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Characterisation of three *Capsicum* species and their phylogenetic relationship

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Abstract

Taxonomical confusion is known to exist in the genus *Capsicum* due to the considerable genetic variability among the members. The characterisation and understanding of the genetic relationship among the *Capsicum* species are therefore important in their breeding programme. This study employed morphological, anatomical, cytological and electrophoretic studies to characterise and elucidate the phylogenetic relationship that exists among the species studied namely: *Capsicum annuum var annuum, C. frutescens and C. chinense*. The cluster analysis of the coded morphological traits employed grouped *C. frutescens* and *C. chinense* on a clade and *C. annuum* on a separate clade. However, the analysis of seed protein profiles of the studied species revealed that C. *annuum* and *C. frutescens* were genetically closed while C. chinense was distantly related to the two species. All the species studied were annuals and facultatively self-fertilized. The species studied possessed many similar characters showing the affinity that exists among them and in addition, they shared some generic bands. The tetracytic and anosocytic stomata were predominant in all *Capsicum* species studied. The presence of starch granules in the leaf parenchyma cells and the paracytic stomata on the abaxial leaf surface of *C. chinense* as well as the jug-shaped midrib of *C. chinense* distinguished this species from the other two species. More so, the absence of trichomes and islets in *C. annuum* made it stand out among the three species. However, the chromosome numbers seem not to be useful taxonomically in this study, as all the species studied possessed the same number of chromosomes though the analysis of the seed protein profile revealed that the species studied were distinct. Therefore, the morphological, anatomical and electrophoretic studies have been proven to be useful in the present study and the study concluded that all the species studied were distinct species from a common origin.

INTRODUCTION

The genus *Capsicum* L., commonly known as pepper, belongs to the Solanaceae (nightshade) family, sub-family Solanoidaeae along with *Lycianthes* (Dunal) Hasal. and the tribe Capsiceae. The family consists of many economically important crops such as peppers, potatoes, tomatoes etc (Eshbaugh, 2012; Jarret *et al.,* 2019; Shiragki *et al.,* 2020; Barboza *et al*., 2022). *Capsicum* consists of 43 species (Barboza *et al.,*2022) including five cultivated species, namely: *C. annuum var. annuum* L., *C. frutescens* L., *C. chinense* Jacq. *C. baccatum* L. *C. pubescens* Ruiz. & Pav.) (Pickersgill,1991; Nicolai *et al.,* 2013; Scaldaferro and Moscone, 2019; Shiragki *et al.,* 2020; Kamar *et al.,* 2021; Parry *et al.,* 2021; Barboza *et al*., 2022; Yan *et al*., 2023). In addition, *C. assamicum* J. Purkay was reported by Kamar *et al.* (2021) as a cultivated species. These species were grouped into 11 clades and two chromosome numbers, 2n=2x=24 and 2n=2x=26 were documented in the genus. The chromosome number, 2n=26 was only recorded in two of the clades, Andean and Atlantic Forest). Though polyploidy is very rare in *Capsicum*, a chromosome number of 2n=48 was discovered in a wild species, *C. annuum var. glabriusculum* (Dunal.) Heiser et Pickersgill (Scaldaferro and Moscone, 2019; Parry *et al.*, 2021. *Capsicum* has a relatively small genome in comparison with *Solanum lycopersicum* L. (Nankolongo *et al*., 2023).

The genus is characterised by shrubby habit, lanceolate or ovate leaves with actinomorphic flowers and its fruits are unique for their pungency though varies among different species and cultivars, depending on the concentration of the group of alkaloid compounds called capsaicinoids present in them while the pungency is absent in the *Capsicum* species with 2n=26 and some other wild species. *Capsicum* originated from intertropical America and the highest diversity of the genus was recorded in the northern and central Andes (Eshbaugh, 2012; Nicolai *et al*., 2013; Wahua *et al*., 2013; Scaldaferro and Moscone, 2019; Usman *et al.,* 2022; Barboza *et al*., 2022;). *Capsicum* is considered

as a monophyletic genus; however, this can only be confirmed upon careful consideration of the two groups with 2n=24 and 2n=26 which are morphologically distinguishable from each other (Eshbaugh, 2012).

Capsicum is one of the most important vegetables consumed in fresh and dry forms globally (Jarret *et al.,* 2019; Scaldaferro and Moscone, 2019; Antannasova *et al.,* 2021; Kamal *et al*., 2021) and they are rich in Vitamins A, B, C, E and K and minerals like magnesium, calcium, iron, copper, folate, thiamine etc. it also consists of flavonoids, saponins, tannins, phlobatannins, cardiac glycosides, combined anthraquinones and free anthraquinones (Wahua et al., 2013; Chakrabarty *et al*., 2017; Mulathagedara *et al.,* 2021; Mladenović *et al*., 2024). The members of the genus are used as spices, pickles or condiments as well as for medicinal, cosmetic and ornamental purposes (Weryszko-Chmielewska and Michalojc, 2011; Shiragki *et al*., 2020, Kamal *et al.,* 2021). Capsaicinoids are documented to have antimicrobial properties and *Capsicum* species are employed for healing purposes such as dropsy, toothache, cough, diarrhoea, sore throat, colic, asthma, muscle cramps, arthritis, headache (Jarret *et al*., 2019; Kamal *et al*., 2021; Mladenović *et al.,* 2024). Also, the capsaicin in *Capsicum* species was documented to have the ability to control metabolism syndrome and associated disorders (Sanati *et al*., 2017).

There is an increasing interest in *Capsicum* as a result of the considerable genetic variability in the genus (Tripodi and Kumar, 2019). More so, taxonomic confusion is known to exist in the genus due to the existence of great genetic diversity within each species in terms of fruit shapes and sizes as well as morphological similarities among the members, consequently leading to misidentification (Eshbaugh, 2012; Toledo-Aguilar *et al*., 2016; Chinnkar and Jadhav, 2020; Liu *et al*., 2023). Furthermore, in the Annuum clade, the three cultivated species (white flowered group): *C. annuum var. annuum, C. frutescens* and *C. chinense* also known as *C. annuum* complex are recognised as a single species by some taxonomist based on their morphological similarities while some treat them as separate and distinct species. In fact, *C. chinense* was considered a cultivated variant of *C. frutescens* in some cases and the relationship between the two is not clear (Pickersgill,1991; Eshbaugh, 2012; Shiragki *et al*., 202; Barboza *et al*., 2022). Moreover, there is a wide range of overlapping morphological traits among these cultivated species and they can easily exchange genes among themselves to produce new varieties which further makes their classification difficult and thereby resulting in their misidentification (Liu *et al.,* 2023).

Characterisation is very crucial in understanding the relationship among *Capsicum* species and the knowledge of this relationship can be adopted in their breeding programme (Ibiza *et al.,* 2012; Parry *et al*., 2021). However, morphological characterisation only cannot be sufficient to delimit these species due to the morphological similarity in the genus and therefore, there is a need to employ more than one method for their characterization (Carvalho *et al*., 2014; Azeez, 2020; Yun *et al.,* 2023; Azeez et al., 2024). Hence, this study employs morphological traits, leaf anatomical characters, chromosome number and seed protein profiles to characterise and elucidate the phylogenetic relationship that exists among three species of *Capsicum* from Nigeria.

MATERIALS AND METHODS

Study Area and Germplasm collection

The project was carried out at the Department of Botany, Obafemi Awolowo University, Ile Ife, Osun State located within the coordinate 7º 31 ‘06’’N 4º 31’22’’ E. Seeds of three species studied (*C. annuum, C. frutescens* and *C. chinense*) were collected from National Horticultural Research Institute (NIHORT), Jericho Reservation Area, Ibadan, Oyo state, Nigeria.

Morphological and reproductive biological studies

Twenty seeds of each of the species were raised and watered in small bowls in the screen house for thirty days. The seedlings were transferred on the 30th day into seven-litre buckets, filled with soil, one plant per bucket in five replicates for each species. The seedlings were watered daily until the end of their life cycle which was marked by senescence. Morphological traits including vegetative and floral traits were observed. They were: the stem colour, leaf and stem shape, flower colour, fruit

size, shape and colour, leaf length, time of growth, opening and closing of flowers, and petiole length. Other characteristics recorded were the number of fruits, the colour of anthers and the stigma of the flowers. In all, 48-character states were coded where 1 denoted present and 0 denoted absent and the coded data was subjected to single linkage cluster analysis.

Anatomical Study

The leaf epidermis, transverse section and venation pattern were studied using matured leaves of the studied species using method adopted by Akinnubi *et al.* (2014). The leaf adaxial and abaxial surfaces were peeled using a sterilized razor blade. The peeled surfaces were stained with Safranin O for 5 minutes and placed gently on the slides. Glycerol was added to make the image clearer under the microscope. Types of stomata, the shape of the epidermal cell, the shape of the anticlinal wall, types of trichomes, as well as the presence or absence of ergastic substances were documented.

For leaf venation studies, the leaves of the three species were boiled with 70% ethanol for 30 minutes separately. The abaxial and adaxial were cleared using sodium hyposulphite. Slides were prepared using Safranin O as a staining agent and glycerol was used for preservation and clearer view of the slides under microscope. The areole shape, vein terminal, and crystal druses were recorded.

Transverse sectioning of the leaves was carried on the mid area of the leaves. The leaves were collected and sterilised with 50% ethanol. Unripe pawpaw was used as the leaf holder so that the microtome holder would not damage the leaf samples. A sledge microtome was used for the sectioning of the laminar and midrib. The sectioned part was stained for 5 minutes with Safranin O and later stained with Asian blue for another 5 minutes. Glycerol was added for a clearer view under the microscope and for the preservation of materials. The cuticle shape, epidermal layer, mesophyll, palisade layer, spongy layer, crystal druses, midrib, epidermal cells, cortex, and vascular bundle were observed and documented.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out at the Department of Animal Science, Obafemi Awolowo University, Ile Ife. The samples were ground with buffer solution, which was later centrifuged for some minutes and later boiled for 5 minutes, allowed to cool then later placed in the freezer. Two types of gels were prepared; the resolving /separating gel and the stacking gel (to arrange all the proteins and for separation purposes). The gel was poured inside an electrophoretic chamber, and layered with distilled water for about 10-15 minutes for polymerization to occur. The comb was inserted to form a well and was left for about 10-15 minutes, the well was loaded with the samples and the empty well was filled with SDS buffer, the samples were moved from the electrophoresis chamber to the tank, running buffer was added to fill up the tank, stain was added and left for hours following the technique of Weber and Osborn (1975). The gel was de-stained with a de-staining solution periodically. The similarity coefficient among the studied species was calculated through the Sokal and Sneath similarity coefficient (1963) as follows:

$$Similarity\ coefficient = \frac{a}{a+b+c} \times 100$$

a= number of common bands between two species

b= number of unique bands in one of the two species

c= number of unique bands in the other species

Then the bands were subjected to single linkage cluster analysis.

Cytological study

The seeds of the *Capsicum* species were planted in Petri dishes separately. Young roots were harvested after 4 to 5 days and pretreated with 8-hydroxyquinoline for 1 hour and then transferred into fixative (mixture of 75% ethanol and 25% acetic acid) for 24 hours. Before the preparation of the slides, the root tips were hydrolysed in 18% HCL for 10 minutes to ensure that the cells were separated. Two to three root tips were excised and squashed on a microscope slide, stained with FLP Orcein and covered with a cover slip using the standard technique adopted by Azeez and Faluyi

(2019). The stained slides were allowed to stay for at least 1 hour after which the excess stain was mopped up with filter paper. The slides are pressed and tapped with a flat end of a pen to ensure flattening of the cells and then viewed under a light microscope. Five good metaphase spreads were obtained for each species to ensure consistency of the chromosome counts and the photomicrograph was taken for each species using an Amscope camera attached to the trinocular microscope.

RESULTS

Morphological and reproductive biological studies

The *Capsicum* species studied were erect herbs, with acute branching and indeterminate growth. Their leaves were glabrous with entire margins and alternately arranged on the stem and branches (figure 1). Purplish pigmentation was observed on the stem of *C. chinense* and *C. frutescens*. The leaf shape was lanceolate in *C. annuum* and *C. frutescens* but cordate in *C. chinense.* The species possessed white petals, cream stigma, whitish style, purplish anthers with adate attachment and whitish powdery pollen. The sepals and pedicel were green. An elongated fruit was observed in *C. frutescens,* triangular in *C. annum* and round in *C. chinense.* The fruits were all red at maturity and the seeds were whitish to creamy. The single linkage cluster analysis of the 48 coded character states grouped *C. frutescens* and *C. chinense* on a clade while C. annuum stood alone (Figure 2).

The seedling emergency occurred 4-5 days after planting. The percentage seed germination of 70% was recorded in *C. annuum,* and *C. chinense* only while 45% was documented in *C. frutescens* 30 days after planting. All three species started flowering four weeks after transplanting. *Capsicum annuum* started fruiting 14 days after flowering while it took *C. chinense* and *C. frutescens* 20 days to start fruiting. The flowers opened between 3:15 am and 4:30 am while they closed around 8:00 pm. The anther dehiscence occurred around 9:30 am and was completed 3-4 days after anthesis. It was observed that the anthers were shorter than the stigma before anthesis and they gradually grew towards the stigma for pollination to occur. Therefore, all the species studied are self-pollinated (Figure 3). In addition, ants were noticed on the leaves of the pepper plants and hence, cross-pollination cannot be ruled out. All three species completed their life cycle between 124 to 130 days making them annuals.

Figure 1: Habits of the *Capsicum* species studied: A. *Capsicum chinense* B. *Capsicum frutescens* C. *Capsicum annum*

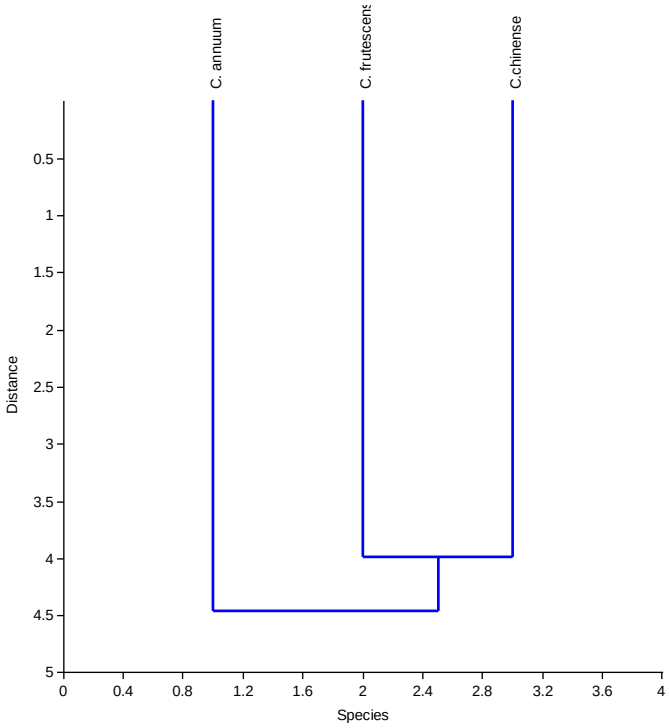

Figure 2: Dendrogram of single linkage cluster analysis of morphological characters

Figure 3: Pollination in *Capsicum chinense* (Typically for all *Capsicum* species studied)
A. Before anther dehiscence B. After anther dehiscence

Anatomical study

Leaf Epidermal peels of Capsicum species studied.

All three *Capsicum* species studied possessed anisoytic and tetracytic stomata on their abaxial and adaxial surfaces. In *C. chinense,* normocytic stomata were also observed on the adaxial surface while paracytic and anomocytic stomata were noted on the abaxial surface. Anomocytic stomata were observed on the abaxial and adaxial surfaces of *C. frutescens* in addition to anisocytic and tetracytic stomata. Hence, the presence of paracytic stomata on the abaxial surface is diagnostic for *C. chinense.* The epidermal cell shape was irregular in all three species while the polygonal cell shape was documented in the adaxial surface of *C. annuum* only. On the adaxial surface of *C. annum*, the anticlinal wall was straight, curved to undulating but curved to wavy on the abaxial surface. In *C. chinense*, the anticlinal wall was undulating and wavy on both abaxial and adaxial surfaces while in *C. frutescens*, it was curved and wavy on abaxial and adaxial surfaces. Trichomes and crystals were not observed on the abaxial and adaxial surfaces of *C. annuum*. On the adaxial surface of *C. chinense*, crystals were absent while on the abaxial surface, crystal druses were deposited on the veins and lamina. Short glandular trichomes were observed on the adaxial and abaxial surfaces of *C. chinense.* Also, short glandular trichomes were observed on the abaxial surface of *C. frutescens.* Crystal druses and crustal sand were found on the abaxial and adaxial surfaces of *C. frutescens.* The absence of trichomes and crystal druses in *C. annuum* is diagnostic for it.

Leaf transverse section of Capsicum species studied.

Cuticle was thin and non-striated in all the *Capsicum* species studied. It was more or less straight in some portions and gently undulating in some portions in *C. annuum* and *C. chinense* while in *C. frutescens,* it was gently undulating. Epidermis was uniseriate in all the species with various cell shapes ranging from circular, cylindrical, oval to short rectangular. The mesophyll was distinguished into palisade and sponge mesophylls. The palisade layer was occupied by 1-2 layers of tightly packed parenchyma cells in all the three *Capsicum* species while the sponge mesophyll was occupied by loosely packed parenchyma cells of different shapes ranging from circular, oval, cylindrical to polygonal. Starch granules of various shapes were deposited in the parenchyma cells of *C. chinense.* Also, certain glands and crystal druses were housed in parenchymal cells of the mesophyll in *C. chinense* and *C. frutescens* respectively.

In the midrib, the cuticle was thin and non-striated in all the species studied. It was pronouncedly undulating in *C. annuum* and *C. chinense* but gently undulating in *C. frutescens*. The midrib was jug-shaped in *C. chinense* which is unique to it. The epidermis was uniseriate with variously shaped cells ranging from circular, cylindrical to short rectangular. The cortex was occupied by variously shaped parenchyma cells ranging from circular, oval, cylindrical, short rectangular to polygonal. The vascular bundles in all three *Capsicum* species were conjoint collateral.

Leaf venation pattern in Capsicum species studied.

The areola in all the species were polygonal in shape. Also, some irregular shapes were observed in *C. chinense* and *C. frutescens* and both had few islets. The absence of islets in *C. annuum* is diagnostic for it. Crystal druses are numerous in all species. The vein terminal was either straight, curved or forkated in all. In addition, a bi-forkated vein terminal was noted in *C. chinense* and is diagnostic for it.

Cytological study

A chromosome number of 2n=24 was observed in each of the *Capsicum* species studied.

Electrophoretic study

The seed protein profiles of the three *Capsicum* species studied showed five bands for *C. chinense* with one unique band; eleven bands for *C. frutescens* with one unique band; and ten bands for *C. annuum* with no unique band. The generic bands were recorded at 0.3, 0.5, 0.7 and 1.9 (Figure 8). The similarity level was very high between *C. frutescens* and *C. annuum* (90.9%). The similarity

level between *C. chinense* and *C. frutescens* was 33.3% while the similarity level between *C. chinense* and *C. annuum* was 36.4%. The single linkage cluster analysis grouped *C. frutescens* and *C. annuum* on a clade and *C. chinense* stood out (Figure 9).

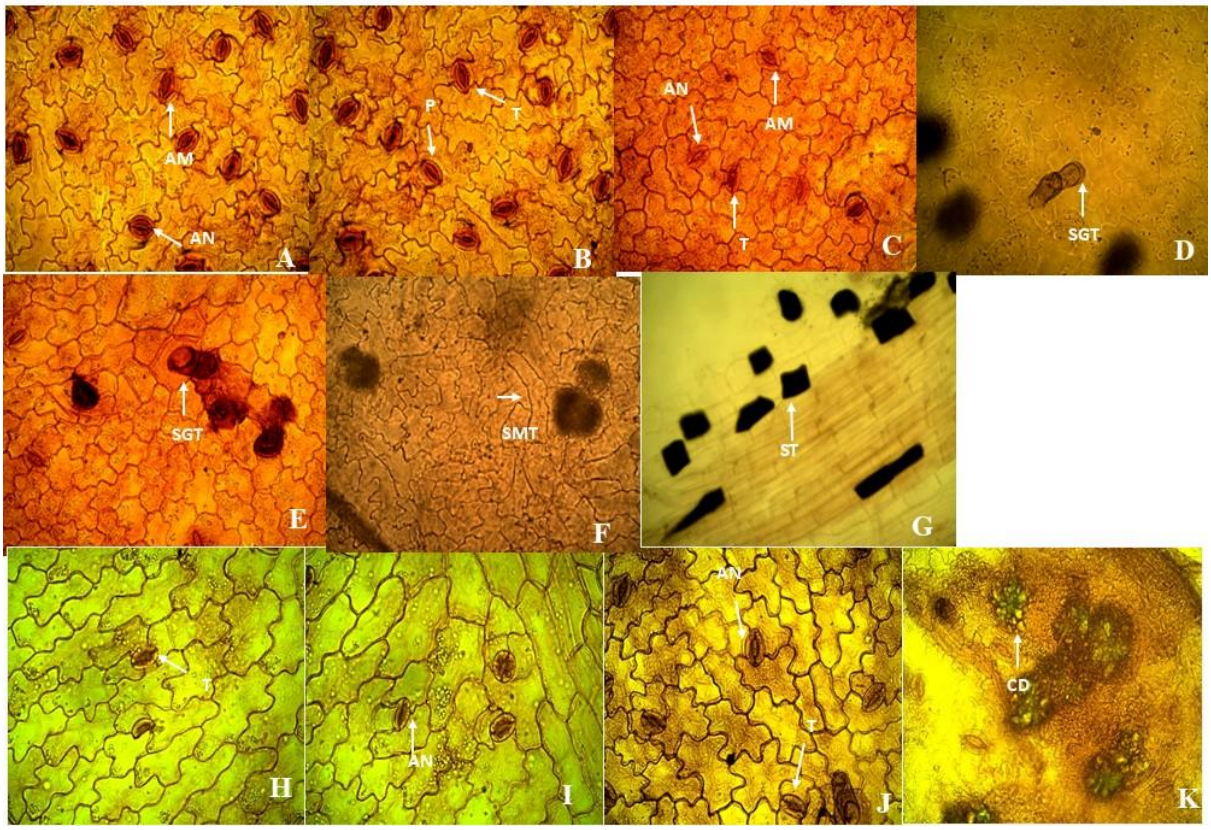

Figure 4: Leaf Epidermal Peels. A. *Capsicum chinense* (abaxial) B. *Capsicum chinense* (abaxial) C. *Capsicum chinense* (adaxial) D. *Capsicum chinense* (abaxial) E. *Capsicum chinense* (adaxial) F. *Capsicum chinense* (abaxial) G. *Capsicum chinense* (abaxial- starch grain arrowed) H. *Capsicum annuum (*abaxial) I. *Capsicum annuum* (adaxial) J. *Capsicum frutescens* (adaxial) K. *Capsicum frutescens* (abaxial).

ABBREVIATION: AM-Anomocytic; AN- Anisocytic; T- Tetracytic; P- Paracytic; SGT- Short

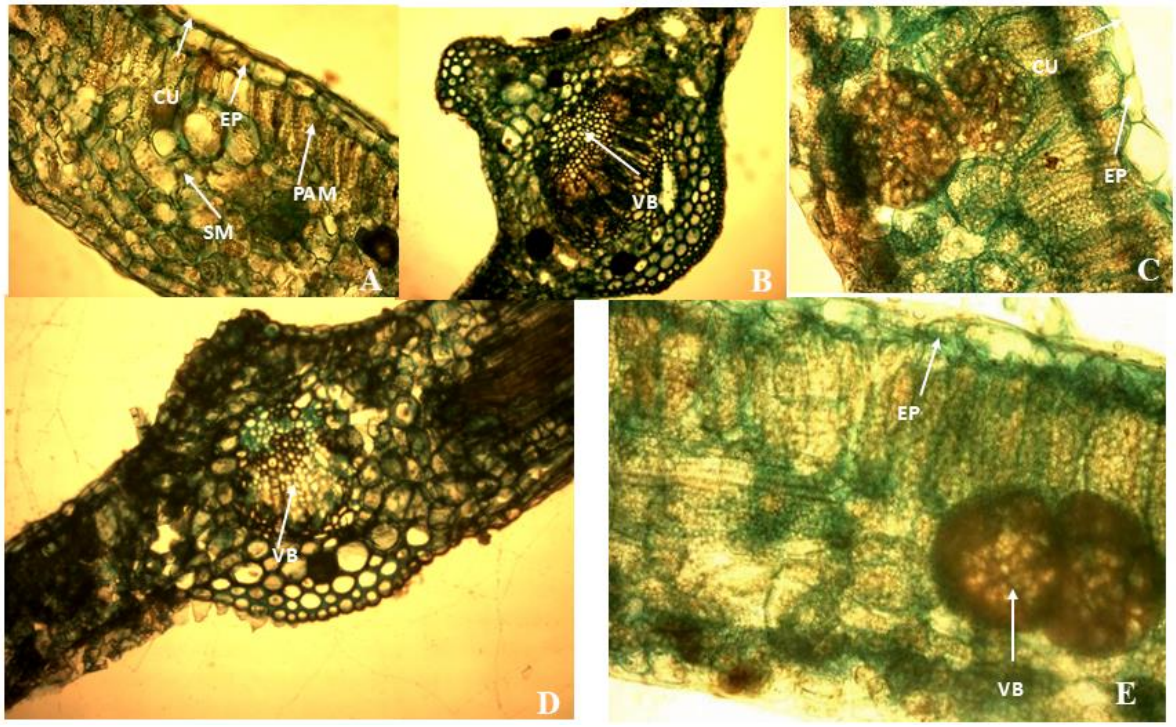

Figure 5: Leaf transverse section of three *Capsicum* species studied. A. *Capsicum chinense* B. *Capsicum chinense* (midrib) C. C*apsicum annuum* D. *Capsicum annuum* (midrib) E. *Capsicum frutescens*

ABBREVIATION: CU-Cuticle, EP- Epidermis, PAM- Palisade Mesophyll; SM- Sponge Mesophyll; VB- Vascular Bundle

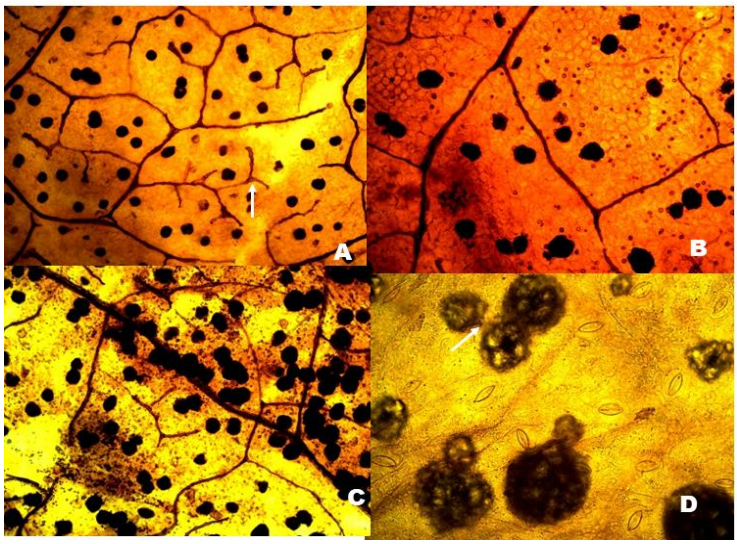

Figure 6: Venation patterns in three Capsicum species studied. A. *Capsicum chinense (*Bi-forkated vein terminal arrowed) B. *Capsicum annuum* C. *Capsicum frutescens* D. *Capsicum frutescens* (Crystal druses arrowed)

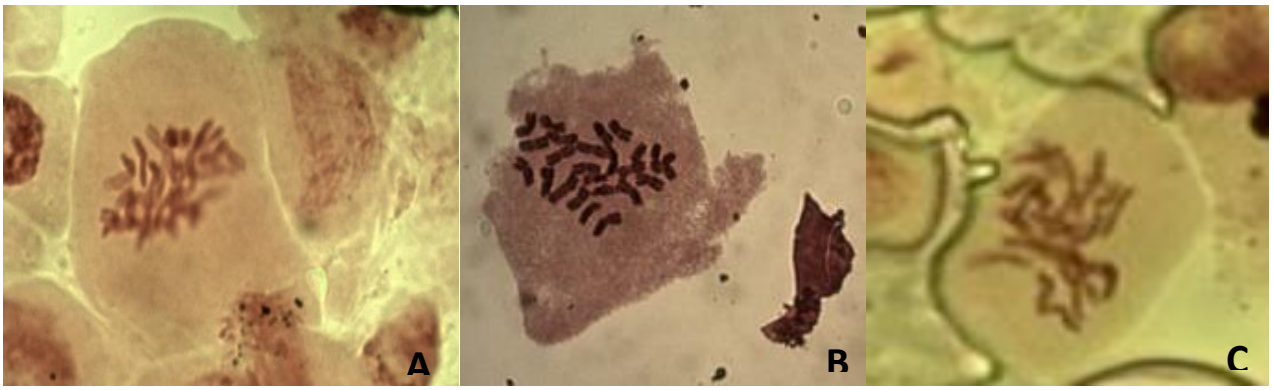

Figure 7: The chromosome numbers in *Capsicum* species studied. A. *Capsicum chinense* (2n=24) B. *Capsicum frutescens* (2n=24) C. *Capsicum annuum* (2n=24)

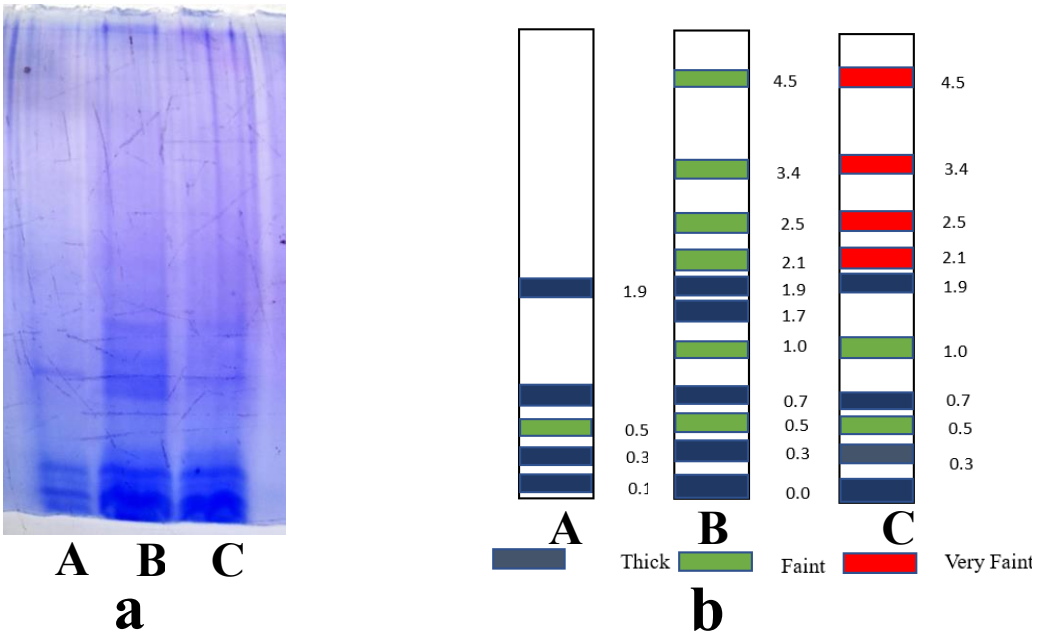

Figure 8: a. Seed protein profiles of the three *Capsicum* species studied b. Schematic diagram of the seed protein profiles of three *Capsicum* species studied. A. *Capsicum chinense* B. *Capsicum frutescens* C. *Capsicum annuum*

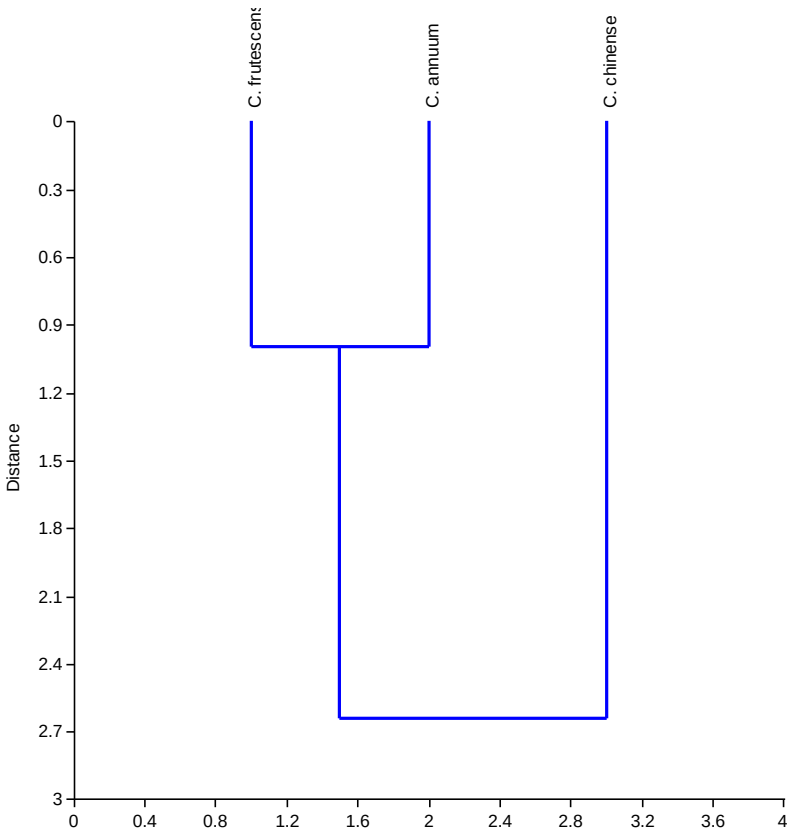

Figure 9: Dendrogram of single linkage cluster analysis of seed protein profiles

DISCUSSION

Characterisation and understanding the phylogenetic relationship among members of a plant group remain the focus of many researchers, and they have employed different combinations of taxonomical tools to achieve these aims as just a tool had been proven to be inadequate (Triploid, 2019; Azeez *et al*., 2022; Azeez *et al.,* 2023). This study employed a combination of morphological, anatomical, cytological and electrophoretic data to delimit the three studied species in the genus *Capsicum.*

The species studied namely: *C. annuum*, *C. frutescens* and *C. chinense* were herbs and annuals. However, Wahua *et al*. (2013) reported short-lived perennials among the accessions of the species they studied. In addition, all the species in this study possessed pentamerous petals whereas Wahua *et al.* (2013) documented hexamerous petals in *C. annuum* along with pentamerous petals. The single linkage cluster analysis of the 48 coded character states from qualitative and quantitative data documented in the present study grouped *C. chinense* with *C. frutescens* while *C. annuum* was separated from them. Similarly, isozyme analysis previously reported showed that *C. chinense* was more closely related to *C. frutescens* than to *C. annuum*. It was opined that *C. chinense* probably evolved from the gene pool *C. frutescens.* Although, the three species were said to have evolved from a common ancestral gene pool (Eshbaugh, 2012). The three species possess an array of similarities in their phenotypes. Furthermore, *C. chinense* and *C. frutescens* belong to Genome A while *C. annuum* belongs to Genome B (Shiragaki *et al.*, 2022) and both the formal species are highly pungent due to the presence of the higher amount of capsaicinoids in them (Barboza *et al*., 2011). The pungency can also be employed to distinguish the species (Usman *et al.,* 2022). In another study by Mulathagedera *et al* (2021), the cluster analysis of quantitative data grouped *C. annuum* with *C. frutescens* while *C. chinense* was separated. On the other hand, the cluster analysis of qualitative data placed *C. chinense* and *C. frutescens* on a clade and *C. annuum* on a separate clade which is in line with the present study. It was documented that a single nucleotide base pair insertion or deletion and substitution within the intron separated *C. chinense* from *C. frutescens* (Triploid, 2019). Also, Parry *et al.* (2021) showed the closeness between *C. chinense* and *C. frutescens.*

The *Capsicum* species studied were self-fertilized, the flowers remained opened for 3-4 days after anthesis which gave room for cross-fertilization. Similarly, Nankolongo *et al.* (2023) reported that the flowers of *Capsicum* species studied remain open 2-3 days after anthesis enabling outcrossing of up to 91% depending on the activities of the pollinating insects. Therefore, the *Capsicum* species was termed a facultative self-fertilized species (Pickersgill, 1991).

Anisocytic and tetracytic stomata were predominant among the *Capsicum* species studied. Likewise, Zhigile *et al*. (2015) reported anisocytic and tetracytic stomata in *Capsicum* species they studied. In addition, anomocytic was noted in *C. chinense* and *C. frutescens* while paracytic was documented only on the abaxial surface of *C. chinense.* However, Wahua *et al.* (2013) and Dias *et al*., (2013) reported anomocytic stomata in *C. annuum*. Therefore, the presence of paracytic stomata on the abaxial surface of *C. chinense* delimits this from the other two *Capsicum* species in this study. In the present study, trichomes were not observed in *C. annuum* though, short glandular trichomes were documented in *C. chinense* and *C. frutescens* whereas, Dias *et al* (2013) documented glandular trichomes in *C. annuum.* The glandular trichomes exude certain substances that protect the plants against pathogens and pests (Dias *et al.,* 2013). In addition, a simple multicellular trichome was sighted in *C. chinense.* Moreover, simple uniseriate trichome was documented in a previous study (Wahua *et al.,* 2013). The species studied possessed conjoint collateral vascular bundles while Wahua *et al*. (2013) and Dias *et al*. (2013) reported bicollateral vascular bundles in their studies on *Capsicum* species.

The three species studied had chromosome number of 2n=24 which is in line in with previous reports (Limaye and Patil, 1989; Barboza *et al*., 2011; Eshbaugh, 2012; Wahua *et al.,* 2013; Scaldaferro and Mescone, 2019). However, 2n=48 was reported in the wild relative and a natural accession of *C. annuum* (da Costa Batista, 2016; Scaldaferro and Mescone, 2019). Even though they all the studied species possessed the same chromosome number, the single linkage cluster analysis of the seed protein profiles grouped the three species into two clades with *C. frutescens* and *C. annuum* on a clade while *C. chinense* occupied the second clade. They all stemmed from a common origin which was evident from several generic bands they shared. *Capsicum annuum* and *C. frutescens* were grouped at a similarity level of about 91% while *C. chinense* shared less than 40% similarity with the formal two according to seed protein profile analysis. Similarly, molecular characterisation carried out on some *Capsicum* species by Dias *et al*. (2013) grouped *C. frutescens* with *C. annuum* while *C. chinense* was on a different clade.

However, from an earlier report, *C. annuum var annuum* had a karyotypic formular of 2acro + 10 met while *C. chinense* and *C. frutescens* had 1acro + 11met. The difference was attributed to a single reciprocal translocation (Triploid, 2019). In a different study, 10m+1sm+1st was reported for *C. annuum var annuum* (Scaldaferro and Mescone, 2019). Furthermore, based on the Stebbins system of classification, the three species were placed in the 2A category except for the purple-flowered accession of *C. annuum* which was placed in 2B (Limaya and Patil, 1989). This showed that a great affinity exists among the studied species with a higher possibility of gene flow among them (Dias *et al.,* 2013). Furthermore, Monteiro *et al.* (2011) showed the great affinity among the three species through hybridization. Moreover, interspecific hybridization among these species has produced fertile or partial fertile hybrids which is very important for the genetic improvement of the *Capsicum* (Triploid, 2019) and the genetic diversity among them is the backbone of the genetic progression (Nicolai *et al.,* 2013). The genetic distance between *C. annuum* and C. chinense revealed in this study was further supported by the result of the interspecific crosses between *C. annuum* and *C. chinense* in which weak hybrids with almost complete arrest of new leaf formation, delayed plant growth, reduction in the upper internodes and abnormality in shoot apical meristem that led to dwarf F_1 plants were obtained as reported by Shiragaki *et al.* (2020). The hybrid weakness however plays an important role in the species formation (Shiragaki *et al*., 2022). Therefore, variation and genetic diversity allow good gene recombination (Mladenovic *et al.,* 2024).

The morphological data employed in this study to characterise the three *Capsicum* species successfully delimited the three species into three distinct species by grouping *C. frutescens* and *C. chinense* on a clade while *C. annuum* occupied a separate clade. On the other, the analysis of the seed

protein profiles of the three species revealed that *C. frutescens* and *C. annuum* were more genetically related while *C. chinense* was distantly related to them. *C. chinense* and *C. annuum* possessed a unique band each, which confirmed their distinctiveness. Many traits were shared by these species and also, they possessed some generic bands; these showed that they all evolved from the same ancestor. However, it was said that morphological characterisation does not always depict the genetic distance among a group of plant species, because the analysis of morphological traits is sometimes superficial and can be influenced by the environment (Carvalho *et al*., 2014), especially quantitative characters. Furthermore, some anatomical characters were shown to be useful in delimiting the species studied in the present study. The presence of starch granules in the leaf parenchyma cells and the paracytic stomata on the abaxial leaf surface of *C. chinense* as well as the jug-shaped midrib of *C. chinense* distinguished this species from the other two species. More so, the absence of trichomes and islets in *C. annuum* made it stand out among the three species. However, the chromosome numbers seem not to be useful taxonomically in this study, as all the species studied possessed the same number of chromosomes though the analysis of the seed protein profile revealed that the species studied were distinct. Therefore, the morphological, anatomical and electrophoretic studies have been proven to be useful in the present study and the study concluded that all the species studied were distinct species from a common origin.

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