Phenotypic characterization of the Rwandan stinging nettle (*Urtica massaica* Mildbr.) with emphasis on leaf morphological differences.

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1. Introduction

The common stinging nettle is a pervasive, wild, herbaceous, and dioecious perennial plant in the family of *Urticaceae*, growing in nitrogen-enriched habitats, widely available in tropical and temperate regions all over the world (Mamta & Preeti, 2014; Ahmed & Parsuraman, 2014). The common stinging nettle is mostly found in moist, damp soils, shady and waste places, non-native grasslands, gravel pits, agricultural fields, and along stream banks. It is believed to have a high potential to meet the nutritional demand of humans for food security. Its crude protein content is bounded from 25.1 to 26.3% and it contains iron, calcium, phosphorus, potassium, sulfur, and magnesium. It is also rich in vitamins A, C, K, D, and B and up to 20% mineral salts, mainly salts of calcium, potassium, silicon, and nitrates (Assefa *et al*., 2013; Dereje *et al*., 2016; Keflie *et al*., 2017). Both drying and cooking methods remove the stinging hairs on leaves. The nettle's nutritive contents from young leaves are traditionally cooked, consumed as a vegetable, and contribute to food security (Di Virgilio *et al*., 2015; Singh & Kali, 2019).The stinging nettle leaves and root powder preparations on market are used for various purposes such as in the treatment of infectious and non-communicable diseases in humans, and even in the stimulation of hair growth. The stinging nettle powder is also commonly found as a component of many shampoos and conditioners, an excellent dietary supplement of poultry, a source of fibers for textiles, and an ingredient in cosmetics (Sharma *et al*., 2018).

The stinging nettle stem is green, erect, hollow solid, fibrous and tough, with occasional thin branches and covered with many stinging hairs and trichomes. The stinging nettle commonly grows between 2 to 4 m tall and is usually found in dense stands. It has simple, serrated green leaves in an opposite pattern, heart-shaped, cordate at the base, and finely toothed. The leaves are 3 to 15 cm long on an erect, wiry green stem. The stinging nettle leaves are covered with stinging hairs when touched injecting irritant chemicals into the skin (Adhikari *et al*., 2016; Bourgeois *et al*., 2016).

The flowers are greenish white or brown and are borne in a terminal cluster at the stem nodes mostly unisexual with male and female flowers on the same or in separate inflorescences, and are wind pollinated. The tiny hard-coated achene nettle fruit is round and contains small dark brown seeds. The root system of the common stinging nettle is made up of a taproot with fine rootlets, which allows it to expand (Joshi *et al*., 2014). The stinging nettle is commonly found in very large patches under favorable conditions (Taylor, 2009). The nettle spreads sexually through seeds and asexually through stoloniferous rhizomes or vegetatively from stem tip cuttings and often forms dense colonies.

Rwanda possesses various species of stinging nettles which have various uses (Nahayo *et al*., 2008). But, the predominant species in East Africa and particularly in Rwanda is believed to be *Urtica massaica* Mildbr.(Grubben, 2004). The majority of the literature describes the genetic diversity of this species and its nutritional potential for both humans and animals (Maniriho *et al*., 2021). However, the information about the morphological characteristics of the stinging nettle in Rwanda remains scanty. Hence there is a need to conduct scientific research to identify the morphological variation of the stinging nettle in its different ecotypes across Rwanda. The main objective of this study was to investigate the phenotypic variation of the Rwandan common stinging nettle (*Urtica massaica* Mildbr.) with emphasis on leaf morphological differences in the lowland, midland, and highland zones of Rwanda. The role of morphological traits in stinging nettle characterization has been intensively investigated elsewhere in the world but it has never been done in Rwanda. Morphological characterization of stinging nettle in Rwanda is very important for the current, and future work as well as for genetic improvement. Phenotypic characterization can also help in the documentation of the genetic variability existing in stinging nettle populations in Rwanda. In fact, morphological traits are important diagnostic features that can be used for distinguishing genotypes.

2. Materials and Methods

2.1 Description of the study area

A field survey and data collection were conducted in September 2021 in twelve Districts of Rwanda through purposive sampling (Figure 1). The sampling sites included four Districts from the highland zone (namely Musanze, Nyabihu, Rubavu, and Rutsiro) where altitudes range between 1800 and 2500 m asl and average annual rainfall range between 1300 and 1600 mm; five Districts) from the midland zone (namely Rulindo, Muhanga, Rubavu, Nyanza and Huye Districts) where altitudes range between 1500 and 2000 m asl and average annual rainfall range between 1000 and 1300 mm; and three Districts from the lowland zone (Rwamagana, Kayonza, and Nyagatare) where altitudes range between 1300 and 1600 m asl and average annual rainfall range between 700 and 1100 mm (Figure 1).

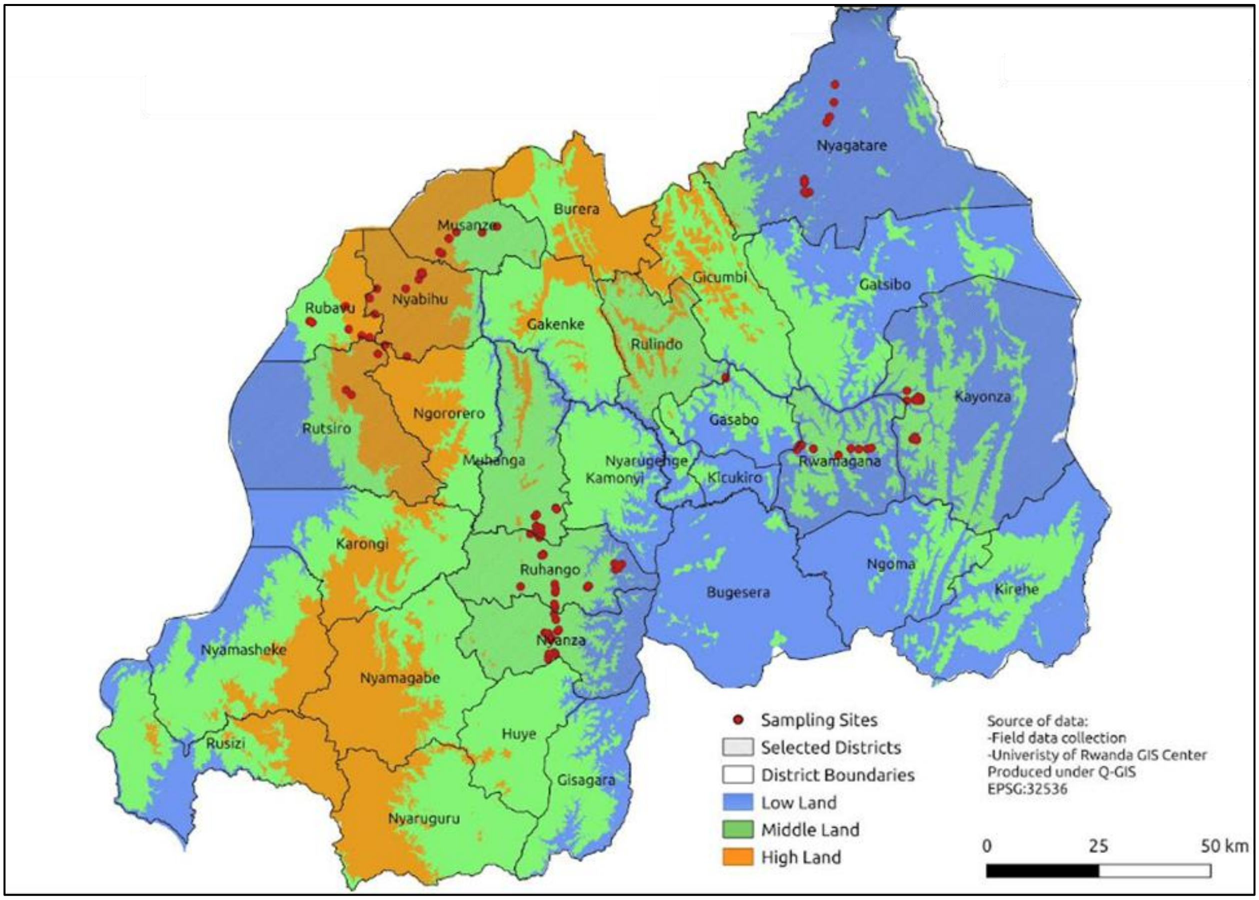

Figure 1: Location of sampling sites (in Lowland, Midland, and Highland zones)

2.2 Collection of relevant data

Qualitative and quantitative data were collected using a checklist of standard morphological descriptors, imaging, and metric data for capturing plant traits. Field surveys across the country in the aforementioned Highland, Midland, and Lowland zones were carried out using a purposive sampling method based on the abundance and availability of different targeted morphological appearances which are useful in the characterization of morphological variation analysis. During fieldwork, some visual features were observed and recorded for the common stinging nettle characterization. These include leaf type, leaf margin, leaf shape, leaf pubescence, presence of stipules, the position of stipules, leaf length, leaf width, leaf surface, leaf color, rooting system, stem posture, stem bark feature, stem stinging nettle abundance, branch posture (tiller), type of flower, type of inflorescence, flower size, flower color, flower composition, the shape of fruits, and seed morphology (Lizawati *et al*., 2018). The quantitative characters including plant height, leaf length, and width, and root length were measured using a measuring tape and the data were later analyzed in the laboratory.

2.3 Imaging and metric data collection of leaves

Images of common stinging nettle leaves were taken using a Nikon D40X camera with an18-55 mm zoom lens in a standardized manner. Early studies showed that the shape of leaves might have a genetic expression (Whitewoods *et al.*, 2020) and could display a divergence along a climate gradient (Bresso *et al.*, 2018; Eisenring *et al.*, 2022). The shape of the leaves is a striking example of the plasticity of plants. Only the dorsal side of all leaf specimens showing prominent veins was photographed. These images were taken on a 20 cm x 15 cm dissection board with a white 21x11 cm paper background. Specimens were centered for the photograph in the same plane as the camera objective lens to avoid optical distortion of the images.The camera was fixed on a vertical support parallel to the ground plane. A scale was included in each picture using plastified millimeter papers of different sizes to allow the acquisition of a scaling factor afterward. A total of 71 leaves were used to collect the data metrics, allowing the detection of size variations between the common stinging nettle's leaf specimens sampled in different locations across Rwanda (Figure 1). Leaves metric data were obtained using Image J software (Schneider *et al.*, 2012) measuring the distances between landmarks (Figure 2).

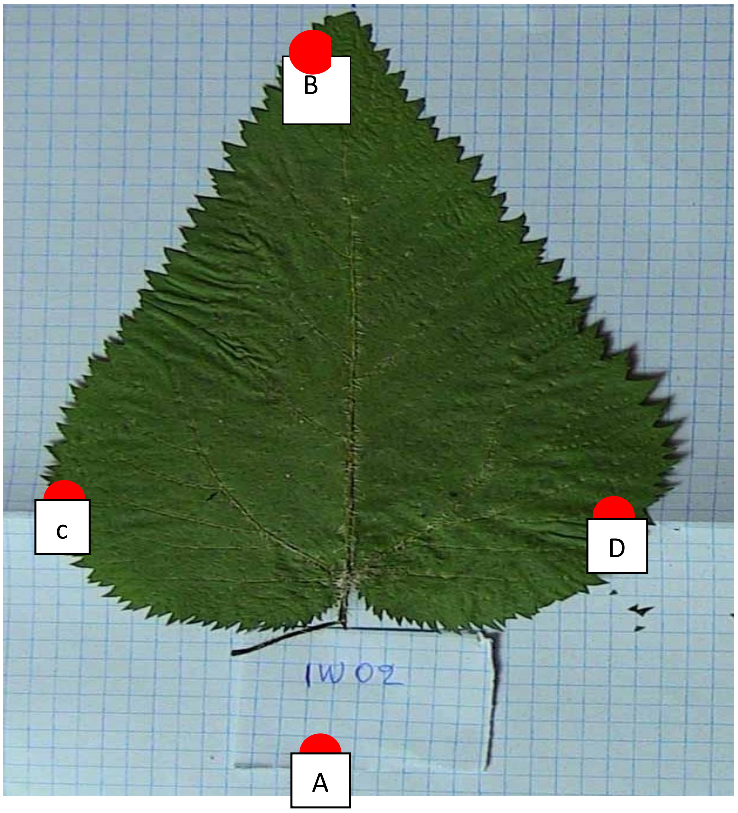

Figure 2: Illustration of collection of data metrics

Key: MV (**Main vein**: a distance between AB); LBV (**left branched vein**: a distance between AC); RBV(**right branched vein**: a distance between AD), and WLR (**width of the leaf**: distance between CD).

In total, eight Operational Taxonomic Units (OTU) were analyzed for the sampled Rwandan common stinging nettle as shown in Table 1.

Table 1: Abbreviations of OTUs and number of specimens used

No	OTUs	Number of specimens	Sampling location	District	Altitude zone
1	IB	13	Bigogwe	Nyabihu	Highland
2	IG	17	Busogo	Musanze	Highland
3	IR	17	Rutsiro	Rutsiro	Highland
4	**IH**	7	Kinihira	Ruhango	Midland
5	IM	3	Muhanga	Muhanga	Midland
6	IW	4	Shyogwe	Muhanga	Midland
7	IJ	7	Barija	Nyagatare	Lowland
8	IZ	3	Zaza	Rwamagana	Lowland
TOTAL		**72**			

Key: IB is specimens from Bigogwe; IG from Busogo; IR from Rutsiro; IJ from Barija; IH From Kinihira; IM from Muhanga; IW from Shyogwe; and IZ from Zaza.

2.4 Analysis of leaf morphological variations

Morphological appearances for phenotypic characterization (Lizawati*et al*., 2018), analysis of variance (ANOVA) for comparing variances across the means of different morphological parameters,and the metric data were recorded in an excel sheet and imported in PAST software for data analysis, then log-transformed (Hammer *et al.*, 2001). To reduce data dimensionality, a principal component analysis (PCA) was run on the linear morphometric dataset of the individual data of the species, and habitats were differently colored (highlighted) in the PAST data table entry. PCA was performed to examine patterns of morphological variation of the species related- habitats types. The test for normality for the linear measurements showed that leaf morphological variations in the species were not normally distributed ($p < 0.05$). Consequently, the linear morphometric data were subjected to a non-parametric test, MANOVA (Anderson, 2001) using PASTA (Hammer *et al.*, 2001). This non-parametric multivariate analysis of variance (NP MANOVA) was used to test for significant differences in the distribution of habitat types for all populations in morpho-space because the assumptions of multivariate normality were not met. The non-parametric MANOVA is an equivalent design to an ANOVA that allows testing multiple factors,and interactions and relies on a permutation procedure.

3. Phenotypic characterization of the Rwandan common stinging nettle

3.1 Morphological descriptors

All the 124 samples collected from the three altitudinal zones (40 from Highland and 45 from Midland and 39 from lowland) were used for qualitative analysis, while 72 samples were used for leaf anatomy analysis, and only 22 samples for quantitative traits analysis. The vegetative traits utilized in studying morphological characterization of stinging nettle in all agroecological zones include plant length, leaf length, leaf width, and root length. The measured nettle plant height varied from about 1 to 4.5 m. The tallest sample of stinging nettle was observed in the samples collected from the midland zone (4.5 m). The stinging nettle plant heights in the samples from highland, midland and lowland were significantly different (F calculated value: 4.70 > F value from table (critical): 3.52).

The average leaf length was highest in the lowland (19 cm) and the lowest was recorded in the Highland (5.14 cm). These differences were significantly different (F calculated value: 10.19 > F value from table: 3.52). The average leaf width was highest in the midland (13.33 cm) and the lowest was in the highland (7.79). However, these differences were not statistically significant (F calculated value: 2.475 < F value from table: 3.52). The average flower size was highest in the lowland (3.14 cm) and lowest in the midland (1.67 cm). However, these differences were also not statistically significant (F calculated value: 1.21 < F value from table: 3.52). The average root length was the highest in the midland (6.67 cm) (Table 2).

In all the studied samples, the leaves were simple, dark green, and facing each other in opposite patterns. The bark of the stinging nettle plant stem was thin at the top and thick at the bottom. The type of shoot growth was erect with branched lateral shoots while the wood anatomy was semi-woody. In morphological appearance, the inflorescence maintains green leaves throughout the year. The leaf pubescence was glandular, the leaf venation was pinnate, the leaf margin was serrated, the phyllotaxy was opposite, and the types of stipules were persistent. All these features are characteristic of *Urtica massaica* Mildbr.

The petiole was moderately long and arises from a leaf axil with two linear stipules at the base. In general, the leaves were ovate to lanceolate in shape, with a shallowly chordate base and acuminate tips. All the above descriptions qualify the surveyed common stinging nettle to be *Urtica massaica* Mildbr.Unfortunately, all the common stinging nettle samples sureveyed then had flowers but no seeds

Table 2: Descriptive morphological features of the common stinging nettle plant samples

Variable	**Class**	**Altitude zones**		
		Highland	**Midland**	**Lowland**
		Frequency (n)	Frequency (n)	Frequency (n)
Plant height (m)	0-2	14	2	2
	2-4	0	1	0
	4-6	0	3	0
	Mean	**1**	**3.3**	**1**
	Std	**0**	**1.97**	**0**
Leaf width (cm)	0-4	6	0	0
	5-9	4	0	1

	10-14	1	4	1
	15-19	3	2	0
	Mean	**7.85**	**13.33**	**9.5**
	Std	**10.64**	**2.6**	**3.54**
Leaf length (cm)	0-4	10	1	0
	5-9	2	0	0
	10-14	0	0	0
	15-19	2	5	2
	Mean	**5.14**	**16.17**	**19**
	Std	**5.91**	**6.94**	**0**
Root length(cm)	0-2	12	2	2
	3-5	0	0	0
	6-8	0	1	0
	9-11	2	3	0
	Mean	**2.29**	**6.67**	**2**
	Std	**3.27**	**4.42**	**0**
Flower size (cm)	0-2	4	8	2
	3-5	1	0	5
	6-8	1	1	0
	Mean	**2.5**	**1.67**	**3.14**
	Std	**2.51**	**4.38**	**2.02**

(a) (b) (c)

Figure 3: Samples of common stinging nettle from a) Highland, b) Midland and c) Lowland

3.2. Leaf morphological variations of collected samples of the common stinging nettle

The measurements illustrating the phenotypic variation of the Rwandan common stinging nettle across surveyed sites in the highland, midland and lowland zones are summarized in Table 3.

Table 3. Measurements of leaf morphological differencesof collected stinging nettle samples.

Zone	Sample site	OTUs	MV (cm)	LBV (cm)	RBV (cm)	WLR (cm)
Highland	**Bigogwe (IB)**	Mean	14.44	7.83	7.69	7.40
		Max	16.25	9.40	8.65	9.51
		Min	11.74	7.27	7.23	6.16
		Std	1.37	0.73	0.53	0.92
Highland	**Busogo (IG)**	Mean	8.92	5.19	4.87	5.19
		Max	10.50	5.87	6.14	6.30
		Min	7.08	4.27	3.81	4.39
		Std	0.92	0.55	0.70	0.49
Highland	**Rutsiro (IR)**	Mean	13.76	7.37	7.21	7.59

		Max	16.07	8.61	8.67	8.85
		Min	9.86	5.40	4.96	5.51
		Std	1.61	0.80	0.91	0.88
Lowland	**Barija (IJ)**	Mean	10.27	5.71	5.38	5.31
		Max	11.61	6.45	6.00	6.48
		Min	8.32	4.83	4.82	4.04
		Std	1.12	0.59	0.45	0.91
Midland	**Ruhango (IH)**	Mean	13.43	7.51	7.34	6.81
		Max	17.72	9.13	10.13	8.42
		min	10.15	5.32	5.27	5.26
		Std	2.80	1.49	1.78	1.18
Midland	**Muhanga (IM)**	Mean	18.09	7.39	8.00	10.37
		Max	19.23	7.65	8.87	11.71
		Min	16.84	6.97	6.80	9.17
		Std	1.20	0.37	1.07	1.28
Midland	**Shyogwe (IW)**	Mean	26.78	12.28	13.81	18.58
		Max	28.62	14.23	15.24	20.30
		Min	24.96	11.25	12.64	17.13
		Std	1.52	1.33	1.13	1.31
Lowland	**Zaza (IZ)**	Mean	22.22	9.83	10.80	15.93
		Max	23.55	10.62	12.73	16.53
		Min	21.16	9.10	9.25	15.14
		Std	1.22	0.76	1.77	0.71

Key: Abbreviations in the brackets were used for analyzing morphospace in OTUs. As defined in Figure 2, MV (Main vein-AB); LBV (left branched vein -AC); RBV (right branched vein-AD and WLR (width of the leaf - CD); and Std (standard deviation).

Different OTUs of the Rwanda common stinging nettle samples collected in the three altitudinal zones differed insize (linear traits were size-corrected) expressed with 95.58 % in PC1 (Figure 4). Their shape differences were expressed with little variation of 3.29 % in PC2. A CVA scatter plot unveiled OTUs in four morphospaces (Figure 4). The convex hills in different colors illustrate the morphospace of each operational taxonomic units studied with acronyms defined in Table 2 as follows IB (sample from Bigogwe in red); IG (from Busogo in purple); IR (from Rutsiro in blue); IJ (from Barija in magenta); IH (from Kinihira in brownish green), IM (from Muhanga in dark red); IW (from Shogwe in yellow); and IZ (from Zaza in green).

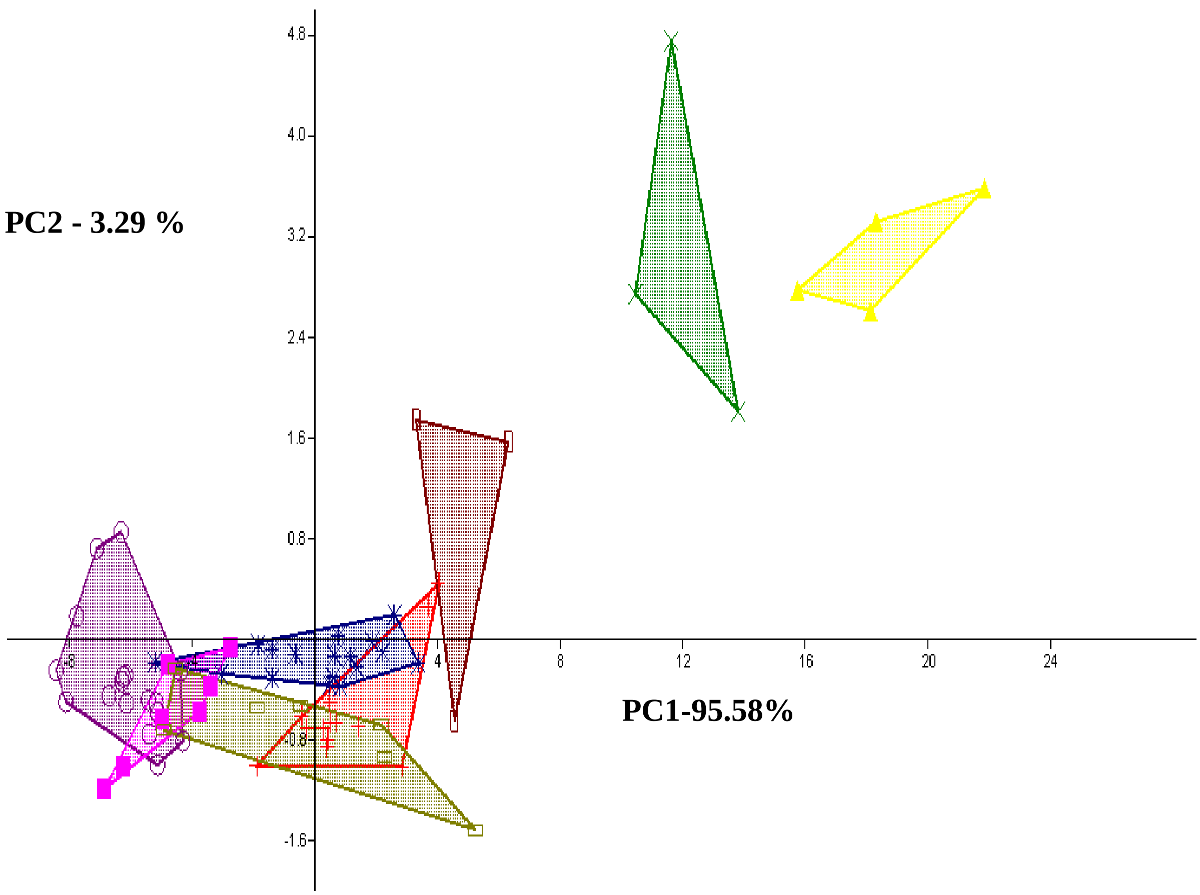

Figure 4: PCA scatter plot of OTUs in morphospaces of the Rwandan stinging nettle leaves

The main vein (MV) was the variable that showed the highest variations among OTUs (Figure 5). Loadings in Figure 5 illustrate how studied parameters of the common stinging nettle samples collected from the three altitudinal zones varied in leaf morphological differences. The non-parametric test MANOVA showed significant differences among OTUs ($p<0.05$). The value for the Wilks' Lambda test was 0.0061 (Df1 = 28; Df2 = 217.8; and F = 24.2) while the value for the Pillai trace test was 2.135 (Df1 = 28; Df2 = 252; and F = 10.3).

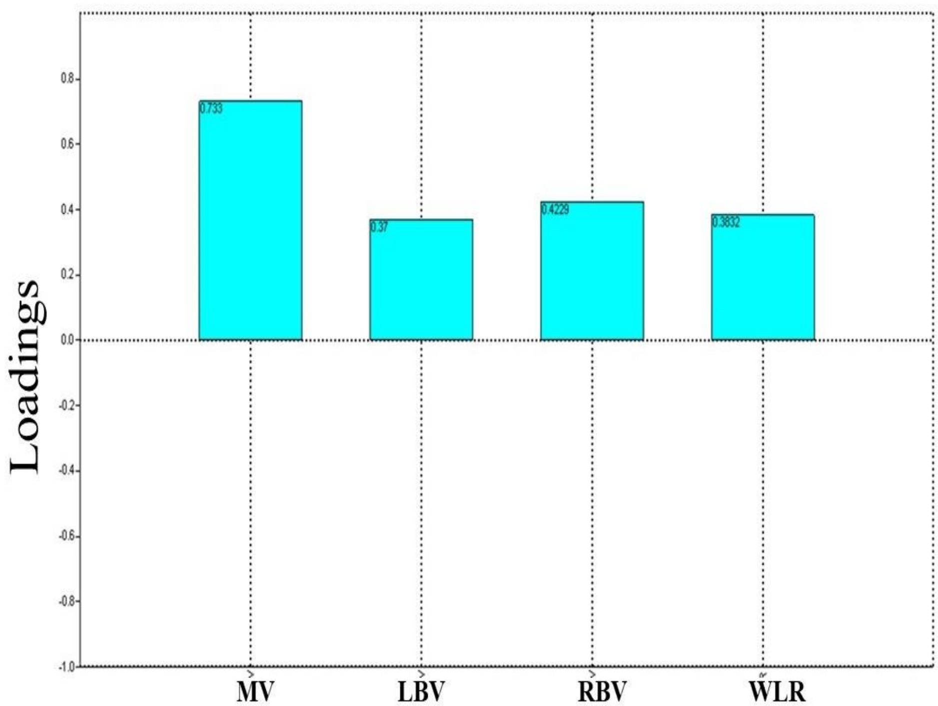

Figure 5. Loadings for studied parameters of the common nettle leaf samples

4. Discussion on the phenotypic characterization of the Rwandan common stinging nettle

Before this study, no information was available regarding the morphological characterization of common stinging nettle (*Urtica massaica* Mildbr.) in Rwanda. The findings reported here were obtained in wild conditions for the highland and in a domesticated form in the midland and lowland. This study has shown that populations of *Urtica massaica* Mildbr. from the study areas have significant variations in morphological descriptors. Abdulkadir &Kusolwa (2020) reported variations in the quantitative traits (plant height and stem length) of *Urtica simensis* from Northern Ethiopia. Singh & Kali (2019) also reported variations in morpho-anatomical and histo-chemical features of *Urtica dioica* L. in India. Vogl & Hartl, (2003) reported that stinging nettle (*U. dioica*) can grow up to 2-4 m tall.

According to Shen *et al.*, (2019), morphological variations like plant height often result from environmental heterogeneity and different selection pressures. In general, plant height increases according to plant population densities due to competition for light (Sangoi *et al.*, 2002; Argenta *et al*., 2001). This is due to a stimulation of apical dominance, which accelerates growth during the vegetative phase due to competition for light. High plant population densities reduce the supply of nitrogen, photosynthates and water to the growing leaves (Zamir *et al.*, 2011). The variations in plant height, leaf length and width in the studied common stinging nettle samples were probably due to the crowding effect of the nettle plant and higher intra-specific competition for resources in their habitats.

The root length was lower in the lowland zone when compared to the midland zone. However, there were no significant differences in the root length between highland and lowland zones. Root systems play a major role in the uptake of water and nutrients from the soil (Hammer *et al.*, 2009).The root length density is reduced in the hardpan soils while soil with lower penetration resistance, and high soil water content enhance greater total root length (Kirkegaard *et al.*, 1992). Root mass allocation is increased, decreased, or canalized with increased density, depending on soil conditions and plant growth stages (Wang *et al.*,2021).

Foliage density varies from dense to intermediate. Intermediate foliage density dominated in medium nitrogen content, and in areas with high intraspecific competition, dense foliage density was noticed in areas with higher nitrogen content and where competition for resources was less. Horizontal and semi-erect leaf attitudes were observed in this study. Three types of leaf attitudes; horizontal, semi-erect and dropping in tomatoes were also noticed by Salim *et al.*, (2020).

The qualitative traits viz leaf type, leaf margin, leaf venation, leaf phyllotaxy, leaf form, leaf shape, leaf pubescence, presence of stipules, the position of stipules, leaf surface, leaf color, internode distance, root type, rooting system, stem posture, stem bark feature, stem stinging nettle abundance, branch posture, type of flower, type of inflorescence, flower color, flower composition, were similar in all zones (Highland, midland and lowland). In many plants, leaf and stem trichomes are thought to deter herbivores from eating the mand may also contribute to resistance against drought and UV injury (Fordycen & Agrawal, 2001).Observations made in this study are similar to a report by Singh & Kali (2019) that showed similar qualitative traits (leaf shape, leaf arrangement and plant growth habit) in study populations of *Urtica dioica* L.

Concerning the size-trait of the four-leaf variables of the *Urtica massaica* Mildbr.examined in this study, the measurements were size related to habitat. There were significant differences in main vein length in highland, midland, and lowland samples of the Rwandan common stinging nettle. This finding is consistent with the one of size-dependent, environmentally-induced changes in leaf traits of a deciduous tree species of *Clausena dunniana* in a subtropical forest (Zheng *et al.*, 2022). This may reveal the adaptation mechanisms of the plant (Jing *et al.*, 2022). The findings suggest that the Rwanda common stinging nettle (*Urtica massaica* Mildbr.) was able to change its morphological features as a result of the environmental diversity (Sharifi *et al.*, 2022), and this phenotypic flexibility is what allowed the plant to successfully establish in different regions of Rwanda. Multivariate statistical analyses revealed that collected samples of *U. massaica* can be divided into three morphological clusters (morphospaces). This result is similar to the finding that showed the phenotypic variation in *Pyrus pyraster* in morphospaces (Vidaković *et al.*, 2022). The length of the main vein exhibited the greatest variability across Rwanda. Similar findings were consistently observed in the first leaf morphology of the *Diospyros lotus* (Samarina *et al.*, 2022).

5. Conclusion

The common stinging nettles can be found all over the world. In Rwanda, the most common stinging nettle species is *Urtica massaica* Mildbr. This study has shown that there were morphological differences, particularly in leaf morphology among samples collected from the three altitudinal zones (Lowland, Midland and Highland). The stinging nettle plant heights and leaf length varied from one site to another and the statistical analysis revealed that average plant heights, as well as average leaf lengths of mature stinging nettle samples from highland, midland and lowland, were significantly different.

In terms of leaf morphology, the most prominent difference was in the main vein of mature stinging nettle leaves. Changes in leaf morphology can be linked to differences in environment and nutrient availability between the three habitats which could have enabled the species to evolve differently to adapt to prevailing conditions.

The observed phenotypic variations among Rwandan common stinging nettle samples from lowland, midland and highland may lead to genetic variations and the development of localized ecotypes. However, the genetic basis of these phenotypic variations needs to be examined in future research to establish their heritability for future populations of the common stinging nettle plant in Rwanda.

Author contributions

Prof. Jean Nduwamungu, Dr. Jean Marie Vianney Senyanzobe & Dr. Charles Ruhimbana : Conceived the ideas, designed the methodology and developed the abstract.

Ms.Marie Claire Ugirabe, Mr.Janvier Mahoro, Ms.Marie Christine Dusingize, Ms.Mary Karungi & Mr.Emmanuel Irimaso : Collected data, designed maps and wrote the manuscript.

Mr.Eric Maniraho : Measured GPS coordinates and kept plant specimens for their identification.

Dr. Philippe Munyandamutsa, Mr. Phenias Nsabimana & Mr.Cyprien Mugemangango : analysed data.

Dr. Canisius Mugunga : red and corrected the manuscript.

All authors contributed to the drafts and approved the final publication.

Acknowledgment

The authors wish to acknowledge the financial support from the National Council for Science and Technology-Rwanda (NCST). The authors also thank the management of the University of Rwanda for providing the infrastructure to carry out this work.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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