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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

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Abstract

Mount Semeru hiking track is a world tourism destination and inhabited by three species of *Anaphalis*. The aims of this research is to get the information of the effectiveness of *trn*L (UAA) intron sequence for analyzing genetic variability of the genus *Anaphalis* populations along Mount Semeru hiking track. The leaf for DNA extraction was collected from several locations along the track. This research contribute to the information that *trn*L (UAA) intron sequences have less power to analyses the genetic variation of the genus *Anaphalis* populations along Mount Semeru hiking track.

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Introduction

The genus *Anaphalis* DC. (Gnaphalieae - Asteraceae) became a part of population that construct the beautiful of Java highland. Bromo Tengger Semeru National Park (BTSNP) inhabited by three species of the genus *Anaphalis* such as *Anaphalis javanica*, *Anaphalis viscida*, and *Anaphalis longifolia* (Backer and van den Brink, 1965; van Steenis, 1972). In Java, genus *Anaphalis* only live on 800 - 3400 asl. (Backer and van den Brink, 1965). Based on Whitmore (1984) this habitat include from lowland forest, lower montane forest (1200 – 1800 asl.), upper montane forest (1800 – 3000 asl.), and subalpine forest (above 3000 asl.). The Java species of *Anaphalis* is a pioner plants and could grow on the slopes of the mountain caldera (Whitten *et. al.*, 1996). *A. Longifolia* is herbaceous and sometime woody at the base while *A. viscida* and *A. Javanica* are shrub (Backer and van den Brink, 1965).

Mount Semeru hiking track is a tourist destination object for beautiful scenery such as Ranukumbolo lake and the summit of Mount Semeru for enjoying the sun rise and crater. Recently, the genus *Anaphalis* in BTSNP threatened by illegal harvesting and exploitation (Hakim, 2011). Tourism activities could impact wildlife in way such as harvest, habitat modification, and pollution (Knight and Cole, 1991). There are many threats to vegetation in protected areas from tourism (Pickering and Hill, 2007), such as vegetation damage/clearing (reduction in height, reduce living biomass, damage to seedlings, change in species composition), spread of weeds, spread of pathogens.

One of the strategy to make the tourism become sustainable are to detect genetic diversity and to get the information about genetic structure for some wild plants especially the genus *Anapahalis* along Mount Semeru hiking track. The genetics and other data from wild populations can be used as a reference populations comparison for restoration populations projects in degraded area (Sutter, 1996; Hakim and Miyakawa, 2013). Moreover, the genetic structure data give a source of consideration in transplantation between adjacent populations to avoid change the gene pool of local populations and confirm the origin of plants collection based on the spatial distribution of DNA haplotype (Honjo *et. al.*, 2004). To assure for the sustainability, monitoring activity could get valuable information from genetic monitoring of wild populations (Schwartz *et. al.*, 2007). Monitoring the existence of certain species can be done by utilizing genetic identification using particular DNA sequence of plants (Thompson and Newmaster, 2014). Genetic diversity need to be considered in conservation program as part of biodiversity preservation.

On the previous study, the *trn*L (UAA) intron already used in many plant genetic research such as to infer the phylogeny of the European gentians (Gielly and Taberlet, 1996). Gielly *et. al.* (2001) use *trn*L (UAA) intron sequence to get the information about geographic isolation and evolution of Mediterranean endemic Cyclamen. Other author use single nucleotide polymorphisms in *trn*L (UAA) intron for precise indentification of *Phyllanthus emblica* and *Phyllanthus indofischeri* in India (Sangeetha *et. al.*, 2010). To the best of our knowledge, *trn*L (UAA) intron sequence variation of *A. Javanica*, *A. Longifolia*, and *A. Viscida* have not yet been reported. The present work was performed to get the information about the effectiveness of *trn*L (UAA) intron sequence variation for detecting genetic variation of the genus *Anaphalis* (*A. Javanica*, *A. Longifolia*, and *A. Viscida*) along Mount Semeru hiking track.

Material and methods

Sites of Study

Leafs of *Anaphalis* spp. for DNA extraction was collected from several location along mount Semeru hiking track start from Ranupani village to the last vegetation community before Mount Semeru Summit at Bromo Tengger Semeru National Park, East Java, Indonesia (Table 1 and in Fig. 1).

Table 1. *Anaphalis trn*L (UAA) intron amplification from sample sites along Mount Semeru hiking track.

No.	Location	*trn*L amplified (+), *trn*L not amplified (-)
1.	*A. viscida* Ranuregulo	+
2.	*A. viscida* Waturejeng	-
3.	*A. longifolia* Waturejeng	+
4.	*A. longifolia* Ayek-ayek	+
5.	*A. viscida* Ayek-ayek	+
6.	*A. viscida* Ranukumbolo	-
7.	*A. longifolia* Ranukumbolo	-
8.	*A. viscida* Jambangan	+
9.	*A. javanica* Jambangan	+
10.	*A. longifolia* Kalimati	+
11.	*A. viscida* Kalimati	-
12.	*A. javanica* Kalimati	-
13.	*A. longifolia* Arcopodo	-
14.	*A. viscida* Arcopodo	-

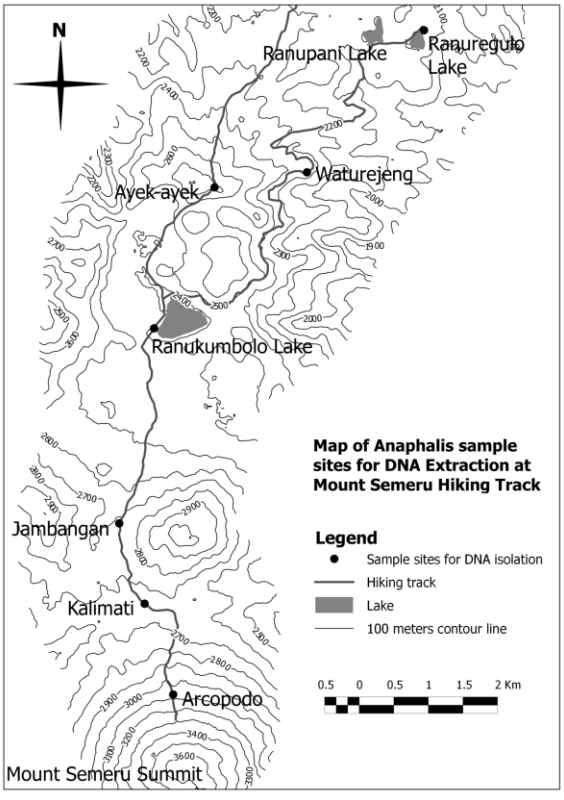

Fig. 1. Map of Anaphalis sample sites for DNA Isolation at Mount Semeru Hiking Track.

DNA Extraction

Total genomic DNA was extracted from young leaf of *Anaphalis* spp. using combination method of Doyle and Dickson (1987) and Deshmukh *et. al.* (2007) with some modification. Young leaf of *Anaphalis* spp. (0,4 gram) was crushed in mortar then added with wash buffer (0,1 M Tris-Cl pH 8, 2% pvp (polyvinilpyrrolidone), 4% 2-mercaptoethanol) until homogen then transferred to 1,5 ml tube and vortexed for about 5 minute then centrifuged at 13.000 rpm for about 5 minutes 4° C. The pellet was added with wash buffer and homogenated until pellet and washbuffer mixed perfectly and vortexed again for 5 minutes and centrifuged at 13.000 rpm for 5 minutes 4°. The treatment with wash buffer was performed for 5 times.

The pellet then was diluted with extraction buffer (0,1 M Tris-Cl pH 8, 0,05 M EDTA pH 8, 0,5 M NaCl, 2% CTAB) and homogenated till pellet and extraction buffer mixed perfectly and then vortexed for 5 minutes and then put in waterbath at 70° C for 15 minutes and then centrifuged at 13.000 rpm for 5 minutes at 4° C.

The supernatant was diluted in CI (Chloroform : Isoamilalcohol 24:1) with the same volume of supernatant then vortexed for 5 minutes and centrifuged at 13.000 rpm for 15 minutes 4° C, this treatment replicated 2 times. The uperphase (supernatant) was transfered to 1,5 ml tube and added with 0,1 ammonium acetat volume of of supernatant and added with absolute ethanol and put in freezer (-20° C) for overnight. The next day, the tube was centrifuged at 13.000 rpm for 15 minutes 4° C. The pellet was diluted in 70% ethanol and put in 2 minutes of room temperature then centrifuged for 13.000 rpm 15 minutes 4° C. The pellet was dried and added with TE buffer 100 µL and stored in freezer (-20° C).

PCR Amplification and Sequence Analysis

The *trn*L (UAA) intron was amplified using universal primer by Taberlet et.al (2007) c: 5'-CGAAATCGGTAGACGCTACG-3' and d: 5'-GGGGATAGAGGGACTTGAAC-3' The PCR was

performed in 30 µL volume reaction mixture that contain 5 µL ddH_2O, 12,5 µL DNA, 2,5 µL primer c (30 pmol/µL), 2,5 µL primer d (30 pmol/µL) and 12,5 µL PCR master mix (intRON Biotechnology). The thermocycling profile was: early denaturation 94 °C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 1 minutes, the final extension of 72 °C for 5 minutes. The PCR products were visualized on 1,5% agarose gel stained with ethidium bromide and were sequenced using 3730XL automated sequencer (Applied Biosystems, Macrogen Inc., Seoul, South Korea) and wer evaluated using ABI Sequence Scanner V.10 (Applied Biosystem). Sequence Analysis using ClustalW (Thompson *et. al.*, 2002) in Bioedit software (Hall, 1999) was performed to get the information of Genetic variation of nucleotide of samples.

Result and discussion

This research could only amplify *Anaphalis* spp. *trn*L (UAA) intron sequence of some sample sites and fail to amplify some of them (Table 1).

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          10        20        30        40        50        60        70        80        90       100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A. javanica Jambangan
CAATCCTGAGCCAAATCACGTTTTCCGAAAACAAACAAAGGTTCAGAAAGCGAAAATCAAAAGGATA
GGTGCAGAGACTCGATGGAAGCTGTTCTAACAA
A. longifolia Ayek-ayek  ..................................................................................
A. longifolia Kalimati  ..................................................................................
A. longifolia Waturejeng  ..................................................................................
A. viscida Ayek-ayek  ..................................................................................
A. viscida Jambangan  ..................................................................................
A. viscida Ranu Regulo  ..................................................................................

         110       120       130       140       150       160       170       180       190       200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A. javanica Jambangan
ATGGAGTTGATTGTCTTACATTGGTAGAGAAATCCTTCTATGGAAACTTCAGAAAAGATGAAGGATA
AACCTGTATACATAATAGAGATACAGAAGAATT
A. longifolia Ayek-ayek  ..................................................................................
A. longifolia Kalimati  ..................................A.................................................
A. longifolia Waturejeng  ..................................................................................
A. viscida Ayek-ayek  ..................................................................................
A. viscida Jambangan  ..................................................................................
A. viscida Ranu Regulo  ..................................................................................

         210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A. javanica Jambangan
GATATGAATCGATTCCATATTGAAGAAAGAATCGAATATTCATTGATCAAACCATTCACTCCATAATC
TGATAGATCTTTTGAAGAACTGATTAATCGGA
A. longifolia Ayek-ayek  ..................................................................................
A. longifolia Kalimati  ..................................................................................
A. longifolia Waturejeng  ..................................................................................
A. viscida Ayek-ayek  ..................................................................................
A. viscida Jambangan  ..................................................................................
A. viscida Ranu Regulo  ..................................................................................

         310       320       330       340       350       360       370       380       390
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A. javanica Jambangan
CGAGAATAAAGATAGAGTCCCGTTCTACATGTCAATACCGGCAACAATGAAATTTATAGTAAGAGGA
AAATCCGTCGATTTCAAAAATCATGAGG
A. longifolia Ayek-ayek  ..................................................................................
A. longifolia Kalimati  ..................................................................................
A. longifolia Waturejeng  ..................................................................................
A. viscida Ayek-ayek  ..................................................................................
A. viscida Jambangan  ..................................................................................
A. viscida Ranu Regulo  ..................................................................................
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Fig. 2. Alignment of *Anaphalis trn*L (UAA) intron Sequences.

The length of *Anaphalis* spp. cpDNA *trn*L (UAA) intron sequence was 395 bp. Alignment of the samples show that there were no deletions, insertions, and only one substitution were found (fig. 2). This research confirm that CpDNA intron *trn*L (UAA) sequences have low variation and hard to be used to distinguish in the species level moreover in the intra-species genetic diversity of *Anaphalis* spp. in the hiking track of Mount Semeru. This result in line with Taberlet *et. al.* (2007) that although *trn*L intron was easy to be amplified but has less ability to detect variation in the species level. Plant identifications using trnL intron DNA sequence in Taberlet *et. al.* (2007) reveal 100% in genus level and 85,44 % in species level for arctic plant.

Other Asteraceae family chloroplast and nuclear DNA sequence analysis reveal less variability. Other research (Blöch *et. al.*, 2010) in genus *Leontopodium* (Asteraceae - Gnaphalieae) using *mat*K and sequence between intron *trn*L to *trn*L/F intergenic spacer reveal low sequence divergence. Nucleotide sequences such as ITS (Internal Transcribed Sequence) and ETS (External Transcribed Sequence) found only small variation in its sequences in *Anaphalis* (Asteraceae - Gnaphalieae) at northern Hemisphere (Nie *et. al.*, 2013). Glenny and Wagstaff (1997) also show that ITS1 and ITS2 sequences have less variation when it used to distinguish endemic *Anaphalis* (Asteraceae - Gnaphalieae) genus in New Zealand.

Other researcher incorporated more than one genetic marker to get the best result in plant DNA sequence analysis research. It was hard to get the best result when only use one plant DNA marker. Newmaster and Ragupathy (2010) used the combination of *rbc*L, *mat*K and *trn*L-F sequences to identify 19 *Biophytum* and 12 *Tripogon* species in the Nilgiri Biosphere Reserve. Saslis-Lagoudakis *et. al.* (2011) incorporated combination of plastid regions *rbcL* and *matK, ndhF-rpL32* intergenic spacer, and nrITS2 and the *trnL-F* intergenic spacer to get the information of the phylogenetic signal in medicinal properties of genus *Pterocarpus* (Leguminosae - Papilionoideae). Honjo *et. al.* (2004) use the combination of five noncoding regions of cpDNA, the spacers between *trn*T and *trn*L, *trn*L and *trn*F, *trn*H and *psb*A, *trn*D and *trn*T, and *trn*L intron to get the data of geographical distribution of *Primula sieboldii* cpDNA haplotype in Japan. This research contribute to the information that *trn*L intron sequences have less power to be used to detect genetic variation of the genus *Anaphalis* along the Mount Semeru hiking track

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