PCR-RFLP and genetic diversity analysis of cpDNA in some species of the genus *Salvia* L.

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ABSTRACT. Long Accurate polymerase chain reaction restriction fragment length polymorphism (LA-PCR-RFLP) of the chloroplast DNA (cpDNA) was used to investigate phylogenetic relationships between 54 species of *Salvia* L. Two *Ocimum* L. species were used as an out group. A pair of universal primers in LA-PCR protocol resulted in long cpDNA of an expected size (~15 kilo base pair (kb)) from each *Salvia* and *Ocimum* species, except *S. x superba* (hybrid; *S. sylvestris* x *S. amplexicaulis* or *S. nemorosa*). LA-PCR products were separately digested with each of *EcoRI*, *HindIII*, *PvuII*, *NdeI*, *SmaI* or *MfeI* restriction enzymes. *S. roemeriana* Scheele showed amplified fragment length difference (AFLD). *S. roemeriana* and/or *S. lyrata* L. could be identified with any of the enzymes used except *PvuII*. Identification of *Ocimum* species from *Salvia* could be carried out with *NdeI*, *SmaI* or *MfeI*. *EcoRI* and *MfeI* exhibited more polymorphic and informative patterns than *HindIII*, *PvuII*, *NdeI* or *SmaI*. LA-PCR-RFLP analysis generated 28 polymorphic restriction fragments. Fragments were visually detected, photographed and used to build a genetic similarity data matrix based on Nei and Li (1979) method. PHYLIP software package (Felsenstein 2005) and the genetic similarity matrix were exploited to construct a phylogenetic tree. The phenogram generated by UPGMA clustering analysis separated *Salvia* species based on genetic distance into distinct groups obviously dependent on origin.

KEYWORDS: Chloroplast DNA, PCR-RFLP, *Salvia*

Salvia L. is the largest genus in the Lamiaceae. *Salvia* species are perennial, biennial, or annual herbs, evergreen or deciduous shrubs (Clebsch 2003). Approximately 900 species of *Salvia* are distributed worldwide and undergone extensive speciation process in three different regions of the world. Central and South America have about 500 species of the genus and Central Asia and Mediterranean area have about 250 species, while eastern Asia has around 90 species. Most members of the *Menthaeae* tribe are characterized by four stamens, but *Salvia* has only two stamens (Claßen-Bockhoff *et al.* 2003). Unusual structure of stamens, diversity in floral morphology, and vegetative habit created some confusion regarding infra-generic boundaries in *Salvia* and was considered monophyletic (Walker and Sytsma 2007). Bentham's study (1848; cited by Walker *et al.* 2004) of *Salvia* separated the genus into 12 sections. Walker *et al.* (2004) studied the chloroplast sequences of *trnL-F* inter-genic region and *rbcL* gene and found that *Salvia* is not monophyletic but comprises at least two and possibly three distinct lineages. These lineages were assigned as Clade I of mainly Old World species, Clade II of mainly New World species and Clade III of Asian species.

Chloroplast genomes as well as other organelle genomes evolve slower than nuclear genomes (Palmer 1987). This conservation phenomenon of organelle genomes was exploited to design universal primers that were capable of

amplifying polymorphic non-coding regions of cpDNA of some algae, bryophytes, pteridophytes, gymnosperms and angiosperms (Taberlet *et al.* 1991), non-coding regions of mitochondrial and cpDNA (Demesure *et al.* 1995), coding regions of cpDNA (Badenes and Parfitt 1995; Tsumura *et al.* 1996) as well as whole chloroplast genomes (Dhingra and Foltá 2005; Ibrahim *et al.* 2007). A database for some of these primers was created by Heinze (2007).

The cpDNA and its universal primers were important for taxonomic, molecular phylogenetics and evolutionary relationships among diverse taxa as well as to develop species-specific markers. Universal primers have been used to study phylogenetic relations (Gielly and Taberlet 1994; Badenes and Parfitt 1995; Tsumura *et al.* 1996; Demesure *et al.* 1996; Parani *et al.* 2000; Wang *et al.* 2000; Cronn *et al.* 2002; Stehlik *et al.* 2002; Stehlik 2002), species identification (Parani *et al.* 2001; Ridgway *et al.* 2003; Tsai *et al.* 2006, 2008), polymorphic chloroplast microsatellites (Powell *et al.* 1995; Cato and Richardson 1996; Parducci *et al.* 2001; Ishii *et al.* 2001; Arroyo-Garcia *et al.* 2002) and genetic diversity (Parducci and Szmids 1999; Huang and Sun 2000; Walker *et al.* 2004).

RFLP analysis of chloroplast genomes is widely used for plant evolutionary studies, particularly at the inter- and intra-specific level and molecular systematics (Strauss and Doerksen 1990; Govindaraju *et al.* 1992; Wang and Szmids 1994; Krupkin *et al.* 1996; Schaal *et al.* 1998;

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Byrne *et al.* 2003). PCR-RFLP was applied for the identification of intra-specific variations in the cpDNA of *Abies alba* (Ziegenhagen *et al.* 1995), *Quercus robur* (Dumolin *et al.* 1995), *Fagus sylvatica* (Demesure *et al.* 1996), *Ginkgo biloba* (Shen *et al.* 2005) and *Quercus glauca* (Shih *et al.* 2006). It has provided evidence for the presence of inter-specific variations in the cpDNA of the genus *Phaseolus* (Vekemans *et al.* 1998), *Abies* (Parducci and Szmidt 1999), Millet (Parani *et al.* 2000), Mangrove (Parani *et al.* 2001), Soybeans (Xu *et al.* 2001), Papayas (Van Droogenbroeck *et al.* 2004), and *Houttuynia* (Wei *et al.* 2005). It was used for inter-generic studies in *Cajanus* (Lakshmi *et al.* 2000).

Some studies relied on coding regions of the cpDNA, others on non-coding regions. Parducci and Szmidt (1999) studied ten different cpDNA regions of the genus *Abies*. Cronn *et al.* (2002) investigated four different cpDNA regions of the genus *Gossypium*. Walker *et al.* (2004) used the inter-genic region of *trnL-F* and *rbcL* gene sequences of the cpDNA to study *Salvia* and related genera. Degrees of polymorphism may vary substantially across the chloroplast genome due to the presence of micro- and macro-structural rearrangements in some chloroplast genomes. For example, small inversions (Hiratsuka *et al.* 1989), insertions and/or deletions (Ogihara *et al.* 1991; Kanno *et al.* 1993; Maier *et al.* 1995), base substitutions (Morton and Clegg 1995), and translocations (Ogihara *et al.* 1988), as well as large inversions such as the case of *Oenothera elata* (Hupfer *et al.* 2000) and *Lotus japonicus* (Kato *et al.* 2000).

The purpose of this study was to explore a long region of the cpDNA of *Salvia* in contrast to most studies that focused on a single short region or short regions of the cpDNA. This long region of the chloroplast genome of *Salvia* covers the area from *psbA* gene to *rps7* gene and of about 15,031bp long. Design of primers and length estimation were based on the chloroplast genome of *Gossypium barbadense* L. (Ibrahim *et al.* 2006).

MATERIALS AND METHODS

Plant Material Young leaf samples were collected from naturally grown *Salvia* (54 species) and two *Ocimum* species in Kyoto Botanical Garden, Japan (Table 1). Leaf samples were stocked at -20°C until needed for experiments.

DNA Isolation Plant Genomic DNA Extraction Miniprep System (Viogene, USA) was used to isolate total genomic DNA from leaf samples. The manufacturer's protocol was followed. Extracted DNA was the template for LA-PCR amplification.

Primers Design The cpDNA sequence of *G. barbadense* was the reference for primers design (Ibrahim *et al.* 2006).

Table 1. Species of *Salvia* and *Ocimum* tested in this study

No	Species	No	Species
1	<i>S. tiliifolia</i> Vahl	29	<i>S. sinaloensis</i> Fern.
2	<i>S. verticillata</i> L.	30	<i>Ocimum basilicum</i> (Sweet Basil)
3	<i>S. microphylla</i> Kunth -1	31	<i>Ocimum basilicum</i> 'Green Ruffles'
4	<i>S. 'purple-majesty'</i>	32	<i>S. farinacea</i> Benth.
5	<i>S. mexicana</i> L.	33	<i>S. patens</i> Cav.
6	<i>S. uliginosa</i> Benth.	34	<i>S. 'Anthony-Parker'</i>
7	<i>S. guaranitica</i> St.-Hil. ex Benth.	35	<i>S. chiapensis</i> Fern.
8	<i>S. elegans</i> Vahl.	36	<i>S. discolor</i> Kunth
9	<i>S. cadmica</i> Boiss.	37	<i>S. staminea</i> M. & A. ex Benth.
10	<i>S. gesneriiflora</i> Lindley & Paxton	38	<i>S. coccinea</i> L.
11	<i>S. microphylla</i> Kunth -2	39	<i>S. nemorosa</i> L.
12	<i>S. leucantha</i> Cav.	40	<i>S. lyrata</i> L.
13	<i>S. 'indigo-Spires'</i>	41	<i>S. chamaedryoides</i> Cav.
14	<i>S. azurea</i> Michx.	42	<i>S. involucrata</i> Cav.
15	<i>S. microphylla</i> Kunth -3	43	<i>S. virgata</i> Jacq.
16	<i>S. madrensis</i> Seemann	44	<i>S. tomentosa</i> Mill.
17	<i>S. microphylla</i> Kunth -4	45	<i>S. x superba</i>
18	<i>S. virgata</i> Jacq.	46	<i>S. officinalis</i> 'Icterina'
19	<i>S. pratensis</i> L.	47	<i>S. officinalis</i> 'Tricolor'
20	<i>S. jurisicii</i> Kosanin	48	<i>S. argentea</i> L.
21	<i>S. apiana</i> Jepson	49	<i>S. japonica</i> Thunb. Ex Murray
22	<i>S. roemeriana</i> Scheele	50	<i>S. glabrescens</i> Makino
23	<i>S. regia</i> Cav.	51	<i>S. nipponica</i> Miq.
24	<i>S. melissodora</i> Lagasca	52	<i>S. glutinosa</i> L.
25	<i>S. buechananii</i> Hedge	53	<i>S. forsskaolii</i> L.
26	<i>S. viridis</i> L.	54	<i>S. microphylla</i> 'violet'
27	<i>S. sclarea</i> L.	55	<i>S. officinalis</i> L.
28	<i>S. splendens</i> Sello ex R. & S.	56	<i>S. greggii</i> Gray

Table 2. Primers used for LA-PCR amplification of cpDNA from *Salvia* and *Ocimum* species

Primer	Sequence	Size (bp)	Position ^a	Expected Size (bp)
F12	CGTAATCGATTAGTTAACATGTTGGTTAACCG	32	(146,617) <i>rps7</i>	
R12	GGTGCCATTATTCCTACTTCTGCAGCTATAGG	32	(01,243) <i>psbA</i>	14,943

^aPosition and gene name on the chloroplast genome sequence of *G. barbadense* (Ibrahim *et al.* 2006)

A pair of primers was selected (Table 2) from the manually designed set that was used before to study cultivated cotton species (Ibrahim *et al.* 2007).

LA-PCR The manufacturer's (Takara Shuzo, Kyoto, Japan) protocol was followed but half of the recommended volumes of reagents were applied in this experiment. LA-PCR amplification of cpDNA was carried out in 200 µl thin-walled tubes using 1.25 units of *TaKaRa* LA *Taq*TM polymerase and specific pair of primers. LA-PCR amplification was run in a Bio-Rad *iCycler* PCR machine (Hercules, CA, USA) under the following conditions: initial denaturizing step at 94°C for 1 min, followed by 30 cycles at two different temperature regimes: 98°C for 10 s and 68°C for 15 min as annealing and extension of primers, then a final extension step was applied at 72°C for 10 min. Successfully amplified LA-PCR-cpDNA was tested against 10 kb DNA marker (λ -*Hind*III digest; Takara Shuzo, Kyoto, Japan), separated by electrophoresis in 0.8% agarose gel in 1xTAE buffer (40mM Tris-acetate and 1mM EDTA), stained with Ethidium bromide (0.5 µg/ml), visualized and photographed under FAS-II UV-illuminator (Toyobo Co. Ltd., Tokyo, Japan).

DNA Digestion After amplification, 2 µl of each LA-PCR-cpDNA were separately digested in total volume of 10 µl with one of six restriction enzymes: *Eco*RI, *Hind*III, *Pvu*II, *Nde*I, *Sma*I or *Mfe*I. The manufacturer's protocol for restriction enzymes was followed (New England BioLabs, UK). Digestion products were then separated along with 1 kb DNA marker (New England BioLabs, UK) by electrophoresis in 1.5% agarose gel in 1xTAE buffer at a constant current of 500 mA and a voltage of 100 V for about 1 h. Thereafter, gels were stained in Ethidium bromide solution, visualized and photographed under UV-illuminator.

Data analysis Restriction fragments were analyzed visually and scored as present (1) or absent (0) for each *Salvia* and *Ocimum* species to generate a data matrix. Seqboot, Restdist, Neighbour, and Consense are components of the PHYLIP software package (Felsenstein 2005). One thousand sets of bootstraps were performed by Seqboot. Restdist was used to calculate the proportion of shared fragments or so called Genetics Similarities (GS) between each pair of *Salvia* and/or *Ocimum* species based on Nei and Li (1979) method, *i.e.* $GS = 2N_{XY} / (N_X + N_Y)$. N_X and N_Y are the total number of fragments scored in species X and Y, respectively, and N_{XY} is the number of shared

fragments between X and Y. The matrix of pair wise (1-GS) values, *i.e.* the proportion of fragments that were not shared, was the source for Neighbor to construct neighbor-joining dendrograms and determine the genetic relationships among *Salvia* and *Ocimum* species. Neighbor generated trees were applied to Consense feature. The final tree was edited with TreeView 1.6.6 (Page 1996).

RESULTS

LA-PCR of cpDNA A pair of primers named F12 and R12 was the start and limit for LA-PCR amplification of a region that spans around 15 kb of the cpDNA from 54 *Salvia* and two *Ocimum* species. Each of 53 *Salvia* species and two *Ocimum* species excluding *S. x superba* produced a single long PCR band of the expected size considering the correspondent region from *psbA* gene to *rps7* gene in the reference chloroplast genome sequence of cotton *G. barbadense* (Ibrahim *et al.* 2006). *S. roemeriana* was the only species that produced slightly longer PCR-cpDNA or visually detectable AFLD (Fig. 1).

LA-PCR-RFLP *Eco*RI, *Mfe*I, *Hind*III, *Pvu*II, *Nde*I or *Sma*I restriction enzymes were used to digest LA-PCR amplified cpDNA of *Salvia* and *Ocimum* species. Restriction enzymes *Eco*RI and *Mfe*I resulted more polymorphic and informative fragments than *Hind*III, *Pvu*II, *Nde*I or *Sma*I. Though, *S. roemeriana* and *S. lyrata* showed different polymorphism, they were easily distinguishable from each other and the rest of species. Therefore, digestion of LA-PCR-cpDNA amplified by F12/R12 primers with one of the enzymes tested except *Pvu*II can be used to identify *S. roemeriana* and/or *S. lyrata* (Fig.2; Lanes 22 and 40). *Ocimum* species showed their difference from *Salvia* when digestion was carried out with *Nde*I, *Sma*I or *Mfe*I (Fig.2; Lanes 30 and 31). Thus, *Nde*I, *Sma*I and *Mfe*I enzymes are useful to identify *Ocimum* species from *Salvia* based on tested part of cpDNA. Shimada and Sugiura (1991) reported that the chloroplast genomes of higher plants are highly conserved. Wagstaff *et al.* (1995) analyzed restriction site variation of cpDNA in Nepetoideae (Labiatae) subfamily and reported *Ocimum* species as a basal genus for *salvia*. Highly conserved cpDNA in higher plants especially related genera may explain the similarity of tested *Ocimum* and *Salvia* species. Fig. 2 illustrates LA-PCR amplified cpDNA digested with *Mfe*I. Table 3 shows restriction enzymes and number of resultant fragments from digestion of amplified LA-PCR cpDNA of *Salvia* and *Ocimum* species.

Fig. 1. Undigested LA-PCR products amplified by F12/R12 primers from the chloroplast genomes of *Salvia* species (Lanes 1~29 and 32~56) and two *Ocimum* species (Lanes 30 and 31). Numbers 1-56 indicate the names of *Salvia* and *Ocimum* species as listed in order in Table 1. The AFLD from *S. roemeriana* (Lane 22) appeared a little longer than PCR products of other species (arrow). M is λ -HindIII digest DNA ladder marker (Takara Shuzo, Kyoto, Japan).

Phylogenetic analysis PHYLIP software package was the tool to analyze the collected data from LA-PCR-RFLP of the cpDNA from *Salvia* and *Ocimum* species. The resultant phylogenetical tree is shown in Fig. 3. The data file was bootstrapped 1000 times before proceeding. *Ocimum* species were an out group.

Fig. 2. LA-PCR-cpDNA amplified from *Salvia* (Lanes 1~29 and 32~48) and *Ocimum* species (Lanes 30~31) digested with *Mfe*I restriction enzyme. Numbers indicate the names of *Salvia* and *Ocimum* species as listed in order in Table 1. M is 1 kb DNA ladder marker (New England BioLabs, UK).

DISCUSSION

Walker *et al.* (2004) grouped *Salvia* into Clade I of mainly Old World species, Clade II of mainly New World species and Clade III of Asian species. From the generated dendrogram, *Salvia* species were clustered into groups based on origin (Clebsch 2003) and was found almost in accordance with Walker *et al.* (2004) report despite the use of different part of the cpDNA. Group I is the basic clade for this collection of *Salvia* and contains five species,

Table 3. Restriction enzymes and number of minimum, maximum, and polymorphic resultant fragments from LA-PCR-RFLP of cpDNA from *Salvia* and *Ocimum* species

Number of fragments	Restriction enzymes					
	<i>EcoRI</i>	<i>HindIII</i>	<i>PvuII</i>	<i>NdeI</i>	<i>SmaI</i>	<i>MfeI</i>
Minimum	8	4	1	2	3	8
Maximum	10	4	1	3	5	10
Polymorphic	9	3	0	3	3	10

Fig. 3 Phylogenetic tree of 53 *Salvia* species and 2 *Ocimum* species obtained by neighbor-joining method based on LA-PCR-cpDNA-RFLP marker-based genetic similarity.

S. forsskaolii, *S. verticillata*, *S. officinalis*, *S. officinalis* ‘Icterina’, and *S. tomentosa* from northern Balkan, Central Europe, Western Asia, northern Mediterranean, and Asia Minor. *S. buchananii* of Mexico was the only species in group II and compose a sister clade with the rest of species

that are designated as group III. Group III was divided into 2 major subgroups; Subgroup III-i is the most sophisticated and diverse clade and was subdivided into many other minor clades. The eastern Asia species include *S. glabrescens*, *S. nipponica*, and *S. japonica* that are

native to Japan were scattered in this subgroup in consistence with a recent study of Japanese *Salvia* (Takano and Okada, 2010). Subgroup III-ia contains only *S. glabrescens* as a basal species for another 2 minor clades of subgroup III-ib. Subgroup III-ib1 includes *S. lyrata* and *S. roemeriana* of North and Central America in one subclade, while the other subclade contains *S. japonica* of Japan as a sister species of another subclade that includes *S. argentea* from southern Europe, *S. viridis* from the Mediterranean up to Iran, *S. sclarea* from the Mediterranean, northern Africa, and Central Asia, *S. pratensis* from Europe, northern Africa, and western Asia, *S. cadmica* from Asia Minor and *S. virgata* from California. Subgroup III-ib2 is of a wide range as reported before (Ibrahim *et al.* 2010). *Salvia nipponica* from Japan was also a basal species to *S. officinalis* 'Tricolor' from northern Mediterranean and Asia Minor and *S. apiana* from California. The presence of *S. apiana* in this subgroup is strange, because in the latest report (Ibrahim *et al.* 2010) it was in a separate subclade and sister to all other species. This could be due to a natural hybridization with Asian species and maternal inheritance of chloroplast genome. *S. officinalis* 'Tricolor' and *S. apiana* species were basal to *S. glutinosa* of Central France, Spain, Italy, Greece and Central Russia, which itself was basal to *S. nemrosa* of Eastern Europe and western Asia and *S. jurisicii* of Europe and southern Balkan area. This might be in consistency with Walker *et al.* (2004) who grouped *S. glutinosa* in a separate clade named III with other five species; *S. digitaloides* from China, *S. fruticosa* from eastern Mediterranean and southern Italy, *S. nubicola* from Afghanistan, Bhutan, India, Pakistan, southeastern Asia and Europe, *S. barrelieri* from North Africa, and *S. hians* from Kashmir. Subgroup III-ii of Latin and Central America species was also divided into subgroup III-iiia that contained only *S. uliginosa* of Brazil, Paraguay and Argentina, subgroup III-iib included 2 minor clades; III-iib1 that accommodates ten species including *S. discolor* as the basic species for this subclade and they are only from South America, Central America, and Mexico. This is in consistence with a recent report that included some of the species in this study (Jenks *et al.* 2010). Group III-iib2 was also subdivided into two minor clades. *Salvia madrensis* and *S. microphylla* were basal to the other subclade of 18 species from South America, Central America and Mexico except *S. virgata* and *S. staminea* from California and Asia Minor up to Iran, respectively. *S. virgata* and *S. staminea* clustered in this last minor clade of group III-iib2 was also reported before in a study included different pair of primers and different part of cpDNA (Ibrahim *et al.* 2010). There are two possibilities for the presence of *S. staminea* in the Latin American group. One possibility is a natural hybridization between *S. staminea* and Latin or Central American species as a maternal donor of chloroplast genome. The other possibility might be a mislabeled plant.

This report shows that *Salvia* species under study can be divided into groups based on only one long PCR product from the chloroplast genome and one restriction enzyme at a time. A combination of two enzymes simultaneously in a double digestion may increase the resolution of results and lead to species specific markers (Parani *et al.* 2001). In general, all species of *Salvia* that included in this study or Ibrahim *et al.* (2010) and the report of Walker *et al.* (2004) were clustered based on origin. *Salvia roemeriana* showed AFLD as well as species-specific pattern against *EcoRI*, *MfeI*, *HindIII*, *NdeI* or *SmaI* restriction enzymes. *Salvia lyrata* expressed the same phenomenon and can also be identified from *S. roemeriana* as well as other species by *EcoRI*, *MfeI*, *HindIII*, *NdeI* or *SmaI* restriction enzymes. *Ocimum* species can be identified from *Salvia* based on the studied part of cpDNA digested with any of *NdeI*, *SmaI* or *MfeI* restriction enzymes. Natural hybridization and maternal inheritance of chloroplast genome might be the reason for the unique cluster of *S. staminea* from Asia Minor and Iran with South and Central America species. This case of *S. staminea* needs further investigation. Based on this report, most species of *Salvia* in Kyoto Botanical Garden are from South America, Central America, the Mediterranean and Asia Minor more than other parts of the world. This could be due to early day's travel of Japanese to South America, Central America and Asia Minor as well.

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