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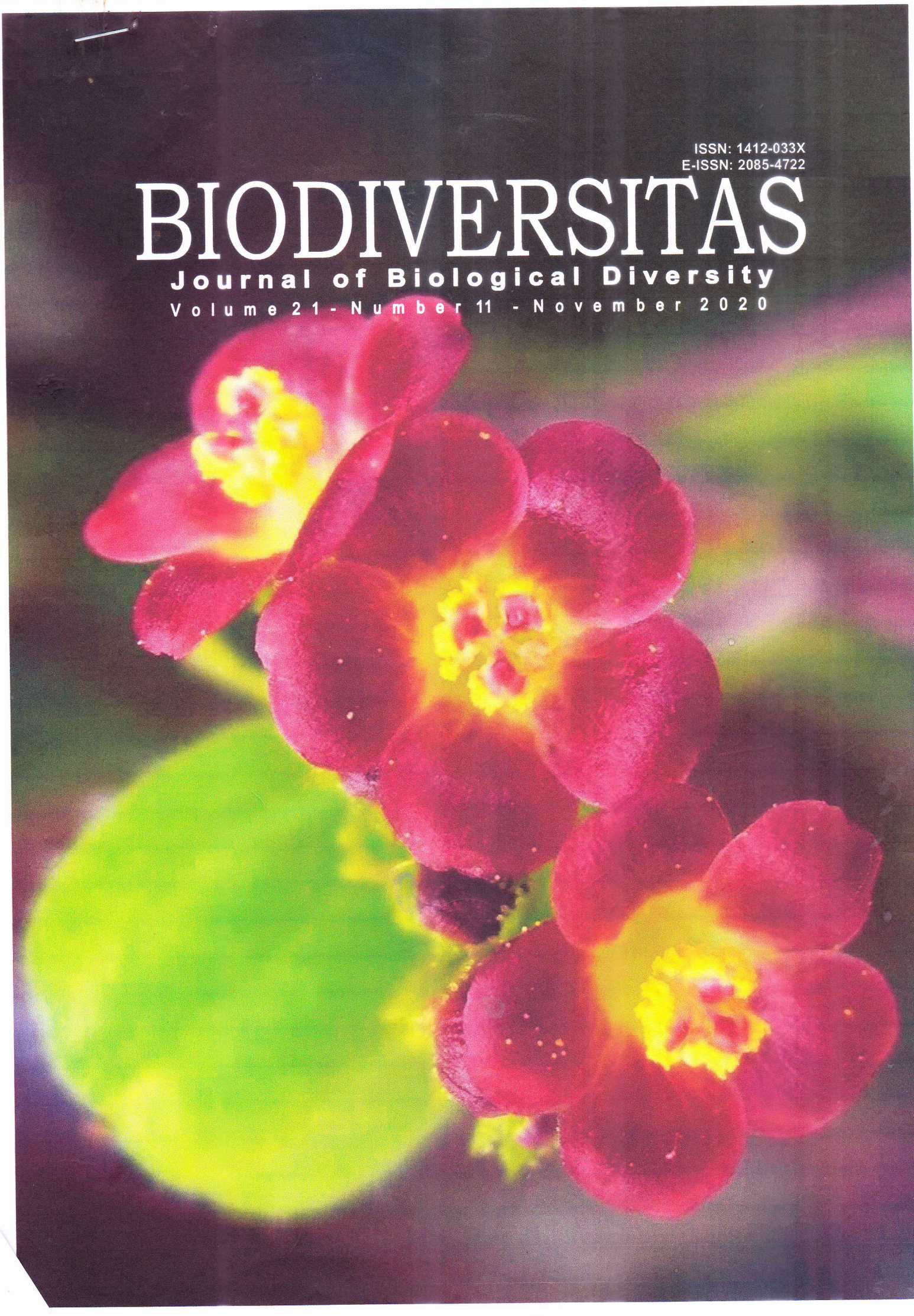

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Chapter in book:

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Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

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Thesis, Dissertation:

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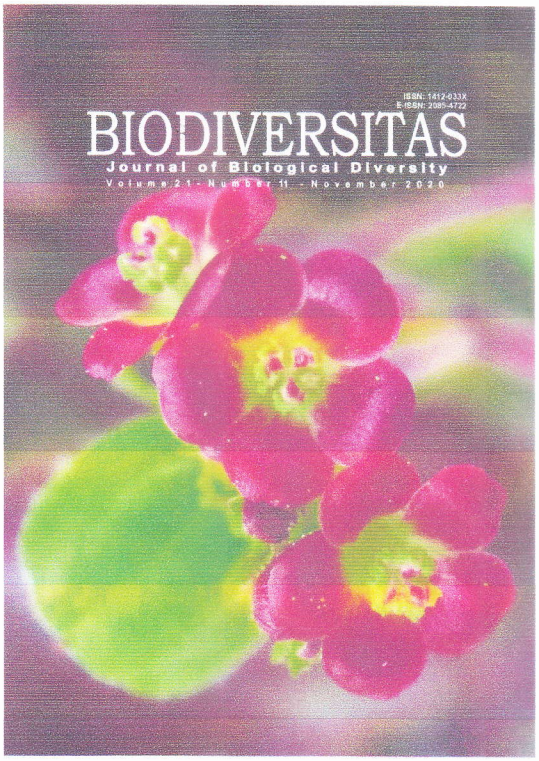

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Molecular characteristics of two phenotypically identical species of Asteraceae based on the intergenic spacer *trn*T(UGU)-*trn*L(UAA)

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Abstract. *Susanto AH, Dwiati M, Pratiwi S. 2020. Molecular characteristics of two phenotypically identical species of Asteraceae based on the intergenic spacer* trn*T(UGU)*-trn*L(UAA). Biodiversitas 21: 5164-5169.* Ogiera (*Eleutheranthera ruderalis*) and nodeweed (*Synedrella nodiflora*) are two different weed species belonging to the family Asteraceae commonly found in tropical regions. At a glance, both species show highly identical morphology, thus leading to difficulty in distinguishing between them. Therefore, molecular data based on particular markers are required. Here, we use an intergenic spacer (IGS) in the cpDNA, i.e., *trn*T(UGU)-*trn*L(UAA), as the molecular marker to reveal the difference between the two species. A pair of PCR universal primers, i.e., B48557 as the forward primer and A49291 as the reverse primer, were employed to amplify the marker. Sequence alignment was performed by the use of ClustalW implemented in Bioedit version 7.0.4.1. The results revealed that some differences with respect to both indel and base substitution were observed. Overall, this led to longer IGS *trn*T(UGU)-*trn*L(UAA) sequences of *E. ruderalis* than those of *S. nodiflora*. Although no direct relationship between the genetic and phenotypic dissimilarities was proven, coincidence seemed likely to exist. This provides molecular evidence that the two phenotypically similar species are genetically different from each other.

Keywords: *Eleutheranthera ruderalis*, genetic differences, IGS *trn*T(UGU)-*trn*L(UAA), *Synedrella nodiflora*

INTRODUCTION

Asteraceae is the largest family of flowering plants throughout the world. It consists of approximately 1,600 genera and 23,000 species, many of which are important for ornamental, medicinal, and economic purposes (Gao et al. 2010). However, some of them are known as invasive weeds (Souza Filho and Takaki 2011), causing detrimental effects on several tropical crops with respect to productivity (Srithi et al. 2017).

Ogiera *(Eleutheranthera ruderalis* (Sw.) Sch.-Bip.*)* and nodeweed *(Synedrella nodiflora* (L.) Gaertn*)* are two widely distributed weed species of Asteraceae in tropical regions. Both species prefer moist and shady places and show highly identical morphology (Choudhury and Mukherjee 2005). For many years, the morphological resemblance between them has led to misidentification of *E. ruderalis* as *S. nodiflora*. In 1996, *E. ruderalis* collected in southern Taiwan was identified as an unknown species of Asteraceae that was phenotypically very similar to *S. nodiflora* (Yang and Hsieh 2006).

S. nodiflora is taxonomically the only member of the genus *Synedrella* (Davidse et al. 2015), although another species, i.e., *S. vialis* (Less.) A. Gray, has been reported in the district of Kangra, India (Lal et al. 2009). However, the latter was then known as *Calyptocarpus vialis* (Less.), the accepted name for another species of Asteraceae. In addition, there was also *S. peduncularis* Benth, which was then proven to be the synonym of *Schizoptera peduncularis* (Benth) S.F. Blake (The Plant List 2013). Despite its widely known role as a weed species, *S. nodiflora* has some potential as a medicinal herb (Adjibode et al. 2015; Amoateng et al. 2017a, b; Sekar et al. 2018), bioinsecticide (Rathi and Gopalakrishnan 2006), and biofungicide (Sanit 2016). Meanwhile, *E. ruderalis* has been used by some indigenous people in West Pasaman, Indonesia to stop wound bleeding. Additionally, the decoction of this plant can increase milk production for breastfeeding mothers and treat high blood pressure (Rizki et al. 2019). Unlike *S. nodiflora*, *E. ruderalis* is not the only species of *Eleutheranthera*, since two other species exist, i.e., *E. tenella* (Kunth) H. Rob. and *E. divaricata* (Rich.) Millsp. (The Plant List 2013).

To support the phenotypic comparison between *E. ruderalis* and *S. nodiflora*, molecular characterization should be performed. An intergenic spacer (IGS) in the cpDNA, i.e., *atp*B-*rbc*L, has been used to genetically distinguish between the two species and reveals some indels and base substitutions (Susanto and Dwiati 2019). Additional evidence employing another molecular marker to confirm these findings is required. Hence, the objective of this study was to genetically compare *E. ruderalis* and *S. nodiflora* using another cpDNA marker, i.e., intergenic spacer (IGS) *trn*T(UGU)-*trn*L(UAA). This marker has become one of the most widely used cpDNA markers for phylogenetic analyses in plants (Quandt et al. 2004; Yousefzadeh et al. 2018) since universal primers have been introduced (Taberlet et al. 1991).

MATERIALS AND METHODS

Sample collection and preparation

Plant samples were collected randomly in January 2020 from several places in Purwokerto City, Central Java, Indonesia, including three *E. ruderalis* and four *S. nodiflora* individuals. All samples were cut at the stems and then put into plastic bottles containing a small amount of water. They were then grown using polybags in the screen house of the Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia until molecular analysis was carried out in the Laboratory of Genetics and Molecular Biology of the institution.

Genomic DNA extraction

Three to five uppermost leaves of all plant samples, from which genomic DNAs would be extracted, were cut at the leaf base and sprayed with 70% ethanol. Genomic DNA extraction was performed following the CTAB method (Doyle and Doyle 1990). Small pieces of leaf of approximately 0.1 g were put into a mortar, and 800 µL of CTAB solution previously incubated in a water bath at 65°C for 30 mins was added. The individual mixture was crushed using a pestle up to homogeneity, and the resulting mixture was placed in a 1.5-mL microtube. This mixture was then incubated in the water bath at 65°C for 1 h, in which the microtube was inverted gently every 10 mins. Hereafter, the mixture was taken from the water bath and allowed to cool down at room temperature for 2 mins, after which 500 µL of CIAA solution was added. The mixture was homogenized gently, vortexed for 5 mins, and then centrifuged at 12,000 rpm for 15 mins. The supernatant was transferred carefully into a new microtube, and 3 M sodium acetate (1/10 supernatant volume) was added and mixed gently. Cold isopropanol of 2/3 total volume was then added and mixed gently by flipping the tube. The mixture was kept in the freezer for 24 h, after which it was centrifuged at 12,000 rpm for 10 min. The supernatant was removed, while the DNA pellet was washed by adding 500 µL of 70% ethanol with gentle flipping of the microtube. The DNA solution was centrifuged at 12,000 rpm for 5 mins, after which the supernatant was removed and the DNA pellet was air-dried. Finally, the DNA pellet was dissolved in 100 µL of TE buffer and stored at 4°C. Genomic DNAs were visualized by electrophoresis in a 1.5% agarose gel, while the concentration and purity were measured using GeneQuant.

Amplification of IGS *trn*T(UGU)-*trn*L(UAA)

The concentration of individual genomic DNA was 5 ng μL^{-1} by diluting it with nuclease-free water (NFW); it was diluted 10x when it was used as a DNA template for PCR. The total volume of the PCR mixture was 11.5 µL, consisting of 4 µL of DNA template, 0.125 µL of forward primer, 0.125 µL of reverse primer, 5 µL of Kapa Taq Polymerase and 2.25 µL of NFW. A pair of universal primers, i.e., B48557 (5'-CATTACAAATGCGATGCTCT-3') as the forward primer and A49291 (5'-TCTACCGATTTCGCCATATC-3') as the reverse primer (Taberlet et al. 1991), were employed. The PCR mixture was spun down before it was placed in a BIO-RAD thermal cycler machine with the reaction conditions as follows: predenaturation at 94°C for 5 mins, followed by 30 PCR cycles consisting of denaturation at 94°C for 45 secs, annealing at 47°C for 45 secs and extension at 72°C for 1 min 30 secs, and termination with a final extension at 72°C for 5 mins. The PCR products were visualized by electrophoresis in a 1% agarose gel.

For sequencing preparation, the PCR mixture was scaled up to a total volume of 46 µL consisting of 8 µL of DNA template, 0.5 µL of forward primer, 0.5 µL of reverse primer, 20 µL of Kapa Taq Polymerase, and 17 µL of NFW. Similar PCR conditions as before were applied.

Sequencing of IGS *trn*T(UGU)-*trn*L(UAA)

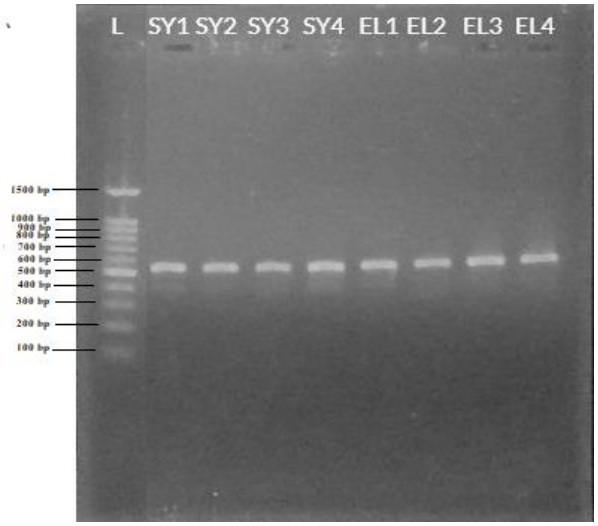
The PCR products were purified using a QIAquick kit (Qiagen, Germany) and were sent to Firstbase, Malaysia for sequencing. The dideoxy DNA automated sequencing method (Sanger et al. 1977) with terminator labeling was employed.

Data analysis

Data for DNA sequences were edited using Bioedit version 7.0.4.1 (Hall 1999) and were checked manually. Blasting was performed to ensure that the sequences were undoubtedly IGS *trn*L(UGU)-*trn*T(UAA) by comparing them with those available in the NCBI database. Sequence alignment was carried out using ClustalW (Thompson et al. 1994), which was also implemented in the Bioedit software. A phylogenetic diagram showing the relationships between the two species and some other species of Asteraceae (extracted from NCBI GenBank) was constructed using the MEGA Program version 6 (Tamura et al. 2016).

RESULTS AND DISCUSSION

All DNA samples were successfully amplified, showing PCR bands of approximately 650 bp (Figure 1). These were slightly shorter than those obtained by the use of IGS *atp*B-*rbc*L primers, where the PCR products of both *S. nodiflora* and *E. ruderalis* samples were 880 bp in size (Susanto and Dwiati 2019). A relatively more similar size of IGS *trn*T-*trn*L was found in some other species, e.g., 712 bp long in *Betonica officinalis* (Lamiaceae) (Thell et al. 2019). However, IGS *trn*T-*trn*L sequences 1,100 bp long from *Citrus madurensis* and *C. depressa* (Rutaceae) were amplified with the same primers as those used in this study (Yahada et al. 2019). Various sizes of IGS *trn*T-*trn*L were inevitably obtained due to the different sequences of IGS *trn*T-*trn*L among the plant species, despite the fact that they were amplified using the same primers.



L = 1 kb ladder
SY1 = *Synedrella nodiflora* 1 EL1 = *Eleutheranthera ruderalis* 1
SY2 = *Synedrella nodiflora* 2 EL2 = *Eleutheranthera ruderalis* 2
SY3 = *Synedrella nodiflora* 3 EL3 = *Eleutheranthera ruderalis* 3
SY4 = *Synedrella nodiflora* 4 EL4 = *Eleutheranthera ruderalis* 4

Figure 1. Amplicons of IGS *trn*T (UGU)-*trn*L(UAA) of *Synedrella nodiflora* (L.) Gaertn and *Eleutheranthera ruderalis* (Sw.) Sch.-Bip.

After manual editing, DNA sequences of 627 bp and 634 bp were obtained from *S. nodiflora* and *E. ruderalis* samples, respectively. When *E. ruderalis* was blasted against the NCBI database, the highest similarity of 98% with IGS *trn*T- *trn*L of *Melanthera nivea* (Asteraceae, accession number AY215953.1) was observed. Similarly, the highest similarity of 92.25% between the DNA sequence of *S. nodiflora* and IGS *trn*T-*trn*L of *Lasianthaea macrocephala* (Asteraceae, accession number AY215953.1) was also found. These findings indicate that all sequences of the PCR products obtained in this study were definitely parts of IGS *trn*T- *trn*L.

Of the four PCR products of *E. ruderalis* samples, one was not successfully sequenced due to its low quality. No difference among the IGS *trn*T-*trn*L sequences of the remaining three samples was observed (Figure 2). However, one of the four IGS *trn*T-*trn*L sequences of *S. nodiflora* samples, i.e., Sample number 4, showed a transversion at base 54, where C in the other three samples was replaced with A (Figure 3). In other words, the IGS *trn*T-*trn*L sequence of this sample showed 99% homology with those of the other three. All IGS *trn*T-*trn*L sequences of both *E. ruderalis* and *S. nodiflora* have now been submitted to NCBI for accession number assignments.

Several differences with respect to both indel and base substitution were observed when IGS *trn*T-*trn*L sequences of *S. nodiflora* and *E. ruderalis* were aligned, as depicted in Figure 4. Very long deletions in some sites of *S. nodiflora* were detected, e.g., from base 9 to 31 and from base 437 to 455 of *E. ruderalis* sequences. In contrast, some relatively shorter deletions in *E. ruderalis* sequences, e.g., from base 175 to 187 and from base 208 to 222, were also identified. These observations fully explain why the IGS *trn*T-*trn*L sequence of *E. ruderalis* is somewhat longer than that of *S. nodiflora*. An opposite result was obtained when another cpDNA marker, i.e., IGS *atp*B-*rbc*L, was employed to compare *E. ruderalis* and *S. nodiflora*. In this case, IGS *atp*B-*rbc*L of *S. nodiflora* appeared 22 bp longer than that of *E. ruderalis* (Susanto and Dwiati 2019). Meanwhile, almost no differences within the IGS *atp*B-*rbc*L sequences of *S. nodiflora* samples collected from several places throughout Java Island were observed. Of 58 samples, only one was grouped into a different haplotype, indicating high connectivity among the species populations on the island (Susanto et al. 2018).

More transversions than transitions seemed likely to occur, which is different from what should commonly be the case. For instance, an average transition/transversion ratio of 2.22 among the three transgenic rice plants was observed. Additionally, average transition/transversion ratios of 2.16, 2.17, and 2.18 were found between the transgenic rice plants and their respective parents (Park et al. 2018). This is because transition occurs more easily than transversion, although the latter has as many as two variations in comparison to the first. Transition is the conversion of purine into purine or pyrimidine to pyrimidine, or in other words, involves a change in a nucleotide base into another of similar shape. Conversely, transversion is a more complicated process because it involves conversion of a nucleotide base into a different shape, i.e., purine into pyrimidine or vice versa.

Figure 2. Sequence alignment of IGS *trn*T(UGU)-*trn*L(UAA) within *Eleutheranthera ruderalis* (Sw.) Sch.-Bip. samples

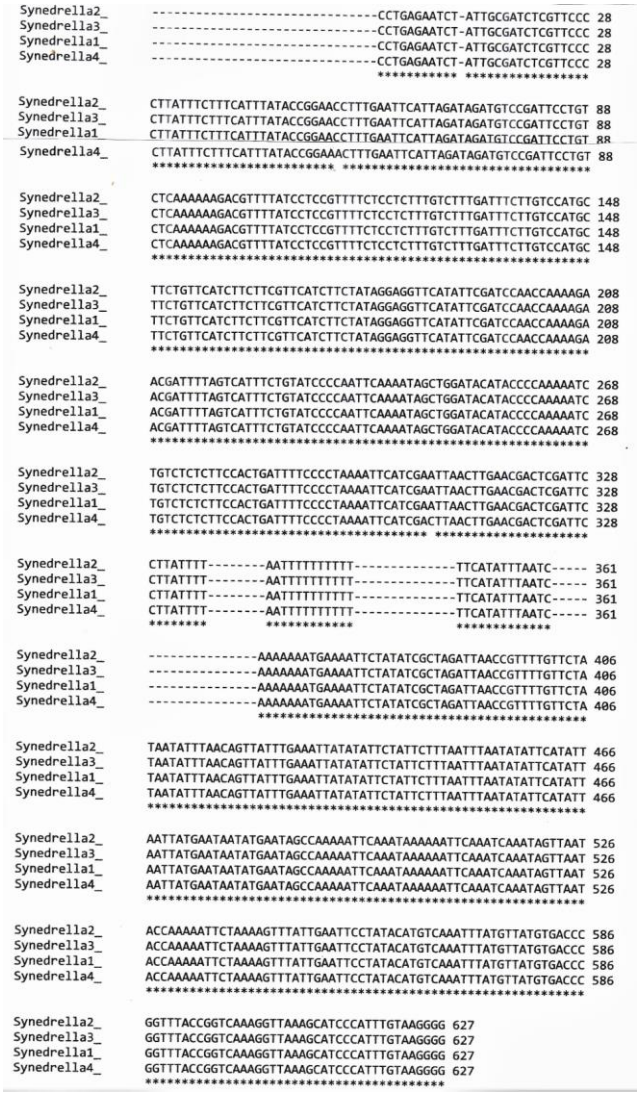


Figure 3. Sequence alignment of IGS *trn*T(UGU)-*trn*L(UAA) within *Synedrella nodiflora* **(**L.) Gaertn samples

Phylogenetic relationships of *S. nodiflora*, *E. ruderalis*, and some other species of Asteraceae from NCBI GenBank are shown in Figure 5. *S. nodiflora* and *E. ruderalis* are genetically very close, with a 93% bootstrap value, while the other species of Asteraceae are relatively more remote from these two species, which supports the fact that *S. nodiflora* and *E. ruderalis* are phenotypically very similar to each other, though some genetic differences exist, as shown in Figure 4.

Although there is no fact proving the relationship between the difference in IGS *trn*T-*trn*L sequences and the dissimilarity in apparent phenotypes of *S. nodiflora* and *E. ruderalis*, a coincidence can at least be considered. This cpDNA marker has been widely used to identify various plant species. Even boreal forest roots in Canada, for example, have been successfully identified by the use of IGS *trn*T-*trn*L, along with two other cpDNA markers, revealing as many as 194 plant species (Metzler 2018). Meanwhile, IGS *trn*T-*trn*L has been one of five cpDNA markers recommended for use in phylogenetic studies and the identification of *Dendrobium* orchids (Zhitao et al. 2017).

Figure 4. Sequence alignment of IGS *trn*T(UGU)-*trn*L(UAA) between *Synedrella nodiflora* (L.) Gaertn and *Eleutheranthera ruderalis* (Sw.) Sch.-Bip. samples

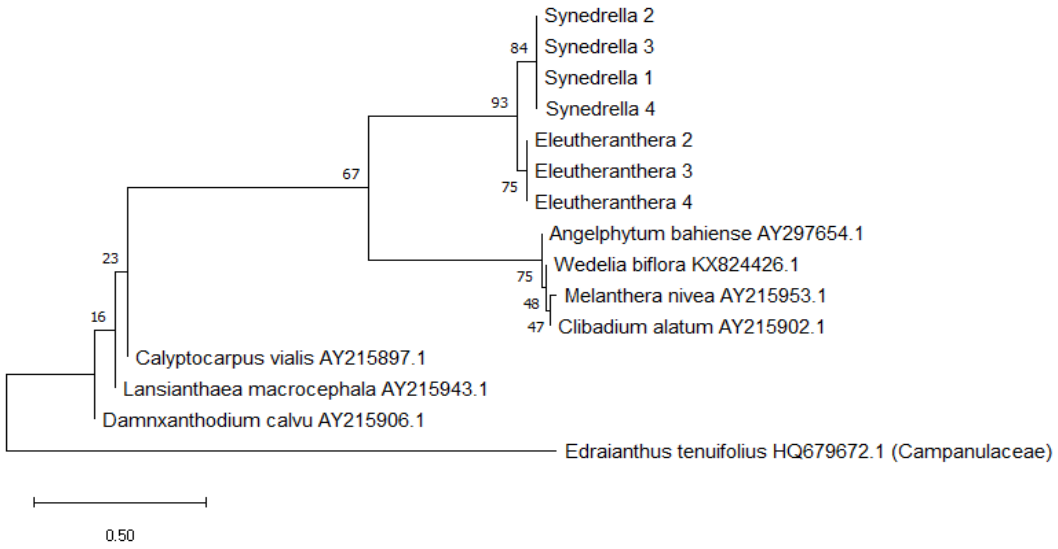


Figure 5. Phylogram using the bootstrapped neighbor-joining method with 1,000 replicates based on 600 bp IGS *trn*T-*trn*L of *Synedrella nodiflora*, *Eleutheranthera ruderalis*, some other species of Asteraceae (GenBank), and a Campanulaceae species (GenBank) as the outgroup

Other cpDNA intergenic spacers have also been used in the identification of numerous plant species. Several species of *Gluta* (Anacardiaceae) have been identified by the use of IGS *trn*L-*trn*F in combination with a cpDNA intron (Roslim and Herman 2017). Two morphologically similar genera of Myrtaceae, i.e., *Syzygium* and *Eugenia*, have been molecularly distinguished by the use of IGS *atp*B-*rbc*L. In addition, the latter was also used to establish the taxonomic status of a formerly confusing species, i.e., *Eugenia boerlagei* Merr., which was then changed to *Syzygium* rather than *Eugenia*. Consequently, the scientific name should also be changed to *Syzygium boerlagei*. However, this replacement was not based on the size of IGS *atp*B-*rbc*L but rather on the GC contents of the marker (Widodo et al. 2019).

The GC contents of IGS *trn*T-*trn*L in *S. nodiflora* and *E. ruderalis* were approximately the same, i.e., 29% and 28%, respectively. Similarly, relatively equal GC contents of IGS *atp*B-*rbc*L in both species were also reported (Susanto and Dwiati 2019). It seems likely that GC content does not relate to the existence of species in a particular environmental condition since *S. nodiflora* and *E. ruderalis* have been found in the same sites of *Stachytarpheta jamaicensis* (Verbenaceae) habitats, with important value indices (IVIs) of 12.80 and 5.57, respectively (Solikin 2019). Nevertheless, a significant correlation between GC content and longitude was reported in *Cyanus*, another Asteraceae genus, where plants growing in west areas showed higher GC contents than those in the east. Moreover, plants growing in the coldest areas with low minimum temperatures tend to have higher GC contents in their genomes (Olsavska et al. 2012).

Overall, cpDNA has been a source of molecular markers commonly used in studies of genetic diversity and the relationship of many plant species populations. Three pairs of primers, i.e., rp11671, NTCP21, and NTCP22, have been employed to amplify several cpDNA regions in sago palm (*Metroxylon sagu* Rottb.) from some populations in Indonesia. Both genetic diversity within populations and genetic differences among populations within islands were observed. Nevertheless, no genetic difference among islands occurred (Abbas et al. 2010). The presence and distribution of SSRs in cpDNA have also been used to study the genetic diversity of olive *(Olea)* species. This in silico study provided a scientific foundation for phylogenetics, evolutionary genetics, and genetic diversity studies on various olive species (Filiz and Koc 2012).

In the case of Asteraceae, cpDNA is generally relatively conserved in terms of gene content, but not in gene structure or tRNA abundance. Therefore, cpDNA is a suitable source of molecular markers to study the evolutionary relationship between species of Asteraceae (Wang et al. 2015). In this study, we obtained some indels and base substitutions distinguishing between *S. nodiflora* and *E. ruderalis.* Despite the lack of evidence of a direct relationship between the molecular and phenotypical differences, this finding reveals some coincidence between the two. In other words, the molecular evidence obtained from this study can show genetic differences between these two phenotypically similar plant species.

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