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Short Communications

Intraspecific Variability and Phenetic Relationships of *Centella asiatica* (L.) Urb. Accessions from Central Java Based on Morphological Characters

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ABSTRACT
Centella asiatica (L.) Urb. is a plant species native to Java and one of the main basic materials in traditional and modern medicine. This study is the first to report the intraspecific variation and taxonomic relationships of *C. asiatica* accession from natural populations in Central Java. The purpose of this study was to reveal phenotypic variations of *C. asiatica* populations and to asses phenetic relationships based on morphological characters. Thirty-two accessions of *C. asiatica* were collected from natural populations from eight mountains in Central Java. Observation on vegetative organs resulted in 25 morphological characters as a basis for assessing phenetic relationships using cluster analysis and principal component analysis. Result of cluster analysis showed that the grouping of accessions was not correlated to the localities from where the samples were collected, although there was a tendency that accessions from the same localities grouped in one cluster. The results of this study confirmed the existence of intraspecies morphological variability in *C. asiatica* which was not affected by geographical aspects. Results of principal component analysis indicated that the grouping of accessions was mainly determined by similarities in petiole color, stolon color, leaf margin, petiole length, stolon length, and leaf color. Given that the characters contributing to the grouping of accessions were mainly qualitative characters, the results indicated a genetic basis underlying phenotypic variations of *C. asiatica* accession.

Centella asiatica (L.) Urb. or Asiatic pennywort is a member of Apiaceae family which has a wide range of local distribution in Indonesia. This species has the ability to grow in various habitats and is well adapted to wide range of altitudes. Global distribution of *C. asiatica* covers tropical Asia, Australia, Africa, South America and the Pacific islands (Parker 2014). *C. asiatica* is well-known as medicinal plant having various medicinal properties such as antioxidant, antigastritic, antitumor, wound healing, immuno-modulatory, and antiproliferative effect (Mariska et al. 2015; Gray et al. 2016). Moreover, Arribas-López et al. (2022) mentioned that the use of *C. asiatica* for wound healing was due to its anti-inflammatory effect, so this species also has the potential for the treatment of rheumatoid arthritis. In a review of the pharmacology of the two major secondary metabolites found in *C. asiatica*, Bandopadhyay et al. (2023) noted that in traditional and modern medicine it is especially targeted on neurological problems and dermatitis. This species has even been used as herbal materials for modern medicine and cosmetic products.

The need for *C. asiatica* for the traditional medicine industry nationally reaches 100 tons per year, with an average local factory needing 25 tons per year, of which only 4 tons can be supplied (Vinolina 2018). At present, fresh materials of Asiatic pennywort are still harvested from nature, and considering the high demand for this species, the uncontrolled harvest from wild natural populations might have negative impact on the scarcity of this plant in nature (Vinolina 2018; Vinolina & Sigalingging 2021).

Although *C. asitica* is widely known as a medicinal plant, until now there has been no cultivation of this species in Indonesia as indicated by data from the Agriculture and Plantation Office of Central Java Province (eData 2019) and the Central Bureau of Statistics (BPS 2023) regarding the production of biopharmaceutical plants. Data from these two government agencies showed that *C. asiatica* is not included as species for biopharmaceutical cultivation. Given that *C. asiatica* has wide distribution and ability to grow in a variety of habitats, there is a potential risk of erroneous sampling of this species used as material for medicinal products. The reason behind this risk is mainly because *C. asiatica* is morphologically similar to other species in the same genus or even those from different genera within the family of Apiaceae as mentioned by Maruzy et al. (2020). Two species from two different genera that have morphological similarities with *C. asiatica* are *Hydrocotyle verticillata* and *Merremia emarginata*, as mentioned by Daminar & Bajo (2013) and Subramanian & Subramanian (2013). Concerning its function as a medicinal ingredient, the risk of erroneous sampling become a serious problem. In an effort to overcome this problem, a study on morphological characterization to reveal intraspecific variations of *C. asiatica* is very important. Such study will provide scientific information as a basis for the authentication of this species.

Previous studies on morphological variations of *C. asiatica* were carried out on samples grown in experimental gardens with various treatments, and observations were made on the effects of those treatments on growth and morphology as reported by Bermawie & Purwiyanti (2008) and Mumtazah et al. (2020). Research on morphology, anatomy, phytochemistry, and molecular analysis for the characterization of *C. asiatica* from natural populations has been reported by Subositi et al. (2016) and Maruzy et al. (2020). These two studies aimed to develop an authentication method for *C. asiatica* as raw material for medicinal products, and were not focused on assessing phenotypic variation. Meanwhile, a study on variations in the morphological characters of *C. asiatica* originating from natural populations has been reported by Vinolina (2019) with samples originating from North Sumatra.

Research on the intraspecific variation of *C. asiatica* from various mountains in Central Java is still lacking. This study aimed to reveal the phenotypic variations of *C. asiatica* based on morphological characterization of natural populations originating from eight mountains in Central Java. Determination of sampling sites is made by considering that C. asiatica is a herb that can grow in a variety of habitats and soil types with an altitude range of up to 2,300 meters above sea level (Parker 2014;

Devkota & Jha 2019). Based on this fact, the selected areas for collecting plant materials in this study are the localities that have geographical characteristics of *C. asiatica* habitat, especially in terms of altitude range, which in this case are represented by eight mountains in Central Java. The results of this study will produce a mapping of morphological diversity and scientific evidence on the phenotypic variations of *C. asiatica* which can be used as a basis for formulating recommendations for cultivation programs of this species to meet the needs of materials for herbal-based medicine. The mapping of morphological characteristics was carried out by conducting comprehensive characterization of *C. asiatica* accessions collected from various geographic locations so as to reflect intraspecific variations. Information regarding the intraspecific variations will be the basis for recommending accessions that are suitable for cultivation of this species as herbal ingredients, since differences in accessions characterized by morphotypes have an impact on the content of secondary metabolites (Rahajanirina et al. 2012; Prasad et al. 2014).

Plant specimens were collected from natural populations in eight mountains in Central Java, namely Mount Lawu, Mount Merapi, Mount Merbabu, Mount Sindoro, Mount Sumbing, Mount Slamet, Mount Prau and Mount Ungaran (Figure 1). The fieldwork was carried out from June 2022 to February 2023. Sampling locations were determined based on data and information on the presence of *C. asiatica* which covers the areas of altitude ranges of this species, namely from 400 - more than 2,000 m asl. Four accessions representing populations of *C. asiatica* from different altitudes were collected, resulting in a total of 32 accessions used in this study (Table 1). Living specimens of *C. asiatica* collected as materials for morphological characterization were those that met the criteria of healthy adult individuals representing the general features of the population. From each population, 2-3 duplicate samples were taken for the purposes of characterization and preparing herbarium specimens. Observation of morphological characters was carried out in the field and at the Plant Systematics Laboratory, Faculty of Biology Universitas Gadjah Mada, and Plant Systematics Laboratory, Laboratory of Traditional Pharmaceutical Ingredients (*Laboratorium Bahan Baku Obat Tradisional*),

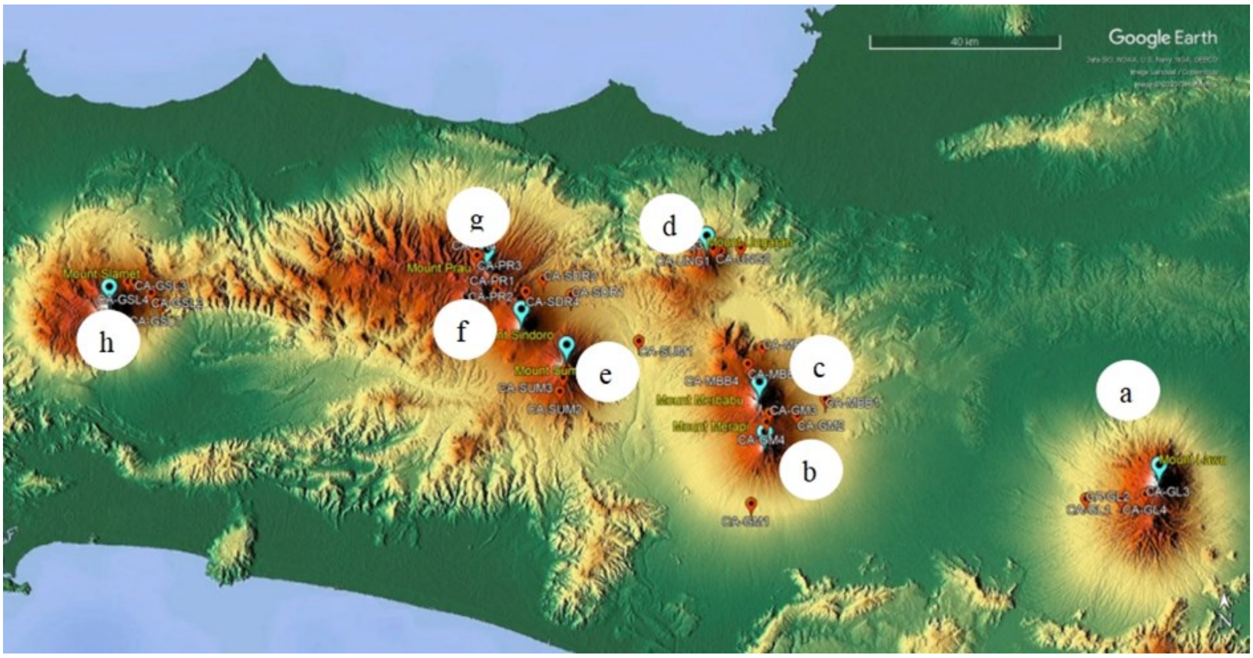

Figure 1. Location of eight mountains in Central Java as sampling sites of C. asiatica accessions. a. Mt. Lawu, b. Mt. Merapi,c. Mt. Merbabu, d. Mt. Ungaran, e. Mt. Sumbing, f. Mt. Sindoro, g. Mt. Prau, h. Mt. Slamet (Google Earth Pro 2023).

Soetarman Co-working Space of National Research and Innovation Agency in Tawangmangu.

Morphological data were obtained by observing characters characterizing the species in Flora of Java (Backer & Brink 1963), The Plant Systematics literature (Simpson 2010), and other characters found in the specimens. Observation of morphological characters was carried out on stolons, petioles, and leaf blades. In this study, no observations were made on flower characters because not all samples were found in flowering phase, but the inflorescence characters can be observed from the remaining parts. All measurements were made based on observation of 10 replicates. Determination of color was carried out referring to RHS Colour Chart 6th version (Royal Horticultural Society 2019).

Table 1. Accessions of *C. asiatica* used in this study.

Accession code	Location	Elevation (m asl)
LWU1	Mount Lawu - Karanganyar Regency	896
LWU2	Mount Lawu - Karanganyar Regency	501
LWU3	Mount Lawu - Karanganyar Regency	1564
LWU4	Mount Lawu - Karanganyar Regency	1759
MRP1	Mount Merapi - Sleman Regency	431
MRP2	Mount Merapi - Boyolali Regency	977
MRP3	Mount Merapi - Boyolali Regency	1585
MRP4	Mount Merapi - Boyolali Regency	1901
MBB1	Mount Merbabu - Boyolali Regency	621
MBB2	Mount Merbabu - Semarang Regency	1075
MBB3	Mount Merbabu - Semarang Regency	1560
MBB4	Mount Merbabu - Semarang Regency	1913
UNG1	Mount Ungaran - Semarang Regency	1064
UNG2	Mount Ungaran - Semarang Regency	1277
UNG3	Mount Ungaran - Semarang Regency	1406
UNG4	Mount Ungaran - Semarang Regency	554
SMB1	Mount Sumbing - Temanggung Regency	493
SMB2	Mount Sumbing - Magelang Regency	1134
SMB3	Mount Sumbing - Magelang Regency	1516
SMB4	Mount Sumbing - Magelang Regency	2080
SDR1	Mount Sindoro - Temanggung Regency	801
SDR2	Mount Sindoro - Temanggung Regency	1027
SDR3	Mount Sindoro - Temanggung Regency	1608
SDR4	Mount Sindoro - Wonosobo Regency	1961
PRU1	Mount Prau - Wonosobo Regency	1345
PRU2	Mount Prau - Wonosobo Regency	1547
PRU3	Mount Prau - Wonosobo Regency	2215
PRU4	Mount Prau - Wonosobo Regency	2280
SLM1	Mount Slamet - Purbalingga Regency	708
SLM2	Mount Slamet - Purbalingga Regency	1055
SLM3	Mount Slamet - Purbalingga Regency	1511
SLM4	Mount Slamet - Purbalingga Regency	1829

Morphological data of *C. asiatica* consisting of qualitative and quantitative characters were analyzed to determine grouping patterns and establish phenetic relationships between accessions using numerical taxonomy methods namely cluster analysis and principal component analysis. The degree of similarity between accessions was determined using the Euclidean distance, followed by clustering using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) method to produce a dendrogram. Assessment of characters contributing to the formation of clusters was done using principal component analysis.

Cluster analysis and principal component analysis were performed using PAST software version 4.13.

Observations on the morphology of 32 accessions of *C.asiatica* resulted in 25 characters used in determination of taxonomic relationships between accessions. Among the 25 characters, 13 were obtained from leaves, 6 from petioles, 4 from stolons, and 2 from inflorescences. Considering that the leaves are the part used as a medicinal ingredient, characterization of this organ is very important and need to be carried out in detail. Leaf shape in all accessions was reniform although there was slight variation when calculating the ratio of the width to the length of the leaf blade. In the four organs observed, the highest variation was found in color, including leaf color, petiole color, and stolon color. The color of the leaf upper surface varied from medium green to light green, while the color on the lower surface varied from medium green to medium yellow-green. Characters that also showed prominent variations in leaves were the leaf margin, namely crenate and dentate. Higher color variations were found in petioles and stolons, ranging from light green, medium green, medium yellow-green, light brown, medium brown to red-brown. The morphology of accessions representing each location, namely from eight mountains in Central Java, is shown in Figure 2. Detail

Figure 2. Morphology of *C. asiatica* representing eight locations of sample origin: (A) Mount Lawu; (B) Mount Merapi; (C) Mount Merbabu; (D) Mount Ungaran; (E) Mount Sumbing; (F) Mount Sindoro; (G) Mount Prau; (H) Mount Slamet.

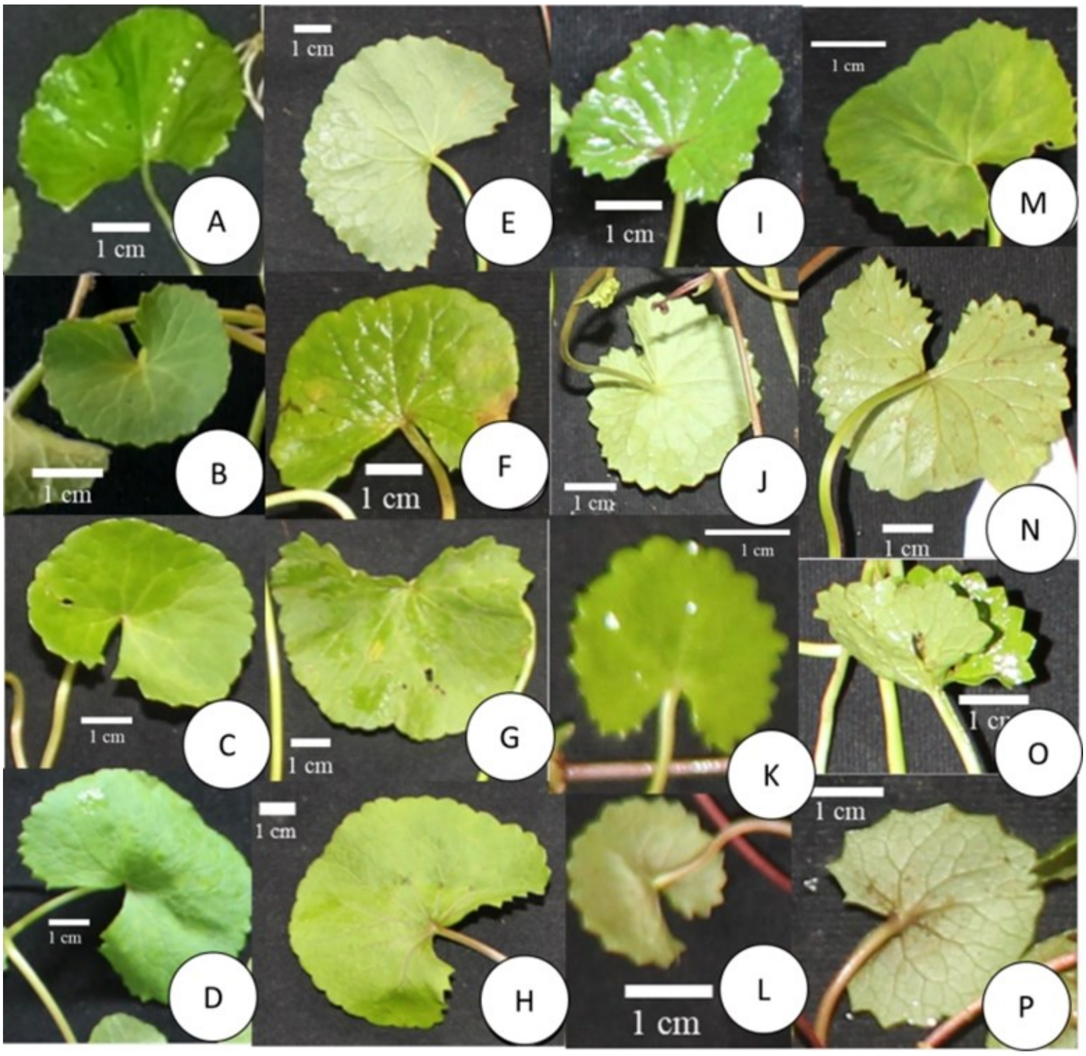

Figure 3. Variations in the shape of the leaf margins of *C. asiatica* representing the several accessions used in this study (Remarks: Leaf margins edged = (A) LWU1; (B) LWU2; (C) LWU3; (D) LWU4; (E) SLM1 ; (F) SLM2; (G) SLM3; (H) SLM4. Leaf margin toothed = (I) MRP4; (J) MBB3; (K) UNG1; (L) UNG2; (M) UNG3; (N) PRU1; (O) PRU2; (P) PRU4).

photographs of *C. asiatica* leaves showing variations on leaf shape and leaf margins of representative accessions are displayed in Figure 3.

Result of cluster analysis based on 25 morphological characters showed the grouping of 32 accessions into three clusters (Figure 4). Cluster I consisted of three accessions, namely one from Mount Sumbing and two from Mount Lawu. Cluster I was formed at a branching point which was clearly separated from clusters II and III. The members of this cluster were characterized by crenate leaf margin as a morphological feature that clearly distinguished it from members of clusters II and III. Apart from the leaf margin, members of cluster I generally had leaves with medium to dark green color on their lower surface, while members of clusters II and III generally had light green in color. In terms of color, the stolons also showed notable differences, in which members of cluster I had stolon with light to medium green in color, while the accessions in clusters II and III showed a more varied range of stolon colors from green, orange, brown to red.

In the dendrogram, 29 of the 32 accessions in this study formed clusters II and III, with relatively closer relationship between the two compared to cluster I. Observations of leaves, petioles, and stolons showed that members of cluster II could be distinguished from members of cluster III especially on the color of the petioles and stolons. The petiole and stolons of accessions in cluster II had a wide range of colors from light green, medium green, medium yellow-green, to medium orange. Meanwhile, the petioles and stolons belonging to cluster III showed color variations from light brown, medium brown, orange-brown, brown-red, and dark purple red to dark purple.

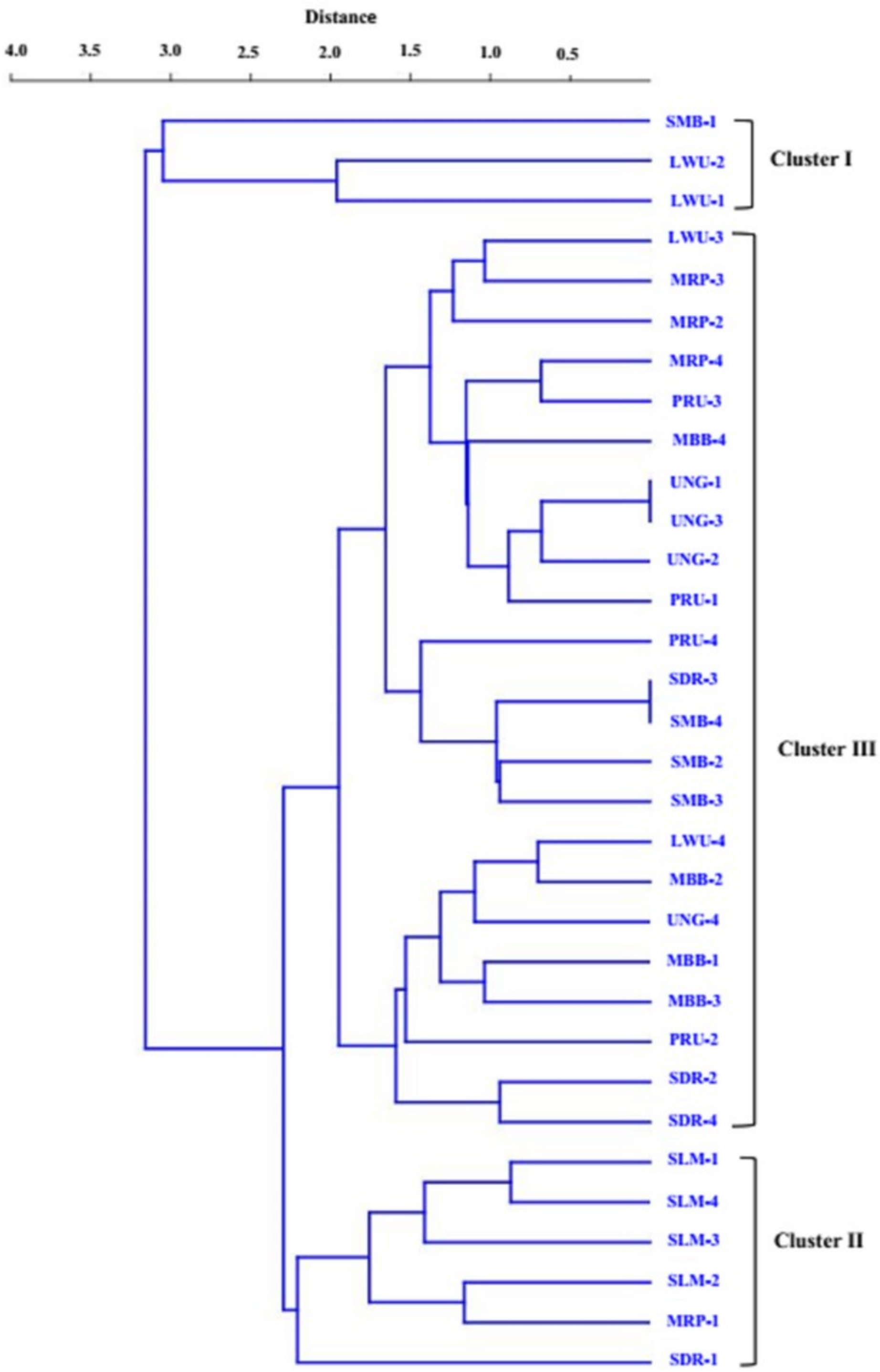

Figure 4. Dendrogram of *C. asiatica* accessions based on morphological characters.

The leaf length varied from 1.5 to 4.1 cm, and most of the accessions have leaves with a length of 1.5-2.3 cm, while nine accessions have 2.4-3.2 cm leaf length, and the longest leaf is found in SMB1 accession with a leaf length of 4.1 cm. Leaf width characters showed variations from 2.8 to 6.3 cm. Most of the accessions have leaf width of 2.8-3.9 cm, ten accessions with leaf width of 4.0-5.1 cm, and the widest leaves are found in accessions SMB1, MBB2 and UNG4. The size of the leaf width in these three accessions is probably caused by exposure to fertilizers or nutrients from the environment, because the three accessions were found in areas close to rice fields. The variation in the length of the petiole was from 5.1 to 18.8 cm, while the variation in the length of the stolon was 7.5 to 23.0 cm.

Based on the observations of morphological variations, and to confirm the contribution of characters in the grouping of accessions, a principal component analysis (PCA) was carried out. In plant taxonomy studies PCA was generally used to provide a basis for recognizing distinguishing characters between groups. The results of principal component analysis were presented as character loadings (Table 2) indicated the contribution of the characters in forming the grouping of accessions. In this study character's loadings that showed an absolute value of > 0.2 were considered as characters that had important roles in grouping *C. asiatica* accessions. In Table 2 there were nine characters that have a relatively large contribution in the grouping of accessions into three clusters as shown in the dendrogram. These characters were leaf width, color of the leaf upper surface, petiole color, petiole length, and stolon length. The role of these characters was indicated by the loadings values of > 0.2 on the first and second axes in Table 2 This result indicated that these five characters were those differentiated cluster I from clusters II and III collectively. The characters that both showed high loadings on both axis 1 and axis 2 were not only considered as having direct contribution to distinguishing cluster I from clusters II and III, but also differentiating between cluster II and cluster III.

The morphological variability found in this study was in line with those reported in several studies from other regions. Research on *C. asiatica* by Sudhakaran (2017) for the purpose of identifying diagnostic characters showed that leaf shape, leaf margin, enlargement at the base of petiole, stolon length, and stolon color were characters to recognize this species. The characterization study of *C.asiatica* for determining potential accessions for cultivation as a medicinal plant material was reported by Chachai et al. (2021) on 30 accessions from Thailand based on 11 morphological and agronomic characters, which showed that variation between accessions was mainly found in leaf number, leaf length, leaf width, shoot number and stolon number. Variation on leaf margin found in this study as a distinguishing character between clusters align with the report of Chua et al. (2022) on characterization of *C. asiatica* based on a computational analysis on leaf morphology which noted that leaf margin were found to be an easily recognizable character for identification. The research was used as a basis for differentiating *C. asiatica* from *Hydrocotyle verticillata*, a species that is often mistakenly recognized as *C. asiatica* because of the similarities between the two species.

In general, the result of cluster analysis showed that accessions originating from the same location, in this case represented by a mountain, were not always placed in the same cluster. In the dendrogram, it can be seen that there were only few accessions that formed groups based on their geographical origin. The accession from the same origin which formed notable groups were three accessions from Mount Ungaran, (UNG-1, UNG-3, UNG-2), and three accessions from Mount Sumbing (SUM-3, SUM-2, SUM-4) which become the members of cluster III. The same phenomenon was found in four accessions from Mount Slamet, all of which were in cluster II. The three accessions from Mount Ungaran were similar to one another, indicated by their position in one branch of the dendrogram. The level of similarity of these three accessions was higher than those of Mount Sumbing and Mount Slamet, which, although they were grouped in the same cluster, they were not in the same branches of the dendrogram. In other words, the grouping of *C. asiatica* accession in this study was influenced more by their morphological similarity, and not by geographical origin. The grouping pattern of accessions that did not match the geographical origin indicated that there was a genetic basis underlying the morphological variability found in *C. asiatica*. Patterns of population grouping that showed no relation to geographical area were also reported in other species, including *Musa* sp. cv. Rastali from Peninsular Malaysia based on cluster analysis and principal component analysis (Putra et al. 2010). The same result was reported in *Cyamopsis tetragonoloba* in which the grouping of samples based on cluster analysis and principal component analysis on morphological characteristics was not related to their geographical origin (Manivannan et al. 2015).

The results of this study indicated that there was notable infraspecies variation in *C. astiatica*, and most of the variability encountered was in qualitative characters. Variations in qualitative characters found in this study, especially leaf margin, petiole color, and stolon color, were morphological characters that were determined by genetic factors. This indication was supported by the results of cluster analysis which showed the grouping of accession which were in general not influenced by the geographic origin of accessions. The results of this study not only confirmed that *C. asiatica* is a species with high morphological variability, but also

Table 2. Character loadings, eigenvalues, and percentage of variance resulted from PCA.

No	Code	Character	PC 1	PC 2
1	LSH	Leaf shape	0.006	-0.069
2	LAP	Leaf apex	0.006	-0.069
3	LMG	leaf margin	**-0.209**	-0.161
4	LBS	Leaf base	0.006	-0.069
5	LLG	Leaf length	0.174	**0.302**
6	LWD	Leaf width	**0.228**	**0.342**
7	LVE	Leaf venation	0.006	-0.069
8	LUC	Color of upper leaf surface	**0.391**	**-0.462**
9	LLC	Color of lower leaf surface	**-0.287**	-0.083
10	LUT	Trichomes on upper leaf surface	-0.015	-0.156
11	LLT	Trichomes on lower leaf surface	0.006	-0.069
12	LWT	Types of trichomes on lower leaf surface	0.006	-0.069
13	LGF	Leaf growth form	0.045	-0.084
14	PBE	Petiole base enlargement	0.006	-0.069
15	PSH	Petiole shape	0.006	-0.069
16	PCL	Petiole color	**-0.486**	**0.332**
17	PLG	Petiole length	**0.299**	**0.493**
18	PGF	Petiole growth form	0.006	-0.069
19	PTR	Trichomes of petiole	-0.032	-0.079
20	SSH	Stolon shape	0.006	-0.069
21	SLG	Stolon length	**0.345**	**0.246**
22	SCL	Stolon color	**-0.424**	0.187
23	STR	Trichomes on stolon	-0.108	-0.048
24	IFT	Inflorescence type	0.006	-0.069
25	IFP	Inflorescence position	0.006	-0.069
Eigenvalues			1.053	0.509
Variance explained (%)			42.284	20.413
Cumulative variance (%)			42.284	62.697

provide a basis for further research to examine whether the variations found can be recognized as indicators of the existence of morphotypes or ecotypes in *C. asiatica*, as reported from previous studies in other regions. Rahajanirina et al. (2012) documented two morphotypes of *C. asiatica* that grew sympatrically in Madagascar, namely the morphotype with small renifrom leaves and the morphotype characterized by large rounded leaves. Similar results were reported by Prasad et al. (2014) in a characterization study of *C. asiatica* collected from various populations growing in the altitudes range of 116 to 2,050 m asl from India, which showed that there were two morphotypes recognized based on the leaves qualitative characters. Ravi et al. (2019) in a study of 39 accessions of *C. asiatica* from eight locations with different altitudes reported that there were variations in all organs examined, and that differences in the color of the petioles and stolons was claimed as genetic expression of the accessions. In this study it was also known that the color variation of the petiole was the same as that of the stolons, which was also found in the study reported here. Meanwhile, the results of Nav et al. (2021) who also used cluster analysis and principal component analysis found that morphological characters that had considerable role in the classification of three *C. asiatica* ecotypes originating from different geographical areas were leaf length, leaf width, petiole diameter, petiole length, and root per node. It could be concluded here that results of this study clearly showed the intraspecific morphological variations of *C. asiatica* which was not influenced by differences in the habitat and geographical origin.

AUTHORS CONTRIBUTION

A.M. designed the research, collected and analyzed the data, and wrote the manuscript; R.S. designed the research, analyzed the data, wrote the manuscript, and supervised all the process.

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CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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