** Scheme of primers used to amplify *trnL* intron and *trnL-trnF* intergenic spacer.

**Figure 2.** Ethidium bromide stained 1% agarose gel showing PCR amplified products for selected legume taxa screened for presence/absence of the *trnL* intron. Lanes 1-11: (1) *Alhagi persarum*, present (625 bp); (2) *Onobrychis pulchella*, present (491 bp); (3) *Ebenus stellata*, present (499 bp); (4,5) *Hedysarum alpinum*, absent (74 bp); (6,7) *Hedysarum boreale*, absent (74 bp); (8) *Hedysarum aculeolatum*, present (423 bp); (9) *Hedysarum hedysaroides*, present (432 bp); (10) *Sulla pallida*, present (428 bp); (11) *Astragalus curvipes*, present (615 bp). M= 100bp ladder.
fied using the universal “c” and “d” primers of Taberlet et al. (1991). In order to sequencing, the whole trnL-trnF region (trnL gene, trnL-trnF intergenic spacer, and a portion of trnF exon) was then amplified using the “c” and “f” primers of Taberlet et al. (1991). Position of all primers used for the amplifi-
Polymerase chain reaction (PCR) was carried out in 20 μl final volume of mixture containing 1.0 μl of template DNA (5 ng/μl), 0.5 μl of each primer (5 pmol/μl), 10 μl of the 2X Taq DNA polymerase master mix Red (Amplicon, Cat. No. 180301, Germany) and 8.0 μl sterile water. PCR cycles consisted of 31 cycles of 50 s at 94ºC for template denaturation, 40 s at 58ºC for primer annealing, and 55 s at 72ºC for primer extension, followed by 7 min at 72ºC for completion of primer extension. PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and were photographed with a UVI gel documentation system (UVItec, Cambridge, UK). The trnL-F region was then sequenced using the ‘Big dye terminator cycle sequencing ready reaction kit’ (Applied Biosystems, USA) with the same “c” and “f” primers in an ABI Prism 3730xl DNA Analyzer (Applied Biosystems, USA).

Phylogenetic analysis: trnL-F sequences for the abovementioned taxa were edited using BioEdit ver. 7.0.9.0 (Hall, 1999) and aligned using ClustalX (Larkin et al., 2007), followed by manual adjustment. Alignment of the dataset required the introduction of numerous single and multiple base indels (insertions/deletions). Positions of indels were treated as missing data. Phylogenetic analysis of the trnL-F dataset was performed by the maximum parsimony method using PAUP* program version 4.0b10 (Swofford, 2002), employing the same search strategy as described previously (Tavakkoli et al., 2010). Astragalus curvipes was chosen as an outgroup.

RESULTS

According to our PCR experiments, the trnLUA intron was inferred to be absent only in four accessions of the two species of Hedysarum, H. alpinum and H. boreale (Fig. 2). The length of trnL gene ranged from 74 bp in H. alpinum and H. boreale to 625 bp in Alhagi persarum. For those species having the intron, size variation differed 116 to 192 bp from the control species.
(Astragalus curvipes), except for Alhagi persarum, where it was 10 bp longer than in A. curvipes. DNA sequencing of the trnL-F region confirmed that the intron was completely missing from H. alpinum and H. boreale, and that it has led to a junction of 5’trnL exon and 3’trnL exon to form an uninterrupted gene (Fig. 3).

**DISCUSSION**

As noted above, DNA sequencing showed that the trnL intron was completely lost in both H. alpinum and H. boreale. Parsimony analysis of trnL-F sequences revealed that these two species with four sampled accessions formed a well supported clade which was sister to H. hedysaroides (Fig. 4). It is fully consistent with our comprehensive phylogenetic analyses of nrDNA ITS, trnL-F and the combined nrDNAITS-trnL-F sequence data for Hedysareae and related taxa (Amirahmadi et al. unpubl. data). This indicates that the intron loss has occurred in the chloroplast genome before the divergence of the two *Hedysarum* species (Fig. 4). The precise loss of an intron has been reported from other chloroplast genes (e.g., Jansen et al., 2008, 2007; Campagna and Downie, 1998; Wallace and Cota, 1996; Downie and Palmer, 1992; Downie et al., 1991; Hiratsuka et al., 1989). The legumes are one of a few angiosperm families that have experienced multiple losses of introns such as rpl2, rps12 and clpP (Jansen et al., 2008 and references therein). The loss of the trnL intron in the two species of Hedysarum is another exemplar that might be **per se** a rare evolutionary event among photosynthetic land plants.

Earlier studies (Simon et al., 2003; Xu et al., 1990) hypothesized that trnL intron in the chloroplast of the land plants has completely lost the self-splicing ability and thus has become dependent on a host factor to facilitate excision. Furthermore, Asakura and Barkan (2007) revealed that Crm Family Member 2 (CFM2), a nucleus-encoded protein, is bound to and enhances the splicing of the trnL intron. Nakamura et al. (1999) reported, however, that chloroplast ribonucleoproteins (cPRNPs) interact with unspliced trnL_{UAA} RNA from tobacco chloroplast extract. One possible mechanism for the loss of trnL intron might be reverse transcription of the spliced RNA, which does not contain intron, followed by homologous recombination of the cDNA with the chloroplast genome at precisely the same position in the common ancestor of the two *Hedysarum* species. Such process has been already reported for the loss of both group I and group II introns in plants, fungi and animals (e.g., Jeffares et al., 2006; Hu 2006; Campagna and Downie, 1998; Downie et al., 1991; Hiratsuka et al., 1989; Dujon, 1989). Other mechanisms of intron loss could be simple genome deletion and in-frame intron deletion (Niu et al., 2005 and references therein).

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**References**


