Distinguishing two morphologically similar species of Asteraceae using a chloroplast DNA marker

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Abstract. *Synedrella nodiflora* (L.) Gaertn and *Calyptocarpus vialis* Less are members of Asteraceae family that morphologically show high similarities. To genetically distinguish between them, a particular molecular marker should be employed. This study aims to present molecular comparison between both species using a chloroplast DNA marker, i.e. *atp*B – *rbc*L IGS. A pair of PCR universal primers was used to amplify the marker. Sequence alignment on the PCR products reveals longer *S. nodiflora* sequence in comparison to that of *C. vialis*. In addition, some transversions and transitions are also observed. This suggests that the two species exhibit considerable genetic difference despite their similar phenotypic appearance.

1. Introduction

Many members of Asteraceae family are recognized for their potentials as ornamental, medicinal, and economic plants[1]. On the other hand, some others are known as invasive weeds[2], resulting in significant loss on several crops with respect to productivity[3].

Some species of Asteraceae family show very high phenotypical similarities causing difficulty in differentiating them from each other. For example, *Calyptocarpus vialis* Less has ever been identified as *Synedrella vialis* (Less.) A. Gray due to its high resemblance to *Synedrella nodiflora*[4]. Nevertheless, *S. vialis* is now changed into *C. vialis* [5] and this is the scientifically accepted name for the species, while *S. nodiflora* has taxonomically been the only species of genus *Synedrella* [6].

Relatively many studies on the potentials of *S. nodiflora*, e.g. as medicinal herbs[7],[8],[9],[10],[11], bioinsecticide[12], biofungicide[13], and detoxificant for heavy metals such as Cu and Pb[14], have been reported. On the other hand, no study has been performed on *C. vialis* potentials to human life. However, this plant species is often called as straggle daisy because of its capacity to grow invasively in various terrestrial habitats[15]. The allelopathy effect of root and leaf extracts of *C. vialis* was reported to strongly inhibit *S. Nodiflora*[16],[17].

Despite its wide distribution over many tropical countries, *S. nodiflora* showed no genetic difference among various altitudes[18], while low genetic difference within *S. nodiflora* populations in Java Island, Indonesia was observed[19]. On the other hand, *C. vialis* is not only spread over tropical regions, but is also distributed throughout subtropical areas as it is native to Mexico or even Texas[20]. It seems likely that *C. vialis* is a self-pollinated species presumably leading to slightly floral morphological differences between the populations in Texas and those in Mexico, especially concerning anther number and corolla lobe number of disk florets[21]. Yet, these phenotypical variations are not sufficiently easy to see unless considerably carefull examination is made. Even the difficulty occurs in the case of distinguishing *C. vialis* and *S. nodiflora*.

The problem with phenotypical discrimination between both species is necessarily overcome by means of molecular comparison using particular genetic markers, some of which are those from chloroplast genome (cpDNA). This source of molecular markers is maternally inherited in agiosperms giving rise to the absence of genetic recombination. Hence, it can be used properly for assessing both intra-specific and inter-specific genetic diversity[22]. An *atp*B – *rbc*L intergenic spacer (IGS) is one of cpDNA markers commonly used to analyze evolutionary history at lower level, since it is a non coding sequence showing high evolution rate[23],[24],[25]. This marker has been used to study population genetic structure of some Chinese endemic plant species revealing high connectivity among populations[26]. Here we present our study on the genetic comparison between *S. nodiflora* and *C. vialis* by the use of *atp*B – *rbc*L IGS as the molecular marker. It is expected from this study to obtain DNA barcoding for the respective species.

2. Materials and Methods

2.1. Plant sampling and preparation

The samples of both *S. nodiflora* and *C. vialis* were collected randomly from some sites in Banyumas Regency, Central Java, Indonesia in May 2020. Five plant individuals were used as samples of the respective species, each of which was taken by removing its roots and put the whole plant into a plastic bottle formerly filled with some water. This was then grown in the glass house of the Faculty of Biology Universitas Jenderal Soedirman. Molecular analysis was performed in the Laboratory of Genetics and Plant Breeding of the Faculty of Agriculture Universitas Gadjah Mada.

2.2. Genomic DNA extraction and marker amplification

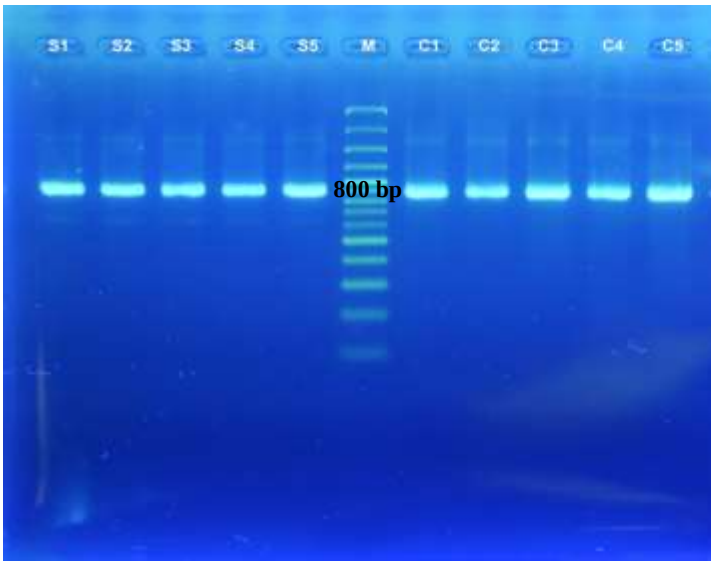
Genomic DNAs were extracted from the uppermost leaves of the plant samples using CTAB method [27]. The extracted DNAs were then used as PCR templates to amplify *atp*B – *rbc*L IGS employing a pair of universal primers, i.e. 5' – ACATCKARTACKGGACCAATAA - 3' as forward primer and 5' - AACACCAGCTTTRAATCCAA - 3' as reverse primer [28]. Individual PCR reaction was performed in a total volume of 10 µl consisting of 2.5 µl genomic DNA; 0.25 µl primers (0.125 µl each primer); 5 µl Gotaq green and 2.25 µl NFW. This reaction mixture was then treated in a PCR condition as follows: pre-denaturation at 94^{o}C for 3 mins, 33 reaction cycles consisting of denaturation at 94^{o}C for 45 secs, primer annealing at 55^{o}C for 45 secs, extension at 72^{o}C for 2 mins respectively, followed by final extension at 72^{o}C for 3 mins and storage at 4^{o}C. Visualization of the PCR products was performed in a 1.5 % agarose gel electrophoresis using 1X TAE buffer run at 75 Volt, 400 mA for 40 mins. After ethidium bromide staining, the gel was exposed to UV transiluminator for documentation.

2.3. DNA sequencing and data analysis

The PCR products were purified using QIAquick kit (Qiagen, Germany), and were sequenced following automated Sanger et al.[29] with terminator labelling. Data on base sequences were edited using Bioedit version 7.0.4.1[30] and were checked manually. Sequence alignment was carried out using ClustalW [31], which was also implemented in the Bioedit version 7.0.4.1.

3. Results and Discussion

All DNA samples were successfully amplified resulting in PCR bands of approximately 800 bp in length as depicted in Figure 1. After manual editing the amplicon sequences were trimmed into only 773 bp long. Blasting to NCBI reveals that those of *S. nodiflora* samples show 99.74% to 99.87% homology with *atp*B – *rbc*L IGS sequences of *S. nodiflora* available in the data base. Meanwhile, somewhat lower percentage of homology, i.e. 95.6% to 95.73%, was observed between amplicon sequences of *C. vialis* samples and *atp*B-*rbc*L IGS in the NCBI genbank (Table 1). This confirms that the PCR products of both *S. nodiflora* and *C. vialis* samples are definitely *atp*B – *rbc*L IGS.



S1 = *Synedrella nodiflora* 1
S2 = *Synedrella nodiflora* 2
S3 = *Synedrella nodiflora* 3
S4 = *Synedrella nodiflora* 4
S5 = *Synedrella nodiflora* 5
M = 1 kb ladder
C1 = *Calyptocarpus vialis* 1
C2 = *Calyptocarpus vialis 2*
C3 = *Calyptocarpus vialis* 3
C4 = *Calyptocarpus vialis* 4
C5 = *Calyptocarpus vialis* 5

Figure 1. Amplicons of *atp*B – *rbc*L IGS *Synedrella nodiflora* (L.) Gaertn and *Calyptocarpus vialis* Less

Table 1. Sequence alignment of *atp*B – *rbc*L IGS of *Synedrella nodiflora* (L.) Gaertn and *Calyptocarpus vialis* Less to NCBI data base

No.	Sequence name	Acession number	% homology		Sequence length (bp)
			Sn	Cv	
1	*Synedrella nodiflora* haplotype 5 *rbc*L-*atp*B	KY983545.1	99.87	95.73	860
2	*Synedrella nodiflora* haplotype 3 *rbc*L-*atp*B	KY983543.1	99.87	95.73	860
3	*Synedrella nodiflora* biovar lumajang *rbc*L-*atp*B	KX096802.1	99.87	95.73	866
4	*Synedrella nodiflora* biovar yogya1 *rbc*L-*atp*B	KX096801.1	99.87	95.73	866
5	*Synedrella nodiflora* haplotype 4 *rbc*L-*atp*B	KY983544.1	99.74	95.60	860

Sn = *Synedrella nodiflora* (L.) Gaertn
Cv = *Calyptocarpus vialis* Less

No difference within *atp*B – *rbc*L IGS sequences of either *S. nodiflora* or *C. vialis* was found. On the other hand, as shown in Table 2 slightly shorter *atp*B – *rbc*L IGS sequence of *C. vialis* in comparison to that of *S. nodiflora* was observed due to several deletions. In addition, some base substitutions were also found, where transversion occurs more frequently rather than transition. Both *S. nodiflora* and *C. vialis atp*B – *rbc*L IGS sequences have now been submitted to NCBI data base for accession numbers.

Table 2. Sequence differences of *atp*B – *rbc*L IGS between *Synedrella nodiflora* (L.) Gaertn and *Calyptocarpus vialis* Less

No.	Species	Site (s)	Sequence (s)	Type of mutation
1	*Synedrella nodiflora*	229	T	transversion
	Calyptocarpus vialis	229	G	
2	*Synedrella nodiflora*	230	T	deletion
	Calyptocarpus vialis	230	-	
3	*Synedrella nodiflora*	406 – 412	ATAGAAA	deletion
	Calyptocarpus vialis	405 – 406	-	
4	*Synedrella nodiflora*	523	C	transversion
	Calyptocarpus vialis	515	A	
5	*Synedrella nodiflora*	609 – 629	TGAAAACATTGAAATAAATAT	deletion
	Calyptocarpus vialis	601 – 602	-	
6	*Synedrella nodiflora*	646	A	transition
	Calyptocarpus vialis	617	T	
7	*Synedrella nodiflora*	661	G	transversion
	Calyptocarpus vialis	632	T	
8	*Synedrella nodiflora*	683	G	transition
	Calyptocarpus vialis	653	A	

The cpDNA marker *atp*B – *rbc*L has also been used previously to distinguish between *S. nodiflora* and another species of Asteraceae, i.e. *Eleutheranthera ruderalis*. These two species are also phenotypically very identical to each other. Nevertheless, by using the molecular marker some genetic differences with respect to indels and base substitutions were observed. Overall, the *atp*B – *rbc*L IGS of *S. nodiflora* was proven somewhat longer than that of *E. ruderalis* [32]. Oppositely, when another cpDNA marker, i.e. *trn*T – *trn*L, was employed to discriminate between both species, the sequence of *S. nodiflora* was found slightly shorter in comparison to that of *E. ruderalis* [33].

The *atp*B – *rbc*L IGS is a non-coding sequence, which is not responsible for a protein synthesis. Hence, it has no any relationship with the existence of some morphological characters observed in the plant individuals. Nevertheless, the difference in *atp*B – *rbc*L IGS sequences between *S. nodiflora* and *C. vialis* can potentially be used as DNA barcoding of the respective species. An intergenic spacer from cpDNA, i.e. *psb*A – *trn*H was used to distinguish several species of *Tolpis* (Asteraceae)[34], while the same cpDNA marker was used to provide an empirical model in the identification of some medicinal plant species of *Sinosenecio* (Asteraceae)[35]. In addition, this cpDNA marker was also used to construct phylogenetic tree among some species of *Anacyclus* (Asteraceae)[36].

Two morphologically similar genera of Myrtaceae, i.e. *Eugenia* and *Syzygium*, have been distinguished genetically employing *atp*B – *rbc*L IGS. By using this molecular marker, a previously confusing species, i.e. *Eugenia boerlagei* Merr, has now been taxonomically grouped into *Syzygium* rather than *Eugenia* leading to renaming this species into *Syzigium boerlagei*. However, this replacement is not based on the size of *atp*B – *rbc*L IGS, but rather depending on the GC content of the marker [37].

4. Conclusion

Despite no direct relationship between *atp*B – *rbc*L IGS and the phenotypic characters of both *S. nodiflora* and *C. vialis*, genetic differences between them were clearly observed. This provides potential DNA barcodes for identification of the two species.

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