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The Biological Diversity of *Sinosenecio* (Asteraceae: Senecioneae) in Thailand

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Abstract The diversity and genetic relationships among and within four populations of sixteen accessions of *Sinosenecio oldhamianus* (Maxim.) B. Nord. from Thailand were analyzed using Sequence-related amplified polymorphism (SRAP) markers. Genomic DNA was extracted from fresh leaf of the samples. Few polymorphic bands were detected by SRAP markers, indicating the genetic diversity of *S. oldhamianus* was low. A total of 213 DNA fragments, varying from 100-2000 bp, were amplified, of which 34 (15.96%) were polymorphic. A dendrogram showing genetic similarities among *S. oldhamianus* was constructed based on polymorphic bands using the SPSS program (version 18). Based on the results from the dendrogram, two clusters could be separated with similarity coefficients ranging from 0.810-1.000. In conclusion genetic markers were found to be powerful tools to analyze the population genetic structure of *S. oldhamianus*. Based on these findings, strategies are proposed for the genetic conservation and management of the species.

Keywords: *Sinosenecio oldhamianus*, SRAP, Genetic diversity, Asteraceae, Thailand

Introduction

The term biological diversity or the shorter biodiversity has various meanings depending on the biological level to which it is applied (Féral, 2002). Traditional approaches to the measurement of biodiversity rely upon the ability to determine differences in morphological characters (Krab *et al*., 1996). However, the characters may be affected by different environments and the stage of species development. The advent of the polymerase chain reaction (PCR) by Sakai *et al*. (1988) has enabled the progress of powerful genetic markers for the measurement of genotypic variation. By detecting genotype, rather than phenotype, genetic markers avoid complicating environmental effects and give ideal tools for assessing genetic diversity, identifying species and other locally adapted forms, patterns of historical biogeography and the

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analysis of ancestry and relatedness (Vanijajiva *et al*., 2005; Agarwal *et al*., 2008; Vanijajiva 2011; Vanijajiva 2012; Korir *et al*., 2013).

Currently, there are several different molecular marker systems available for species identification. Molecular markers could exhibit sufficient polymorphism and provide potential tools for effective and efficient genetic evaluation of important traits in many species. Sequence-related amplified polymorphism (SRAP) is a novel molecular marker first introduced by Li and Qurios (2001). The SRAP method consists of preferential amplification of open reading frames (ORFs) using PCR, and the observed polymorphism basically originates in the variation of the length of these introns, promoters, and spacers, both among population and among species. Presently, SRAP had been applied extensively in genetic diversity analysis, and comparative genetics of different species and other fields (Agarwal *et al*., 2008; Soleimani *et al*., 2012; Li *et al*., 2013).

Sinosenecio B. Nord. is a perennial herbaceous genus of Asteraceae-Senecioneae characterized by its palmately veined, petiolate leaves with the lamina distinct from the petiole, ecalyculate involucres, corymbose-paniculate, radiate, yellow-flowered, anthers ecaudate, endothecium polarized and cylindrical filament collars (Nordenstam, 1978). In the tribe Senecioneae, *Sinosenecio* is usually considered to be most closely related to *Nemosenecio* and *Tephroseris* (Nordenstam 1978, 2007; Liu and Yang, 2011). The genus consists of about 40 species all occurring in China, only two extending into Myanmar, Vietnam which one species, *Sinosenecio oldhamianus* (Maxim.) B. Nord., has only been recorded in Thailand (Nordenstam 2007; Koyama, 1988). Up to now there is no report of measuring genetic diversity *Sinosenecio* by SRAP markers. The objective of the present study is to assess the genetic variation among and within populations of *S. oldhamianus* from Thailand, and to evaluate the efficiency of SRAP markers in this genus.

Materials and methods

Plant materials

Field observations in Thailand, mainly in Loei province were made from September to December 2011, from January to February 2012 and from April to June 2013. Sixteen accessions of *Sinosenecio oldhamianus* used in this experiment were collected from four populations at Chiengkan on the bank of Me Kong of Loei province. Identification of plants into species was done by observing morphological characteristics, checking references and comparing with herbarium specimens at several herbaria. Then, the names were verified to the correct botanical names by way of taxonomic study. The detailed

description of each species was done, followed by the construction key to species (Koyama, 1988).

DNA isolations were carried out using fresh leaf of samples from 16 accessions collected in Loei province (Table 1). All leaf tissue sampled were stored in plastic zipper bags containing silica gel for shipping or transport to the laboratory then immediately frozen and stored at -20 ^{o}C until DNA extraction. Voucher specimens of all accessions were deposited in the Phranakhon Rajabhat University Herbarium.

Table 1. Samples of *Sinosenecio oldhamianus* used in this study with the coordinates and number

Sample number	Sample site		Vouchers
	Latitude (North)	Longitude (East)	
Si1	N 17 ^{o} 89'81.55"	E 101^{o} 66'57.55"	OVSi0405-12
Si2	N 17 ^{o} 89'81.15"	E 101^{o} 66'56.78"	OVSi0505-12
Si3	N 17 ^{o} 89'81.24"	E 101^{o} 66'56.85"	OVSi0705-12
Si4	N 17 ^{o} 89'81.28"	E 101^{o} 66'57.22"	OVSi0805-12
Si5	N 17 ^{o} 89'80.55"	E 101^{o} 66'57.55"	OVSi0905-12
Si6	N 17 ^{o} 89'61.15"	E 101^{o} 66'66.78"	OVSi1305-12
Si7	N 17 ^{o} 89'61.24"	E 101^{o} 66'66.55"	OVSi1905-12
Si8	N 17 ^{o} 89'71.28"	E 101^{o} 66'67.82"	OVSi1805-12
Si9	N 17 ^{o} 88'72.45"	E 101^{o} 65'62.24"	OVSi0606-12
Si10	N 17 ^{o} 88'33.75"	E 101^{o} 65'63.33"	OVSi0706-12
Si11	N 17 ^{o} 88'32.78"	E 101^{o} 65'62.52"	OVSi0806-12
Si12	N 17 ^{o} 88'32.60"	E 101^{o} 65'62.42"	OVSi2105-12
Si13	N 17 ^{o} 88'32.65"	E 101^{o} 65'72.24"	OVSi2505-12
Si14	N 17 ^{o} 88'33.45"	E 101^{o} 65'73.42"	OVSi1306-12
Si15	N 17 ^{o} 88'32.88"	E 101^{o} 65'72.52"	OVSi1506-12
Si16	N 17 ^{o} 88'32.70"	E 101^{o} 65'72.32"	OVSi1906-12

Genomic DNA isolation

Genomic DNA was extracted from the leaves of 16 accessions using the CTAB method with minor modification (Vanijajiva, 2012). The leaves (500 mg) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris–HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol)] 500 µl was added and the solution was incubated at 60 C^{o} for 30 min. The homogenate was mixed with 25:24:1 phenol: chloroform: isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the upper aqueous layer was transferred to a new tube. RNA was removed by treating with 2.5 µl of the RNase (10 µg/µl) for 30 min at 37 ^{o}C. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the

solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with 70% ethanol. Following this, the DNA was extracted using CTAB DNA extraction protocol without RNase. The process was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. DNA quality and quantity were determined on 0.8% agarose gel. The DNA was stored at -20 C^o, for further use as templates for PCR amplification. The quality of DNA was also evaluated by reading the absorbance at 260 and 280 nm.

SRAP analysis

Primers pairs used in this study were synthesized by Ward Medic Ltd., Part. Thailand (Table 2). The PCR was performed using a Thermohybaid Px2 (Roche Molecular Systems, Inc., USA). The PCR reaction mixtures (25 µl total volume) consisted of 10x Reaction Buffer, 100 ng template DNA, 0.6 mM dNTP mixture, 5 mM $MgCl_2$, 1 unit of Taq polymerase and 0.6 µM of each primers. The SRAP amplification conditions were 5 min initial denaturation at 94 ^oC and 5 cycles consisting of 1 min denaturation at 94 ^oC, 1 min primer annealing at 35 ^oC, and 2 min extension at 72 ^oC. In the following 30 cycles, the annealing temperature was increased to 50 ^oC and a final 8 min extension at 72 ^oC.

The SRAP products were all analysed by agarose (1.8% w/v) gel electrophoresis at 150 A for 30 minutes in 0.04 M TAE (Tris–acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml). The gels were viewed and photographed by Bio-Imaging System (Syngene, Genegenuis). To determine SRAP profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

Table 2. SRAP primers used in this study

Forward primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
		Em6	GACTGCGTACGAATTGCA

Gel scoring and data analysis

Only strong and reproducible SRAP bands were scored. Different observed patterns were scored as discrete variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. The SPSS (version 18) data analysis package was used for the statistical analyses. Relationships among individuals were determined by the distance matrix method. Nei and Li's Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix (Nei and Li 1979). A principal component analysis (PCA) was also conducted using a genetic distance matrix obtained from the binary data set. It was negated and rescaled (0–1), using the Euclidean distance between pair-wise comparison of individuals (Ludwig and Reynolds, 1988).

Results and discussions

Morphology and ecology

For morphological identification, Description: *Sinosenecio oldhamianus* (Maxim.) B.Nord., Opera Bot. 44: 50 (1978). – *Senecio oldhamianus* Maximowicz, Bull. Acad. Imp. Sci. Saint-Pétersbourg 16: 219. (1871); *Senecio martini* Vaniot – *Senecio savatieri* Franchet – *Sinosenecio savatieri* (Franchet) B. Nordenstam. Type: China, Zhejiang, *Oldham* 58 (syntype K!), *Oldham* 62 (syntype K!, isosyntype PE!).

The plant is annual or biennial herb, rhizomatous, with leafy stems. *Stems* are solitary or few, erect, 40-80 cm or taller, sparsely villous and arachnoid, glabrescent to subglabrous. *Leaves* withers by anthesis, long petiolate; lower stem leaf petioles 3-6 cm, white arachnoid, basally somewhat expanded; blade adaxially green, ovate-orbicular or suborbicular, 3-5(-8) × 3-6 cm, membranous, abaxially white arachnoid, sometimes ± glabrescent, adaxially sparsely arachnoid or subglabrous, palmately 5-veined, base cordate, margin shallowly to deeply duplicate-dentate or duplicate-serrate with mucronulate teeth, apex obtuse to acute, acuminate and mucronulate. Upper leaves shortly petiolate, smaller, with ovate or ovate-deltoid, basally cuneate blades; uppermost leaves ovate or ovate-lanceolate. *Capitula* are numerous, arranged in compound terminal corymbs; peduncles 1.5-3 cm, slender, sparsely pubescent, usually with a basal linear bract. Involucres broadly campanulate, 3-4 × 2.5-4 mm, not calyculate; phyllaries ca. 13, oblong-lanceolate, ca. 1 mm wide, herbaceous, with membranous margins, white arachnoid or puberulent to glabrous, apically acuminate and sometimes purplish. *Ray florets* ca. 13; corolla tube 2-2.5 mm, glabrous; lamina yellow, oblong, 8-9 × 1-2 mm, 4-veined,

apically 3-denticulate, obtuse. *Disk florets* are numerous; corolla yellow, 3-3.5 mm, with 1.5-1.8 mm tube and campanulate limb; lobes ovate-oblong, ca. 1 mm, apically acute. *Anthers* oblong, 0.8-0.9 mm, basally obtuse, appendages ovate-oblong. Style branches recurved. *Cypselas* cylindric, ca. 1.5 mm, smooth and glabrous in ray florets, puberulent and papillate in disk florets; pappus absent in ray florets, in disk florets white, 3-3.5 mm.

In Thailand *S. oldhamianus* only found at Chiengkan on the bank Me Kong of Loei province, along streamsides, wet rocky and grassy places, margins of cultivated fields; alt. 100-210 m.

DNA isolation

DNA extracted from *S. oldhamianus* leaf using a modified Vanijajiva (2012) gave a good and sufficient quality DNA for PCR reaction, and the amount of DNA extracted from the accessions ranged from 129 to 192 µg/g fresh weight leaf material. The ratios of A260/A280 varied from 1.75 to 1.87. The quality of DNA was also tested by PCR, which confirmed that the DNAs were suitable for PCR reaction.

Genetic diversity

SRAP is a new marker technique based on PCR and it has good repeatability and stability. In this study, it is the first time to analyse the genetic variation of *Sinosenecio* using SRAP marker. The results showed that SRAP is an economic, efficient, and reliable technique. A total of 30 different primer combinations were employed using five forward and six reverse primers. About 28 of them could produce clear and reproducible bands and were chosen in the following study. This study clearly showed that there is low genetic variation within and among *S. oldhamianus* populations in Thailand. A total of 213 bands ranging from 100 to 2000 bp were observed (Table 3).

Table 3. Polymorphism obtained by SRAP analysis of *Sinosenecio oldhamianus*

Primer combination	Total bands	Polymorphic bands	Percentage of polymorphism
Me1/ Em1	7	2	28.57
Me1/ Em2	11	2	18.18
Me1/ Em3	9	1	11.11
Me1/ Em4	7	1	14.28
Me1/ Em5	6	2	33.33
Me1/ Em6	7	1	14.28

Primer combination	Total bands	Polymorphic bands	Percentage of polymorphism
Me2/ Em1	7	2	28.57
Me2/ Em3	5	0	00.00
Me2/ Em4	9	0	00.00
Me2/ Em5	8	2	25.00
Me2/ Em6	6	0	00.00
Me3/ Em1	7	1	14.28
Me3/ Em2	8	1	12.50
Me3/ Em3	11	2	18.18
Me3/ Em4	9	2	22.22
Me3/ Em5	8	1	12.50
Me3/ Em6	6	0	00.00
Me4/ Em1	4	0	00.00
Me4/ Em2	9	1	11.11
Me4/ Em3	7	2	28.57
Me4/ Em4	7	1	14.28
Me4/ Em6	8	1	12.50
Me5/ Em1	10	2	20.00
Me5/ Em2	7	1	14.28
Me5/ Em3	9	2	22.22
Me5/ Em4	6	0	00.00
Me5/ Em5	9	2	22.22
Me5/ Em6	6	2	33.33
Total	213	34	15.96

The genetic similarity among sixteen accessions examined using SRAP patterns and Nei and Li's Dice similarity coefficients for pair-wise comparison between individuals is shown in Table 4. The similarity coefficient ranges from 0.810 to 1.000.

Based on SRAP pattern, genetic distances among sixteen samples were calculated and Cluster analysis by principal component analysis (PCA) was constructed. The PCA consisted of two major clusters agree with sample location (Fig. 1). The first cluster contained a group of the eight samples of Si1-Si8. The second group is made up of Si9-Si16.

Table 4. Pair-wise genetic similarity of 16 samples of *Sinosenecio oldhamianus* according to the index of Nei and Li (1979)

	Si1	Si2	Si3	Si4	Si5	Si6	Si7	Si8	Si9	Si10	Si11	Si12	Si13	Si14	Si15	Si16
Si1	1.000															
Si2	.974	1.000														
Si3	.974	.947	1.000													
Si4	.947	.919	.919	1.000												
Si5	.947	.919	.919	1.000	1.000											
Si6	.889	.919	.919	.889	.889	1.000										
Si7	.889	.919	.919	.889	.889	1.000	1.000									
Si8	.919	.947	.947	.919	.919	.974	.974	1.000								
Si9	.824	.857	.857	.824	.824	.889	.889	.919	1.000							
Si10	.850	.810	.810	.850	.850	.867	.867	.810	.924	1.000						
Si11	.850	.810	.810	.850	.850	.867	.867	.810	.924	1.000	1.000					
Si12	.867	.810	.810	.867	.867	.824	.824	.878	.979	.989	.989	1.000				
Si13	.867	.810	.810	.867	.867	.824	.824	.878	.989	.989	.989	1.000	1.000			
Si14	.824	.878	.857	.824	.824	.850	.850	.878	.989	.950	.950	.950	.950	1.000		
Si15	.824	.878	.857	.820	.850	.824	.824	.878	.989	.924	.974	.974	.974	.989	1.000	
Si16	.824	.878	.857	.820	.850	.824	.824	.878	.989	.950	.8950	.924	.924	.947	.947	1.000

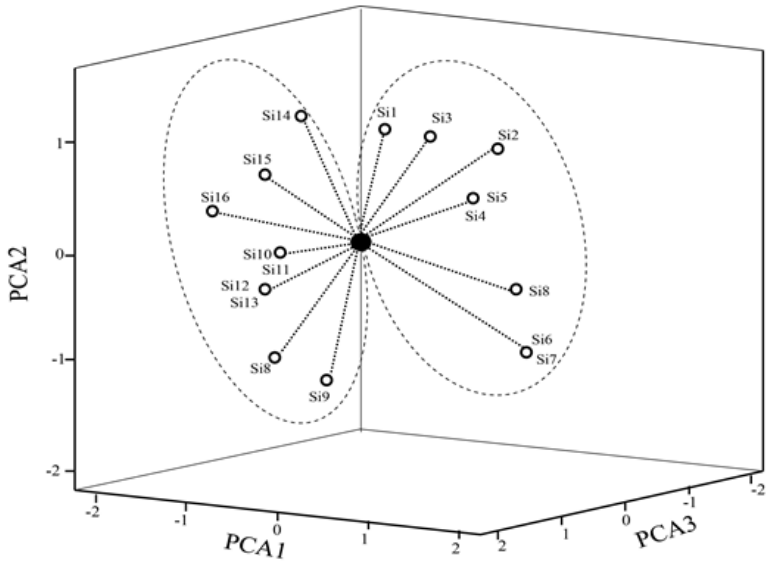

Fig. 1. Plot of PCA analysis of genetic similarity of 16 samples of *Sinosenecio oldhamianus* obtained from SRAP, showing groups individual plants

Conservation implications

Understanding of the levels and distribution of genetic diversity is important for planning conservation strategies for threatened and endangered species (Hamrick, 1983). Loss of genetic diversity could be lead to a decrease in a species' ability to survive environmental changes and demographic fluctuations both in short and in long term (Ellstrand and Elam, 1993; Milligan *et al*., 1994). *S. oldhamianus* is an endanger species to Thailand, and it has isolated populations only in Loei Province and is considered to have a high priority for the conservation of biodiversity in Thailand. The genetic structure of *S. oldhamianus* can be summarized as low genetic diversity, apparently results from its past evolutionary processes, as well as from population distribution and limited gene flow mainly due to habitat fragmentation and loss. The conservation of endangered species depends on maintaining a significant population size (Zhang *et al*., 2005). Given the extremely limited number of individuals of *S. oldhamianus*, it is necessary to protect all the existing populations and individuals *in situ* in order to preserve as much genetic variation as possible. Alternatively, with information of its genetic structure available, a suitable strategy for sampling and propagation could be formulated when *ex situ* conservation is carried out. Although a small number of *ex situ* individuals can preserve most of the genetic variation in this endangered species.

Conclusion

The SRAP technique has proven to be successful in supporting the taxonomical identification and providing the genetic structure of *S. oldhamianus*. Although the low genetic variation of *S. oldhamianus* in Thailand was discovered here, comparison of genetic diversity of *S. oldhamianus* from different parts of the world, particularly from related region such as Vietnam and Myanmar and China is required in order to understand the overall genetic structure of this species. Therefore, more researches in the future is necessary. Further investigation of genomic DNA using technique such as SRAP together with sequence data from DNA regions will be useful in prospect to detect more information of species dispersal and genetic structure of *S. oldhamianus*.

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