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# Short Communication: Analysis of simple sequence repeats variations for the assessment of genotypic diversity in *Centella asiatica* (L.) Urb.

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**Abstract.** *Maruzy A, Susandarini R. 2024. Short Communication: Analysis of simple sequence repeats variations for the assessment of genotypic diversity in* Centella asiatica (*L.*) *Urb. Biodiversitas 25: 734-740. Centella asiatica* (L.) Urb. is a perennial plant widely known as herbal ingredient in traditional and modern medicine, as well as in the cosmetics industry. This herbaceous species grows in various types of habitat, and is known for its morphological variations among populations, indicating the existence of genotypic variation. In this study, the assessment of genotypic diversity using simple sequence repeats (SSR) was carried out on *C. asiatica* accessions from 32 populations collected from eight mountains in Central Java. Representative individuals from four populations grow at different altitudes were sampled in each mountain. Amplification of genomic DNA using the SSR primer mCaCIR002 produced a single amplicon of 180 bp. The results of SSR analysis showed that there is high genotypic diversity in *C. asiatica* which is characterized by variations in the number of microsatellite repeats of $(CT)_{10}$ to $(CT)_{19}$. Variations in the number of repeats are mainly caused by insertions and deletions in DNA sequences at the positions 102-145. Haplotype analysis also showed high genetic diversity within and between populations of *C. asiatica*, indicated by the haplotype diversity of 1.00 in all populations. The haplotype network analysis indicated that there was no gene flow between the four populations at each mountain where *C. asiatica* populations grow. The results of this study provide evidence for the role of SSR as a suitable molecular marker for studying genotypic diversity at the intraspecific level, and confirming the morphological variability in this species.

**Keywords:** *Centella asiatica*, genotype diversity, microsatellite, molecular marker

## INTRODUCTION

*Centella asiatica* (L.) Urban or asiatic pennywort is a perennial herb with the ability to grow in wide range of habitats. *Centella asiatica* is known as medicinal plant species with many beneficial properties including antioxidant, antigastritis, antitumor, wound healing, and immunomodulator (Mariska et al. 2015; Gray et al. 2016). Most of the plant materials used to meet the needs of *C. asiatica* for herbal-based medicine and industries is still relies on harvesting the leaves from natural populations. This raises the risk of mistakes in collecting materials from the nature because there are other species that have similar morphology, such as *Merremia emarginata* and *Hydrocotyle verticillata*. In addition, the wide range of habitats can give rise to intraspecies variations in *C. asiatica*. A study conducted by Nav et al. (2021) showed that *C. asitica* in Iran has variations in morphology and secondary metabolites content that are recognizable as ecotypes. Other studies indicated the existence of intraspecific morphological variation were reported from different countries, including Prasad et al. (2014) who mentioned two morphotypes in India, and Chachai et al. (2021) who reported intraspecies variation of *C. asiatica* in Thailand. The occurrence of different morphotypes and ecotypes in this species underlies the need to study intraspecies variation in *C. asiatica* using molecular markers to assess the genotypic diversity in this species.

Molecular markers have been used for genetic diversity analysis, and for evaluating the relationship between phenotypic and genotypic variations (Garrido-Cardenas et al. 2018). Molecular markers have major advantage as being not influenced by the environment compared to morphology, which make them useful for accurate characterization and identification of plants (Jiao et al. 2018). Molecular markers widely used in plant systematics studies are those derived from DNA variations, both from the nuclear and chloroplast genomes (Lee et al. 2017). Moreover, molecular markers in the form of variations in DNA sequences between individuals representing a population are commonly related to the expression of particular traits (Amiteye 2021), and thus useful in the analysis of genetic diversity at intraspecies level. A number of studies on the use of different molecular markers in *C. asiatica* have been reported. Molecular characterization of *C. asiatica* accessions collected from populations from six geographical locations in India using ALFP fingerprinting technique was reported by Prasad et al. (2014). The use of simple sequence repeats in *C. asiatica* with the aim of testing its reproducibility and transferability to *C. javanica* and species from other genera in the Apiaceae

family was carried out by Sahu et al. (2015). Meanwhile, the application inter simple sequence repeats for species identification in the genus *Centella* was reported by Alqahtani et al. (2017) who successfully distinguished *C. asiatica*, *C. cordifolia*, and *C. erecta* which have morphological similarities.

One of the molecular markers that has been successfully used in species identification and plant population analysis is Simple Sequence Repeats (SSR) from nuclear DNA (Mehrotra and Goyal 2014; Mathavaraj and Sabu 2021). The role of SSR to reveal intraspecific variation and genetic relationships which have implications for determining conservation and breeding strategies has been reported in *Moringa oleifera* (Popoola et al. 2017). The use of SSR on *C. asiatica* was reported by Rohini et al. (2019) who used fingerprinting techniques to analyze genetic variation in accessions that showed morphological differences. The effectiveness of SSR in the analysis of genetic diversity at the intraspecies level was reported by Xu et al. (2021) on *Brassica oleracea*, who noted that SSR is useful for cultivar identification. Based on the results of previous studies on the use of SSR for genetic diversity analysis, it is evident that this molecular marker has high polymorphism suitable to be applied at the intraspecies level. SSR also useful to detect genetic variation at the intraspecies level and phylogenetic relationships in genus *Raphanus* (Li et al. 2021).

A study by Mathavaraj et al. (2018) used simple sequence repeats for genetic diversity analysis in 30 *C. asiatica* accessions, while Rohini et al. (2019) used SSR for genotypic characterization on 16 *C. asiatica* genotypes from India. The fact that *C. asiatica* populations have inherent morphological variations become a strong basis for the application of molecular markers for the assessment of genotypic diversity in *C. asiatica*. In this study SSR is used to assess genotypic diversity of *C. asiatica* populations from various geographical areas in Central Java.

## MATERIALS AND METHODS

### Collection of plant materials

Plant materials used for DNA isolation were fresh leaves collected from 32 populations on 8 mountains in Central Java (Table 1). Collection of plant materials from the field was carried out from February to April 2022. In each population, three individual plants were collected for morphological observation, and at the same time the leaves were taken for isolation of genomic DNA for molecular analysis using SSR marker. Voucher specimens were stored in the form of dried collections at the Bureau for Organization and Human Resources, National Research and Innovation Agency, Tawangmangu, Central Java.

**Table 1**. *Centella asiatica* accessions from 32 populations in Central Java Province, Indonesia used in this study

| Accession code | Mountain area | Location | Elevation (m asl) |
|---|---|---|---|
| LWU1 | Mount Lawu | Jumog Waterfall, Karanganyar District | 896 |
| LWU2 | Mount Lawu | Toh Kuning Village, Karanganyar District | 501 |
| LWU3 | Mount Lawu | Cemoro Kandang hiking track, Karanganyar District | 1,564 |
| LWU4 | Mount Lawu | Cemoro Kandang hiking track, Karanganyar District | 1759 |
| MRP1 | Mount Merapi | Kledokan Hamlet, Umbulmartani, Sleman District | 431 |
| MRP2 | Mount Merapi | Cepogo, Boyolali District | 977 |
| MRP3 | Mount Merapi | Samiran, Selo Sub-district, Boyolali District | 1,585 |
| MRP4 | Mount Merapi | Lencoh, Selo Sub-districtBoyolali District | 1,901 |
| MBB1 | Mount Merbabu | Sidomulyo, Ampel Sub-district, Boyolali District | 621 |
| MBB2 | Mount Merbabu | Sumogawe, Getasan Sub-district, Semarang District | 1,075 |
| MBB3 | Mount Merbabu | Cuntel hiking track, Semarang District | 1,560 |
| MBB4 | Mount Merbabu | Cuntek hijing track, Semarang District | 1,913 |
| UNG1 | Mount Ungaran | Sidomukti Village, Bandungan, Semarang District | 1,064 |
| UNG2 | Mount Ungaran | Sidomukti Village, Bandungan, Semarang District | 1,277 |
| UNG3 | Mount Ungaran | Sidomukti Village, Bandungan, Semarang District | 1,406 |
| UNG4 | Mount Ungaran | Sidomukti Village, Bandungan, Semarang District | 554 |
| SMB1 | Mount Sumbing | Parakan Sub-district,Temanggung District | 493 |
| SMB2 | Mount Sumbing | Kajoran Sub-district, Magelang District | 1,134 |
| SMB3 | Mount Sumbing | Kajoran Sub-district, Magelang District | 1,516 |
| SMB4 | Mount Sumbing | Kajoran Sub-district, Magelang District | 2,080 |
| SDR1 | Mount Sindoro | Parakan Sub-district,Temanggung District | 801 |
| SDR2 | Mount Sindoro | Ngadireho Sub-district, Temanggung District | 1,027 |
| SDR3 | Mount Sindoro | Candiroto Sub-district, Temanggung District | 1,608 |
| SDR4 | Mount Sindoro | Sigedang hiking track,Wonosobo District | 1,961 |
| PRU1 | Mount Prau | Kejajar Sub-district, Wonosobo District | 1,345 |
| PRU2 | Mount Prau | Kejajar Sub-district, Wonosobo District | 1,547 |
| PRU3 | Mount Prau | Dieng Kulon, Wonosobo District | 2,215 |
| PRU4 | Mount Prau | Kalilembu hiking track,Wonosobo District | 2,280 |
| SLM1 | Mount Slamet | Mrebet Sub-district, Purbalingga District | 708 |
| SLM2 | Mount Slamet | Karangrejo Sub-district, Purbalingga District | 1,055 |
| SLM3 | Mount Slamet | Bambangan hiking track, Purbalingga District | 1,511 |
| SLM4 | Mount Slamet | Bambangan hiking track, Purbalingga District | 1,829 |

**DNA isolation and PCR procedures**

Isolation of genomic DNA was done using Geneaid™ DNA Isolation Kit according to manufacturer's procedure. Quantification of DNA was carried out using Thermo Scientific™ Multiskan Sky Microplate Spectrophotometer. The genomic DNA quality was measured based on its absorbance at wavelengths of 260 nm and 280 nm (λ260/280). Amplification of DNA using SSR primers of mCaCIR002 (Mathavaraj and Sabu 2021): 5'-CCACAGGTAACACCG AAT-3' (forward) and 5'-GCACTTGCACTATCTGGAA-3' (reverse) was performed at a total volume of 50 µL consisted of 30 ng/µL of genomic DNA, 10 µM of each primers (forward and reverse), 25 µL of PCR buffer (MyTaq HS Red Mix), and 22 µL of nuclease-free water. The PCR was done using Bio-Rad™ T100 thermal cycler using amplification program of pre-denaturation at 95°C for minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 10 seconds, and final extension at 95°C for 5 minutes. Visualization of PCR products was carried out on agarose gel stained with SYBR dye, with electrophoresis programmed at 100 Volt for 30 minutes. The agarose gel was documented using Gel Documentation System, and the image was saved as jpeg format. The DNA sequencing using Sanger method was performed at 1st Base Laboratory, Malaysia. This study aims to reveal SSR (microsatellite) variability as a molecular marker for assessing genotypic diversity, and SSR variability in the form of differences in the number of motif repeats (CTn) can only be detected through analysis of DNA sequences.

**Analysis of DNA sequences**

The alignment of SSR sequences was carried out using MEGA 11 for the analysis of microsatellite variations. Analysis of genetic variation was done using Mesquite, MEGA, DnaSP, and NoteTab Light software as well as the Network program to generate a haplotype network.

## RESULTS AND DISCUSSION

**Variation in microsatellite repeat number**

Amplification of genomic DNA using the SSR primers mCaCIR002 resulted in single fragment of 180 bp for all 32 accessions of *C. asiatica*. Analysis of SSR sequences showed variations on $(CT)_n$ repeats observable at the position of 102-145. The microsatellite motifs of $(CT)_n$ varies from 10 to 19 repeats, with the most frequent one is $(CT)_{18}$ (Table 2). The analysis of variation on SSR sequences as a high-throughput molecular technique is claimed to be more accurate compared to SSR fingerprinting technique due to its ability to detect variations among individuals, groups, or populations (Viruel et al. 2018; Li et al. 2021). The SSR sequences amplified using mCaCIR002 primers in this study showed high variability in the number of $(CT)_n$ repeats representing microsatellite polymorphism become strong evidence on the intraspecific variability in *C. asiatica*. Previous studies showed that microsatellites polymorphism caused by mutations can be used as genetic markers for evolutionary studies, phylogenetic analysis, gene flow, paternity analysis, and population structure analysis (Milner et al. 2013; Mehrotra and Goyal 2014; Putman and Carbone 2014; Vieira et al. 2016; Susandarini et al. 2020).

The microsatellite polymorphisms examined in this study in the form of reduction or addition of repeat units among different accessions was in line with Vieira et al. (2016) who mentioned that different individuals might show microsatellite variations indicated by differences in the number of repeats unit. Based on the alignment of SSR sequences from 32 accessions, it is evident that differences in microsatellite length were caused by substitutions, deletions and insertions at various positions. Among the three causes of variation, the insertions and deletions are more common than nucleotide substitutions. Insertions and deletions generally occured at the end of microsatellite repeats, especially at the 3' end. Putman and Carbone (2014) noted that changes in microsatellite length are generally caused by temporary dissociation of DNA strands during the replication process, and resulting in misalignment during the formation of double-stranded DNA. In this study, analysis of SSR polymorphism was carried out using sequencing method. In this regard, genotypic variability revealed from the SSR sequencing method produces better resolution than those obtained from DNA fingerprinting profiles, as mentioned by Šarhanová et al. (2018).

Variations in the number of microsatellite sequence repeats $(CT)_n$ found in this study indicate genotypic diversity in *C. asiatic*a. The results of this study support the use of microsatellites in taxonomic study of plant taxa in Apiaceae family such as *Foeniculum vulgare* and *Daucus carota* (Maksylewicz and Baranski 2013; Scariolo et al. 2022). The knowledge of genotypic diversity is very important to provide genetic profiles in terms of population structure and allele richness that is useful for breeders in using genetic resources in plant species of interest. In this regard, Mathavaraj and Sabu (2021) noted that plant genetic variation at intraspecific level found in natural populations has important role in facing various environmental conditions. Moreover, the success of any genetic conservation program depends on accurate documentation of variations within and between populations.

The molecular marker used in this study is part of the Expressed Sequence Tags-Simple Sequence Repeat (EST-SSR), as confirmed by Sahu et al. (2015), that $(CT)_n$ repeats were the most dominant motif accounting for 64.03% of all dinucleotide repeats, and occupying a proportion of 10.6% of all SSRs. A high percentage of $(CT)_n$ repeats in ESTs was also found in pineapple with 33.2% all SSRs, and 86.5% of all dinucleotide repeats (Wöhrmann and Weising 2011). Results of this study showed that variations in microsatellite sequences have the potential to be developed into molecular markers at intraspecies level. The application of intraspecies markers can be used to differentiate between cultivars or natural populations of *C. asiatica*. An indication for this potential can be seen in *C. asiatica* accessions from Mount Merapi (MRP1, MRP2, MRP3, MRP4) and Mount Merbabu

(MBB2, MBB3, MBB4), in which populations from the two volcanoes located in close geographical area have similarity in microsatellite type of $(CT)_{18}$. This finding was in line with previous studies indicated that microsatellites can be developed into specific markers (Sahu et al. 2015; Wang et al. 2021).

**Genetic profiles of *Centella asiatica* based on SSR**

Analysis of genetic variation using DnaSP showed that the 32 populations of *C. asiatica* have four haplotypes in each geographical areas represented by different mountains. This means that the number of haplotypes correspond to the number of populations. The haplotype diversity of each geographical areas is 1.00, and nucleotide diversity ranges from 0.146 to 0.288 (Table 3). The haplotype diversity value of 1.00 indicated high haplotype variation between populations. This result is in line with those reported by (Mathavaraj and Sabu 2021) on *C. asiatica* from India. High haplotype variation indicates high genetic diversity, which is very important for plants species in the process of adaptation and long-term survival to environmental changes. In addition, high genetic diversity in a population will reduce the risk of species extinction through the ability to survive or adapt to environmental changes (Nonić and Šijačić-Nikolić 2021; Mubarok et al. 2023). This is in accordance with the result of this study, that *C. asiatica* has the ability to grow in various types of habitats and a wide altitude range.

Result of haplotype network analysis in this study (Figure 1) revealed that *C. asiatica* populations in Central Java tend to be close to each other and are connected by different, but not overlapping, mutation lines. In the haplotype network, all populations of *C. asiatica* clearly have their own haplotype, with no haplotype sharing observable between populations. In other words, the analysis of SSR as nuclear DNA markers generated unique haplotype for each population (Storz 2002), and this finding indicated the lack of gene flow between populations. Based on the haplotype network, it is clear that the populations of the eight mountains are different, and based on their position in Figure 1, there are no indications on the relative proximity of populations. In other words, the haplotype network did not show which populations are relatively closer to the other populations from the eight mountains observed in this study. The specific haplotype identities in the four populations on each mountain are also unique and different from each other, and no haplotype sharing was detected. Only the populations from Mount Paru are comparatively closer one to another as indicated by its position on the same network branch.

**Table 2.** Variations on the length of microsatellite $(CT)_n$ repeats

| Accession | Nucleotide sequence at the position of 102 to 145 | Number of $(CT)_n$ |
|---|---|---|
| LWU1 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| LWU2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| LWU3 | TC-CCTCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 19 |
| LWU4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCCC | 17 |
| MRP1 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| MRP2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| MRP3 | TG-CCGCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| MRP4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| MBB1 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCCC | 17 |
| MBB2 | AC-GCACTCTCTCTCTCTCTCTCTCTCTCTACTCTCTC | 18 |
| MBB3 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| MBB4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| UNG1 | GC-CGCCTCTCTCTCTCTCTCTCTCTC----CCCATTAAGTA | 11 |
| UNG2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| UNG3 | GC-CGCCTCTCTCTCTCTCTCTCTCTC----CCCATTAAGTT | 11 |
| UNG4 | GC-CGCCTCTCTCTCTCTCTCTCTCTC------CCCTATATT | 12 |
| SUM1 | GC-CGCCTCTCTCTCTCTCTCTCTCTC------CATTAAGTT | 11 |
| SUM2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCT---CTCTCTCTCTC | 17 |
| SUM3 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CCCTCTAA-TA | 15 |
| SUM4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCC---CCACCTAAGTA | 12 |
| SDR1 | GC-CGCCTCTCTCTCTCTCTCTCTCTC----CCCATTAAGTT | 11 |
| SDR2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| SDR3 | GC-CGCCTCTCTCTCTCTCTCTCTCTC--------CCAATTA | 11 |
| SDR4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTCTCTC | 18 |
| PRU1 | GC-CGCCTCTCTCTCTCTCTCTCTCTCCC------CATTAAGTT | 10 |
| PRU2 | GCTCGCCTCTCTCTCTCTCTCTCTCTCTCTCCCCCATTAATA | 13 |
| PRU3 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCTCCCCATTAAGTA | 13 |
| PRU4 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCTCCCCATTAAGTT | 13 |
| SLM1 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCTCCCCATTAAGTT | 13 |
| SLM2 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCTCCCCATTAAGTT | 13 |
| SLM3 | GC-CGCCTCTCTCTCTCTCTCTCTCTCTCT-CTCTCTACTTC | 17 |
| SLM4 | GC-CGCCTCTCTCTCTCTCTCTCTCCC------CATTAAGTT | 11 |

**Table 3**. Genetic profiles of *Centella asiatica* inferred from analysis of SSR mCaCIR002 sequences

| Parameter | Geographical area | | | | | | | | All accessions |
|---|---|---|---|---|---|---|---|---|---|
| | Mount Lawu | Mount Merapi | Mount Merbabu | Mount Ungaran | Mount Sumbing | Mount Sindoro | Mount Prau | Mount Slamet | |
| n | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| Nh | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 32 |
| S | 52 | 55 | 64 | 61 | 56 | 47 | 36 | 40 | 67 |
| Hd | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| π | 0.200 | 0.176 | 0.288 | 0.264 | 0.262 | 0.206 | 0146 | 0.153 | 0.288 |

Notes: n: Number of population, Nh: Number of haplotype, S: Number of polymorphic sites, Hd: Haplotype diversity, π: Nucleotide diversity

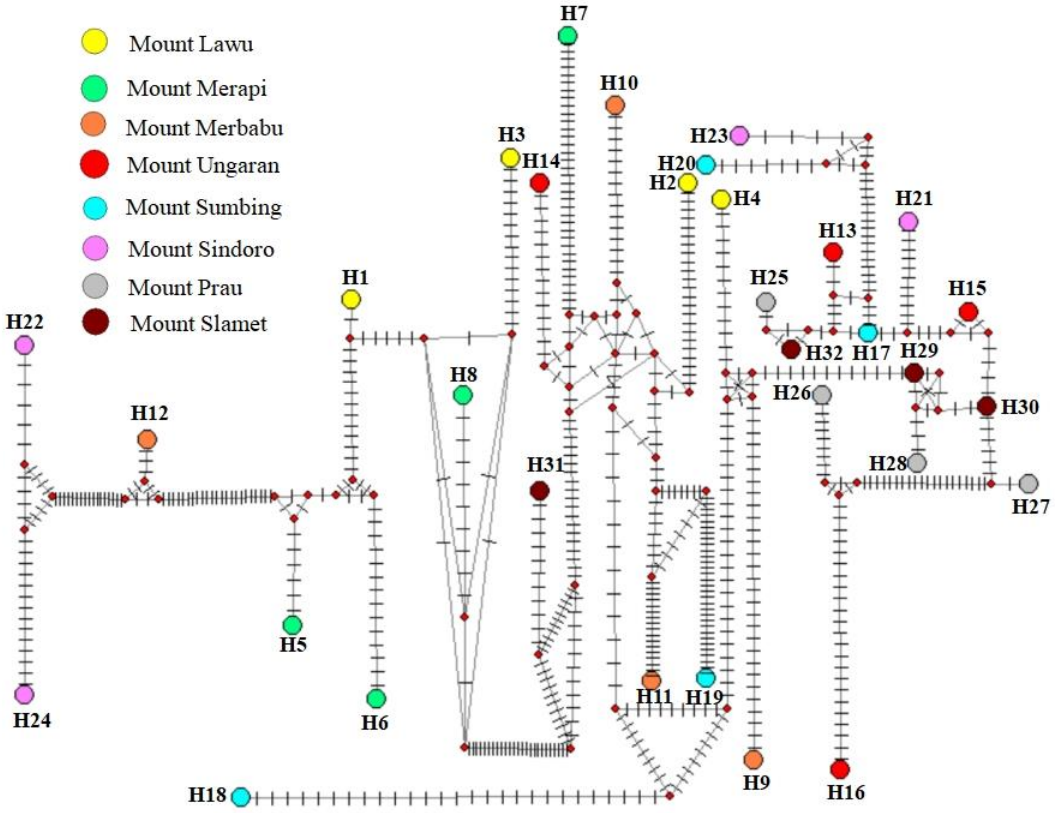


**Figure 1**. Haplotype network of *Centella asiatica* populations

The haplotype network analysis is useful to describe the relationship between haplotypes or DNA sequences in a population (intrapopulation) or among populations within a particular species (intraspecies) as mentioned by Teacher and Griffiths (2011) and Paradis (2018). The application of haplotype networks for analyzing genetic diversity in plant populations within one species and for visualizing genealogical relationships at the intraspecies level was noted by Hapsari et al. (2020). Haplotype network analysis based on SSR by Kongjaimun et al. (2023) is reported to be useful in tracing the origins and history of *Lablab purpureus* domestication. Results of these studies show the suitability of haplotype network analysis to evaluate genetic diversity at the intraspecies level which is useful in taxonomy, for breeding programs in cultivated plants, and for determining appropriate conservation based on phylogenetic relationship.

In conclusion, sequence analysis on SSR provides evidence of genotypic diversity in *C. asiatica* as indicated by variations in the number of dinucleotide repeats unit of $(CT)_n$. Populations originating from the same localities tend to have consistent numbers of microsatellite repeats, and there are indications of differences in microsatellite repeat profiles in populations from different geographic areas. Genotypic diversity was also demonstrated through haplotype analysis which showed that *C. asiatica* populations originating from the same geographic area did not show haplotype sharing. The results of this study provide a basis for the conservation of natural populations of *C. asiatica*, and open

opportunities for further studies to reveal the relationship between genotype and secondary metabolite content, considering that *C. asiatica* is a plant species used as herbal ingredient in medicine and cosmetic industries.

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