International Journal on Advanced Science Engineering Information Technology

Vol.9 (2019) No. 5
ISSN: 2088-5334

The Detection of *Anaphalis* spp. Genetic Diversity Based on Molecular Character (using ITS, ETS, and EST-SSR markers)

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Abstract*— *Anaphalis* is the natural vegetation component in mountainous areas and is found in volcanic soils. *Anaphalis* is one of the species which existence is currently presumed to decrease and is feared to be extinct in nature. The purpose of this study was to detect genetic diversity of *Anaphalis* spp. in Bromo Tengger Semeru National Park (BTSNP) based on molecular characters by using ITS, ETS, and EST-SSR markers in supporting the conservation aspects of *Anaphalis* genetically. This research method was carried out by exploring the existence of *Anaphalis* populations, their coordinates marked by GPS, and leaf samples collected as a source of molecular analysis material. PCR products from ITS and ETS markers were sequenced, and the results' phylogenetic were analyzed using the MEGA6 program. PCR products from EST-SSR markers were performed by scoring DNA bands (allel) and both variations and genetics population were analyzed by using the POPGENE 1.32 program. *Anaphalis* populations found in BTSNP are *Anaphalis javanica, A. longifolia,* and *A. viscida*. The genetic diversity of *Anaphalis* spp. in BTSNP has polymorphism potential that is high enough, which is 57% (ITS) and 31% (ETS-partial), with a maximum likelihood phylogenetic tree topology which is monophyletic separated into four clusters consisting one cluster outgroup and three others being an ingroup. Whereas based on the genetic diversity value of the EST-SSR sequences in *Anaphalis* spp. in BTSNP shows that only *A. longifolia* populations in the Ranu Kumbolo area that have a high genetic diversity value (0.024) compared to the other two *Anaphalis* species. The highest genetic distance of *Anaphalis* spp. BTSNP in *A. longifolia* is found in the population of Penanjakan and Mt. Batok areas (0.040) with the smallest gene flow rate (0.428). Further research is needed to obtain a more complete picture of *Anaphalis* genetic diversity by using more molecular markers, bigger population numbers, more individuals and in bigger populations in other conservation areas in supporting the *Anaphalis* conservation strategy program.

***Keywords*— *Anaphalis*; conservation; external transcribed spacer; expressed sequence-tag simple sequence repeat; internal transcribed spacer.**

I. INTRODUCTION

Anaphalis is a plant that belongs to the Asteraceae family and can only grow in mountainous areas so that it is known as the montana zone and sub alpine zone plant. The genus *Anaphalis* is distributed and spread globally on this earth [1-6]. *Anaphalis* has an important contribution to the ecological process, and is one of the pioneer plants in mountainous ecosystems that can thrive well on mountain volcanic soils [7].

Anaphalis is currently suspected of being reduced in nature and is feared to be extinct soon. International Union for Conservation of Nature 3.1 (IUCN 3.1) that is in charged in the world conservation status classifies that the conservation status of *Anaphalis* belongs to critically endangered-Threatened or is a plant in a threatened condition, so that further scientific review is needed to support *Anaphalis* conservation aspects. One of the important aspects that needs to be studied in the preservation of *Anaphalis* is the genetic diversity of *Anaphalis*. Data related to genetic diversity from *Anaphalis* is very limited and not yet maximally recognized so that the conservation effort is difficult to do. The aspect of genetic diversity needs to be investigated because it has a close relationship with the ability for the plants to live (fitness) and the plant species population to adapt.

Bromo Tengger Semeru National Park (BTSNP) is one of the most important conservation areas in Java, and is located in East Java, Indonesia, with an ecosystem that is rich in biodiversity. BTSNP region is one of the habitats for *Anaphalis* and has a very high diversity of *Anaphalis* species. It is proofed with the discovery of three out of four

types of *Anaphalis* in Java [8]. However, its presence in the BTSNP area is increasingly threatened due to the active eruption of Mount Bromo [9]; forest fires; the activities of indigenous people who inhabit the National Park (Tengger people) which can be in the form of agricultural activities [10]; economy, and culture; and BTSNP as a favourite tourist location [11].

Some molecular based studies have been successfully carried out on *Anaphalis* and the Asteraceae Family by using molecular markers derived from nuclear ribosomal DNA (nrDNA), namely the Internal Transcribed Spacer (ITS) region [2]; and External Transcribed Spacers (ETS) [5]; and some use Simple Sequence Repeat (SSR) [12], [13]. The ITS area has proven to be a useful source of molecular information for genetic variation and phylogenetic studies in the Angiosperms plant group [14]; and RAPD marker for phylogenetic analysis [15], [16]. The use of Expressed Sequence-Tag Simple Sequence Repeat (EST-SSR) molecular markers has been successfully carried out in various studies on genetic diversity in several plant genera included in the Asteraceae Family [17], [18]. Molecular markers SSR or EST-SSR are frequently used to analyze genetic diversity for genetic conservation [19-21].

The research related to the existence of *Anaphalis* in BTSNP on a molecular basis is very limited. So, information about these plants is very little, especially those that will be useful later for the conservation of *Anaphalis*, so research on genetic diversity is needed as initial information for the conservation of this plant. The purpose of this study was to detect genetic diversity of *Anaphalis* spp. in BTSNP based on molecular characters by using ITS, ETS, and EST-SSR markers to support aspects of genetic *Anaphalis* conservation.

II. Materials and Methods

A. Research Area and Sample Collection

This research was carried out in the BTSNP area by using the *Anaphalis* population exploration and verification method. Interviews with local residents and BTSNP officers were also conducted to obtain information on *Anaphalis* population areas. Location of the *Anaphalis* population is marked by GPS coordinates. At each location, morphological checks and vegetation analysis were calculated. Then, leaf samples were also collected as a source of genetic diversity analysis material. Therefore, description of the research location can be seen in Fig. 1.

Observation for vegetation analysis is aimed to determine the type of dominant organism in a community by using calculation parameters obtained from the results of an Important Value Index (IVI) from *Anaphalis*. IVI is obtained from the sum of the relative values of density and frequency in a community. IVI (equation 1) is used to ease the interpretation of the results of vegetation analysis.

$$Important\ Value\ Index(\mathrm{IVI}) = RD + RF \quad (1)$$

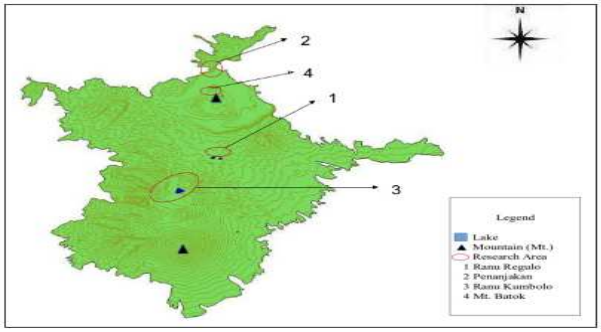


Fig. 1. The Map of Research Location in BTSNP

B. Observation of Genetic Diversity

Analysis of genetic diversity was done by isolating DNA from the *Anaphalis* genome, sequence amplification, and sequencing (carried out at ITS and ETS markers) on leaf samples that had been collected at each research location. DNA isolation and extraction were carried out by following the manual instruction in the Genomic DNA Purification Kit (Geneaid). Sequence amplification using the PCR technique is applied by using primers for amplification. The primers used in this study are ITS and ETS derived from nuclear DNA, and EST-SSR primers are also used from non-coding DNA regions, namely SSR expressed. Primary ITS4 and ITS5 were used to amplify the nuclear ITS areas. The ETS1F primers and 18S-IGS were used to amplify the nuclear ETS region. The primers used to amplify EST-SSR were the primers that succeeded in the amplification of *Chrysantemum* cultivars. Sequence amplification was carried out on each type of *Anaphalis* for each primer used in the study and carried out by PCR technique. The PCR annealing program to amplify sequences as listed in Table I.

TABLE I
Sequence Amplification Using The PCR Technique

Primer	Program Annealing PCR				
	Pre-denaturation	Denaturation	Annealing	Extension	Final Extention
ITS	95ºC, 5 minute (1 cycle)	95ºC, 1 minute	55ºC, 1 minute	72ºC, 1 minute	72ºC, 5 minute (1 cycle)
		30 siklus			
ITS5 (F) 5'- GGAAGTAAAAGTCGTAACAAGG -3' ITS4 (R) 5'- TCCTCCGCTTATTGATATGC -3'					
ETS	95ºC, 5 minute (1 cycle)	95ºC, 1 minute	55ºC, 1 minute	72ºC, 1 minute	72ºC, 5 minute (1 cycle)
		30 siklus			
ETS1F (F) 5'-CTTTTTGTGCATAATGTATATATATAGGGGG-3' 18S-IGS (R) 5' GAGACAAGCATATGACTACTGGCAGGATCAACCAG-3'					
EST-SSR	94ºC, 10 minute (1 cycle)	94ºC, 30 second	56ºC, 30 second	72ºC, 30 second	72ºC, 10 minute (1 cycle)
		35 cycles			
CM07 (F) 5'- TGG GTG TAG AAG GTG AGT CAA -3' CM07 (R) 5'- ACG CAC CAT GTG AAT TGT GT -3'					

The PCR reaction in this study uses 2x PCR Master Mix Solution (i-TaqTM) (Intron). The component contained in 2x PCR Master Mix Solution (i-TaqTM) is 2.5mM for each dNTPs, i-TaqTM DNA Polymerase (5 U / µl), 1x PCR reaction buffer, 1x loading gel buffer. The addition of BSA in the PCR reaction component is needed as a driver PCR master mix can work optimally. PCR products were visualized on agarose gels that had been added with etidium bromide (EtBr) on the GelDoc UV - trans illuminator device. The PCR products (from ITS and ETS primers) are then sent to the First Base (Malaysia) sequencing laboratory for DNA sequencing. The amplification product volume for sequencing was 40 µL and sequencing using the same primer pairs for each primer on PCR amplification. The composition of the PCR reaction as listed in Table II.

TABLE II
PCR REACTION COMPOSITION

PCR Reaction Composition	Volume reaction (20µl)
2x PCR *Master Mix Solution* (i-Taq^{TM})	10 µl
Template DNA	2 µl
Primer *Forward* (10 pmol/µl)	1 µl
Primer *Reverse* (10 pmol/µl)	1 µl
Destilated water (ddH2O)	5,5 µl
BSA (10mg/ml)	0,5 µl

C. Molecular Data Analysis

Molecular data analysis is divided into data analysis in the form of sequences of sequencing results using ITS and ETS primers, and analysis of EST-SSR results. Data sequences obtained from the ITS and ETS sequences obtained were in-group samples, then the ITS and ETS *Helichrysum asperum* sequence data were used as an outgroup originating from the GenBank database (NCBI). Analysis of ITS and ETS sequence data sequencing products was evaluated using the sequence scanner program and also using the Basic Local Alignment Search Tool (BLAST) program at GenBank. Sequence analysis is performed using ClustalW on the Bioedit software. The results of the sequence analysis are then analyzed again to make a phylogenetic tree using the MEGA6 program. Phylogenetic trees are constructed using the Maximum Likelihood algorithm method in the MEGA6 program. Evaluation of phylogenetic trees was done by bootstrapping analysis of 1000 repetitions.

Analysis of the EST-SSR results was carried out by describing the pattern of the amplification tape. The results of the amplification band with EST-SSR primers are called alleles, allele variations are the number and size of alleles (bp). Alleles are converted in binary data format through scoring (a score of 1 for DNA bands that are present and a score of 0 for DNA bands that are not present). Furthermore, genetic populations were analyzed using the POPGENE 1.32 analysis program with several parameters including: percentage of polymorphic loci (PPL), Shannon phenotypic diversity index (I), Nei's (H) genetic diversity, heterozygosity in subpopulations (HS) and total heterozygosity in the population (HT), gene flow (Nm), and genetic differentiation coefficient (GST). Gene flow (Nm) values sre calculated using the formula Nm = 0.5 (1-GST / GST).

III. RESULT AND DISCUSSION

A. Distribution and Characteristics of Anaphalis spp. in BTSNP

The results of identification of *Anaphalis* spp. found that there were three species of *Anaphalis* found in TNBTS namely *Anaphalis javanica*, *A. longifolia*, and *A. viscida*, but it did not find *A. maxima*. These results are the same as those found by [8]. The species found showed a different character.

Anaphalis spp. which were found in each study area formed habitat zones of *Anaphalis* spp. in certain areas. This zone is formed from the position points found *Anaphalis* spp. which geographical coordinates are marked using GPS (Fig. 2). The identified *Anaphalis* population in the Ranu Regulo is *Anaphalis javanica* population which is found at altitudes ranging from 2118-2123 meters above sea level (masl). *Anaphalis* populations in the Penanjakan area found were *Anaphalis javanica*, *A. longifolia*, and *A. viscida* populations which were found at altitudes ranging from 2627-2767 masl. The identified *Anaphalis* populations in the Ranu Kumbolo area were *Anaphalis javanica*, *A. longifolia*, and *A. viscida* populations which were found at altitudes ranging from 2383-2546 masl. The population of *Anaphalis* in the Batok Mountain (Mt.) area found was the population of *Anaphalis longifolia* located at altitudes ranging from 2155-2183 masl.

The distribution zone of *Anaphalis* spp. in TNBTS generally shows that *Anaphalis* plants can grow well in open areas, grasslands, slopes of volcanic ash, and areas that get direct sunlight. This is in line with [1] who explained that *Anaphalis* plants can grow in open places with direct sunlight, mixed forests shaded by tree canopies and grasslands, slopes of volcanic ash, and not shady places.

The area around the Ranu Regulo in the northern part of the lake (ranu) has open fields overgrown by *Anaphalis javanica* populations. In this area is an area that gets direct sunlight because there is no canopy of other plants higher than *A. javanica* that can cover *A. javanica* plants. This is consistent with the statement of [1] that *A. javanica* does not like the shade which is often shaded by other higher plant canopies.

The identified *Anaphalis* populations in the Penanjakan area were *Anaphalis javanica*, *A. longifolia*, and *A. viscida* populations. In addition, every type of *Anaphalis* is present together in the population. *Anaphalis javanica* and *A. viscida* usually grow in sunny places, whereas *A. longifolia* usually grows under the auspices of the dense canopy of *Acasia decurens* so that the portion of sunlight obtained by *Anaphalis longifolia* plants is lower. *A. longifolia* is thought to be more tolerant of the canopy shade of trees which results in less direct sunlight. This is in line with [1] who stated that *A. longifolia* lives in dense canopy mix forests and *Casuarina junghuhniana* forests.

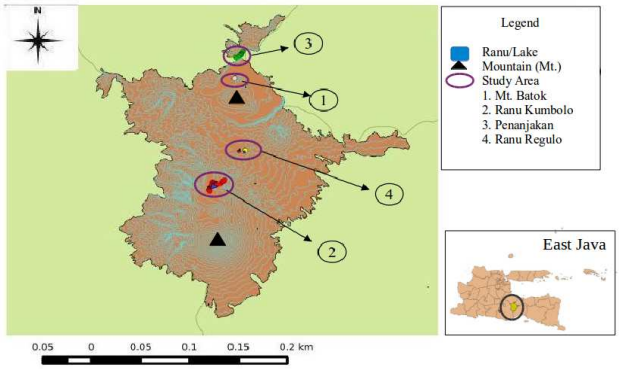
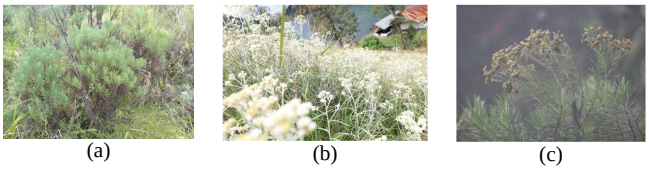


Fig. 2. Coordinates where the *Anaphalis* spp. found

The conditions around the Ranu Kumbolo area are open savannah and there are only a few mountain cypresses (*C. junghuhniana*) on several sides of the lake's edge (ranu). *Anaphalis* populations in the Ranu Kumbolo area found were *A. javanica, A. longifolia,* and *A. viscida* populations. The plants of *A. javanica* and *A. viscida* are more often found in places with lots of sun. *A. viscida* is thought to be unable to grow optimally if it is under the canopy of dense leafy trees, so that the amount is very limited. However, *A. viscida* grows optimally in open field areas that are exposed to direct sunlight or even if there is a canopy of shade trees, the canopy is not too dense and it still exposed to sunlight. In regards to this, [1] explained that *Anaphalis javanica* and *A. viscida* grow in open areas that with enough sunlight such as on mountain tops, vacant land between *C. junghuhniana* forests, grasslands, and bushes.

The area around Mount Batok is an area that is very close to Mount Bromo which is still actively releasing volcanic ash so that it just allows very few plants to live. Plants that can live in the area of Mount Batok are plants that are very tolerant of exposure of volcanic ash produced by Mount Bromo. Meanwhile, *Anaphalis longifolia* is one of the pioneer plants that can be found in the Batok Mountain area in the northwestern part, which is an area that is slightly exposed to Mount Bromo volcanic ash, but it is found very little. *A. longifolia* plants are thought to be very tolerant of volcanic ash and usually grow at altitudes above 1200masl. Related to this, [1] agreed that *Anaphalis longifolia* grows on mountain peaks, cliff walls and grows at an altitude of 1200-2850masl. It is apparently often found as a pioneer plant on lava rocks and volcanic ash slopes. Reference [8] found that *A. longifolia* can survive at an altitude of 1000-2000 masl.

Anaphalis is an alpine /montane zone endemic plant in various high mountains in this archipelago. *Anaphalis* plants which grow in a limited area make this plant grow depending on temperature and humidity at altitudes above 2000masl. According to [7], *Anaphalis* is a pioneer plant of young volcanic soil that is able to survive on barren land, and this plant does not like shade, and it invites specific insects that live in mountain areas as pollinators of *Anaphalis* plants. *Anaphalis* spp. in BTSNP shows a different character (Fig. 3).



(a) (b) (c)

Fig. 3. Variety of *Anaphalis* in BTSNP (a) *Anaphalis javanica*, (b) *A. longifolia*, (c) *A. viscida*

Anaphalis population distribution has a clustered pattern which is a distribution pattern that is commonly found in nature. It is predicted that this pattern occurred due to the vegetative reproductive pattern of an individual member of the *Anaphalis* population. In addition, it is also caused by the adaptation carried out by *Anaphalis* plants in a limited growing habitat. The spread of *Anaphalis* species in BTS area is found based on certain height and grows on flat land contour /soil with a certain slope (cliff). *A. javanica* is mostly found at an altitude of 2561-2691m above sea level. *A. javanica* thrives and is more widely distributed on slopes and upper cliffs that are difficult to reach. Generally, these plants grow more fertile and form more populations on the cliff. This is due to the growing nature of *Anaphalis* which like to grow to get sunlight without the shade of other plants. At the same time, *A. longifolia* is often found at an altitude of 2670-2755masl. In fact, there is one plant population of *A. longifolia* along with *A. javanica* at an altitude of 2665m above sea level and commonly found to grow on slopes/cliffs. *A. viscida* is not found in the population of *A. viscida* itself. However, the population of *A. viscida* is found together with other *Anaphalis* plants. This plant is sometimes found together with *A. longifolia* or with *A. javanica*, even with *A. javanica* and *A. longifolia*. Moreover, population of *A. viscida* was found at an altitude of 2584-2711masl. This height included in the range of heights found *A. javanica* and *A. longifolia*, so that *A. viscida* can grow together in the population.

Anaphalis plants can usually grow at the top of mountains, subalpine forests and are found at an altitude of 2000-3400masl. Each type of *Anaphalis* can grow at different heights. *A. javanica* is found at an altitude of 2000-3400masl, while *A. longifolia* grows on mountain peaks, cliff walls and at an altitude of 800-2850 masl. However, *A. viscida* grows at an altitude of 1800-3300 masl. According to [1] *A. viscida* is only found in mountains with a dry Munson climate while *A. longifolia* grows on grasslands, on slopes, along footpaths, in mixed and evergreen forests. It often grows as pioneers in lava rocks and slopes. volcanic ash slope, at an altitude of 1200-2850masl and rarely found at altitudes below 800masl.

Spatial distribution results show that the three species found in the BTS volcanic zone. Mount Bromo, which is within the BTS area, is one of the most active mountains in recent decades with the last eruption occurring in 2016 which resulted in damage to vegetation and disrupting tourist activities. The volcanic activity of Mount Bromo is considered to be a significant contributor to the existing composition in Penajakan and Batok Mountain. This is allegedly due to Penanjakan and Mt. Batok is an area that gets significant volcanic material from the Mt. eruption process. Bromo, so ash and sand will cover the soil and vegetation in this area after the eruption process. Soil types

in BTSNP are regosol and litosol originating from intermedia volcanic ash and sand up to bases with very tight permeability properties and the top layer is very sensitive to erosion. Regosol soil types are usually found in different climate and heights, and contain plant nutrients (phosphate and potassium) [22]. The thing is that, the soil structure, climate, and altitude of an area will affect the growth of plants in that particular area. Moreover, *Anaphalis* growth is also influenced by climate, altitude, and soil structure. Increasing the height of an area will reduce the climate in the area so that the process of soil formation will slow down and plant roots will grow shallower. Soil differences will affect existing vegetation but are not limited by other factors such as climate and changes in seed dispersing animal communities. According to [23], there are two of the most important climatic variables that influence soil formation; they are soil temperature and humidity (soil moisture). The soil moisture depends on several factors, namely rainfall intensity, variability of seasonal rainfall, slope aspects (topography), organisms in the soil, and soil texture/permeability of parent material.

Vegetation habitat profile of *Anaphalis* spp. has a diverse vegetation composition but has similarities in general aspects of the soil. Different vegetation structures in each study area are thought to be due to different soil functions, rainfall intensity, fires in natural habitats, and the introduction of exotic plant species. The abundance of weeds in the study area is a natural disturbance condition of the *Anaphalis* habitat, but also found an abundance of exotic plant species in the *Anaphalis* habitat under study. The abundance of exotic plant species contributes to forest fires, one of which is caused by the dry leaves of these plants. Reference [24] recently found many exotic plant species and invasive species plants in mountainous areas. *Anaphalis* in the BTSNP area is thought to have been threatened, especially from fire. Forest fires usually occur together with the dry season and dry biomass of plants in certain areas. Dried plant biomass is one of the important elements of any forest fire incident, especially in the grasslands. Species that are usually found in grassland areas are *Imperata cylindrica, Pteridium* spp., and *Agrostis* spp., which belong to the group of flammable plants in the dry season period. *Anaphalis* spp. in BTSNP grows on volcanic soils with high plant pressure of invasive species. Cleaning of invasive species in *Anaphalis* habitat is highly recommended as a strategy to increase *Anaphalis* spp. as an endemic species with a limited population in BTSNP.

The results of vegetation analysis at each observation location found that many invasive and exotic plants grow and inhabit the vegetation of *Anaphalis* population indicated by high IVI values. The Importance Value Index (IVI) is the importance value of a plant species and its role in the community. Organisms with high IVI have an important role in a community that will form dominance among species. Plants with the highest IVI value are included as one of the factors that inhibit the growth of *Anaphalis* because they are thought to inhibit the absorption of nutrients from the soil for *Anaphalis* growth. The plants that were found to have the highest INP were grasses and weed types namely *Agrostis* spp., *Imperata cylindrica*, and *Pteridium* spp.

B. Genetic Diversity of Anaphalis spp. di TNBTS based on molecular markers (ITS, ETS, dan EST-SSR)

The DNA sequence of the ITS and ETS regions of the entire *Anaphalis* sample showed a high variation so that it could be used as an important character to establish (reconstruct) genetic relationships in phylogenetic trees. The phylogenetic tree reconstruction in this study used the maximum likelihood method which was analyzed by the MEGA6 program (Fig. 4 and Fig. 5).

Anaphalis genetic relationship analysis results in BTSNP based on ITS and ETS sequences using the maximum likelihood algorithm showed that *Anaphalis* phylogenetic trees belong to a monophyletic group and separated from *Helichrysum asperum*. *Anaphalis* spp. in the BTSNP found were thought to originate from the same ancestors and can be seen in the results of phylogenetic tree reconstruction including monophyletic groups. As described by [25], a monophyletic group is a group that consists of a common ancestor plus all the descendants of that ancestor. Relationship to the phylogenetic system is a novelty value from the same ancestors.

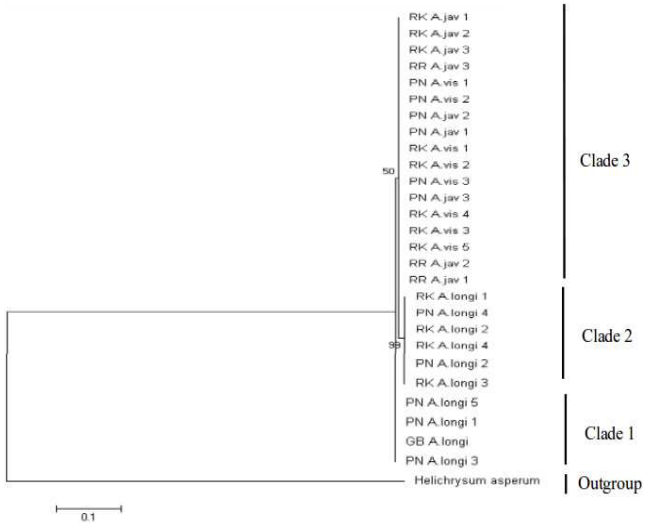


Fig. 4. Maximum Likelihood (ML) phylogenetic tree of *Anaphalis* at Bromo Tengger Semeru National Park based on ITS sequences. The value on the branch represent bootstrap value based on 1000 replicate.

Phylogenetic trees based on ITS and ETS sequences produce trees whose branches are separated into four clones (clusters) with the composition of one clad being outgroup and the other three being ingroup. Each clad gives an illustration of the speciation process that occurred during the evolution of the plant. In phylogenetic trees based on ITS sequences (Fig. 4), they produce three clade with *A. longifolia* separated in two clade. Clade one and clade two consist of *A. longifolia* from different locations. Meanwhile, the third clad consists of *A. javanica* and *A. viscida* (Fig. 4). The results of this phylogenetic tree topology showed that *A. longifolia* has similarity and closeness of sequences among individual samples of *A. longifolia* found at this study site, thus making this species more stable in phylogenetic tree groups, with a bootstraps value of 99%. The phylogeny tree topology produced based on the analysis used differed in the value of the bootstrap and the number of monophyletic groups that had clad

robustness or consistency. According to [26], monophyletic groups have one common ancestor and all of their descendants are from that ancestor. This causes members in the monophyletic group to be considered to have a very close relationship and are assumed to carry the same genetic and biochemical characteristics or patterns altogether.

The phylogenetic tree based on the ETS sequence also produced three clads consisting of in-group samples (Fig. 5). In this phylogenetic tree, *A. longifolia* was also found to be more stable in the phylogenetic tree group contained in the clad two, but not all types of *A. longifolia* samples were found here. Clade one and clade three were identified to be incongruent in *A. longifolia*, *A. javanica*, and *A. viscida* samples. Similar results (incongruence) were also found by [4] in phylogenetic trees where there are two species of the genus *Anaphalis* at the same branching.

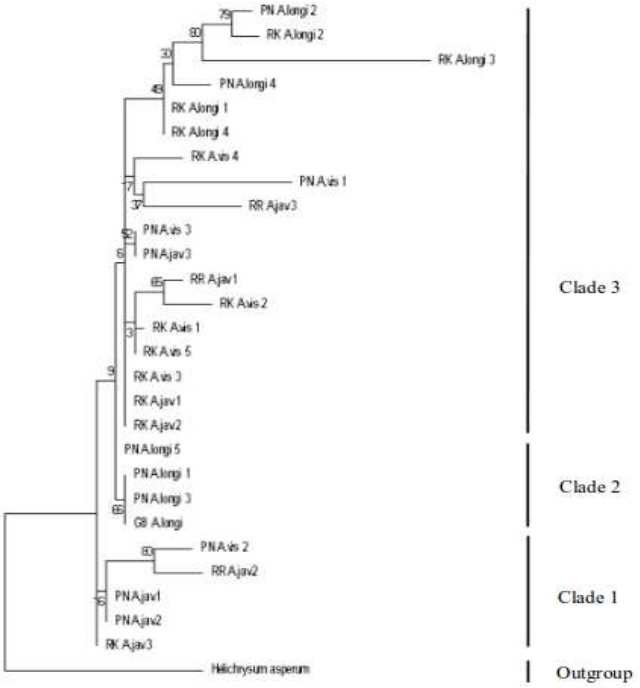


Fig. 5. Maximum Likelihood (ML) phylogenetic tree of *Anaphalis* at Bromo Tengger Semeru National Park based on ETS sequences. The value on the branch represent bootstrap value based on 1000 replicate.

Phylogenetic tree analysis can describe an evolutionary process that occurs in a sample being compared / examined. The process is called the speciation process which shows a group in branching phylogenetic trees that are more primitive or more advanced in the evolutionary process. In accordance with [27], phylogenetic trees consist of nodes and branches where each node describes the speciation process during evolution. The length of a branch means that more changes occur in the gene marker during the evolutionary process, resulting in the species in the branch can be said to be more advanced.

Phylogenetic trees produced based on ITS and ETS sequences appear incongruent in their branching, which is possible due to variations in DNA sequences in each sample, the polymorphism of the nrDNA sequences, and the distribution and fragmentation of *Anaphalis* growing habitats. According to [4], discrepancies arising in phylogenetic relationships in the genus can occur due to low sequence resolution, hybridization, and trans-specific nrDNA polymorphisms. Habitat fragmentation and heterogeneity can also be influenced by climate change (climatic change). In addition, the presence of varying distribution in different groups of organisms can also represent a general form in plant evolution [28].

The results of the analysis based on EST-SSR found some variations in the amplification band (allele). Variation of alleles indicates that there was genetic variation at the genome level. The results of the analysis of genetic diversity showed that there was genetic diversity in the population of Ranu Regulo, Penanjakan, Ranu Kumbolo, and Mt. Batok BTSNP. Analysis of genetic diversity was performed on each type of *Anaphalis* found in each population. The genetic diversity value of *Anaphalis* spp. BTSNP can be seen in Table III below.

TABLE III
THE RESULTS OF GENETIC DIVERSITY ANALYSIS OF *ANAPHALIS* SPP. IN BTSNP

Species	Population site	Amount of sample	N_a	N_e	*H*	*I*
A. javanica	Ranu Regulo	3	1.049	1.034	0.019	0.028
	Penanjakan	3	1.024	1.023	0.012	0.017
	Ranu Kumbolo	3	1.000	1.000	0.000	0.000
A. longifolia	Penanjakan	5	1.049	1.011	0.009	0.016
	Ranu Kumbolo	4	1.049	1.049	0.024	0.034
	Mt. Batok	1	1.000	1.000	0.000	0.000
A. viscida	Penanjakan	3	1.000	1.000	0.000	0.000
	Ranu Kumbolo	5	1.024	1.034	0.012	0.017

Notes: N_a: Average number of alleles observed; N_e: average number of effective alleles; *H*: Nei heterozygosity/genetic diversity; *I*: Shannon Index average

In general, the results of *Anaphalis* spp. BTSNP has low genetic diversity information. The highest value of genetic diversity was obtained in the population of *A. longifolia* in the Ranu Kumbolo area. While, the lowest value of genetic diversity was obtained in the population of *A. javanica* in the Ranu Kumbolo area, the population of *A. longifolia* in the Mt. Batok and population of *A. viscida* in the Penanjakan area. The value of genetic diversity is low because the number of samples found in this population was very small. This was also supported by the spatial distribution data of this study, that very few plant species were found at the geographical coordinates. In addition to spatial distribution, the presence of environmental variations also affected the adaptability of plants to grow well.

The low value of genetic diversity in *Anaphalis* in BTSNP indicates that *Anaphalis* does not have a high adaptation to environmental conditions, thus allowing

Anaphalis vulnerable to extinction in nature. The influence of environmental conditions for *Anaphalis* is thought to originate from several factors including climate (temperature), adequate sunlight for growth, soil conditions and nutrient content in the soil that affect plant reproduction, and geographical position. Soil temperature, availability of the element Nitrogen (N) can cause a species to differentiate. Reference [29] makes it clear that the high genetic diversity of an organism can indicate that the organism has a great opportunity in adapting to environmental changes, and vice versa.

Heterozygosity value of *Anaphalis* spp. BTSNP based on genetic diversity analysis can be seen in the following Table IV. Heterozygosity value of *Anaphalis* spp. TNBTS also has a low value. The highest total heterozygosity values were found in *A. longifolia* population and followed by *A. javanica* and *A. viscida*. The value of heterozygosity between populations (D_{ST}) in *A. longifolia* is higher than the value of heterozygosity in populations (H_S) indicating that the genetic variation of *A. longifolia* between populations is higher than the genetic variation in the population. This may be due to the relationship of geographic distances between *A. longifolia* populations so that the gene flow that occurs is also low. Genetic quality needs to be considered because it is related to the capacity to adapt to the growth environment.

TABLE IV
HETEROZYGOSITY AND GENE FLOW VALUES FROM THE ANALYSIS OF GENETIC DIVERSITY *ANAPHALIS* SPP. IN BTSNP

Spesies	Amount of sample	H_T	H_S	D_{ST}	G_{ST}	N_M
A. javanica	9	0.020	0.011	0.009	0.474	0.555
A. longifolia	10	0.024	0.011	0.013	0.538	0.428
A. viscida	8	0.008	0.006	0.002	0.288	1.236

Note: H_T: total population heterozygosity (H_S + D_{ST}) value; H_S: heterozygosity values in the population; D_{ST}: heterozygosity values between populations; G_{ST}: genetic differentiation between populations; N_M : gene flow

A high G_{ST} value of *A. longifolia* will result in a low gene flow rate. This is due to reproductive isolation and geographical isolation which are limiting factors between populations. Reference [30] stated that a high G_{ST} value among populations will indicate a low level of gene flow, and vice versa. The highest genetic distance was found in *A. longifolia* population in Penanjakan and Mt. Batok areas (0.040), while the lowest was in *A. viscida* in Penanjakan and Ranu Kumbolo areas (0.004).

The population of *A. longifolia* in the Ranu Kumbolo area has the highest value of genetic diversity which can be indicated as one of the *Anaphalis* diversity centers in BTSNP and requires serious protection and attention in its preservation. Efforts to conserve and protect and strengthen *Anaphalis* populations are based on the individuals in each population, and the genetic property conditions of each population.

IV. CONCLUSION

The genetic diversity of *Anaphalis* spp. in BTSNP has a fairly low value that occurs within and between populations of each *Anaphalis* species. These results illustrate that there is a need for a conservation strategy activity for *Anaphalis* populations in BTSNP. Moreover, further research is also needed to obtain a more complete picture of *Anaphalis* genetic diversity by using more molecular markers with population numbers and the number of individuals in more populations with various populations in other conservation areas in support of the *Anaphalis* conservation strategy program.

ACKNOWLEDGMENT

The authors would like to acknowledge the ministry of research, technology, and higher education, Republic of Indonesia (KEMENRISTEKDIKTI) for the funding support of the research.

REFERENCES

[1] C. G. G. J. Van Steenis, A. Hamzah, and M. Toha, *The Mountain Flora of Java*, Second edition, Leiden: Brill Academic Publisher, 2006.

[2] D. Glenny, and S. Wagstaff, "Evolution and Biogeography of New Zealand Anaphalis (Asteraceae: Gnaphalieae) inferred from rDNA sequences," *New Zealand Journal of Botany*, vol. 35, pp. 441-449, 1997.

[3] D. Wahyudi. (2010) *Distribusi dan kerapatan Edelweiss (A. javanica) di Gunung Batok Taman Nasional Bromo Tengger Semeru*. [Online]. Avilable: ejournal.uin-malang.ac.id/index.php/bio/article/view/1694.

[4] Z. L. Nie, V. A. Funk, H. Sun, T. Deng, Y. Meng, and J. Wen, "Molecular phylogeny of *Anaphalis* (Asteraceae, Gnaphalieae) with biogeographic implications in the Northern Hemisphere," *J. Plant Res*, vol. 126, pp. 17-32, 2013.

[5] A. Taufiq, Syamsuardi, A. Arbain, T. Maideliza, Mansyurdin, and Nurainas, "Analisis Morfometri dan Biologi Reproduksi *Anaphalis javanica* dan *A. longifolia* (Asteraceae) di Sumatera Barat," *Floribunda*, vol. 4, pp. 161-168, 2013.

[6] H. Prakasa, A. Z. Akmal, W. A. Guci, and S. Edi, "Analisis Habitat (Anaphalis longifolia (Blume) Blume ex DC.) di Sumatera Utara," *Jurnal Biosains*, vol. 4, pp. 78, 2018.

[7] T. Whitten, R. E. Soeriaatmadja, and S. A. Afif, *Ekologi Jawa dan Bali. Seri Ekologi Indonesia Jilid II*, Terjemahan S.N. Kartikasari, T. B. Utami, and A. Widyantoro, Jakarta: Prenhallindo, 1999.

[8] L. H. Kurniawan, L. Hakim, and E. L. Arumingtyas, "Effectiveness of *trn*L (UAA) intron sequence for detecting genetic variation of *Anaphalis* spp. along Mount Semeru hiking track, Bromo Tengger Semeru National Park Indonesia," *Journal of Biodiversity and Environmental Sciences (JBES)*, vol. 5, pp. 501-507, 2014.

[9] T. A. Rachmawati, D. Rahmawati, and Rachmansyah, "Disaster risk analysis of Mount Bromo eruption after the 2015 eruption in Sukapura District," M*ATEC Web of Conferences*, vol. 229, p. 01015, 2018.

[10] S. Sumarmi, "The Local Genius of Tengger People in Conserving Forest and Increasing Economic Benefits Using Agroforestry System," In *IOP Conference Series: Earth and Environmental Science*, vol. 145, 2018.

[11] A. Purnomo, I. N. Ruja, and L. Y. Irawan, "Typology of Tourist Bromo Tengger Semeru National Park as a Basic Planning Integrated Tourism Design," In *IOP Conference Series: Earth and Environmental Science*, vol. 145, 2018.

[12] D. Gutiérrez-Larruscain, T. M. Ferreras, M. M. Martínez-Ortega, E. Rico, and S. Andrés-Sánchez, "SSR markers for Filago subg. Filago (Gnaphalieae: Asteraceae) and cross-amplification in three other subgenera," *Applications in Plant Sciences*, 6, 2018.

[13] M. Jemelková, M. Kitner, E. Křístková, I. Doležalová, and A.Lebeda, "Genetic variability and distance between Lactuca serriola L. populations from Sweden and Slovenia assessed by SSR and AFLP markers," *Acta Bot. Croat*, vol. 77, pp. 172–180, 2018.

[14] S. Ghanbari, B. A. Fakheri, M. R. Naghavi, and N. Mahdinezhad, "Evaluating phylogenetic relationships in the Lilium family using the ITS marker," *J Plant Biotechnol*, vol. 45, pp. 236–241, 2018.
[15] I. Suliansyah, Yusniwati, I. Dwipa, "Genetic Diversity and Association amongst West Sumatra Brown Rice Genotype Based on Morphological and Molecular Markers," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 8, pp. 610-615, 2018.
[16] U. Sholikhah, Parjanto, T. Handoyo, A. Yunus, "Genetic Diversity of Black and Aromatic Rice Cultivar (Oryza sativa L.) from Various Regions in Indonesia using Random Amplified Polymorphic DNA Markers (RAPD)," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 9, pp. 1046-1051, 2019.
[17] Y. Yang, S. Huang, Y. Wang, L. Gui, Y. Liu, X. Huang, G. Chen, F. Tan, and J. Wang, "Development of EST-SSR markers for Pluchea indica (Asteraceae) and cross-amplification in related species," *Applications in Plant Sciences*, vol. 6, 2018.
[18] S. Stojnic, E. V. Avramidou, B. Fussi, M. Westergren, S. Orlovi´c, B. Matovi´c, B. Trudi´c, M. Kraigher, F. A. Aravanopoulos, and M. Konnert, "Assessment of Genetic Diversity and Population Genetic Structure of Norway Spruce (Picea abies (L.) Karsten) at Its Southern Lineage in Europe. Implications for Conservation of Forest Genetic Resources," *Forests*, vol. 10, pp. 258, 2019.
[19] A. Mohamad, A. N. Alhasnawi, A. A. Kadhimi, A. Isahak, W. M. W. Yusoff, C. M. Z. C. Radziah, "DNA Isolation and Optimization of ISSR-PCR Reaction System in Oryza sativa L," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 7, pp. 2264-2272, 2017.
[20] D. M. Czarnecki, R. M. Nageswara, J. G. Norcini, F. G. Gmitter, and Z. Deng, "Genetic Diversity and Differentiation among Natural, Production, and Introduced Populations of the Narrowly Endemic Species Coreopsis leavenworthii (Asteraceae)," *Journal of the American Society for Horticultural Science*, vol. 13, pp. 234–241, 2019.
[21] Y. Zhao, B. Pan, and M. Zhang M, "Phylogeography and conservation genetics of the endangered Tugarinovia mongolica (Asteraceae) from Inner Mongolia, Northwest China," *PLoS ONE*, vol. 14, pp. E0211696, 2019.
[22] S. Hardjowigeno. Klasifikasi *Tanah dan Pedogenesis*. Edisi Revisi. Cetakan ke-2. Akademika Pressindo, 2016.
[23] M. L. Rayes, *Morfologi Dan Klasifikasi Tanah*, Malang: Universitas Brawijaya Press, 2017.
[24] L. Hakim, B. Rahardi, and A. Rachmansyah, "Checklist of flora along tourist trail to Mt. Lamongan, East Java (Indonesia): misconception of restoration and ecotourism programs in mountain region?," *Journal of Degraded and Mining Lands Management*, vol. 5, pp. 1299. 2018.
[25] M. G. Simpson, *Plant Systematics*, Second edition, USA: Academic Press, Elsevier, 2010.
[26] D. A. Rahayu, and E.D. *Nugroho*, *Biologi molekuler dalam perspektif konservasi*, Yogyakarta: Plantaxia, 2015.
[27] J. W. Ochieng, A.W Muigai, and G.N. Ude, "Phylogenetic in plant biotechnology: principles, obstacles, and opportunities for the resource poor," *Afr J Biotechnol*, vol. 6, pp. 639-649, 2007.
[28] Z. L. Nie, V. A. Funk, Y. Meng, T. Deng, H. Sun, and J. Wen, "Recent assembly of the global herbaceous flora: evidence from the paper daisies (Asteraceae: Gnapalieae)," *New Phytologist*, vol. 209, pp. 1795-1806, 2016.
[29] R. Rambey, A. Susilowati, and N. Anna, "Genetic diversity of mindi (Melia azedarach) from community forest in Deli Serdang, North Sumatera, Indonesia revealed by microsatellite marker," *Biodiversitas*, vol. 201, pp. 1708-1712, 2019.
[30] R. Frankham, J. D. Ballou, and D. A. Briscoe, D. A. 2002. *Introduction to conservation genetics*, Cambridge University Press, UK, 2002