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DNA BARCODING EDELWEISS (*Anaphalis longifolia*) ASAL SUMATERA UTARA MENGGUNAKAN SEKUEN GEN *maturase* K

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ABSTRACT

Anaphalis longifolia merupakan anggota dari family Asteraceae yang tersebar di dataran tinggi Eropa, Amerika, hingga Asia. Penelitian tentang tanaman ini masih terbatas pada studi habitat, sedangkan penelitian terkait identifikasi molekuler masih belum dilakukan. Penelitian ini bertujuan untuk menganalisis DNA barcode dari *A. longifolia* menggunakan sekuen *mat*K gene. Sampel yang diperoleh dari Sumatera Utara kemudian di Isolasi DNA, di amplifikasi menggunakan primer spesifik, lalu disequencing. Hasil sequencing dianalisis menggunakan program Molecular Evolution Genetics Analysis (MEGA) Version X. Hasil penelitian menunjukkan bahwa sekuen *mat*K gen berhasil diamplifikasi pada panjang 800-850 kb. Hasil analisis pohon filogenetik menunjukkan bahwa sekuen *mat*K gene dapat mengelompokkan *A. longifolia*. Pada sekuen *mat*K gene *A. longifolia*, AT content lebih tinggi dibandingkan dengan GC conten. Jarak genetik yang diperoleh berkisar 0-0.0014. Hasil analisis alignment sekuen *mat*K gene menunjukkan terdapat 1521 karakter yang dapat diamati, 1403 karakter conserved site, 118 karakter variable site, 9 karakter parsimony informative site, dan 7 karakter single nucleotide polymorphism (SNP) site. Sekuen *mat*K gene dapat digunakan sebagai DNA barcoding untuk mengidentifikasi *A. longifolia*. Hasil penelitian ini diharapkan dapat memberikan informasi penting dalam konservasi *A. longifolia*.

Keywords: *Anaphalis longifolia, mat*K, DNA barcoding, Sumatera Utara

DNA BARCODING OF EDELWEISS (*Anaphalis longifolia*) OF NORTH SUMATRAN ORIGIN USING SEQUENCE *Maturase* K GENE

ABSTRACT

Anaphalis longifolia is a member of the Asteraceae family found throughout the highlands of Europe, America and Asia. Studies on this plant is still limited to their habitat, whereas its molecular identification is largely unknown. This study aims to analyze the DNA barcode of *A. longifolia* using the *mat*K gene sequence. DNA of the samples obtained from North Sumatra were isolated and then amplified using specific primers, and eventually sequenced. The results of sequencing were analyzed using the Molecular Evolution Genetics Analysis (MEGA) program Version X. The results showed that the *mat*K gene sequence was successfully amplified at a length of 800-850 kb. The results of the phylogenetic tree analysis show that the *mat*K gene sequence is capable of classifying *A. longifolia*. In the *A. longifolia mat*K gene sequence, the AT content was

higher than GC. The genetic distance obtained from the sequencing ranges from 0-0.0014. The results of matK gene sequence alignment analysis show that there were 1521 observable characteristics, 1403 conserved site characteristics, 118 site variable characteristics, 9 parsimony informative site characteristics, and 7 single nucleotide polymorphism (SNP) site characteristics. The *matK* gene sequence can be used in DNA barcoding to identify *A. longifolia*. The results of this study are expected to provide important information in *A. longifolia* conservation attempts.

Keywords: *Anaphalis longifolia, mat*K, *DNA barcoding, North Sumatra*

Introduction

Anaphalis is a member of the Asteraceae family (Tjitrosoedirdjo, 2002) widespread across mountainous areas in the continents of Europe, America, and even Asia (Chanchani *et al.*, 2011). *Anaphalis* thrives at an altitude between 800 to 3400 asl (Backer & van den Brink, 1965). Most of this genus can be found in the highlands and mountains (Prakasa *et al.*, 2018). Due to its ability to thrive in a nutrient-poor environment, *Anaphalis* is considered to have high ecological value (Aliadi *et al.*, 1990).

From 2001 to 2019, North Sumatra has lost 23% (1.33 Mha) of its tree coverage, which is equivalent to the ability to absorb 549 Mt of CO_2 emissions. Mandailing Natal Regency is the region with the most reduction in tree coverage (147 Kha) (Global Forest Watch, 2020). Forest degradation and climate change are the main causes of the increasing difficulty of finding Anaphalis. In addition to its habitat in critical environments, *A. longifolia* is also a plant with low seed viability, making it difficult to conserve. Besides that, *A. longifolia* also has very slow growth.

Plant identification using barcoding DNA is one tool that can be used in conservation efforts. Barcoding DNA is used to identify, inventory and study specimens to understand species diversity and evaluate the genetic variability of species (Krishna Krishnamurthy & Francis, 2012). With barcoding DNA, researchers can identify species more quickly and thoroughly in order to take the appropriate action for establishing the right scale for conservation. (Francis *et al.*, 2010).

The *mat*K gene is a plant DNA barcode recommended by Barcode of Life (CboL). It is one of the fastest growing plastid encoders and consistently shows high levels of discrimination towards angiosperm species. (Fazekas *et al.*, 2008; Lahaye *et al.*, 2008a). (Li *et al.*, 2011) stated that a combination of plastid markers including rbcl, *mat*K and trnH-psbA could be used as DNA barcoding with a success rate of 87.1-92.7%. According to (Hollingsworth *et al.*, 2009), *mat*K markers can be used to identify angiosperms (90%), gymnosperms (83%), and cryptogams (10%). In Indonesia, the *mat*K marker has been used for identifying Aeridinae (Orchidaceae) subtribe. (Topik *et al.*, 2005), *Myristica fragrans* (Tallei & Kolondam, 2015), Dipterocarpaceae (Harnelly *et al.*, 2018), palm (Abbas *et al.*, 2020), and Andrographis paniculata (Arif *et al.*, 2019).

Anaphalis longifolia is a member of the genus Anaphalis (Koster, 1941). IUCN Redlist (2008) classified *Anaphalis spp* as threatened or endangered plants. Research related to *A. longifolia* is still limited to its ecological status and distribution (Taufiq *et al.*, 2013). The use of *mat*K gene DNA barcoding to identify this species has not yet been done before. This study aims to analyze the potential of the *matK* gene as barcoding DNA of *A. longifolia* plants from North Sumatra. This research is expected to provide important information on how to identify *A. longifolia* in its conservation effort in North Sumatra.

Materials and Methods

A. longifolia Samples

2 samples of A. longifolia were obtained from Taman Eden in Toba Samosir regency and Bukit Sipiso Piso in Simalungun regency, North Sumatra. A total of 8 data from NCBI were used ingroup, namely that of *Anaphalis margaritacea* (HQ594564), *Anaphalis aureopunctata* (MH659925.1), *Anaphalis aureopunctata* (MH714290.1), *Anaphalis sinica* (MH659676.1), *Anaphalis sinica* (KX148081.1), *Anaphalis margaritacea* (MG224815), *Anaphalis margaritacea* (HM445632), and *Helianthus annuus* (AY215805.1) outgroup.

DNA Extraction

Fresh leaves from A. longifolia were extracted using the Geneaid Plant DNA Isolation Kit following the kit protocol. A total of 100 mg of plant leaf tissue was crushed and then put into a 1.5 ml microcentrifuge tube and added 400 µl of lysis buffer GP1 and 5 µl of RNase A. After homogenizing and incubating at 60°C for 10 minutes, 200 l of elution buffer and 100 µl of GP2 buffer were added. The mixture was then transferred to a column filter in a 2 ml collection tube and then centrifuged at a rate of 1,000xg for 1 minute. The column filter containing

the supernatant was removed and replaced with a new column filter. The solution in the collection tube was moved to a new filter column and 150% buffer GP3 was added to the solution volume. GD Column and the collection tube were then centrifuged at a speed of 16,000 x g for 2 minutes. DNA was washed 2 times using W1 buffer and wash buffer. The DNA in the GD column was then eluted using a 100 µl elution buffer which had been heated at 60°C. The collection tube was then replaced with a microcentrifuge tube. After being centrifuged again at a speed of 16,000 x g for 30 seconds, the DNA that had entered the 1.5 ml microsentrifuge tube was then stored at -20°C.

DNA Amplification

The *mat*K sequence was amplified using matK-F 5'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3 'and matK-R 5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3' primers (Ki-Joong Kim, School of Life Sciences and Biotechnology, Korea University, Korea, unpublished). Amplification was carried out using the MyTaq HS Red Mix (Bioline) kit with a total reaction of 25 µl (2.5 µl of DNA template; 2.5 l matK-F primer; 2.5 µl of matK-R primer; 5 µl of distilled water; 12.5 µl of PCR Mix). Amplification of the *mat*K sequence was carried out at the predenaturation stage at 97°C for 5 minutes, denaturation at 94°C for 1.5 minutes, annealing at 52°C for 1 minute and extension at 72°C for 1 minute. PCR results were visualized using agarose gel. PCR products which showed clear DNA bands were to be sent to FirstBase DNA Sequencing Service in Singapore for sequencing.

Data Analysis

The sequencing results in the form of a chromatogram were edited using Bioedit 7.0.1 to obtain a consensus sequence based on the conservative sequences generated from the primary sequencing results of matK-F and matK-R. The consensus sequence that has been obtained was then aligned using Basic Local Alignment Search Tool (BAST) from the National Center for Biotechnology Information. Data with high similarity to the sample were included in the phylogenetic tree analysis. Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) X program (Kumar *et al.*, 2018). Analyzes were performed to calculate the percentage of similarity, GC content, and genetic distance.

Result and Discussion

We succeeded in amplifying the *mat*K gene sequence from the total genome of *A. Longifolia,* which would then be analyzed as DNA barcode (Figure 1). The amplification results ranged from 800-850 kb.

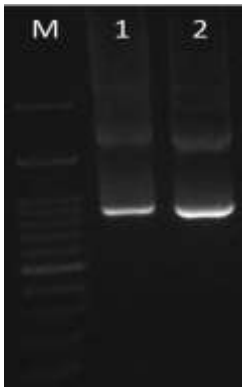

Figure 1. The results of visualization of A. longifolia *mat*K gene PCR product using 1% agarose gel with 1 kb marker

PCR products that have shown positive visualization results using agarose gel were then sequenced. After the sequencing results were analyzed using BLAST on NCBI, it was found that data with high similarity to the sample were primarily from the Asteraceae family. Species with a high level of similarity according to BLAST analysis were *Anaphalis margitacea* (99.87%), *Anaphalioides mariae* (99.87%), *Anaphalis hancockii* (99.75%), *Helichrysum felinum* (99.62%) *Anahalis aureopunctata* (99.40%), and *Anaphalis sinica* (99.15%). Phylogenetic studies show that Anaphalis is very close to Helichrysum and Pseudognaphalium (Smissen *et al.*, 2011; Ward *et al.*, 2009). The results of the BLAST analysis, which showed species variation, indicated that the *mat*K marker is not every effective when used as DNA barcoding on *A. longifolia*. Some researchers suggested a combination of the *mat*K and rbcl markers in determining DNA barcode (Saarela *et al.*, 2013; Wattoo *et al.*, 2016; Hollingsworth *et al.*, 2009; Techen *et al.*, 2014).

In a previous study using the same primer as this one, the said primer has succeeded in identifying 35% herbarium specimens and 45% fresh specimens consisting of 900 vascular plants from 51 families, 24 orders, 147 genera, and 312 species. (Kuzmina *et al.*, 2012). The use of this primer on Indonesian plants has been performed on Annonaceae (18 samples), Apocynaceae (7 samples), Dipterocarpaceae (12 samples), Lauraceae (6 samples), Meliaceae (7 samples), Moraceae (7 samples), Myristicaceae (12 samples), Phyllanthaceae (1 sample), Primulaceae (4 samples), and Rubiaceae (7 samples) plants (Dean *et al.*, 2018). Especially in Sumatra, this primer has

successfully amplified 441 species, 97 families and 40 orders of flowering plants (Amandita *et al.*, 2019).

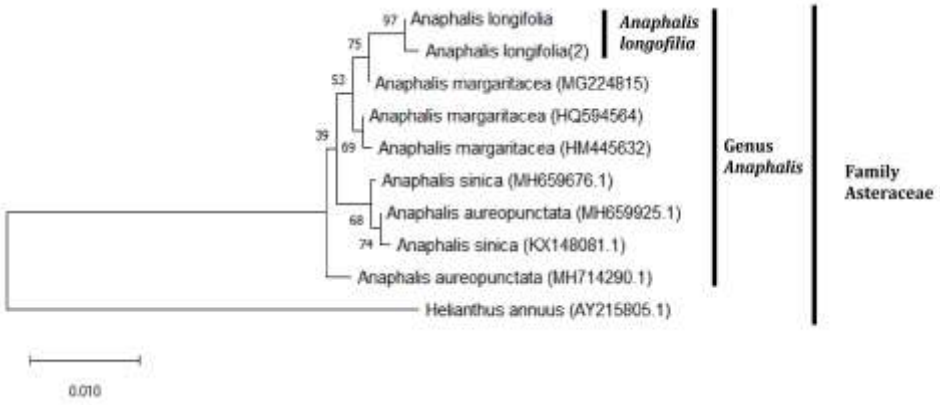

Figure 2. Phylogenetic tree based on the *mat*K gene sequence of *Anaphalis longifolia* with the *Helianthus annuus* outgroup reconstructed using the Neighbor Joining method, with the evolutionary distance calculated using the Kimura 2-parameters method (Kimura, 1980). Percentage of species-replicating trees under bootstrap test (1000 replicates)) (Felsenstein, 1985).

The phylogenetic tree in Figure 2 shows that the *mat*K gene sequence in *A. longifolia* can distinguish this species from other *Anaphalis* genus and from *Helianthus annuus* as an outgroup of the Asteraceae family. This shows that the *mat*K gene has the potential to be used as DNA barcoding for *A. longifolia*, but it is less effective when used to distinguish between species in the *anaphalis* genus.

Analysis using clustal W in the MEGA X application shows that there are 1521 characteristics that can be observed. From this data, there are 1403 conserved sites characteristics, 118 site variables characteristics, and 9 parsimony informative sites characteristics. Phylogenetic analysis using the *mat*K gene sequence has more parsimony informative sites than genes in other chloroplasts (Müller *et al.*, 2006; Barthet & Hilu, 2007).

Table 1. Composition of nucleotides, AT and GC content of the *mat*K gene sequence in *Anaphalis longifolia*

Species	T	C	A	G	Total	GC (%)	AT (%)
Anaphalis margaritacea (HQ594564)	36.34	17.41	29.48	16.77	787	34.18	65.82
Helianthus annuus (AY215805.1)	36.92	16.60	29.48	17.00	1506	33.60	66.40
Anaphalis aureopunctata (MH659925.1)	36.88	17.36	29.28	16.48	789	33.84	66.16
Anaphalis aureopunctata (MH714290.1)	36.38	17.49	29.58	16.55	852	34.04	65.96
Anaphalis sinica (MH659676.1)	37.01	17.52	28.65	16.82	862	34.34	65.66
Anaphalis sinica (KX148081.1)	37.28	16.24	30.05	16.44	1521	32.68	67.32
Anaphalis margaritacea (MG224815)	36.53	17.24	29.63	16.60	783	33.84	66.16
Anaphalis margaritacea (HM445632)	36.89	16.34	30.04	16.73	1518	33.07	66.93
Anaphalis longifolia	36.32	17.68	29.51	16.49	837	34.17	65.83
Anaphalis longifolia (2)	36.24	17.53	29.65	16.59	850	34.12	65.88
Average	36.75	16.99	29.60	16.66	1030.5	33.65	66.35

The results of the *matK* sequence analysis on *A. longifolia* show that the AT content was higher than the GC content in the Asteraceae family (Table 1). Variation in GC content is a key genome feature due to being closely related to the fundamental elements of genome organization in an organism (Eyre-Walker & Hurst, 2001; Mukhopadhyay *et al.*, 2007). Genomes rich in GC show higher gene density, higher mutation rates conservation level, and higher rates of recombination level compared to regions lacking in GC (Niu *et al.*, 2017). GC content from 65 accessions of Edelweiss (*Leontopodium*) from the Himalayan/Tibet centre using nuclear ribosomal (ITS and ETS) and plastid (*matK* and *trnL_F*) sequences ranged from 43-52% (Blöch *et al.*, 2010). *Anapalis selengensis* genome has 37.46% GC content and 62.54% AT content (Meng *et al.*, 2019).

Table 2. Genetic distance between *Anaphalis longifolia* and species from the Anaphalis genus and the Asteraceae family

No	Species	1	2	3	4	5	6	7	8	9
1	*Anaphalis longifolia*	1								
2	*Anaphalis longifolia* (2)	0.000	1							
3	*Anaphalis margaritacea* (HQ594564)	0.003	0.006	1						
4	*Anaphalis margaritacea* (MG224815)	0.001	0.001	0.000	1					
5	*Anaphalis margaritacea* (HM445632)	0.006	0.009	0.000	0.000	1				
6	*Anaphalis aureopunctata* (MH659925.1)	0.006	0.006	0.005	0.005	0.005	1			
7	*Anaphalis aureopunctata* (MH714290.1)	0.006	0.010	0.004	0.004	0.008	0.006	1		
8	*Anaphalis sinica* (MH659676.1)	0.009	0.012	0.006	0.006	0.006	0.001	0.008	1	
9	*Anaphalis sinica* (KX148081.1)	0.011	0.014	0.005	0.005	0.006	0.000	0.011	0.001	1
10	*Helianthus annuus* (AY215805.1)	0.070	0.074	0.069	0.070	0.072	0.072	0.069	0.067	0.074

The genetic distance analysis of *Anaphalis longifolia* with species from the *Anaphalis* genus and the Asteraceae family showed that the distances between species in the genus *Anaphalis* ranged between 0-0.014 (Table 2). The highest variation was found in *Anaphalis sinica* and the lowest was in *Anaphalis longifolia*. The genetic distance between the *Anaphalis* genus and *Helianthus annuus* as an outgroup ranged from 0.067 to 0.074. The genetic distance in the *Leontopodium* ITS region ranged from 0.2% to 6.8% (Blöch *et al.*, 2010). A study by Ade *et al.* (2019) who analyzed the genetic distance of Anaphalis spp (*A. javanica, A. longifolia,* and *A. viscida*) based on molecular characteristics (ITS, ETS, and EST-SSR markers) showed that the genetic distance was between 0.004 to 0.040, indicating small genetic distance between species in the *Anaphalis* genus.

Molecular-based research on *Anaphalis* has been successfully carried out using the internal transcribed spacer (ITS) marker (Glenny & Wagstaff, 1997; Blöch *et al.*, 2010) and external transcribed spacer (ETS) (Blöch *et al.*, 2010; Nie *et al.*, 2013; Ade *et al.*, 2019), EST-SSR markers (Ade *et al.*, 2019), *trnL* (Blöch *et al.*, 2010; Kurniawan *et al.*, 2014), and *mat*K (Blöch *et al.*, 2010). Molecular-based research on *Anaphalis* using the *matK* marker is still very rare. The *matK* gene is a marker that can be used as a DNA barcode and is recommended by Barcode of Life (CboL) for its consistent ability to show high discrimination level between angiosperm species) (Lahaye *et al.*, 2008a).

In this study, the results of alignment of the *matK* gene sequence from 9 data on *Anaphalis* genus indicated that there were 7 single nucleotide polymorphism (SNP) sites (Table 3), namely sites 492, 504, 505, 506, 1061, 1068, and 1176. In the *A. longifolia* species, no SNP site was detected. SNP for defined genetic location were determined in at least 1% of the population (Kim & Misra, 2007). SNP is one of the stable genetic polymorphisms in a genome and can be used to analyze differences between closely related species (Germano & Klein, 1999; Yamamoto *et al.*, 2010). *matK* and rbcl showed high sequence quality, but only provided a few SNP sites (Huang *et al.*, 2014) and therefore highly suitable for use in identifying species (Hollingsworth *et al.*, 2009).

Table 3. Single nucleotide polymorphism in the *Anaphalis* genus

Sample	Nucleotide Base Site						
	4 9 2	5 0 4	5 0 5	5 0 6	1 0 1	1 0 8	1 1 6
A. margaritacea (HQ594564)	-	A	G	A	T	C	G
A. aureopunctata (MH659925.1)	-	-	-	-	.	.	.
A. aureopunctata (MH714290.1)	A	.	.	.	A	A	.
A. sinica (MH659676.1)	C	.	.	.	.	.	T
A. sinica (KX148081.1)	C	.	.	.	.	.	.
A. margaritacea (MG224815)	-	-	-	-	.	.	.
A. margaritacea (HM445632)	C	.	.	.	.	.	.
A. longifolia	-	-	-	-	.	.	.
A. longifolia (2)	-	G	A	T	.	.	.

In this study, the *matK* gene sequence in *A. longifolia* has the potentials to be used as DNA barcode. Lahaye *et al.* (2008) proposed that *matK* could potentially become DNA barcode in plants. The *matK* gene sequence is one of the fastest growing sequences of the plastid genome (Hilu & Liang, 1997) and possibly the closest plant analogue to the COI gene sequence in animal DNA barcodes (Hollingsworth *et al.*, 2011). The *matK* gene sequence can be very difficult to amplify by PCR using existing primary sets, especially in non-angiosperm plants (Hollingsworth *et al.*, 2011).

DNA barcoding, which is widely applied in taxonomic research today, is invaluable for understanding species boundaries, community ecology, evolution and biodiversity conservation (Kress *et al.*, 2015). Currently, conservationists have adopted DNA barcodes as a tool in the field of conservation (Chakraborty *et al.*, 2014; Joly *et al.*, 2014). DNA Barcoding enables the identification species boundaries to be used as clues in determining target conservation habitats (Faith, 1992). When the DNA barcode for each species is complete, comparative measure of phylogenetic diversity will become the standard metric for assessment in determining conservation strategies (Kress *et al.*,

2015). In addition, the development of DNA barcode research is also used for the identification and detection of illegally-traded endangered species (Lahaye *et al.*, 2008b). There will be increased use of DNA barcode in the future, mainly because the available technology will become simpler and cheaper (Kress *et al.*, 2015).

Conclusion

The results showed that the *matK* gene sequence can be amplified at a length of 800-850 bp. In the *A. longifolia mat*K gene sequence, the AT content was higher than the GC content. The resulting genetic distance ranged between 0-0.0014. The alignment results of the *mat*K gene sequence showed that there are 1521 observable characteristics, including 1403 conserved site characteristics, 118 site variable characteristics, 9 parsimony informative site characteristics, and 7 single nucleotide polymorphism (SNP) site characteristics. This suggests that the *mat*K gene sequence has the potential for development as DNA barcoding to identify *A. longifolia*.

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