Discrimination and Authentication of *Eclipta prostrata* and *E. alba* Based on the Complete Chloroplast Genomes

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ABSTRACT *Eclipta prostrata* and *E. alba* are annual herbal medicinal plants and have been used as Chinese medicinal tonics. Both species are widely distributed in tropical and subtropical regions as well as in Korea. Both species have similar morphological features but *E. alba* has smoother leaf blade margins compared with *E. prostrata*. Although both species are utilized as oriental medicines, *E. prostrata* is more widely used than *E. alba*. Morphological semblances have confounded identification of either species. Here, we report the complete chloroplast genomes of both species to provide an authentication system between the two species and understand their diversity. Both chloroplast genomes were 151,733–151,757 bp long and composed of a large single copy (83,285–83,300 bp), a small single copy (18,283–18,346 bp), and a pair of inverted repeats (25,075–25,063 bp). Gene annotation revealed 80 protein coding genes, 30 tRNA genes and four rRNA genes. A phylogenetic analysis revealed that the genus *Eclipta* is grouped with *Heliantheae* tribe species in the Asteraceae family. A comparative analysis verified 29 InDels and 58 SNPs between chloroplast genomes of *E. prostrata* and *E. alba*. The low chloroplast genome sequence diversity indicates that both species are really close to each other and are not completely diverged yet. We developed six DNA markers that distinguish *E. prostrata* and *E. alba* based on the polymorphisms of chloroplast genomes between *E. prostrata* and *E. alba*. The chloroplast genome sequences and the molecular markers generated in this study will be useful for further research of *Eclipta* species and accurate classification of medicinal herbs.

Keywords *Eclipta prostrata*, *E. alba*, Medicinal herbs, Chloroplast sequence, Molecular marker

INTRODUCTION

Eclipta prostrata is an annual herbaceous plant belonging to the family Asteraceae which includes 1,620 genera and 23,600 species (Mithun *et al.* 2011). It is distributed in tropical and subtropical regions including South America, Asia, Africa, and south of the central region in Korea (Chokotia *et al.* 2013). It has tap roots and many brown branches. Also, it has two types of flowers, ray

florets and disc florets, with involucre of bracts that arrange in two lines (Neeraja and Margaret 2012). *E. alba* is also included in the family Asteraceae and has very similar shapes with *E. prostrata* except for smoother margins of leaf blades. *E. prostrata* has been used as a medicinal plant owing to compounds like Eclalbasaponin I and wedelolactone found in the plant which have antitumor and cirrhosis and hepatitis curing effects (Dalal *et al.* 2010; Liu *et al.* 2012). In addition, it has been used as resources for

Received November 20, 2017; Revised November 22, 2017; Accepted November 22, 2017; Published December 1, 2017

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Chinese tonics to cure loose teeth, tinnitus, hemoptysis, hematuria and uterine bleeding (Chinese Pharmacopoeia Commission 2010). Similarly, *E. alba* has been used as a medicinal plant owing to its flavonoids that could induce anagen, which helps blackening hairs (Datta *et al.* 2009). Due to similar morphological traits and widely shared habitats (Baskaran and Jayabalan 2005; Dhaka and Kothari 2005), *E. alba* is often mixed with *E. prostrata* (Muruganatham *et al.* 2009), which cause confusion in the academic world as well as the markets (Neeraja and Margaret 2012). Therefore, it is necessary to develop a high-throughput system to discriminate them for efficient genetic research and sustain its market value.

Chloroplast genomes are maternally-inherited (Cheng *et al.* 2005; Dong *et al.* 2012), rarely apt to occur recombination and mutation events. Chloroplast genome possesses significant interspecific variations and has widely been used for classification of plant species. Interspecific taxonomic studies have been conducted based on chloroplast genomes in *Scalesia affinis*, *Abies*, and *Citrus* species (Parducci *et al.* 2001; Nielsen *et al.* 2004; Cheng *et al.* 2005). Chloroplast DNA markers are still a useful tool for species classification. Recently, some researches had been performed to discriminate species with molecular markers based on chloroplast genomes of *Cynanchum wilfordii*, *Cynanchum auriculatum* and *Polygonum multiflorum* (Kim *et al.* 2013; Jang *et al.* 2015; Kim *et al.* 2015c; Park *et al.* 2015).

Previously, we reported the chloroplast genome for *E. prostrata* (Park *et al.* 2016). In this study, we assembled the complete chloroplast genome of *E. alba*. In addition, we have conducted a comparative phylogenetic analysis of *E. alba* and *E. prostrata* along with 10 species that belong to the Asteraceae family. In addition, we show genome-level diversity between the closely related *Eclipta* species and have developed polymorphic markers that could efficiently distinguish *E. alba* and *E. prostrata* and thus can practically be applied for authentication of those species for adequate use of these medicinal herbs.

MATERIALS AND METHODS

Plant materials and genome sequencing

E. prostrata and *E. alba* plants used in this study were collected from HanTaek Botanical Garden (Yongin, Korea, www.hantaek.co.kr) and Jeju Island, respectively. Specimens of dried *Eclipta* plant tissues were provided by Ministry of Food and Drug Safety. Genomic DNAs of *E. prostrata* and *E. alba* were extracted from leaf tissues by a modified cetyltrimethylammonium bromide (CTAB) method (Allen *et al.* 2006), and the DNA of the specimens were extracted from each of the whole, stem, and leaf tissues by Genomic Plus DNA Prep kit (Inclone, Korea). The whole tissues of specimens were composed of stems, leaves, and flowers. The quality and quantity of extracted DNA were checked using Nanodrop ND-1000 (Thermo scientific, Wilmington, USA). Paired-end sequencing (PE) was conducted using Illumina MiSeq platform by Lab-Genomics (www.labgenomics.co.kr, Seongnam, Korea).

Chloroplast genome assembly

Raw paired-end (PE) reads were trimmed and *de novo* assembled using the method as described by Kim *et al.* (2015a, 2015b). From initial assembly, contigs representing chloroplast genome were retrieved, ordered, and combined into a single sequence by comparing chloroplast genome sequence of *Centaurea diffusa* (KJ690264). The assembled sequence was manually corrected and gap-filled by a series of PE read mapping. The assembled chloroplast genome was annotated using DOGMA (<http://dogma.cccb.utexas.edu>) and manually corrected with BLAST search.

Phylogenetic analysis

Phylogenetic analysis was conducted using multiple sequence alignments of 29 protein coding gene sequences (*psaI/J*, *psbB/C/D/H/I/K/T*, *petA/B/D/G/N/L*, *atpA/F/H/I*, *ndhB*, *rpoB/C2*, *rps2/7/18*, *rpl2*, *accD*, *ycf2/4*) in 12 chloroplast genomes (*Chrysanthemum × morifolium*, JQ362483; *Chrysanthemum indicum*, JN867589; *Artemisia montana*, KF887960; *Artemisia frigida*, JX293720; *Aster spathulifolius*, KF279514; *Leontopodium leirolepis*, KM267636; *Helianthus annuus*, DQ383815; *Eclipta prostrata*, KU361242; *Eclipta alba*, MF993496; *Guizotia*

abyssinica, EU549769; *Jacobaea vulgaris*, HQ234669; *Lactuca sativa*, AP007232) belonging to the Asteraceae family. Phylogenetic tree was constructed using MEGA6.0 (Tamura *et al.* 2013) with the parameters of neighbor-joining method and 1000 bootstrap.

Comparison of inter-species level and development of molecular marker

Sequence variations were identified by mVISTA program (<http://genome.lbl.gov/vista/mvista/submit.shtml>) and the polymorphic regions were found using MAFFT

Fig. 1. Chloroplast genome map of *E. alba* and inter-species polymorphic DNA markers. Chloroplast genome map of *E. alba* was generated using OGDRAW (<http://ogdraw.mpimp-golm.mpg.de/>). Genes transcribed clockwise and counterclockwise are indicated on the outside and inside of the large circle, respectively. The four parts of the chloroplast genome and GC content are indicated on the inner circle. Red and blue bars in the inner circle indicate InDels and SNPs, respectively, identified between *E. prostrata* and *E. alba* chloroplast genome sequences. Black arrows represent the target regions for InDel markers and the purple arrow indicates SNP marker to differentiate *E. prostrata* and *E. alba*.

program (<http://mafft.cbrc.jp/alignment/software>). The two types of molecular markers, InDel and SNP, were developed based on the polymorphic sites in chloroplast genomes of *E. prostrata* and *E. alba*. The primers were designed using NCBI Primer-blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). PCR reaction for InDel markers was conducted in a total volume of 25 µL. The reaction mixtures consisted of 20 ng of template DNA, 1x PCR buffer (Vivagen, South Korea), 2.5 mM dNTP (Vivagen, South Korea), 10 pmol of each primer, and 2 units/µL of *Taq* DNA polymerase. PCR conditions were as follows: 5 minutes at 95°C, following 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C, 30 seconds at 72°C, and a final extension at 72°C for 5 minutes. PCR amplicons were checked on 3% agarose gel in condition of being stained by Inclone™ Safe Gel stain. Then they were visualized under UV-trans-illuminator with a gel documentation system.

PCR reaction for SNP markers was performed in a total volume of 20 µL. The mixtures were composed of 20 ng of template DNA, 1x PCR buffer (Vivagen, South Korea), 2.5 mM dNTP (Vivagen, South Korea), 10 pmol of each primer, 2 units/µL of *Taq* DNA polymerase, and fluorescent dye SYTO 9 (Roche Diagnostics, IN). The PCR conditions were as follows: 5 minutes at 95°C, following 35 cycles of 20 seconds at 95°C, 20 seconds at 58°C, 30 seconds at 72°C. Then, HRM analysis was conducted using LightCycler 480 (Roche Applied Science) with the conditions that were as follows: 1 minute at 95°C, 1 minute at 40°C, 5 seconds at 70°C and the temperature increased to 90°C, then cooled down to 40°C as fluorescence acquisition.

RESULTS

Complete chloroplast genome and inter-species polymorphism between *E. alba* and *E. prostrata*

The completed chloroplast genome of *E. alba* is a circular molecule of 151,733 bp long and has typical quadripartite structure composed of a large single copy (LSC) region of 83,300 bp, small single copy (SSC) region of 18,283 bp and a pair of inverted repeats (IRa and IRb) of 25,075 bp (Fig. 1, Table 1). A total of 114 genes including 80 protein coding genes, 30 tRNA genes, and four rRNA genes (Table 2) were annotated and the GC content of the chloroplast genome was 37.49%.

In comparison, the overall chloroplast genome size of *E. alba* showed 24-bp shorter than that of *E. prostrata*. *E. alba* is 15-bp and 24-bp longer than *E. prostrata* in the LSC and IR regions, respectively. In the SSC region, *E. alba* is 63-bp shorter than *E. prostrata*. A total of 58 SNPs and 29 InDels were identified between *E. alba* and *E. prostrata*. Among the polymorphic sites, 39 SNPs and 29 InDels are identified at intergenic regions and remaining 19 SNPs were identified in the genic regions.

Phylogenetic analysis

Phylogenetic analysis was conducted using protein coding gene sequences in the chloroplast genomes of *E. prostrata*, *E. alba* and 10 species from the Asteraceae family. *E. prostrata* and *E. alba* showed very narrow genetic diversity as being grouped together. As expected, this result showed that *E. prostrata* and *E. alba* were grouped with *Heliantheae* tribe species (Fig. 2) and exhibited close relationship to the species in *Senecioneae* and *Cichorieae* in the Asteraceae family. The clade, including *Eclipta* species, showed a sister relationship with species belonging to the *Astereae*, *Gnaphalieae* and *Anthemideae*.

Table 1. Summary of NGS data and chloroplast genomes of *E. alba* and *E. prostrata*.

Collected species	Raw data bases (bp)	Cp genome coverage (x)	Cp length (bp)	GenBank accessions	LSC length (bp)	IR length (bp)	SSC length (bp)
<i>E. alba</i>	1,027,482,009	89.31	151,733	MF993496	83,300	25,075	18,283
<i>E. prostrata</i> ²⁾	1,473,824,997	317.69	151,757	KU361242	83,285	25,063	18,346

²⁾Reported by Park *et al.* (2016).

Table 2. Information of annotated genes in chloroplast genome of *E. alba*.

Gene types	Gene list
Photosystem I	<i>psaA</i> , B, C, I, J
Photosystem II	<i>psbA</i> , B, C, D, E, F, H, I, J, K, L, M, N, T, Z
Cytochrome b6/f complex	<i>petA</i> , B, D, G, N, L
ATP synthase	<i>atp</i> A, B, E, F, H, I
NADH oxidoreductase	<i>ndhA</i> , B, C, D, E, F, G, H, I, J, K
RubisCO large subunit	<i>rbcL</i>
RNA polymerase	<i>rpoA</i> , B, C1, C2
Ribosomal proteins (SSU)	<i>rps</i> 2, 3, 4, 7, 8, 11, 12, 14, 15, 16, 18, 19
Ribosomal proteins (LSU)	<i>rpl</i> 2, 14, 16, 20, 22,23, 32, 33, 36
clpP, matK	<i>clpP</i> , <i>matK</i>
Other genes	<i>ccsA</i> , <i>cemA</i> , <i>accD</i> , <i>infA</i>
Hypothetical chloroplast reading frames	<i>ycf1</i> , 2, 3, 4, 15
Transfer RNAs	<i>trnA</i> -UGC, C-GCA, D-GUC, E-UUC, F-GAA, fM-CAU, G-GCC, G-UCC, H-GUG, I-CAU, I-GAU, K-UUU, L-CAA, L-UAA, L-UAG, M-CAU, N-GUU, P-UGG, Q-UUG, R-ACG, R-UCU, S-GCU, S-GGA, S-UGA, T-GGU, T-UGU, V-GAC, V-UAC, W-CCA, Y-GUA
Ribosomal RNAs	<i>rrn</i> 4.5, 5, 16, 23

Fig. 2. Phylogenetic analysis of *E. prostrata* and *E. alba*. The tree was generated with protein coding gene sequences in chloroplast genomes of *E. prostrata*, *E. alba* and 10 species belonging to the Asteraceae family by multiple alignment using MAFFT (<http://mafft.cbrc.jp/alignment/server/index.html>) and a neighbor-joining (NJ) analysis using MEGA 6.0 (Tamura *et al.* 2013). Numbers in the nodes are bootstrap support values (>50%) from 1000 replicates.

Molecular markers to distinguish *E. alba* and *E. prostrata*

Six inter-species polymorphic markers were developed

from polymorphic regions (Table 3). The PCR amplicons showed 266/284 bp, 283/267 bp, and 230/284 bp difference (*E. prostrata*/*E. alba*) for three InDel markers, ep_01, ep_02 and ep_03, respectively, as expected (Fig. 3).

Table 3. Details of molecular markers developed in this study.

Type	Marker name	Target region		Primer sequence	Variation	Product size (bp)
InDel	ep_01	<i>atpI-atpH</i>	F	TGTCAAGGGTTAGACGCATCC	18 bp	266/284
			R	TGTCCCGAATCGCTCTTTGA		
	ep_02	<i>clpP</i>	F	AGAACCAGCAGGTTGATGGA	16 bp	283/267
			R	TTCCTCCGAAAGGAAGGGTG		
	ep_03	<i>ycf1-ndhF</i>	F	TCGATGCAACAGCAAGATGC	54 bp	230/284
			R	AAATCATTAGGGGGTGGACG		
SNP	ep_hrm_01	<i>ccsA</i>	F	GTGGCGACTCTAGGCTTTCT	G/A	179
			R	TCGCCGTTGAGACAAGATT		
	ep_hrm_02	<i>ycf1</i>	F	TCTCTACGACGTTTAGACGATAAAA	T/G	155
			R	AAGCACCAAAGTAATCAAAGATACC		
	ep_hrm_03	<i>petB</i>	F	TATTGCCGCTTCTTACTGCG	T/C	146
			R	ACTATAGTTCCTACCCCAAGTGAT

Fig. 3. Three InDel-based markers to discriminate between *E. prostrata* and *E. alba*. (a) A 18-bp InDel in *atpI-atpH* and (d) its PCR products for *E. prostrata* and *E. alba*. (b) A 16-bp InDel in *clpP* and (e) its PCR products for *E. prostrata* and *E. alba*. (c) A 54-bp InDel in *ycf1-ndhF* and (f) its PCR products for *E. prostrata* (EP) and *E. alba* (EA). M: 100-bp DNA ladder.

Meanwhile, three SNP markers, ep_hrm_01, ep_hrm_02, and ep_hrm_03 discriminated *E. prostrata* from *E. alba* based on HRM analysis (Fig. 4).

Application of InDel markers to randomly collected *Eclipta* herbal medicinal products

Three InDel-based markers were applied to five random

specimens provided by Ministry of Food and Drug Safety to authenticate their source whether from *E. prostrata* or *E. alba*. The specimens include collections of dried plant tissues which utilized as oriental medicines. PCR analysis conducted with the InDel-based markers applying to the each form (bulk form, stem form, leaf form, another leaf form) of all samples. Both sizes of PCR amplicons,

Fig. 4. Three SNP-based markers to distinguish *E. prostrata* and *E. alba*. Alignment of nucleotide sequences to show the SNPs (arrow) in (a) *ccsA*, (b) *ycf1* and (c) *petB* gene of chloroplast genomes of *E. prostrata* and *E. alba*, and (d-f) HRM analysis to detect each of the three SNPs. M: 100-bp DNA ladder.

Fig. 5. Application of InDel markers to five bulk *Eclipta* samples. The three InDel markers, (a) ep_01, (b) ep_02, (c) ep_03 were applied to *E. prostrata*, *E. alba*, and five random specimens in each form of bulk, stem, and leaves. M: 100bp ladder, EP: *E. prostrata*, EA: *E. alba*, 01-05: The names of the random specimens, B: bulk form, S: stem form, L1: leaf form, L2: another leaf form.

266/284 bp, 283/267 bp, and 230/284 bp differences, were shown for three InDel markers, respectively in the sample 01. (Fig. 5). The results indicated that these medicinal herbs are being sold with the state of mixing in the markets.

DISCUSSION

Discrimination of *E. alba* and *E. prostrata* with six molecular markers

Despite different medicinal effects of *E. alba* from those of *E. prostrata*, it has been confused often with *E. prostrata* in the market and academic world because of their similar morphology and widely shared distribution (Baskaran and Jayabalan 2005; Dhaka and Kothari 2005). Thus, it is essential to develop a unique system to investigate *E. prostrata* and *E. alba* to protect against false trading (Neeraja and Margaret 2012).

Here, we generated the complete chloroplast genome of *E. alba* and identified 87 inter-species polymorphic sites, 58 SNPs and 29 InDels, between *E. prostrata* and *E. alba*. These polymorphic regions could be applied to phylogenetic analysis and development of molecular markers for discrimination of *E. prostrata* and *E. alba*. Phylogenetic analysis revealed that *E. prostrata* and *E. alba* are really close and grouped with *Heliantheae* species (Fig. 2). Overall phylogenetic result is consistent with a previous study (Park *et al.* 2016).

In this study, we report six markers that can be applied for classification of *E. prostrata* and *E. alba*, and for authentication of five random specimens of dried *Eclipta* plant tissues. Three InDel-based markers successfully distinguished *E. prostrata* from *E. alba* and three SNP markers effectively divided the melting patterns of *E. prostrata* from *E. alba* with HRM analysis. Overall, the results suggest that these molecular markers could contribute to ascertain the exact medicinal resources between those species.

Authentication of *Eclipta* products with three InDel-based markers

For further validation of InDel-based markers and authentication of random *Eclipta* medicinal herbs, we

conducted PCR analysis with the InDel-based markers developed in this study (Figs. 4 and 5). All InDel-based markers efficiently discriminated *E. prostrata* from *E. alba* as previously proved in this research. However, sample no. 01 showed both genotypes for the markers, indicating that the products contain tissues from both species (Fig. 5).

Through this analysis, we presented an instance of admixture between *E. prostrata* and *E. alba* in the market. Both species show very similar morphology and also very low sequence level genetic diversity for complete chloroplast genomes. Relatively abundant intra-species polymorphisms are identified in *Pedicularis chamissonis*, *Panax ginseng*, *Scutellaria baicalensis* and *Rhus chinensis* (Fujii *et al.* 1997; Kim *et al.* 2015a; Jiang *et al.* 2017; Joh *et al.* 2017; Kim *et al.* 2017). We found very low diversity between both species with their sequence variations, suggesting an on-going genome divergence of the two species. Because of the similar morphology and the sequence level genome similarity, nevertheless, it is required to expand this research by investigating more *Eclipta* species for solid conclusion and for establishment of clear discrimination criteria.

ACKNOWLEDGEMENTS

This research was supported by a grant from Ministry of Food and Drug Safety in 2015 (15172MFDS246) and the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (NRF-2015M3A9A5030733).

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