Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Unraveling the secrets of *Eclipta alba* (L.) Hassk.: a comprehensive study of morpho-anatomy and DNA barcoding

Desvendando os segredos de *Eclipta alba* (L.) Hassk.: um estudo abrangente de morfoanatomia e código de barras de DNA

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Abstract

Safety regarding herbal products is very necessary; therefore, routine identification of raw materials should be performed to ensure that the raw materials used in pharmaceutical products are suitable for their intended use. In order for the identification-related data obtained to be accurate, the identification of various kinds of markers is also very necessary. The purpose of this study was to describe the characteristics of *Eclipta alba* (L.) Hassk. based on qualitative morpho-anatomical markers and quantitative DNA coding. The morphology of this plant has herbaceous habit with a taproot and a stem with branches that appear from the middle. Leaves are single type imperfectly arranged oppositely, lanceolatus, finely serrated on the edges, tapered at the base, pointed at the end, and have a pinnate and hairy leaf surface. The flowers consist of ray flowers and tube flowers with a cup shape. Meanwhile, in terms of anatomy, *E. alba* has aerenchyma, which are scattered in the cortex of the root and stem. In addition, there are anisocytic stomata, glandular trichomes, and non-glandural trichomes with an elongated shape accompanied by ornamentation found on the leaf epidermis. The results of sequence alignment and phylogenetic tree reconstruction show that the sample plants are closely related to species in the genus Eclipta.

Keywords: *Eclipta alba*, DNA barcode, morpho-anatomy, *matK*, *rbcL*.

Resumo

A segurança em relação aos produtos fitoterápicos é um fator de extrema importância, assim, a identificação rotineira dos materiais deve ser realizada para garantir que as matérias-primas utilizadas nos produtos farmacêuticos sejam adequadas ao uso pretendido. Para que os dados relacionados à identificação obtidos sejam precisos, a identificação de vários categorias de marcadores também é necessária. O objetivo deste estudo foi descrever as características de *Eclipta alba* (L.) Hassk. com base em marcadores morfoanatômicos qualitativos e codificação quantitativa de DNA. A morfologia desta planta tem hábito herbáceo com raiz principal e caule com ramos que surgem a partir do meio. As folhas são de tipo único, dispostas de maneira imperfeita e oposta, lanceoladas, finamente serrilhadas nas bordas, afiladas na base, pontiagudas nas extremidades e possuem superfície foliar pinada e peluda. As flores consistem em flores raiadas e flores tubulares em formato de taça. Enquanto isso, em relação à anatomia, *E. alba* possui aerênquimas, que estão espalhados no córtex da raiz e do caule. Além disso, existem estômatos anisocíticos, tricomas glandulares e tricomas não glandulares de formato alongado acompanhados de ornamentação encontrada na epiderme das folhas. Os resultados do alinhamento de sequências e da reconstrução filogenética da árvore mostram que as plantas amostrais estão intimamente relacionadas com espécies do gênero Eclipta.

Palavras-chave: Eclipta alba, código de barras de DNA, morfoanatomia, matK, rbcL.

1. Introduction

In the modern era, herbal medicine is starting to be forgotten because people are generally more familiar with several medicines that are practical and easy to obtain. Even though there are many herbal medicines or traditional ingredients around them, it is very easy to process them (Kurniadi and Ahmad, 2015). Herbal medicine has advantages over modern chemical-based drugs, including being safer (Sari, 2006) and boosting the

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Received: April 28, 2023 – Accepted: July 21, 2023

body's immune system (Nurmalina, 2012). In today's era, the use of herbal products and the global market for herbal product have also started and continue to grow. One of the various standards that must be met in the phytopharma industry is the selection of the right raw botanical source before the production process is carried out.

Routine testing or identification of raw materials should be performed to ensure that the raw materials used in pharmaceutical products are suitable for their intended use. This is because many medicinal plants have similar macro-structural morphologies among species within the same genus. Adulterated, counterfeit, and substitute products pose serious safety issues (Osathanunkul et al., 2018). On the other hand, the emergence of new phenotypes experienced by organisms can arise due to stimuli from the environment (Xue et al., 2019). On the basis of this statement, depending solely on morphological inspection and vernacular names may result in mislabeled botanical specimens (De Boer et al., 2014). Therefore, it is very important to identify the botanical species accurately for the safety authentication of herbal plant products.

One of the herbal plants that has been used for a long time is *Eclipta alba* (L.) Hassk., used in the cultures of the Indian subcontinent (Jahan et al., 2014), Thailand (Tewtrakul et al., 2007), Egypt (Boulos et al., 1984), the Philippines (Dan and Nhu, 1990), etc. Compound extracts from the *E. alba* plant have various medical benefits, including: the antidiabetic effect of ethanolic extract has been investigated for possible beneficial effects against hyperglycemia and diabetic nephropathy (Jahan et al., 2014); treating liver diseases, eye ailments, and hair-related disorders (Yadav et al., 2017). However, to maximize the utilization of *E. alpha*, there must be proper morphological and anatomical descriptions for the labeling of herbal plant species. According to Sharma et al. (2017), in order to ensure the use of only genuine and uniform material for such herbal drugs, work on plant identifying features assumes vital significance.

The use of plant organs as identification keys is indeed common, but it can cause concern considering that morpho-anatomical features are strongly influenced by the environment. A molecular approach, such as DNA barcoding, develops into conclusive authentication and taxonomic assignment for phytopreparation quality assurance (Ulrich-Merzenich et al., 2007; Patwardhan et al., 2014). DNA barcoding is a tool for rapid species identification based on DNA sequence, which compares the entire genome structure and expression between two specimens, so this method offers new answers to questions that were previously outside the reach of traditional scientific disciplines (Kress and Erickson, 2008). For land plants, the most commonly recommended core DNA barcoding markers are the two coding regions within the chloroplast, parts of the gene *rbcL* (the large subunit of the rubisco enzyme gene) and *matK* (the chloroplast gene encoding maturase protein) (de Vere et al., 2015). Nevertheless, the limited data provide molecular markers, especially for *E. alba* subjected to *rbcL* and *matK* barcodes.

Several previous studies that described the identifying features of *E. alba* explained the characteristics of these plants in general, such as the morphological study by Kumari et al. (2021), who describe *E. alba* as reaching 30 –50 cm in height with a sessile single leaf with a lanceolatus shape which has a length of 4 –10 cm and a width of 0.8 – 2 cm. The floral heads are solitary and white, and their diameter is 6 – 8 mm. Previous molecular research conducted by Kim et al. (2017) managed to analyze the chloroplast genome of *E. alba* and its closest relative (*Eclipta prostrata*), and gene annotation revealed 80 protein-coding genes, 30 tRNA genes, and four rRNA genes. The results of the study by Kim et al. (2017) also revealed a low diversity of nucleotide sequences in *E. alba* and *E. prostrata*.

To provide comprehensive data for the authentication of *E. alba* in pharmaceutical applications and to enhance previous taxonomic research across multiple disciplines, a qualitative and quantitative analysis will be conducted on E. alba specimens from Taman Husada Graha Famili. This analysis will encapsulate the examination of morpho-anatomical characteristics as well as DNA barcoding. It is hoped that the data obtained from this study can be used as a reference for making comparisons with other species so that errors in the authentication of medicinal plants can be kept to a minimum.

2. Materials and Methods

2.1. Plant preparation

*Eclipta alb*a was collected from Taman Husada Graha Famili, Surabaya. The plant was authenticated in the Purwodadi Botanical Garden Herbarium, Indonesia Science Institute, and the voucher specimen was deposited in the Laboratory of Plant Systematics, Department of Biology, Faculty of Science and Technology, Universitas Airlangga (Vourcher no. EA03102021).

2.2. Morpho-anatomy slide preparation and observation

Morpho-anatomical markers were characterized descriptively with morphological characters using all organs and anatomical characters using vegetative organs. Anatomical preparations were made using the paraffin method (Purnobasuki et al., 2017), in which each vegetative organ gets a different coloring. Safranin staining for root and stem and fast green staining for leaf slides (Purnamasari et al., 2016). The paraffin method consists of several steps: collecting plants from the field, washing, fixing, dehydrating, dealcoholizing, infiltrating, blocking in pure paraffin, slicing, gluing, staining, and covering (Santos et al., 2016; Susetyarini et al., 2020).

2.3. DNA barcode analysis

Three DNA samples from *Eclipta alba* leaves were extracted using the Plant DNA Genomic Kit, following the manufacturer's steps. The extracted DNA samples were then amplified using a pair of primers specifically for the Asteraceae family (*rbcL*: Forward 5'

AAGTTCCTCCACCGAACTGTAG 3'; Reverse 5' TACTGCGG GTACATGCGAAG 3' and *matK*: Forward 5' TGGTTCAGGCT CTTCGCTATTG 3'; Reverse 5' CTGATAAATCGGCCCAAATCGC 3'), this primer was previously has been successfully used from several studies such as *Sonchus arvensis* (Wahyuni et al., 2019), *Achillea millefolium* (Ilham et al., 2022), *Cosmos caudatus* (Purnobasuki et al., 2022), and *Pluchea indica* (Wahyuni et al., 2022).

The PCR materials were mixed into a container with a total volume of 35 µL consisting of: 17.5 µL GoTaq® Green Master Mix, 1.5 µL of each forward and reverse primer with a concentration of 350 – 500 nM, 50 ng of DNA template (5 µL), and 9.5 µL of nuclease-free water. The amplification using PCR was arranged into 5 stages, including one cycle of the denaturation stage at 94°C for 5 minutes, 35 cycles with 3 different stages (denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds), and one cycle of final extension taking place at 72°C for 5 minutes. Confirmation of successful amplification was verified with 1% agarose (Promega, USA) before sending the amplicons to the Magrogen Inc. (Korea) for sequencing.

Forward and reverse sequences were processed using several software programs such as Bioedit, Mega X, and Geneious 2021.0.3, to produce mature consensus data and phylogenetic trees. The sequences were then aligned to the GenBank nucleotide database using NCBI's MEGABLAST (Morgulis et al., 2008), applying default parameters such as score, percentage identity, e-value, and query cover. The phylogenetic tree reconstruction was built using the neighbor-joining tree model with 1000 bootstrap replications in Mega X software using sample plants and several plants from the NCBI GenBank database (Table 1). The phylogenetic tree has two scenarios: the *rbcL* tree and the *matK* tree. To determine the sequences used, an alignment process was carried out to compare sequence similarities from various organisms. After that, the sequence that has the highest percentage of similarities among all the organisms that have been compared is selected. Thus, the sequence with the highest level of identity was chosen to be analyzed and used in this study.

3. Results and Discussion

3.1. Morpho-anatomy characterization

Eclipta alba is an annual herbaceous plant (Udayashankar et al., 2019; Umesh and Kumudhavalli, 2020) (Figures 1A and 1B). In general, this plant lives in tropical areas and can be found in marshy soils, roadsides, and waste places. *E. alba* has a bright brown or grayish cylindrical taproot that is pointed at the end and has fine hairs on its surface (Figure 1C). Leaves of *E. alba* are single-type, imperfectly attached to the stem, only have lammina and petiole, are arranged oppositely (there are two leaves per node along the stem and facing each other), are lanceolatus, finely serrated on the edges, tapered at the base (acuminatus), pointed at the end (acutus), and have a pinnate and hairy leaf surface (Tjitrosoepomo, 2010; Prasad et al., 2012; Paliwal, 2017; Suryabhan, 2019). The leaves of this plant are bifacial, in which the adaxial side has a darker color than the abaxial side (Figures 1D and 1E). According to Wang et al. (2008, 2011), it is generally believed that the adaxial side of the leaf plays a major role in accessing carbon from the air due to its higher photosynthetic abilities. This plant has a single stem base but many spreading branches exist in the middle until the upper part, often rooting at the lowest nodes. The stem is also purple-brown, cylindrical in shape with longitudinal ridges, and has many hairy (trichomes) patches on its surface (Figure 1F). A compound type of flower with a cup shape has eight involucral bracts with an ovate, obtuse, or acute shape (Figure 1G). The cup flower of this plant consists of two types of flowers, namely the ray flowers, or ribbon flowers, and the tube flowers or disc flowers (Figure 1I). Compound flower located in the axils of the leaves and at the end of the stem, and elongated cylindrical flower stalks (Tjitrosoepomo, 2010) (Figure 1H). Ray flowers ligulate with a small ligule; these flowers are not toothed and are arranged in a spread almost along the bractea with a white color. Similar to the morphological characteristics of the *Eclipta prostrata* flower (Krisdiyanti and Batoro, 2019), the ray flowers are female because they only have sepals, petals, and a pistil. Disc flowers

Table 1. Plant sequences downloaded from GenBank.

rbcL	***Acesssion Number***	***matK***	***Accession Number***
Eclipta prostrata	MH767494.1	*Eclipta alba*	LC565053.1
Eclipta alba	NC_039774.1	*Eclipta alba*	MF993496.1
Eclipta alba	MF993496.1	*Eclipta alba*	NC_039774.1
Eclipta prostrata	MF135472.1	*Eclipta prostrata*	AY215789.1
Eclipta prostrata	MF135376.1	*Eclipta prostrata*	HM989761.1
Eclipta prostrata	KU361242.1	*Eclipta prostrata*	KU361242.1
Eclipta prostrata	NC_030773.1	*Eclipta prostrata*	MF770218.1
Eclipta prostrata	AY215108.1	*Eclipta prostrata*	MF770220.1
Rojasianthe superba	AY215172.1	*Eclipta prostrata*	MH767803.1
Dyscritothammus mirandae	AY215105.1	*Eclipta prostrata*	NC_030773.1
Sphagneticola calendulacea	NC_039346.1	*Sphagneticola calendulacea*	KY828438.1
Rudbeckia hirta	MN601467.1	*Sphagneticola calendulacea*	NC_039346.1

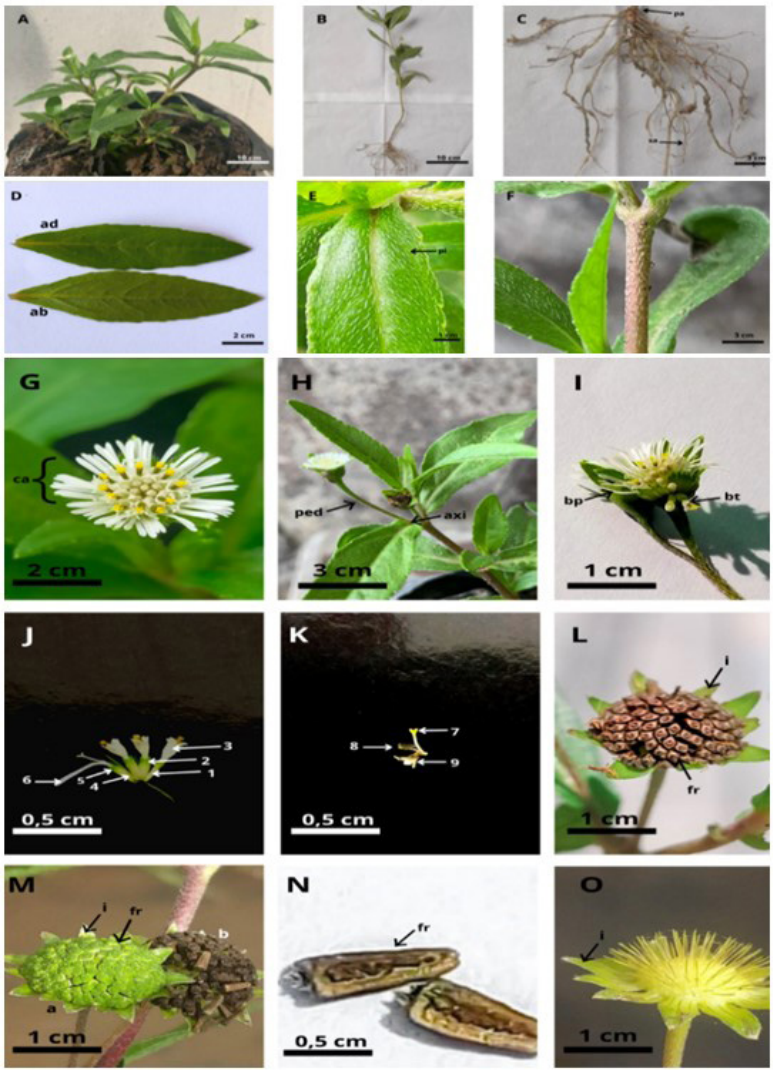

Figure 1. Morphological of all parts of the *Eclipta alba* (L.) Hassk. plant. A. Habits in a pot; B. Habits outside the pot; C. Tap root, pa: root base, sa: root fibers; D. Leaf view adaxial (ad) and abaxial (ab); E. Leaf surface with pilosus, pi: pilosus; F. Main stem; G. Cup flower. ca: cup shape; H. Flower layout, ped: peduncle, axi: leaf axil; I. Compound flowers consisting of ribbon flower (bp) and disc flower (bt); J. Disc flower (1. Ovarium, 2. Pappus, 3. Corolla) and ribbon flower (4. Ovarium, 5. Pappus, 6. Corolla) without involucral bracts; K. Sexual organs (7. Pistillum, 8. Stamen 9. Tube flower (fertile flower); L. Old compound fruit; M. Young (a) and old compound fruit (b), i: involucrum, fr: fruit; N. Achenium; O: Receptaculum, i:involucrum.

are tubular in shape with a 4-toothed corolla, 5 stamens, and a bicarpel pistil with an inferior ovary (Suryabhan, 2019). Because tube flowers have both genitals, this type is classified as a sissy flower (Figures 1J and 1K). Fruits are compound brackets composed of barren fruits on the edges and fertile fruits on the inside or center (Figures 1L and 1M). Fertile fruits are oval in shape, tapered at one end with a black color and narrow wings; each fruit contains 1 seed (Figure 1N). Black seeds are needle-shaped, hairy, and do not contain endosperm (Tjitrosoepomo, 2010; Suryabhan, 2019; Khairullah et al., 2021).

Asteraceae flowers have complexity because their arrangements consist of different flowers. To make it easier to study flowers in this family, especially *E. alba*, a flower diagram is used (Figure 2). According to Figure 2, ribbon flowers of *E. alba* are flowers that have zygomorphic symmetry and have sepals that reduce to pappus, three petals that are attached to each other and the presence of 2 gynesium. The arrangement of the parts of the ribbon flowers can be written as follows: Br↑♀KpapusC(3) A0G(2). Meanwhile, the tube flowers have actinomorphic symmetry and have sepals that reduce to pappus; 4 petals and 4 androeciums attached to each other, and the presence of two gynesiums, attached to the base of the flower. The arrangement of the parts of the ribbon flowers can be written as follows: Br *☿ KpappusC(4) A(4)G(2). Morphologically, *E. alba* is close to *E. prostata*. A comparison of morphological characteristics between the

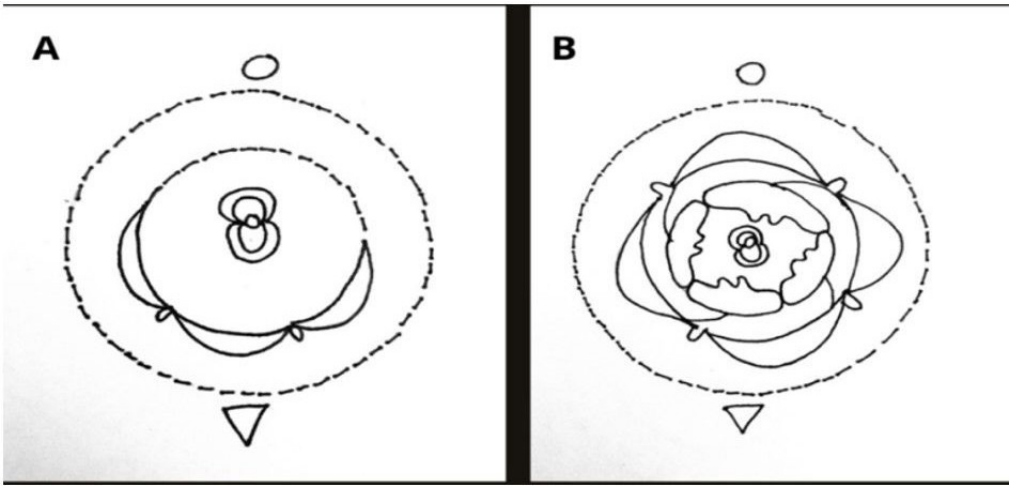

Figure 2. *Eclipta alba* (L.) Hassk. flower diagram, A. Ribbon flower; B. Disc flower.

two plants can be seen in Table 2. Based on this comparison, the difference between the two species is in the color of the stem and the base of the leaf. According to Kim et al. (2017), both species have similar morphological features, but *E. alba* has smoother leaf blade margins compared to *E. prostrata*. Because they share the same morphological characteristics and share the same habitat (Baskaran and Jayabalan, 2005; Dhaka and Kothari, 2005), *E. alba* is often mixed with *E. prostrata* (Murugananthan et al., 2009). These things can cause confusion in the academic sector as well as the market (Neeraja and Margaret, 2012). It seems that distinguishing *E. alba* and *E. prostrata* using morphological markers is very difficult because the differences are very minor.

Following the morphological description, the anatomical structure of the root, stem, and leaf of *E. alba* was observed (Figure 3). The root anatomy of *E. alba* in this study is identical to that of a similar plant studied by Rahaman and Sultana (2019); its histology is composed of epidermis, hypodermis, cortex, aerenchyma, endodermis, and stele (Figure 3A). The epidermis is composed of a single layer of cells that are tightly packed together so that there is no space between cells, and below this layer is the hypodermis. The cortical zone has the widest size among the tissues in the root, where several layers of cells are attached to each other in certain areas and other areas are not occupied by cells at all; these areas are known as air cavities (aerenchyma). Aerenchyma forms gas-conducting tubes that provide the root with oxygen under hypoxic conditions (Mühlenbock et al., 2007). The formation of cortical aerenchyma in the root is thought to occur by either lysogeny pathway; lysigenous gas-spaces are formed because of the senescence of specific cells in the primary cortex followed by their death due to autolysis (Joshi and Kumar, 2012). A different thing was found in Suryabhan's study (2019), in which little or no aerenchyma was found in the cortex tissue. The cortical region is composed of thin-walled square or rectangular cells. Furthermore, on the inside of the cortex, there is an endodermis, which consists of a single layer of parenchymatous cells that are interconnected in a cylindrical shape, separating the cortical parenchyma from the central stele, each cell having a barrel-like shape. Vascular bundles are naturally arranged collaterally, where phloem lies outside (occupying a tiny area) and xylem lies inside (occupying almost the entire area of the section). The xylem of *E. alba* is the exarch type; the protoxylem arrangement is located at the edges while the metaxylem is in the middle.

The cross section of the stem of *E. alba* bears a histological similarity to the study conducted by Khan et al. (2013) and Rahaman and Sultana (2019), with a generally circular shape that consists of the epidermis, hypodermis, cortex with aerenchyma, vascular bundles, and pith (Figures 3B and 3C). The epidermis is composed of a single layer of cells that are tightly connected with thick cell walls that do not allow space between cells. The cells that make up this tissue are quadrangular in shape. Under the epidermis, there is a hypodermis, which is the top part of the cortex. According to Peterson (1989), the hypodermis is the upper part of the cortex since it is derived from the ground meristem. This tissue is characterized by several layers of cells (3-5 layers) of collenchyma with tangential thickening; the cells are compactly arranged without intercellular cavities. The cortical tissue in the stem has several differences from the root, including that the thickness or diameter of the cortex is thinner, and the gas chambers are much smaller in size. Aerenchyma on the stem also belongs to different species, but still to one genus, such as *Eclipta prostata* (Sharma et al., 2017). In the deepest part of the cortex, there is a waxy endodermis arranged in a circle without gaps between cells, and the cells are barrel-shaped. The vascular bundles are scattered in an open collateral type, with cambium tissue separating the phloem and xylem. Phloem is composed of sieve tube elements, companion cells, and phloem parenchyma, while xylem is composed of many xylem vessels, xylem fibers, and xylem parenchyma. Pith is the tissue with the largest diameter, consisting of parenchyma cells that are hexagonal in shape.

Table 2. Morphological characteristics among genus *Eclipta*.

Morphology Comparison	*Eclipta alba* from this study	*Eclipta prostrata* (Krisdiyanti and Batoro, 2019)	*Eclipta prostrata* (Tzonev, 2007)
Root	Tap root	Tap root	Not listed
Stem			
a. Surface color	Purple-brown	green-brown	Not listed
b. Branch	✓	✓	✓
c.Trichomes (pilosus)	✓	✓	✓
d. Shape	Cylindrical	Not listed	Not listed
e. Texture	Longitudinal ridges	Not listed	Not listed
Leaf			
a. Shape	Lanceolate	Oblong-lanceolate	Oblong-lanceolate
b. Edge	Serrate	Serrate	Serrate
c. Arrangement	Opposite	Opposite	Opposite
d. Base	Acuminatus	Attenuate	Acute
e. Tip	Acute	Acute	Acute-acuminate
f.Trichomes (pilosus)	✓	✓	✓
g.Connected with stem	Imperfectly attached, sessile use petiole	Imperfectly attached, sessile use petiole	Imperfectly attached, sessile use petiole
Flower			
a.Place	Axil of the leaf and at the end of the stem	Not listed	Axil of the leaf and at the end of the stem
b.Involucral bracts	Eight involucral bracts with an ovate, obtuse or acute shape	7-8 involucral bracts	Involucral bracts herbaceous, ovate, acute, appressed-pubescent
c. Disc flower	Perfect, 4 corollas are white with a tubular shape; 4 stamens fused together; and the stigma is branched in a bifid fashion	Perfect, 4 corollas are white with a tubular shape; 4 stamens fused together; and the stigma is branched in a bifid fashion	Perfect, not explained in detail
d. Ribbon flower	Pistillate, white ray, ligule	Pistillate, white ray, ligule	Pistillate, white ray, ligule

The transverse section of the leaf through the midrib region showed several histological features similar to those of Prasad et al. (2012), which consist of epidermal tissue with several accessory organs, mesophyll tissue, and tissues in the midrib area (Figure 3D). From the outermost tissue, the upper epidermis and lower epidermis are made of a single layer of tightly packed cells with a rectangular cell wall. On both sides of the epidermis are also found several types of accessory organs, such as trichomes and stomata. These two accessory organs experience changes in quality and quantity when the environment changes, as reported by Rai and Agrawal (2020), whose observation of leaf surfaces showed an increase in stomatal and trichome densities, suggesting the adaptive resilience of the plants against UV-B. The mesophyll area is composed of palisade tissue and spongy tissue. Palisade tissue is formed from a single layer of cells that are radially elongated and compact, so that the spaces between cells are very sparse. The palisade tissue is arranged lengthwise under the upper epidermis but terminates at the midrib area. Spongy tissue is made up of parenchymatous cells that are round or oval and loosely arranged, which causes intercellular spaces to be frequently present. The midrib area consists of various tissues, such as 2-3 layers of tightly packed cells composing collenchyma, which is located just below the epidermis, and vascular tissue, which is in the middle area. The xylem is on the adaxial side, while the phloem is on the abaxial side.

There are two variations of trichomes in *E. alba*, namely glandular trichomes and non-glandular trichomes. Trichomes have an important role, especially for the plant itself, pharmacy, and taxonomy. The abundance of trichome types may aid in taxonomic studies of genera, species, and plant families (Metcalfe and Chalk, 1950; Carpenter, 1999; Krak and Mraz, 2008; Hayat et al., 2009). Trichomes have a structure and vary from species to species, so their characteristics can be used for classification purposes (Perveen et al., 2016). The non-glandular trichomes of *E. alba* have anatomical similarities to *Eclipta prostrata* (Perveen et al., 2016; Rusli et al., 2019; Shafira and Salamah, 2020), which are uniseriate and multicellular with a rounded base and tapered top and have an ornament unique

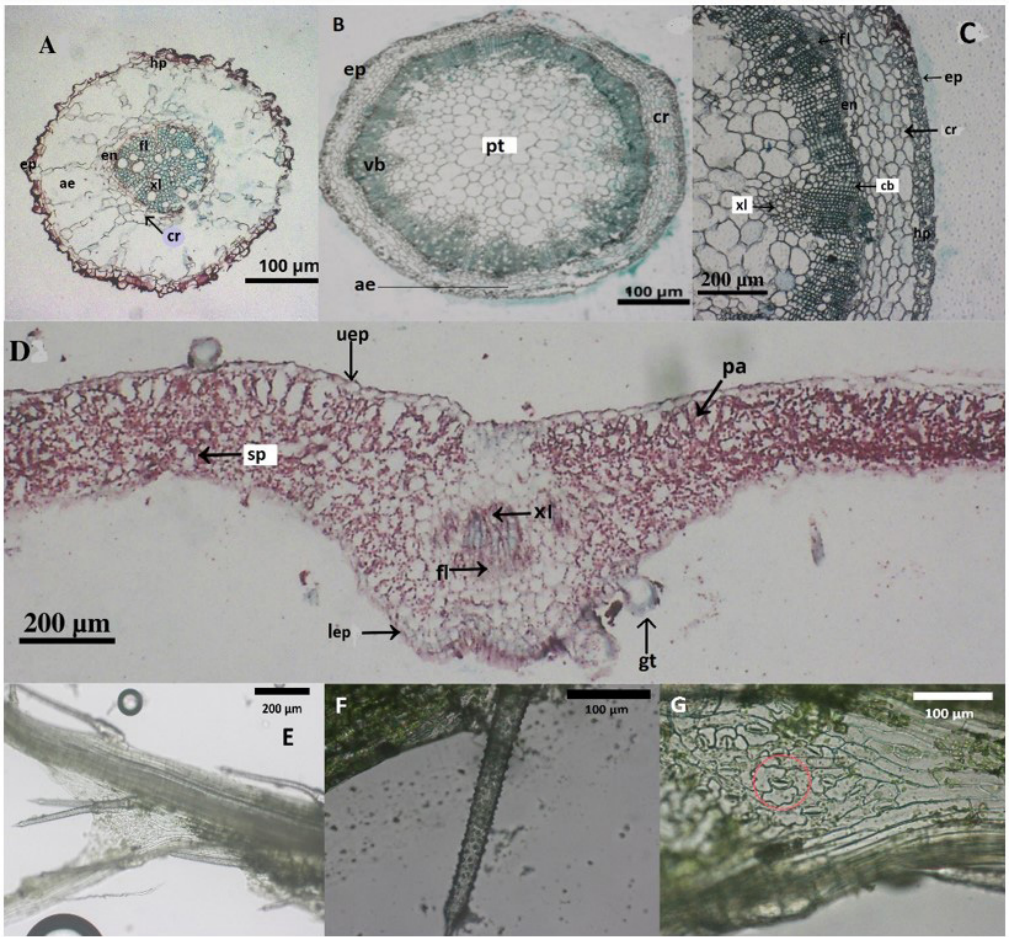

Figure 3. Anatomical characters of *Eclipta alba* (L.) Hassk. A. Root; B-C. Stem; D. Leaf.; E-F. Non-glandular trichomes; G. Stomata. Ep: epidermis, hp: hypodermis, en: endodermis; fl: floem, xl: xylem, cr: cortex, ae: aerenchyma, vb: vascular bundle, pt: pith, cb: cambium, uep: upper epidermis, lep: lower epidermis, pa: palisade, sp: spongy tissue, gt: glandular trichome.

along the surface of the trichomes (Figures 3E and 3F). According to Rasyid et al. (2022), the non-glandular trichomes of *E. alba* are simple multicellular types that have more than two cells with echinate ornamentation and a pointed end on the apex. Meanwhile, the glandular trichomes from the studied plants bear similarity to similar plants studied by Prasad et al. (2012) and *Eclipta prostata* (Sharma et al., 2017) (Figure 3D). Glandular trichomes have an important role because they are considered natural producers of essential oils and the secretion of these trichomes produces a pleasant-smelling product that has a variety of important potentials, including as a crucial medical compound (Perveen et al., 2016). The stomata of this plant are of the anisocytic type (Figure 3G) (Prasad et al., 2012; Khan et al., 2013; Rahaman and Sultana, 2019); this type is also found in *E. prostata* (Rusli et al., 2019). This type of stomata is surrounded by three subsidiary epidermal cells, of which one is markedly smaller than the others. If we review Table 3 by comparing *E. alba* with other species in the same genus, such as *E. prostrata*, the histological differences appear not to be too significant. Both stem and leaf histology have the same characteristics.

3.2. DNA barcoding

3.2.1. Visualization of electrophoresis results

A visualization of *Eclipta alba* DNA using agarose gel can be seen in Figure 4. The amplification results show thick, white bands that glow clearly without any smear phenomenon. Smear occurs due to RNA, protein, or other contaminants, which are then carried away in the electrophoresis process (Aulia et al., 2021). According to Ilham et al. (2022), DNA bands that are thick and single or clumped (not spread out) indicate high concentrations and the total extracted DNA is intact, while DNA bands that appear to be spread out indicate bonds between DNA molecules that were broken during the extraction process, so that the DNA genome is cut into smaller pieces. In the *rbcL* gene, it appears that the DNA bands have a size of approximately 500 bp, while the bands based on the *matK* gene have a slightly larger size, 750 bp. The size of the *rbcL* and *matK* gene bands in *E. alba* is the same size as that of the following plants: *Achillea millefolium* (Ilham et al., 2022) and *Cosmos caudatus* (Purnobasuki et al., 2022).

Table 3. Anatomical characteristics among genus *Eclipta*.

Anatomy comparison	*Eclipta alba*	*Eclipta prostrata* (Sharma et al., 2017)	*Eclipta prostrata* (Rusli et al., 2019)
Stem			
a. Epidermis	Uniseriate	Uniseriate	Not listed
b. Cortex	Contains aerenchyma	Contains Aerenchyma	Not listed
c. Vascular bundle	Open collateral	Open collateral	Not listed
d . Pith	consist of large, thin-walled, rounded cells	Consists of large, thin-walled, rounded cells	Not listed
Leaf			
a.Epidermis	Uniseriate	Uniseriate	Not listed
b. Trichomes	Contains glandular and non-glandular trichomes of the same type and shape	Contains glandular and non-glandular trichomes of the same type and shape	Contains glandular and non-glandular trichomes of the same type and shape
c. Stomata	Anomocytic	Not listed	Anomocytic
c. Palisade tissue	Single layered, compact, radially elongated, and terminates at the midrib area	Single layered, compact, radially elongated, and terminates at the midrib area	Not listed
d. Spongy tissue	round or oval in shape and loosely arranged	round or oval in shape and loosely arranged	Not listed

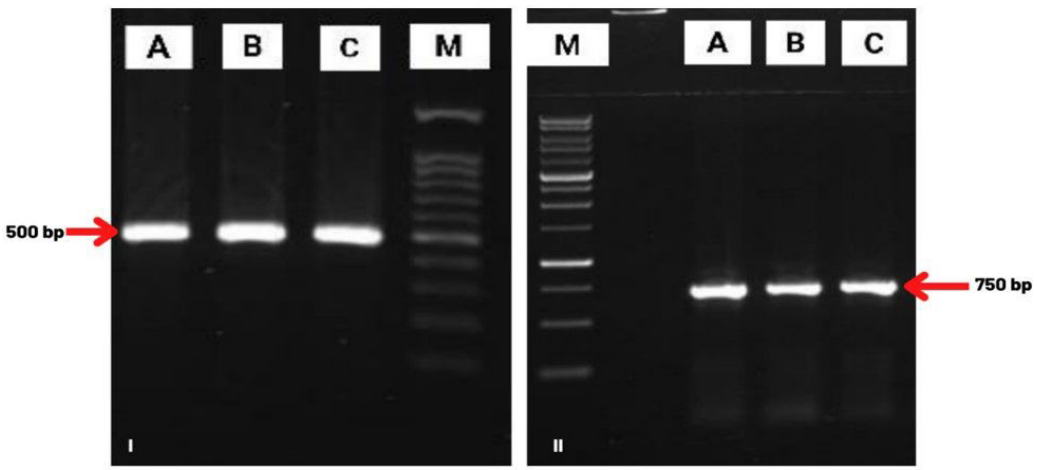

Figure 4. The results of visualization of *Eclipta alba* (L.) Hassk. DNA based on markers (I) *rbcL* gene (500bp) and (II) *matK* gene (750 bp).

3.2.2. DNA sequencing and alignment

The sequences of the three *Eclipta alba* samples based on the *rbcL* gene can be seen in Supplementary Table S1 while the three sequences based on the *matK* gene can be seen in Supplentary Table S2. The *rbcL* gene sequence of three samples of *E. alba* is composed of 29.4–29.6% adenine, 19.2–19.3% guanine, 25.6–25.8% thymine, and 24.5–25.4 cytosine. The first sample *rbcL* sequence has a sequence length of 477 bp while the second sequence is 483 bp, and the third has a size of 480 bp. Meanwhile, the *matK* gene sequences consist of 27.5–27.8% adenine, 17.0–17.3% guanine, 37.1–37.5% thymine, and 17.7–18.0% cytosine. Each *matK* gene sequence has a size of 707 bp (sample 1), 708 bp (sample 2), and 699 bp (sample 3). Adenine and thymine seem to dominate the overall percentage of nitrogenous bases that make up the *rbcL* and *matK* genes, this is quite natural because these two nitrogen bases are the largest constituents of the mitochondrial and chloroplast genomes (Smith et al., 2011). These sequenced numbers have a range that is not much different from Suparman's (2013) statement that the length measures of the *rbcL* and *matK* genes range from 500–1400 bp, so they can provide many characters for phylogenetic studies.

The results of the alignment of *E. alba* sequences from Taman Husada Graha Famili using both the *rbcL* and *matK* gene markers through the BLAST program can be seen in Supplementary Table S3. The alignment based on the *rbcL* marker shows that the sample plants have the highest

kinship with *Eclipta prostrata* MH767494.1 than with plants of the same species (*Eclipta alba* NC_039774.1). It seems that the *rbcL* marker has not been able to distinguish very accurately at the species level. According to Bangol et al. (2014), the *rbcL* gene has an advantage in terms of ease of amplification, but on the other hand, this gene has a low resolution to be able to distinguish several closely related species. Determining the kinship between sequences aligned using the BLAST program can use five indicators: namely score, max score, e-value, query cover, and percentage identity. The higher the quantity of the five indicators, the higher the genetic kinship shown. All sequences from GenBank that have been aligned with the sample plants show a percentage identity value above 98%. According to Perwitasari et al. (2020), the higher the percentage identity obtained, the higher the level of homology between the two sequences. High homology indicates low genetic variation among the species. The estimated value (E-value) is zero (0.0), indicating the alignment of all sequences is significant.

A little different thing is shown by the *matK* gene-based alignment: species that have a high degree of similarity to the sample plants come from the same species. These data have shown that the *matK* gene marker can genetically discriminate up to the species level with fairly accurate results. The *matK* gene has high difficulty in amplifying but can provide higher resolution in comparing plant species (Bangol et al., 2014). The highest genetic closeness was achieved by *E. alba* accession number NC_039774.1 with a percentage identity of 99.86%, an e-value of 0.0, and a query cover of 100%. The accuracy of the *matK* gene marker is due to several reasons. According to Barthet and Hilu (2007), in the case of a protein-coding gene, *matK* has an unusual evolutionary mode and tempo, including relatively high substitution rates at both the nucleotide and amino acid levels. The *matK* gene has ideal size, a high rate of substitution, a large proportion of variation at the nucleic acid level at the first and second codon positions, a low transition/transversion ratio, and the presence of mutationally conserved sectors. These features of the *matK* gene are exploited to resolve family and species-level relationships (Selvaraj et al., 2008).

In addition, various alignment methods were employed to explore the similarities between sequences, including comparisons of sequence homology and genetic variation, both within and between species (Misener and Krawetz, 2000; Meshoul et al., 2005). The results can be seen in Supplementary Figures S1 and S2. Based on Supplementary Figure S1, comparison between samples based on the *rbcL* gene marker shows the presence of nucleotide variations. In sample number one, there are variations at nucleotide numbers 747, 816, 836, 982, 1107, 1128, 1140, 1170, and 1185. In some of these numbers, the nucleotide symbols do not use the nucleotide symbols in general, such as A, G, C, and T; otherwise, they use other symbols such as S in nucleotide numbers 836, 1107, and 1140; and K in numbers 982, and 1128.

The nucleotide symbol K denotes guanine or thymine, while the nucleotide S refers to guanine or cytosine. In sample number two, the genetic variation is found at nucleotide number 1174 with the symbol S. The existence of nitrogen bases in DNA sequences with symbols other than A, G, T, and C is generally known as the phenomenon of nitrogen base degeneration. According to Choi et al. (2019), degenerate bases are combinations of the four DNA bases that can be inserted at any base site within a sequence. Degenerate bases are located in the DNA sequence when nucleotides are mixed at a specific position in the DNA sequence. For example, in the sequence 'AWC', 'W' indicates a combination of A and T; thus, two types of nucleotide variants exist in the pool of molecules: 'AAC' and 'ATC'. Meanwhile, according to Forsdyke (2017), the genetic code is degenerate (redundant), and any one of a set of "synonymous" three-base codons can encode the same amino acid. In the first *Eclipta alba* sample, nitrogen base degeneration was found in the ASC, KCC, GTS, CCK, and GGS sequences, namely:

- ASC produces two variant sequences: AGC (sherine) and ACC (threonine)
- KCC produces two sequence variants: GCC (alanine) and TCC (serine)
- GTS produces two sequence variants: GTG (valine) and GTC (valine)
- CCK produces two sequence variants: CCG (proline) CCT (proline)
- GGS produces two sequence variants: GGG (glycine) GGC (glycine)

Whereas a similar phenomenon was found in sample number two in the SAG sequence, which produced two nucleotide variants, namely, GAG (glutamate) and CAG (glutamine). Comparisons between sample plants with *E. alba* NC_039774.1 and *Eclipta prostrata* MH767494.1 show the same variations in nucleotide numbers 737 and 738. Meanwhile, comparing *Rudbeckia hirta* MN601467.1, there are nucleotide variations at numbers 747, 841, and 1065. Different things happened in the comparison based on the *matK* gene. Based on Figure 5, the comparison between the sample plants does not show any variation at all. *E. alba* NC_039774.1, *E. prostrata* KU361242.1, and *Sphagneticola calendulacea* NC_039346.1 display variations at nucleotide number 339. *S. calendulacea* NC_039346.1 also shows variations at nucleotide number 499.

The alignment comparison in supplementary Figures S1 and S2 provides information that the genetic variation based on the *rbcL* gene has an abundance compared to the genetic variation using the *matK* marker. This is because *rbcL* is one of the conserved chloroplast genes (a structural characteristic that is maintained) (Perwitasari et al., 2020). Although the plastid *rbcL* gene sequence is highly conserved, the enzyme encoded by this gene has played a role in plant adaptation to changing environments across geological time and continues to do so across recent geographical space (Yao et al., 2019).

The alignment result this time is like the result from the previous BLAST process. Genetic variations that appear are generally caused by mutation. Basically, mutations can occur naturally or due to external factors. Natural mutation occurs due to errors in copying DNA molecules during DNA replication, while mutation due to external factors occurs due to radiation or exposure to chemicals. Even though the plant body has a DNA repair system, t mutation events still occur, and sometimes they accumulate, and

are carried over to the next generation. These mutations will result in evolution (Toha, 2011).

Mutations that occur both between sample plants and plants from other species are classified as substitution mutations, or more precisely, transition and transversion. Mutation-based transition bias may be caused by the spontaneous deamination of cytosines to thymines (Rohs et al., 2009). Meanwhile, transversion mutations are likely generated by one or several translational polymerases that ensure the replication bypass of basic sites that failed to be corrected by the classical base excision repair pathway after excision of uracils by uracil glycosylases (Delbos et al., 2005).

3.2.3. Phylogenetic tree

The phylogenetic tree reconstruction in this study used the neighbor-joining method. According to Dharmayanti (2011), the neighbor-joining tree method selects sequences that, when combined, will provide the best estimate of the length of the branch that closest reflects the real distance between the sequences. The phylogenetic tree was tested statistically using the bootstrap method with 1000 replications. Hall (2001) states that a bootstrap value of 100 to 1000 replications is used to estimate the confidence level of a phylogenetic tree. The reconstructed phylogenetic tree using *rbcL* and *matK* gene markers can be seen in Figures 5 and 6. Based on Figure 6, the three sample plants are not in the same cluster; this may be due to the phenomenon of degeneration of nitrogen bases in the *E. alba* sample, which has been described in Figure 6 previously. So, sample number 1 is more inclined to form clusters with the genera *Rojasianthe, Dyscritothammus,* and *Rudbeckia*. Samples 2 and 3 are closely related to several plants from the genus *Eclipta*, although they do

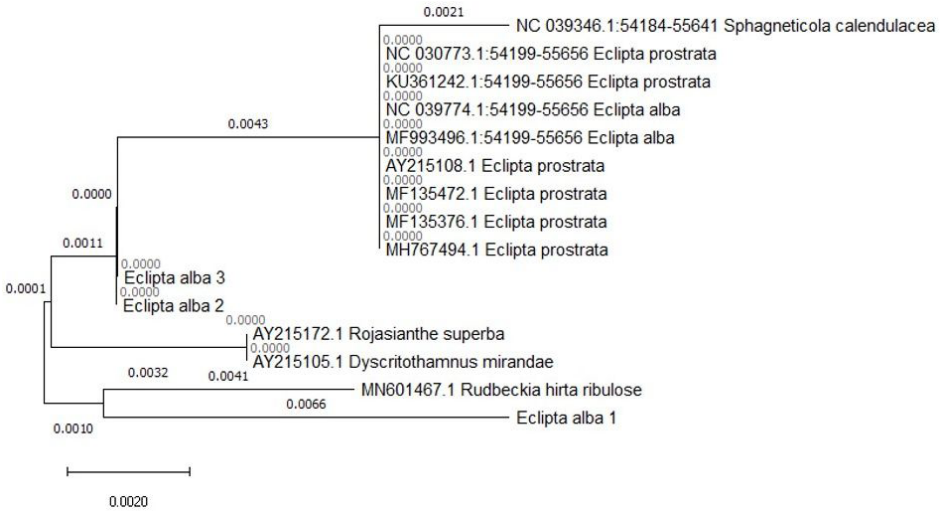

Figure 5. Phylogenetic tree based on *matK* marker.

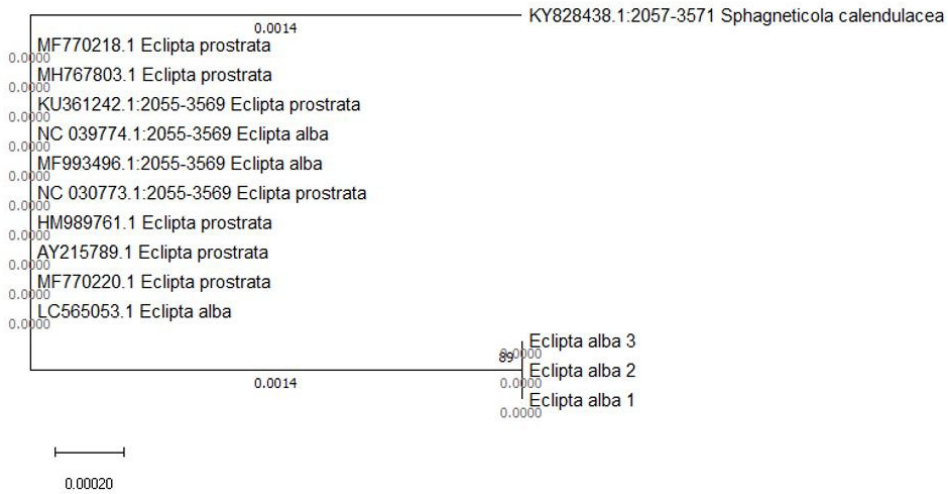

Figure 6. Phylogenetic tree based on *rbcL* marker.

not form the same cluster. The explanation for this case is still since the *rbcL* gene is still unable to differentiate at the species level (Kolondam et al., 2012). The role of the *rbcL* gene, which encodes the RuBisCO protein, is thought to cause this gene sequence to have a low mutation rate compared to other barcode genes in cpDNA, so that the level of similarity between species is quite high (Rahayu and Jannah, 2019).

Different things occur in the *matK* gene-based phylogenetic tree. From Figure 5, all sample plants are in the same cluster, together with similar species and *Eclipta prostrata*. As a coding gene, the very high rate of evolution of the *matK* gene makes this gene usable in phylogenetic reconstruction at the taxonomic level of ordo and family, and sometimes also at the taxonomic level of genus, species, and subspecies (Jing et al., 2011). Therefore, the *matK* gene is widely used in taxonomic and phylogenetic studies, both intraspecies and interspecies in angiosperms.

It can be concluded that *E. alba* has its own morphological, anatomical, and DNA barcoding characteristics. The morphology of this plant has a herbaceous habit with a taproot, a stem with branches that appear from the middle to the top, and roots that can appear at the lowest nodes. Leaves of *E. alba* are single-type, imperfectly attached to the stem; only have lamina and petiole, arranged oppositely, lanceolate, finely serrated on the edges, tapered at the base, pointed at the end, and a pinnate and hairy leaf surface. The flowers are arranged in a compound consisting of ray flowers and tube flowers with a cup shape. Meanwhile, in terms of anatomy, *E. alba* has aerenchyma, which is scattered in the cortex of the root and stem. In addition, there are anisocytic stomata, glandular trichomes, and non-glandular trichomes with an elongated shape accompanied by ornamentation found on the leaf epidermis. The results of sequence alignment and phylogenetic tree reconstruction show that the sample plants are closely related to species in the genus *Eclipta*. The anomaly in sample *rbcL* number 1 is caused by the phenomenon of nitrogen base degeneration.

Acknowledgements

The authors thank to Plant Medicinal Garden "Taman Husada Graha Famili" Surabaya, East Java Indonesia for providing sample plants. This study was financially supported by Universitas Airlangga, Indonesia 2021 Fiscal Year, following The Decree of Rector of Universitas Airlangga Number: 1089/UN3.15/PT/2022.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Comparison result between the sample sequences and the plant sequences from GenBank based on rbcL gene (Ea 1, Ea2, Ea3 : sample plants; Eax : *Eclipta alba* NC_039774.1; Ep : Eclipta prostrata MF135472.1; Rh : *Rudbeckia hirta* MN601467.1).

Figure S2. Comparison result between the sample sequences and the plant sequences from GenBank based on matK gene (Ea1, Ea2, Ea3 : sample plants; Eay : *Eclipa alba* NC_039774.1; Epy : *Eclipta prostrata* KU361242.1; Sc : *Sphagneticola calendulacea* NC_039346.1).

Table S1. Results of *Eclipta alba* DNA sequence analysis based on rbcL gene marker

Table S2. Results of *Eclipta alba* DNA sequence analysis based on matK gene marker

Table S3. Alignment results of *Eclipta alba* using BLAST

This material is available as part of the online article from https://doi.org/10.1590/1519-6984.274315

Abbreviations

DNA: Deoxyribonucleic Acid, matK: maturase-K, rbcL: ribulose-biphosphate carboxylase large subunit, CBOL: Consortium for Barcode of Life, PCR: Polymerase Chain Reaction, BLAST: Basic Local Alignment Search Tool.