Molecular Comparison between Two Similar Asteraceae Species (Synedrella nodiflora (L.) Gaertn. and Eleutheranthera ruderalis (Swartz) Sch._Bip) by the Use of trnL(UAA) - trnF(GAA) Intergenic Spacer

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Molecular Comparison between Two Similar Asteraceae Species (*Synedrella nodiflora* (L.) Gaertn. and *Eleutheranthera ruderalis* (Swartz) Sch._Bip) by the Use of *trn*L(UAA) - *trn*F(GAA) Intergenic Spacer

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ABSTRACT

Synedrella nodiflora (L.) Gaertn and *Eleutheranthera ruderalis* (Swartz) Sch.-Bip. are two Asteraceae species showing very similar morphological appearances. Both are broad-leaf weed species in some crops throughout many tropical areas. Nevertheless, the individual species can be potentially utilized for human life, in which better knowledge on their phenotypical and molecular characters should be available. This study aims to provide scientific information in distinguishing the two identical species using a molecular marker, i.e. *trn*L(UAA) – *trn*F(GAA) intergenic spacer (IGS), which has been frequently used to identify genetic differences at a low levels of plant taxa. Plant samples were collected randomly from several Banyumas Regency, Central Java, Indonesia. Genomic DNAs were extracted using a CTAB method, followed by PCR amplification of the marker employing a pair of universal primers, e B49873 as a forward primer and f A50272 as a reverse primer. Sequencing of the amplicons was carried out using the automated Sanger method with labeled terminators. The results showed that *trn*L (UAA) - *trn*F (GAA) IGS in *S. nodiflora* and *E. ruderalis* were 312 bp and 317 bp in length, respectively. Sequence alignment between *trn*L (UAA) - *trn*F(GAA) IGS of both species showed several indels and base substitutions. Despite no direct correlation between these genetic differences and some observed phenotypic differences, this finding provides molecular data distinguishing both species, potentially used for DNA barcoding.

Keywords: *Eleutheranthera ruderalis* (Swartz) Sch.-Bip., *Synedrella nodiflora* (L.) Gaertn, *trnL (UAA) - trnF (GAA) IGS*

1. INTRODUCTION

Synedrella nodiflora (L.) Gaertn and *Eleutheranthera ruderalis* (Swartz) Sch.-Bip are two different plant species belonging to the Asteraceae family, very identical in their external morphology, thus frequently causing some difficulties in distinguishing them [1]. Even for many years, *E. ruderalis* had been misidentified as *S. nodiflora.* This was mainly because *E. ruderalis* could only be distinguished from *S. nodiflora* due to the absence of ray florets and pappus. Oppositely, pubescent and angled achenes were only found in *E. ruderalis* [2]. Both species originate from tropical America and spread over other regions, including Asia [2,3]. They are often seen as broad-leaf weeds in several crops with some severe problems concerning productivity [4], although some potentials of *S. nodiflora* as medicinal herbs [5], bioinsecticide [6], bio fungicide [7], and detoxificant for heavy metals [8] have been reported. On the other hand, very little is known about the potential use of *E. ruderalis* for human life, and it was even described as nothing from an economic point of view [1]. However, it has phytosociological been listed as one species of roadside vegetation moderately tolerant to the considerably polluted aerial environments. Hence, it can be explored as a phytoremediation agent in an emerging cost-effective eco-technology [9,10].

The utilization of *S. nodiflora* and *E. ruderalis* should be supported by better knowledge of their respective phenotypical and molecular characters. In particular, this becomes important, avoiding misidentification due to their highly identical appearances. A comparative study on morpho-anatomical traits between the two species has been previously reported. Both herbs' more distinguishable phenotypical character is the habitus, which is erect for *S. nodiflora* and decumbent for *E. ruderalis* [1,11]. In addition, molecular comparison between them using a cpDNA marker, *atp*B – *rbc*L intergenic spacer (IGS), has also been performed, revealing longer sequences of the marker in *S. nodiflora* than those observed in *E. ruderalis* [12]. Oppositely, longer sequences in *E. ruderalis* than those in *S. nodiflora* were found when another cpDNA marker, *trn*T(UGU) – *trn*L(UAA) IGS, was used [13]. Hence, some other genetic markers should be employed in completing the molecular characterization.

One of the most widely used molecular markers to detect genetic differences at lower levels of plant taxa is *trn*L(UAA) – *trn*F(GAA), an intergenic spacer in the cpDNA. The sequence has been successfully used to characterize the genetic differences among closely related plant species, e.g. between *Nymphaea kakaduensis* and *N. violacea* (Nymphaeaceae) [14]. A non-coding region with no responsibility for protein synthesis shows a high evolution rate [15]. As well as belonging to the single copy part of cpDNA, this marker has a relatively small size, thus enabling it to be easily amplified and sequenced [16,17,18].

This study aims to provide scientific information in distinguishing the two identical species of the Asteraceae family using *trn*L(UAA) – *trn*F(GAA) (IGS). The molecular comparison will complete those available with other genetic markers. Some supporting data on morpho-anatomical characters are also included.

2. MATERIALS AND METHODS

The study was conducted in the Laboratory of Genetics and Molecular Biology and the Laboratory of Plant Physiology, the Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto. Four individual samples of either *S. nodiflora* or *E. ruderalis* were collected randomly from Banyumas Regency, Central Java, Indonesia. Genomic DNAs were extracted from 0.1 g fresh truly expanded leaves following the CTAB method [18]. These were then used as PCR templates to amplify *trn*L(UAA) – *trn*F(GAA) IGS in a PTC-100 programable thermal cycler. The PCR condition was as follows: pre-denaturation at 94ºC for 5 mins, proceed by 30 reaction cycles consisting respectively of denaturing at 94ºC for 45 sec, annealing at 47ºC for 45 sec, extension at 72ºC for 1 min 30 sec, and terminated with a final extension at 72ºC for 5 min. A total volume of 11.5 µL PCR mixture containing 4 µL (20 ng) template DNA, 0.125 µL forward primer, 0.125 µL reverse primer, 5 µL Kappa Taq polymerase, and 2.25 µL nuclease-free water was used. A pair of universal primers, namely e B49873 (5'-GGTTCAAGTCCCTCTATCCC-3') as a forward primer and f A50272 (5'-ATTTGAACTGGTGACACGAG-3') as a reverse primer, were employed [20].

The amplicons produced were visualized on a 1.5% agarose gel electrophoresis using a 1x TAE buffer solution. Electrophoresis was run at 80 V, 400 mA for 50 mins before gel staining with ethidium bromide and documentation under ultraviolet transilluminator. Sequencing of the amplicons was performed in Firstbase Malaysia using the automated dideoxy method [21] with terminator labeling. The sequences obtained were manually edited using BioEdit version 7.0.4.1 [22]. The edited sequences were then blasted to the NCBI database to make sure that they were the *trn*L(UAA) – *trn*F(GAA) IGS. Sequence alignment was carried out using Clustal W [23], also implemented in the BioEdit software. Data on morpho-anatomical characters were obtained by measuring and visually observing them. They included habitus, plant height, foliar size and shape, number of flowers per capitulum, number, and types of trichomes.

3. RESULTS AND DISCUSSION

Assessment of the quality of the extracted genomic DNAs using electrophoretic visualization indicates that they all could serve properly as PCR templates. The purity (A260nm/280nm ratio) of the DNAs ranges from 1.816 in *S. nodiflora* sample 3 to 1.974 in *E. ruderalis* sample 2. In contrast, the concentration ranges from 5.54 ng/µL in *E. ruderalis* sample 1 to 24.78 ng/µL in *S. nodiflora* sample 2. Then, the amplicons thus produced are depicted in Figure 1.

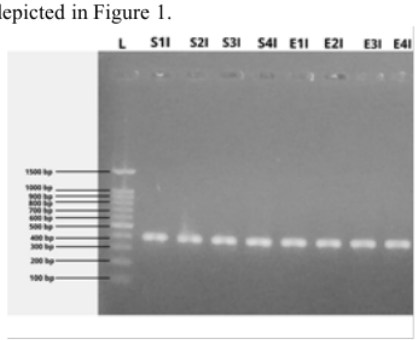

Figure 1. PCR amplicons of *Synedrella nodiflora* and *Eleutheranthera ruderalis* samples (L = DNA ladder; S1I – S4I = *S. nodiflora* 1 – 4; E1I – E4I = *E. ruderalis* 1 – 4)

Table 1. Blasting analysis of *trn*L(UAA) – *trn*F(GAA) IGS to NCBI database

Query	Subject	Query Cover (%)	Identity (%)	Accession number
S. nodi-flora	*Stomatanthes oblongifolious*	76	86.97	KP901245.1
	Synedrella nodiflora	74	86.59	AY216243.1
	Lasianthaea macrocephala	74	86.59	AY 216195.1
E. ruderalis	*Eclipta prostate*	75	86.64	KP208928.1
	Ajania breviloba	73	86.22	EF577324.1
	Dittrichia viscosa	78	85.02	GU818017.1

It can be seen in Figure 1 that all the amplicons are approximately 400 bp in size. This leads to the preliminary presumption that they are *trn*L(UAA) – *trn*F(GAA) IGS since about similar sizes of the IGS in some other Asteraceae species have been reported. For example, *trn*L(UAA) – *trn*F(GAA) IGS of 452 bp and 387 bp were found in *Conyza sumatrensis* and *Gnaphalium purpureum*, respectively [24]. In addition, those of 453 bp and 514 bp were observed in several species of *Chrysanthemum* [25] and *Tanacetum* [26].

Two of the eight amplicons obtained, i.e., E1I and E2I, could not be sequenced due to the faint bands produced when re-PCR was scaled up before sequencing. On the other six sequences, manual editing results in a fragment size of 312 bp in *S. nodiflora* and 317 bp in *E. ruderalis*. Then, blasting to NCBI database reveals that the sequences of *S. nodiflora* samples have identity up to 86.97% with available *trn*L(UAA) – *trn*F(GAA) IGS, while those of *E. ruderalis* show up to 86.64% identity with the IGS. Therefore, it can be ensured that the sequences of both species are undoubtedly *trn*L(UAA) – *trn*F(GAA) IGS. The results of the blasting analysis are shown in Table 1.

It is shown in Table 1 that one of the sequences blasted with *trn*L(UAA) – *trn*F(GAA) IGS of *S. nodiflora* is also that of *S. nodiflora* (Acc no. AY216243.1, unpublished). Both sequences reveal only 86.59% identity with a query cover of 74%. This is strongly assumed due to the different pairs of primers that have been used, resulting in slightly different amplified parts.

No difference was observed within *trn*L(UAA) – *trn*F(GAA) IGS sequences of *S. nodiflora* samples. Neither was within those of *E. ruderalis* samples. However, there were some insertions-deletions (indels) and base substitutions of *trn*L(UAA) – *trn*F(GAA) IGS between the two species. Sequence alignments of either within or between species are presented in Figure 2.

Long deletions in *S. nodiflora* (sites 320 – 326) and *E. ruderalis* (sites 168 – 171) were observed. Overall, however, more deletions occurred in *S. nodiflora* than those in *E. ruderalis* giving rise to 5 bp longer *trn*L(UAA) – *trn*F(GAA) IGS of *E. ruderalis* rather than that of *S. nodiflora*. This was also the case with *trn*T(UGU) – *trn*L(UAA) IGS, where it was somewhat longer in *E. ruderalis* than in *S. nodiflora* [13]. Nevertheless, different results were observed with another IGS, i.e., *atp*B – *rbc*L, where *S. nodiflora* showed a 22 bp longer sequence than *E. ruderalis* [12].

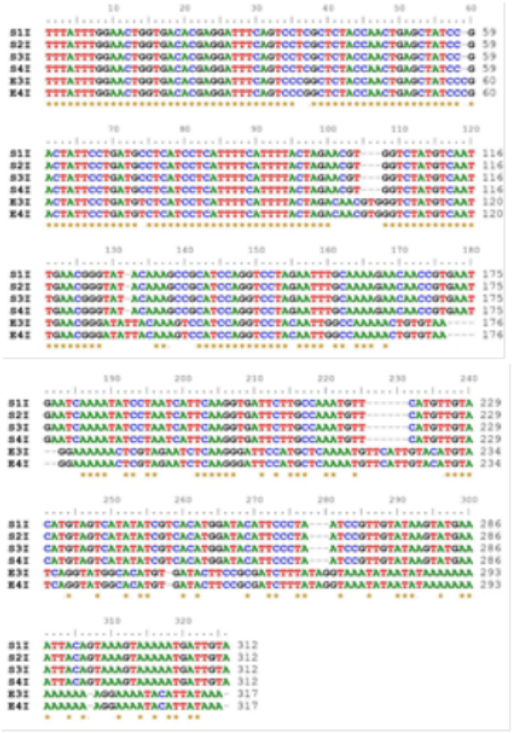

Figure 2. Sequence alignment of *trn*L(UAA) – *trn*F(GAA) IGS of *Synedrella nodiflora* and *Eleutheranthera ruderalis* (S1I – S4I = *S. nodiflora* 1 – 4; E3I – E4I = *E. ruderalis* 3 – 4; * = identical nucleotide site among six samples)

Base substitutions, either transitions or transversions, occurred, e.g., T in *S. nodiflora* was in replace of C in *E. ruderalis* at site 36 and C in *S. nodiflora* was in replace of G in *E. ruderalis* at site 37. As a whole, more transversions than transitions were observed. A similar result was reported in *trn*L(UAA) – *trn*F(GAA) IGS of *Quercus suber* [27]. This is normal due to the higher possibilities of transversions than those of transitions [28]. Yet, transversion is more energy-consuming in comparison to transition due to the more complicated alteration in the molecular structure of the nucleotide base, in which purine is changed into pyrimidine and vice versa [29]. Considering *S. nodiflora* as the more recently mutating species compared to *E. ruderalis*, all the mutation types are summarized in Table 2.

Table 2. Types of mutations in *trn*L(UAA) – *trn*F(GAA) IGS of *Synedrella nodiflora* compared to that of *Eleutheranthera ruderalis*

No.	Type of mutation	Sites
1	Insertion	142; 168 – 171; 185 – 186; 242; 253
2	Deletion	59; 101 – 102; 109; 129; 133; 260; 320 – 326
3	Transition	36; 175; 183; 195; 202; 210; 213; 219; 224; 227; 229; 230; 247; 268; 275; 276; 277; 283; 287; 300; 305; 310; 311; 313; 317; 318
4	Transversion	37; 161; 179; 181; 191; 192; 200; 201; 209; 211; 215; 220; 223; 226; 228; 246; 251; 266; 270; 272; 274; 278; 279; 281; 282; 293; 296; 298; 301; 306; 312; 315

The phylogenetic tree constructed among samples showed that all *S. nodiflora* individuals were grouped into one cluster, and so were all *E. ruderalis* samples. However, both *S. nodiflora* and *E.ruderalis* were much closer to each other than to other Asteraceae species, i.e., *Stomatanthes oblongifolius* (Figure 3). This supports morphological characterization, where the two species are phenotypically identical [1].

Corresponding to previous studies, many resemblances between *S. nodiflora* and *E. ruderalis* in morphological characters were observed. The differences existed only concerning habitus, plant height, leaf size and texture, and the number of flowers per capitulum (Table 3).

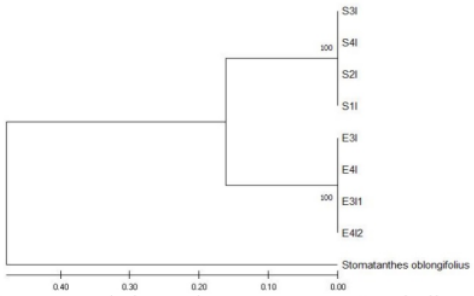

Figure 3. Phylogenetic tree among *Synedrella nodiflora* and *Eleutheranthera ruderalis* samples

Table 3. Some morphological characters of *Synedrella nodiflora* and *Eleutheranthera ruderalis*

Characters	*S. nodiflora*	*E. ruderalis*
Habitus	erect	decumbent
Plant height (cm)	25.47 ± 8.09	20.25 ± 5.54
Leaf shape	ellipse	ellipse
Leaf length (cm)	5.60 ± 1.62	3.70 ± 1.68
Leaf width (cm)	3.00 ± 0.52	1.80 ± 2.04
Leaf thickness (cm)	0.50 ± 0.14	0.70 ± 0.08
Apex folii	acutus	acutus
Basis folii	acuminatus	acuminatus
Margo folii	serratus	serratus
Nervatio	penninervis	penninervis
Leaf surface	glabrous	pubescent
Leaf upper surface colour	dark green	dark green
Leaf lower surface colour	light green	light green
Number of flowers per capitulum	14 (9 disc florets, 5 ray florets)	8 (all are disc

An earlier study on the morphological comparison between the two species revealed similar results, in that the differences were particularly concerning the habitus and plant height [30]. Regarding the number of flowers per capitulum, our results confirm that heterogamy capitulum is typical in *S. nodiflora*, while *E. ruderalis* has homogamy capitulum [31].

The difference in leaf surface texture between *S. nodiflora* and *E. ruderalis* was likely related to the number of trichomes. Anatomical examination showed that more trichomes were present in *E. ruderalis* than to those in *S. nodiflora*, causing pubescent leaf surface in *E. ruderalis* and glabrous in *S. nodiflora*. Glandular trichomes were found in both species, though much less than non-glandular trichomes observed. This approves a previous study on several Asteraceae species that non-glandular trichomes are found more than glandular trichomes [32].

Although no direct correlation between differences in *trn*L(UAA) – *trn*F(GAA) IGS and those in some morpho-anatomical characters is observed, this finding

may contribute to the completion of molecular data in distinguishing *S. nodiflora* and *E. ruderalis*. Both sequences have been submitted to NCBI GenBank and are awaiting accession numbers. Combined with some other molecular markers, this can be possibly used for DNA barcoding of the respective species.

AUTHORS' CONTRIBUTIONS

Murni Dwiati designed the research idea, analyzed morpho-anatomical data, and took foremost responsibility for the project, Indrawati collected data, Agus Hery Susanto analyzed molecular data and wrote the manuscript.

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