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Genetic Difference between Two Phenotypically Similar Members of Asteraceae By the Use of Intergenic Spacer *atp*B – *rbc*L

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Abstract

Two Asteraceae species, i.e. *Synedrella nodiflora* (L.) Gaertn and *Eleutheranthea ruderalis* (Swartz) Sch.-Bpi. are phenotypically similar with each other, although some differences in morphological and anatomical traits are apparently observable. Molecular comparison using particular marker is required to support a phenotype-based study that previously reported. Chloroplast DNA marker, . *atp*B – *rbc*L IGS, was used to identify genetic difference between both species. Six samples of the respective species were collected randomly from some places in Banyumas Regency, Central Java, Indonesia. Amplification of the marker was performed employing a pair of universal primers. Sequence alignment on the PCR products showed that no difference in *atp*B – *rbc*L IGS sequences, either within *S. nodiflora* or *E. ruderalis* samples was observed. On the other hands, several deletions and base substitution in both *S. nodiflora* and *E. ruderalis* were detected when alignment was made between both species. This result suggests that they reveal a convincing genetic difference. Inspite of no direct correlation between this genetic and some visible phenotypic differences, this finding provides preliminary scientific background on the phenotypic traits of both species, which are often difficult to find at a rapid observation.

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INTRODUCTION

Synedrella nodiflora (L.) Gaertn and *Eleutheranthea ruderalis* (Swartz) Sch.-Bpi. are two species belonging to the family of Asteraceae, which morphologically and anatomically resemble each other (Choudhury & Mukherjee, 2005). This has ever led to misidentification of *E. ruderalis* as *S. nodiflora* in a sufficiently long period of time. In 1996, *E. ruderalis* was collected in southern Taiwan as an unknown species of Asteraceae with very similar phenotype to that of *S. nodiflora* (Sheng-Zehn Yang & Gaung-Pu Hsieh, 2006).

Both *S. nodiflora* and *E. ruderalis* are now found as broad leaf weeds in several crops, though the first has been reported to possess some potentials as medicinal herbs (Adjibode et al., 2015; Amoateng et al., 2015; Amoateng et al., 2017sedative and analgesic effects. Preliminary studies conducted in animals, SNE significantly decreased stereotypic behaviours suggesting antipsychotic potential. Coupled with the central nervous system depressant effects of SNE, we hypothesized that it may have utility in the management of psychosis. The present study therefore investigated the antipsychotic potential of the SNE in several murine models of psychosis. Method: The primary central nervous system activities of SNE (30-3000 mg/kg, p.oa; Amoateng et al., 2017b), bioinsecticide (Belmain et al., 2001; Rathi & Gopalakrishnan, 2006; Rathi & Gopalakrishnan, 2010), biofungicide (Sawatdikarn, 2016) and detoxificant for heavy metals such as Cu and Pb (Prekeyi & Oghenekevwe, 2007). On the other hands, no study has been performed on *E. ruderalis* potentials to human life. There is just a report that this species is frequently used by local people in West Pasaman Indonesia as a traditional medicinal herb for relieving wound (Rizki & Fernando, 2019). Nevertheless, this species is phytosociologically considered as tolerant enough to stressed environmental condition, so that, it is said to have a sufficiently high ecological potential in natural communities (Ray & George, 2009).

Taxonomically, *S. nodiflora* has been the only species of genus *Synedrella* despite its world wide distribution over many tropical areas (Chauhan & Johnson, 2008). Another species of *Synedrella* has ever been reported, i.e. *S. vialis* (Less.) A. Gray, which grows in some parts of Kangra district, India (Lal et al., 2015). This finding, however, was proved to be the same as *Calyptocarpus vialis* (Less.), a taxonomically accepted name for another Asteraceae species. Another species that has been reported, i.e. *S. peduncularis* Benth (Asteraceae), which is then known as the synonym of *Schizoptera peduncularis* (Benth) S.F. Blake. Hence, *S. nodiflora* has so far been the only member of genus *Synedrella,* which is taxonomically accepted (The Plant List, 2013).

Like *S. nodiflora*, which originates from tropical America, *E. ruderalis* is then spread to many other regions, such as South, East, Southeast Asia and even Papua New Guinea (Sheng-Zehn Yang & Gaung-Pu Hsieh, 2006). However, this species is not the only member of genus *Eleutheranthera*, since two other species, i.e. *E. tenella* (Kunth) H. Rob. and *E. divaricata* (Rich.) Millsp., are known (The Plant List, 2013).

A comparative study between *S. nodiflora* and *E. ruderalis* by the use of several morphological and anatomical markers has been reported. In general, both species showed many phenotypic similarities, although some characters were clearly distinguishable (Choudhury & Mukherjee, 2005).

To support the phenotypic study, molecular characterization employing a particular genetic marker was needed. One of the markers that can be used is intergenic spacer (IGS) *atp*B – *rbc*L, which is a non coding sequence in chloroplast genome. As a region unresponsible to any protein synthesis, *atp*B – *rbc*L IGS has some parts with high evolution rate (Chiang & Schaal, 2000). Such marker is suitable for evolutionary history analysis in lower level, e.g. species, genus and family (Shaw et al., 2014)comparing the number of genetic differences found in 107 NC-cpDNA regions and matK. We surveyed Web of Science for the plant phylogeographic literature between 2007 and 2013 to assess how NC-cpDNA has been used at the intraspecific level. KEY RESULTS Several regions are consistently the most variable across angiosperm lineages: ndhF-rpl32, rpl32-trnL((UAG. Hence, the purpose of this study was to identify genetic difference between *S. nodiflora* and *E. ruderalis* by the use of *atp*B – *rbc*L IGS sequences as the molecular marker. This study was expected to provide the basic information on some genetic background causing slightly phenotypic difference between both species.

METHODS

Collecting plant samples

Both *S. nodiflora* and *E. ruderalis* samples were collected in April 2019 from some places in Banyumas Regency, Central Java, Indonesia. The six samples of the respective species were taken randomly from the areas. Each sample was pulled out up to its roots and then put into a plastic bottle previously filled with a little water. The

samples were then grown in the screen house of Fakultas Biologi Universitas Jenderal Soedirman. Molecular analysis was carried out in the Laboratory of Molecular Genetics of the institution.

Extraction of genomic DNAs

Genomic DNAs were extracted from the uppermost leaves following CTAB method (Doyle & Doyle, 1990). Individual leaf of 0.1 g was cut into small pieces and put into a 1.5 mL microtube. Then, 800 µL CTAB buffer previously heated at 65°C for 30 mins was added. The leaf pieces were crushed and powdered by using minibeadbeater for four mins. Afterward, the sample was put into a waterbath of 65°C for 60 mins, in which the microtube was turned upside down in every 10 mins. The sample was then taken from the waterbath and allowed to cool down at room temperature for two mins, after which, 500 µl chloroform-isoamylalcohol (CIAA) was added. It was mixed gently and vortexed for five mins. The mixture was then centrifuged at 12,000 rpm for 15 mins, and the supernatant was moved carefully into a new microtube, where 3M sodium acetate of 1/10 supernatant volume was added and mixed gently. Cold isopropanol of 2/3 total volume (sodium acetate plus supernatant) was then added to the mixture and mixed gently by flipping the tube. This mixture was then kept in the freezer for 24 hours. The sample was centrifuged at 12,000 rpm for 10 mins, after which, the supernatant was discarded and the DNA pellet was washed with 500 µL ethanol 70% by flipping the tube. The mixture was centrifuged again at 12,000 rpm for five mins, after which, the supernatant was discarded and the DNA pellet was air dried. The DNA pellet was then dissolved into 100 µL TE buffer and kept at 4°C before quantification was performed by using GeneQuant.

Amplification of IGS *atp*B – *rbc*L

The extracted DNAs were used as PCR templates to amplify *atp*B – *rbc*L IGS using a pair of universal primers, i.e. 5' – ACATCKARTACKGGACCA ATAA - 3' as forward primer and 5' - AACACCAGCTTTRAATCCAA - 3' as reverse primer (Chiang et al., 1998). Each PCR reaction was performed in a total volume of 10 µl comprising 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 subjected to a PCR condition as follows: pre-denaturation at 94°C for 3 mins, 33 reaction cycles consisting of denaturation at 94°C for 45 secs, primer annealing at 55°C for 45 secs, extension at 72°C for 2 mins respectively, followed by final extension at 72°C for 3 mins and storage at 4°C. The PCR products were visualized in a 1.5 % agarose gel electrophoresis using 1X TAE buffer, which was run at 75 Volt, 400 mA for 40 mins. After being stained with ethidium bromide, the gel was exposed to UV transiluminator for documentation.

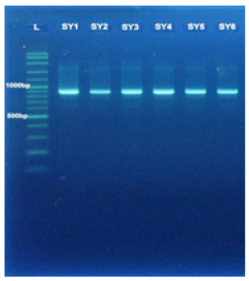
Sequencing and data analysis

The PCR products were purified using QIAquick kit (Qiagen, Germany), and were sequenced following automated dideoxy method (Sanger et al., 1977) with terminator labelling. Data on base sequences were edited using Bioedit version 7.0.4.1 (Hall, 1999) and were checked manually. Sequence alignment was carried out using ClustalW (Thompson et al., 1994), which was also implemented in the Bioedit version 7.0.4.1.

RESULTS AND DISCUSSION

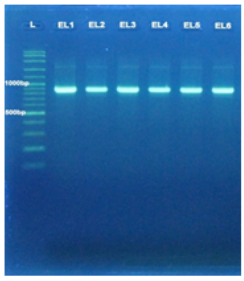
PCR products of both *S. nodiflora* and *E. ruderalis* samples that are presented in Figure 1 showing bands of approximately 880 bp in size. However, these were pruned into only 834 bp length after manual editing. NCBI blasting reveals that those of *S. nodiflora* samples show 99% to 100% homology with *atp*B – *rbc*L IGS sequences of *S. nodiflora* available in the data base with accession numbers of KX096801.1, KX096802.1, KY983543.1, KY983544.1, KY983545.1 and MF285608.1 submitted by Susanto (2018). This means that the PCR products of *S. nodiflora* are undoubtedly *atp*B – *rbc*L IGS. Similarly, sequence alignment of the amplicons resulting from *E. ruderalis* samples with those from *S. nodiflora* shows 96% homology in range 1 and 94% homology in range 2 (Figure 2), ensuring them as also *atp*B – *rbc*L IGS sequences.

Within the six *S. nodiflora* samples, no difference in *atp*B – *rbc*L IGS sequences was observed. There were also no difference found in *atp*B – *rbc*L IGS sequences within the six *E. ruderalis* samples. The *atp*B – *rbc*L IGS sequences of both *S. nodiflora* and *E. ruderalis* have now been submitted to NCBI database for accession numbers.



L = DNA ladder
SY1 – 6 = *Synedrella nodiflora* samples number 1 to 6

a



L = DNA ladder
EL1 – 6 = *Eleutheranthera ruderalis* samples number 1 to 6

b

Figure 1. Amplicons of IGS *atp*B – *rbc*L, a) *Synedrella nodiflora* (L.) Gaertn, b) *Eleutheranthea ruderalis* (Swartz) Sch.-Bpi.

Sequence alignment between *atp*B – *rbc*L IGS of *S. nodiflora* and that of *E. ruderalis* shows differences in some sites as depicted in Figure 2. Some insertion-deletion (indel) mutation between both sequences are observed. A 33 bp long base deletion in *E. ruderalis* starting from site 660 occurs, while several relatively shorter base deletions are observed in *S. nodiflora*, e.g that starts from site 775. Overall, this makes *E. ruderalis* has 22 bp shorter *atp*B – *rbc*L IGS compared to that of *S. nodiflora.*

In addition to indel, some base substitutions, either transition or tranversion, between both sequences are also detected. For instance, adenine in site 139 of *S. nodiflora* is substituted by guanine site 132 of *E. ruderalis*, indicating the transition to occur. On the other hands, tranversion can be seen in site 88 of *S. nodiflora* or site 81 of *E. ruderalis*, where cytosine is in replace of adenine.

Despite no evidence on the correlation between the differences in *atp*B – *rbc*L IGS sequence of both species and some observable phenotypical dissimilarity between them, this could at least be viewed as a coincidence. Widodo et al. (2019) used *atp*B – *rbc*L IGS to distinguish between two morphologically similar genera, i.e. *Eugenia* and *Syzygium*. This genetic marker was also used to determine the taxonomical status of a previously bewildering species, i.e. *Eugenia boerlagei* Merr. (Myrtaceae), where it was proved to be grouped into *Syzygium* rather than *Eugenia*. Hence, the species name should be changed into *Syzigium boerlagei*. This conclusion was not based on the size of *atp*B – *rbc*L IGS, since there was no obvious correlation between the length of the marker and the genera. Instead, the taxonomical status replacement was based on the GC content of the marker.

The GCcontents of *atp*B – *rbc*L IGS in *S. nodiflora* and *E. ruderalis* are 28% and 29% respectively, which means that they do not show significant difference. On the other hands, both species were ever found in the same sites of *Stachytarpheta jamaicensis* (Verbenaceae) habitats with important value index (IVI) of 12.8 and 5.57 respectively (Solikin, 2019). It shows that the almost equal GCcontents between both species do not affect their presence in a particular environmental condition as different IVIs were observed. However, different facts were reported in another Asteraceae genus, i.e. *Cyanus*, where GCcontent was found significantly correlated with longitude, in which plants growing in west areas showed higher GC contents than those in east areas. In addition, plants with GC-rich genomes were concentrated in the coldest areas with low minimum temperature (Olšavská et al., 2012).

In general, chloroplast genome of Asteraceae is relatively conservative with respect to gene content, although it is not the case in gene structure and tRNA abundance. Hence, chloroplast genome is an appropriate source of molecular markers to study the evolutionary relationship between species of the family (Wang et al., 2015). Similarly, chloroplast genome provides suitable markers to determine interspecies relationships in *Pistacia* (Anacardiaceae). This is because evolutionary processes in the chloroplast genome occur more slowly than those in nuclear genome (Talebi et al., 2016).

*Atp*B – *rbc*L IGS, in a combination with *trn*L – *trn*F IGS, has been used to reveal the ancestry of *Impatiens* spp. (Balsaminaceae) in South India. Based on these molecular markers, it was suggested that *Impatiens* in South India was originated from two independent dispersal events, i.e. one from Southeast Asian ancestor and the other from African affinities (Shajitha et al., 2016). As well, *atp*B – *rbc*L IGS, along with some other molecular markers from chloroplast genome, has proved to change the current sectional classification of genus *Musa* (Musaceae), which was based on chromosome number and morphological characteristics (Lamare et al., 2017).

High variation of *atp*B – *rbc*L IGS sequences has been shown in some populations of Alis-

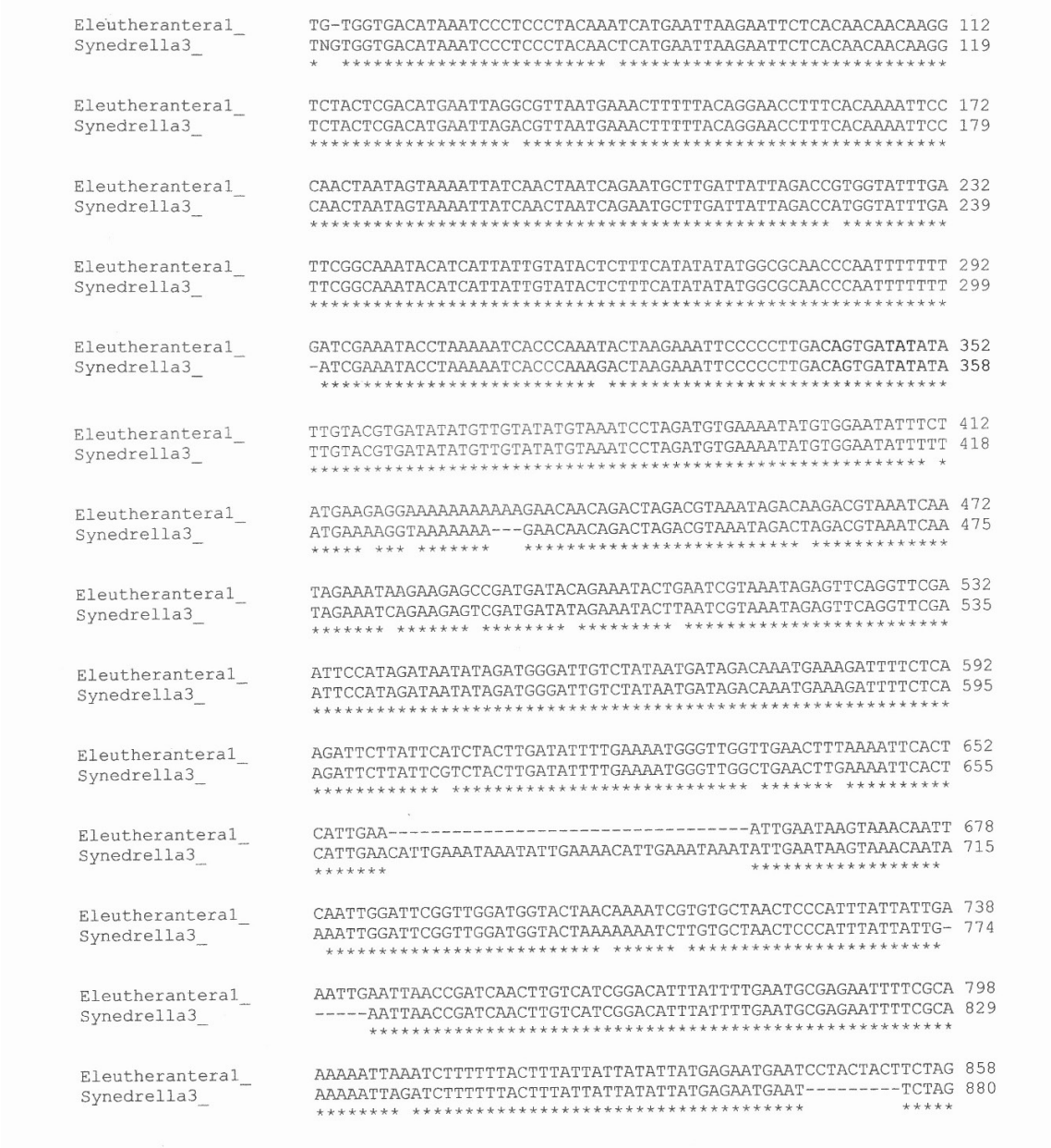


Figure 2. Sequence alignment of *atp*B – *rbc*L IGS between *Synedrella nodiflora* and *Eleutheranthera ruderalis*

mataceae species in China, i.e. *Sagittaria trifolia* (Chen et al., 2008), *S. potamogetifolia* (Tan et al., 2008) and *S. lichuanensis* (Liu et al., 2010). Similarly, high variation of atpB – rbcL IGS in the populations of *Hygrophila pogonocalyx* (Acanthaceae) in Taiwan (Huang et al., 2005) and *Ceriops tagal* (Rhizophoraceae) in Southeast Asia (Liao et al., 2007) were also reported. Oppositely, this marker exhibited low variation in the population of *S. nodiflora* in Java Island (Susanto et al., 2018).

In this study we find that *atp*B – *rbc*L IGS sequences of *S. nodiflora* and *E. ruderalis* showed several differences in some sites. This genetic characterization has never been previously reported thus providing a novel information that accomplishes phenotypic characterization of both species. Furthermore, this finding can be used to support molecular taxonomy study of the Asteraceae members.

CONCLUSION

Several genetic differences with respect of either base substitution or insertion-deletion between *S. nodiflora* and *E. ruderalis* were observed. Though no direct correlation has been proved, this genetic difference supports some phenotypically distinguishable traits between both species,

which are frequently difficult to find at a glance.

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