Systematics of *Crabbea* Harv. (Acanthaceae) in southern Africa

By

Nelson Alexander Mendes de Gouveia

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Supervisor: Dr. L. Joubert

Co-supervisor: Dr. M. Jackson

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“For a star to be born, there is one thing that must happen: a gaseous nebula must collapse. So collapse. Crumble. This is not your destruction. This is your birth.”

~ Zoe Skylar ~

“Be humble, for you are made of earth. Be noble, for you are made of stars.”

~ Serbian Proverb ~

“If I can just make it through this week.”

~ Alexander de Gouveia ~

LIST OF ABBREVIATIONS

A

A	Aperture
AIC	Akaike Information Criterion
AW	Anticlinal Wall

B

B	Breadth
BAWN	Barlerieae, Andrographideae, Whitfielidieae and *Neuracanthus*
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
BOT	Botswana
BP's	Base Pairs
BRAHMS	Botanical Research and Herbarium Management System
BS	Bootstrap

C

CAW	Cystolith Attachment Width
$ChCl_3$/IAA	Chloroform/Isoamylalcohol
CI	Consistency Index
cpDNA	Chloroplast DNA
CS	Cuticular Striations
CTAB	Cetyltrimethylammonium Bromide

D

DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl Sulphoxide

DNA	Deoxyribonucleic Acid
	E
E	Equatorial Width
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
	F
FS	Free State
	G
G	Gauteng
GA	Glutaraldehyde
GC	Guard Cell
GIS	Global Information System
GPS	Global Positioning System
GT	Glandular Trichome
GW	Gemma Width
	I
ICL	Individual Crystal Length
ICW	Individual Crystal Width
IPNI	International Plant Names Index
ITS	Internal Transcribed Spacer
	K
KZN	KwaZulu-Natal

L

L	Length
LES	Lesotho
LM	Light Microscope
LP	Limpopo
LU	Lumen

M

M	Mpumalanga
MCMC	Markov Chain Monte Carlo
MH	Murus Height
MOZ	Mozambique
MP	Maximum Parsimony
MW	Murus Width
MYA	Million Years Ago

N

NAM	Namibia
NaOH	Sodium Hydroxide
NC	Northern Cape
NGS	Next Generation sequencing
nrDNA	Nuclear Ribosomal Deoxyribonucleic Acid
NW	North West
NWD	New World

O

OWD	Old Wolrd

P

P	Polar Axis
PCR	Polymerase Chain Reaction
PHT	Partition Homogeneity Test
PP	Posterior Probability
PV	Primary Vein
PVP	Polyvinylpyrrolidone
PW	Periclinal Wall
PS	Plant Sciences

R

RI	Retention Index
rRNA	Ribosomal Ribonucleic Acid

S

SC	Subsidary Cells
SD	Standard Deviation
SCT	Simple Cone-shaped Trichomes
SEM	Scanning Electron Microscope
SL	Stomatal Ledges
SNT	Simple Needle-shaped Trichomes
SP	Stomatal Pore
SV	Secondary Vein
SW	Swaziland

T

TBR	Tree Bisection and Reconnection
TL	Total Length

TMAC	Tetramethyl Ammonium Chloride
Tris-HCl	Tris-hydroxymethyl Aminomethane

U

UFS	University of the Free State
UV	Ultraviolet

W

WC	Western Cape

Z

ZIM	Zimbabwe

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SUMMARY

The purpose of this study is to provide an updated taxonomic revision and a molecular phylogenetic investigation of *Crabbea* Harv. (Acanthaceae) in southern Africa. The taxonomic component of this study entailed a detailed analysis of anatomical, macromorphological and micromorphological data and appropriate descriptions. Updated distribution maps of each southern African *Crabbea* species is presented and detailed habitat and ecological information is also provided. Four different identification keys were constructed using leaf anatomy, leaf micromorphology, pollen micromorphology and macromorphology. Type literature, type material and nomenclature for each investigated *Crabbea* species is critically reviewed. In cases where holotype material could not be located and/or identified, appropriate isotypes, lectotypes, syntypes and/or neotypes were assigned and/or confirmed. Additional herbarium specimens, on loan and electronic scans, from various European and South African herbaria were studied to construct identification keys, species descriptions, distribution maps and obtain ecological and habitat information. Fresh material was collected for each investigated species.

The investigated *Crabbea* species are all small to medium-sized herbs with cymose inflorescences and corolla being two-lipped, zygomorphic, funnel-shaped with paired, raised bosses. The corolla tube is largely creamish-white but light pink corolla tubes are occasionally found. Growth form, root appearance, stem orientation, position, texture and leaf shape and indumentum are important for species-level identification.

Leaf micromorphological characters are both significant on species level. The occurrence of both amphistomatic and hypostomatic leaves among the investigated species are characteristic of Acanthaceae and could be effectively used to distinguish the investigated *Crabbea* species from each other.

This study provides a first detailed analysis of *Crabbea* cystoliths. Cystolith attachment width on the adaxial leaf surface proves to be the best character state to split the southern African *Crabbea* into two groups. The groupings obtained were similar to that of the leaf micromorphology groupings.

Pollen micromorphology divided *Crabbea* into two groups based on the absence or presence of murus and lumin. However, this character set yielded a different grouping from the leaf micromorphology and anatomy character sets. Pollen grain morphology for certain *Crabbea* species either remained constant over a geographic range, or varied between and within populations.

Macromorphology could key-out all species, except *C. cirsioides* and *C. nana*. This character set displays a similar grouping to that of the pollen micromorphology character set.

The molecular phylogenetic component of this study resulted in the first molecular investigation of the phylogeny for the southern African *Crabbea* species. The phylogeny is primarily based on the two chloroplast DNA sequences *trn*L-*trn*F and *rps*16; however, anatomical and morphological characters are also included in the phylogeny to increase the resolution of the tree in absence of the ITS sequences.

Molecular phylogenetic results suggest that *C. velutina* is the first diverging southern African *Crabbea* species, from the larger *Crabbea* clade, consisting of *C. acaulis*, *C. angustifolia*, *C. cirsioides*, *C. galpinii*, *C. ovalifolia* and *C. pedunculata*. Within the larger *Crabbea* clade, *C. acaulis* forms a distinct clade as well as *C. galpinii* and *C. pedunculata*. The molecular results confirm the close relationship between *C. galpinii* and *C. pedunculata*. Moreover, within the larger *Crabbea* clade, molecular data could not clearly resolve and group the sprawling *Crabbea* species into distinct clades, as in the case of *C. angustifolia*, *C. cirsioides* and *C. ovalifolia*.

The end result of this systematic study provides a new insight into the classification of the southern African *Crabbea* species and the genus *Crabbea*. *Crabbea galpinii* and *C. pedunculata* are confirmed as two separate, sister species and *C. nana* is now regarded as a synonym of *C. cirsioides.* Seven *Crabbea* species are recognised in southern Africa.

Keywords: Acanthaceae; Barlerieae; *Crabbea*; Systematics; ITS; *trn*L-*trn*F; *rps*16; Cystoliths; Micromorphology; Macromorphology

CHAPTER 1

INTRODUCTION

1.1 Diversity and distribution

Crabbea Harv. is a widespread African, endemic genus with a significant portion of its members distributed in southern Africa (Balkwill and Welman, 2000; Thulin, 2007). The genus forms part of the tribe Barlerieae and is classified under the subfamily Acanthoideae, Acanthaceae (McDade *et al*., 2008) in the order Lamiales (Schäferhoff *et al*., 2010).

Acanthaceae is represented by 350 genera and 4 350 species (Koekemoer *et al*., 2014) which are mostly herbs or shrubs (Woodland, 1991). This family is regarded as one of the 12 most diverse plant families in the world (Tripp and Fatimah, 2012). In southern Africa, Acanthaceae is composed of 43 genera and 373 species, making it the largest southern African family from the Lamiales (Koekemoer *et al*., 2014). The subfamily Acanthoideae accounts for 95% of the Acanthaceae species (Scotland *et al*., 1995).

This family occupies mainly tropical and subtropical habitats (Hutchinson, 1969; Balkwill and Welman, 2000; Heywood *et al*., 2007) with fewer representatives in more temperate regions (Heywood *et al*., 2007). Acanthaceae is distributed across the entire African continent and most of South America; however, Argentina and Chile lack Acanthaceae. Furthermore, Acanthaceae has a relatively narrow distribution range in North America, Europe and Asia, concentrated mainly in the southern parts of these continents and stretching through the Malay Archipelago to the northern parts of Australia (Stevens, 2001 onwards; Heywood *et al*., 2007).

Prominent genera within Acanthaceae include *Justicia* L. (600–700 species), *Barleria* L. (300 species), *Ruellia* L. (250 species) and *Thunbergia* Retz. (100 species) (Woodland, 1991; Heywood *et al*., 2007; Koekemoer *et al*., 2014). *Crabbea* is a significantly smaller genus compared to the above-mentioned genera, having only 16 species (Thulin, 2007).

Crabbea is distributed from southern Africa to the Democratic Republic of the Congo and along the east coast of Africa to tropical east Africa - Ethiopia and Somalia. In South Africa, the genus is restricted to the eastern, central and northern provinces (Welman, 2003). Many *Crabbea* herbarium specimens fail to provide detailed locality and habitat information concerning individual plant specimens. The lack of precise locality and habitat information creates the need for further research. All the southern African *Crabbea* species have a red list status of least concern (Raimondo *et al*., 2009); however, in the light of the lack in data on the distribution and habitat of the species, their conservation status may need to be re-evaluated.

1.2 Economic and cultural significance

The Acanthaceae has economic and cultural importance in both horticulture and traditional medicine. Certain species within *Barleria*, *Justicia* and *Thunbergia* are cultivated as garden ornamentals (Koekemoer *et al*., 2014). *Aphelandra squarrosa* Nees and *Justicia brandegeeana* Wassh. & L.B.Sm. are popular house plants due to corolla shape and colour, leaf shape and colour and vein colour (Hennessy, 2010).

Koekemoer *et al*. (2014) explain that southern African Acanthaceae has limited local medicinal value in treating ailments such as coughs, diarrhoea and fevers. Hutchings (1989) investigated the ethnobotanical or traditional medicinal uses of various South African plant species and/or families among Sesotho, Xhosa and Zulu cultures. Several Acanthaceae species were included in the report. *Thunbergia natalensis* Hook. and *Thunbergia venosa* C.B.Clarke are used as a natural aphrodisiac. *Thubergia venosa* is also used to treat nervous and psychological ailments such as nightmares, states of believed bewitchment and hysteria. *Barleria ovata* E. Mey. ex Nees is used as a natural aphrodisiac and is used to improve overall health and strengthen immunity.

Various authors have investigated the ethnobotanical significance of *Crabbea* (Smith, 1895; Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Arnold *et al*., 2002; Fowler, 2007; Moffett, 2010; Kirby, 2013; Eshete *et al*., 2016). However, to date, no *Crabbea*

species is cultivated extensively or grown in mass production, therefore, suggesting that the genus has a more cultural and/or ethnobotanical than commercial value.

1.3 Previous taxonomic treatments of *Crabbea*

Crabbea has received attention in seven taxonomic accounts in the past, each revising *Crabbea* from a particular geographical area across Africa: southern Africa (Harvey, 1842; Nees von Esenbeck, 1847; Buys, 1982; Burkill and Clarke, 1899–1900; Clarke, 1901); Somalia (Thulin, 2007) and south tropical Africa (excluding Angola) (Vollesen, 2015).

Buys (1982) provided the most recent revision of the southern African *Crabbea* species. This taxonomic revision recognises five species and two subspecies (Table 1). Results from this taxonomic account were never validly published, therefore, stressing the importance of a new revision to confirm the findings of Buys (1982).

The classification of the southern African *Crabbea* species by Welman (2003) primarily follows Buys (1982), though the two subspecies of *Crabbea nana* (Nees) Nees which were proposed by Buys (1983) are not recognised by Welman (2003) (Table 1).

In his treatment of *Crabbea* in the Flora Zambesiaca, Vollesen (2015) emphasized that *Crabbea* "species are closely related and often difficult to separate." Vollesen's (2015) treatment of *Crabbea* includes all the South African species, except *Crabbea acaulis* N.E.Br. but differs from the treatments by Buys (1982) and Wellman (2003). Species recognised by Vollesen (2015), Buys (1982) and Wellman (2003), include *C. nana* and *C. velutina* S.Moore (Table 1). However, Vollesen (2015) did not recognise the two subspecies of *C. nana*, which were proposed by Buys (1982). In addition, Vollesen (2015) subsumed three species recognised by Buys (1982) and Wellman (2003), *C. angustifolia* Nees, *C. hirsuta* Harv. and *C. ovalifolia* Ficalho & Hiern, under *C. cirsioides* Nees (Nees).

Table 1 Classification of *Crabbea* following Buys (1982), Welman (2003) and Vollesen (2015).

Buys (1982)		**Welman (2003)**	**Vollesen (2015)**
***Crabbea* species**	***Crabbea* subspecies**	***Crabbea* species**	***Crabbea* species**
***C. acaulis* N.E.Br.**		***C. acaulis* N.E.Br.**	**Not included in revision.**
***C. angustifolia* Nees** = *C. undulatifolia* Engl.		***C. angustifolia* Nees** = *C. undulatifolia* Engl.	***C. cirsioides* (Nees) Nees** = *C. angustifolia** Nees = *C. hirsuta** Harv. = *C. nana* (Nees) Nees *sensu* Burkill and Clarke (1899–1899) and Clarke (1901) = *C. ovalifolia** Ficalho & Hiern = *C. robusta* N.E.Br. = *C. undulatifolia* Engl. = *Ruellia cirsioides* Nees
***C. hirsuta* Harv.** = *C. cirsioides* (Nees) Nees = *C. robusta* N.E.Br.		***C. hirsuta*** = *C. cirsioides* (Nees) Nees = *C. robusta* N.E.Br.	
***C. ovalifolia* Ficalho & Hiern**		***C. ovalifolia* Ficalho & Hiern**	
***C. nana* (Nees) Nees**	***C. nana* subsp. *galpinii* (C.B.Clarke) Voorendyk** = *C. galpinii* C.B.Clarke	***C. galpinii* C.B.Clarke**	***C. nana* (Nees) Nees** = *C. galpinii* C.B.Clarke = *C. pedunculata* N.E.Br. = *R. nana* Nees
	***C. nana* subsp. *nana* (Nees) Voorendyk** = *C. pedunculata* N.E.Br.	***C. nana* (Nees) Nees** = *C. pedunculata* N.E.Br.	
***C. velutina* S.Moore** = *C. reticulata* C.B.Clarke		***C. velutina* S.Moore** = *C. reticulata* C.B.Clarke	***C. velutina* S.Moore** = *C. reticulata* C.B.Clarke

* In the taxonomic treatment of Vollesen (2015), three of the *Crabbea* species recognized in taxonomic treatments of the genus in southern African, namely *C. angustifolia, C. hirsuta* and *C. ovalifolia,* are subsumed under *C. cirsioides.*

Thulin (2007) revised three genera, *Acanthostelma* Bidgood & Brummitt, *Crabbea* and *Golaea* Chiov., based on the shared morphological features among the three genera. Shared features included densely bracteate spherical heads and fan-shaped stigmas. The end result was the description of two new species and the synonomisation of two monotypic genera, *Golaea* and *Acanthostelma* with *Crabbea*. Prior to Thulin (2007), Scotland and Vollesen (2000) recorded that *Golaea* and *Acanthostelma* were monotypic genera and unplaced within the Acanthaceae. The molecular work of McDade *et al.* (2008) placed both genera in the tribe Barlerieae, close to *Crabbea*.

Previous taxonomic treatments lacked a detailed analysis of the significance of micromorphological and anatomical features at genus and species level classification of *Crabbea.* Molecular work has also not received attention in past accounts. However, each treatment did mention the trichome complement. Anatomical and palynological work on southern African *Crabbea* was only reported in the unpublished taxonomic treatment by Buys (1982), while palynology was also reviewed for the Somalian *Crabbea* species by Thulin (2007). Therefore, a re-evaluation of the nomenclature, morphological and anatomical data in combination with molecular data is required for the publication of a generic revision.

1.4 Motivation and aim

The primary aim of this study is to provide a taxonomic revision of *Crabbea* in southern Africa with the focus on reviewing the number of accepted species and subspecies, correcting and reviewing nomenclature, type specimens and synonyms. The proposed classification of Buys (1982), and updated nomenclature proposed by Vollesen (2015), will be evaluated by reviewing all aspects of the taxa included in Buys' (1982) treatment. However, for the purpose of this evaluation of species boundaries, *C. galpinii* C.B.Clarke, *C. nana* and *C. pedunculata* N.E.Br. will be treated as distinct species, as opposed to the species *C. nana* with two subspecies, as proposed by Buys (1982) (Table 1). For the eight *Crabbea* species included in this treatment, suitable morphological, micromorphological and anatomical characters for identification and

classification will be evaluated and used to produce identification keys and descriptions. Updated distribution maps will be produced for all species and molecular phylogenetics will be used for the first time to develop a better understanding of *Crabbea* species relationships in southern Africa.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and historical review

Carl Linnaeus described the genus *Acanthus* L. (Linnaeus, 1753), which later became the type genus of Jussieu's (1789) Acanthaceae. The genus name "*Acanthus*" was derived and modified from the Greek word, "*acanth*-" meaning "spiny" or "thorny" (Stearn, 1983) which was consistent with the spiny, thorny or hairy appearance of most members of this family.

Nees von Esenbeck (1847) provided the first revision of the entire distribution range of Acanthaceae. He split the family into two distinct groups based on the absence (anechmatacantheae) or presence (echmatacantheae) of retinacula - hook-like outgrowths of the funicles, which hold the seed to the fruit of the Acanthaceae (Table 2.1). Eleven tribes were recognised. Nelsonieae and Thunbergieae constituted the anechmatacantheae group. The echmatacantheae group consisted of 9 tribes - Acantheae, Andrographideae, Aphelandreae, Barlerieae, Dicliptereae, Eranthemeae, Gendarusseae, Hygrophileae and Ruellieae. *Crabbea* was one of the 36 genera classified under the Ruellieae tribe. Calyx, corolla, stamen, capsule morphology and bract indumentum were characters used to delimit *Crabbea* from the remaining genera.

Bentham (1876) rearranged the family into five tribes based on a combination of corolla aestivation, seed and ovule characteristics. Acantheae, Justiceae, Nelsonieae, Ruellieae and Thunbergieae were the recognised tribes, with Justicieae and Ruellieae being further divided into subtribes (Table 2.2). *Crabbea* was placed within the subtribe Barlerieae, tribe Justicieae. The subtribe Barlerieae was distinguished from the other Justicieae subtribes by corolla lobe number (5, rather than 2), corolla type (labiate, wide spread, imbricate, concealed), stamen number (4), anther compartments (2) and ovules per ovary (2, rarely 4). *Crabbea* was identified based on calyx, inflorescence and bract characteristics.

Table 2.1 Classification of Acanthaceae *sensu* Nees von Esenbeck (1847).

Family	**Group**	**Tribes of the Echmatacantheae Group**
Acanthaceae	Anechmatacantheae	
	Echmatacantheae	Acantheae Andrographideae Aphelandreae Dicliptereae Eranthemeae Gendarusseae Hygrophileae Barlerieae **Ruellieae** **(*Crabbea*)**

Table 2.2 Classification of Acanthaceae *sensu* Bentham (1876).

Family	Tribes	Subtribes of the Justicieae
Acanthaceae	Thunbergieae	
	Nelsonieae	
	Ruellieae	
	Acantheae	
	Justicieae	Andrographideae
		Asystasieae
		Dicliptereae
		Eranthemeae
		Eujusticieae
		Barlerieae
		(*Crabbea*)

Clarke (1885), like Bentham (1876), also recognised five tribes within Acanthaceae, namely Acantheae, Justicieae, Nelsonieae, Ruellieae and Thunbergieae (Table 2.3). Clarke (1885) reported that the seeds of Nelsonieae and Thunbergieae lacked retinacula and were not hard compared to the remaining tribes. The assessment of the absence of retinacula in Nelsonieae and Thunbergieae of Clarke (1885) was congruent with that of Nees von Esenbeck (1847). Clarke (1885) also maintained further division of Justiceae and Ruellieae into subtribes. However, the subdivision of Justiceae *sensu* Clarke (1885) differed from that of Bentham (1876) in two aspects. Firstly, Justicieae *sensu* Clarke (1885) included only four subtribes, namely Andrographideae, Asystasieae, Barlerieae and Eujusticieae. The tribes Diclipteреae and Eranthemeae did not form part of Clarke‘s (1885) Justiceae. Secondly, Clarke (1885) did not include *Crabbea* in the revision; therefore, the position of *Crabbea* within Acanthaceae *sensu* Clarke (1885) remains unknown.

Lindau (1895) reorganised Acanthaceae into four subfamilies namely Acanthoideae, Mendoncioideae, Nelsonioideae and Thunbergioideae (Table 2.4). The presence or absence of retinacula, retinacula shape and ovule numbers were used to delimit the four subfamilies from each other. Genera characterised by retinaculate fruits were grouped under Acanthoideae. Genera lacking retinaculate fruits were classified as Mendoncioideae, Nelsonioideae or Thunbergioideae. The distinction between retinaculate vs. non-retinaculate groups of Lindau (1895) also followed the classification of Nees von Esenbeck (1847) and Clarke (1885). Corolla aestivation was used to divide the Acanthoideae into two groups. Tribes with an imbricate (overlapping sepals or petals) aestivation pattern were grouped as “Imbricatae” and tribes with a contorted (sepals or petals overlapping adjacent sepals or petals one side only) aestivation pattern were grouped as “Contortae.” Pollen morphology was used in addition to corolla aestivation to further classify taxa within the two groups. Eleven types of pollen grains were identified. Barlerieae and Ruellieae were classified under the Contortae group. Within Contortae, *ripen* “ribbed pollen”, *stachel* “spiky pollen”, *spagen* “sponge pollen”, and/or *waben* “honeycomb pollen” occurred. Barlerieae and Ruellieae were recorded as having *waben* “honeycomb pollen.” Lindau (1895) delimited *Crabbea* from other genera

Table 2.3 Classification of Acanthaceae *sensu* Clarke (1885).

Family	Tribes	Subtribes of the Justicieae
Acanthaceae	Acantheae	
	Nelsonieae	
	Ruellieae	
	Thunbergieae	
	Justicieae	Andrographideae Asystasieae Eujusticieae Barlerieae

Table 2.4 Classification of Acanthaceae *sensu* Lindau (1895).

Family	Subfamilies	Groups within Acanthoideae	Tribes within Contortae
Acanthaceae	Mendoncioideae		
	Nelsonioideae		
	Thunbergioideae		
	Acanthoideae	Imbricatae	
		Contortae	Hygrophileae
			Louteridieae
			Petalidieae
			Ruellieae
			Strobilantheae
			Trichanthereae
			Barlerieae (*Crabbea*)

within the Barlerieae tribe by referring to inflorescence type, bract and calyx morphology.

Burkill and Clarke (1899–1900) organised Acanthaceae into five tribes namely Acantheae, Justicieae, Nelsonieae, Ruellieae and Thunbergieae (Table 2.5), effectively reinstating the tribal classification of Bentham (1876) and Clarke (1885). The classification of the various tribes was based on a combination of characters significant to each tribe. Furthermore, the characters and their character states were not consistent and/or comparable among the five tribes. Burkill and Clarke (1899–1900) placed *Crabbea* under the subtribe Tetrandrae (four fertile stamens; anther cells with rounded bases), tribe Justiceae (corolla 2/5-lobed, one lobe wholly within and one wholly without in the bud; seeds without hygroscopic hairs). Morphological features used to distinguish *Crabbea* from related genera within Justiceae include stamen number (4), number of ovules per ovary/locule (3 or more) and corolla lobe structure (2-lipped).

Clarke (1901) divided Acanthaceae into four tribes namely Acantheae, Justicieae, Ruellieae and Thunbergieae (Table 2.6). Nelsonieae of Burkill and Clarke (1899–1900) was not included in the revision. Thunbergieae was characterised by not having retinaculate fruits, which complemented the classification of Thunbergieae *sensu* Nees von Esenbeck (1847), Clarke (1885), Lindau (1895) and Burkill and Clarke (1899–1900). *Crabbea* was organized under the subtribe Tetrandrae, tribe Justicieae based on stamen number (4), flower arrangement (dense compound heads) and number of ovules per cell (3). Clarke (1901) used the same features as in Burkill and Clarke (1899–1900) to circumscribe *Crabbea*. However, Clarke (1901) added that *Crabbea* flowers form dense compound heads, a description that was absent from the circumscription of this genus by Burkill and Clarke (1899–1900).

Bremekamp (1965) made a radical change in the circumscription of the Acanthaceae, working mainly on the Asian Acanthaceae. Taxa with non-retinaculate fruits were removed from Acanthaceae and either synonymised under existing families or classified as new families. As a result, Nelsonioideae was synonymised under Scrophulariaceae. The status of both Thunbergioideae and Mendoncioideae was raised to family level, Thunbergiaceae and Mendonciaceae. Bremekamp's (1965) view of Lindau's (1895)

Table 2.5 Classification of Acanthaceae *sensu* Burkill and Clarke (1899–1900).

Family	Tribes	Subtribes of Justiceae
Acanthaceae	Acantheae	
	Nelsonieae	
	Ruellieae	
	Thunbergieae	
	Justicieae	Barlerieae
		Eranthemeae
		Eu-Justicieae
		Tetrandreae (*Crabbea*)

Table 2.6 Classification of Acanthaceae *sensu* Clarke (1901).

Family	Tribes	Subtribes of Justicieae
Acanthaceae	Acantheae	
	Ruellieae	
	Thunbergieae	
	Justicieae	Barlerieae Eranthemeae Eu-Justicieae **Tetrandreae** **(*Crabbea*)**

Thunbergioideae and Mendoncioideae, was that the two subfamilies had a greater affinity with Bigoniaceae and Pedaliaceae rather than Acanthoideae itself. The presence of retinaculate fruits was used to delimit Acanthaceae from closely related families. Balkwill and Getliffe-Norris (1988) integrated and organised the southern African Acanthaceae to that of Bremekamp's (1965) classification (Table 2.7). Subdivisions within Acanthaceae resulted in the formation of two distinct groups, Acanthoideae and Ruellioideae. The Acanthoideae lineage was delimited by the presence of monothecate anthers and colpate pollen grains. Cystoliths and articulated stems were used as diagnostic features of the Ruellioideae. Quincuncial or decussate aestivation is significant to the subtribe Barleriinae. Pollen morphology was also incorporated in the classification of Acanthaceae *sensu* Bremekamp (1965). The Acanthoideae consisted of five tribes and Ruellioideae seven. The positions of a number of genera were not clarified, especially in the case of Justicieae Bremekamp (1965).

Balkwill and Getliffe-Norris (1988) focused on the classification of the southern African Acanthaceae (Table 2.8). Characters reviewed in their classification include inflorescence type, corolla shape and aestivation, disc shape - nectariferous tissue positioned at ovary base, androecium features - stamen number, staminodes, filament length and fusion, thecae number, disposition and appendages, fruit shape, seed indumentum and palynology. Results obtained are similar to those of Bremekamp (1965) in the sense that Nelsonioideae and Thunbergioideae are excluded from the classification, and that Acanthoideae and Ruellioideae were the two recognised subfamilies. Ruellioideae was represented by three tribes, namely Justicieae, Neuracantheae and Ruellieae. Barleriinae, Petalidiinae and Ruelliinae were placed firmly within Ruellieae, with the position of Hygrophilinae and Lepidathidinae under question within the tribe. Pollen morphology (globose, reticulate - Barleriinae and Ruelliinae vs. prolate, banded pollen - Petalidiinae) and corolla symmetry (zygomorphic - Barleriinae vs. actinomorphic or subactinomorphic - Ruelliinae vs. subactinomorphic - Petalidiinae) are two consistent characters that could be used to distinguish the three subtribes. Balkwill and Getliffe-Norris (1988) proposes that *Crabbea* should be positioned within Ruelliinae on the basis of pollen similarity with that of *Ruellia*, having

Table 2.7 Classification of Acanthaceae *sensu* Bremekamp (1965) given by Balkwill and Getliffe-Norris (1988).

Family	**Subfamily**	**Tribes of the Ruellioideae**	**Subtribes of the Ruellieae**
Acanthaceae	Acanthoideae		
	Ruellioideae	Justicieae Lepidagathideae **Ruellieae**	Hygrophilinae Petalidiinae Ruelliinae **Barleriinae (*Crabbea*)**

Table 2.8 Classification of Acanthaceae *sensu* Balkwill and Getliffe-Norris (1988).

Family	**Subfamily**	**Tribes of the Ruellioideae**	**Subtribes of the Ruellieae**
Acanthaceae	Acanthoideae		
	Ruellioideae	Justicieae Neuracantheae **Ruellieae**	Barleriinae Hygrophilinae Ledagathidinae Petalidiinae **Ruelliinae (*Crabbea*)**

didynamous stamens and two inner petals being opposed and not adjacent. The position of *Crabbea* within Ruelliinae *sensu* Balkwill and Getliffe-Norris (1988) differs from that of Bremekamp (1965) with *Crabbea* positioned within the subtribe Barleriinae.

Scotland (1990) rejected Bremekamp's (1965) classification and again included taxa with non-retinaculate fruit within the Acanthaceae. However, Acanthaceae *sensu* Scotland (1990) was similar to previous classification systems. For example, retinacula and explosive fruits were used to delimit Acanthoideae from Mendoncioideae, Nelsonioideae and Thunbergioideae. Acanthaceae *sensu* Scotland (1990) constituted the four subfamilies recognised by Lindau (1895). Additionally, anatomical features such as hygroscopic hairs on seeds, the presence or absence of cystoliths, corolla aestivation and monothecate anthers were used to classify genera within Acanthoideae.

The taxonomic position of the Barlerieae *sensu* Lindau (1895) differs from the classification given by Scotland (1990) and Scotland *et al.* (1994). Initially, Barlerieae, including *Crabbea*, was organised under the Contortea Group of Lindau (1895). The findings of Scotland (1990) resulted in taxa within the Barlerieae lineage being divided into one of two groups, Barlerieae (unified by quincuncial corolla aestivation) or Contortae less Barlerieae (left contorted aestivation). Scotland *et al*. (1994) reported that *Barleria, Crabbea* and related genera have a quincuncial corolla aestivation.

Scotland and Vollesen (2000) provided an in-depth analysis of corolla aestivation, pollen micromorphology, molecular data obtained from chloroplast and nuclear genomes as well as a suite of potentially most informative morphological characters. Eleven morphological characters were identified as being most informative, based on a combination of pollen, cystolith, corolla aestivation, androecium and fruit characters. Additionally, Scotland and Vollesen (2000) only used representative genera for their analysis. Results from the combined morphological matrix tree revealed three distinct subfamilies, namely Acanthoideae, Nelsonioideae and Thunbergioideae. Acanthoideae was divided into two tribes namely Acantheae and Ruellieae. The latter tribe was grouped into four smaller subtribes (Table 2.9). Twenty of the 221 Acanthaceae genera could not be placed within respective tribes, subtribes and subfamilies.

Table 2.9 Classification of Acanthaceae *sensu* Scotland and Vollesen (2000).

Family	**Subfamilies**	**Tribes within the subfamily Acanthoideae**	**Subtribes within the tribe Ruellieae**
Acanthaceae	Nelsonioideae Thunbergioideae		
	Acanthoideae	Acantheae	
		Ruellieae	Andrographinae Justicinae Ruelliinae **Barleriinae (*Crabbea*)**

Recently, McDade *et al*. (2008) reported that the Acanthaceae is composed of three subfamilies, namely Acanthoideae, Nelsonioideae, Thunbergioideae and the genus *Avicennia* L. (mangroves). The Acanthoideae is described as the retinaculate clade, based on the presence of retinaculate fruits and constitutes the following tribes: Acantheae (cystoliths absent), Andrographideae, Barlerieae, Justicieae, Ruellieae, Whitfieldieae and two genera, *Lankesteria* Lindl. and *Neuracanthus* Nees (cystoliths present) (McDade *et al*., 2008). Stevens (2001 onwards) follows the tribal classification of Acanthaceae of McDade *et al*. (2008), but divides the Acanthaceae into four subfamilies, namely Acanthoideae, Avicennioideae, Nelsonioideae and Thunbergioideae. Acantheae, Andrographideae, Barlerieae, Justicieae, *Nemacanthus,* Ruellieae and Whitfieldieae form part of the subfamily Acanthoideae (Stevens, 2001 onwards).

2.2 Phylogenetic study of Acanthaceae and *Crabbea*

Plant systematics focuses on the reconstruction of phylogenetic relationships among species, genera and taxa at higher taxonomic levels. The necessity for plant systematics is driven by the immense diversity and plasticity displayed in plant morphologies (Palmer *et al*., 1988).

Scotland *et al*. (1995) constructed the first detailed phylogeny for Acanthaceae using two chloroplast markers, *ndh*F and *rcb*L. The *ndh*F trees suggested that *Barleria* and *Crabbea* were sister taxa within Acanthoideae, with Thunbergioideae being sister to Acanthoideae, and Nelsonioideae the most distantly placed from Acanthoideae. The most parsimonious tree for the *rcb*L region showed that *Barleria* is positioned within Acanthoideae: with Thunbergioideae positioned in between Acanthoideae. Nelsonioideae was placed in a lineage separate from the Acanthoideae-Thunbergioideae Clade. *Crabbea rcb*L sequences were not obtained. The topology of the *rbc*L tree for Acanthaceae suggests that this region is a slow evolving gene region, explaining why Thunbergioideae was placed within Acanthoideae.

McDade and Moody (1999) used the *trn*L-*trn*F gene region to establish the phylogenetic relationships across Acanthaceae. Molecular data revealed three major groupings namely: Acanthoideae (constituting *Acanthus*, *Barleria*, *Ruellia* and *Justicia* lineages); Thunbergioideae (represented by *Mendoncia* Vell. ex Vand. and *Thunbergia*) was sister to Acanthoideae and Nelsonioideae (represented by *Elytraria* Michx.) was most distantly related to Acanthoideae. The molecular data of McDade and Moody (1999) complemented the molecular data of Scotland *et al*. (1995).

McDade *et al*. (2000b) obtained sequences from both Internal Transcribed Spacer (ITS) and *trn*L-*trn*F gene regions, also in an attempt to investigate the evolutionary relationships across Acanthaceae. The resolution obtained from the ITS and combined ITS and *trn*L-*trn*F phylogenetic trees were congruent with the molecular findings of McDade and Moody (1999) and Scotland *et al*. (1995).

Manktelow *et al.* (2001) determined the phylogenetic position of the tribe Whitfieldieae using molecular (*trn*L-*trn*F and *ndhF*) and morphological data. Their phylogenies revealed that Whitfieldieae was sister to the tribe Barlerieae within Acanthoideae. Thunbergioideae was placed as sister to Acanthoideae and the Nelsonioideae being placed more distant to Acanthoideae. These results agreed with the molecular findings of Scotland *et al*. (1995), McDade and Moody (1999) and McDade *et al*. (2000b).

Schwarzbach and McDade (2002) clarified the evolutionary position of the mangrove family, Avicenniaceae (*Avecinnia*) using *trn*L-*trn*F, *rcb*L and ITS. The combined molecular results revealed that Avicenniaceae belongs within Acanthaceae and is sister to Acanthoideae. This resulted in Thunbergioideae and Nelsonioideae being placed further from Acanthoideae and, therefore, “modifying” previous molecular findings.

McDade *et al*. (2008) provided a comprehensive molecular investigation of the evolutionary relationships of the lineages that constitute Acanthaceae by combining four chloroplast markers (*trn*L-*trn*F, *rps*16, *trn*S-*trn*G and *trn*T-*trn*L) and ITS. The overall topology for the family revealed the same layout as Schwarzbach and McDade (2002). The phylogenetic position of *Crabbea* was verified. The genus was positioned within the *Barleria* Clade (Barlerieae) and showed a close affinity to *Acanthostelma* (= *Crabbea*),

Barleria, *Goleae* (= *Crabbea*) and *Lasiocladus* Bojer ex Nees (Thulin, 2007) (Figure 2.1).

2.2.1 Gene regions used in Acanthaceae phylogenetic studies

2.2.1.1 ITS

Within the nucleus, nuclear ribosomal deoxyribonucleic acid (nrDNA) in land plants is organised into many copies of tandem repeats of coding subunits namely 18S–5.8S–26S (Blattner, 1999; Small *et al*., 2004) and 5S (Judd *et al*, 1999; Small *et al*., 2004). Ribosomal genes are widely distributed across the genome in several hundred to thousand copies (Judd *et al.*, 1999). The three coding subunits (18S, 5.8S and 26S) are separated by two spacer regions namely ITS–1 and ITS–2 (Musters *et al*., 1990; Blattner, 1999; Judd *et al.*, 1999) (Figure 2.2). Additionally, the two spacers form part of the transcriptional unit but are not included in the final ribosomal ribonucleic acid (rRNA) product (Blattner, 1999). The spacers function in the maturation of the primary transcripts by bringing the subunit boundaries together, thereby allowing the three subunits to be processed (Musters *et al*., 1990). The whole set of genes are transcribed as a single unit (Judd *et al.*, 1999). The ITS region in Eukaryotes has significant variation in sequence composition, despite being under strong evolutionary constraints (Musters *et al.*, 1990). The complete ITS region is about 500–700 base pairs (bp's) in length (Álvarez and Wendel, 2003). Primers used to amplify the ITS region are given by Blattner (1999).

The advantages of using ITS in phylogenetic studies are that nuclear genomes/gene regions have a more rapid evolutionary rate compared to the chloroplast genome (Small *et al*., 2004). Ribosomal gene regions are distributed widely across the plant genome. ITS sequences in an individual are derived from two parents, which is useful to detect possible hybridisation events (Baldwin *et al*., 1995). ITS sequences can be easily amplified using a number of universal or species specific primers available for this gene region (Blattner, 1999). These primers are used for both plants and fungi (Baldwin *et al*.,

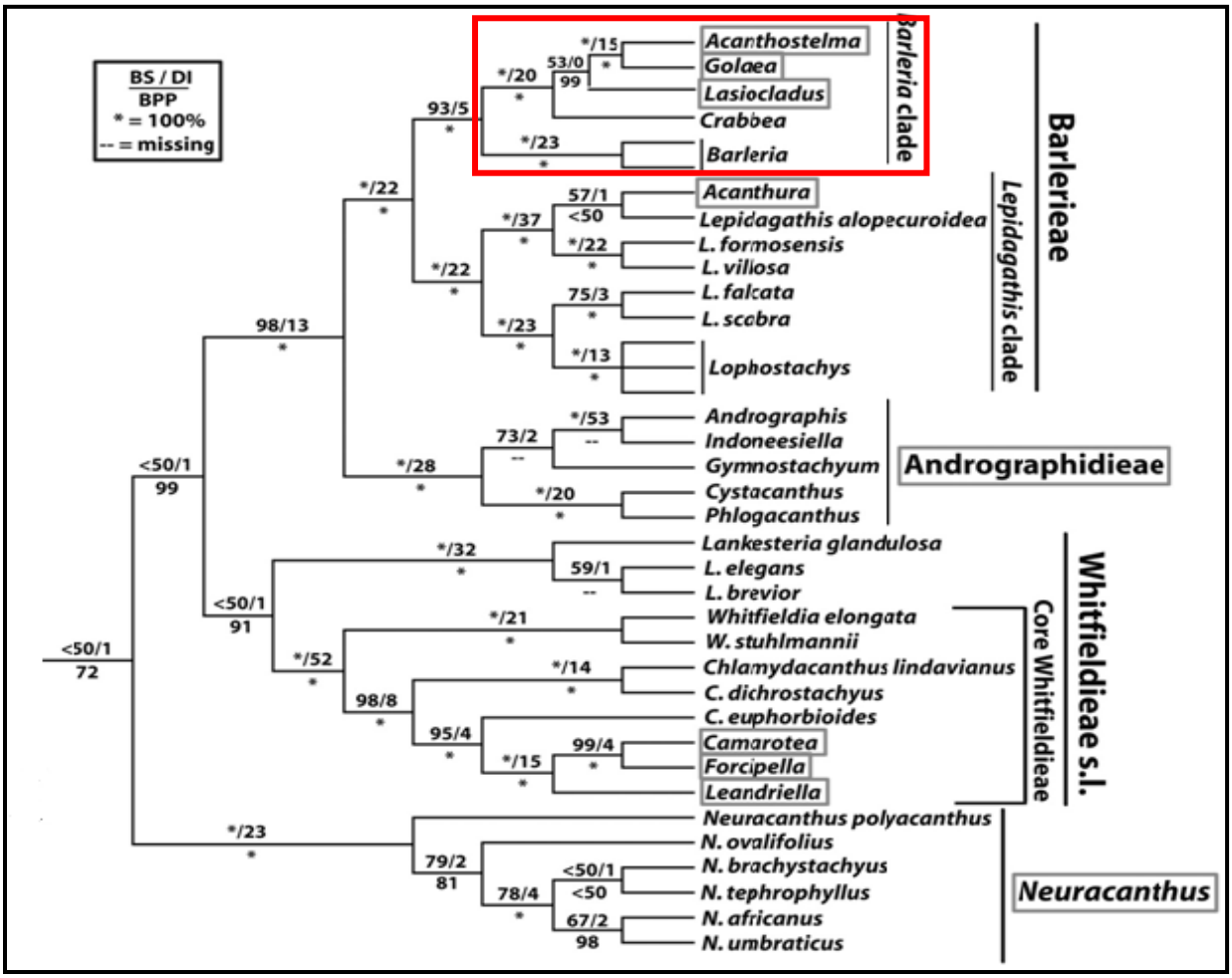

Figure 2.1 The phylogenetic position of *Crabbea* (Barlerieae: Acanthoideae) by McDade *et al.* (2008), with the *Barleria* clade highlighted in red.

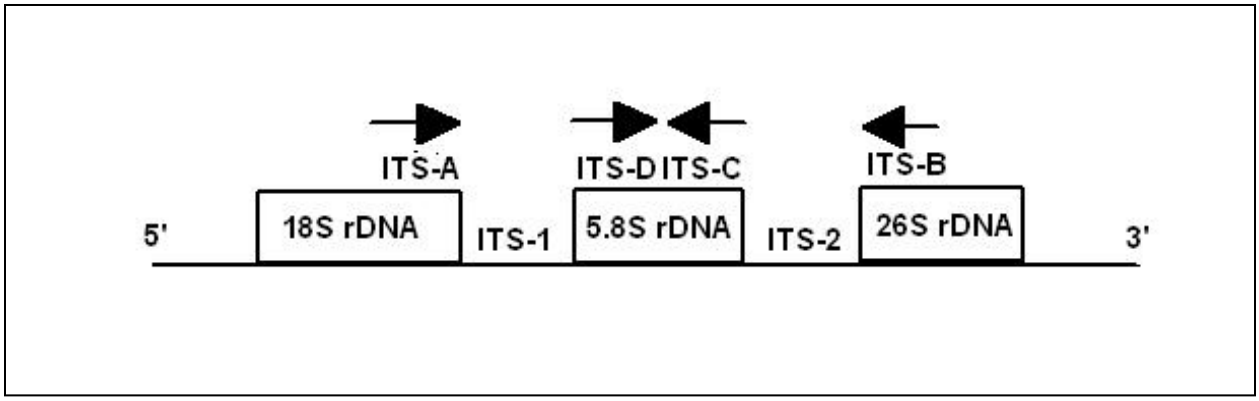

Figure 2.2 The ITS region consisting of three subunits and two internal transcribed spacer regions with the directions of the primers indicated by arrows, as adapted from Blattner (1999).

1995). The ITS region is useful to infer phylogenetic relationships at family level and below (Baldwin *et al*., 1995; Stuessy, 2009).

There are limitations that must be taken into account when working with ITS. Variation among angiosperm ITS sequences are mostly derived from point mutations. Thus, deletions and insertions may complicate ITS sequence alignments, resulting in the formation of gaps so that positional homologies may be kept in their original state. Additionally, ITS sequences are difficult to align when testing for relationships among species grouped under different families (Baldwin *et al*., 1995). Ribosomal gene sequences are subjected to concerted evolution. It is found that the individual copies of rDNA genes evolve at the same rate and undergo gene mutations which can lead to homogenisation, therefore, reducing sequence variation among gene regions (Zimmer *et al*., 1980; Álvarez and Wendel, 2003).

2.2.1.2 *trn*L-*trn*F

The *trn*T-*trn*F gene region consists of three non-coding regions, namely an intergenic spacer between the genes *trn*T (UGU) and *trn*L (UAA) 5'; the *trn*L (UAA) intron and an additional intergenic spacer between *trn*L (UAA) 3' and *trn*F (GAA) (Taberlet *et al*., 1991) (Figure 2.3). The length of the *trn*L 3' intron is between 461–510 bp's long and the spacer about 226–383 bp's long (McDade and Moody, 1999). Primers used to amplify the *trn*L-*trn*F region are given by Taberlet *et al*. (1991).

The advantages of using the *trn*L-*trn*F region in phylogenetic studies is that these sequences can be easily amplified with universal primers (Taberlet *et al*., 1991). Moreover, the small size of the *trn*L-*trn*F spacer allows for easy amplification by designated primers (Gielly and Taberlet, 1994). Phylogenetic studies focusing on *trn*L intron and the *trn*L-*trn*F spacer sequences have shown that the *trn*L-*trn*F spacer has more parsimony-informative characters than the *trn*L intron, despite that the intron is larger in size (Shaw *et al*., 2005).

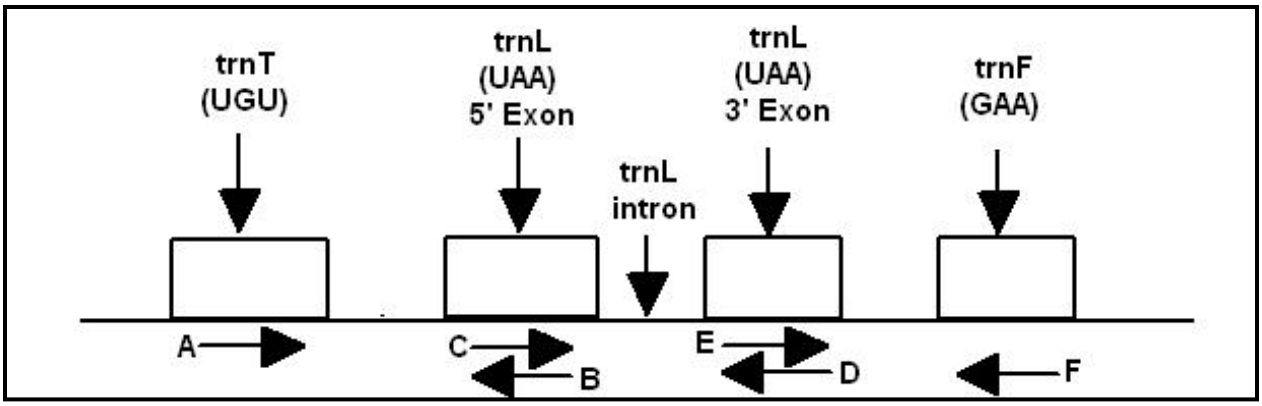

Figure 2.3 The *trn*T-*trn*F chloroplast region, with primer directions indicated by arrows, as adapted from Taberlet *et al*. (1991).

2.2.1.3 *rps*16 intron

The *rps*16 gene is located in the chloroplast genome and codes for the S16 ribosomal protein (Neuhaus *et al*., 1989). A group II intron interrupts the *rps*16 gene (Downie and Palmer, 1992) (Figure 2.4). Amongst angiosperms, the *rps*16 intron varies between 707 to 951 bp's (Oxelman *et al*., 1997).

The *rps*16 intron is a useful molecular tool to infer phylogenetic relationships as this region can be easily isolated and amplified from plant specimens. Furthermore, normal sequencing protocols can be used easily to sequence the *rps*16 intron template (Oxelman *et al.,* 1997; Downie and Katz-Downie, 1999) and sequences can be aligned with relative ease (Oxelman *et al.,* 1997). Additionally, Downie and Katz-Downie (1999) state that the exon-specific primers of the *rps*16 were designed to be used on all angiosperms, thus, allowing easy amplification of the *rps*16 intron. Downie *et al*. (1996) tested the sequence variation of the *rps*16 intron among tobacco (*Nicotiana tabacum* L.) and rice (*Oryza sativa* L.) and results showed that the *rps*16 intron is highly divergent with 67% similarity between the two species.

Caution should be kept in mind when attempting to use the *rps*16 intron for phylogenetic studies. It has been found that the *rps*16 intron is absent from certain angiosperm taxa and, therefore, cannot be used in comparative phylogenetic analysis across all angiosperms. For example, *Pisum sativum* L. lacks the *rps*16 gene (Nagano *et al*., 1991).

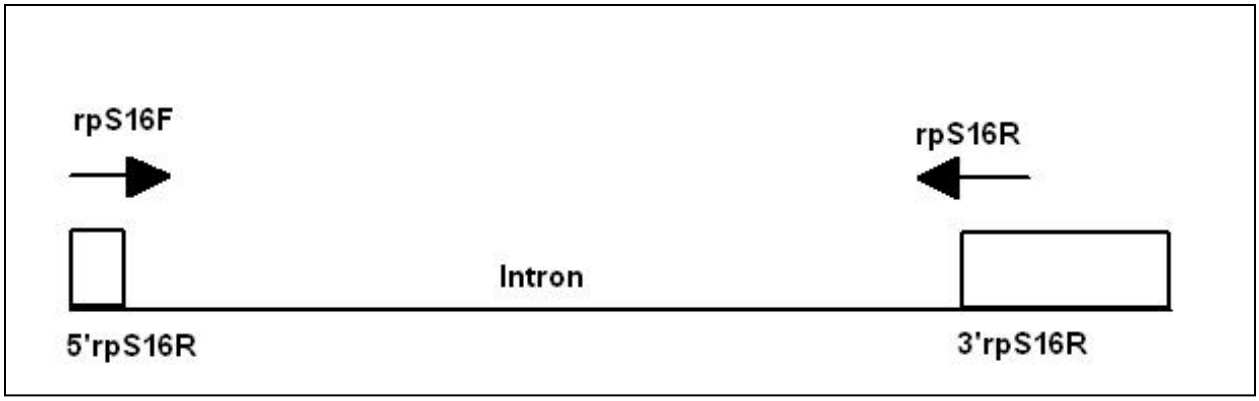

Figure 2.4 The *rps*16 intron with the direction of the primers indicated by arrows, as adapted from Shaw *et al.* (2005), based on the *Nicotiana* L. chloroplast genome of Wakasugi *et al*. (1998).

CHAPTER 3

LEAF MICROMORPHOLOGY

3.1 Introduction

Leaves are regarded as an important source of taxonomic evidence as they can be readily collected from fresh and preserved material and are more often available than flowers, which are limited to a short period during a year (Stuessy, 2009). Leaf character states, such as leaf shape and texture, have proven useful as diagnostic characters (Radford *et al*., 1974). Three southern African *Crabbea* species have been named based on their leaf character states, as in the case of *C. angustifolia*: “narrow leaves” (Nees von Esenbeck, 1847), *C. ovalifolia*: “oval leaves” (Ficalho and Hiern, 1881) and *C. velutina*: “velvet-like” texture over the leaf surface (Moore, 1894).

When describing leaf micromorphology, features of the plant surface architecture may be organised into four categories namely, cellular arrangement, primary, secondary and tertiary sculpture. A description of cellular arrangement on the epidermal surfaces of foliage leaves focus on how cells are organised across the lamina surface. Details of primary sculpture entail the analysis of epidermal cell shape. This constitutes a combination of characters such as anticlinal and periclinal wall curvature, cell outline and cell boundary relief. Primary structure may be of systematic value on lower taxonomic levels. The study of secondary sculpture focuses on cuticular striations and folds across the epidermal surface. Tertiary sculpture results from epicuticular secretion of waxes and related substances (Barthlott, 1981). Epicuticular waxes differ in appearance (crusty, smooth or fissured layers), shape, size, orientation and thickness (Barthlott *et al*., 1998). Tertiary sculpture may be taxon specific and, hence, of diagnostic value on lower taxonomic levels (Barthlott, 1981; Barthlott *et al*., 1998).

Leaves among Acanthaceae taxa are either amphistomatic or hypostomatic (Solereder, 1908; Metcalfe and Chalk, 1950a). Acanthaceae stomata are of the “caryophyllaceous” type, namely diacytic (Solereder, 1908; Metcalfe and Chalk, 1950a) (Figure 3.1). The

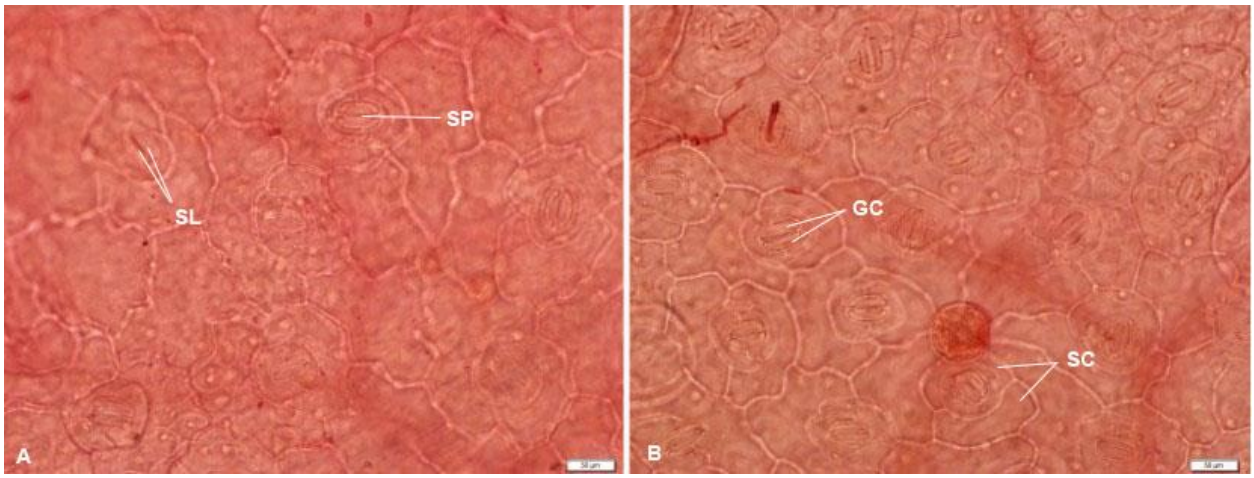

Figure 3.1 Diacytic stomata found on the (A) adaxial and (B) abaxial leaf surfaces of *C. ovalifolia*. **Legend:** GC **=** Guard cells; SC = Subsidary cells; SL = Stomatal ledges; SP = Stomatal pore. **Specimen:** *de Gouveia 136* (BLFU). **Scale bar** = 50 µm.

homogeneity in stomata type in Acanthaceae is a result of stomatal features being under genetic control and stomatal characters can, thus, be used to group taxa at higher taxonomic levels (Payne, 1979; Croxdale, 2000). Stomatal classification depends on a suite of characters such as stomatal pore size, arrangement and distribution, guard cell shape, subsidiary cell arrangement and shape (Wilkinson, 1979; Ash *et al*., 1999).

Trichomes are plant epidermal outgrowths displaying immense variation in appearance and function (Theobald *et al*., 1950; Beentje, 2010). Examples of trichome appearance and functional diversity can be based on the following characters: glandular or eglandular trichomes; the degree of branching and occurrence (Metcalfe and Chalk, 1950b; Beentjie, 2010). Glandular trichomes are useful for higher taxonomic levels from subfamily-level and up. Eglandular trichomes are used at lower taxonomic levels from genus-level and below (Ahmad, 1978). Both glandular and eglandular trichomes have been reported in the Acanthaceae (Metcalfe and Chalk, 1950a; Ahmad, 1978).

Previous taxonomic studies of *Crabbea* have primarily been based on macro and micromorphological characters to classify and identify *Crabbea* taxa, including indumentum of leaves (Harvey, 1842; Nees von Esenbeck, 1847; Burkill and Clarke, 1899–1900; Clarke, 1901; Buys, 1982; Thulin, 2007; Vollesen, 2015). Of all treatments, the taxonomic account of Buys (1982) provides the most information on *Crabbea* leaf micromorphology, incorporating cuticular, stomatal and trichome characters.

The aim of this chapter is to determine the diagnostic leaf micromorphological features that can be used to circumscribe southern African *Crabbea* at genus and species level. This will be achieved by examination of epidermal, cuticular, stomatal and trichome characteristics.

3.2 Materials and Methods

Fresh leaves were fixed and leaves from herbarium vouchers were rehydrated in 3% (v/v) phosphate-buffered glutaraldehyde (GA) for a minimum of 48 hr. Due to time and financial constraints a maximum of three specimens from geographically separate

population of each *Crabbea* species were included in this analysis. Since destructive sampling of most herbarium material was not allowed, sampling could only be done from populations visited during two field trips, which meant that rare or geographically isolated species are represented by only two specimens each. The specimens used for each *Crabbea* species is provided in Table 3.1.

Following fixation, leaf samples were dissected into 5 x 5 mm pieces in distilled water and dehydrated in an alcohol series. The dehydration series was conducted as follows: 30%, 50%, 70%, 95% and 100% (v/v) ethanol (EtOH). Leaves were dehydrated in each alcohol phase for 30 minutes, with the 100% (v/v) EtOH dehydration step repeated four times. After successful dehydration, leaf samples were critical point dried using the Tousimis Samdri®-795 Critical Point Dryer. Dried leaf samples were mounted on clean aluminium stubs with epoxy glue and silver paint was applied to the corners of each leaf sample to increase conductivity. All samples were sputter coated with gold, ± 60 nm thick, using the BioRad Scanning Electron Microscope (SEM) Coating System. Micrographs were taken using the Shimadzu Superscan SSX-550 SEM at 10 kV and a working distance range of 9–17 mm. All preparation for and analysis using SEM was done at the Centre for Confocal Microscopy, University of the Free State (UFS), Bloemfontein.

To determine the stomatal type of *Crabbea,* the Olympus® BX 53 Light Microscope (LM), with a mounted Olympus® DP 72 Camera, at X 20, was used. Measurements were taken using the software package, cellSens® v.1.4.1. Leaf micromorphological descriptions follow the terminology of Beentje (2010) (Figure 3.2).

Table 3.1 *Crabbea* voucher specimens used for leaf micromorphology analysis.

Herbarium	Species	Collector name and number	Collection date
BLFU	*C. acaulis*	A. de Gouveia 80	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 149	28-03-2015
BLFU	*C. angustifolia*	A. de Gouveia 74	21-03-2015
BLFU	*C. angustifolia*	J.E. Burrows and S.E. Burrows 1438	07-03-2015
PRE	*C. angustifolia*	R. Leendertz 6561	00-01-1909
BLFU	*C. cirsioides*	A. de Gouveia 88	22-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 142	28-03-2015
BLFU	*C. galpinii*	A. de Gouveia 169 C	20-11-2015
BLFU	*C. galpinii*	A. de Gouveia 170	21-11-2015
BLFU	*C. galpinii*	A. de Gouveia 171	21-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 81	22-03-2015
BLFU	*C. pedunculata*	A. de Gouveia 176	25-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 177	26-11-2015
BLFU	*C. nana*	A. de Gouveia 146	28-03-2015
BLFU	*C. nana*	A. de Gouveia 179	26-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 136	27-03-2015
BLFU	*C. ovalifolia*	A. de Gouveia 174	23-11-2015
BLFU	*C. velutina*	A. de Gouveia 114	25-03-2015
BLFU	*C. velutina*	A. de Gouveia 163	19-11-2015

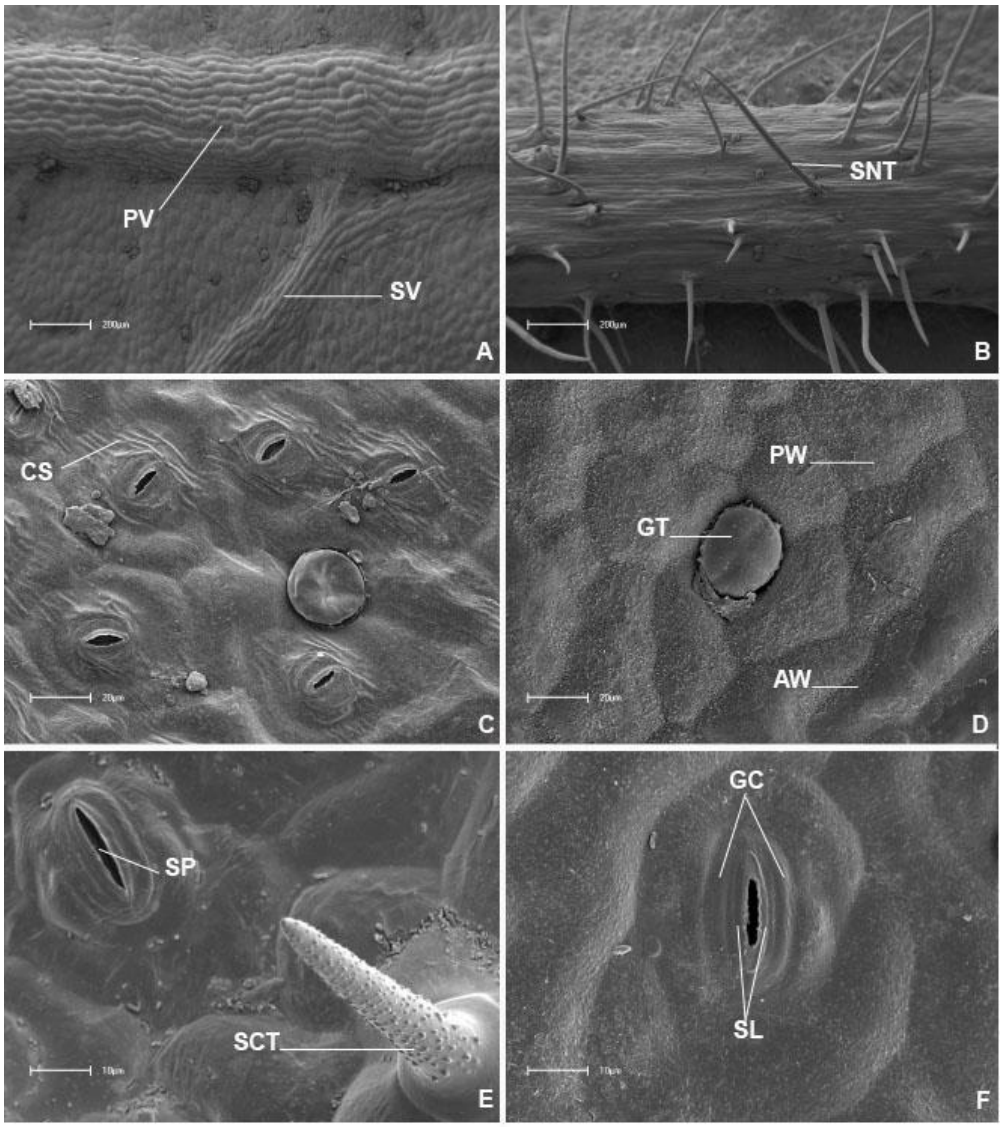

Figure 3.2 Features used to describe *Crabbea* leaf micromorphology. **In:** (A) *C. pedunculata* - adaxial surface with primary and secondary veins; (B) *C. cirsioides* - abaxial surface with raised primary vein and simple needle-shaped trichomes with acute apices; (C) *C. angustifolia* - abaxial surface with cuticular striations surrounding stomata; (D) *C. galpinii* - adaxial leaf surface with subsessile, globose glandular trichomes; convex periclinal walls and sunken anticlinal walls; (E) *C. velutina* - abaxial surface with raised stomata and narrow stomatal pore and simple, cone-shaped ornamented trichome with smooth/polished base; (F) *C. ovalifolia* - adaxial leaf surface with narrow guard cells and narrow stomatal ledges surrounding the stomatal pore. **Legend:** AW = anticlinal wall; CS = cuticular striations; GC = guard cell; GT = glandular trichome; PV = primary vein; PW = periclinal wall; SL = stomatal ledges; SP = stomatal pore and SCT = simple cone-shaped trichome; SNT = simple needle-shaped trichome; SV = secondary vein. **Specimens:** (A) *de Gouveia 81* (BLFU); (B) *de Gouveia 142* (BLFU); (C) *Leendertz 6561* (PRE); (D) *de Gouveia 171* (BLFU); (E) de *Gouveia 114* (BLFU); *de Gouveia 136* (BLFU).

3.3 Results

3.3.1 Genus description of leaf micromorphology of southern African *Crabbea* species

On the **adaxial leaf surface**, **epidermal cells** are isodiametric or rarely elongate and polygonal. **Periclinal walls** are convex. **Anticlinal walls** are sunken, straight to arched, rarely sinuous. The **cuticle** is smooth to granular or rarely striate. **Simple trichomes** are rarely absent; trichomes are filiform to needle-shaped with acute apices, rarely cone-shaped and ornamented on the surface with smooth bases; inserted on the primary or secondary veins or in intercostal areas. **Glandular trichomes** are subsessile, globose and smooth.

On the **abaxial leaf surface, epidermal cells** are isodiametric and polygonal. **Periclinal walls** are convex. **Anticlinal walls** are sunken, straight to arched and sinuous. The **cuticle** is mostly granular, rarely smooth or striate. **Simple trichomes** are rarely absent, when present, they are filiform to needle-shaped with acute apices, rarely cone-shaped, ornamented on the surface with smooth bases; inserted on primary or secondary veins or in intercostal areas. **Glandular trichomes** are subsessile, globose and smooth.

Leaves are amphistomatic or hypostomatic.

The **stomata** on the **adaxial leaf surface** are diacytic, levelled with the epidermal surface, 81–196 stomata per mm^2. **Stomatal pores** are 10.53–15.14 µm long. **Stomatal ledges** are narrow, 14.88–17.66 µm long and 1.84–2.60 µm wide. **Guard cells** are 23.3–24.95 µm long and 3.75–4.87 µm wide (Table 3.2).

The **stomata** on the **abaxial leaf surface** are diacytic, raised above or level with the epidermal surface, 106–428 stomata per mm^2. **Stomatal pores** are 8.85–14.58 µm long. **Stomatal ledges** are narrow, 12.48–19.02 µm long and 1.59–2.92 µm wide. **Guard cells** are 16.88–25.85 µm long and 2.72–4.74 µm wide (Table 3.3).

3.3.2 Leaf micromorphology measurements of the southern African *Crabbea* species

Table 3.2 Measurements of the stomatal characters on the adaxial leaf surface of southern African *Crabbea* species*.

Species	Leaves	Stomatal Density (/mm^2) ± (SD)	Stomatal pore length (µm) ± (SD)	Stomatal ledge length (µm) ± (SD)	Stomatal ledge width (µm) ± (SD)	Guard cell length (µm) ± (SD)	Guard cell width (µm) ± (SD)
C. acaulis	Amphistomatic	196 ± 56	12.19 ± 2.39	14.88 ± 2.44	2.60 ± 0.73	23.30 ± 1.39	4.24 ± 0.52
C. angustifolia	Amphistomatic	136 ± 44	13.56 ± 3.02	17.66 ± 1.39	2.59 ± 0.69	24.77 ± 1.34	3.75 ± 1.15
C. cirsioides	Amphistomatic	81 ± 13	15.14 ± 1.20	17.46 ± 1.22	2.32 ± 0.43	23.86 ± 1.49	4.01 ± 0.98
C. galpinii	Hypostomatic	0	0	0	0	0	0
C. nana	Amphistomatic	165 ± 22	10.98 ± 2.88	15.92 ± 5.37	1.84 ± 0.53	23.93 ± 8.20	4.34 ± 1.35
C. ovalifolia	Amphistomatic	113 ± 47	10.53 ± 2.76	15.98 ± 4.09	2.16 ± 0.77	24.95 ± 6.79	4.87 ± 1.36
C. pedunculata	Hypostomatic	0	0	0	0	0	0
C. velutina	Hypostomatic	0	0	0	0	0	0

*The standard deviation (SD) is provided next to the average value for a particular measurement.

Table 3.3 Measurements of the stomatal characters on the abaxial leaf surface of southern African *Crabbea* species*.

Species	Leaves	stomatal density (/mm^2) ± (SD)	Stomatal pore length (µm) ± (SD)	Stomatal ledge length (µm) ± (SD)	Stomatal ledge width (µm) ± (SD)	Guard cell length (µm) ± (SD)	Guard cell width (µm) ± (SD)
C. acaulis	Amphistomatic	306 ± 25	11.95 ± 3.43	14.83 ± 2.78	2.92 ± 0.39	23.51 ± 4.95	3.27 ± 0.85
C. angustifolia	Amphistomatic	245 ± 72	14.16 ± 3.05	19.02 ± 2.09	2.17 ± 0.50	25.85 ± 3.63	4.74 ± 1.39
C. cirsioides	Amphistomatic	282 ± 55	14.58 ± 1.80	17.61 ± 0.78	2.79 ± 0.35	23.43 ± 1.28	4.25 ± 0.94
C. galpinii	Hypostomatic	239 ± 82	12.94 ± 2.68	16.65 ± 1.68	1.98 ± 0.37	24.75 ± 2.24	4.54 ± 0.98
C. nana	Amphistomatic	240 ± 13	8.85 ± 3.60	12.48 ± 2.80	1.59 ± 0.34	16.88 ± 1.42	2.72 ± 0.48
C. ovalifolia	Amphistomatic	106 ± 68	11.34 ± 3.18	17.53 ± 2.35	2.18 ± 0.57	25.20 ± 2.63	4.37 ± 0.84
C. pedunculata	Hypostomatic	203 ± 29	13.58 ± 1.92	16.68 ± 0.70	2.61 ± 0.63	25.20 ± 2.50	3.87 ± 1.15
C. velutina	Hypostomatic	428 ± 31	10.58 ± 3.16	14.07 ± 2.88	1.82 ± 0.40	18.76 ± 2.52	3.50 ± 0.79

*The SD is provided next to the average value for a particular measurement.

3.3.3 Description of leaf micromorphology among southern species of African *Crabbea*

3.3.3.1 *Crabbea acaulis*

On the **adaxial leaf surface, simple trichomes** are present on intercostal areas and filiform to needle-shaped with acute apices (Figure 3.3.1 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.1 A). The **epidermal cells** are elongated and polygonal (Figure 3.3.1 B). **Anticlinal walls** are straight to arched (Figure 3.3.1 B). The **cuticle** is strongly granular (Figure 3.3.1 C, D).

On the **abaxial leaf surface**, **simple trichomes** are present on the primary vein and filiform to needle-shaped with acute apices (Figure 3.3.2 A). **Simple conical trichomes** are absent (Figure 3.3.2 A). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.2 B). **Anticlinal walls** are straight to arched (Figure 3.3.2 B). The **cuticle** is strongly granular (Fig. 3.3.2 C, D).

The leaves are **amphistomatic** (Figures 3.3.1; 3.3.2; Tables 3.2; 3.3).

On the **adaxial leaf surface**, **stomatal density** is 196 stomata per mm^2**. Stomata** are levelled with the epidermal surface (Figure 3.3.1 B–D). **Guard cells** are 21.4–26.2 µm long and 3.00–5.06 µm wide. **Stomatal pores** are 8.26–15.7 µm long. **Stomatal ledges** are 11.3–18 µm long and 1.7–3.93 µm wide (Figure 3.3.1 D; Table 3.2).

On the **abaxial leaf surface**, **stomatal density** is 306 stomata per mm^2**. Stomata** are raised on the epidermal surface (Figure 3.3.2 B–D). **Guard cells** are 19.6–33.5 µm long and 1.92–5.06 µm wide. **Stomatal pores** are 6.93–16.6 µm long. **Stomatal ledges** are 11.5–18.2 µm long and 2.03–3.56 µm wide (Figure 3.3.2 D; Table 3.3).

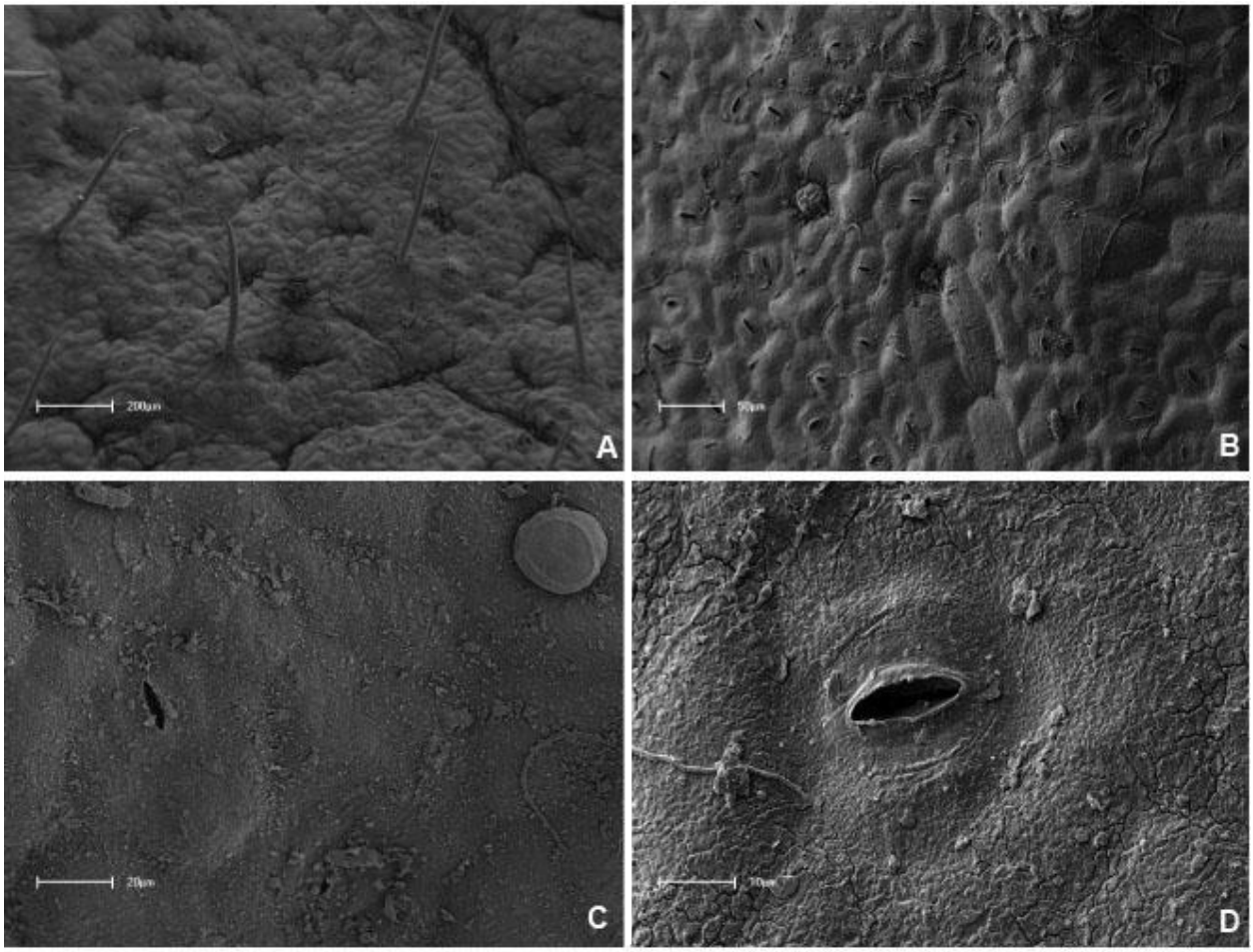

Figure 3.3.1 Adaxial leaf surface of *Crabbea acaulis*. **In:** (A) epidermis covered with simple trichomes; (B, C) stomata levelled with the epidermal surface, sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome, granular cuticle; (D) stomata with narrow stomatal ledges and epidermal cells strongly covered by a granular cuticle. **Specimens:** (A, C) *de Gouveia 149* (BLFU); (B, D) *de Gouveia 80* (BLFU).

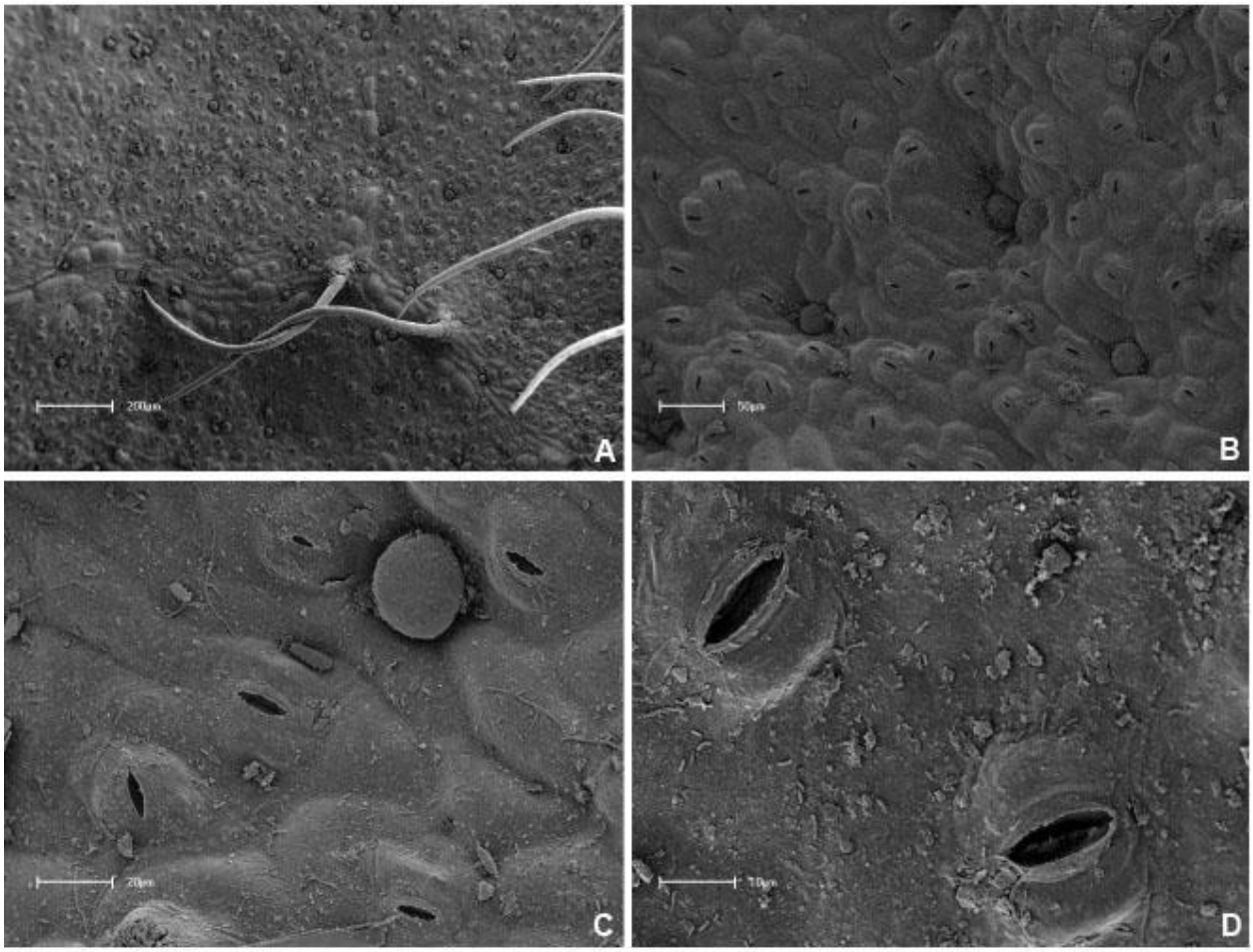

Figure 3.3.2 Abaxial leaf surface of *Crabbea acaulis*. **In:** (A) epidermis covered with simple, filiform to needle-shaped trichomes (B, C) stomata raised on the epidermal surface, glandular trichomes, sunken, globose and smooth anticlinal walls, convex periclinal walls; (D) stomata with narrow stomatal ledges and epidermal cells strongly covered by a granular cuticle. **Specimens:** (A, C) *de Gouveia 149* (BLFU); (B, D) *de Gouveia 80* (BLFU).

3.3.3.2 *Crabbea angustifolia*

On the **adaxial leaf surface**, **simple trichomes** are absent (Figure 3.3.3 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.3 A). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.3 B). **Anticlinal walls** are straight to arched (Figure 3.3.3 B). The **cuticle** is weakly granular (Figure 3.3.3 C, D).

On the **abaxial surface**, **simple trichomes** are filiform to needle-shaped, positioned on the primary vein (Figure 3.3.4 A). **Simple cone-shaped trichomes** ornamented and are sparsely distributed across the epidermal surface (Figure 3.3.4 B). **Anticlinal walls** are arched and sinuous (Figure 3.3.4 B). **Cuticular striations** cover the epidermal surface (Figure 3.3.4 C). The **cuticle** is weakly granular (Figure 3.3.4 C, D).

The leaves are **amphistomatic** (Figures 3.3.3; 3.3.4; Tables 3.2; 3.3).

On the **adaxial leaf surface**, **stomatal density** is 136 stomata per mm^2**. Stomata** are levelled with the epidermal surface (Figure 3.3.3 B–D). **Guard cells** are 22.7–26.5 µm long and 2.10–5.86 µm wide. **Stomatal pores** are 9.4–15.8 µm long. **Stomatal ledges** are 16.0–20.6 µm long and 1.56–4.04 µm wide (Figure 3.3.3 D; Table 3.2).

On the **abaxial leaf surface**, **stomatal density** is 245 stomata per mm^2**. Stomata** are weakly raised on the epidermal surface (Figure 3.3.4 B, C, D). **Guard cells** are 22.1–33.3 µm long and 1.30–5.86 µm wide. **Stomatal pores** are 10.7–17.4 µm long. **Stomatal ledges** are 16.9–22.3 µm long and 1.57–3.13 µm wide (Figure 3.3.4 D; Table 3.3).

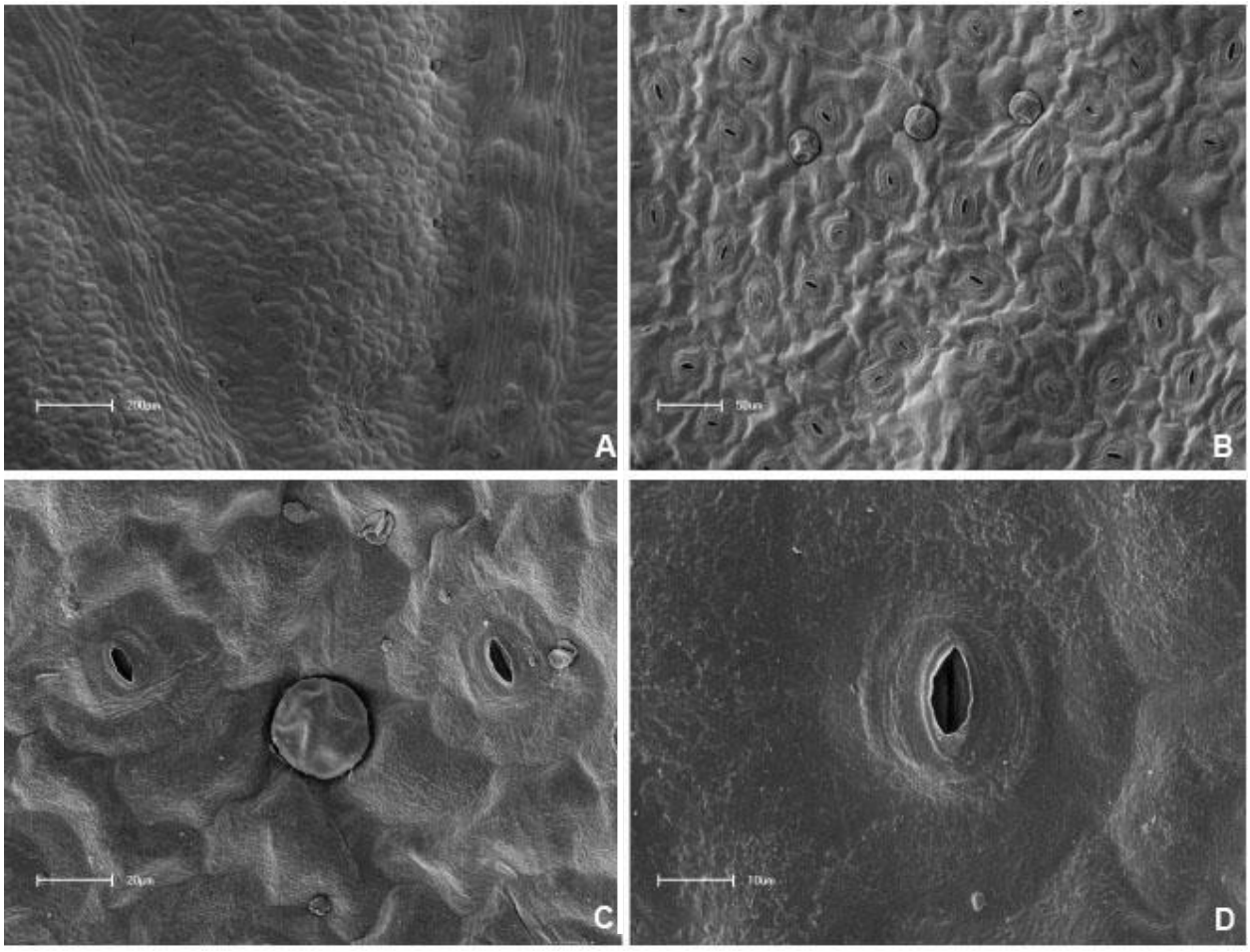

Figure 3.3.3 Adaxial leaf surface of *Crabbea angustifolia*. **In:** (A) adaxial epidermal surface devoid of simple trichomes; (B, C) stomata levelled with the epidermal surface, sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth glandular trichome; (D) stomata with narrow stomatal ledges, cuticle weakly granular. **Specimens:** (A, B) *Burrows and Burrows 14638* (BLFU); (C) *Leendertz 6561* (PRE); (D) *de Gouveia 74* (BLFU).

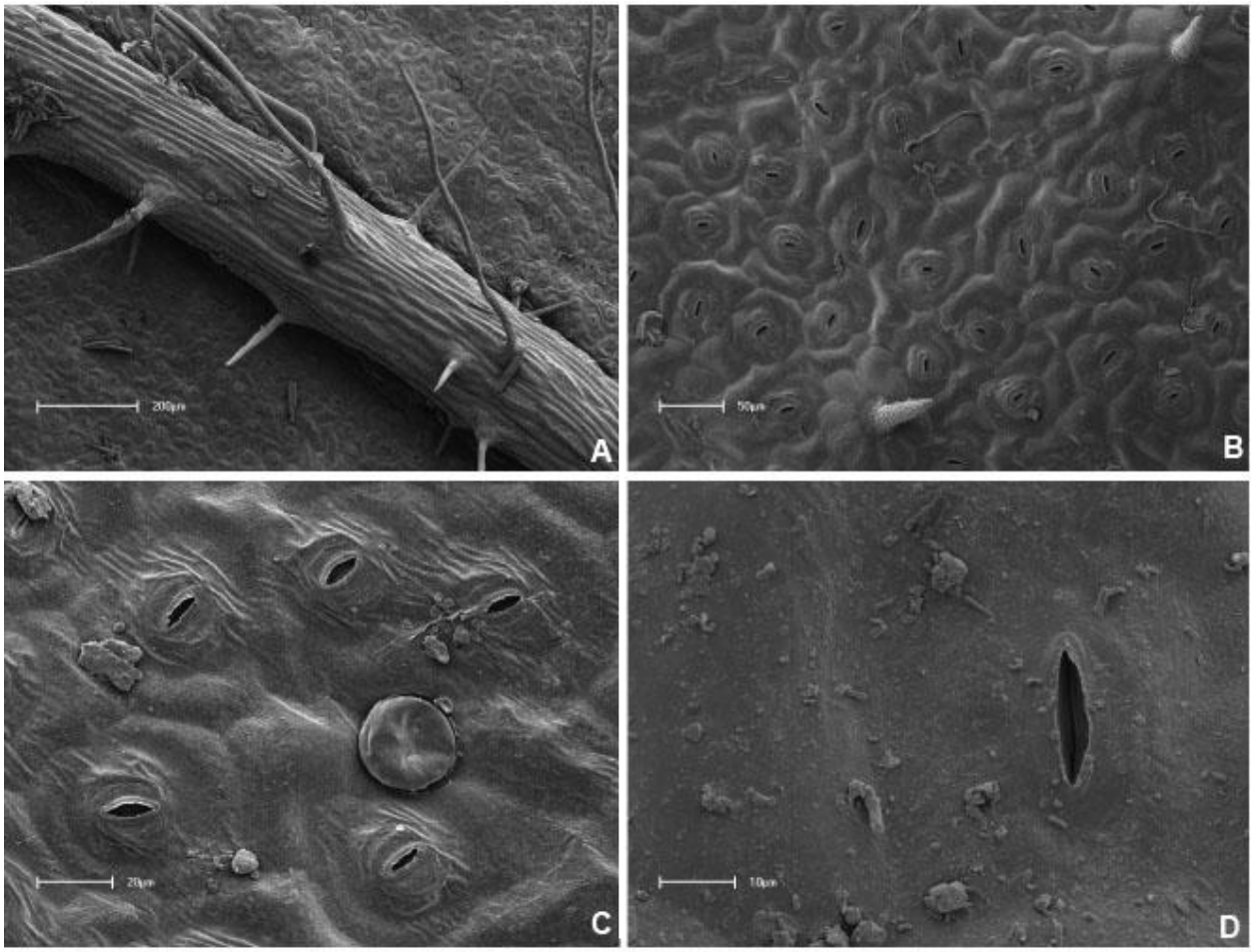

Figure 3.3.4 Abaxial leaf surface of *Crabbea angustifolia*. **In:** (A) simple needle-shaped trichomes on primary vein (B) cone-shaped trichomes sparsely distributed across the epidermal surface; (B, C) stomata weakly raised on the epidermal surface, sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome and epidermis covered by cuticular striations; (D) stomata with narrow stomatal ledges and epidermal cells weakly covered by a granular cuticle. **Specimens:** (A, C) *Leendertz 6561* (PRE); (B) *Burrows and Burrows 14638* (BLFU); (D) *de Gouveia 74* (BLFU).

3.3.3.3 *Crabbea cirsioides*

On the **adaxial leaf surface**, **simple trichomes** are filiform to needle-shaped with acute apices, present on the primary vein (Figure 3.3.5 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.5 B). The **epidermal cells** are isodiametric, polygonal and elongate (Figure 3.3.5 B, C). **Anticlinal walls** are straight to weakly arched (Figure 3.3.5 B, C). The **cuticle** is weakly granular (Figure 3.3.5 C, D).

On the **abaxial surface**, **simple trichomes** are filiform to needle-shaped with acute apices, present on the primary vein (Figure 3.3.6 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.6 B). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.6 B, C). **Anticlinal walls** are weakly arched, sinuous (Figure 3.3.6 B, C). The **cuticle** is smooth (Figure 3.3.6 C, D)

The leaves are **amphistomatic** (Figures 3.3.5; 3.3.6; Tables 3.2; 3.3).

On the **adaxial leaf surface**, **stomatal density** is 80 stomata per mm^2**. Stomata** are levelled with the epidermal surface (Figure 3.3.5 B–D). **Guard cells** are 22–26.2 µm long and 2.73–5.61 µm wide. **Stomatal pores** are 14.4–16.8 µm long. **Stomatal ledges** are 15.6–19.4 µm long and 1.65–3.13 µm wide (Figure 3.3.5 D; Table 3.2).

On the **abaxial leaf surface**, **stomatal density** is 282 stomata per mm^2**. Stomata** are raised on the epidermal surface (Figure 3.3.6 B, C). **Guard cells** are 20.7–25.8 µm long and 3–6.05 µm wide. **Stomatal pores** are 11.4–16.9 µm long. **Stomatal ledges** are 16.9–22.3 µm long and 1.57–3.13 µm wide (Figure 3.3.6 D; Table 3.3).

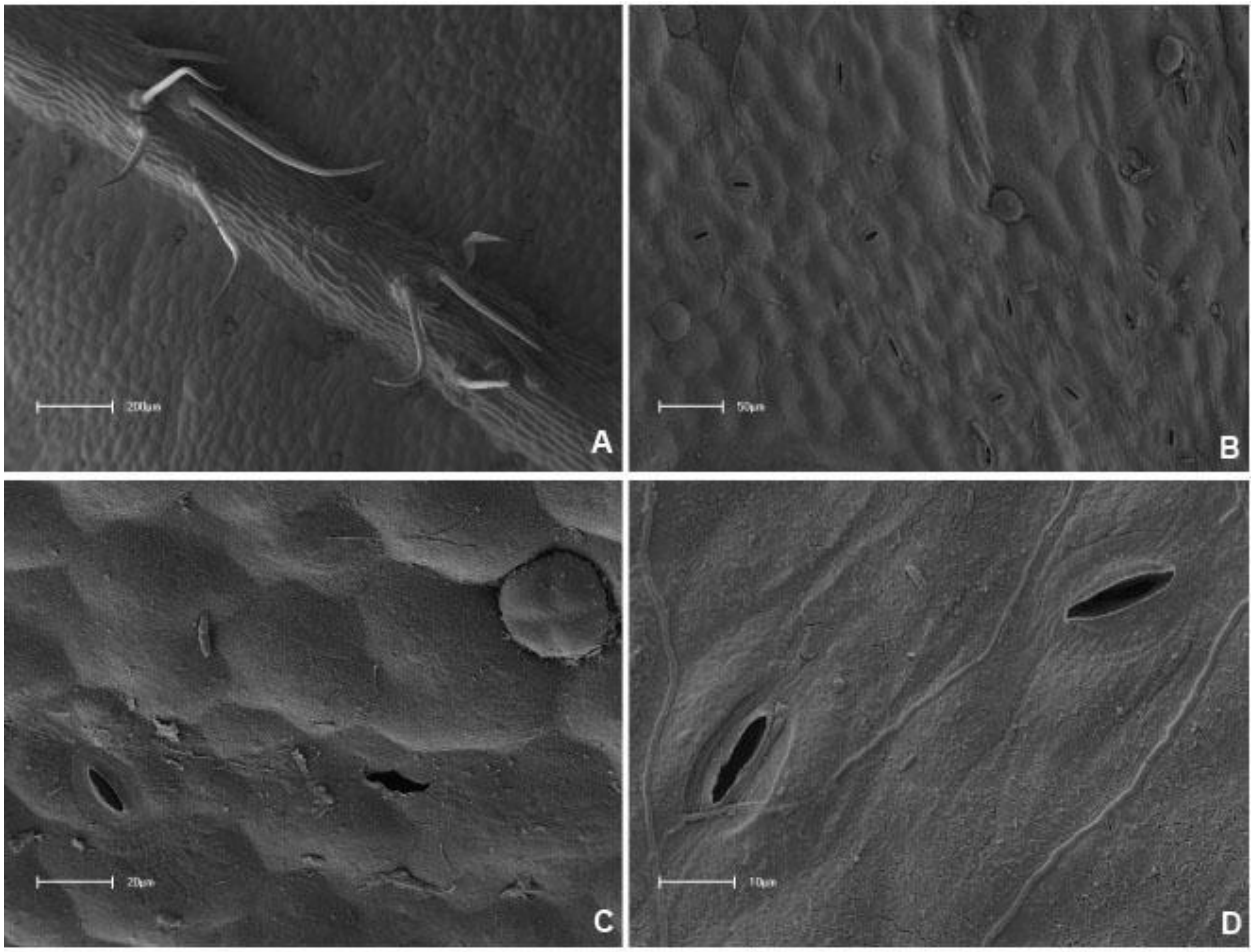

Figure 3.3.5 Adaxial leaf surface of *Crabbea cirsioides*. **In:** (A) primary vein covered with simple needle-shaped trichomes; (B, C) stomata levelled with the epidermal surface, subsessile, globose, smooth, glandular trichome, epidermal cells with sunken anticlinal walls, convex periclinal walls; (D) stomata with narrow stomatal ledges, epidermal cells with a weakly covered granular surface. **Specimens:** (A, C) *de Gouveia 142* (BLFU); (B, D) *de Gouveia 88* (BLFU).

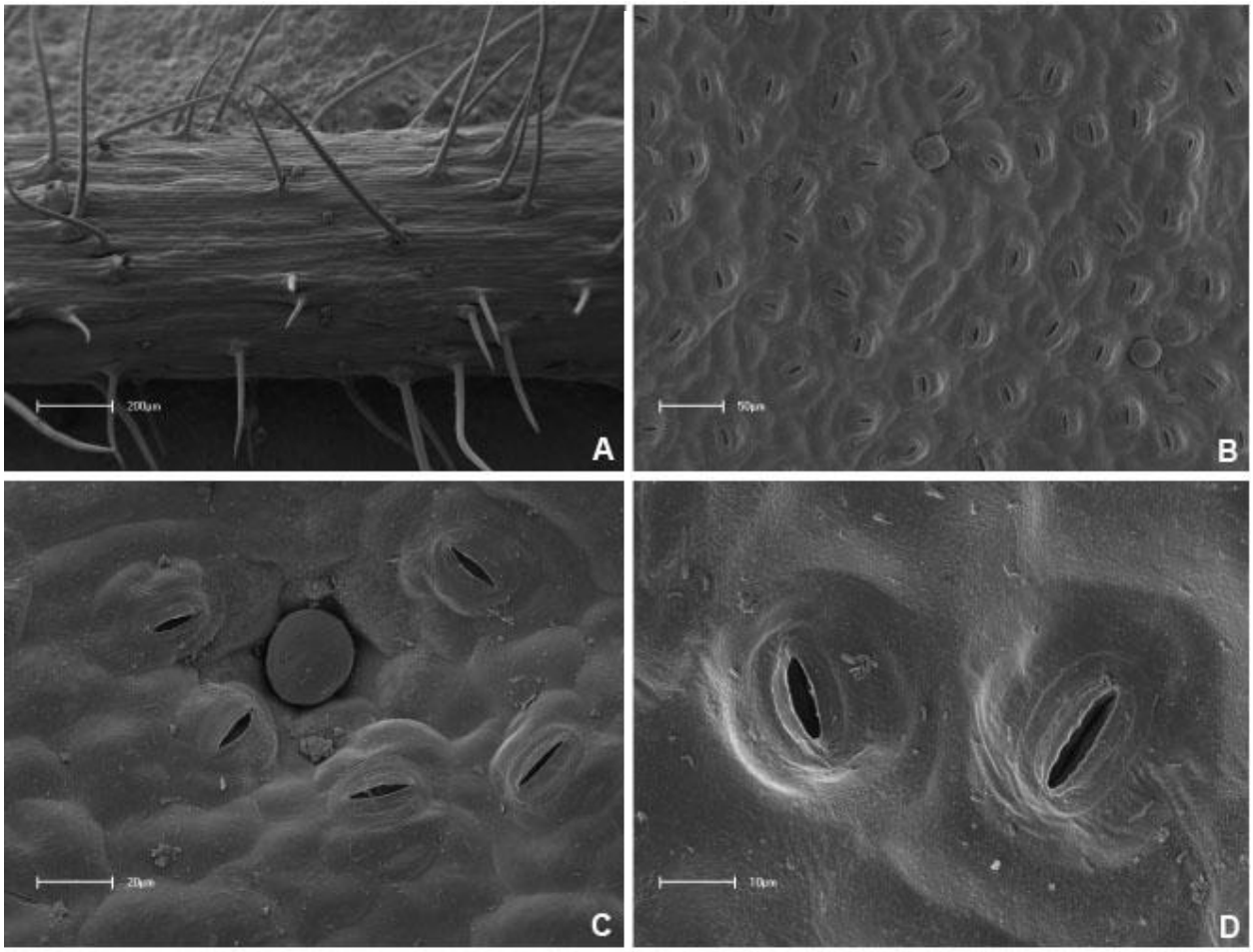

Figure 3.3.6 Abaxial leaf surface of *Crabbea cirsioides*. **In:** (A) simple, needle-shaped trichomes on primary vein; (B, C) stomata raised from the epidermal surface, epidermal cells with sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichomes; (D) stomata with narrow stomatal ledges, epidermal cells with a smooth cuticle. **Specimens:** (A, C) *de Gouveia 142* (BLFU); (B, D) *de Gouveia 88* (BLFU).

3.3.3.4 *Crabbea galpinii*

On the **adaxial leaf surface**, **simple trichomes** are absent. **Simple cone-shaped trichomes** are absent (Figure 3.3.7 A, B). The **epidermal cells** are isodiametric and polygonal. **Anticlinal walls** are straight to weakly arched. The **cuticle** is weakly granular (Figure 3.3.7 C, D).

On the **abaxial surface**, **simple trichomes** are absent (Figure 3.3.8 A, B). **Simple cone-shaped trichomes** are absent (Figure 3.3.8 A, B). **Anticlinal walls** are arched and sinuous. The **cuticle** is smooth (Figure 3.3.8 B–D).

The leaves are **hypostomatic** (Figures 3.3.7; 3.3.8; Tables 3.2; 3.3).

On the **abaxial leaf surface**, **stomatal density** is 239 stomata per mm^2**. Stomata** are levelled with the epidermal surface (Figure 3.3.8 C, D). **Guard cells** are 20.8–26.9 µm long and 3.26–6.64 µm wide. **Stomatal pores** are 8.66–16 µm long. **Stomatal ledges** are 14.3–19.1 µm long and 1.56–2.43 µm wide (Figure 3.3.8 D; Table 3.3).

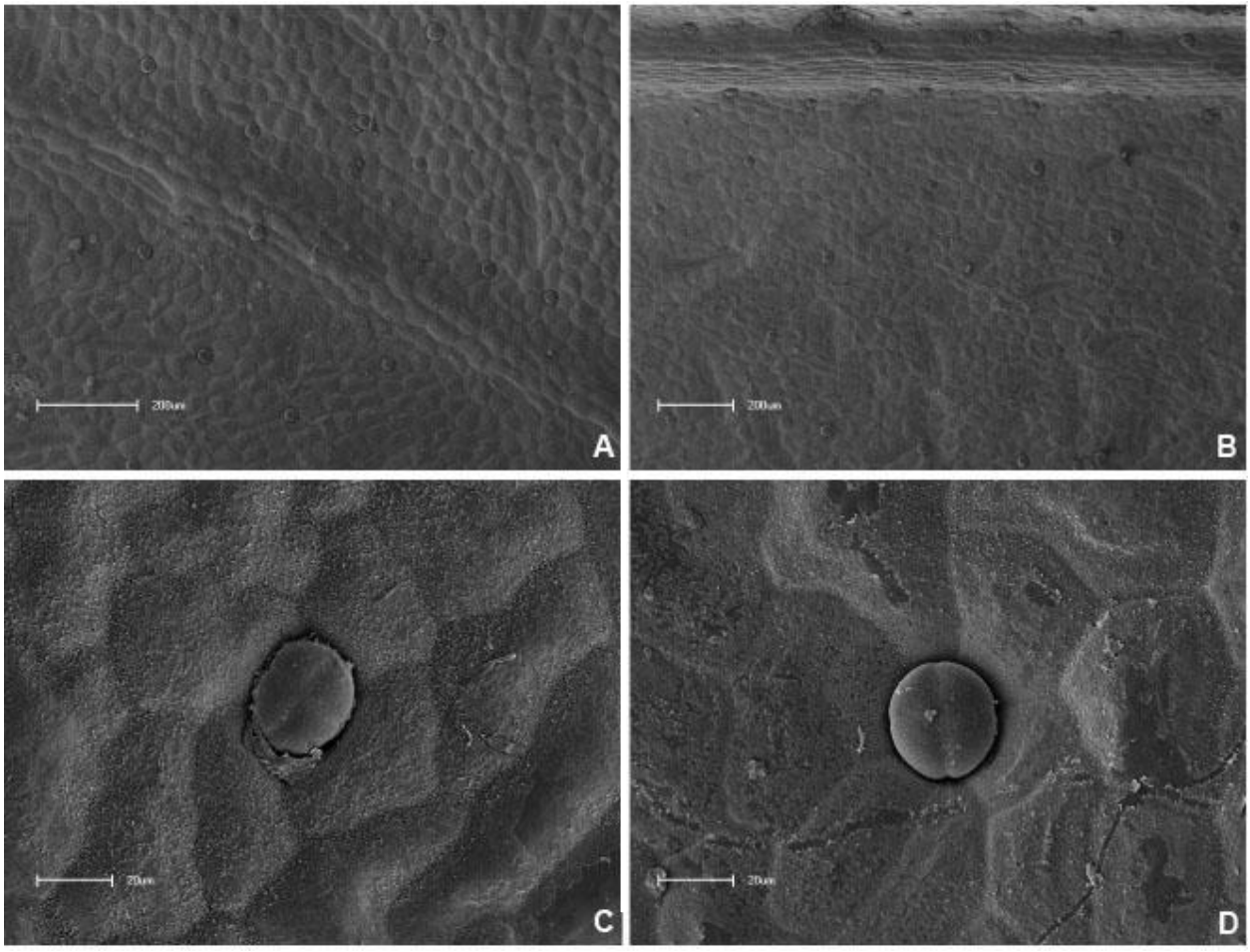

Figure 3.3.7 Adaxial leaf surface of *Crabbea galpinii.* **In:** (A, B) glandular trichomes on primary veins, simple trichomes absent, epidermal cells with sunken anticlinal walls and convex periclinal walls; (C, D) subsessile, globose, smooth, glandular trichomes, epidermis covered by a weakly granular cuticle. **Specimens:** (A, C) *de Gouveia 170* (BLFU); (B) *de Gouveia 171* (BLFU); (D) *de Gouveia 169 C* (BLFU).

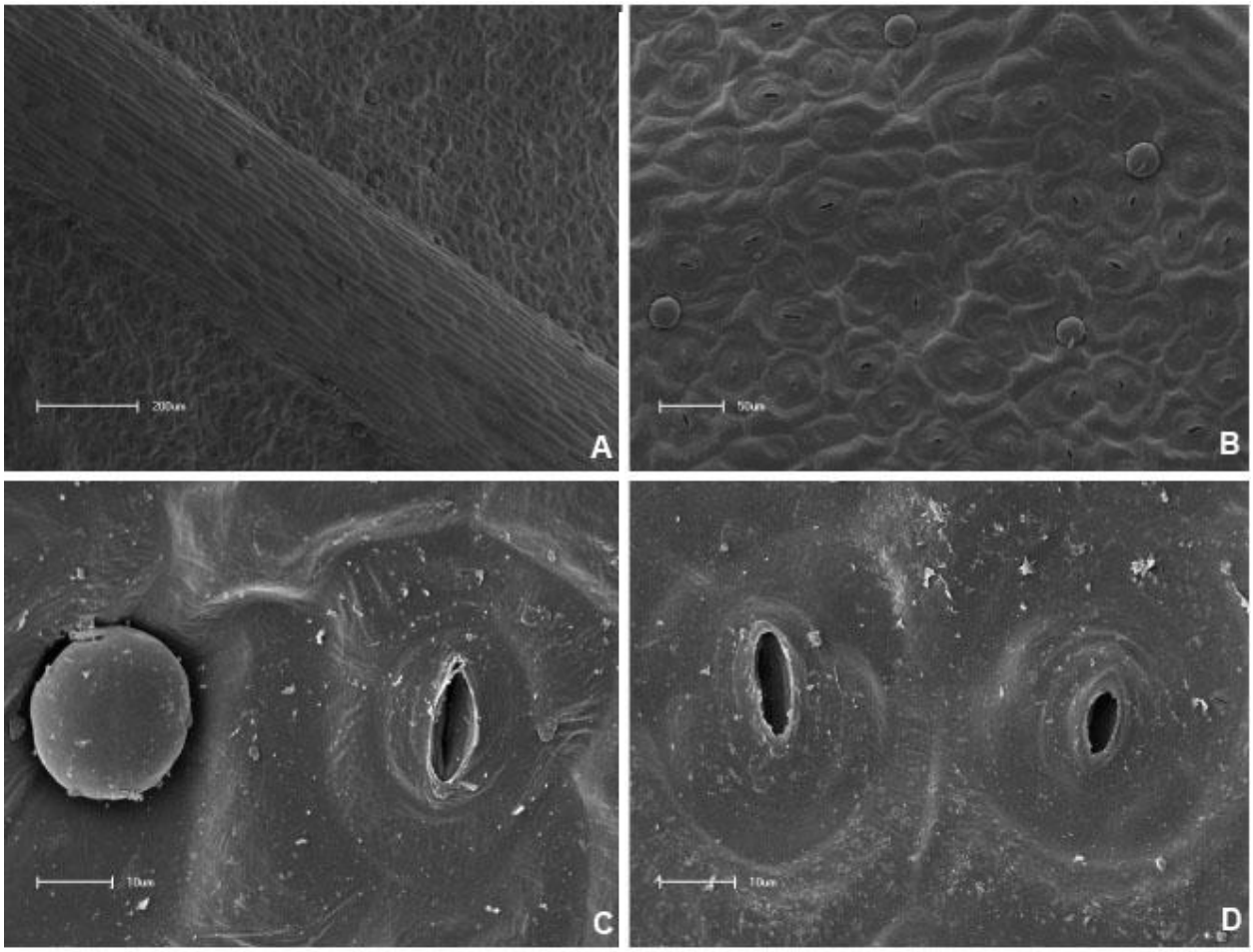

Figure 3.3.8 Abaxial leaf surface of *Crabbea galpinii*. **In:** (A) primary veins devoid of simple trichomes; (B, C) sunken anticlinal walls and concave periclinal walls, subsessile, globose, smooth glandular trichomes; (D) stomata levelled with the epidermal surface, with narrow stomatal ledges and epidermal cells with a smooth cuticular surface. **Specimens:** (A) *de Gouveia 171* (BLFU); (B, D) *de Gouveia 169C* (BLFU); (C) *de Gouveia 170* (BLFU).

3.3.3.5 *Crabbea nana*

On the **adaxial leaf surface**, **simple trichomes** are absent (Figure 3.3.9 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.9 B). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.9 B, C). **Anticlinal walls** are straight to weakly arched, sinuous (Figure 3.3.9 B, C). The **cuticle** is granular (Figure 3.3.9 C, D).

On the **abaxial surface**, **simple trichomes** are filiform to needle-shaped with acute apices, present on the primary vein (Figure 3.3.10 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.10 B). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.10 B, C). **Anticlinal walls** are straight to weakly arched, sinuous (Figure 3.3.10 B, C). The **cuticle** is granular (Fig. 3.3.10 C, D).

The leaves are **amphistomatic** (Figures 3.3.9; 3.3.10; Table 3.2; 3.3).

On the **adaxial leaf surface**, **stomatal density** is 165 stomata per mm^2**. Stomata** are levelled with the epidermal surface (Figure 3.3.9 B–D). **Guard cells** are 20.5–26.4 µm long and 3.13–5.34 µm wide. **Stomatal pores** are 7.03–13.8 µm long. **Stomatal ledges** are 14.5–18.2 µm long and 1.57–2.08 µm wide (Figure 3.3.9 D; Table 3.2).

On the **abaxial leaf surface**, **stomatal density** is 240 stomata per mm^2**. Stomata** are raised on the epidermal surface (Figure 3.3.10 B, C). **Guard cells** are 14.7–19 µm long and 1.95–3.17 µm wide. **Stomatal pores** are 4.3–14.6 µm long. **Stomatal ledges** are 8.37–16.6 µm long and 1.04–2.02 µm wide (Figure 3.3.10 D; Table 3.3).

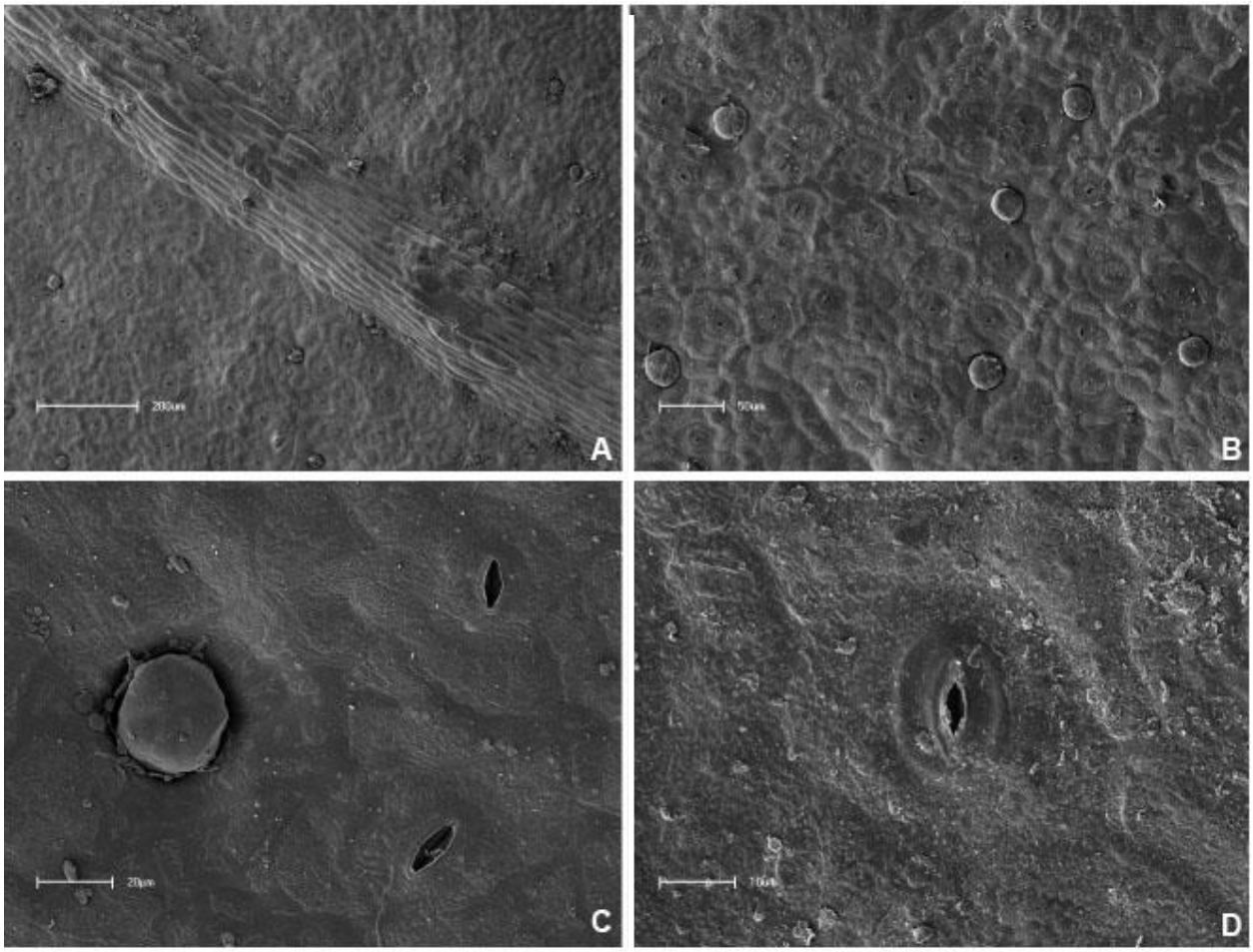

Figure 3.3.9 Adaxial leaf surface of *Crabbea nana*. **In:** (A) primary vein devoid of simple trichomes; (B, C) stomata levelled with the epidermal surface, epidermal cells with sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome; (D) stoma with narrow stomatal ledges and epidermal cells with a granular cuticle. **Specimens:** (A, B, D) *de Gouveia 179* (BLFU); (C) *de Gouveia 146* (BLFU).

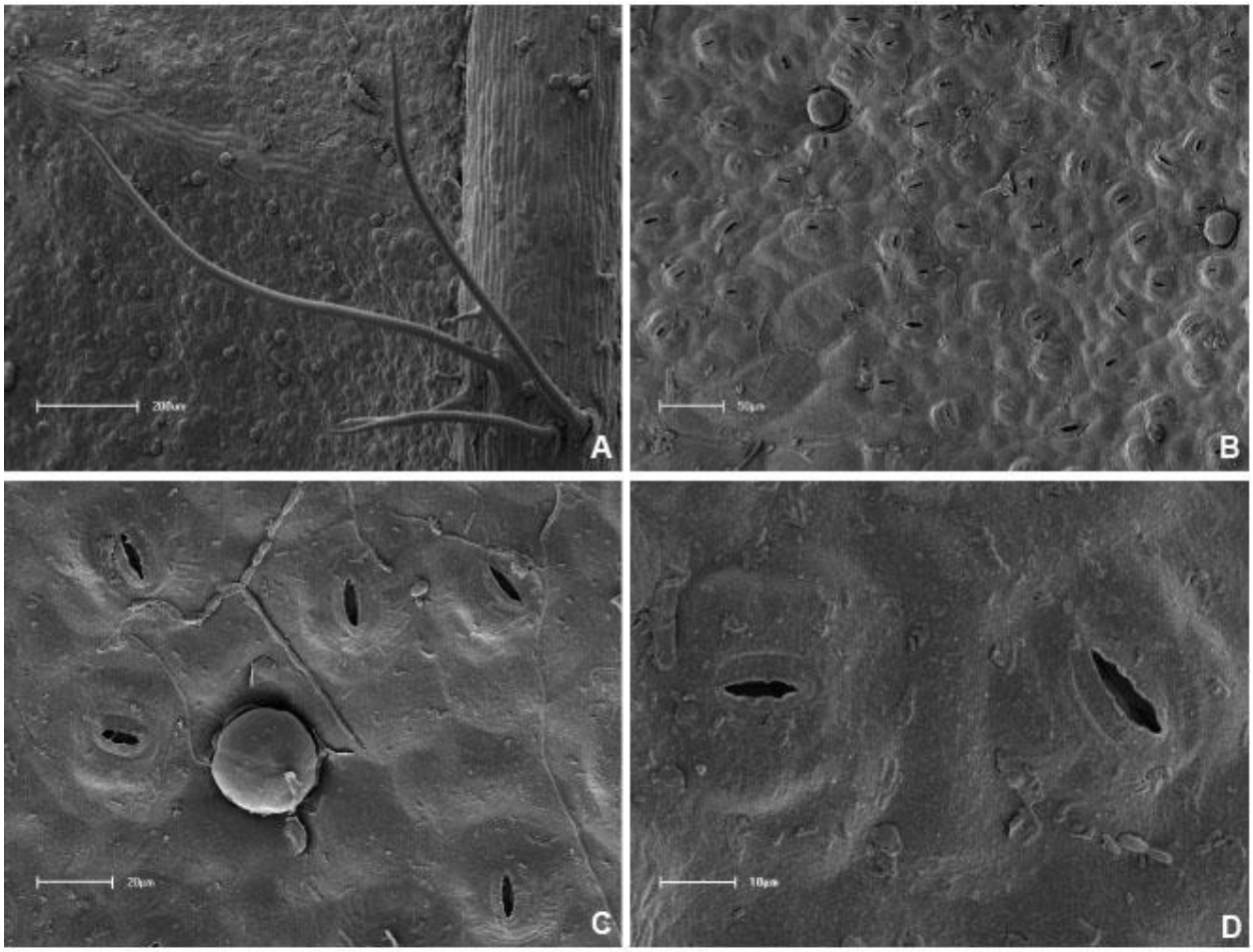

Figure 3.3.10 Abaxial leaf surface of *Crabbea nana*. **In:** (A) simple needle-shaped trichomes on primary vein; (B, C) stomata raised on the epidermal cells, epidermal cells with sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome; (D) stomata with narrow stomatal ledges and epidermal cells with a strongly granular cuticle. **Specimens:** (A) *de Gouveia 179* (BLFU); (B–D) *de Gouveia 146* (BLFU).

3.3.3.6 *Crabbea ovalifolia*

On the **adaxial leaf surface**, **simple trichomes** are filiform to needle-shaped with acute apices, present on the primary vein (Figure 3.3.11 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.11 B). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.11 B, C). **Anticlinal walls** are straight to weakly arched (Figure 3.3.11 B, C). The **cuticle** is weakly granular (Figure 3.3.11 C, D).

On the **abaxial surface**, **simple trichomes** are filiform to needle-shaped with acute apices, present on the primary vein (Figure 3.3.12 A). **Simple cone-shaped trichomes** are absent (Figure 3.3.12 B). The **epidermal cells** are isodiametric and polygonal (Figure 3.3.12 B, C). **Anticlinal walls** are straight to weakly arched (Figure 3.3.12 B, C). The **cuticle** is smooth (Figure 3.3.12 C, D).

The leaves are **amphistomatic** (Figures 3.3.11; 3.3.12; Tables 3.2; 3.3).

On the **adaxial leaf surface**, **stomatal density** is 113 stomata per mm^2. **Stomata** are levelled with the epidermal surface (Figure 3.3.11 B–D). **Guard cells** are 19.8–28.8 µm long and 3.91–5.47 µm wide. **Stomatal pores** are 6.8–13.4 µm long. **Stomatal ledges** are 14.1–18.9 µm long and 1.7–2.75 µm wide (Figure 3.3.11 D; Table 3.2).

On the **abaxial leaf surface**, **stomatal density** is 106 stomata per mm^2. **Stomata** are raised on the epidermal surface (Figure 3.3.12 B, C). **Guard cells** are 21.7–28.3 µm long and 3.4–5.86 µm wide. **Stomatal pores** are 7.32–15.1 µm long. **Stomatal ledges** are 14.6–20.9 µm long and 1.18–2.76 µm wide (Figure 3.3.12 D; Table 3.3).

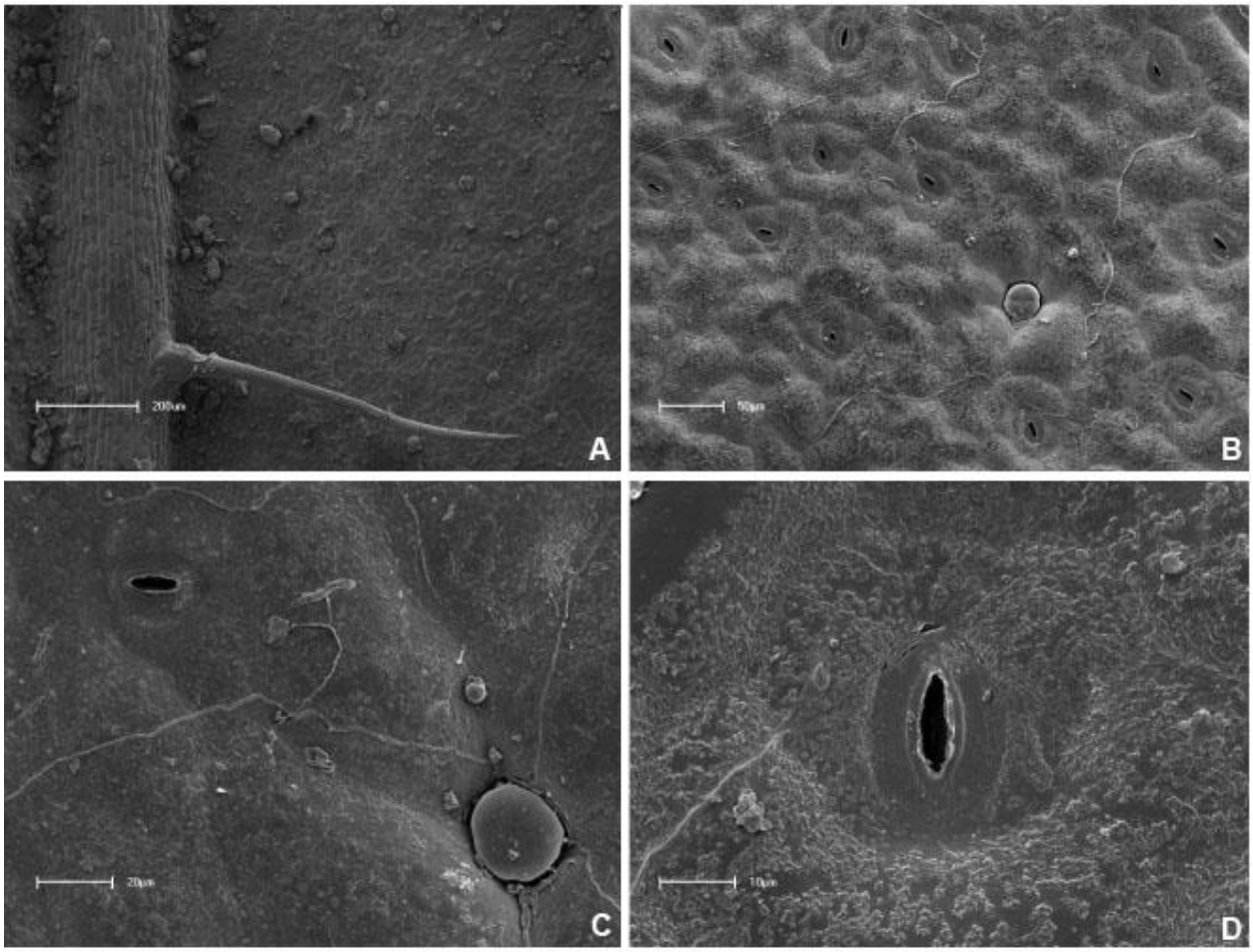

Figure 3.3.11 Adaxial leaf surface of *Crabbea ovalifolia*. **In:** (A) simple needle-shaped trichome on primary vein; (B, C) stomata levelled on the epidermis, epidermal cells with sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome; (D) stoma with narrow stomatal ledges and epidermal cells with a granular cuticle. **Specimens:** (A) *de Gouveia 174* (BLFU); (B–D) *de Gouveia 136* (BLFU).

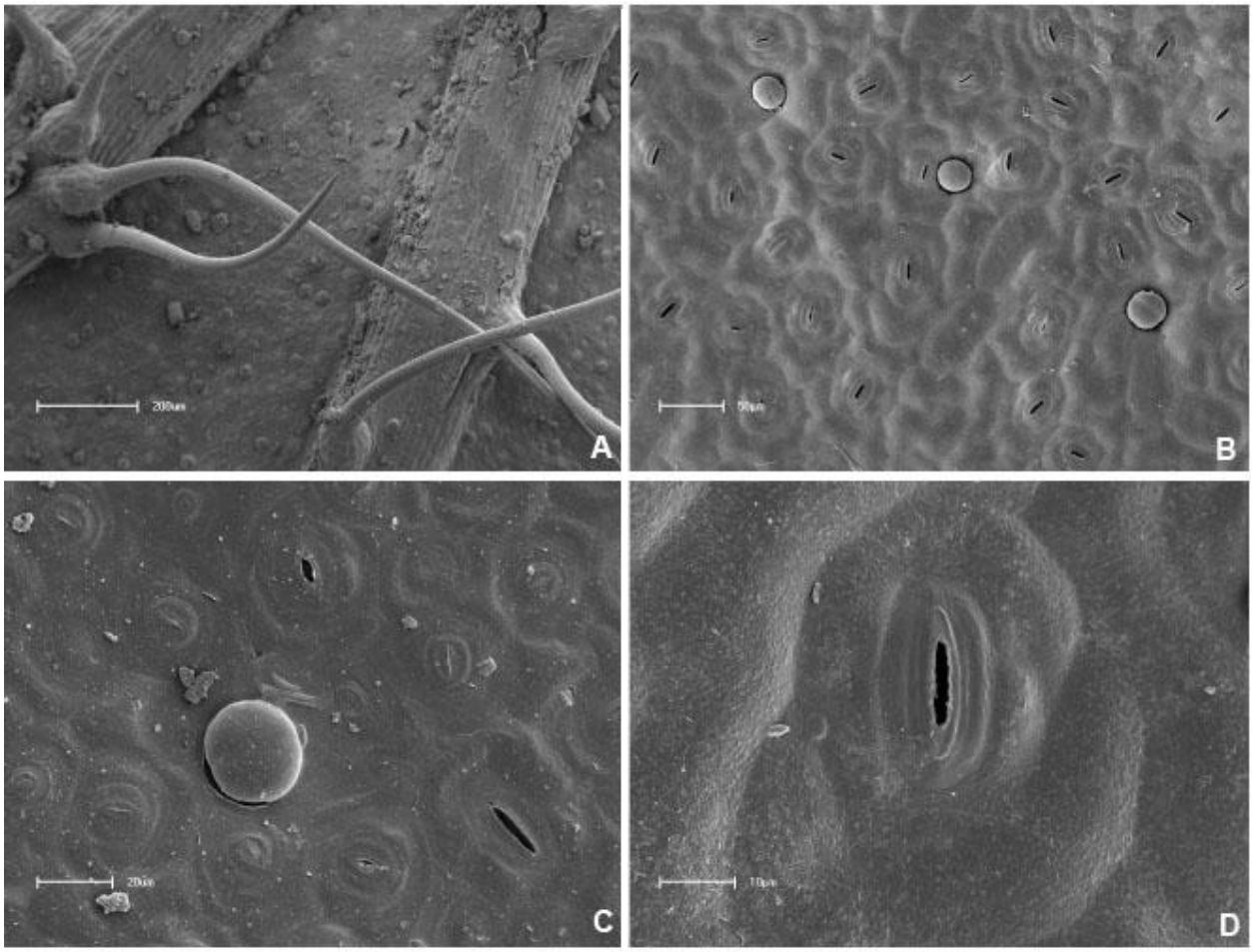

Figure 3.3.12 Abaxial leaf surface of *Crabbea ovalifolia*. **In:** (A) simple needle-shaped trichomes on primary and secondary veins; (B, C) stomata levelled with the epidermis, epidermal cells with sunken anticlinal walls and convex periclinal walls, subsessile, globose, smooth, glandular trichome; (D) stoma with narrow stomatal ledges and epidermal cells with a smooth cuticle. **Specimens:** (A, C) *de Gouveia 174* (BLFU); (B, D) *de Gouveia136* (BLFU).

3.3.3.7 *Crabbea pedunculata*

On the **adaxial leaf surface**, **simple trichomes** are absent. **Simple cone-shaped trichomes** are absent (Figure 3.3.13 A, B). The **epidermal cells** are isodiametric and polygonal. **Anticlinal walls** are straight to weakly arched. The **cuticle** is weakly striated and weakly granular (Figure 3.3.13 C, D).

On the **abaxial surface**, **simple trichomes** are absent from the epidermal surface. **Simple cone-shaped trichomes** are absent (Figure 3.3.14 A, B). **Anticlinal walls** are arched and sinuous (Figure 3.3.14 C). The **cuticle** is weakly striated (Figure 3.3.14 C, D).

The leaves are **hypostomatic** (Figures 3.3.13; 3.3.14; Tables 3.2; 3.3).

On the **abaxial leaf surface**, **stomatal density** is 203 stomata per mm^2. **Stomata** are levelled with the epidermal surface (Figure 3.3.14 C, D). **Guard cells** are 21–29.3 µm long and 2.1–5.98 µm wide. **Stomatal pores** are 10–15.5 µm long. **Stomatal ledges** are 15.2–17.6 µm long and 1.57–3.91 µm wide (Figure 3.3.14 D; Table 3.3).

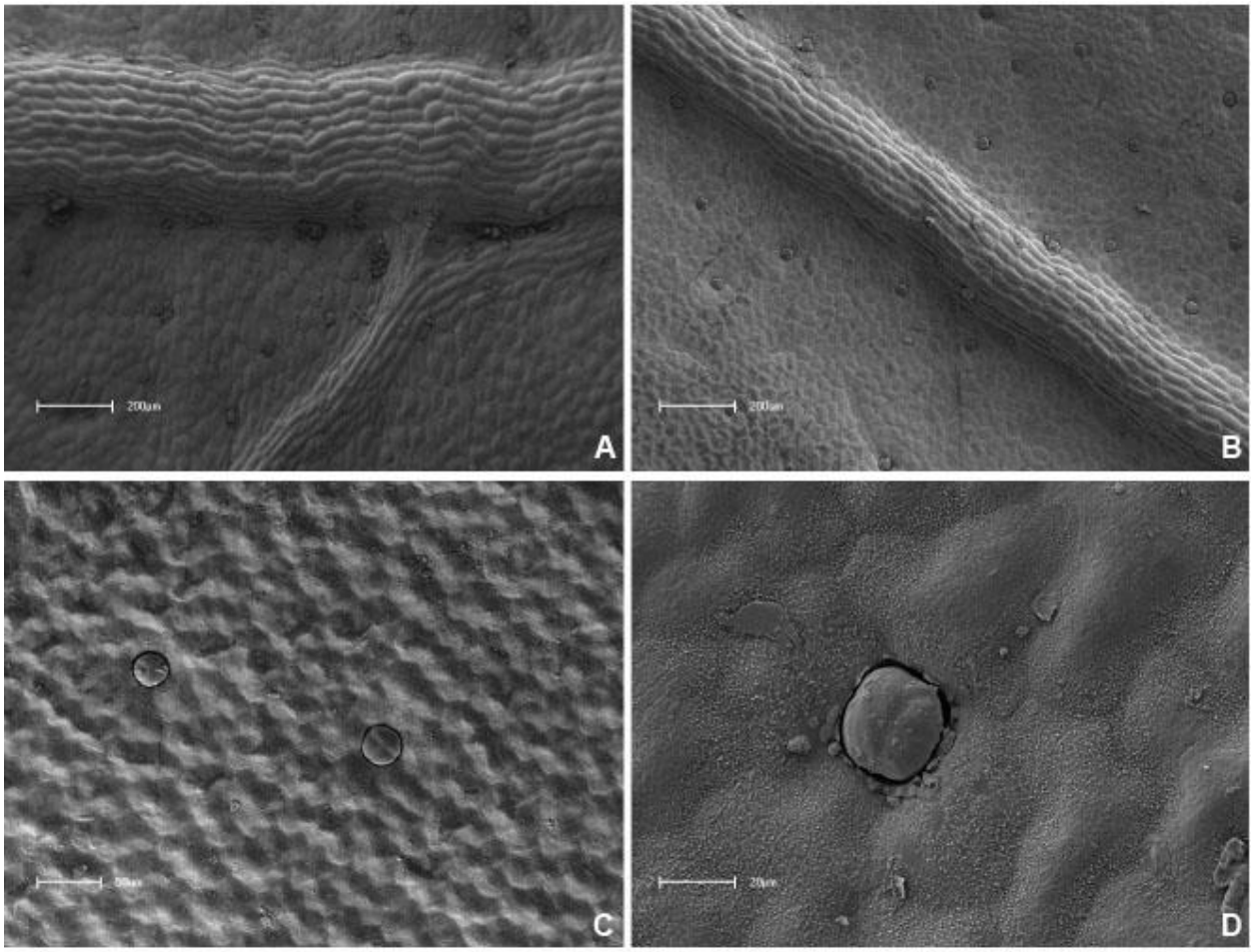

Figure 3.3.13 Adaxial leaf surface of *Crabbea pedunculata*. **In:** (A, B) simple trichomes absent from entire leaf surface; (C) epidermal cells with sunken anticlinal walls, concave periclinal walls and cuticular striations; (D) subsessile, globose, smooth, glandular trichome and epidermal cells with a weakly granular cuticular surface. **Specimens:** (A, D) *de Gouveia 81* (BLFU); (B) *de Gouveia 176* (BLFU); (C) *de Gouveia 177* (BLFU).

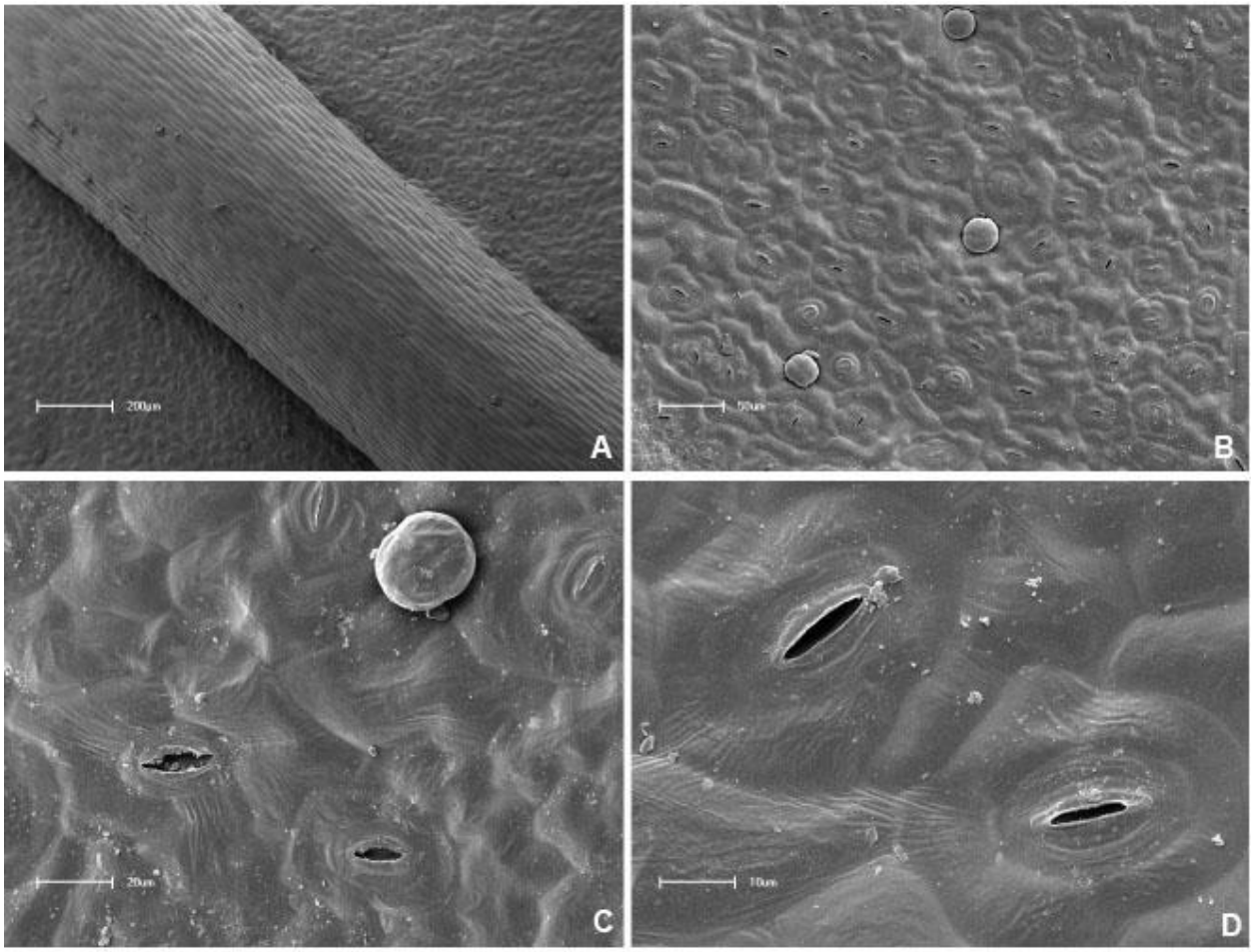

Figure 3.3.14 Abaxial leaf surface of *Crabbea pedunculata*. **In:** (A) simple trichomes absent from primary vein; (B, C) epidermal cells with sunken anticlinal walls, concave periclinal walls, subsessile, globose, smooth, glandular trichome; (C, D) stomata with narrow stomatal ledges, levelled with the epidermal surface and epidermal cells with weak cuticular striations. **Specimens:** (A) *de Gouveia 81* (BLFU); (B, D) *de Gouveia 176* (BLFU); (C) *de Gouveia 177* (BLFU).

3.3.3.8 *Crabbea velutina*

On the **adaxial leaf surface**, **simple trichomes** are filiform to needle-shaped, present on the primary vein (Figure 3.3.15 A). **Simple, cone-shaped trichomes** are ornamented with smooth bases are present on the epidermal surface (Figure 3.3.15 A, C). The **epidermal cells** are isodiametric and polygonal. **Anticlinal walls** are straight to weakly arched (Figure 3.3.15 B). The **cuticle** is weakly granular (Figure 3.3.15 C, D).

On the **abaxial surface**, **simple trichomes** are filiform and needle-shaped with acute apices, located on the primary and secondary veins (Figure 3.3.16 A). **Simple, cone-shaped trichomes** are ornamented with smooth bases are found across the surface. **Anticlinal walls** are arched and sinuous (Figure 3.3.16 B, C). Epidermal cells have a smooth cuticle (Figure 3.3.16 C, D).

The leaves are **hypostomatic** (Figures 3.3.15; 3.3.16; Tables 3.2; 3.3).

On the **abaxial leaf surface**, **stomatal density** is 428 stomata per mm^2. **Stomata** are raised on the epidermal surface (Figure 3.3.16 B–D). **Guard cells** are 15.9–22.7 µm long and 2.66–5.50 µm wide. **Stomatal pores** are 7.08–16.1 µm long. **Stomatal ledges** are 11–19.8 µm long and 1.23–5.5 µm wide (Figure 3.3.16 D; Table 3.3).

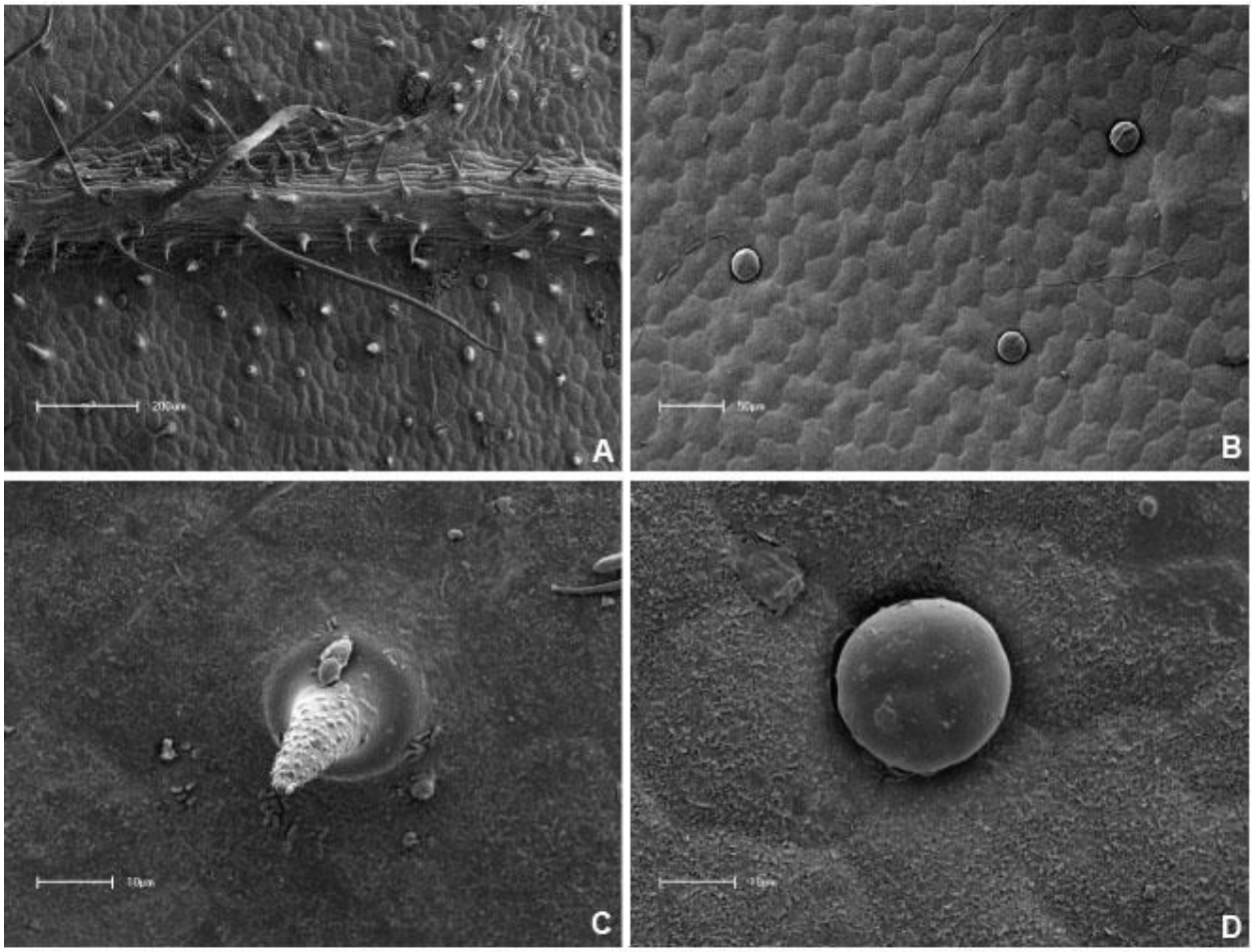

Figure 3.3.15 Adaxial leaf surface of *Crabbea velutina*. **In:** (A) simple needle-shaped trichomes concentrated on primary vein and cone-shaped on adjacent epidermal surface; (B) epidermal cells with sunken anticlinal walls and convex periclinal walls; (C) simple, ornamented cone-shaped trichome with smooth base; (D) subsessile, globose, smooth, glandular trichome and epidermal cells with a granular cuticle. **Specimens:** (A) *de Gouveia 163* (BLFU); (B–D) *de Gouveia 114* (BLFU).

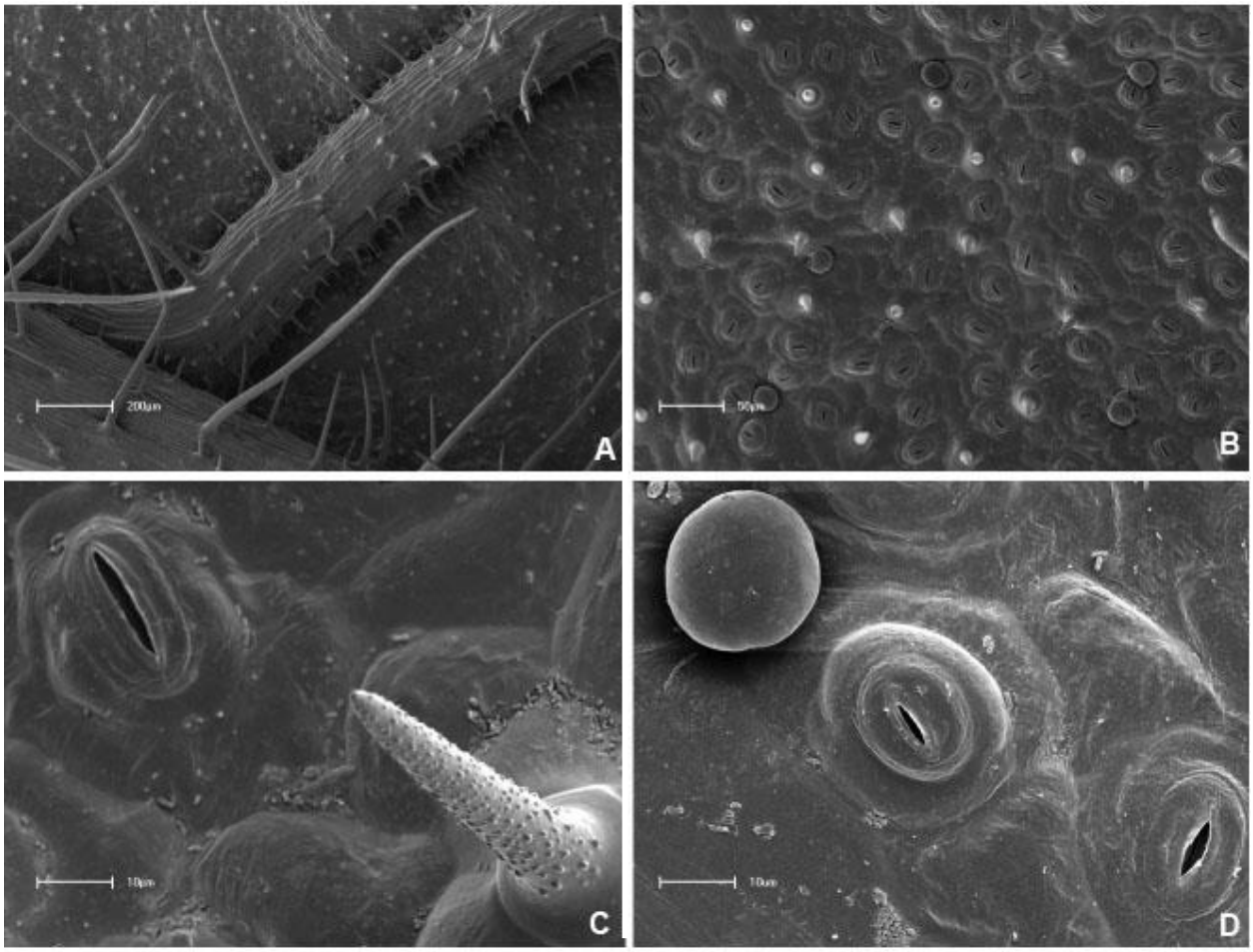

Figure 3.3.16 Abaxial leaf surface of *Crabbea velutina*. **In:** (A) primary and secondary veins and epidermal surface covered with simple needle-shaped trichomes and cone-shaped trichomes found on adjacent epidermal surface; (B) stomata raised from the epidermal surface, epidermal cells with sunken anticlinal walls and concave periclinal walls; (C) stomata raised, simple ornamented, cone-shaped trichome smooth base; (D) subsessile, globose, smooth, glandular trichome, stomata with narrow stomatal ledges and epidermal cells with a smooth cuticle. **Specimens:** (A–C) *de Gouveia 114* (BLFU); (D) *de Gouveia 163* (BLFU).

3.4 Discussion and Conclusion

Overall adaxial and abaxial epidermal characteristics are rather uniform across the southern African *Crabbea* species, with only slight variations on species-level. Adaxial epidermal features that vary slightly include: anticlinal wall structure (arched vs. sinuous vs. straight); epidermal cell shape (elongate vs. non-elongate cells) and cuticular texture (smooth vs. granular or striate) (Figures 3.3.1–3.3.16) For example, *C. pedunculata* is the only species which has a striated cuticle on the adaxial epidermal surface (Figure 3.3.13). Both *C. angustifolia* and *C. pedunculata* are characterized by having cuticular striations on the abaxial leaf surface (Figures 3.3.4; 3.3.14). Additionally, the abaxial cuticular texture varies among other *Crabbea* species, ranging from smooth (*C. galpinii*, *C. cirsioides*, *C. ovalifolia* and *C. velutina*) to granular (*C. acaulis* and *C. nana*) (Figures 3.3.2; 3.3.6; 3.3.8; 3.3.10; 3.3.12; 3.3.16). Buys (1982) found that the southern African *Crabbea* had a corrugated cuticle. However, results obtained from this study reveal the opposite. Possible factors contributing to the corrugated cuticle in Buys' (1982) report include: dry environmental conditions when specimens were collected; preparation and preservation methods and the use of old herbarium specimens implemented throughout the study of Buys (1982). In our study, 17 out of the 19 specimens used for leaf micromorphology analysis are from fresh material, which may have influenced the absence of a corrugated cuticle.

Stomatal arrangement across the leaf surface divides *Crabbea* into two distinct groups namely, species with amphistomatic or hypostomatic leaves. *Crabbea acaulis, C. angustifolia*, *C. cirsioides*, *C. nana* and *C. ovalifolia* have amphistomatic leaves (Figures 3.3.1–3.3.6; 3.3.9–3.3.12). Hypostomatic leaves are diagnostic for *C. galpinii*, *C. pedunculata* and *C. velutina* (Figures 3.3.7; 3.3.8; 3.3.13–3.3.16), confirming a close affinity between the former two species, as proposed in Buys' (1982) classification. However stomatal arrangement contradicts the synonymy of the two hypostomatic species, *C. galpinii* and *C. pedunculata*, with the amphistomatic *C. nana,* which was proposed by Buys (1982).

Stomatal density can vary among individuals of the same species (Al Afas *et al*., 2006). Light, humidity, water availability and atmospheric CO_2 concentration are abiotic factors

that influence stomatal density (Woodward and Kelly, 1995). In all investigated species with amphistomatic leaves, the abaxial leaf surface always displayed a higher stomatal density than the adaxial surface (Tables 3.2; 3.3). In *C. cirsioides*, the adaxial stomatal density was the lowest of all amphistomatic southern African *Crabbea* with 81 stomata per mm^2 (Table 3.2). Abaxial stomatal densities among hypostomatic and amphistomatic species are similar. *Crabbea velutina* has the greatest abaxial density of all southern African *Crabbea* with 428 stomata per mm^2 (Table 3.3). This is the first account where stomatal distribution and density was compared for the southern African *Crabbea*.

Stomata on the abaxial leaf surface are either raised above or level with the epidermal surface. *Crabbea acaulis*, *C. angustifolia*, *C. cirsioides*, *C. nana*, *C. ovalifolia* and *C. velutina* have raised stomata on the abaxial leaf surface (Figures 3.3.2; 3.3.4; 3.3.6; 3.3.10; 3.3.12; 3.3.16), while *C. galpinii* and *C. pedunculata a*re characterised by stomata which are level with the epidermal surface (Figures 3.3.8; 3.3.14). Buys (1982) found that *C. cirsioides*, has raised stomata, a finding confirmed by the present study. By contrast, results from the present study showed that *C. ovalifolia* stomata are level with the epidermal surface, as opposed to raised stomata reported by Buys (1982).

Glandular and eglandular trichomes were found on both *Crabbea* leaf surfaces. However, glandular trichomes were consistently present on both surfaces and were morphologically uniform in all *Crabbea* species. This suggests that glandular trichomes are only significant on genus-level and higher (Ahmad, 1978). Glandular trichomes were also observed by Buys (1982).

Eglandular, simple trichomes were either present or absent among the investigated *Crabbea* species. Eglandular trichomes are mainly concentrated on the veins and leaf margins of Acanthaceae (Ahmad, 1978). Among the southern African *Crabbea* species, eglandular trichomes are more concentrated on the veins on the abaxial leaf surface (Figures 3.3.2; 3.3.4; 3.3.6; 3.3.10; 3.3.12; 3.3.16). Eglandular trichomes are completely absent from the leaves of *C. galpinii* and *C. pedunculata* (Figures 3.3.7; 3.3.8; 3.3.13; 3.3.14). Thus, the absence or presence of eglandular trichomes is taxonomically significant for *Crabbea* on species-level, confirming the results of Buys (1982).

Crabbea velutina is also the most distinct from all other southern African *Crabbea* species, as three types of trichomes (one glandular and two types of simple, eglandular trichomes) can be found on both lamina surfaces (Figures 3.3.15; 3.3.16). The two types of simple trichomes include filiform to needle-shaped and ornamented, cone-shaped trichomes (Figures 3.3.15; 3.3.16), characters which were also reported by Buys (1982).

In the identification key supplied in section 3.5, *C. acaulis*, *C. cirsioides* and *C. nana* are grouped together. Comparison of the growth form of the three species (rosette-form - *C. acaulis* vs. decumbent - *C. cirsioides*, and *C. nana*) and the leaf micro-morphology suggests that *C. cirsioides* [= *C. hirsuta*] and *C. nana*, as circumscribed by Buys (1982), may be one species. The two species have similar leaf micromorphology, suggesting close affinity between the two (Figures 3.3.5; 3.3.6; 3.3.9; 3.3.10). Although the mean density of stomata on the leaf surfaces of *C. cirsioides* and *C. nana* differ more than among some other species, this character is not reliable in identifying species boundaries since it has been shown to be highly variable among individuals of the same species and is likely strongly influenced by environmental conditions (Woodward and Kelly, 1995; Al Afas *et al*., 2006). The obtained results show that most of the southern African *Crabbea* species can be distinguished based on a combination of stomatal arrangement and trichome density, type and the distribution as well as cuticular striations and texture. This study provides the first southern African *Crabbea* leaf micromorphology identification key. The identification key may be used as a basis when constructing a leaf micromorphology identification key for the entire genus.

3.5 Leaf micromorphology identification key for the southern African of *Crabbea*

1A Leaves amphistomatic:

2A Cuticular striations and conical trichomes present on abaxial leaf surface:

C. angustifolia

2B Cuticular striations and conical trichomes absent from abaxial leaf surface:

3A Abaxial leaf stomata level with the epidermal surface:

C. ovalifolia

3B Abaxial leaf stomata raised above the epidermal surface:

C. acaulis

C. cirsioides

C. nana

1B Leaves hypostomatic:

4A Simple trichomes present on both leaf surfaces; abaxial stomata raised from the epidermal surface:

C. velutina

4B Simple trichomes absent from both leaf surfaces; abaxial stomata levelled with the epidermal surface:

5A Cuticular striations absent from adaxial leaf surface:

C. galpinii

5B Cuticular striations present on adaxial leaf surface:

C. pedunculate

CHAPTER 4

CYSTOLITHS

4.1 Introduction

Cystoliths are calcium carbonate crystals, occasionally composed of silica, formed on ingrowths of epidermal cell walls of specialised cells called lithocysts (Metcalfe and Chalk, 1983; Lawrence, 2008). Cystoliths may be found in the epidermis of leaves, stems and sometimes roots (Metcalfe and Chalk, 1983; Scotland and Vollesen, 2000). The function and origin of cystoliths are much debated. The majority of cystolith-bearing plants occur in the tropics, where soils are relatively calcium-poor. One hypothesis suggests that lithocysts store calcium which can later be used by the plant (Stahl, 1920). Calcium has two main functions within the plant: firstly, calcium maintains the integrity of the mitotic spindle during cell division and, secondly, calcium sustains the physical structure and normal functioning of membranes. Calcium also acts as a secondary messenger in a variety of environmental and hormonal responses (Hopkins and Hüner, 2009). Crystals, such as cystoliths, may serve as a deterrent against grazing by large herbivores and insects. Shape, size, location and quantity of crystals determine defence efficiency and resilience (da Costa *et al*., 2009).

Highly restricted among angiosperms, cystoliths are diagnostic of Acanthaceae, Cannabaceae, Moraceae and Utricaceae (Metcalfe and Chalk, 1983; Scotland and Vollesen, 2000). Cystoliths form an important component of any taxonomic account focusing on Acanthaceae, since this is the only family within the Lamiales that contain cystoliths (Metcalfe and Chalk, 1950a; Scotland and Vollesen, 2000). Acanthaceae cystoliths have received attention in previous studies which have shown that these cystoliths vary in shape, size, colour and occurrence (Inamdar, 1970; Inamdar *et al*., 1990; Patil and Patil, 2011; Choopan and Grote, 2015) and are of taxonomic value at genus and species level (Metcalfe and Chalk, 1950a).

Karlstrom (1980) organised cystoliths into eight categories based on occurrence, size and shape. The shape of a cystolith is primarily determined by its length/breadth (L/B) ratio (Inamdar *et al.*, 1990).

Ahmad (1975) studied cystoliths in the tribe Barlerieae focusing on a few representative species in two genera, *Barleria* and *Lepidagathis* Willd. Cystoliths of *Barleria* are commonly found in the epidermal tissue, and are paired and frequently arranged in groups or chains of three or more. *Lepidagathis* cystoliths are also paired and sparsely to densely distributed within the leaf epidermis. The presence of paired cystoliths within *Barleria* was also confirmed by Inamdar *et al.* (1990) and Patil and Patil (2011). Inamdar *et al*. (1990) recorded the cystolith density on the leaf epidermal surfaces of a number of Acanthaceae genera. It was found that cystolith density varies across the Acanthaceae from 2–26 cystoliths per mm^2.

McDade *et al*. (2008) established that Acanthaceae taxa bearing cystoliths form a highly supported monophyletic clade. The Cystolith Clade is positioned within the subfamily Acanthoideae, including all tribes in this subfamily but one, Acantheae. *Crabbea*, being a member of Barlerieae, is included within the Cystolith Clade (Figure 4.1).

In spite of the taxonomic importance of cystoliths in the Cystolith Clade, three of the seven taxonomic accounts of *Crabbea* did not include cystoliths in their investigations (Harvey, 1842; Nees von Esenbeck, 1847; Clarke, 1901). Buys (1982) briefly mentioned the cystoliths of only three southern African *Crabbea* species namely *C. angustifolia*, *C. cirsioides* [= *C. hirsuta*] and *C. ovalifolia*. Buys (1982) showed that cystoliths are found within lithocysts of both adaxial and abaxial leaf epidermal tissue; have an irregular shape and that each crystal occupies the entire space within a lithocyst. *Crabbea* cystoliths, like those found in *Barleria*, may be paired (Metcalfe and Chalk, 1950a). Burkill and Clarke (1899–1900) and Vollesen (2015) only mention that cystoliths are conspicuous on the leaf surfaces of the investigated *Crabbea* species but gives no detail on shape, size and density. Thulin's (2007) revision briefly mentions the presence of cystoliths in one of the four *Crabbea* species, *C. albolutea* Thulin, from Somalia, which has "rounded to shortly oblong cystoliths."

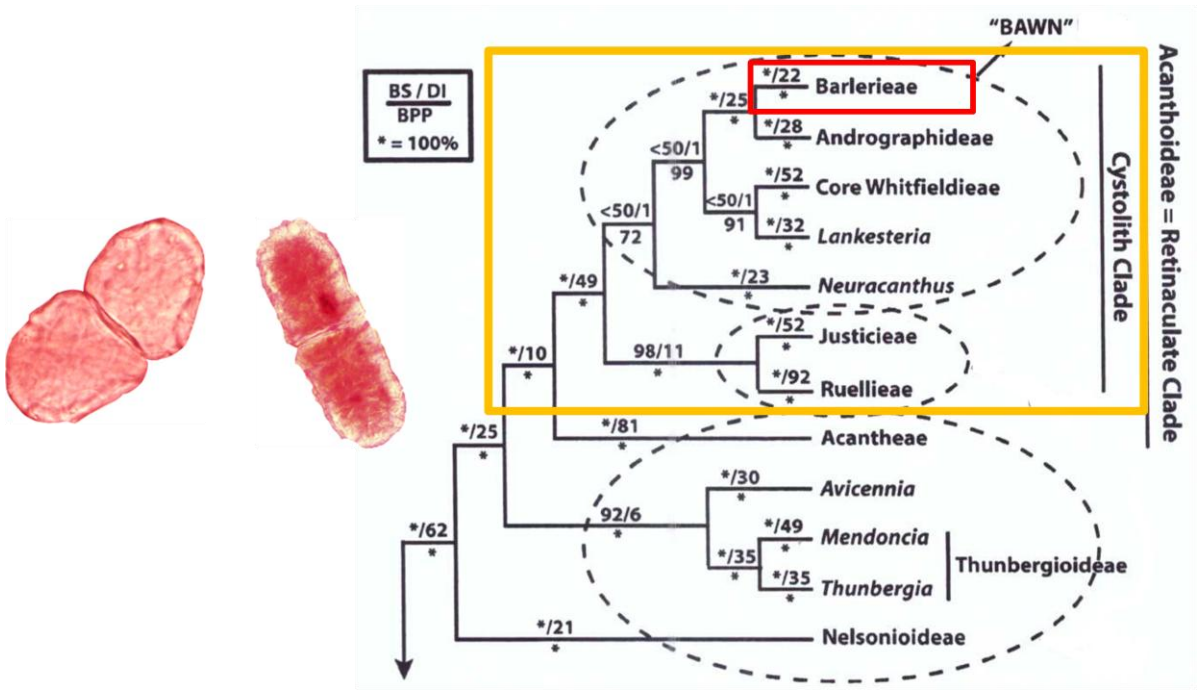

Figure 4.1 The distribution of cystoliths within Acanthaceae, with the Cystolith Clade (orange box) and the presence of paired cystoliths (red box) indicated, as adapted from McDade *et al.* (2008).

The aim of this chapter is to investigate the cystoliths of *Crabbea* in southern Africa and to determine if cystoliths can be used to confirm the position of *Crabbea* within Barlerieae.

4.2 Materials and Methods

Fresh leaves were preserved in 3% phosphate-buffered GA and leaves from herbarium vouchers were rehydrated for 48 hrs in 3% phosphate-buffered GA. Compared to leaf micromorphology, more *Crabbea* specimens were examined due to the relatively low cost involved in preparing the leaf material for light microscopy compared to scanning electon microscopy. The number of samples used per *Crabbea* species are indicated in Table 4.1.

Prior to dissection, leaf material was rinsed properly with distilled water. Dissected leaf material was dehydrated through an EtOH series of 30% (v/v), 50% (v/v) and 70% (v/v). Samples were dehydrated in each EtOH phase for 30 min and the 70% (v/v) EtOH phase was repeated twice.

Leaf samples were prepared using a modified leaf clearing technique as described by Arnott (1959). Dissected leaf tissue was placed in a 5% sodium hydroxide (NaOH) solution and stored at 37°C for 48 hrs in a Memmert Standard Paraffin Oven. After 24 hours, the 5% NaOH solution was replaced with fresh 5% NaOH solution, thus, ensuring efficient leaching of pigments. Leaf samples were thoroughly rinsed with distilled water and left in a chloral hydrate solution (50g $C_2H_3Cl_3O_2$: 30 cc distilled water) for 24 hrs at 37°C. Leaves were washed with distilled water three times in four hour intervals over a 12 hr period and subsequently stored in 70% (v/v) EtOH, at room temperature, overnight.

Leaves were stained with Safranin O (1% Safranin O and 50% (v/v) EtOH). Leaves were stained for 60 min, at room temperature. To remove the staining medium, leaf material was rinsed for a minute in 70% (v/v) EtOH. Following rinsing, leaf material was further dehydrated, at room temperature, according to the following dehydration series:

Table 4.1 Southern African *Crabbea* specimens used to investigate cystoliths.

Herbarium	*Crabbea* species	Collector name and number	Collection date
BLFU	*C. acaulis*	A. de Gouveia 80	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 121	25-03-2015
BLFU	*C. acaulis*	A. de Gouveia 149	28-03-2015
BLFU	*C. angustifolia*	J.E. Burrows and S.E. Burrows 14638	07-03-2015
PRE	*C. angustifolia*	R. Leendertz 6551	00-01-1909
BLFU	*C. angustifolia*	A. de Gouveia 74	21-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 88	22-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 124	25-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 127	26-03-2015
BLFU	*C. cirsioides*	A. de Gouveia142	28-03-2015
BLFU	*C. galpinii*	A. de Gouveia 169 C	20-11-2015
BLFU	*C. galpinii*	A. de Gouveia 170	21-11-2015
BLFU	*C. galpinii*	A. de Gouveia 180	21-11-2015
BLFU	*C. nana*	A. de Gouveia 146	28-03-2015
BLFU	*C. nana*	A. de Gouveia 149	27-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 81	22-03-2015
BLFU	*C. pedunculata*	A. de Gouveia 94	23-03-2015
BLFU	*C. pedunculata*	A. de Gouveia 176	25-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 177	26-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 136	27-03-2015
BLFU	*C. ovalifolia*	A. de Gouveia 174	23-11-2015
BLFU	*C. velutina*	A. de Gouveia 114	25-03-2015
BLFU	*C. velutina*	A. de Gouveia 163	19-11-2015
BLFU	*C. velutina*	A. de Gouveia 164	19-11-2015

95% (v/v) EtOH, 100% (v/v) EtOH, half 100% (v/v) EtOH-half 100% xylene, and 100% xylene. Each dehydration step took 30 min; however, the 100% xylene phase was repeated twice, with each interval spanning 60 min. Leaves were mounted on glass microscope slides and cover slips were mounted using Entallan® and Neo-Clear®, according to manufacturer's specifications.

Cystoliths were photographed using the Olympus® BX 53 LM, with a mounted Olympus® DP 72 Camera, at X 20. Measurements were taken using the software package, cellSens® v.1.4.1. Cytolith micrographs were captured at the Department of Plant Sciences (PS), UFS.

Terminology used to describe cystolith shape and appearance is based on Karlstrom (1980) (Table 4.2) and Beentje (2010). Anatomical characteristics used to describe *Crabbea* cystoliths are provided in Figure 4.2.

Cystolith density was determined in the following steps: For each image of cystoliths in the leaf epidermis, the surface area (mm^2) represented by the image was calculated and the number of cystoliths counted. For each specimen, three different areas of the leaf surface was analysed and the average cystolith density was determined. For each species, the average number of cystoliths per mm^2 was determined for a minimum of two specimens (Table 4.1).

Table 4.2 Terminology used to describe cystolith shape and size, according to Karlstrom (1980).

Appearance		Breadth (B) (μm)	Length (L) (μm)	L/B ratio
A	Elongated, narrow, tapering at one end	≤ 21		≥ 4
B	Elongated, broad, tapering at one end	≥ 22		≥ 4
C	Elongated, tapering at both ends			≥ 4
D	Elongated, narrow, blunt at both ends	≤ 20	≥ 80	
E	Elongated, broad, blunt at both ends	≥ 21	≥ 80	
F	Short, narrow, blunt at both ends	≤ 80		> 5
G	Short, broad, blunt at both ends		≤ 2	
H	Irregular	N/A	N/A	N/A

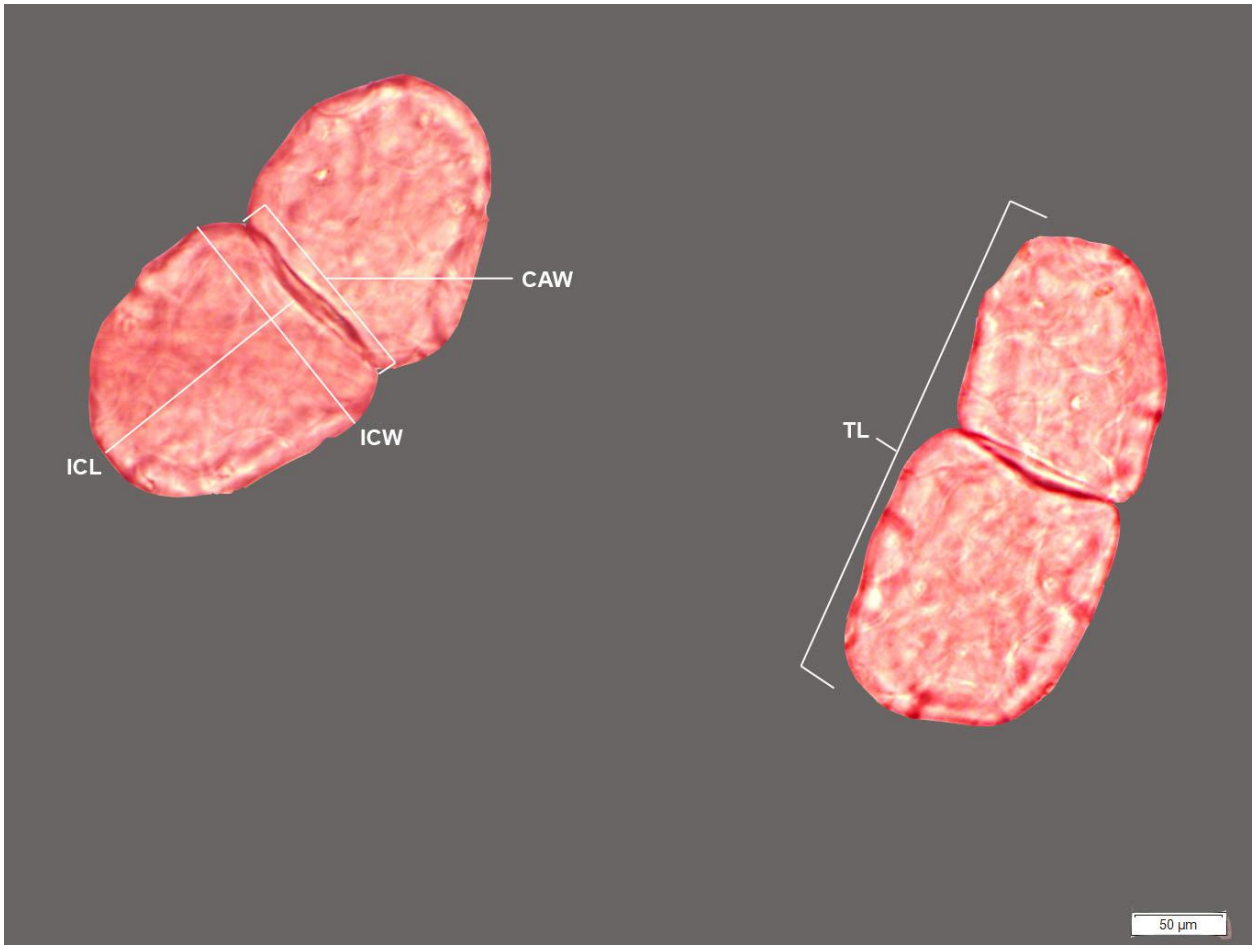

Figure 4.2 Measurements used to describe the paired cystoliths of the southern African *Crabbea* species. **Legend:** CAW = cystolith attachment width; ICL = individual crystal length; ICW = individual crystal width; TL = total length. **Specimen:** *de Gouveia 136* (BLFU).

4.3 Results

4.3.1 Genus description of cystoliths in the leaf epidermis of southern African *Crabbea* species

Crystals are always paired; **crystal pairs** are elliptic, occasionally narrowly elliptic to oblong, weakly arched. **Individual crystals** are ovate, occasionally broadly ovate, rarely narrowly ovate to oblong. **Apices** are blunt and obtuse.

Cystoliths are relatively evenly distributed throughout the adaxial (4–10 cystoliths per mm^2) and abaxial (4–11 cystoliths per mm^2) epidermis of leaves. The **total length** of the cystoliths varies from 174.67–289.91 µm on adaxial leaf epidermis and 180.26–272.14 µm on the abaxial leaf epidermis. The **Individual crystal** range from 84.32–148.18 x 38.14–83.88 µm on the adaxial leaf epidermis and 86.14–137.93 x 37.79–86.60 µm on the abaxial leaf epidermis. The cystolith **attachment width** on both adaxial (28.57–72.30 µm) and abaxial (27.55–66.96 µm) leaf surfaces are similar (Tables 4.3; 4.4).

4.3.2 Leaf cystolith measurements of the southern African *Crabbea* species

Table 4.3 Cystolith density and size in the adaxial leaf epidermis of southern African *Crabbea* species.

Species	Density (cystoliths per mm^2) ± (SD)	Total Length (TL) (µm) ± (SD)	Cystolith attachment width (CAW) (µm) ± (SD)	TL/ CAW Ratio	Individual crystal length (ICL) (µm) ± (SD)	Individual crystal width (ICW) (µm) ± (SD)	Cystolith shape *sensu* Karlstrom (1980)
C. acaulis	8 ± 5.73	269.98 ± 68.57	72.30 ± 12.52	3.73	120.94 ± 37.43	83.88 ± 19.79	Elongated, broad, blunt at both ends
C. angustifolia	10 ± 5.18	262.03 ± 47.00	60.20 ± 16.00	4.35	130.08 ± 24.82	74.46 ± 15.93	Elongated, broad, blunt at both ends
C. cirsioides	7 ± 1.35	289.91 ± 72.29	62.81 ± 15.33	4.62	148.18 ± 44.94	80.53 ± 17.66	Elongated, broad, blunt at both ends
C. galpinii	10 ±1.17	231.33 ± 53.85	36.06 ± 10.37	6.42	116.32 ± 29.20	51.21 ± 8.56	Elongated, broad, blunt at both ends
C. nana	5 ± 2.24	257.29 ± 46.36	71.78 ± 14.42	3.58	130.07 ± 25.29	90.06 ± 15.66	Elongated, broad, blunt at both ends
C. pedunculata	4 ± 1.36	194.53 ± 65.52	28.57 ± 7.05	6.81	96.38 ± 32.09	38.14 ± 8.27	Elongated, broad, blunt at both ends
C. ovalifolia	4 ± 1.36	248.86 ± 54.05	59.44 ± 16.29	4.19	123.06 ± 29.47	73.49 ± 14.36	Elongated, broad, blunt at both ends
C. velutina	7 ± 6.51	174.67 ± 44.79	47.13 ± 6.60	3.71	84.32 ± 26.40	55.20 ± 7.02	Elongated, broad, blunt at both ends

*The SD is provided next to the average value for a particular measurement.

Table 4.4 Cystolith density and size in the abaxial leaf epidermis of southern African *Crabbea* species*.

Species	Density (cystoliths per mm^2) ± (SD)	Total Length (TL) (µm) ± (SD)	Cystolith attachment width (CAW) (µm) ± (SD)	TL/ CAW Ratio	Individual crystal length (ICL) (µm) ± (SD)	Individual crystal width (ICW) (µm) ± (SD)	Cystolith shape *sensu* Karlstrom (1980)
C. acaulis	8 ± 2.95	206.99 ± 87	59.79 ± 13.22	3.46	107.33 ± 43.89	79.07 ± 12.12	Elongated, broad, blunt at both ends
C. angustifolia	11 ± 6.72	197.40 ± 53.92	45.69 ± 9.42	4.32	98.19 ± 28.94	63.10 ± 16.56	Elongated, broad, blunt at both ends
C. cirsioides	5 ± 1.91	272.14 ± 97.22	59.72 ± 16.81	4.56	137.93 ± 52.26	74.61 ± 17.27	Elongated, broad, blunt at both ends
C. galpinii	6 ± 1.17	180.26 ± 50.69	32.30 ± 7.61	5.58	89.61 ± 29.36	44.95 ± 13.93	Elongated, broad, blunt at both ends
C. nana	5 ± 2.24	211.87 ± 64.25	58.06 ± 13.95	3.65	107.19 ± 31.80	69.80 ± 12.26	Elongated, broad, blunt at both ends
C. pedunculata	4 ± 2.34	167.78 ± 59.11	27.55 ± 8.54	6.09	86.14 ± 30.57	37.79 ± 11.08	Elongated, broad, blunt at both ends
C. ovalifolia	4 ± 1.36	221.20 ± 97.57	66.96 ± 37.66	3.30	110.75 ± 47.57	86.60 ± 47.06	Elongated, broad, blunt at both ends
C. velutina	9 ± 2.24	197.40 ± 62.86	47.59 ± 13.06	4.15	100.67 ± 31.83	55.48 ± 13.99	Elongated, broad, blunt at both ends

*The SD is provided next to the average value for a particular measurement.

4.3.3 Species description of cystoliths among southern African *Crabbea*

4.3.3.1 *Crabbea acaulis*

On the adaxial leaf surface **paired crystals** are elliptic (Figure 4.3.1 A–D). **Individual crystals** are ovate (Figure 4.3.1 A–D).

On the abaxial leaf surface **paired crystals** are elliptic (Figure 4.3.2 A–D). **Individual crystals** are ovate (Figure 4.3.2 A, B) to broadly ovate (Figure 4.3.2 C, D).

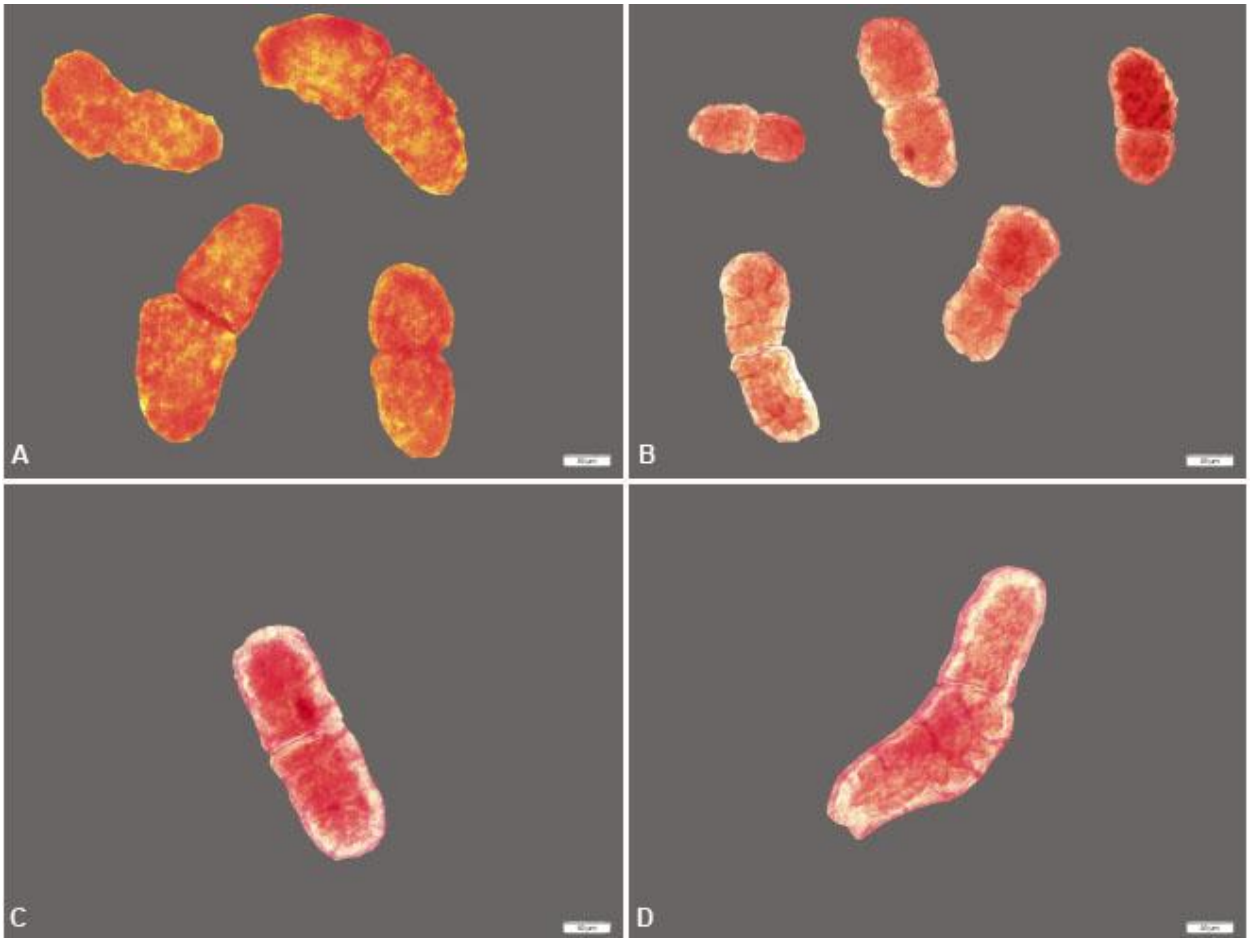

Figure 4.3.1 Adaxial leaf cystoliths of *Crabbea acaulis*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape, weakly arched and obtuse apices, individual crystals are ovate. **Specimens:** (A) *de Gouveia 80* (BLFU); (B) *de Gouveia 121* (BLFU); (C, D) *de Gouveia 149* (BLFU).

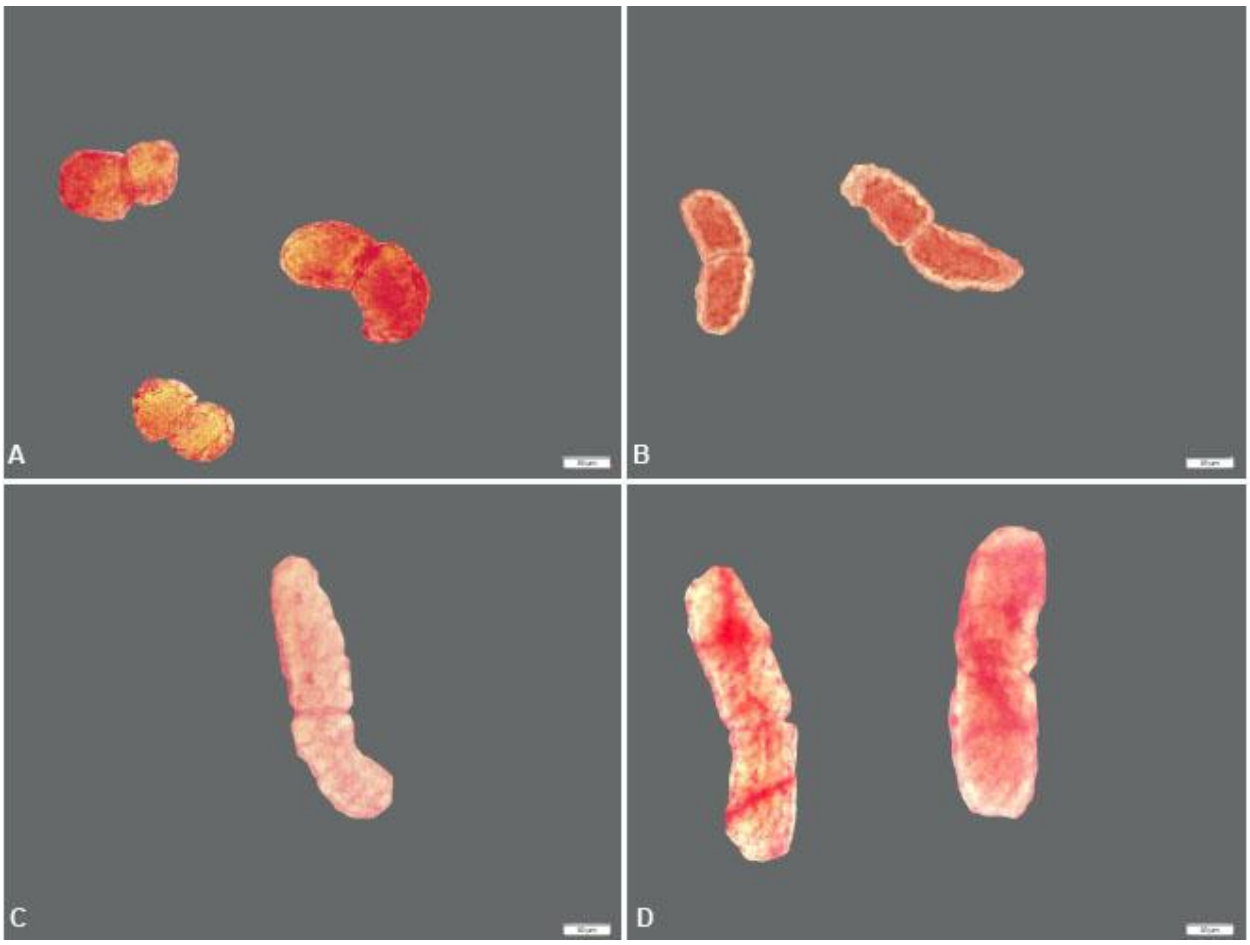

Figure 4.3.2 Abaxial leaf cystoliths of *Crabbea acaulis*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape, weakly arched and obtuse apices, individual crystals mostly ovate. **Specimens:** (A) *de Gouveia 80* (BLFU); (B) *de Gouveia 121* (BLFU); (C, D) *de Gouveia 149* (BLFU).

4.3.3.2 *Crabbea angustifolia*

On the adaxial leaf surface **paired crystals** are elliptic (Figure 4.3.3 A, B, C) to oblong (Figure 4.3.3 D). **Individual crystals** are broadly ovate (Figure 4.3.3 A–D).

On the abaxial leaf surface **paired crystals** are elliptic (Figure 4.3.4 A–C) to oblong (Figure 4.3.4 D). **Individual crystals** are broadly ovate (Figure 4.3.4 A, D) to ovate (Figure 4.3.4 B, C).

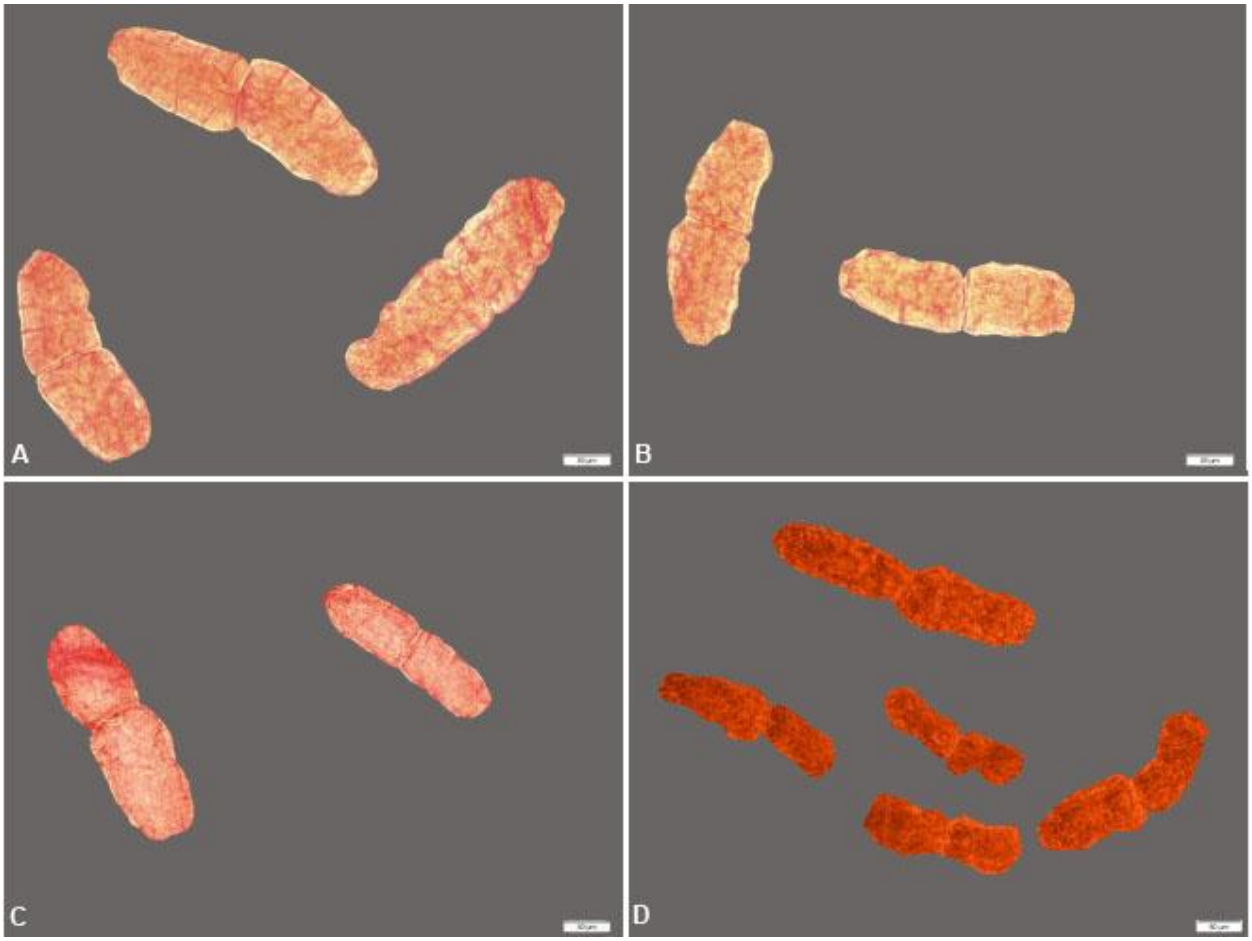

Figure 4.3.3 Adaxial leaf cystoliths of *Crabbea angustifolia*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape, weakly arched and obtuse apices, individual crystals are broadly ovate. **Specimens:** (A, B) *de Gouveia 74* (BLFU); (C) *Burrows and Burrows 14638* (BLFU); (D) *Leendertz 6561* (PRE).

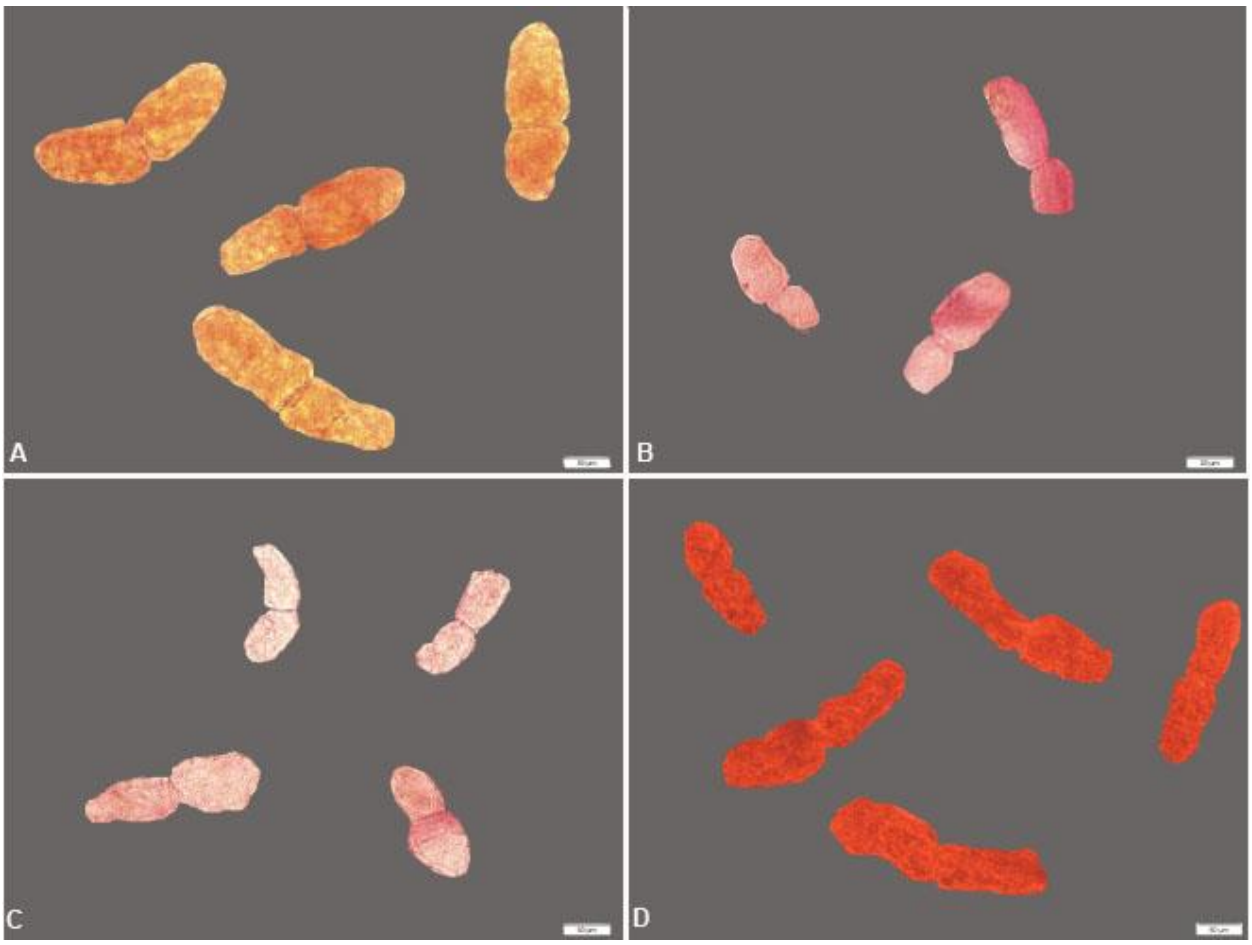

Figure 4.3.4 Abaxial leaf cystoliths of *Crabbea angustifolia*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate to broadly ovate. **Specimens:** (A) *de Gouveia 74* (BLFU); (B, C) *Burrows and Burrows 14638* (BLFU); (D) *Leendertz 6561* (PRE).

4.3.3.3 *Crabbea cirsioides*

On the adaxial leaf surface **paired crystals** are elliptic (Figure 4.3.5 A) and narrowly elliptic to oblong (Figure 4.3.5 B–D). **Individual crystals** are ovate (Figure 4.3.5 A) to broadly ovate (Figure 4.3.5 B–D).

On the abaxial leaf surface **paired crystals** are elliptic (Figure 4.3.6 A–D). **Individual crystals** are ovate (Figure 4.3.6 A, D) to broadly ovate (Figure 4.3.6 B–D).

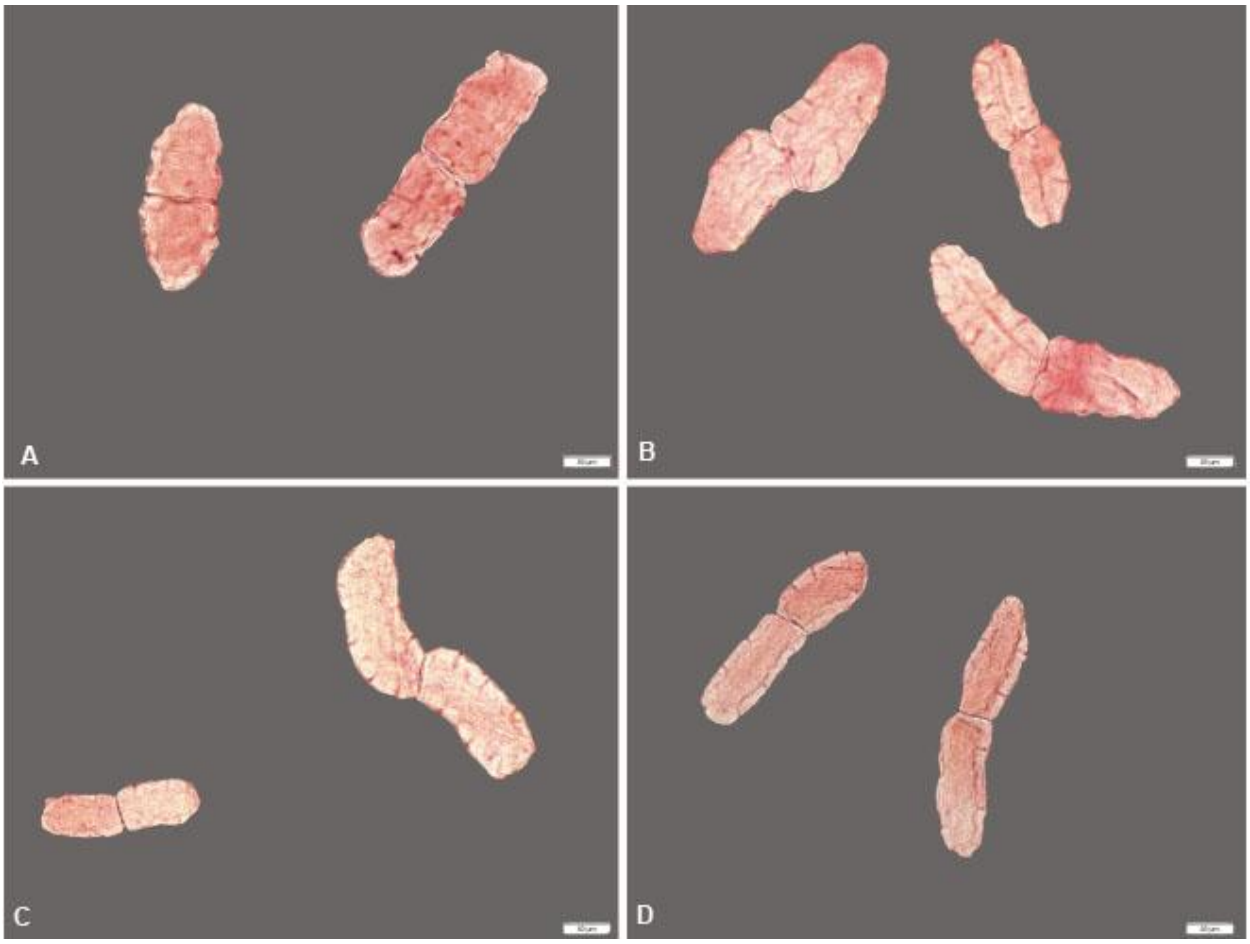

Figure 4.3.5 Adaxial leaf cystoliths of *Crabbea cirsioides.* **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystal ovate to broadly ovate. **Specimens:** (A) *de Gouveia 88* (BLFU); (B) *de Gouveia 124* (BLFU); (C) *de Gouveia 127* (BLFU); (D) *de Gouveia 142* (BLFU).

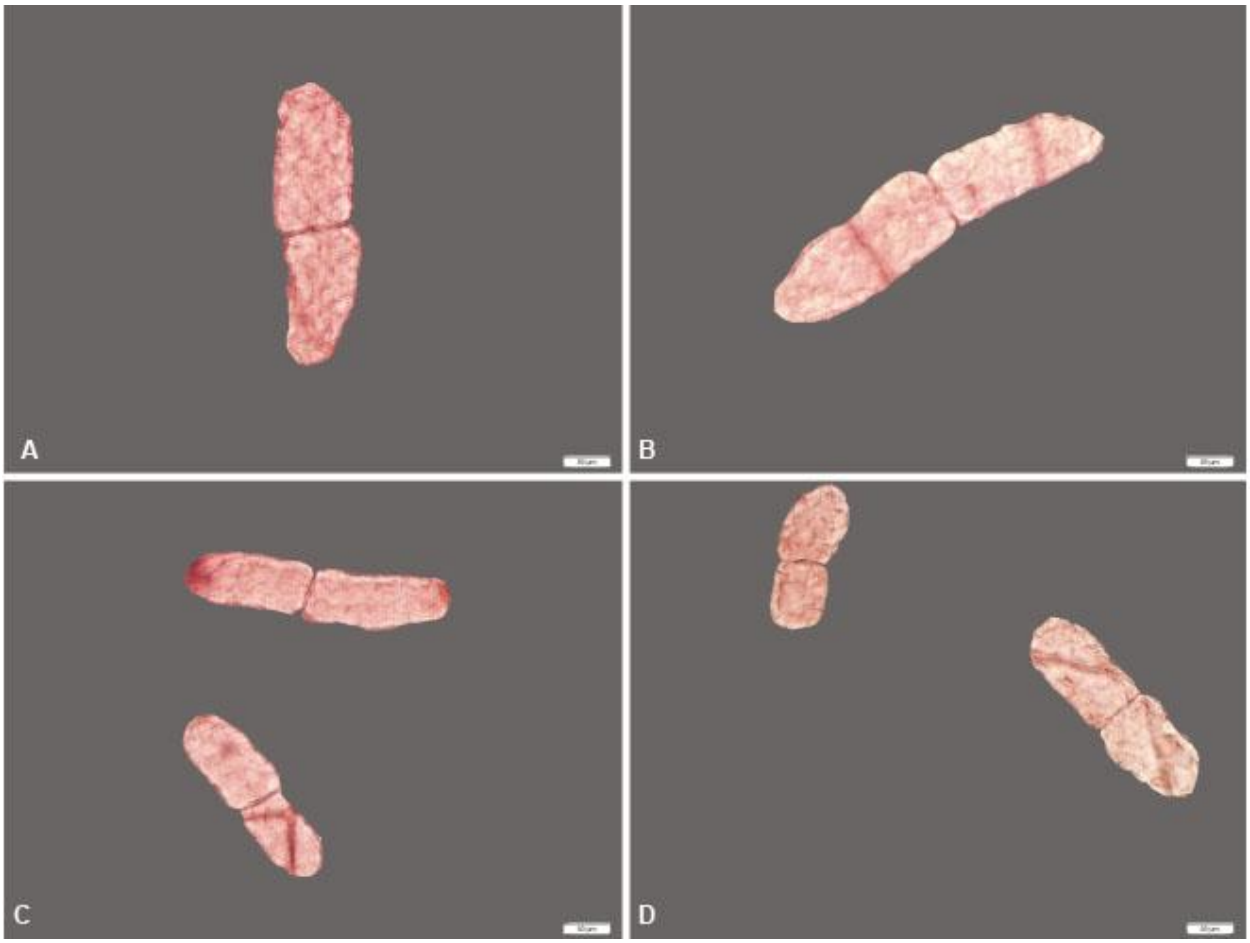

Figure 4.3.6 Abaxial leaf cystoliths of *Crabbea cirsioides.* **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate to broadly ovate. **Specimens:** (A) *de Gouveia 88* (BLFU); (B) *de Gouveia 124* (BLFU); (C) *de Gouveia 127* (BLFU); (D) *de Gouveia 142* (BLFU).

4.3.3.4 *Crabbea galpinii*

On the adaxial leaf surface **paired crystals** are narrowly elliptic to oblong (Figure 4.3.7 A–D), rarely linear (Figure 4.3.7 B, C). **Individual crystals** are oblong (Figure 4.3.7 A–D) or ovate (Figure 4.3.7 A, B, D).

On the abaxial leaf surface **paired crystals** are narrowly elliptic to oblong (Figure 4.3.8 A–D). **Individual crystals** are ovate (Figure 4.3.8 A) to oblong (Figure 4.3.8 C–D).

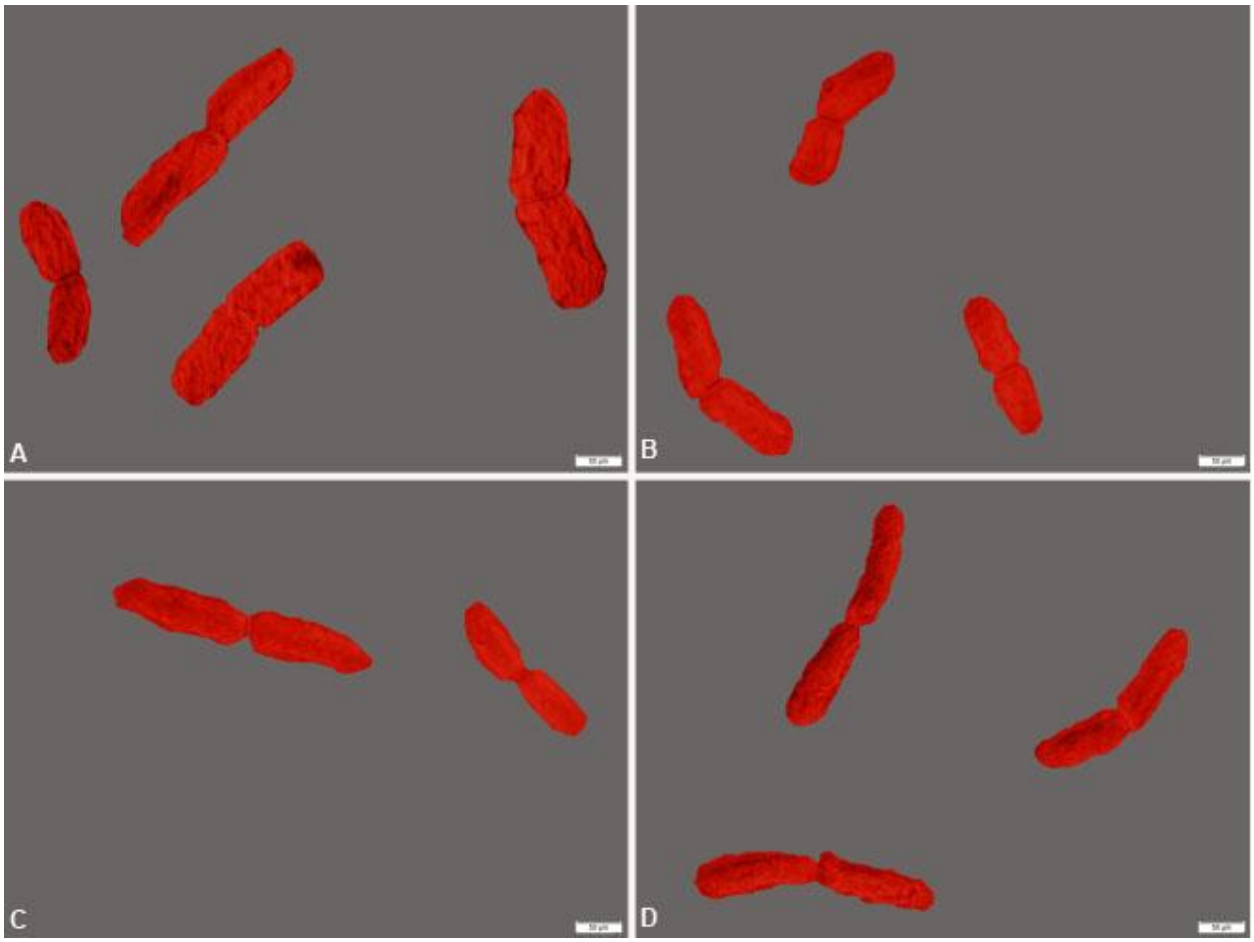

Figure 4.3.7 Adaxial leaf cystoliths of *Crabbea galpinii*. **In:** (A–D) paired cystoliths with an overall narrowly elliptic to oblong shape, rarely linear, narrow attachment widths and obtuse apices, individual crystals are oblong and ovate. **Specimens:** (A) *de Gouveia 169C* (BLFU); (B, C) *de Gouveia 170* (BLFU); (D) *de Gouveia 171* (BLFU).

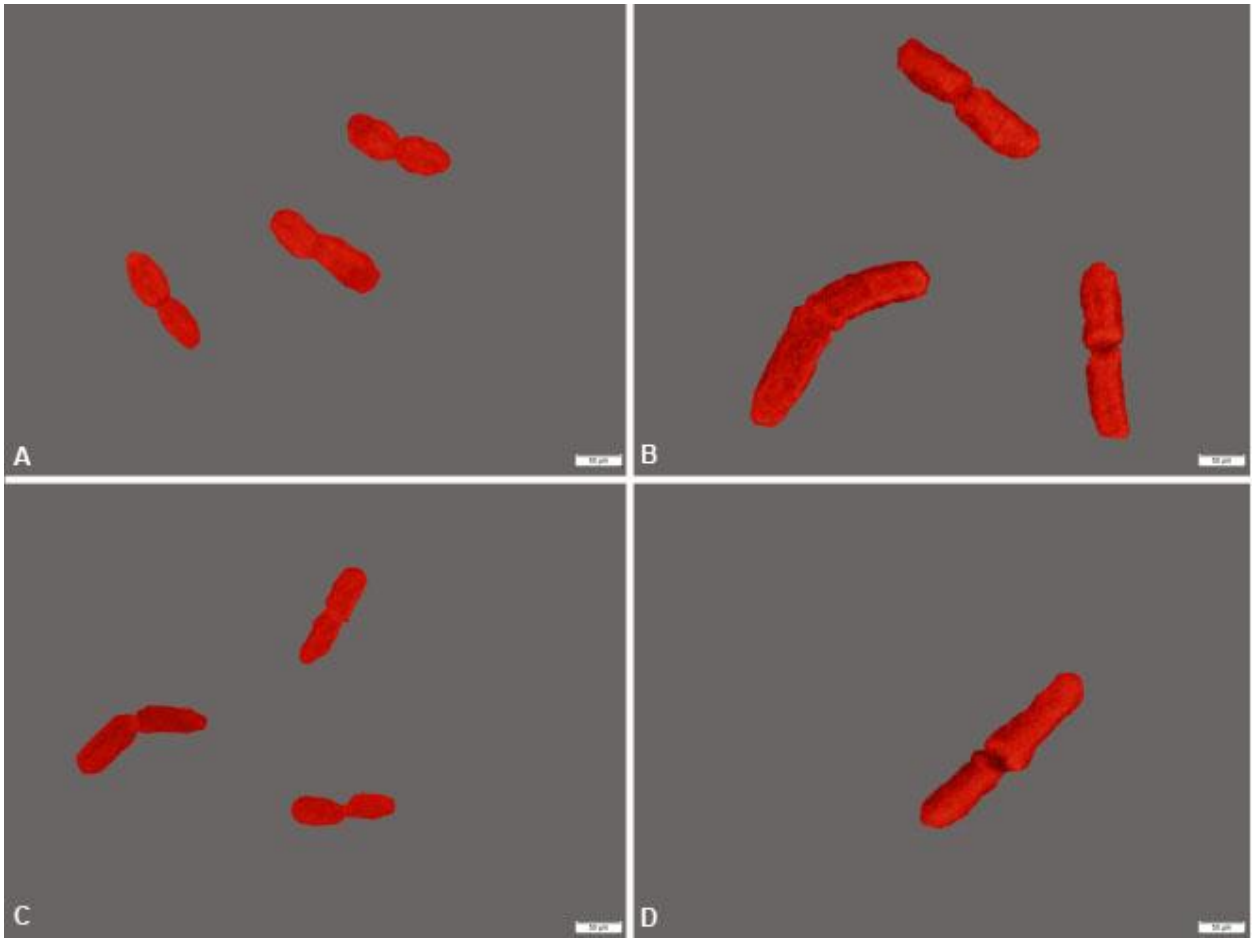

Figure 4.3.8 Abaxial leaf cystoliths of *Crabbea galpinii*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape, narrow attachment widths and obtuse apices. Individual crystals mostly elliptic, rarely orbicular. **Specimens:** (A) *de Gouveia 169C* (BLFU); (B) *de Gouveia 170* (BLFU); (C, D) *de Gouveia 171* (BLFU).

4.3.3.5 *Crabbea nana*

On the adaxial leaf surface **paired crystals** are elliptic to oblong (Figure 4.3.9 A–D). **Individual crystals** are ovate (Figure 4.3.9 A–D).

On the abaxial leaf surface **paired crystals** are elliptic (Figure 4.3.10 A; B) to oblong (Figure 4.3.10 C), arched (Figure 4.3.10 D). **Individual crystals** are ovate to broadly ovate (Figure 4.3.10 A–D).

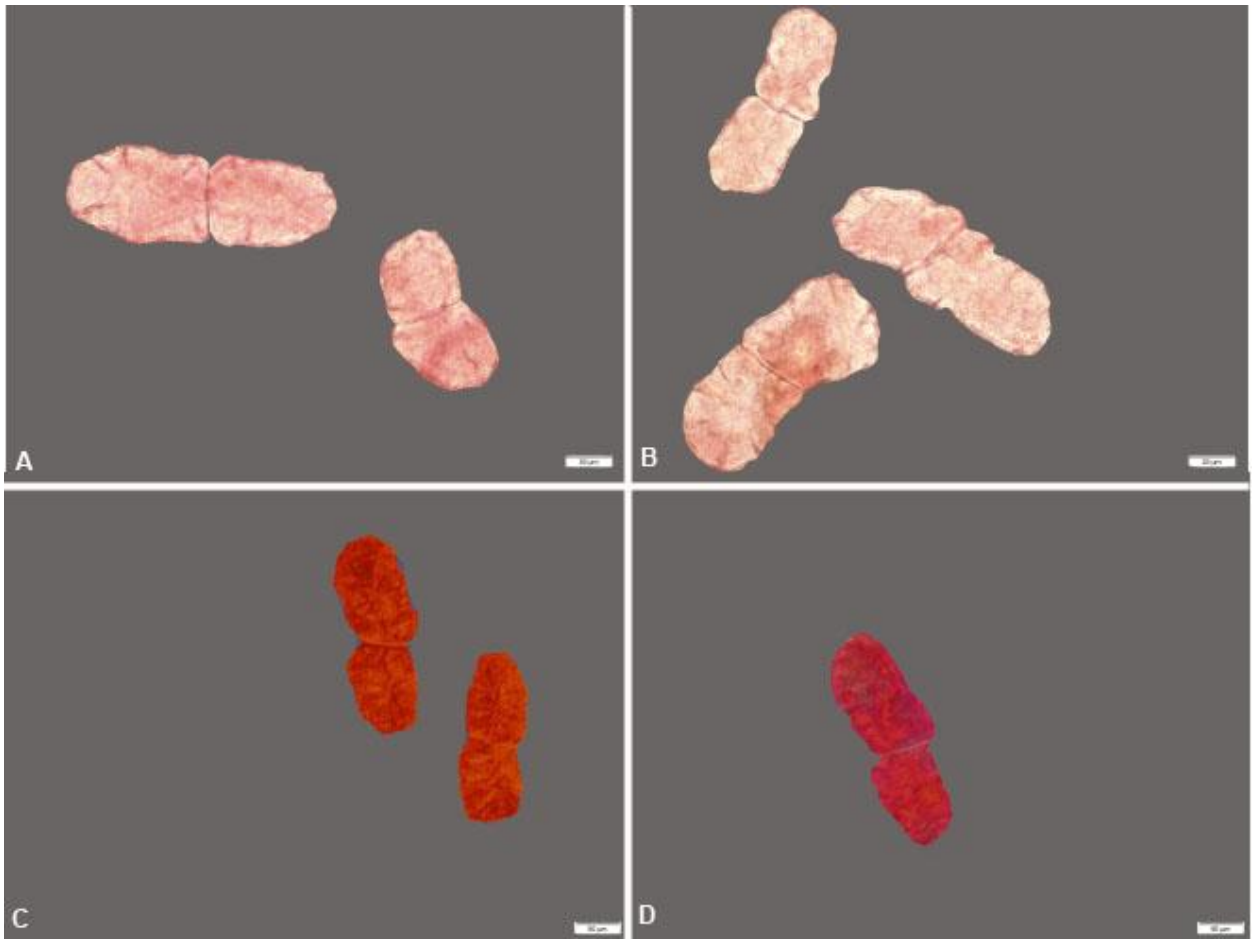

Figure 4.3.9 Adaxial leaf cystoliths of *Crabbea nana*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate. **Specimens:** (A, B) *de Gouveia 146* (BLFU); (C, D) *de Gouveia 179* (BLFU).

Figure 4.3.10 Abaxial leaf cystoliths of *Crabbea nana*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate to broadly ovate. **Specimens:** (A, B) *de Gouveia 146* (BLFU); (C, D) *de Gouveia 179* (BLFU).

4.3.3.6 *Crabbea ovalifolia*

On the adaxial leaf surface **paired crystals** are elliptic (Figure 4.3.11 A, B) to oblong (Figure 4.3.11 C, D). **Individual crystals** are ovate (Figure 4.3.11 A–C) to narrowly ovate (Figure 4.3.11 D).

On the abaxial leaf surface **paired crystals** are elliptic (Figure 4.3.12 A, B) to oblong (Figure 4.3.12 C, D). **Individual crystals** are ovate (Figure 4.3.12 A–C) to narrowly ovate (Figure 4.3.12 D).

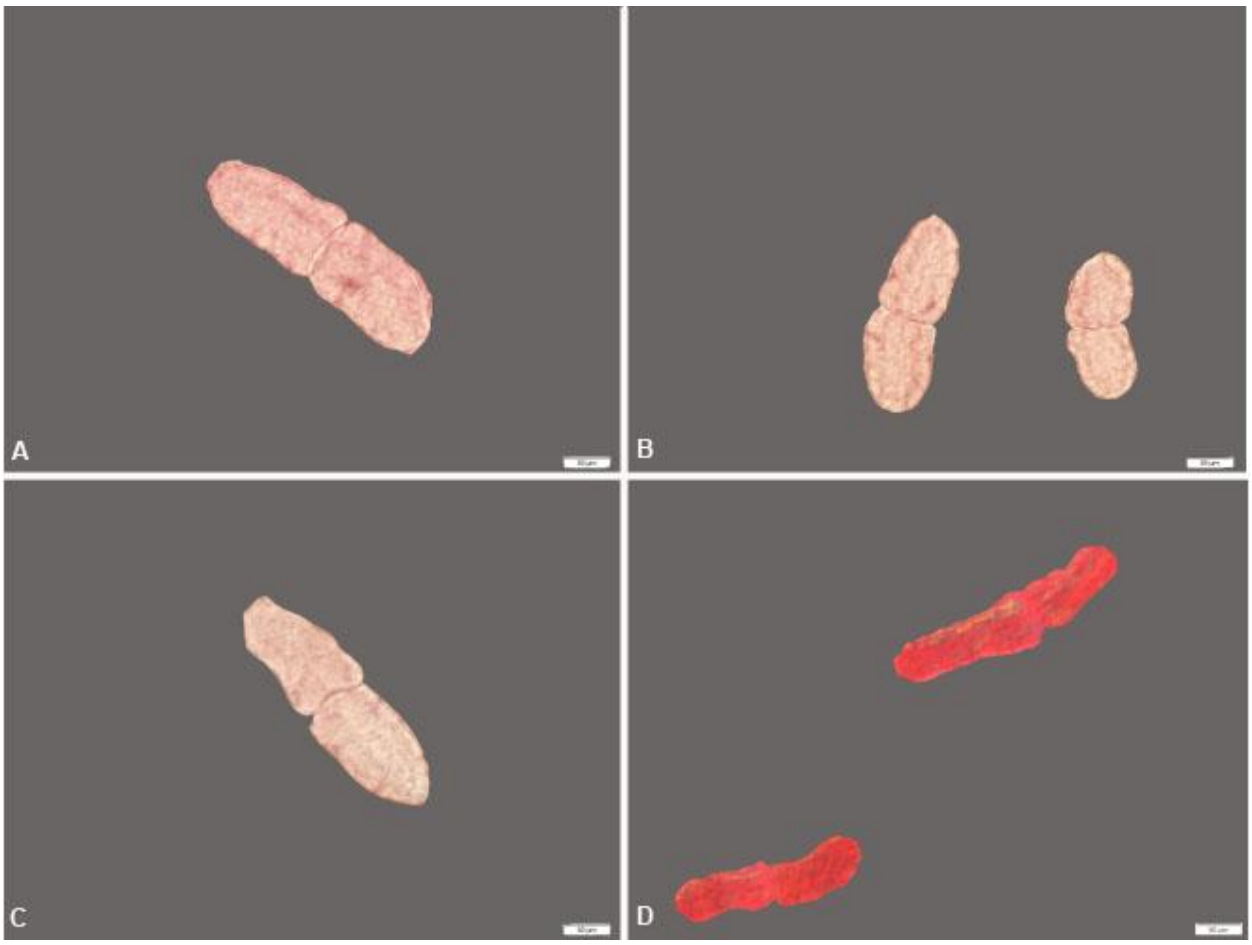

Figure 4.3.11 Adaxial leaf cystoliths of *Crabbea ovalifolia*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate to narrowly ovate. **Specimens:** (A, B, C) *de Gouveia 136* (BLFU); (D) *de Gouveia 174* (BLFU).

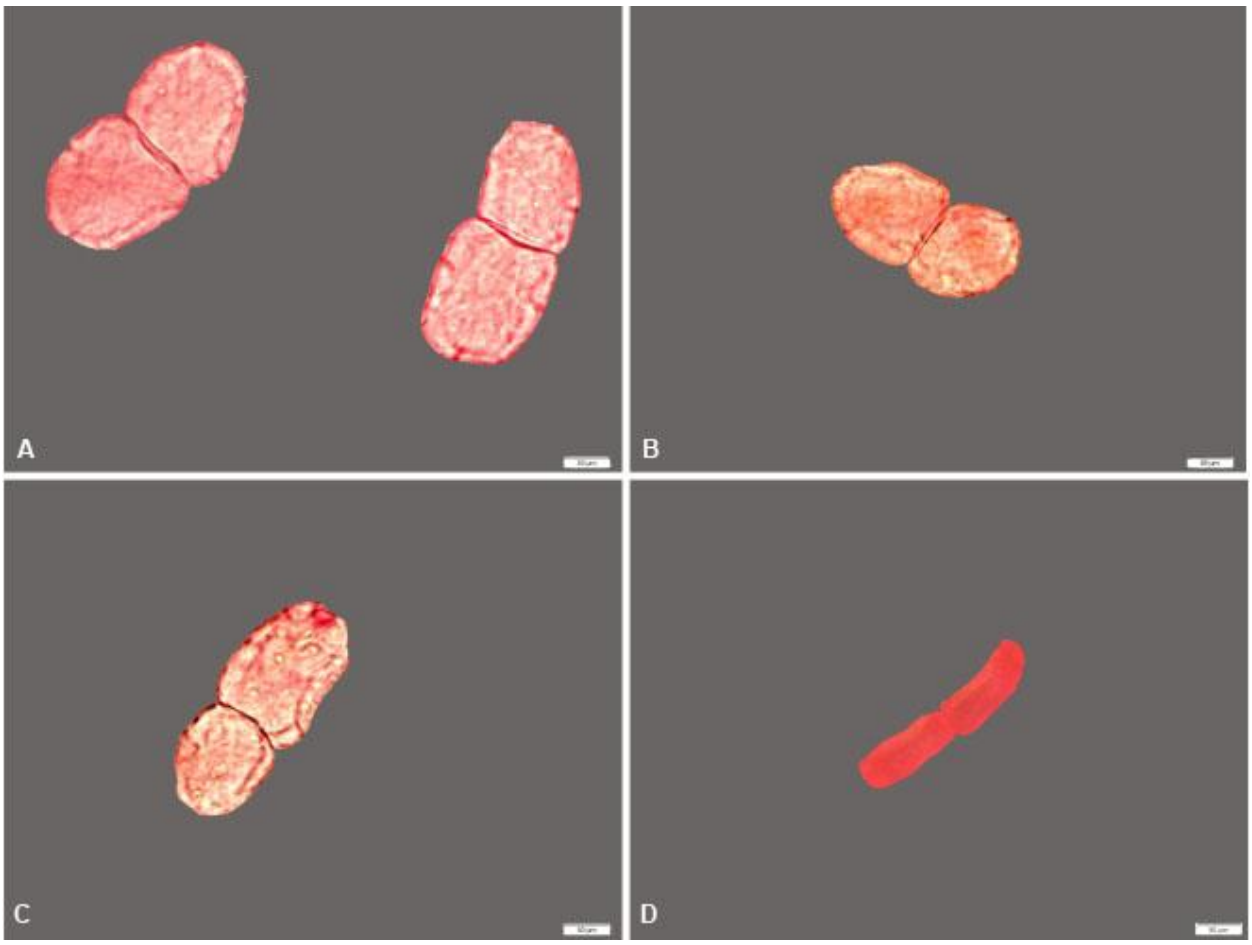

Figure 4.3.12 Abaxial leaf cystoliths of *Crabbea ovalifolia*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate to narrowly ovate. **Specimens:** (A, B, C) *de Gouveia 136* (BLFU); (D) *de Gouveia 174* (BLFU).

4.3.3.7 *Crabbea pedunculata*

On the adaxial leaf surface **paired crystals** are narrowly elliptic to oblong (Figure 4.3.13 A–D). **Individual crystals** are narrowly ovate (Figure 4.3.13 A–D).

On the abaxial leaf surface **paired crystals** are oblong (Figure 4.3.14 A, D) to elliptic (Figure 4.3.14 B, D). **Individual crystals** are narrowly ovate (Figure 4.3.14 A–D).

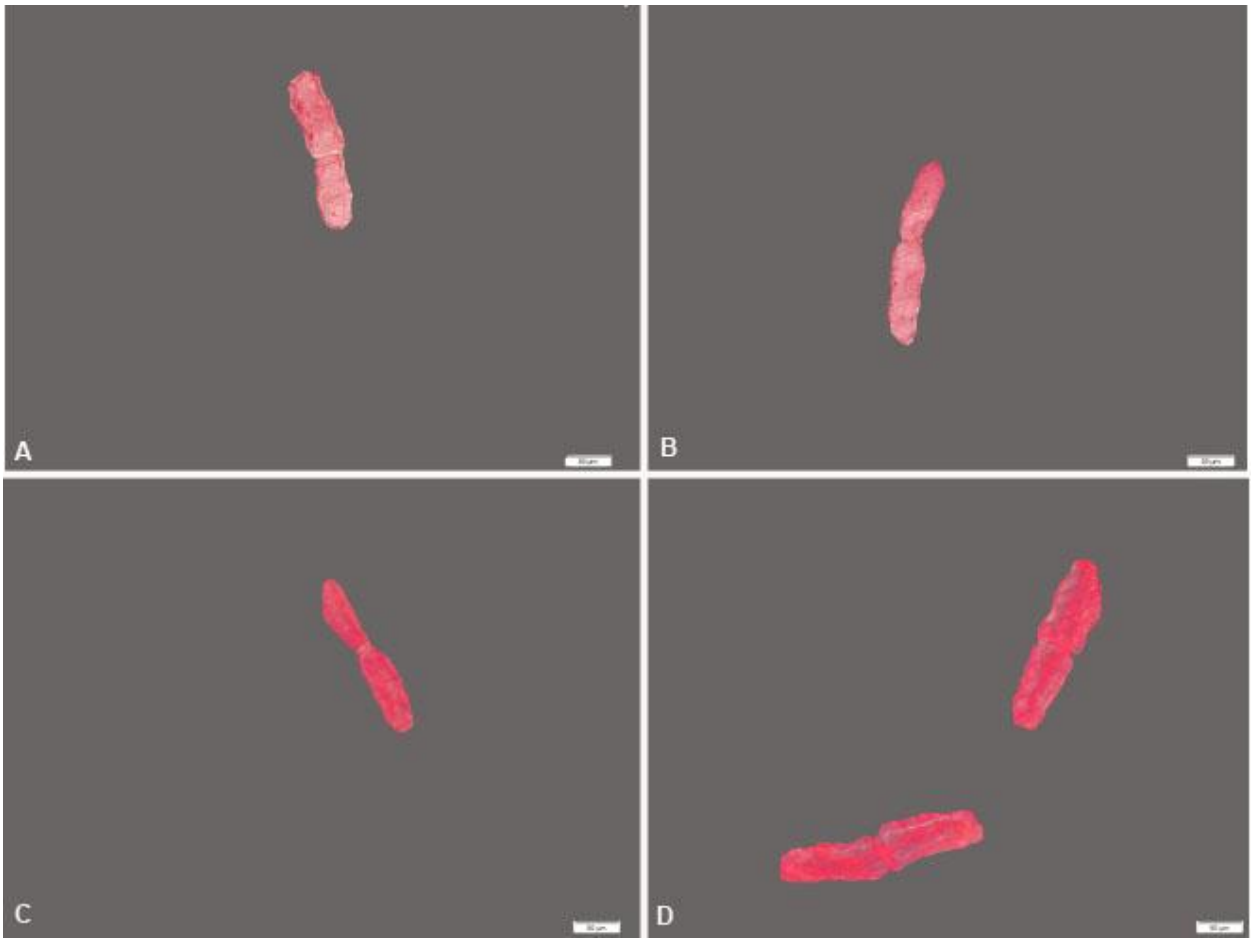

Figure 4.3.13 Adaxial leaf cystoliths of *Crabbea pedunculata*. **In:** (A–D) paired cystoliths with an overall narrowly elliptic to oblong shape, narrow attachment widths and obtuse apices, individual crystal are narrowly ovate. **Specimens:** (A, B) *de Gouveia 81* (BLFU); (C) *de Gouveia 176* (BLFU); (D) *de Gouveia 177* (BLFU).

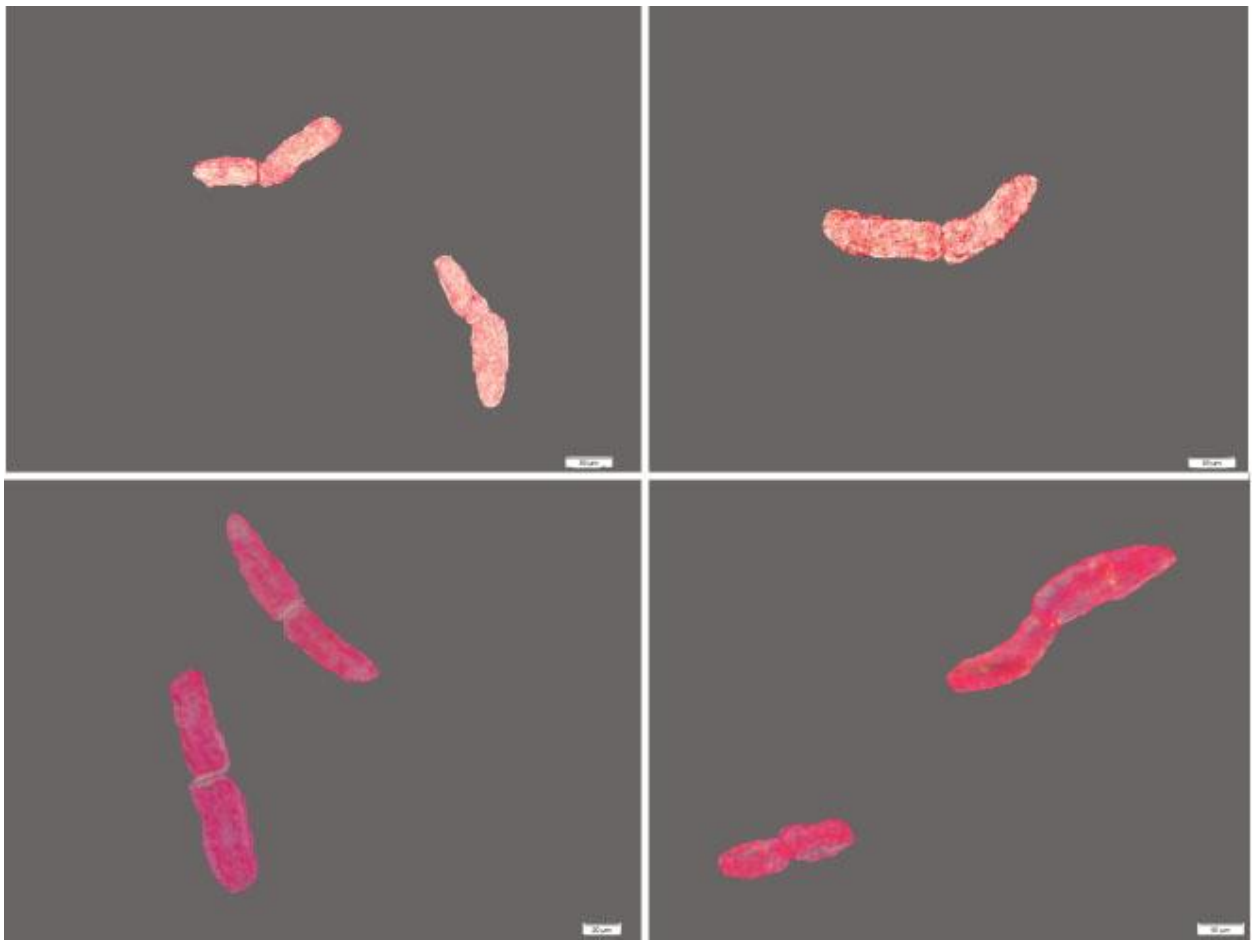

Figure 4.3.14 Abaxial leaf cystoliths of *Crabbea pedunculata*. **In:** (A–D) paired cystoliths with an overall elliptic to oblong shape, narrow attachment widths and obtuse apices, individual crystal are narrowly ovate. **Specimens:** (A) *de Gouveia 81* (BLFU); (B) *de Gouveia 176* (BLFU); (C, D) *de Gouveia 177* (BLFU).

4.3.3.8 *Crabbea velutina*

On the adaxial leaf surface **paired crystals** are elliptic to oblong (Figures 4.3.15 A–D). **Individual crystals** are ovate (Figure 4.3.15 A–D).

On the abaxial leaf surface **paired crystals** are elliptic to oblong (Figures 4.3.16 A–D). **Individual crystals** are ovate (Figures 4.3.16 A–D).

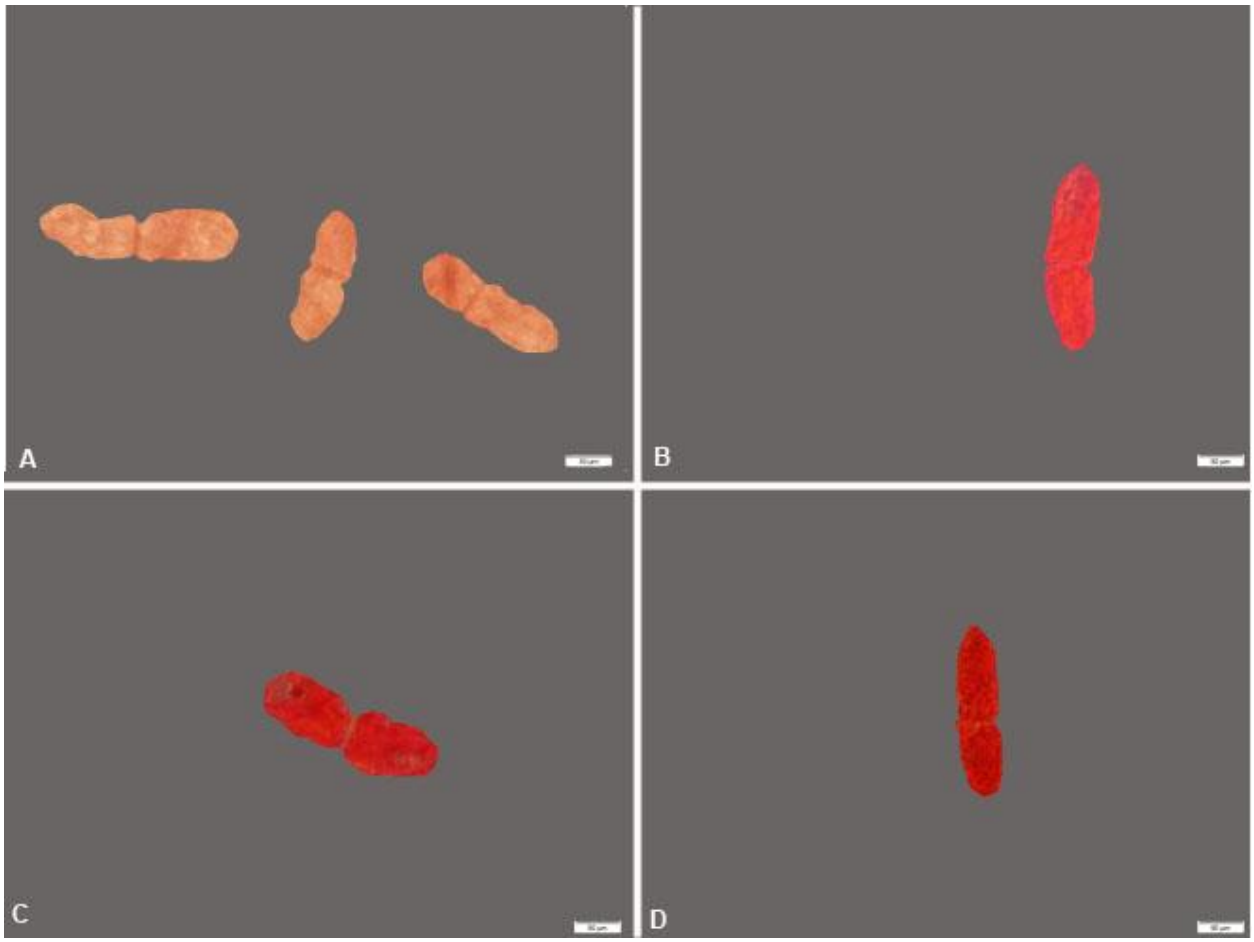

Figure 4.3.15 Adaxial leaf cystoliths of *Crabbea velutina*. **In:** (A–D) cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate. **Specimens:** (A) *de Gouveia 114* (BLFU); (B) *de Gouveia 163* (BLFU); (C, D) *de Gouveia 164* (BLFU).

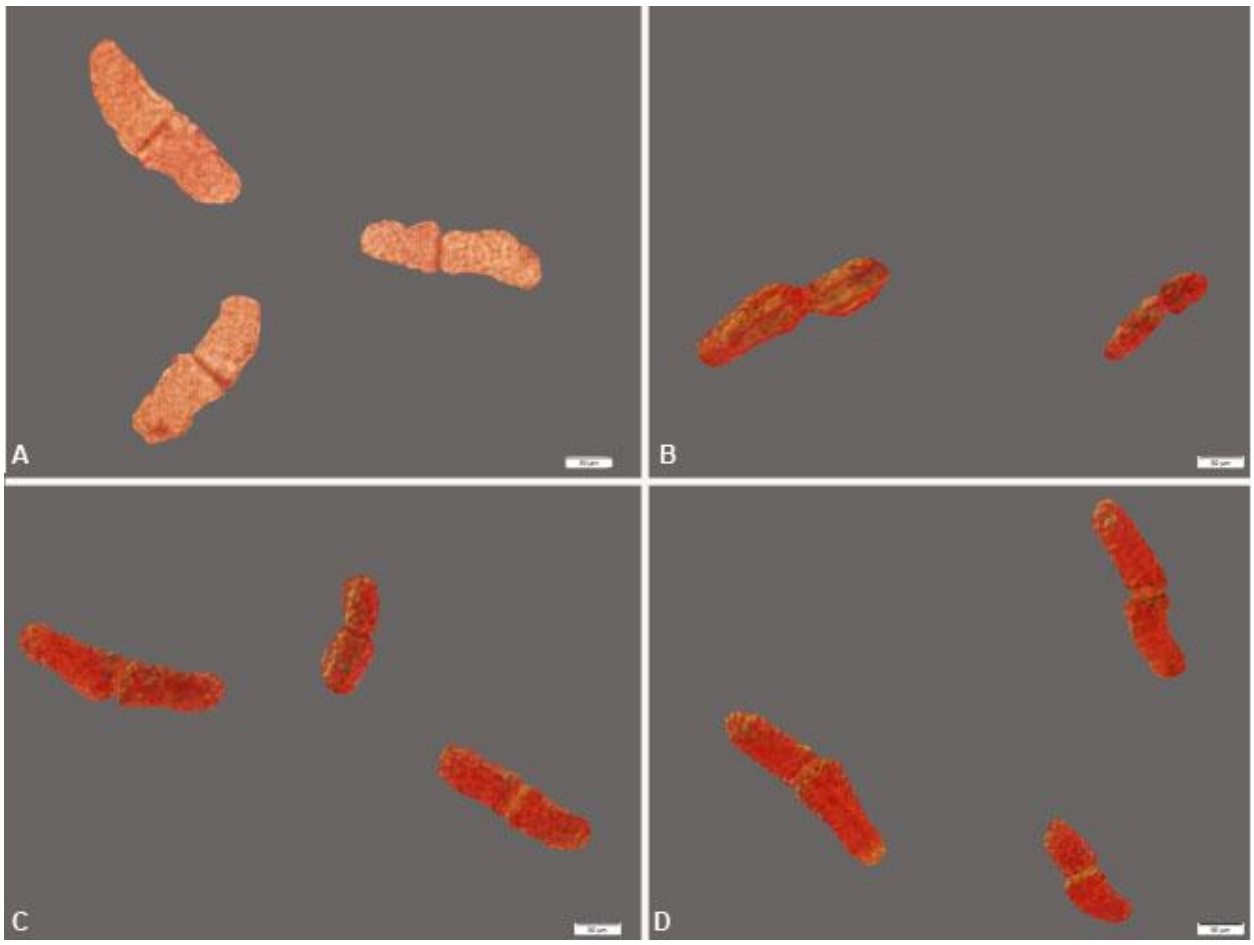

Figure 4.3.16 Abaxial leaf cystoliths of *Crabbea velutina*. **In:** (A–D) cystoliths with an overall elliptic to oblong shape and obtuse apices, individual crystals are ovate. **Specimens:** (A) *de Gouveia 114* (BLFU); (B) *de Gouveia 163* (BLFU); (C, D) *de Gouveia 164* (BLFU).

4.4 Discussion and Conclusion

The presence of paired cystoliths in *Crabbea* confirms the findings of Metcalfe and Chalk (1950a), indicating that paired cystoliths are significant on subtribal level as such cystoliths have been recorded among various members of the Barlerieae (Metcalfe and Chalk, 1950a; Ahmad, 1975; Inamdar *et al*, 1990; Patil and Patil, 2011).

Findings from this study expand on the data on cystoliths presented by Buys (1982), as cystoliths were only mentioned for three *Crabbea* species namely, *C. angustifolia*, *C. hirsuta* [=*C. cirsioides*] and *C. ovalifolia* and were not described in detail. The present study can confirm the presence of cystoliths among the three taxa mentioned by Buys (1982) as well as the remaining southern African species.

Southern African *Crabbea* cystolith shape *sensu* Karlstrom (1980) on both adaxial and abaxial leaf epidermal surfaces can be interpreted as “elongated, broad [being] blunt at both ends” (Table 4.2). Cystolith characteristics are relatively uniform across the southern African *Crabbea* species. Adaxial and abaxial leaf cystolith features that remain constant over the sampled geographic range, and are possibly significant on genus level: include the presence of paired, weakly arched cystoliths with an overall elliptic to oblong shape and ovate individual crystals with obtuse and/or blunt apices (Figures 4.3.1–4.3.16). *Crabbea galpinii* and *C. pedunculata* have a unique oblong individual crystal shape on both leaf surfaces, which is not found among the remaining *Crabbea* species (Figures 4.3.7; 4.3.8; 4.3.13; 4.3.14).

Results from cystolith measurements suggest that adaxial leaf cystolith attachment width is an important character for division of the southern African *Crabbea* species into two distinct groups, those with an average adaxial leaf cystolith attachment width > 50 µm (*C. acaulis*, *C. angustifolia*, *C. cirsioides*, *C. nana* and *C. ovalifolia*) and those with an attachment width of < 50 µm (*C. galpinii*, *C. pedunculata* and *C. velutina*) (Table 4.3). Using the adaxial leaf cystolith attachment of 50 µm as the reference point, cystoliths of *Crabbea* taxa with an adaxial leaf cystolith attachment width less than 50 µm are generally narrowly elliptic to oblong, as in the case *C. galpinii*, *C. pedunculata* and *C. velutina.* Cystoliths of *Crabbea* taxa with an adaxial leaf cystolith attachment width more

than 50 µm display a generally elliptic to oblong shape, for example *C. acaulis*, *C. angustifolia*, *C. cirsioides*, *C. nana* and *C. ovalifolia*.

Cystolith density on the adaxial and abaxial leaf epidermal surfaces is relatively similar among the investigated *Crabbea* species (Tables 4.3; 4.4). *Crabbea angustifolia* and *C. galpinii* have the greatest concentration of cystoliths on the adaxial leaf epidermal surface, both bearing 10 cystoliths per mm^2 (Table 4.3). The highest cystolith concentration on the abaxial leaf epidermal surface is found in *C. angustifolia* with 11 cystoliths per mm^2 (Table 4.4). The cystolith density for the sampled *Barleria* and *Lepidagathis* species range from 3–4 and 15–18 cystoliths per mm^2, respectively (Inamdar *et al*., 1990). However, Inamdar *et al*. (1990) do not investigate the difference in cystolith density across both leaf epidermal surfaces but rather give one, average value. To determine how *Crabbea* leaf epidermal cystolith density compares to *Barleria* and *Lepidagathis* on both leaf surfaces, additional sampling of both mentioned genera is needed as well as the sampling of cystoliths of the remaining *Crabbea* species outside southern Africa.

In conclusion, to effectively determine the significance of the various characters and their character states with Crabbea, the entire genus must receive further leaf micromorphology treatment. Additionally, other genera within Barlerieae must also receive further leaf micromorphology analysis to determine/confirm whether the suite of leaf micormorphology characters investigated in this study are significant on genus and/or species level. Cystolith characteristics for the southern African species of *Crabbea* were more significant on genus than species level for the southern African species of *Crabbea*. This is supported by the cystolith identification provided in section 4.5, as the cystolith identification key recognises four groups. Of the four groups, only one species, *C. velutina* could be keyed-out successfully. The remaining southern African *Crabbea* species were placed in larger groups containing two or three *Crabbea* species. For cystolith characters to be significant on species level, anatomical data focusing on measurements are proven relatively useful as the investigated *Crabbea* species could be placed into four distinct groups successfully using this data. This study

provides the first comprehensive investigation of the significance of cystolith characters and character states for the genus *Crabbea*.

4.5 Cystolith identification key for the southern African species of *Crabbea*

1A Average cystolith attachment width > 50 µm for crystals on the adaxial leaf surface:

2A Cystolith density > 7 cystoliths per mm^2 on the abaxial leaf surface:

C. acaulis

C. angustifolia

2B Cystolith density < 7 cystoliths per mm^2 on the abaxial leaf surface:

C. cirsioides

C. nana

C. ovalifolia

1B Average cystolith attachment width < 50 µm for crystals on the adaxial leaf surface:

3A Average attachment width < 40 µm and TL/CAW ratio > 5 for crystals on the abaxial leaf surface:

C. galpinii

C. pedunculata

3B Average attachment width > 40 µm and TL/CAW ratio < 5 for crystals on the abaxial leaf surface:

C. velutina

CHAPTER 5

POLLEN MICROMORPHOLOGY

5.1 Introduction

Palynology entails the study of plant spores and pollen grains, focusing specifically on the outer pollen and spore surface and not the live interior (Erdtman, 1966; 1969; Walker and Doyle, 1975; Stuessy, 2009). The value of incorporating pollen grains in a systematic study is that large quantities can be easily extracted from both fresh and herbarium material. Additionally, pollen grains offer an array of characters that can be used to study plants on all taxonomic levels (Walker and Doyle, 1975).

The classification of Acanthaceae *sensu* Lindau (1895) recognised 11 different pollen types. Barlerieae and Ruellieae pollen, more specifically *Barleria* and *Ruellia*, were very similar and where described as *waben pollen* “honeycomb pollen”, characterised by the presence of muri. Bremekamp (1965) emphasized that only *Barleria* and *Crabbea* pollen share similarity with that of *Ruellia* and that the remaining Barlerieae pollen, from other lineages were heterogeneous. Bremekamp (1965) found that the pollen of lineages closely related to *Barleria* and *Crabbea* such as *Lepidagathis* differed in a number of characters: being more ellipsoidal in shape than the spheroidal type of *Barleria*; being small, rather than large, as in the case of *Barleria*; differing in aperture type, 3-colporate rather than the 3-porate nature of *Barleria* and muri, being a prominent feature of *Barleria* pollen, were not prominent in *Lepidagathis*.

Acanthaceae pollen wall architecture displays great diversity in micromorphology and is, thus, of systematic and taxonomic value (Lindau, 1895; Walker and Doyle, 1975; Cairne and Scotland, 2000; Scotland and Vollesen, 2000; House and Balkwill, 2016). This was confirmed in a study by Scotland and Vollesen (2000) in which pollen micromorphology was used in conjunction with additional anatomical and micromorphological characters to classify taxa constituting the entire Acanthaceae. Results obtained showed that pollen characteristics vary across the family on both tribal and genus level. Pollen grains

from larger, closely related genera, such as *Barleria*, *Lepidagathis* and *Ruellia,* were examined from more than one species. Palynological findings indicated that pollen micromorphology for these genera varies on species level as well. However, for *Crabbea*, only *C. velutina* was used as the representative species of the genus. Therefore, no indication is given as to whether *Crabbea* pollen micromorphology varies or remains constant on genus level in their study. Cairne and Scotland (2000) and Scotland and Vollesen (2000) found that Acanthaceae pollen micromorphology is better studied at genus level than at higher taxonomic levels to achieve proper and informative analyses.

Comparison of the molecular analysis of Acanthaceae by McDade *et al*. (2008) and palynological results of Scotland and Vollesen (2000) showed that taxa placed under the tribe Barlerieae were supported by both molecular and palynological data. Taxa which were examined in both studies that show such correlation are: *Barleria*, *Crabbea*, *Lepidagathis* and *Lophostachys* Pohl. Phylogenetically, these taxa are positioned in a highly supported monophyletic Barlerieae Clade (McDade *et al*., 2008). From which palynological results indicate that the sampled taxa may be grouped together based on the strong presence of muri. Muri are also observed among other tribes within the subfamily Acanthoideae, suggesting that muri may be regarded as taxonomically informative on subfamily level. Tripp and Fatimah (2012) investigated the anatomy, morphology and phylogeny of *Satanocrater* Schweinf., found in tropical east Africa. Palynological data confirmed the presence of the following features across the entire genus: the presence of muri, lumina devoid of gemmae, three aperturate, weakly colporate to porate and spheroidal pollen grains. The presence of muri among the *Satanocrater* species supports the classification of this genus within the subfamily Acanthoideae.

Pollen micromorphology has not been included in five of the seven taxonomic treatments of *Crabbea* (Harvey, 1842; Nees von Esenbeck, 1847; Burkill and Clarke, 1899–1900; Clarke, 1901; Vollesen, 2015). The taxonomic accounts of Buys (1982) and Thulin (2007) are the only two revisions that investigated the pollen micromorphology of *Crabbea*. Buys (1982) found that southern African *Crabbea* could be classified as

having either reticulate pollen or gemmate pollen. Thulin (2007) compared the SEM micrographs of *C. acaulis* (Furness, 1998) and *C. velutina* (Scotland and Vollesen, 2000) and found that *Crabbea* pollen displays variation in terms of gemma distribution on the lumina surface. Moreover, Thulin (2007) observed that the pollen grains of *C. acaulis* and *C. velutina* are both porate and reticulate. The Somali *Crabbea*, *C. albolutea* and *C. pinnatifida* Thulin are also porate, with verrucose reticulum and gemmae present in the lumina, character states found in both *Acanthostelma* and *Crabbea*.

The aim of this chapter is to establish the significant pollen micromorphology characters for the southern African *Crabbea* at genus and species level. This will be achieved by the investigation of overall pollen shape and size, murus height and width, lumina, gemma density and size and aperture size and shape.

5.2 Materials and Methods

Fresh flowers and/or buds used for pollen micromorphological examination were preserved in 3% (v/v) phosphate-buffered GA or 70% (v/v) EtOH. Flowers and buds sampled from herbarium specimens were rehydrated in 3% (v/v) phosphate-buffered GA for 48 hrs. It is emphasized that only mature buds and young flowers were used to collect pollen grains for micromorphology analysis, thus, ensuring that fully developed pollen grains are examined. The number of samples used to examine the pollen micromorphology of each investigated *Crabbea* species is presented in Table 5.1.

Pollen was removed from dissected anther thecae in 95% (v/v) EtOH and the Acetolysis Method (Erdtman, 1960) was followed to prepare pollen for SEM and LM. After acetolysis, pollen was prepared for SEM by rinsing the acetolysed pollen in glacial acetic acid, washing twice with distilled water and followed by 95% (v/v) ethanol. Prior to mounting on aluminium stubs, stubs were cleared and cleaned with 100% (v/v) ethanol. Stubs with pollen samples were sputter-coated with gold, ± 60 nm thick, using the BioRad SEM Coating System and studied using the Shimadzu Superscan SSX-550

Table 5.1 *Crabbea* voucher specimens examined for pollen micromorphology.

Herbarium	Species	Collector name and number	Fixative	Collection date
BLFU	*C. acaulis*	A. de Gouveia 78	3% (v/v) GA	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 124	3% (v/v) GA	25-03-2015
BLFU	*C. acaulis*	A. de Gouveia 151	3% (v/v) GA	28-03-2015
BLFU	*C. angustifolia*	J.E. Burrows and S.E. Burrows 1438	3% (v/v) GA	07-03-2015
PRE	*C. angustifolia*	F.A. Rogers 18718	70% (v/v) EtOH	00-04-1916
BLFU	*C. galpinii*	A. de Gouveia 169 C	70% (v/v) EtOH	20-11-2015
BLFU	*C. galpinii*	A. de Gouveia 170	70% (v/v) EtOH	21-11-1205
BLFU	*C. galpinii*	A. de Gouveia 171	70% (v/v) EtOH	21-11-2015
BLFU	*C. hirsuta*	A. de Gouveia 88	3% (v/v) GA	22-03-2015
BLFU	*C. hirsuta*	A. de Gouveia 124	3% (v/v) GA	25-03-2015
BLFU	*C. hirsuta*	A. de Gouveia 132	3% (v/v) GA	26-03-2015
BLFU	*C. nana*	A. de Gouveia 179	70% (v/v) EtOH	27-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 173	3% (v/v) GA	22-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 174	3% (v/v) GA	22-11-2015
PRE	*C. ovalifolia*	N. van Rooyen 2224	70% (v/v) EtOH	27-12-1979
BLFU	*C. pedunculata*	A. de Gouveia 176	70% (v/v) EtOH	25-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 177	70% (v/v) EtOH	26-11-2015
BLFU	*C. velutina*	A. de Gouveia 157	70% (v/v) EtOH	17-11-2015
BLFU	*C. velutina*	A. de Gouveia 163	70% (v/v) EtOH	17-11-2015
BLFU	*C. velutina*	A. de Gouveia 164	70% (v/v) EtOH	19-11-2015

SEM at 10 kV and a working distance of 14–16 mm. Sputter coating and SEM investigation took place at the Centre for Confocal Microscopy, UFS.

For LM analysis, a few drops of 50% (v/v) glycerine with dissolved phenol crystals were added to the remaining pollen in each centrifuge tube to prevent fungal and/or bacterial growth. Once dried, samples were mounted on slides with glycerol jelly, following the protocol of Reitsma (1969). Pollen grains were photographed using an Olympus BX 53 LM, with a mounted Olympus DP 72 Camera, at X 60 magnification, using immersion oil. Measurements were recorded using the software package cellSens v.1.4.1, at the department of PS.

The following pollen grain measurements were taken: aperture diameter (A); equatorial width (E); gemma width (GW); murus height (MH); murus width (MW) and polar axis length (P) (Figure 5.1). Average P/E ratios of each species were also determined. Measurements were done for at least three pollen grains of each specimen.

Pollen grain terminology follows Punt *et al*. (2007) and P/E ratios, shapes and sizes follow Walker and Doyle (1975). Pollen grain size is determined by the length of the longest pollen grain axis (Walker and Doyle, 1975).

5.3 Results

5.3.1 Genus description of *Crabbea* pollen micromorphology in southern Africa

Pollen unit: Monad. **Size:** Medium (25–49 µm) to large (50–99 µm). **Shape:** Oblate spheroidal (P/E ratio: 0.88–0.99), prolate spheroidal (P/E ratio: 1.01–1.14), or subprolate (P/E ratio: 1.15–1.33). **Aperture:** Simple, tri-porate, circular and equatorial. **Muri:** Rarely absent, when present, continuous or rarely discontinuous, reticulate, straight, undulate to slightly curved. **Lumen:** Formed where muri are continuous, variable in shape and size, rarely uniform in shape. **Gemmae:** Globose, rarely oblong, variable in size and density, located in lumina, enclosed by muri, or dispersed over the pollen surface where muri are lacking (Tables 5.2; 5.3).

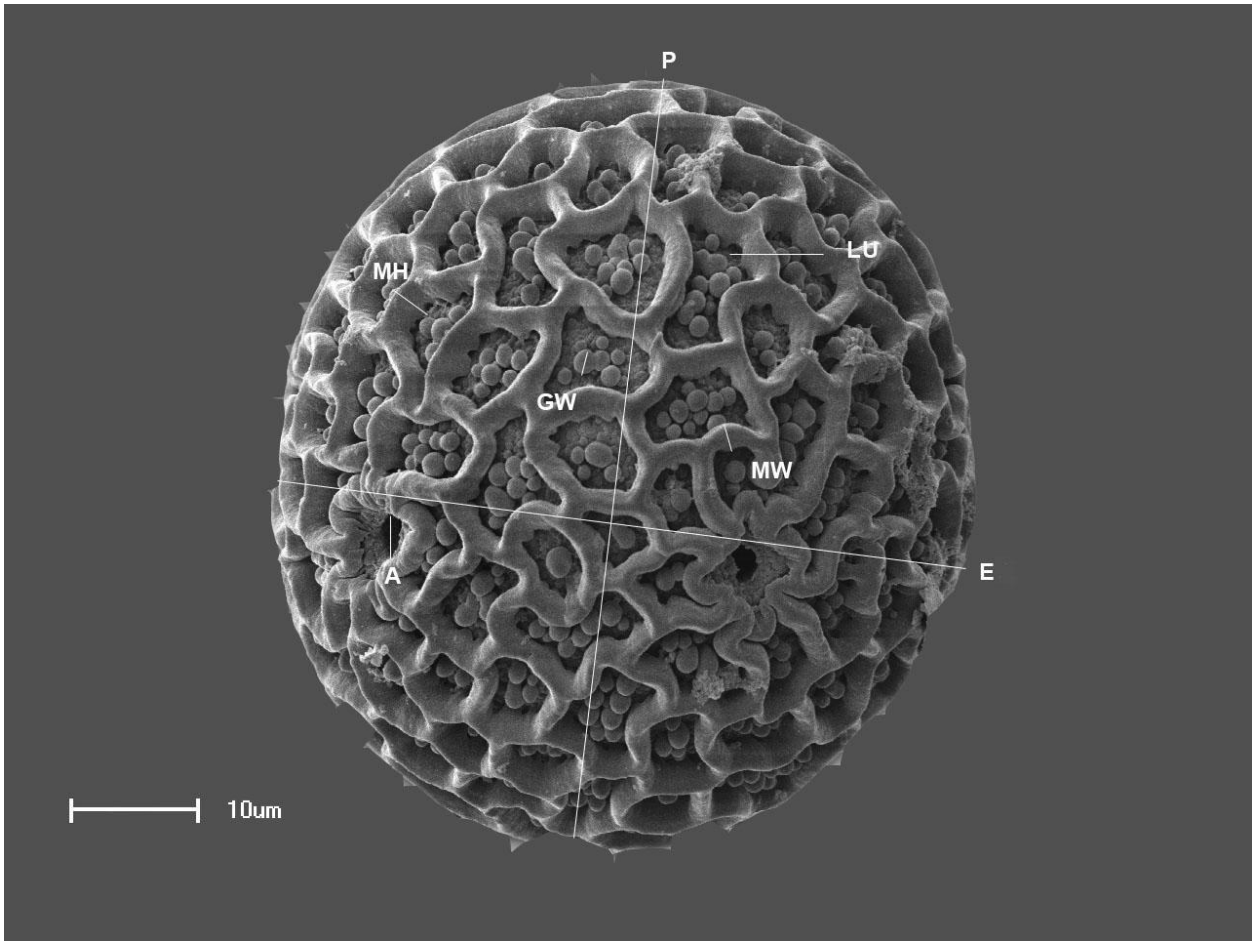

Figure 5.1 Measurements used to determine the dimensions of various features of *Crabbea* pollen. **Legend:** (A) aperture; (E) equatorial width; (GW) gemma width; (LU) lumen; (MH) murus height; (MW) murus width; (P) polar axis length. **Specimen:** *de Gouveia 78* (BLFU).

5.3.2 Pollen micromorphology measurements of the southern African *Crabbea* species

Table 5.2 Average pollen dimensions of southern African *Crabbea*[*/**].

Species	Polar axis Length (P) (µm)	Equatorial width (E) (µm)	Pollen grain size*	P/E ratio	Pollen grain shape
C. acaulis	60.02 ± 7.39	57.16 ± 7.34	Large	1.05	Prolate spheroidal
C. angustifolia	61.06 ± 5.54	52.35 ± 7.31	Large	1.17	Subprolate
C. cirsioides	60.16 ± 14.39	51.38 ± 5.34	Large	1.17	Subprolate
C. galpinii	46.55 ± 8.25	46.55 ± 3.88	Medium	1.01	Prolate spheroidal
C. nana	39.64 ± 2.72	40.42 ± 2.11	Medium	0.98	Oblate spheroidal
C. ovalifolia	62.57 ± 0.74	61.80 ± 7.03	Large	1.02	Prolate spheroidal
C. pedunculata	45.28 ± 5.22	44.70 ± 2.05	Medium	1.01	Prolate spheroidal
C. velutina	67.80 ± 1.95	69.14 ± 5.28	Large	0.98	Oblate spheroidal

* Pollen size is determined on the length of the longest pollen grain axis: medium-sized pollen = 25–49µm; large pollen = 50–99 µm. Pollen grain size measured according to Walker and Doyle (1975).

**The SD is provided next to the average value for a particular measurement.

Table 5.3 Average pollen dimensions of southern African *Crabbea* continued**.

Species	Aperture diameter (A) (µm)	Gemma width (GW) (µm)	Murus height (MH) (µm)	Murus width (MW) (µm)
C. acaulis	3.89 ± 1.39	1.59 ± 0.43	2.47 ± 0.43	1.99 ± 0.43
C. angustifolia	3.82 ± 1.20	1.73 ± 0.34	2.97 ± 0.38	2.19 ± 0.43
C. cirsioides	4.08 ± 1.11	1.60 ± 0.32	2.76 ± 0.59	1.97 ± 0.42
C. galpinii	3.39 ± 1.43	2.19 ± 0.45	N/A***	N/A***
C. nana	3.82 ± 1.18	1.77 ± 0.56	2.36 ± 0.44	1.86 ± 0.31
C. ovalifolia	4.83 ± 2.03	1.78 ± 0.59	3.18 ± 1.23	1.73 ± 0.39
C. pedunculata	3.79 ± 0.98	2.30 ± 0.56	N/A**	N/A**
C. velutina	3.10 ± 1.70	1.89 ± 0.35	5.42 ± 1.09	1.97 ± 0.45

**The SD is provided next to the average value for a particular measurement.

*** Muri absent from the pollen of *C. galpinii* and *C. pedunculata*.

5.3.3 Species description of pollen among southern African *Crabbea*

5.3.3.1 *Crabbea acaulis*

Shape: Prolate spheroidal. **Size:** Large (Table 5.2). **Muri:** Continuous or rarely discontinuous, reticulate, curved, variable within and among individuals. **Lumen:** Present when muri are continuous, variable in shape and size. **Gemmae:** Globose, variable in size and density (Figures 5.2.1 A–F).

5.3.3.2 *Crabbea angustifolia*

Shape: Subprolate. **Size:** Large (Table 5.2). **Muri:** Continuous, reticulate, undulate, uniform in individuals and slightly variable among different individuals. **Lumen:** Present, variable in shape and size. **Gemmae:** Globose, sparsely distributed over pollen surface (Figures 5.2.2 A–F).

5.3.3.3 *Crabbea cirsioides*

Shape: Subprolate. **Size:** Large (Table 5.2). **Muri:** Mostly continuous (Figure 5.2.3 A–C), sometimes discontinuous (Figure 5.2.3 D, F) or absent (Figure 5.2.3 E), reticulate, undulate to curved, variable among individuals. **Lumen:** Present when muri are continuous (Figures 5.2.3 A–C). **Gemmae:** Globose, vary in density across pollen surface (Figures 5.2.3 A–F).

5.3.3.4 *Crabbea galpinii*

Shape: Prolate spheroidal. **Size:** Medium (Table 5.2). **Muri:** Absent. **Lumen:** Absent. **Gemmae:** Globose, rarely oblong, densely covering pollen surface (Figures 5.2.4 A–F).

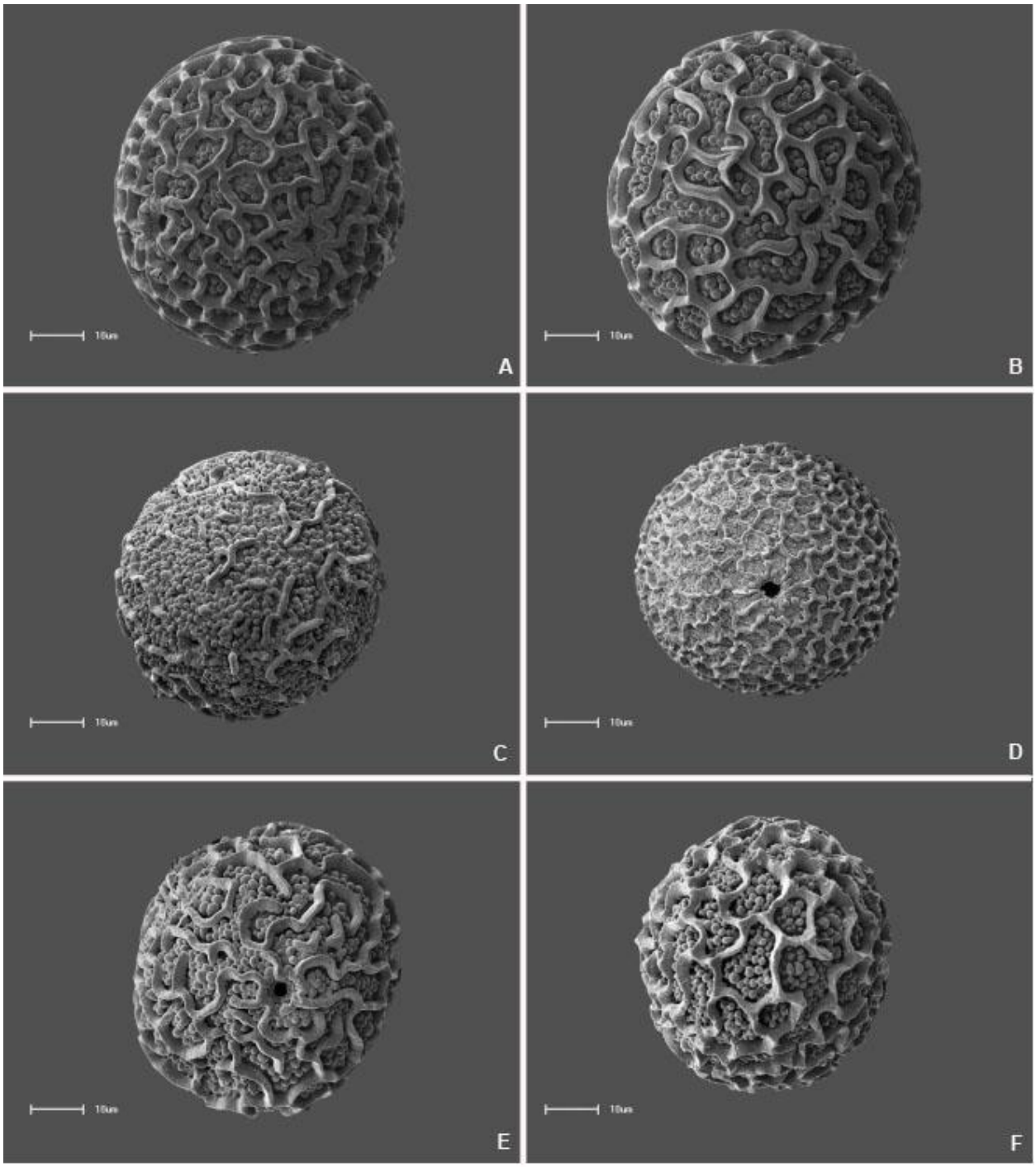

Figure 5.2.1 SEM micrographs of *C. acaulis* pollen. **In:** (A, B) muri and lumina uninterrupted and reticulate, gemmae densely arranged within lumina; (C) muri and lumina interrupted, gemmae densely arranged within lumina; (D) gemmae sparsely arranged within lumina, aperture porate; (E, F) muri interrupted and reticulate, lumina interrupted, gemmae densely arranged within lumina. **Specimens:** (A, B) *de Gouveia 78* (BLFU); (C, D) *de Gouveia 123* (BLFU); (E, F) *de Gouveia 151* (BLFU).

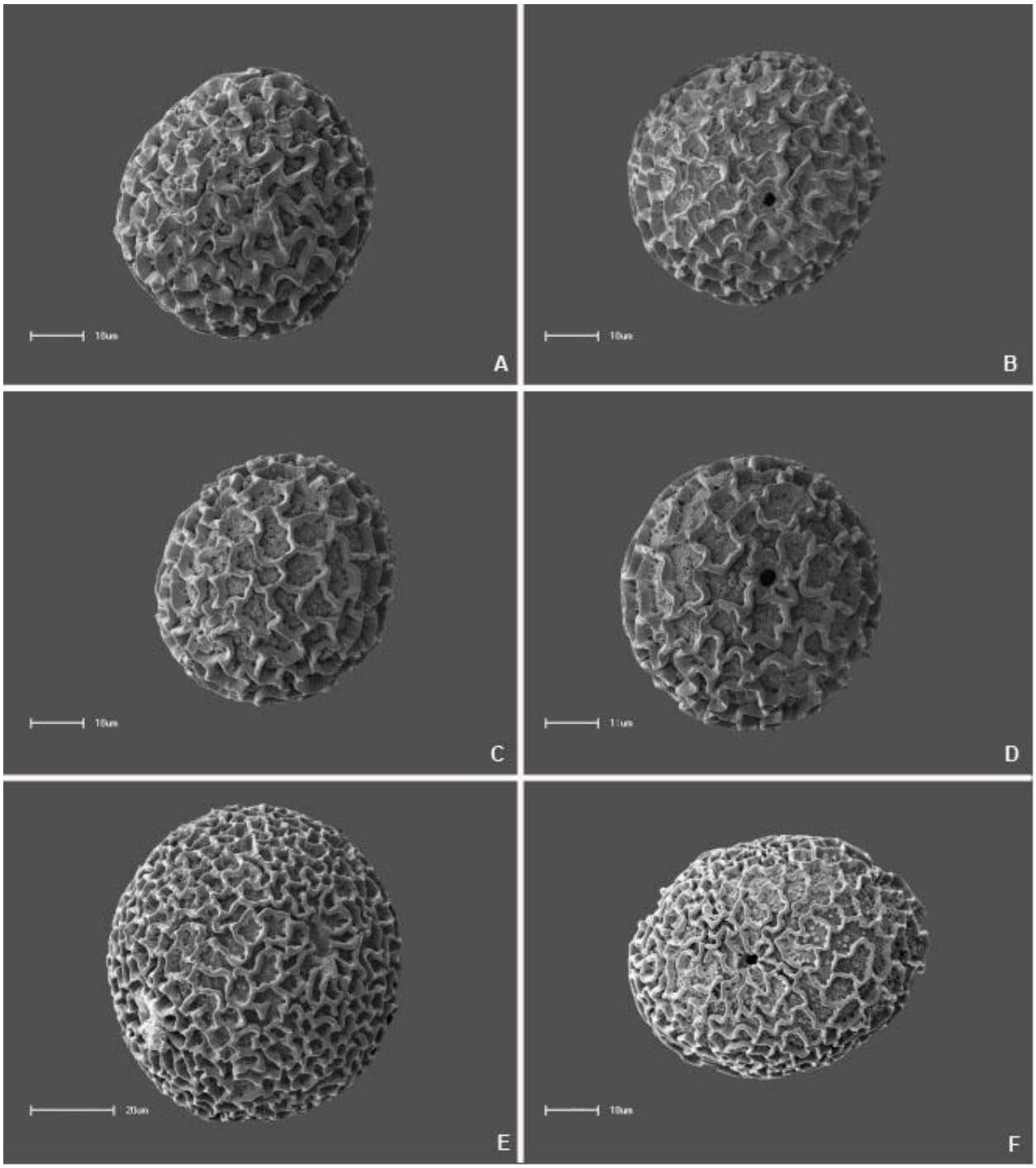

Figure 5.2.2 SEM micrographs of *C. angustifolia* pollen. **In:** (A–B) muri uninterrupted, reticulate and undulate, gemmae sparsely distributed within lumina; (C–F) muri uninterrupted, reticulate and undulate, gemmae sparsely distributed within lumina, apertures are porate. **Specimens:** (A–D) *Burrows* and *Burrows 14638* (BLFU); (E, F) *Rogers 18718* (PRE).

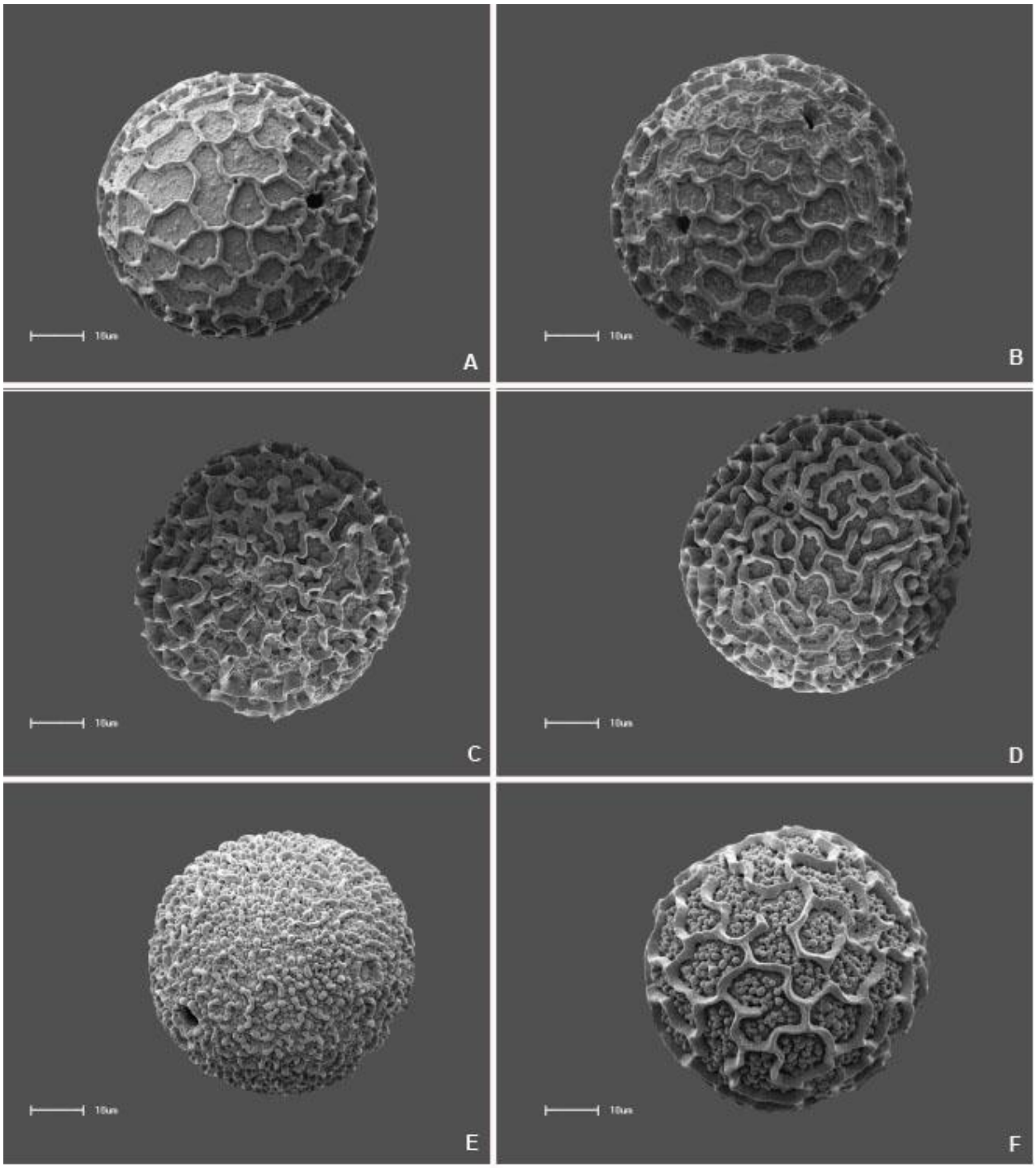

Figure 5.2.3 SEM micrographs of *C. cirsioides* pollen. **In:** (A–D) muri uninterrupted and reticulate, lumina uninterrupted, gemmae sparsely arranged within lumina, apertures porate; (E) muri absent and lumina absent, aperture porate; (F) muri interrupted and reticulating, lumina interrupted, gemmae densely arranged within lumina, apertures porate. **Specimens:** (A, B) *de Gouveia 88* (BLFU); (C, D) *de Gouveia 124* (BLFU); (E, F) *de Gouveia 132* (BLFU).

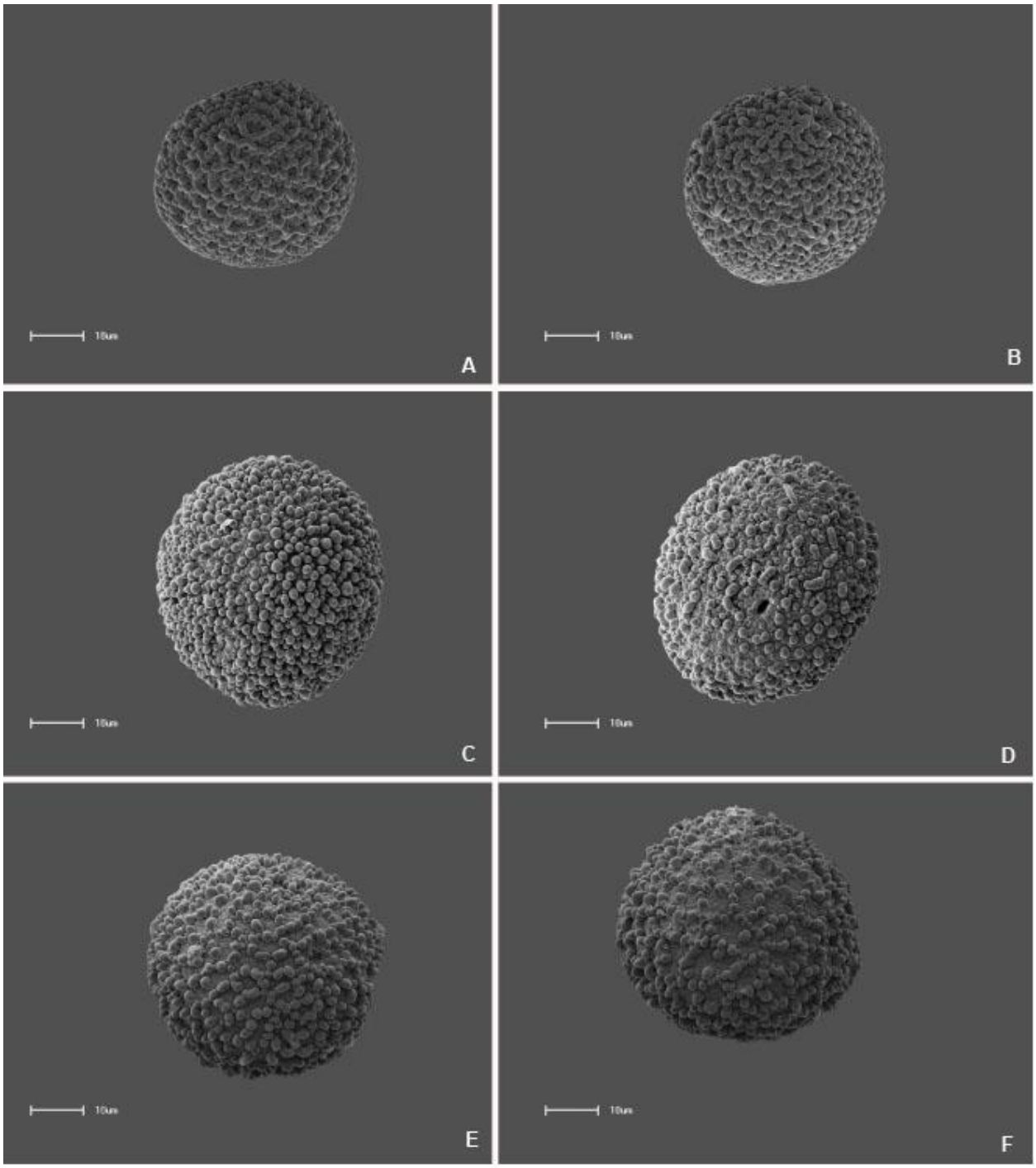

Figure 5.2.4 SEM micrographs of *C. galpinii* pollen. **In:** (A–F) muri absent and lumina absent, gemmae globose to oblong, largely covering pollen grain. **Specimens:** (A, B) *de Gouveia 169 C* (BLFU); (C, D) *de Gouveia 170* (BLFU); (E; F) *de Gouveia 171* (BLFU).

5.3.3.5 *Crabbea nana*

Shape: Oblate spheroidal (Figures 5.2.5 A–F). **Size:** Medium (Table 5.2). **Muri:** Continuous (Figures 5.2.5 B, C, E, F), sometimes absent (Figures 5.2.5 A, D), reticulate. **Lumen:** Present when muri are continuous, variable in shape and size (Figures 5.2.5 B, C, E, F). **Gemmae:** Globose, densely distributed over the pollen surface (Figures 5.2.5 A–F).

5.3.3.6 *Crabbea ovalifolia*

Shape: Prolate spheroidal. **Size:** Large (Table 5.2). **Muri:** Continuous, reticulate, slightly curved. **Lumen:** Present, variable in shape and size (Figure 5.2.6 A, B). **Gemmae:** Globose, densely (Figure 5.2.6 A) to sparsely covering pollen surface (Figure 5.2.6 B).

5.3.3.7 *Crabbea pedunculata*

Shape: Prolate spheroidal. **Size:** Medium (Table 5.2). **Muri:** Absent. **Lumen:** Absent. **Gemmae:** Globose, rarely oblong, densely covering the pollen surface (Figures 5.2.7 A–F).

5.3.3.8 *Crabbea velutina*

Shape: Oblate spheroidal. **Size:** Large (Table 5.2). **Muri:** Continuous, reticulate, undulate. **Lumen:** Present, uniform. **Gemmae:** Globose, densely covering lumen (Figure 5.2.8 A–F).

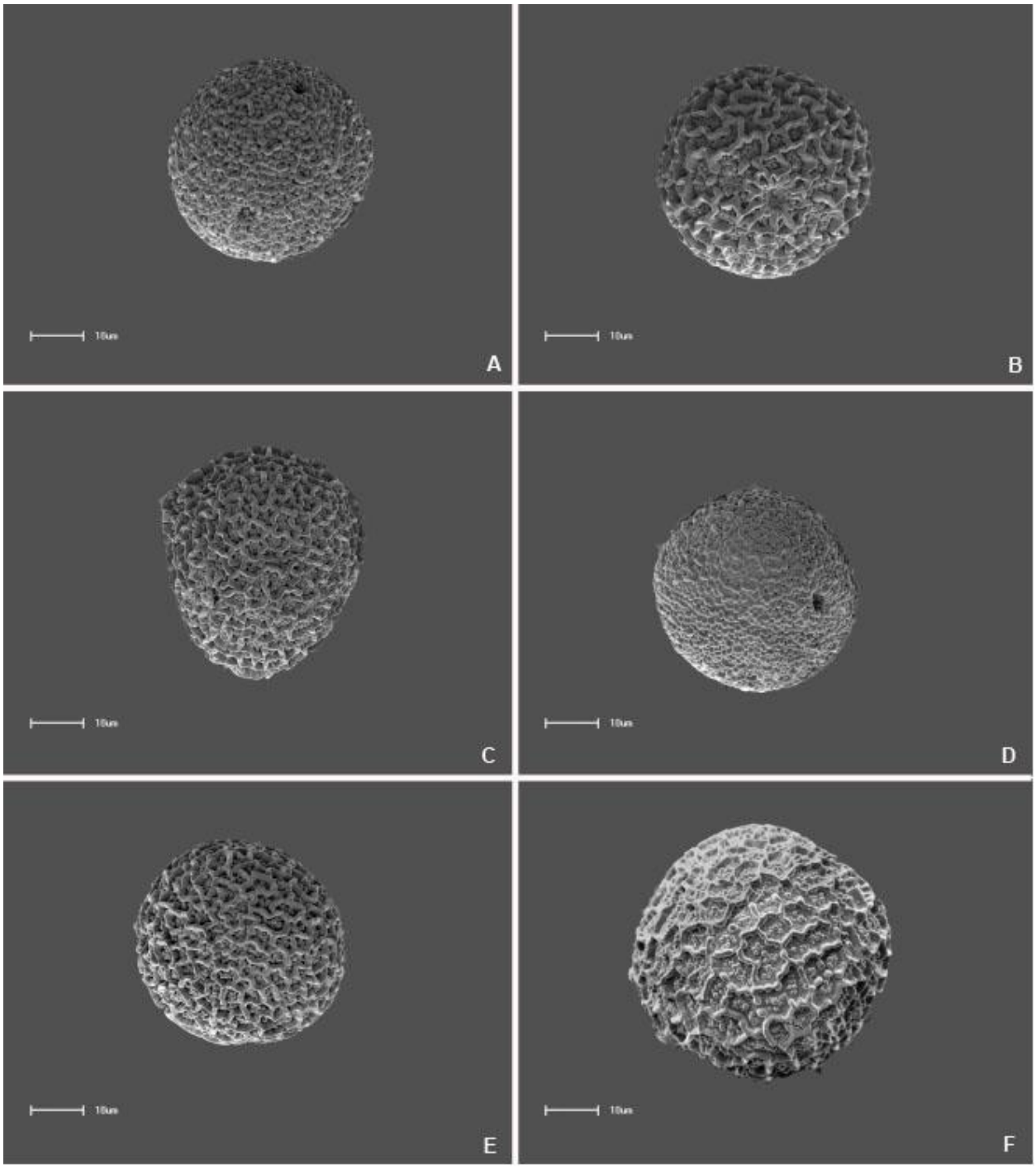

Figure 5.2.5 SEM micrographs of *C. nana* pollen. **In:** (A) muri and lumina absent, gemmae covering pollen grain; (B, C, E) muri uninterrupted and reticulate, lumina uninterrupted, gemmae densely arranged within lumina; (D) muri and lumina absent, gemmae covering pollen grain; (F) muri interrupted and reticulate, lumina interrupted, gemmae sparsely arranged within lumina. **Specimens:** (A–F) *de Gouveia 179* (BLFU).

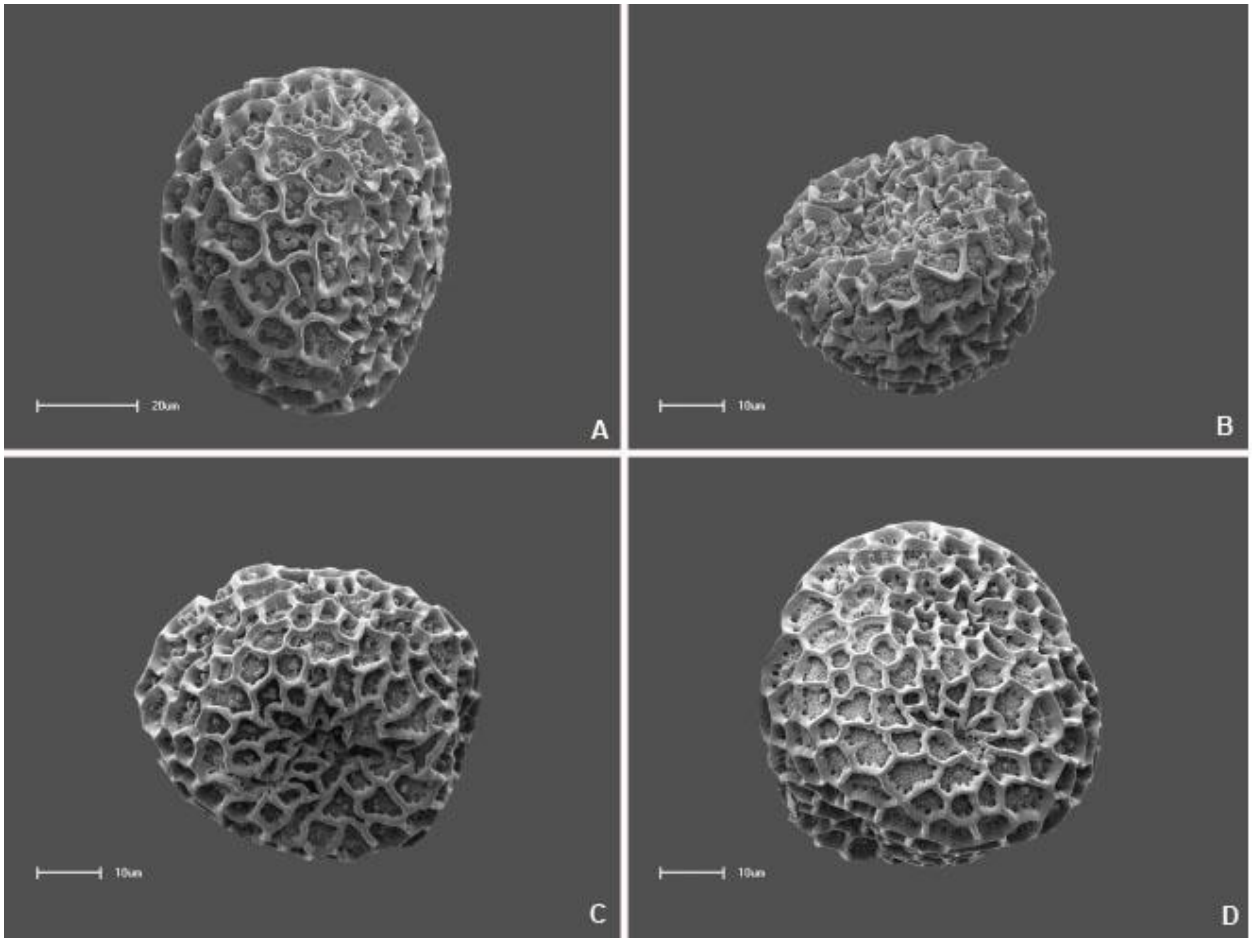

Figure 5.2.6 SEM micrographs of *C. ovalifolia* pollen. **In:** (A–D) muri uninterrupted, reticulate and slightly curved, lumina uninterrupted, pollen grains partly collapsed; (A) gemmae densely covering lumina; (B–D) gemmae sparsely covering lumina. **Specimens:** (A) *de Gouveia 173* (BLFU); (B) *de Gouveia 174* (BLFU); (C, D) *van Rooyen 2224* (PRE).

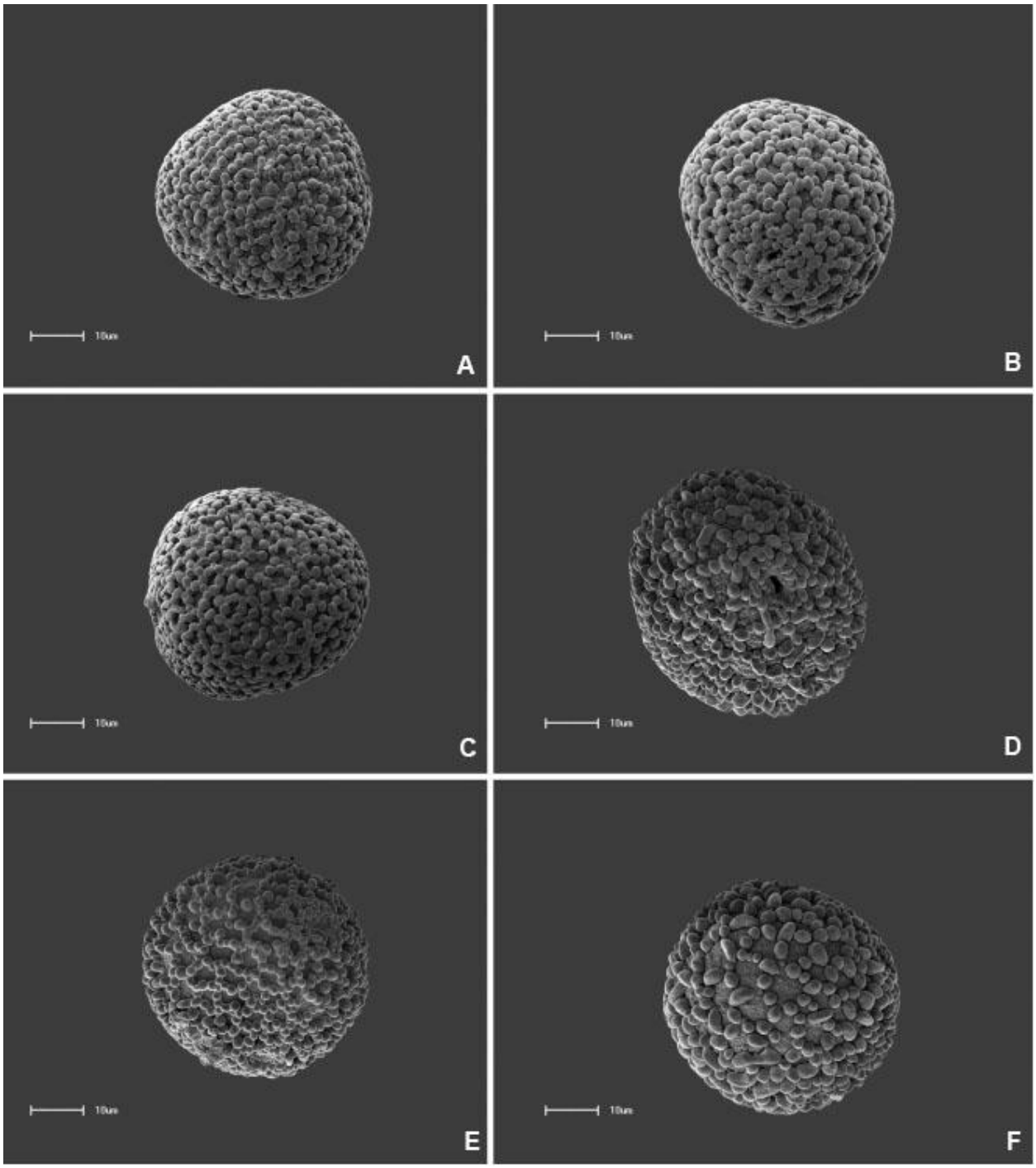

Figure 5.2.7 SEM micrographs of *C. pedunculata* pollen. **In:** (A–F) muri absent and lumina absent, gemmae globose, rarely oblong, densely covering the pollen surface. **Specimens:** (A–C) *de Gouveia 176* (BLFU); (D–F) *de Gouveia 177* (BLFU).

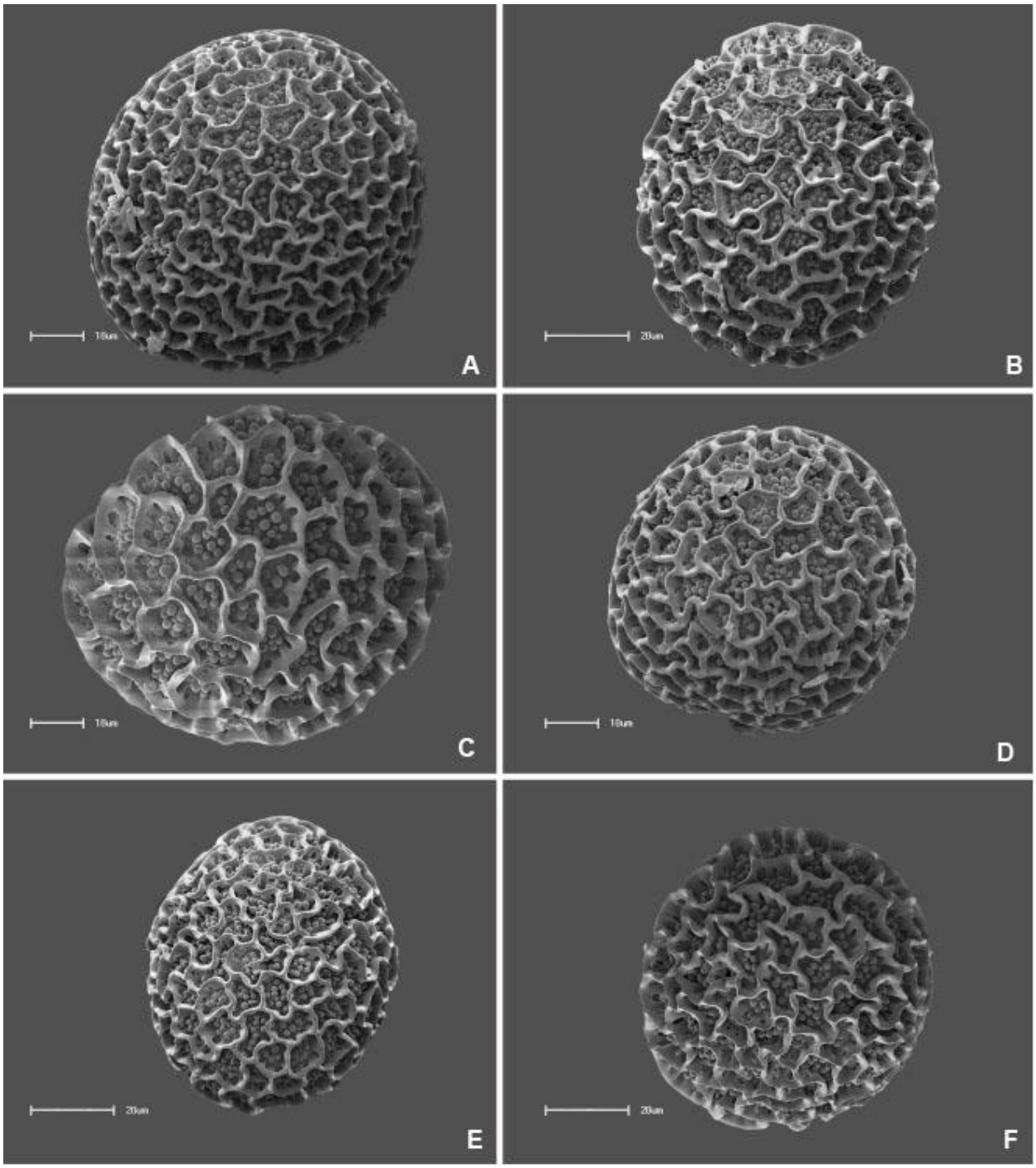

Figure 5.2.8 SEM micrographs of *C. velutina* pollen. **In:** (A–F) muri uninterrupted, reticulate and undulate, lumen variable in shape and size, gemmae densely covering lumina. **Specimens:** (A, B) *de Gouveia 157* (BLFU); (C, D) *de Gouveia 163* (BLFU); (E, F) *de Gouveia 164* (BLFU).

5.4 Discussion and Conclusion

Results from the present study show that pollen characteristics may be of limited taxonomic value at species level for the southern African *Crabbea*. This is proven in the pollen identification key generated in section 5.5. The identification key only recognises four groups. Pollen characters that remain constant in all investigated specimens include: globose, tri-porate pollen grains with simple apertures that are equatorially positioned. Buys (1982) reports that *C. acaulis* is tetra-aperturate whereas Raj (1961) finds that *C. acaulis* is bi-aperturate. The difference in aperture number in *C. acaulis* may be attributed to the location/population where pollen was collected for *C. acaulis* palynological analysis.

Scotland and Vollesen (2000) and McDade *et al*. (2008) highlight the value of muri in tribal classification of the Acanthaceae. However, results of the present study show that within southern African *Crabbea*, there is significant variation in the appearance of muri and that these do not occur consistently in all members of the genus. With such variation present at genus level, muri should be used with caution in classification at higher taxonomic levels.

Erdtman (1966) suggests that, within a genus, more than one pollen type may be found, and this is confirmed by results of the present study. Based on palynological data, the southern African *Crabbea* species can be divided into two main groups which are identified here and in the study of Buys (1982). The first group, characterized by the presence of muri, *sensu* Lindau (1895) *waben* “honey comb” pollen, can be further divided into two, based on continuous versus discontinuous muri. Taxa with muri include: *C. acaulis*, *C. angustifolia*, *C. cirsioides*, *C. nana*, *C. ovalifolia* and *C. velutina* (Figures 5.2.1–5.2.3; 5.2.5; 5.2.6; 5.2.8). The absence of muri from the pollen grain surface can be used to delimit the second group, consisting of *C. galpinii* and *C. pedunculata* (Figures 5.2.4; 5.2.7). The variation in pollen grain morphology of the southern African *Crabbea* species confirmed in this study, and the variation among the Somali *Crabbea* species of Thulin (2007) may suggest that *Crabbea* pollen micromorphology varies across the entire genus. This will be confirmed with the addition of the remaining *Crabbea* species north of southern Africa.

In some *Crabbea* species, such as *C. angustifolia*, *C. galpinii*, *C. ovalifolia*, *C. pedunculata* and *C. velutina* pollen characters such as the presence or absence of muri remain constant over the sampled geographical range of these species (Figures 5.2.2; 5.2.4; 5.2.6–5.2.8)

In other species, however, much variation in pollen characters from geographically separate populations was observed. In different populations of *C. acaulis*, *C. cirsioides* and *C. nana*, for instance, muri may be present or absent in different individuals or populations of the same species (Figures 5.2.1; 5.2.3; 5.2.5). This suggests that wider sampling of populations of species, in which pollen characters appear to remain constant, may reveal a greater degree of intraspecific variation. Scotland (1992) advises that pollen micromorphology cannot be used on its own to classify taxa but should be used in conjunction with additional character sets. Results from the present study further confirm the findings of Buys (1982), showing that gemma density and shape varied among the southern African *Crabbea* representatives (Figures 5.2.1–5.2.8)

Pollen grain size does not remain constant at a specific taxonomic level (Bell, 1959; Fægri *et al.*, 1989). Fægri *et al.* (1989) points out that "[pollen grain] size is even more dependent on the history of the grain than is shape." Factors that may influence pollen grain size, include: nutrition, when the plant was still growing (Bell, 1959), moisture availability (Fægri *et al.*, 1989), pollen preparation method (Walker and Doyle, 1975) and other chemical treatments (Reitsma, 1969; Hesse and Waha, 1989) and the age/maturity of the pollen grains.

Keeping the above mentioned in mind (Table 5.1), when analysing the dimensions of various *Crabbea* pollen features, the following observations were made: if pollen size is classified based on the length of the longest axis, as proposed by Walker and Doyle (1975), the pollen of the majority of the investigated *Crabbea* species is large, 50–99 µm. Pollen with muri are generally larger than pollen grains devoid of muri (Tables 5.2). Overall P/E values indicate that southern African *Crabbea* has three pollen types: oblate spheroidal (*C. nana* and *C. velutina*), prolate spheroidal (*C. acaulis*, *C. galpinii*, *C. ovalifolia* and *C. pedunculata*) and subprolate (*C. angustifolia* and *C. cirsioides*) (Table 5.2). Aperture diameter is similar for all *Crabbea* species. However, *C. galpinii* has the

smallest average aperture diameter, 3.39 µm and *C. ovalifolia* the largest, 4.83 µm (Table 5.3). *Crabbea velutina* has the largest average murus height recorded for all southern African *Crabbea* species, 5.42 µm (Table 5.3). Murus width is most significant to *C. angustifolia*, having an average murus width of more than 2 µm (Table 5.3).

Palynological results for *Crabbea velutina* proved congruent with the findings of Buys (1982) and Scotland and Vollesen (2000). Palynological characteristics that were found on the *C. velutina* grain were described as: “sub-spherical, tri-porate, open reticulate tectum with scattered granules of exine in lumina”. Buys (1982) made similar conclusions concerning *C. velutina* pollen, and described the overall pollen appearance as having “reticulate tecta [muri] with an uneven granular [gemmae] distribution of various lengths.” Both the current study and that of Buys (1982) agree that *C. velutina* has the largest pollen grains of all southern Africa *Crabbea* species (Table 5.2).

In conclusion, *Crabbea* palynological data generated from this study confirmed the phylogenetic position of *Crabbea* within Barlerieae by the presence of muri among the majority of taxa. Muri characteristics proved to be most useful to delimit *Crabbea* pollen on species level. On the other hand, muri should be used with caution as a diagnostic tool at species level, as it varies in appearance from specimens collected from one population and between populations of the same species. Thus, the value of these characters in delimiting the genus can only be determined through a wider sampling of pollen in all *Crabbea* species over their geographic ranges and comparison with pollen characters in all closely related genera. This study provides the first attempt to construct identification keys for the southern African *Crabbea* using pollen micromorphology.

5.5 Identification key to the southern African species of *Crabbea* based on pollen micromorphology

1A Muri and lumina usually present, but when absent, gemma width < 2 µm:

2A Both polar axis length and equatorial diameter > 65 µm; murus height > 5 µm:

C. velutina

2B Either polar axis length or equatorial diameter < 65 µm; murus height < 5 µm:

3A Murus width > 2µm:

C. angustifolia

3B Murus width < 2µm:

C. acaulis

C. cirsioides

C. nana

C. ovalifolia

1B Muri and lumina absent; gemma width > 2 µm:

C. galpinii

C. pedunculata

CHAPTER 6

PHYLOGENY

6.1 Introduction

Initially, plant systematics relied mainly on morphological trait analysis for classification (Sivarajan, 1991). Morphological trait analysis has proved successful in describing new species, identifying species in the wild and constructing identification keys (Abdelaziz *et al*., 2011). At present, molecular data forms an essential component in a systematic study, especially when there is a significant degree of morphological similarity among the members of the group under study and when questions concerning the taxonomic status of a species arise (Bickford *et al*., 2006; Knowles and Carstens, 2007).

When collecting molecular data for DNA sequence analysis, different gene regions from different genomes, chloroplast DNA (cpDNA) and nrDNA, should be combined. Sequence combinations should incorporate a minimum of three different gene regions to increase the resolution of the topology of the phylogenetic tree under study (Stuessy, 2009).

In many cases, to improve phylogenetic resolution, molecular data can be combined with additional data sets (Bickford *et al*., 2006). The molecular work done on Acanthaceae *sensu lato*, lays the foundation whereby molecular and morphological data can be combined to determine the taxonomic significance of various characters within Acanthaceae (McDade *et al*., 2008; Abdel-Hameed *et al*., 2015). Molecular, morphological and anatomical data have been incorporated in a number of Acanthaceae systematic studies and the results placed and/or confirm the position of specific lineages within Acanthaceae *sensu lato* (McDade *et al*., 2000a; Scotland and Vollesen, 2000; Manktelow *et al*., 2001; McDade *et al*., 2005; 2008; 2012; Tripp and Fatimah, 2012; Abdel-Hameed *et al*., 2015).

All previous systematic studies on *Crabbea* were based on traditional taxonomy, wherein morphological and anatomical characters were used for classification of *Crabbea* on genus and species level (Harvey, 1842; Nees von Esenbeck, 1847; Burkill and Clarke, 1899–1900; Clarke, 1901; Buys, 1982; Thulin, 2007; Vollesen, 2015).

No extensive molecular work has been done on species level for *Crabbea*. A few *Crabbea* species have been included in phylogenetic studies of the Acanthaceae to determine the genus' evolutionary position among other genera. Phylogenetic data revealed that *Crabbea* is sister to the genus *Barleria*, tribe Barlerieae, subfamily Acanthoideae (Scotland *et al.*, 1995; Scotland and Vollesen, 2000; Manktelow *et al*., 2001; McDade *et al*., 2008; Tripp and Fatimah, 2012; Tripp and McDade, 2014). In all these studies either *C. acaulis* or *C. velutina* was included as representative of the genus. A total of eight *Crabbea* DNA sequences are currently available on GENBANK®, where seven are derived from cpDNA and one is an incomplete ITS sequence.

In a phylogenetic study, two main issues concerning Acanthaceae ITS have been identified by previous molecular analyses. Firstly, Acanthaceae is associated with a variety of fungi (McDade *et al*., 2000b). Therefore, when universal ITS primers are used to amplify the region for Acanthaceae, the possibility does exist that fungal DNA can also be amplified. Secondly, the ITS region among numerous Acanthaceae taxa is very CG-rich (McDade *et al*., 2000b). Three and two regions in ITS-1 and ITS-2, respectively, have a poly-C or poly-G rich region (McDade *et al*., 2000b). Gene regions with a CG-rich composition, more specifically at their -5' and -3' primer binding regions, are difficult to amplify even when additional reagents such as dimethyl sulphoxide (DMSO) are added (Sahdev *et al*., 2007). Additionally, CG-rich templates have strong secondary structures that hinder denaturation and primer annealing (Hubé *et al*., 2005). Complications with DNA sequencing in Acanthaceae are described in detail by McDade *et al*. (2000b).

The aim of this chapter is to reconstruct the phylogenetic history of the southern African *Crabbea* species. Initially, the study was planned to include three gene regions, *rps*16, *trn*L-*trn*F and ITS. However, complications were experienced with ITS. Therefore, morphological and anatomical data were also incorporated with molecular data in an attempt to improve resolution of species boundaries and understand evolutionary patterns among the investigated *Crabbea* species.

6.2 Materials and methods

6.2.1 DNA extraction and purification

Fresh leaf material was obtained from specimens collected in the field, dried and preserved in silica gel and/or sampled from herbarium specimens (Table 6.1). DNA was isolated and purified with a modified Cetyltrimethylammonium Bromide (CTAB) Method (Doyle and Doyle, 1987) with additional modifications (McDade *et al*., 2000b). Leaf material was ground to a powder using a Qiagen® TissueLyzer. To each sample, 750 µL of CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-hydroxymethyl aminomethane (Tris-HCl) pH 8.0, 20 mM Ethylenediaminetetraacetic acid (EDTA) pH 0.8, 1.4 M NaCl, Diethyl pyrocarbonate (DEPC) water, 0.2% (v/v) ß-mercapto-ethanol and 3% (w/v) Polyvinylpyrrolidone (PVP)] was added. All the samples were incubated in a water bath at 65°C for 1 hr.

Following incubation, DNA was extracted with 500 µL (v/v) chloroform/isoamylalcohol ($ChCl_3$/IAA) (24:1). The DNA was precipitated from the aqueous phase by centrifugation at 12 000 *g* for 10 min at 4°C. This extraction step was repeated in accordance with McDade *et al*. (2000b). The DNA was precipitated from the aqueous phase with 500 µL isopropanol by incubation at room temperature for 1 hr. The samples were centrifuged at 12 000 *g* for 10 min at 4°C and the supernatant was discarded. The DNA pellets were washed with 500 µL 70% (v/v) EtOH and left overnight at -20°C.

Samples were centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant was discarded and the DNA pellet was air-dried for 30 min at room temperature. The DNA was resuspended in 200 µL TE buffer (10 mM Tris-HCl pH 8.0 and 1mM EDTA) and left for 1 hr at 4°C, after which, 2 µL of RNase (10 mg/mL) was added and samples were incubated for 2 hrs at 37°C. Following incubation, 20 µL of 7.5 M ammonium acetate and 200 µL (v/v) $ChCl_3$/IAA (24:1) was added. Samples were centrifuged at 12 000 *g* for 10 min at 4°C. The DNA was precipitated from the aqueous phase by adding 500 µL ice-cold 100% EtOH and left overnight at 4°C.

Table 6.1 *Crabbea* voucher specimens used for phylogenetic analysis.

Herbarium	Species	Collector name and number	Collection date
BLFU	*C. acaulis*	A. de Gouveia 80	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 121	25-03-2015
BLFU	*C. acaulis*	A. de Gouveia 149	28-03-2015
BLFU	*C. angustifolia*	A. de Gouveia 74	21-03-2015
BLFU	*C. angustifolia*	J.E. Burrows and S.E. Burrows 1438	07-03-2015
BLFU	*C. angustifolia*	H.M. Steyn 2294	05-03-2016
BLFU	*C. cirsioides*	A. de Gouveia 88	22-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 124	25-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 128	26-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 143	28-03-2015
BLFU	*C. cirsioides*	H.M. Steyn 2288	06-03-2016
BLFU	*C. cirsioides* [=*C. nana*]	A. de Gouveia 146	28-03-2015
BLFU	*C. galpinii*	A. de Gouveia 169 C	20-11-2015
BLFU	*C. galpinii*	A. de Gouveia 170	21-11-2015
BLFU	*C. galpinii*	A. de Gouveia 171	21-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 136	27-03-2015
BLFU	*C. ovalifolia*	A. de Gouveia 174	23-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 81	22-03-2015
BLFU	*C. pedunculata*	A. de Gouveia 176	25-11-2015
BLFU	*C. velutina*	A. de Gouveia 110	25-03-2015
BLFU	*C. velutina*	A. de Gouveia 113	25-03-2015
BLFU	*C. velutina*	A. de Gouveia 163	19-11-2015

Samples were centrifuged at 12 000 *g* for 15 min at 4°C and the supernatant discarded. The DNA pellets were washed twice by adding 500 µL ice-cold 70% (v/v) EtOH and centrifuged at 12 000 *g* for 10 min at 4°C. Pellets were air-dried and resuspended in 50 µL TE buffer and stored overnight at 4°C.

The quality and quantity of the purified DNA was examined by separating 5 µL of each sample on a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide (EtBr) in 0.5 x TAE buffer [20 mM Tris-HCl pH 8.0, 0.28% (v/v) acetic acid and 0.5 mM EDTA pH 8.0]. Prior to separation, each DNA sample was mixed with loading buffer [0.015% (w/v) bromophenol blue and 2.5% (w/v) ficoll]. The gel was separated for 40 min at 80 $V.cm^{-1}$.

The DNA was visualised with ultraviolet (UV) light illumination and photographed with the Gel Doc XR™ documentation system (Bio-Rad). DNA concentration was confirmed using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific) and expressed as ng/µL. The DNA samples were diluted accordingly to a final concentration of 20 ng/µL.

6.2.2 Amplification and purification of gene regions

The *Crabbea rps*16 gene region was amplified with primer sets “ACA5’rps16” and “ACA3’rps16” (McDade *et al*., 2005) which are specifically designed for Acanthaceae species and the *trn*L-*trn*F region was amplified with “c” and “f” (Taberlet *et al*., 1991). For the nuclear ITS gene region, initially “ITS-A” and “ITS-B” were used (Blattner, 1999) (Table 6.2).

Optimal amplification parameters for all three gene regions were determined by performing gradient reactions. The regions were amplified using EmeraldAmp® MAX HS polymerase chain reaction (PCR) Master Mix (Takara Biotechnology (Dalian) Co., Ltd.), according to manufacturer’s specifications. Amplification parameters for all three gene regions were set as follows: 20 ng of genomic DNA, 50 pmol of the forward and reverse primers respectively, and 2 x EmeraldAmp® Master Mix. Reaction conditions were set as follows: 30 cycles of 98°C for 10 sec; 62°C (*rps*16), 64°C (*trn*L-*trn*F) and 49°C (ITS) for 30 sec and 72°C for 1 min. Five µL of the PCR product was separated on a 1% (w/v) agarose gel, prepared and separated as described in 6.2.1.

Table 6.2 Nucleotide sequences of primers used in this study.

Gene region	Primer name	Primer sequence	Forward/Reverse	Reference
*rps*16	ACA5'-rps16	5'-GAGGACARRATCCGTTGTGGAT-3'	Forward	McDade *et al*. (2005)
*rps*16	ACA3'-rps16	5'-AGACGGCTCATTGGGATA-3'	Reverse	McDade *et al*. (2005)
*trn*L-*trn*F	C	5'-CGAAATCGGTAGACGCTACG-3'	Forward	Taberlet *et al*. (1991)
*trn*L-*trn*F	F	5'-ATTTGAACTGGTGACACGAG-3'	Reverse	Taberlet *et al*. (1991)
ITS	ITS-A	5'-GGAAGGAGAAGTCGTAACAAGG-3'	Forward	Blattner (1999)
ITS	ITS-B	5'-CTTTTCCTCCGCTTATTGATATG-3'	Reverse	Blattner (1999)
ITS	ITS-C	5'-GCAATTCACACCAAGTATCGC-3'	Reverse	Blattner (1999)
ITS	ITS-D	5'-CTCTCGGCAACGGATATCTCG-3'	Forward	Blattner (1999)
ITS	C26A	5'-GTTTCTTTTCCTCCGCT-3'	Forward	Wen and Zimmer (1996)
ITS	N-nc18S10	5'-AGGAGAAGTCGTAACAAG-3'	Reverse	Wen and Zimmer (1996)
ITS	MJ23	5'-CAAGGAAAACCGAAACGAAG-3'	Forward	EU528885
ITS	MJ24	5'-GAGCAGTTCAACCACCACTG-3'	Reverse	EU528885
Plasmid	17mer	5'-GTTTTCCCAGTCACGAC-3'	Forward	Puc/M13

The remaining PCR product was purified by using the Favorgen® PCR/Gel Purification Kit (Favorgen Biotech Corporation) following the manufacturer's instructions. The quality and quantity of the purified product was verified by separating 5 µL per DNA sample on a 1% (w/v) agarose gel as prepared in 6.2.1.

6.2.3 DNA sequencing

Purified PCR products were sequenced using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer's parameters. DNA fragments were sequenced using the forward primers for each gene region. Each sequencing reaction consisted of 10 ng DNA, 3.2 pmol primer, 1 µL premix and 2 µL buffer. The amplification regime was set as: one cycle of 96°C for 1 min, 25 cycles of 96°C for 1 min, 50°C for 5 sec and 60°C for 4 min.

The sequenced PCR products were purified by adding 5 µL EDTA and two volumes of 100% ethanol to each reaction. The samples were incubated at room temperature for 15 min. The tubes were centrifuged for 15 min at 12 000*g* at 4°C. The supernatant was discarded and washed with 70% (v/v) ethanol and recentrifuged for 5 min at 12 000*g* at 4°C and the pellet dried. The nucleotide composition of each sample was determined by using the Applied Biosystems 3130xl Genetic Analyzer.

6.2.4 Troubleshooting with ITS

ITS amplification proved to be very difficult to optimize from every *Crabbea* specimen. A variety of different enzyme kits, primer combinations, annealing temperatures, different DNA concentrations, extended PCR elongation times, the addition of 5% (v/v) DMSO or 100mM Tetramethyl Ammonium Chloride (TMAC) were tested to optimize the amplification of a single ITS fragment. Initially, the universal ITS-A and ITS-B primers (Blattner, 1999) were used which resulted in multiple fragments. A primer set more specific to Acanthaceae species, namely "C26A" and "N-nc18S10" (Wen and Zimmer, 1996; McDade *et al*., 2000b; McDade *et al*., 2008) (Table 6.2) was used. Multiple or no amplifications were observed. To overcome this barrier, an attempt was made to amplify the ITS-1 and ITS-2 spacer regions separately with the following primer set combinations: "C26A" and "ITS-C" (yielded multiple fragments and/or smears) and "ITS-

D" and "N-nc18S10" (resulted in no amplification) (Wen and Zimmer, 1996; Blattner, 1999) (Table 6.2). Lastly, a *Crabbea*-specific ITS primer set "MJ23" and "MJ24" was developed using Primer 3 input (v0.4.0) (http://www.bioinfo.ut.ee/primer3-0.4.0/primer3/) with the only *Crabbea* ITS sequence available on GENBANK® from *Crabbea acaulis* (EU528885) as template. However, this sequence is only a partial sequence. Multiple fragments were obtained when attempting to amplify the ITS region with MJ23 and MJ24. These fragments were then ligated (pGEM-Teasy®) and transformed to *Escherichia coli* (*E. coli*). The plasmid DNA was extracted and the "17mer" primer (Table 6.2) was used to sequence the cloned fragments. However, no DNA sequences were obtained as none of the sequencing reactions were successful. In a last attempt to obtain ITS sequences from the cloned fragments, the plasmid was sequenced with the forward primer "C26A" (Wen and Zimmer, 1996). Fragments were successfully sequenced with perfect electropherograms but the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated similarity to various fungal DNA.

In the end, the CG-richness of *Crabbea* ITS was regarded as the main problem in the amplification of this region, even with a variety of different enzymes, primer combinations and DNA concentrations. When fragments were cloned and sequences were obtained, the close association of Acanthaceae/*Crabbea* species with various fungal species proved to hinder all our attempts to obtain *Crabbea* ITS. Therefore, due to this reason and time constraints, ITS was excluded from this study.

6.2.5 DNA sequence editing, alignment and outgroups

The identity of each sequence was determined with BLAST. Chromas Lite v.2.01 (Technelysium Pty. Ltd), Mega v.5.2 (Tamura *et al*., 2011) and BioEdit v.7.1.5.0 (Tom Hall, Ibis Biosciences) were used to determine sequence quality and to do sequence editing. MAFFT v.6.956b was used for sequence alignment.

Three outgroups were chosen for the phylogenetic study, varying in phylogenetic distance from *Crabbea* (McDade *et al*., 2008) and all sequences were obtained from GENBANK. The chosen outgroup species were: *Andrographis paniculata* Nees (***rps*16:**

EU5289006; ***trn*L-*trn*F:** EU528914), *Barleria repens* Nees (***rps*16:** EU529011; ***trn*L-*trn*F:** EU528915) and *Thunbergia erecta* (Benth.) T.Anders (***rps*16:** EU529065; ***trn*L-*trn*F:** AF061821).

6.2.6 Phylogenetic analyses

6.2.6.1 Bayesian Inference analysis

A nucleotide substitution model for the Bayesian inference (BI) analysis was determined using the Akaike Information Criterion (AIC) in jModeltest v 2.1.1 (Guindon and Gascuel, 2003; Darriba *et al*., 2012). The model best suited for the combined molecular data set was GTR+G. MrBayes v.3.1.2 was used for BI analysis (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The analysis was initiated from a random tree and employed four Markov Chain Monte Carlo (MCMC) chains. The search was set to 2 000 000 generations, whereby every 500th generation was sampled. To ensure the accuracy of the two runs, the average standard deviation of split frequencies was examined and the analysis was stopped at a value below 0.01. The first 1 000 of the sampled trees were discarded as burnin before analysis.

6.2.6.2 Maximum parsimony analysis

Maximum parsimony (MP) analysis was performed using PAUP* 4.0b10 (Swofford, 2003). A combined tree for *rps*16 and *trn*L-*trn*F gene regions was constructed. Gaps were recorded as missing data and all characters were weighted equally. The analysis was set as follows: a heuristic search was done with 1 000 addition-sequence replicates and branch swapping to set to tree bisection and reconnection (TBR). Only 10 trees were saved per replicate. To obtain clade support values, nonparametric bootstrapping was performed using 1 000 replicates with 100 random addition-sequence replicates and TBR branch swapping.

6.2.6.3 Combined DNA and morphological analysis

Initially, the trees generated for both BI and MP analyses were solely based on molecular chloroplast data. The overall topology yielded a large polytomy, containing all but one *Crabbea* species, *C. velutina* (Figure 6.1). To possibly resolve the species

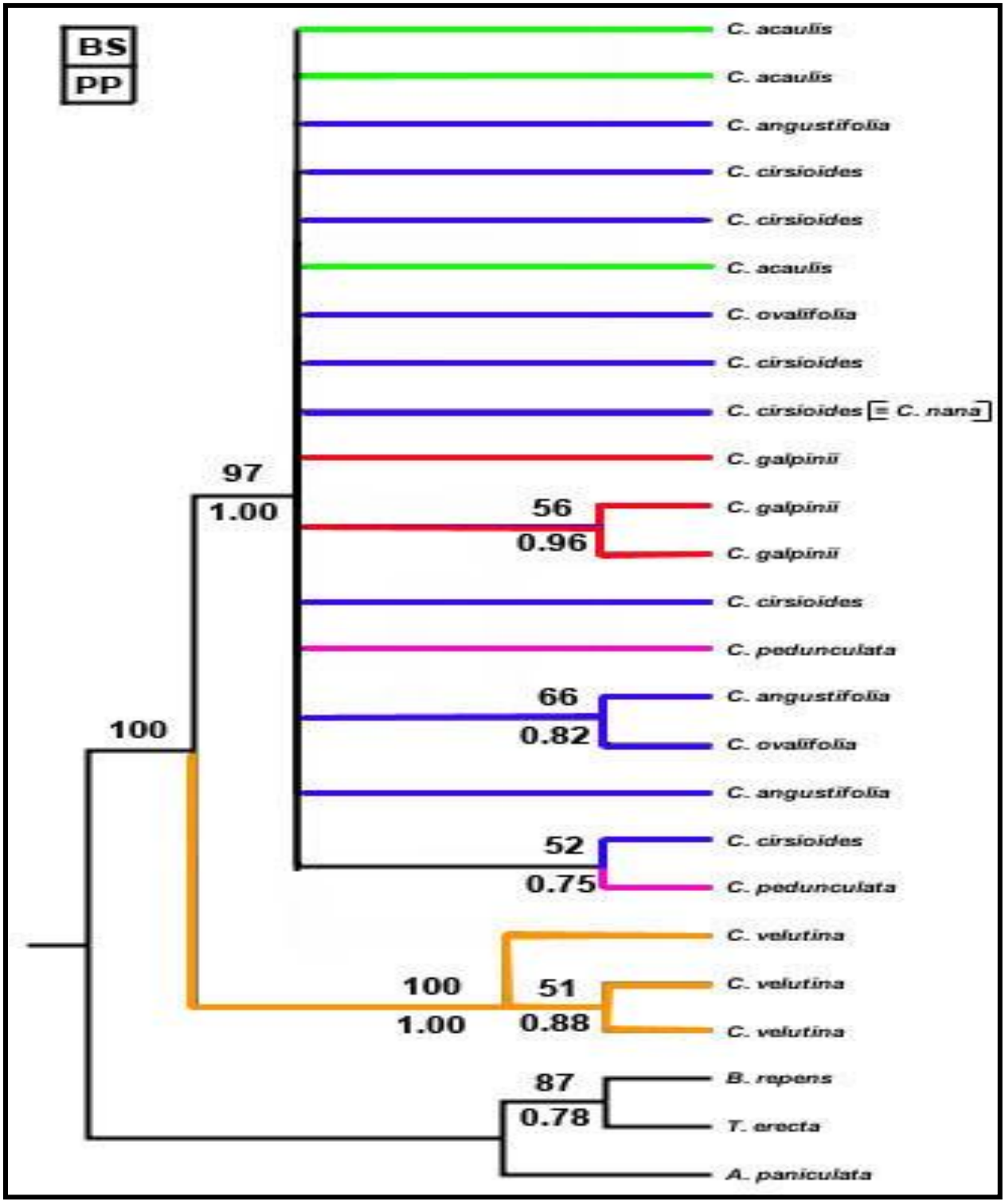

Figure 6.1 Initial phylogenetic tree obtained using only chloroplast (*trn*L-*trn*F and *rps*16) sequences.

relationship better, a morphological matrix was constructed using anatomical, macro- and micromorphological characters and their states (Tables 6.3; 6.4). The combined morphology and molecular data matrix was subjected to the partition homogeneity test (PHT) to establish congruency below the accepted value. Although the PHT score was below the accepted value, many authors (McDade *et al*. 2000a; Kiel *et al*. 2006; McDade *et al*. 2008; 2012) constructed phylogenies combining cpDNA and nrDNA, despite the unfavorable PHT. In the above-mentioned studies, combining the two data sets improved the resolution and the interpretation of the evolutionary relationships among the lineages under investigation. Therefore, in this study, the datasets were also combined.

The choice of morphological and anatomical characters chosen to be used in conjunction with the molecular data set was based on the identification keys provided in sections: 3.5 (leaf micromorphology); 4.5 (cystoliths); 5.5 (pollen micromorphology) and 7.4 (macromorphology). The groupings obtained, in each identification key, were based on character states that remained constant over the sampled geographical range and are easily recognized upon examination. However, it should be emphasized that the chosen characters were only significant for the southern African species of *Crabbea*. To determine the whether the chosen characters and charcter states are significant at species or genus level for *Crabbea*, the entire genus must be sampled, examined and have their leaf micromorphology, cystoliths, pollen micromorphology and macromorphology analyzed.

Both BI and MP analyses for the combined DNA and morphology data set were done as described in both 6.2.6.1 and 6.2.6.2, respectively. The model best suited for the newly combined data set was SYM+G (Guindon and Gascuel, 2003; Darriba *et al*., 2012).

Table 6.3 Southern African *Crabbea* morphological and anatomical characters and character states used in this study.

***Crabbea* species character**	**Character states**			
	0	**1**	**2**	**3**
Habit	Erect	Decumbent/procumbent	Rosette	
Roots	Tuberous	Fibrous		
Stem position	Above ground	Below ground		
Stem texture	Leathery	Herbaceous		
Stem orientation	Vertical	Horizontal		
Leaf shape	Oblanceolate	Lanceolate	Linear	Elliptic-oval
Leaf indumentum	Velutinous	Pilose, hirsute, puberlous	Glabrous	
Leaf base	Strongly attenuate	Attenuate		
Bract indumentum	Strongly hairy	Moderately hairy	Weakly hairy	
Peduncle	Prominent	Inconspicuous		
Corolla tube colour	Creamy-white	White to light pinkish-white		
Glandular trichomes on corolla tube	Absent	Present		
Capsule surface	Not colliculate	Colliculate		
Stomatal distribution on leaves	Hypostomatic	Amphistomatic		
Abaxial leaf stomata	Raised	Not raised		
Presence of cone-shaped trichomes	Both leaf surfaces	Not present on both leaf surfaces		
Average murus height	> 5 µm	< 5 µm	Absent	
Average gemma width	> 2 µm	< 2 µm		
Adaxial leaf cystolith attachment width	< 50 µm	> 50 µm		
Total crystal length on abaxial surface	< 200 µm	> 200 µm		

Table 6.4 The 20-character morphological matrix for the southern African *Crabbea*, with anatomical, macromorphological and micromorphological characters and their character states.

Taxon		Morphological matrix code
Ingroup	*C. acaulis*	20100311111001011011
	C. angustifolia	10001211110001011010
	C. cirsioides	10001111010001011011
	C. cirsioides [= *C. nana*]	10001111110001011011
	C. galpinii	01010221200100112100
	C. ovalifolia	10001311110001011011
	C. pedunculata	01010020201010112100
	C. velutina	00000000000000000000
Outgroup	*A. paniculata*	????????????????????
	B. repens	????????????????????
	T. erecta	????????????????????

6.3 Results

6.3.1 DNA extraction and PCR amplification

The DNA samples extracted from both fresh and herbarium specimens were intact fragments and of very good quality. Both chloroplast genes were easily amplified and the expected sizes were *rps*16 (± 960 bp) and *trn*L-*trn*F (± 470 bp).

6.3.2 DNA sequencing and nucleotide alignments

The cpDNA sequences were of good quality, with electropherograms having mostly single peaks. The sequences for both *rps*16 and *trn*L-*trn*F gene regions BLAST with *Crabbea* or *Barleria* and *Crabbea*, respectively. Nucleotide sequences for all *Crabbea* species were aligned for each gene region accordingly. The combined data matrix consisted of a total of 1242 characters: 1–20 bp (morphology and anatomy); 21–421 bp (*trn*L-*trn*F) and 422–1244 bp (*rps*16).

Both gene regions could be aligned with relative ease. Sequence alignments for both gene regions had gaps but the gaps were primarily created with the inclusion of the three outgroup species. Three DNA sequences, from three different *Crabbea* species, were missing from the DNA matrix: one of *trn*L-*trn*F (*C. acaulis*) and two of *rps*16 (*C. cirsioides* and *C. velutina*). The morphological matrix was complete for all the *Crabbea* species under investigation. However, a morphological matrix could not be constructed for the outgroup species as no herbarium material and/or voucher specimens for these species were available at the time of phylogenetic analysis.

6.3.3 Phylogenetic trees

A most parsimonious tree was constructed for the morphological and molecular combined data matrix. Of the 1242 characters available, 976 characters were parsimony informative and 266 parsimony uninformative. Uninformative characters were excluded. Bootstrap (BS) values above 50% were retained and shown on the tree accordingly. The consistency index (CI) value was 0.951 and retention index (RI) value was 0.936. The BI phylogenetic tree has a similar topology to the MP tree.

Therefore, the MP and BI trees were combined by indicating the BS values above the branch and the posterior probability (PP) values are indicated on the same tree (Figure 6.2).

On the combined morphology and anatomical tree, five major groupings can be observed within southern African *Crabbea. Crabbea velutina* is the first diverging *Crabbea* species in Clade A (BS = 68, PP = 0.88) (Figure 6.2). The remaining groupings are discussed in section 6.4.

6.4 Discussion and Conclusion

Initially, a phylogenetic tree was constructed using only two cpDNA regions (*trn*L-*trn*F and *rps*16). However, the obtained tree had low resolution and did not completely resolve the evolutionary relationships among the seven southern African *Crabbea* species (Figure 6.1). *Crabbea velutina* was first to diverge and the remaining six *Crabbea* species were grouped together in a polytomy (Figure 6.1).

The phylogenetic tree, resulting from combined molecular and morphological datasets, had improved resolution and could better clarify the evolutionary relationships among the seven investigated *Crabbea* species (Figure 6.2).

The combined tree was effectively rooted with all three outgroups and all sampled *Crabbea* species positioned within the ingroup, suggesting *Crabbea* as a monophyletic group (BS = 100; PP = 1.00) (Figure 6.2).

Furthermore, it can be deduced that the *C. velutina* was the first southern African *Crabbea* species to diverge from the larger ingroup and forms a well supported clade distinct from the major *Crabbea* clade (BS = 68; PP = 0.88) (Clade A; Figure 6.2). The remaining southern African *Crabbea* species form part of a larger clade (BS = 57; PP = 0.99) (Clade B; Figure 6.2) in which three notable groupings occurred. *Crabbea galpinii* and *C. pedunculata* form a separate and well-supported clade (BS = 87; PP = 0.88), suggesting that the two species are sister taxa (Clade C; Figure 6.2). The second group

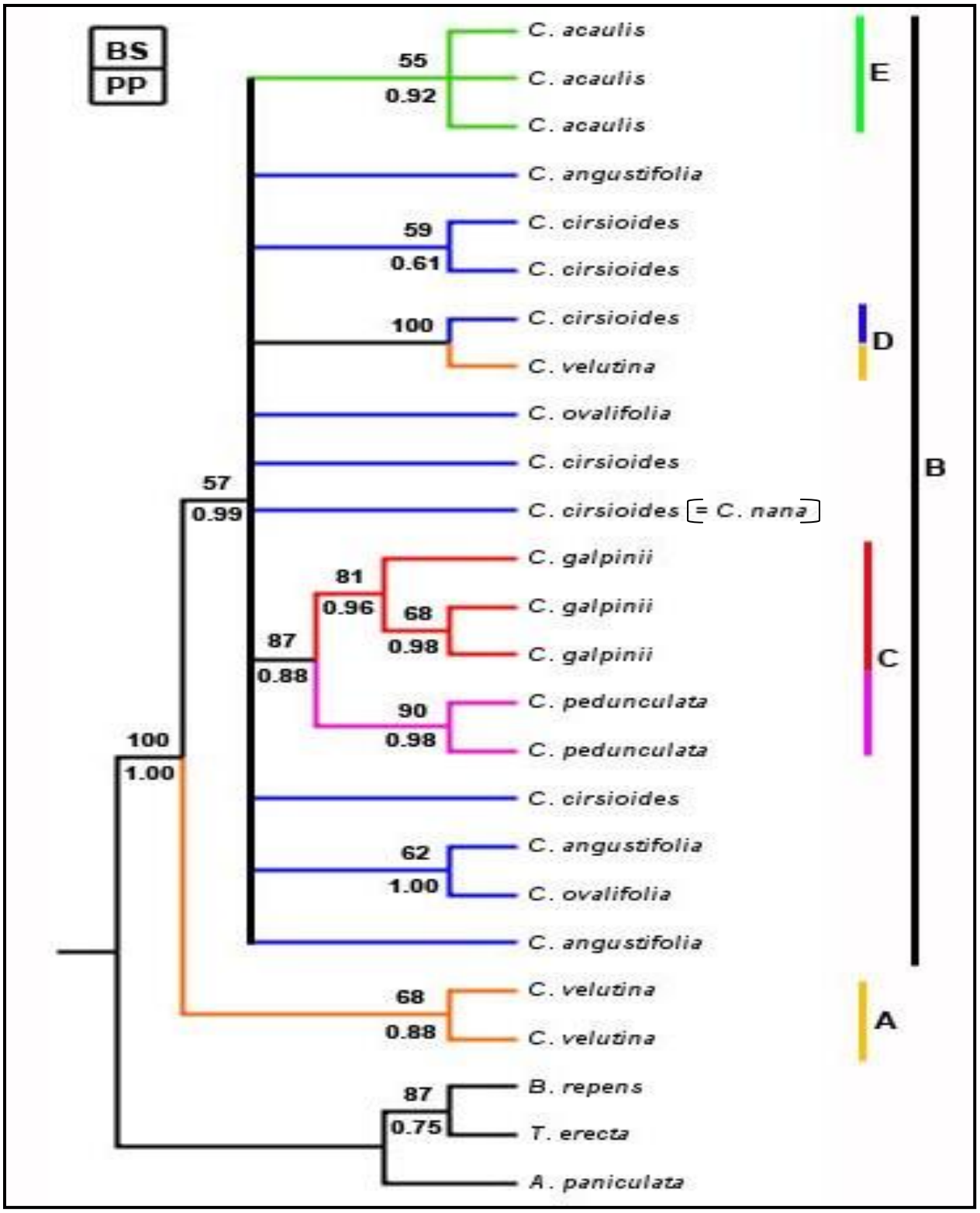

Figure 6.2 The most parsimonious tree generated from the combined molecular and morphological data set. Bootstrap values are indicated above the branches and PP values below.

includes a single specimen of both *C. cirsioides* and *C. velutina* (Clade D; Figure 6.2). A possible reason for the grouping together of these two specimens is the lack of *rps*16, therefore, the close relationship is only based on morphology and *trn*L-*trn*F data. *Crabbea cirsioides* and *C. velutina* have nine out of 20 morphological characters in common, ranging from root appearance (tuberous); stem position, texture and orientation (above ground, vertical and leathery); bract indumentum (strongly hairy); corolla colour (cream–white); absence of stalked, glandular trichomes on the corolla surface; capsule surface (not colliculate); abaxial leaf stomata position (raised above epidermal layer) and average gemmae width (< 2 µm) (Tables 6.3; 6.4). *Crabbea acaulis* was placed in its own distinct group (BS = 55; PP = 0.92) (Clade E; Figure 6.2).

The rate of evolution varies among gene regions and genomes. For example, chloroplast coding gene regions (*mat*K) evolve slower than introns (*trn*L) and intergenic spacers (*trn*L–*trn*F) (Small *et al*., 2004). However, non-coding chloroplast genes (*trn*L and *trn*L–*trn*F) are more useful to study plant relationships at genus and species levels (Gielly and Taberlet, 1994). Non-coding chloroplast regions experience limited selective pressures and, as a result, evolve faster than coding chloroplast gene regions (Wolfe *et al*., 1987) and may allow the delimitation of taxa at lower taxonomic levels (Palmer *et al*., 1988). Despite not being able to obtain ITS DNA sequences for this genus, the combination of the chloroplast data with the morphological data proved to be a good alternative to establish a first but basic phylogeny of the southern African *Crabbea*.

The combined molecular and morphological trees could resolve the phylogeny of the southern African *Crabbea* on genus and species level to some extent. The monophyly of *Crabbea* will be further confirmed once all members within the genus can be included in a molecular phylogeny. It is has been effectively proven that *C. velutina* is the first species to diverge from the larger southern African *Crabbea* group. The lack of ITS sequences does detract significantly from the resolution of the topology obtained. However, all techniques and modifications used in an attempt to optimize and sequence ITS can be used as a reference point for further molecular investigation. The combined phylogeny provides the first insight into the evolutionary history of *Crabbea* in southern Africa.

CHAPTER 7

TAXONOMIC TREATMENT

7.1 Introduction

Classification, description, identification and nomenclature constitute the four main principals of plant taxonomy. These principals allow us to understand and interpret plant biodiversity (Jones and Luchsinger, 1987).

The aim of this chapter is to critically review the taxonomy of *Crabbea* in southern Africa. This will be achieved by providing clear genus and species descriptions; identifying diagnostic characters for the genus and each southern African *Crabbea* species; reviewing the nomenclature and typification.

7.2 Materials and Methods

7.2.1 Taxonomic treatment of the genus and species

Crabbea specimens investigated in this study were sampled only from the southern African region - Botswana, Lesotho, Namibia, South Africa and Swaziland. However, some of the species also have populations north and outside southern Africa such as *C. cirsioides*, *C. ovalifolia* and *C. velutina*. A map of the region is given in Figure 7.1.

Fieldwork took place during the summer season across three South African provinces, namely KwaZulu-Natal, Limpopo and Mpumalanga. Fresh material was collected for all the southern African *Crabbea* species from multiple populations. *Crabbea* specimens collected in the field, were preserved, prepared and mounted on non-acidic mounting paper, following standard herbarium protocols (Victor *et al*., 2004). The prepared voucher specimens are housed at the BLFU Herbarium, Department of PS, UFS. Duplicate *Crabbea* voucher specimens will be donated to the PRE Herbarium upon completion of the study. Herbarium specimens, on loan and scans, from various herbaria across Europe and South Africa were also examined (Table 7.1) (Addendix 1).

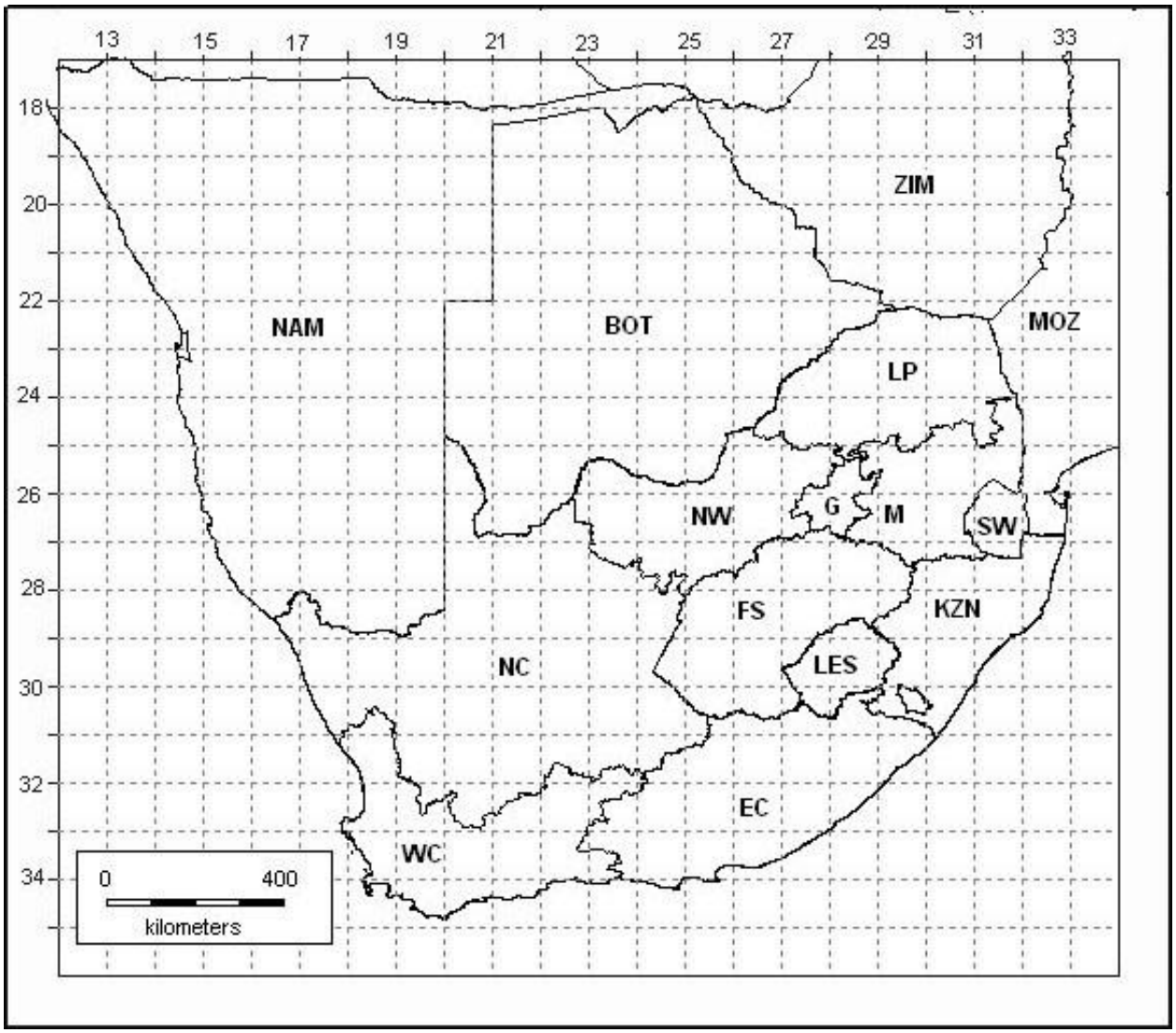

Figure 7.1 Map representing the neighbouring countries of South Africa and the nine provinces of South Africa. Countries neighbouring South Africa, BOT: Botswana; LES: Lesotho; NAM: Namibia; SW: Swaziland; Moz: Mozambique and Zim: Zimbabwe. Provinces of South Africa, EC: Eastern Cape; FS: Free State; G: Gauteng; KZN: KwaZulu-Natal; LP: Limpopo; M: Mpumalanga; NC: Northern Cape; NW: North West; WC: Western Cape.

Table 7.1 List of herbaria providing *Crabbea* specimens on loan or specimen records and scans. Herbarium acronyms are provided by Holmgren *et al*. (1990).

Herbarium	Herbarium name and location
B	Herbarium, Botanischer Garten und Botanisches Museum Berlin-Dahlem, **Berlin, Germany**.
BLFU	Geo Potts Herbarium, Department of Plant Sciences, University of the Free State, **Bloemfontein, South Africa**.
BM	Herbarium, Department of Botany, The Natural History Museum, **London, England**.
BOL	Bolus Herbarium, Botany Department, University of Cape Town, **Cape Town, South Africa**.
BR	Herbarium, Nationale Plantentuin van België, Jardin Botanique National de Belgique, Domein van Bouchout, **Meise, Belgium**.
GRA	Selmar Shonland Herbarium, Albany Museum, **Grahamstown, South Africa.**
GZU	Herbarium, Institut für Botanik, Karl-Franzens-Universität Graz, **Graz, Austria**.
K	Herbarium, Royal Botanic Gardens, Kew, **Richmond, England**.
LISU	Herbarium, Instituto Botânico, Faculdade de Ciências, **Lisbon, Portugal**.
NU	Bews Herbarium, Botany Department, University of Natal, **Pietermartizburg, South Africa**.
PRE	National Herbarium, Botanical Research Institute, **Pretoria, South Africa**.
S	Herbarium, Botany Department, Swedish Museum of Natural History, **Stockholm, Sweden**.
SAM	South African Museum Herbarium, **Cape Town, South Africa**.
TCD	Herbarium, School of Botany, Trinity College, **Dublin, Ireland**.

Herbarium acronyms follow Holmgren *et al*. (1990). Voucher specimens were studied extensively for vegetative and floral characters and character states. Habitat and distribution characteristics were also analysed from the available voucher specimens.

Type material identification and/or confirmation were made possible by referring to the JSTOR Plant Sciences' (2016) electronic data base (www.plants.jstor.org) and Stafleu and Cowan (1976). Original publications containing type material information were accessed and studied on the International Plant Names Index (IPNI) (2015) data base (www.ipni.org). Authors of plant names are according to Brummitt and Powell (1992). Typification and classification of the various southern African *Crabbea* species follow the rules of McNiell *et al*. (2011). Cases where the holotypes were destroyed, as that of the bombing of Berlin (B) during the Second World War, are indicated with the symbol "†".

The Botanical Research and Herbarium Management System (BRAHMS) database was used to capture herbarium specimen data, using BRAHMS v6.50 (http://.herbaria.plants.ox.ac.uk/bol/) (Filer, 2009). Distribution maps were also constructed to determine the distribution range across southern Africa for the genus and for each individual *Crabbea* species using the geographic information system (GIS) software, DIVA v.5.2.0.2 (http://www.diva-gis.org) (Hijmans *et al*., 2005). The majority of studied herbarium specimens did not supply global positioning system (GPS) coordinates, and Google Earth was referred to for georeferencing.

Terminology used to describe the various characters and character states is from Beentje (2010) and leaf terminology follows Ash *et al*. (1999) and Beentje (2010).

7.2.2 Floral, capsule and seed anatomy and morphology

The majority of flowers examined in this study was fresh and collected in the field and were preserved in 3% phosphate-buffered GA. In some cases, flowers were sampled from herbarium specimens and rehydrated in 3% phosphate-buffered GA for 48 hours (Table 7.2). Floral dissections were made under the Nikon SMZ645 LM with a mounted Nikon C-W10X/22 lens, at the Department of PS, UFS.

Table 7.2 Voucher specimens investigated for floral, capsule and seed anatomy and morphology.

Herbarium	Species	Collector name and number	Collection date
BLFU	*C. acaulis*	A. de Gouveia 78	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 80	22-03-2015
BLFU	*C. acaulis*	A. de Gouveia 123	25-03-2015
BLFU	*C. acaulis*	A. de Gouveia 149	28-03-2015
BLFU	*C. acaulis*	A. de Gouveia 180	24-11-2015
BLFU	*C. angustifolia*	A. de Gouveia 75	21-03-2015
BLFU	*C. angustifolia*	J.E. Burrows and S.E. Burrows 1438	07-03-2015
BLFU	*C. angustifolia*	H.M. Steyn 2294	05-03-2016
PRE	*C. angustifolia*	N.P. Barker 535	11-02-1989
PRE	*C. angustifolia*	F.A. Rogers 18718	00-04-1916
BLFU	*C. cirsioides*	A. de Gouveia 87	22-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 88	22-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 124	25-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 128	26-03-2015
BLFU	*C. cirsioides*	A. de Gouveia 132	26-03-2015
BLFU	*C. cirsioides*	P.J. du Preez 823	15-04-1987
BLFU	*C. cirsioides*	G. Potts 2576	00-03-1917
BLFU	*C. galpinii*	A. de Gouveia 169C	20-11-2015
BLFU	*C. galpinii*	A. de Gouveia 170	21-11-2015
BLFU	*C. galpinii*	A. de Gouveia 171	21-11-2015
PRE	*C. galpinii*	R. Pott-Leendertz 5663	00-12-1916
BLFU	*C. nana*	A. de Gouveia 146	28-03-2015
BLFU	*C. nana*	A. de Gouveia 179	27-11-2015
BLFU	*C. ovalifolia*	A. de Gouveia 138	27-03-2015
BLFU	*C. ovalifolia*	A. de Gouveia 173	22-11-2015
PRE	*C. ovalifolia*	J.E. Repton 1220	06-02-1938
PRE	*C. ovalifolia*	N. van Rooyen 2224	27-12-1979
BLFU	*C. pedunculata*	A. de Gouveia 84	22-03-2015
BLFU	*C. pedunculata*	A. de Gouveia 176	25-11-2015
BLFU	*C. pedunculata*	A. de Gouveia 177	26-11-2015
BLFU	*C. velutina*	A. de Gouveia 163	17-11-2015
BLFU	*C. velutina*	A. de Gouveia 164	19-11-2015
PRE	*C. velutina*	H.P. van der Schijf 2306	24-02-1953

Capsule and seed anatomy and morphology were sampled directly from fresh and herbarium specimens. Dissections, measurements and description of characters and character states were made using the light microscope as described above.

Floral, capsule and seed terminology follows Beentje (2010).

7.3 Generic treatment of *Crabbea* in southern Africa

Crabbea Harv., in London Journal of Botany, 1: 26–29 (1842); Nees, 11: 162–163 (1847); Burkill and C.B.Clarke, 5: 118–119 (1899–1900); C.B.Clarke, 5(1): 38–40 (1901); Ross: 324 (1972); Retief and Herman: 220–221 (1997); Welman: 97 (2003); Thulin, 24(5): 501–506 (2007); Vollesen, 8(6): 12–19 (2015).

Type: *Crabbea cirsioides* (Nees) Nees [= *Crabbea hirsuta* Harv.] [Note 1]

Description

Perennial, decumbent or procumbent, occasionally erect, rarely rosulate herbs. **Roots** tuberous, occasionally fibrous, 17–112 mm long. **Stems** above ground, rarely subterranean, horizontally or vertically orientated, multi-stemmed, rarely single stemmed, (7) 11–705 (774) mm long; leathery, occasionally herbaceous, scabrous or glabrous, occasionally puberulous, rarely velutinous; immature stems reddish-brown, occasionally greenish-brown, rarely light green; mature stems reddish-brown, rarely brown or green. **Leaves** evenly distributed along stem, rarely rosette-form, densely packed around stem, adpressed to the ground, decussately opposite; length:width ratio 1:0.09–1:0.60; axils glabrous, occasionally puberulous or slightly puberulous, rarely arachnoid or pilose; petioles light to dark green, occasionally brown, rarely orange-brown, grooved, 2–22 mm long; blades simple, narrowly to broadly lanceolate, narrowly oblanceolate to oblanceolate, elliptic-ovate or elliptic-obovate, occasionally linear, rarely oblong or orbicular, 16–147 (168) x 3–45 mm; texture leathery, occasionally herbaceous, cystoliths conspicuous, puberulous or glabrous, occasionally scabrid, rarely hirsute, dark green adaxially and abaxially; apex broadly acute or obtuse, occasionally narrowly acute; base attenuate; venation pinnate, eucamptodromous, 2–10 secondary veins on either side of primary vein; on the adaxial leaf surface not raised above epidermal surface, green or lime, rarely brown, glabrous or puberulous, rarely slightly puberulous, on the abaxial leaf surface raised above epidermal surface, green or lime, rarely brown or yellow, glabrous or puberulous, rarely pilose or velutinous; margins entire, occasionally crenulate or dentulate, puberulous, occasionally glabrous,

rarely slightly puberulous. **Inflorescences** distributed along stem, rarely at stem apex, cymose, compact; peduncle 1–122 (190) mm long, rarely sessile, dark brown, rarely orange-brown or green, puberulous or glabrous, rarely velutinous, determinate. **Bracts** lanceolate, occasionally ovate, solitary, erect, crowded together, overlapping, 9–45 x 4–19 mm; hirsute, occasionally glabrous to slightly puberulous, rarely hispid, light to dark green; apex narrowly acute, base rounded; veins green or purple. **Flowers** zygomorphic, perfect, bisexual. **Buds** obovoid to tubular, weakly striated, quincuncial, 3–12 mm long, puberulent, rarely with stalked, glandular trichomes, creamy-white, rarely light yellow. **Sepals** pentamerous, simple, ± 1 mm partly fused at base, narrowly lanceolate, 6–15 x 1 mm, light green, pilose, rarely velutinous; apex narrowly acute, rarely colliculate. **Corolla** funnel-shaped, striate, 14–18 x 2–3 mm, creamy-white, occasionally white or light pinkish white, puberulent at upper half of corolla tube or entirely puberulent, occasionally glabrous at base, stalked glandular trichomes rarely covering entire surface; lobes deeply divided, pentamerous, bilabiate, upper lip 2-lobed, lower lip 3-lobed, 4–9 x 2–7 mm, obovate, oblong, convex, slightly puberulent, rarely glabrous; bosses paired, raised, yellow, occasionally golden-yellow, positioned on lower lips of corolla tube mouth, running down length of corolla tube and ending at the point of staminal insertion, puberulent trichomes concentrated below bosses inside corolla mouth, occasionally puberulent trichomes running down the entire length inside corolla tube. **Stamens** 4, didynamous, inserted ± 2–7 mm from corolla tube base; anthers basifixed, introse, bilocular, creamy-white, occasionally white, puberulent, 1–2 mm long; filaments 3–8 mm long, creamy-yellow, occasionally cream, glabrous. **Ovaries** pyriform to tubular, rarely ovoid, 2–4 x 1–2 mm, superior, bilocular, glabrous. **Style** orange or reddish-orange, rarely creamy-green, 2–10 mm long, glabrous, puberulent at base. **Stigma** perpendicular to style, 1–2 x 1–2 mm, ovate with pointed tip, glabrous. **Capsules** tubular with pointed apex, esipitate, 6–11 mm long, golden yellow or light green, polished, smooth, rarely colliculate, bilocular. **Seeds** flattened, ovate or elliptic, occasionally orbicular, 2–3 mm long, orange or light yellow, puberulous to velutinous.

Diagnostic features

In southern Africa, the genera most similar to *Crabbea* are *Barleria* and *Lepidagathis*. *Crabbea* can be distinguished from *Barleria* based on the five calyx lobes and didynamous stamens in t6he former, as opposed to four calyx lobes and non-didynamous stamens in the latter. Distinction between *Crabbea* and *Lepidagthis* is based on the presence of prominent yellow bosses on the lower lip (at least in the southern African *Crabbea* species) and the absence of a rugula from the upper lip in *Crabbea*, whilke yellow bosses are absent from the lower lip of *Lepidathis*, though this genus does have rugula in the upper lip.

Crabbea is also closely allied with a number of Malagasy endemic genera, *Lasiocladus*, *Periblema* DC. (= *Boutonia* DC.), *Pericalypta* Benoist and *Pseudodicliptera* Benoist. It is unlikely that these genera will be confused with *Crabbea* in the field, due to the distinct geographic ranges. However, when specimens from mainland Africa and Madagascar are encountered, the Malagasy genera can be distinguished from most of the southern African *Crabbea* species based on inflorescence morphology. The Malagasy genera all have lax, few flowered cymes, subtended by a few, non-spinescent bracts. By contrast, the most southern African *Crabbea* species have dense heads, surrounded by large, usually spinescent bracts. Of the southern African *Crabbea* species, only *C. galpinii* has lax, few-flowered inflorescences and non-spinescent bracts.

Distribution and ecology

Crabbea is distributed from southern Africa, along the central interior and east coast of Africa up to the Horn of Africa.

In southern Africa, most *Crabbea* species are primarily found in South Africa, where it is found in all provinces, except the Northern and Western Cape. Mpumalanga has the largest *Crabbea* diversity/concentration, with all southern African species occurring in the province (Figure 7.2). The grassland and savannah biomes primarily support *Crabbea* habitat in southern Africa. However, *Crabbea* is also distributed in the Albany Thicket and Indian Costal Belt biomes. Southern African *Crabbea* species can be found in a variety of habitats, ranging from full sun to semi-shade conditions, open plains,

valleys, close to cliff edges and hill slopes. The associated geology is predominantly dolerite and granite. Loamy, sandy and stony soils are associated with the southern African *Crabbea* species. The genus can be found over a broad altitude range, starting at 10 up to 1900 m above sea level. *Crabbea* species are concentrated in small to medium-sized populations within a given area. In many cases, more than one species of *Crabbe*a may be found growing together or in the vicinity of another *Crabbea* species. Flowering occurs in the summer months and initiated by summer rains.

Notes

Note 1:

The name, *Crabbea*, was first published in 1838 by Harvey, and was based on a specimen named *Crabbea pungens* Harv. This species was later moved to *Barleria* and the name *Crabbea sensu* Harvey (1838) should, therefore, be a synonym of *Barleria* - *Crabbea* Harv. (1838) should be rejected. However, in Harvey (1842) a new description and type species are presented under the genus name, *Crabbea*.

No distinct anatomical, morphological or geographic feature was used to name the genus *Crabbea*. Instead, the genus was named after the English poet and nature enthusiast, George Crabbe (1754–1832) (Buys, 1982).

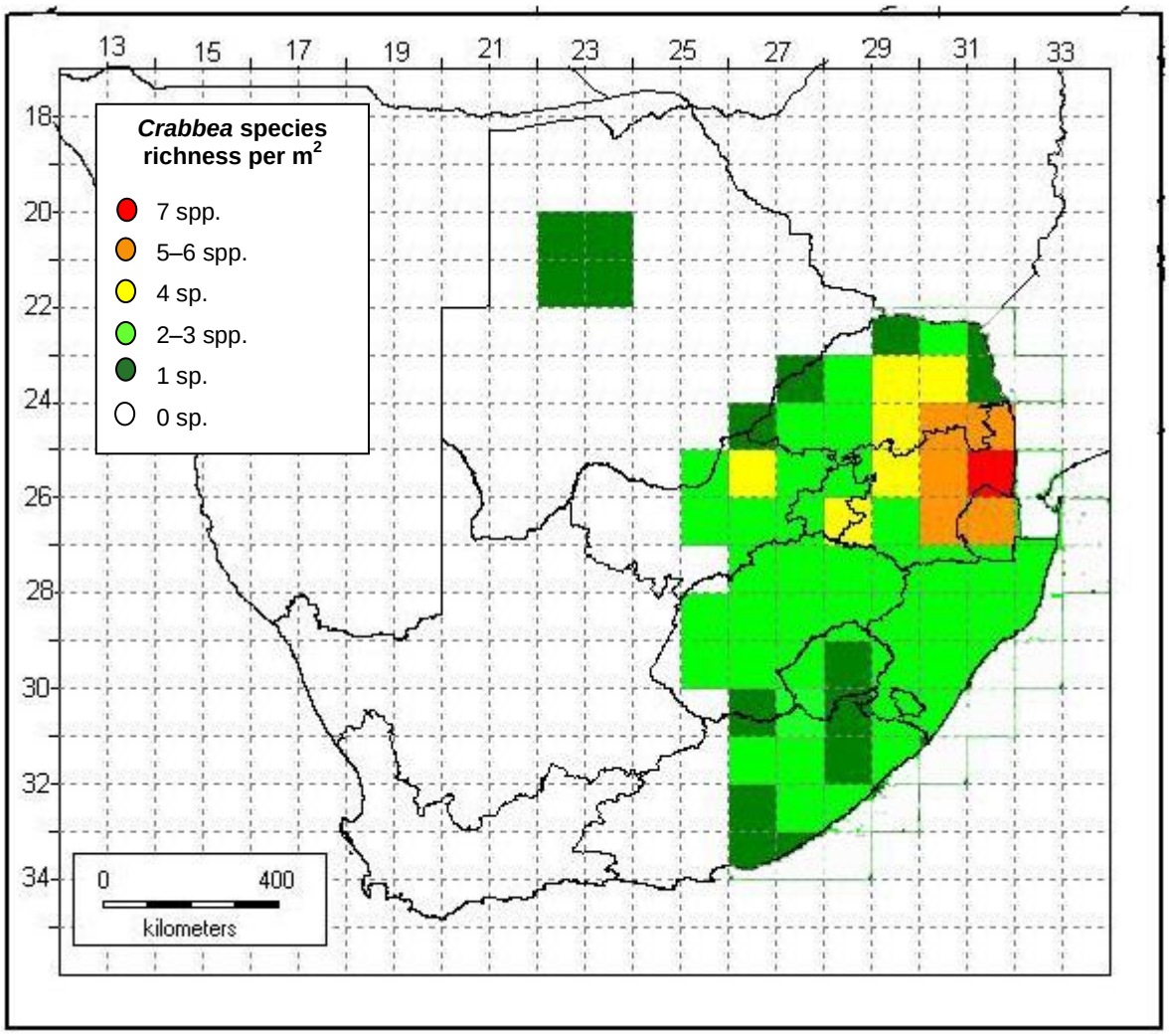

Figure 7.2 Species richness of *Crabbea* in southern Africa.

7.4 Identification key to the southern African *Crabbea* species

1A Roots fibrous; stems herbaceous; leaves herbaceous:

2A Leaves linear, narrowly lanceolate or narrowly oblanceolate; leaf length width ratio 1:0.11; corolla with stalked, glandular trichomes; corolla lobes creamy-white; capsules smooth; seeds orbicular; found in full sun conditions: ***C. galpinii***

2B Leaves oblanceolate, elliptic-ovate or oblong; leaf length width ratio 1:0.31; corolla without stalked, glandular trichomes corolla lobes white to light pinkish-white; capsules colliculate; seeds ovate; found in full sun conditions: ***C. pedunculata***

1B Roots tuberous; stems leathery; leaves leathery:

3A Stem orientation vertical:

4A Plants rosulate, lacking aerial stems; inflorescences at stem apex, sessile, peduncle rarely 1–2 (6) mm long; ovaries pyriform to tubular: ***C. acaulis***

4B Plants not rosulate, aerial, erect or trailing stems; Inflorescences along stem length, peduncle 11–122 (190) mm long; ovaries ovoid: ***C. velutina***

3B Stem orientation horizontal:

5A Leaves linear to narrowly lanceolate; leaf length width ratio less than 1:0.20; leaf apex narrowly acute; leaf margins either undulate or not undulate:

C. angustifolia

5B Leaves lanceolate to broadly lanceolate, oblanceolate, elliptic, elliptic-ovate or elliptic-obovate, rarely oblong; leaf length width ratio more than 1:0.20; leaf apex broadly acute to obtuse; leaves margins always not undulate:

6A Leaves lanceolate to broadly lanceolate or elliptic, occasionally oblanceolate, rarely oblong; leaves strigose, scabrid or hirsute; leaf length width ratio 1:0.32; puberulent trichomes concentrated on upper half of corolla tube, lower half glabrous; bosses yellow; style creamy-green; seeds elliptic: ***C. cirsioides***

6B Leaves elliptic-ovate, elliptic-obovate; leaves puberulous, rarely glabrous; leaf length width ratio 1:0.50; puberulent trichomes across entire corolla tube; bosses golden-yellow; style reddish-orange; seeds ovate: ***C. ovalifolia***

7.5 Species nomenclature, description and ecology

Of the eight species investigated for anatomical and micromorphological diagnostic traits, all but two could also be clearly distinguished based on macromorphology. However, there was no clear discontinuity in the range of morphological characters in specimens of *C. cirsioides* and *C. nana*. This, combined with a review of the type specimens and literature regarding the two species, led to the decision to synonymise *C. nana* with *C. cirsioides*. Consequently, seven species are recognized in southern Africa and their taxonomic treatments are presented here:

7.5.1 ***Crabbea acaulis*** N.E.Br., in Bulletin of Miscellaneous Information, Kew, 10: 436 (1908); Retief and Herman: 220 (1997); Welman: 97 (2003).

Type: South Africa, Jeppestown Ridges, near Johannesburg, 6 000 ft [Gauteng Province], *Gilfillan in Herb. Galpin 6245* (K-scan! holotype [K000394693]).

Description

Rosulate. **Roots** tuberous, 18–89 mm long. **Stems** subterranean, vertically orientated, single-stemmed, 7–51 mm long; leathery, scabrous to puberulous; immature stems reddish-brown; mature stems reddish-brown or brown. **Leaves** rosette-form, densely packed around stem, adpressing the ground surface; length:width ratio 1:0.60; axils glabrous or puberulous; petioles light to dark green, 2–10 mm long; blades elliptic-ovate, occasionally lanceolate or orbicular, 16–80 x 13–49 mm; leathery, puberulous or scabrid, occasionally glabrous adaxially and abaxially; apex broadly acute or obtuse; venation 3–8 secondary veins on either side of primary vein; green or lime, glabrous, occasionally puberulous adaxially, green or lime, glabrous or puberulous abaxially; margins entire, occasionally crenulate, puberulous. **Inflorescences** at stem apex; sessile, peduncle rarely 1–2 (6) mm long, orange-brown, puberulous or glabrous. **Bracts** lanceolate, 9–25 x 4–11 (19) mm, hirsute. **Buds** 8 mm long, puberlent, creamy-white. **Sepals** narrowly lanceolate, 10–15 x 1 mm, colliculate around apex. **Corolla** 14–

16 x 2 mm, white, occasionally light pink, puberlent, trichomes concentrated on upper half of corolla tube, lower half glabrous; lobes 4 x 2–5 mm, weakly puberlent; bosses golden-yellow, puberlent, trichomes concentrated below nectar guides inside corolla mouth. **Stamens** inserted ± 4–5 mm from corolla tube base; anthers white, 2 mm long; filaments 4–5 mm long, creamy-yellow. **Ovaries** pyriform to tubular, 2–4 x 1–2 mm. **Style** orange, 6–8 mm long. **Stigma** 1–2 x 1 mm. **Capsule** 7–9 mm long golden-yellow or light green, smooth. **Seeds** elliptic, 2–3 mm long, light yellow (Figure 7.3.1 A–E).

Diagnostic features

Southern African *Crabbea* species that can be confused with *C. acaulis*: None.

This is the only southern African species of *Crabbea* with a rosette growth form. *Crabbea acaulis* leaves are dark green, leathery and densely packed around the stem, adpressing the ground. The infloresences are positioned at the stem apex and largely sessile. The corolla is white and occasionally light pink.

Ecology, distribution and habitat

Crabbea acaulis has the second largest distribution range in South Africa, around the central interior and eastern South Africa (Figure 7.3.2). This species is commonly associated and/or found growing near and/or among *C. cirsioides*, which is the most widely distributed species of *Crabbea* in South Africa. This species grows at altitudes ranging from 1100–1900 m under full sun conditions. Moisture availability does not determine the habitat of this species; thus, *C. acaulis* can be found close to water bodies such as rivers or in areas where moisture availability is limited. This species can be found in open plains, valleys, along mountain and hill slopes as well as near cliff edges growing in dark stony soils. Dry highveld and mesic highveld grassland bioregions support *C. acaulis.* populations (Figure 7.3.1 F). *Crabbea acaulis* is associated with dolomite and granite. Flowering occurs mostly during late summer from January up to April.

Figure 7.3.1 Photographs of *C. acaulis* **in:** (A) tuberous roots; (B) rosette herb; leaves elliptic-obovate, adpressing the ground; (C) bracts lanceolate and hirsute; (D; E) white, funnel-shaped corolla tubes with paired, yellow bosses; (F) habitat, growing on hill slopes in full sun conditions. **Photos:** Dr. L. Joubert.

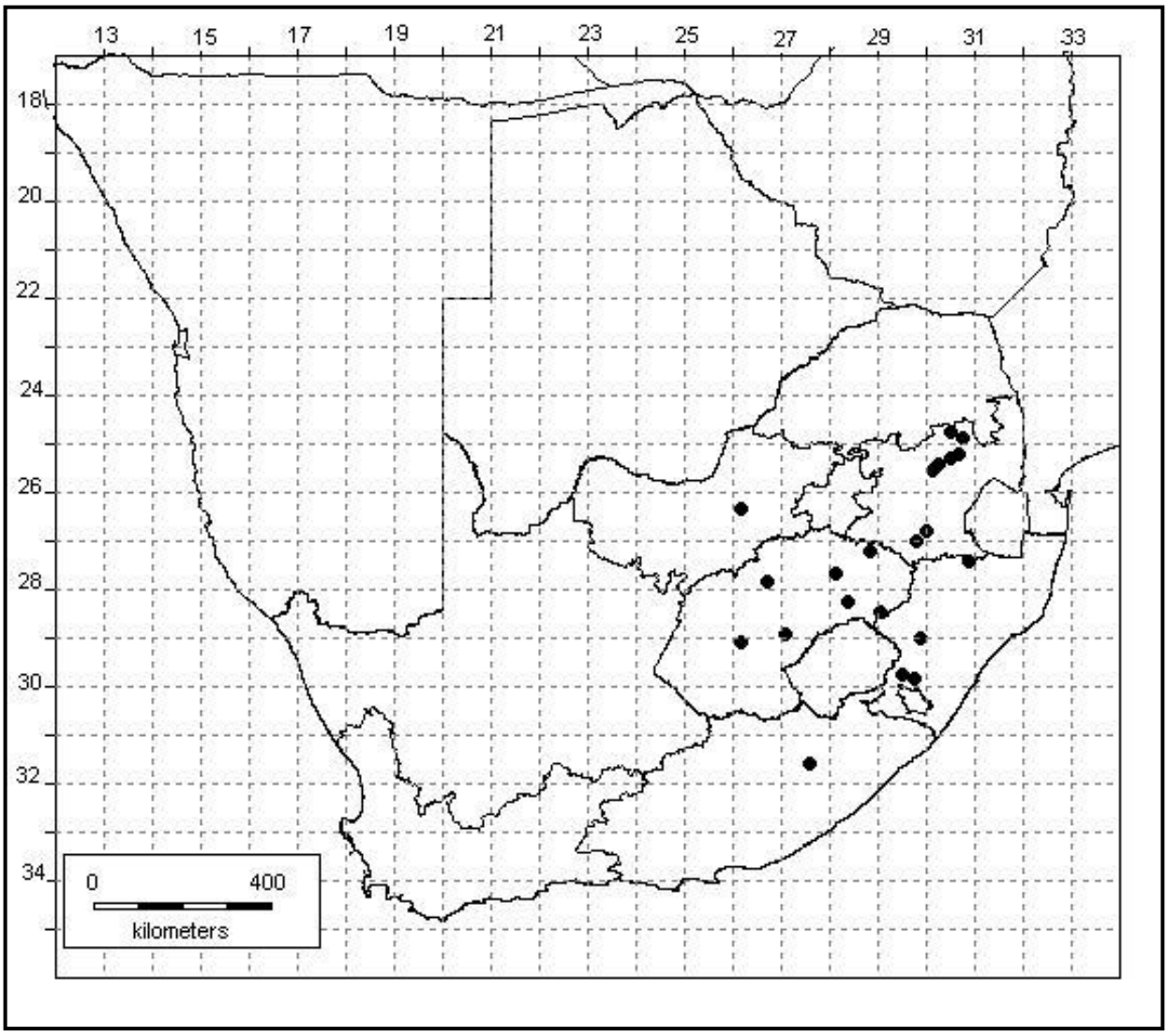

Figure 7.3.2 Known distribution range of *C. acaulis* in southern Africa.

Vernacular name(s) and ethnobotanical use(s)

Sotho: Letswejane, Mereko, Morotowapoho (Moffett, 2010).

Crabbea acaulis is applied in a magical sense and allegedly used to accumulate wealth and prosperity (Arnold *et al*., 2002).

Representative specimens

—25°18’20” S, 30°29’59” E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Kransview Road, 24 November 2015, *de Gouveia*, *A*. *180* (BLFU).

—25°25'33" S, 30°15'32" E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, past rock pool and opposite chalet nr. 2, on foothill of mountain slope, near fence, 22 March 2015, *de Gouveia*, *A*. *77* (BLFU); *de Gouveia*, *A*. *78* (BLFU); *de Gouveia*, *A*. *79* (BLFU); *de Gouveia*, *A*. *80* (BLFU).

—26°46'33" S, 29°59'31" E: Mpumalanga, Amersfoort, N11, turn off into Familiehoek Road, between Ermelo and Amersfoort, 28 March 2015, *de Gouveia*, *A*. *149* (BLFU);

—24°52'53" S, 30°45'37" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *121* (BLFU); *de Gouveia*, *A*. *122* (BLFU); *de Gouveia*, *A*. *123* (BLFU).

7.5.2 ***Crabbea angustifolia*** Nees, in Prodromus Systematis Naturalis Regni Vegetabilis, 11: 163 (1847); Harv. 1: 40 (1859), T.Anderson, 7: 32 (1864); C.B.Clarke, 5(1): 39 (1901); Ross: 324 (1972); Retief and Herman: 220 (1997); Welman: 97 (2003).

Type: South Africa, Magaliesberg [North West Province], *Burke, J. 405* (K-scan! holotype [K000394685]; PRE-scan! isotype [PRE0126663-0]). Note 1, Note 2

= *C. undulatifolia* Engl. in Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 10: 263 (1889).

Type: Betschuanaland [Botswana], Grootfontein, *Marloth, H.W.R. 1079* (B†, holotype); Betschuanaland [Botswana], near Mafikeng [North West Province], *Bolus, F. 6413* (BOL-scan! neotype designated here [BOL138566]). Note 3

Description

Decumbent or procumbent. **Roots** tuberous, (17) 37–75 mm long. **Stems** above ground, horizontally orientated, multi-stemmed (25) 43–690 (705) mm long; leathery, scabrous to puberulous, rarely glabrous; immature stems reddish-brown; mature stems reddish-brown. **Leaves** evenly distributed along stem; length:width ratio 1:0.09; axils glabrous or puberulous; petiole light to dark green, 2–11 mm; blades linear or narrowly lanceolate, 16–35 x 4–11 mm; apices narrowly acute; leathery, puberulous, occasionally glabrous adaxially and abaxially; venation 2–7 secondary veins on either side of primary vein, green or lime, glabrous or slightly puberulous adaxially, green or lime, glabrous or puberulous abaxially; margins entire, denticulate, crenulate, occasionally undulate, puberulous, occasionally glabrous. **Inflorescences** along stem length; peduncle 2–11 mm long, dark brown, puberulous or glabrous. **Bracts** lanceolate, occasionally ovate, 12–35 x 3–10 mm, hirsute. **Buds** 6–9 mm long, puberulent, creamy-white. **Sepals** 6–14 x 1 mm, pilose to velutinous. **Corolla** 14–15 x 2 mm, creamy-white, puberulent, trichomes concentrated on upper half of corolla tube, lower half glabrous; lobes 5 x 2–5 mm, weakly puberlent; bosses yellow, puberulent trichomes concentrated below bosses inside corolla mouth. **Stamens** inserted ± 4 mm from corolla tube base; anthers

creamy-white, 2 mm long; filaments 4–8 mm long, creamy-yellow. **Ovaries** pyriform-tubular, 3 x 1 mm. **Style** orange-red, 7–8 mm long. **Stigma** 1 x 1 mm. **Capsule** 9–11 mm long, golden-yellow or light green, smooth. **Seeds** orbicular, 2–3 mm long, light yellow, puberulous (Figure 7.3.3 A–E).

Diagnostic features

Southern African *Crabbea* species similar to *C. angustifolia*: *C. cirsioides* and *C. ovalifolia.*

Crabbea angustifolia is one of three decumbent and/or procumbent *Crabbea* species of southern Africa. The leaf blades are linear to narrowly lanceolate with narrowly acute apices. The leave length:width ratio is 1:0.09 - significantly smaller than *C. cirsioides* (1:0.32) and *C. ovalifolia* (1:0.50). Seed shape is orbicular.

Crabbea cirsioides has lanceolate to broadly lanceolate or elliptic, occasionally oblanceolate, rarely oblong leaves with broadly acute to obtuse leaf apices. The seeds are elliptic.

Crabbea ovalifolia has elliptic-ovate, elliptic-obovate leaves with leaf apices being broadly acute to obtuse. The seeds are ovate.

Ecology, distribution and habitat

Crabbea angustifolia is distributed mainly in the central interior of South Africa: Free State, Gauteng, southern Limpopo, North West, Mpumalanga and Swaziland, around an altitudinal range of 600–1700 m (Figure 7.3.4). Moisture availability is seasonal in *C. angustifolia* habitats, occurring mostly in summer. *Crabbea angustifolia* can be found in the open bushveld or dry to mesic highveld grassland on hills and ridges, usually associated with dark, red, loamy, stony and granitic soil or dolerite (Figure 7.3.3 F). The peak flowering season occurs from January to April; however, flowering may occur as early as November.

Figure 7.3.3 Photographs of *C. angustifolia* **in:** (A) roots tuberous; (B; C) procumbent, decumbent herb; (D) bracts lanceolate and hirsute; (E) leaves linear to narrowly lanceolate with a narrowly acute apex and puberlent leaf margins; (F) habitat, growing in open plains in full sun conditions. **Photos:** Dr. L. Joubert.

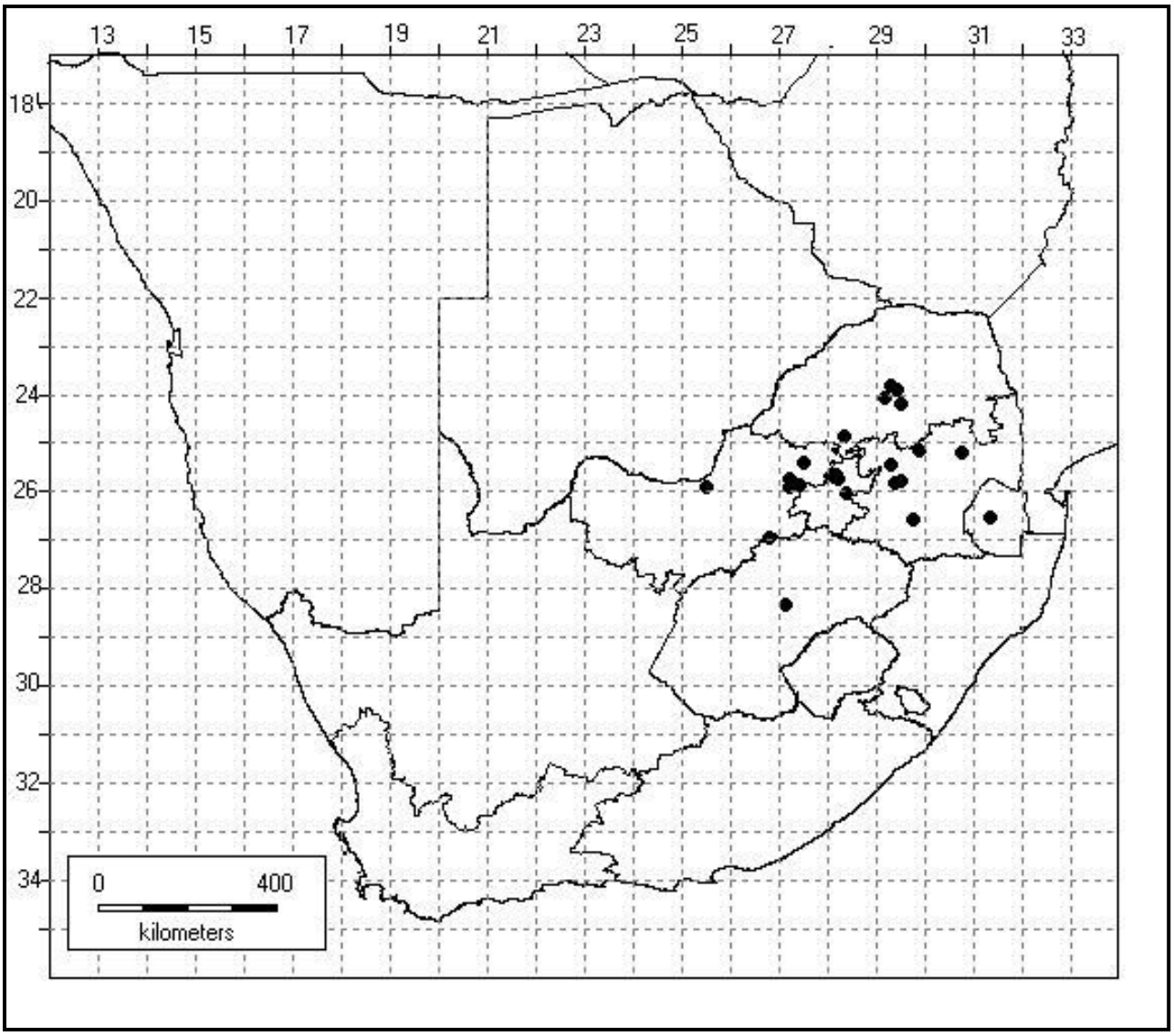

Figure 7.3.4 Known distribution of *C. angustifolia* in southern Africa.

Vernacular name(s) and ethnobotanical use(s)

English: Prickle head, Sheep's Tail (Kirby, 2013).

Tswana: Thôsanathunya (Kirby, 2013).

Crabbea angustifolia is regarded as having magical and/or medicinal properties; used for protection against lightning; used in treating boils and poultice (Arnold *et al*., 2002; Kirby, 2013).

Notes

Note 1:

Nees von Esenbeck (1847) incorrectly reported the collector's name as William J. Burchell (405) but indicated that the specimen forms part of Hooker's Herbarium. Hooker's collection is housed at Kew Herbarium, where Hooker worked. The specimen identified in Hooker's Herbarium is a specimen collected by Joseph Burke with a collector's number, 405, corresponding to that reported in the type description.

Note 2:

Vollesen (2015) classified *C. angustifolia* as a synonym of *C. cirsioides*. The synonomy of *C. angustifolia* under *C. cirsioides* is supported by the absence of a prominent peduncle: "inflorescences subsessile or with peduncle to 0.5(1) cm long."

Note 3:

The holotype for *C. undulatifolia* has been destroyed during the Berlin Bombings of World War 2 (Vollesen, 2015).

Representative specimens

—25°26'28" S, 29°18'48" E: Mpumalanga, Groblersdal, Loskop Dam Nature Reserve, *Steyn, H.M. 2294* (BLFU).

—25°36'56" S, 29°18'39" E: North West, Brits, "Beestekraal" Game Reserve, 11 February 1989, *Barker, N.P. 535* (PRE [PRE0732028-0]).

—25°39'48" S, 27°11'17" E: North West, Rustenburg, April 1916, *Rogers, F.A.* 18718 (PRE [PRE0125146-0]).

—25°47'53" S, 29°22'44" E: Mpumalanga, Middleburg, R555, turn off to Olifants River Lodge, ± 2 km from turnoff, 21 March 2015, *de Gouveia, A. 74* (BLFU); *de Gouveia, A. 75* (BLFU).

—25°50’42” S, 27°24’47” E: North West, Rustenburg, Hamerkop Private Nature Reserve, *Burrows, J.E.* and *Burrows, S.E. 14638* (BLFU).

7.5.3 ***Crabbea cirsioides*** (Nees) Nees, in Prodromus Systematis Naturalis Regni Vegetabilis, 11: 163 (1847); Vollesen, 8(6): 17 (2015). [Note 1] **Basionym:** ***Ruellia cirsioides*** Nees, Acanthaceae, in Linnaea. Ein Journal für die Botanik in ihrem ganzen Umfange, 15: 354 (1841).

Type: South Africa, Fort Beaufort to the Kat River, foot of Chumi Mountains [Eastern Cape], *Ecklon, C.F. s.n.* (GZU-scan! holotype [GZU000249781]).

= ***Crabbea hirsuta*** Harv., in London Journal of Botany 1: 27 (1842); Nees, 11: 163 (1847); T.Anderson, 7: 32 (1864); Burkill and C.B.Clarke, 5: 119 (1899–1900); C.B.Clarke, 5(1): 39 (1901); E.Phillips, 16(1–3): 233 (1917–1933); Ross: 324 (1972); Retief and Herman: 220 (1997); Welman: 97 (2003).

Type: South Africa, Natal [KwaZulu-Natal], *Williamson, J. s.n.* (K-scan! holotype [K000394688]; K-scan! isotype [K000394690]).

= ***C. nana*** (Nees) Nees, in Prodromus Systematis Naturalis Regni Vegetabilis, 11: 162–163 (1847); T.Anderson, 7: 32 (1864); Burkill and C.B.Clarke, 5: 118 (1899–1900); C.B.Clarke, 5(1): 38 (1901); H.Durand and T.Durand: 426 (1909); de Wild.: 199 (1921); Ross: 324 (1972); Retief and Herman: 220 (1997); Welman: 97 (2003); Vollesen, 8(6): 16 (2015). **Basionym:** ***Ruellia nana*** Nees, Acanthaceae, in Linnaea. Ein Journal für die Botanik in ihrem ganzen Umfange, 15: 355 (1841).

Type: South Africa, Tambukiland, rising banks of the Zwarte-Key River [Eastern Cape], 3 000–5 000 ft, *Ecklon, C.F. s.n.* (GZU-scan! lectotype designated by Vollesen (2015) [GZU000261868]; GZU-scan! syntype designated here [GZU000249783]). [Note 2]

= ***C. robusta*** N.E.Br., in C.B.Clarke, Flora Capensis 5(1): 39 (1901); Ross: 324 (1972).

Type: Swaziland, Horo Concession, 2 000 ft, *Galpin, E.E. 1265* (K-scan! holotype [K000394692]; PRE-scan! isotype [PRE0125220-0]). [Note 3]

Description

Decumbent or procumbent. **Roots** tuberous, 26–88 mm long. **Stems** above ground, horizontally orientated, multi-stemmed, (12), 23–368 (400) mm long; leathery, scabrous to puberulous; immature stems reddish-brown; mature stems reddish-brown. **Leaves** evenly distributed along stem; length:width ratio 1:0.32; axils glabrous or pilose; petiole light to dark green or brown, 2–17 mm long; blades lanceolate to broadly lanceolate, elliptic, occasionally oblanceolate or oblong, 29–123 (143) x 10–45 mm; leathery, strigose, scabrid or hirsute adaxially and abaxially; apex broadly acute or obtuse; venation 2–10 secondary veins on either side of primary vein, green, lime or brown, glabrous or puberulous adaxially, green, lime or brown, glabrous or puberulous abaxially; margins entire or weakly crenulate, occasionally denticulate, puberulous. **Inflorescences** along stem length, peduncle 2–14 mm long, dark brown, puberulous or glabrous. **Bracts** lanceolate, rarely ovate, 16–45 x 5–12 (17) mm, hirsute. **Buds** 3–8 mm long, puberulent, creamy-white. **Sepals** 9–15 x 1 mm, pilose, rarely colliculate at apex. **Corolla** 14–18 x 2 mm, creamy-white, puberulent trichomes concentrated on upper half of corolla tube, lower half glabrous; lobes 5–9 x 2–6 mm, weakly puberulent; bosses yellow, puberulent trichomes concentrated below bosses inside corolla mouth. **Stamens** inserted ± 4–6 mm from corolla base; anthers creamy-white, 1–2 mm long; filaments 3–5 mm long, creamy-yellow. **Ovaries** pyriform-tubular, 2–3 x 2 mm. **Style** creamy-green, (2) 7–8 mm long. **Stigma** 1 x 1–2 mm. **Capsule** 9–11 mm long, golden-yellow, smooth. **Seeds** elliptic, 2–3 mm long, light yellow (Figure 7.3.5 A–E).

Diagnostic features

Southern African *Crabbea* species similar to *C. cirsioides*: *C. angustifolia* and *C. ovalifolia*.

Crabbea cirsioides, along with *C. angustifolia* and *C. ovalifolia*, are decumbent and/ or procumbent *Crabbea* species. In *C.* cirsioides, leaves are lanceolate to broadly lanceolate or elliptic, occasionally oblanceolate, rarely oblong. In terms of size, *C. cirsioides* has the largest leaves of the three decumbent/procumbent species: 29–123 (143) x 10–45 mm, with a leaf length:width ratio of 1:0.32. The seeds are elliptic.

Figure 7.3.5 Photographs of *C. cirsioides* **in:** (A) roots tuberous; (B–C) decumbent or procumbent herb with lanceolate to broadly lanceolate leaves; (D–E) corolla lobes funnel-shaped, creamy-white, with raised, paired yellow bosses; (E) bracts hirsute with narrowly acute apices; (F) habitat, growing in open plains, near cliff edges between rocky outgroups under full sun conditions. **Photos:** Dr. L. Joubert.

Crabbea angustifolia has linear to narrowly lanceolate leaves with a leaf length:width ratio of 1:0.09. Leaf dimensions for *C. angustifolia* measures at 16–35 x 4–11 mm. Seeds are orbicular.

Crabbea ovalifolia is distinguished from *C. cirsioides* by having elliptic-obovate or elliptic-ovate shaped leaves with a length:width ratio of 1:0.50. Leaf blade dimensions for *C. ovalifolia* are (19) 30–97 x 5–38 mm. Seeds are ovate.

Ecology, distribution and habitat

Of all southern African *Crabbea* species, *C. cirsioides* has the broadest distribution range in South Africa, found in all provinces, except the Northern and Western Cape (Figure 7.3.6). The altitudinal range varies between 80–1700 m above sea level. The broad distribution range of *C. cirsioides* allows this species to occupy a variety of biomes and habitats. *Crabbea cirsioides* can be found in the grassland, savannah, Albany Thicket and Indian Coastal Belt Biomes. This species grows under semi-shade to full sun conditions in open and closed woodlands, open and dry grasslands, bushlands, close to wetlands, open plains, valley, hill slopes and near cliff edges. Moisture availability is not a determining factor of *C. cirsioides*, thus, *C. cirsioides* can be associated with or without water bodies. Geology associated with *C. cirsioides* includes granite, dolerite, harzburgite and pyroxenite. Sandy, loamy, rocky and stony soils support *C. cirsioides* habitat (Figure 7.3.5 F). *Crabbea acaulis* is most commonly associated with *C. cirsioides*, followed by *C. pedunculata.* Flowering season starts at September and peaking from January to March and ends around April–May.

Vernacular name(s) and ethnobotanical use(s)

Sotho: Letswejana “a small breast”; Mereko; Morotoapoho (Phillips, 1917–1933; Moffett, 2010)

Xhosa: Manzasana (Pooley, 2005)

Zulu: Ihlasi; Umusa (Pooley, 2005)

African: Ubu-Hlungu bedila; Ubu-Hlungu besiyeawu (Smith, 1895)

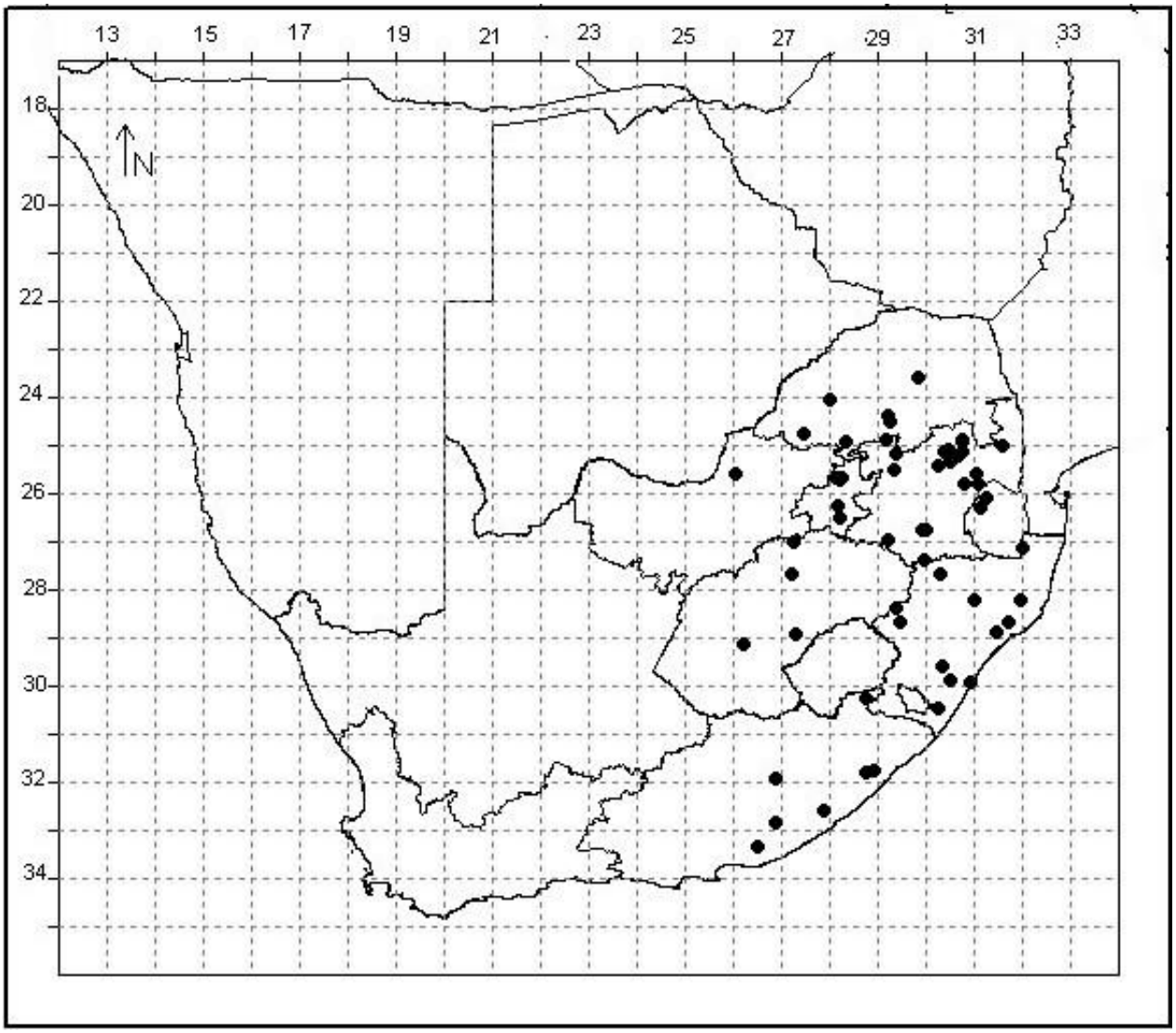

Figure 7.3.6 Known distribution of *C. cirsioides* in southern Africa.

Crabbea hirsuta [= *C. cirsioides*] is used to treat growths and swellings such as swollen glands and potentially cancerous lumps (Hutchings, 1989). This species is also used to treat snake and spider bites, blood poisoning from diseased meat as well as tooth aches. The entire plant is formed into a paste and applied externally to the infected spot. However, if paste is applied internally, a small amount would be used; thus, implying that consumption in large quantities may be lethal (Smith, 1895). The Pedi use burnt and the powdered root of *C. hirsuta* [= *C. cirsioides*] to rub over a hydrocephalic child (Watt and Breyer-Brandwijk, 1962). The Basotho use *C. hirsuta* [= *C. cirsioides*] to reduce pain among babies (Moffett, 2010). Additionally, *C. hirsuta* [= *C. cirsioides*] is used as muti by the Sotho and Zulu groups (Watt and Breyer-Brandwijk, 1962).

Crabbea nana [= *C. cirsioides*] is used treat growths and swellings, tooth ache, sore gums and snake bites. The species is also believed to bring fortune and good luck (Hutchings, 1989).

Notes

Note 1:

Vollesen (2015) grouped a number of southern African *Crabbea* species under *C. cirsioides*, namely *C. angustifolia, C. hirsuta*, *C. ovalifolia, C. robusta* and *C. undulatifolia.* Peduncle length was used to group the five mentioned taxa under *C. cirsioides*. In the identification key provided by Vollesen (2015), *C. cirsioides* is distinguished from *C. nana* using the following lead: “inflorescence subsessile or with peduncle to 0.5(1) cm long.” *Crabbea nana* is keyed out from *C. cirsioides* using the following guide: “inflorescence with peduncle 1–5(15) cm long.”

Buys (1982) regarded *C. cirsioides* as a synonym of *C. hirsuta*. This classification is also observed by Welman (2003).

Note 2:

Buys (1982) used the wrong specimen as the holotype for *R. nana* (= *C. nana*) from Kew Herbarium (K000394680) using a specimen collected by Carl L.P. Zeyher and not

C.F. Ecklon. However, Buys (1982) includes in the *C. nana* protologue that the chosen type specimen for *C. nana* most likely originates from Ecklon's original collection.

Prior to this study, *C. nana* was regarded as an accepted southern African *Crabbea* species. However, comparison of the type literature by Nees von Esenbeck (1841) to that of Harvey (1842) reports similar species descriptions. Additionally, type material of *C. cirsioides* [= *R. cirsioides*] (GZU000249781) and *C. nana* [= *R. nana*] (GZU000261868). The *C. nana* voucher specimens examined in this study could not be distinguished from *C. cirsioides*. Therefore, *C. nana* should become a synonym of *C. cirsioides*.

Note 3:

Welman (2003) reports that the classification of *C. robusta* under *C. hirsuta* is according to Buys (1982), Vollesen (2015) correctly classifies *C. robusta* and *C. hirsuta* as synonyms of *C. cirsioides.*

Representative specimens

—25°13'07" S, 30°40'29" E: Mpumalanga, Sabie, unnamed dirt road, south of Sabie, 26 March 2015, de *Gouveia*, *A*. *132* (BLFU).

—25°07'04" S, 30°45'51" E: Mpumalanga, Sabie, R37 to Mashinging, opposite pine plantations, about 5 km south of Sabie, 26 March 2015, *de Gouveia*, *A*. *128* (BLFU).

—24°52'32" S, 30°45'34" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *124* (BLFU).

—25°25'31" S, 30°15'26" E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, Welgedacht Farm, before rock dam, next to path near car park and chalet nr. 2, 22 March 2015, *de Gouveia*, *A*. *87* (BLFU); *de Gouveia*, *A*. *88* (BLFU).

—26°44'51" S, 30°00'10" E: Mpumalanga, Ermelo, Familiehoek Road between Ermelo and Uitspanning, next to dirt road and fence, about 20 m from Vaal River Bridge, 28 March 2015, *de Gouveia*, *A*. *146* (BLFU); *de Gouveia*, *A*. *179* (BLFU).

—28°55’24’’ S, 27°17’17’’ E: Free State, Excelsior, Korannaberg, 15 April 1987, *du Preez, P.J. 823* (BLFU).

—29°06’37’’ S, 26°13’27’’ E: Free State, Bloemfontein, top of Naval Hill, 00 March 1917, *Potts, G*. *2576* (BLFU).

7.5.4 ***Crabbea galpinii*** C.B.Clarke, in Flora Capensis 5(1): 40 (1901); Retief and Herman: 220 (1997); Welman: 97 (2003).

Type: South Africa, Barberton [Mpumalanga], *Galpin, E.E. 1148* (K-scan! holotype [K000394679]; PRE-scan! isotype [PRE0125191-0]). Note 1

Description

Erect. **Roots** fibrous. **Stems** above ground, vertically orientated, multi-stemmed (17) 31–149 (774) mm long; herbaceous, glabrous; immature stems reddish-brown; mature stems reddish-brown. **Leaves** evenly distributed along stem; length:width ratio 1:0.11; axils glabrous or weakly puberulous; petioles light to dark green or brown, 2–6 mm long; blades linear, occasionally narrowly lanceolate or narrowly oblanceolate, (26) 42–78 x 3–10 mm; herbaceous, glabrous adaxially and abaxially; apex narrowly acute; venation 2–6 secondary veins on either side of primary vein, green or lime, glabrous adaxially, green or lime, glabrous abaxially; margins entire, glabrous. **Inflorescences** along stem length; peduncle 4–65 mm long, green, weakly puberulous. **Bracts** lanceolate, 9–27 x 4–11 mm; glabrous to weakly puberulous. **Buds** 5–11 mm long; covered in stalked glandular trichomes, light yellow. **Sepals** narrowly lanceolate, 7–11 x 1 mm, pilose. **Corolla** 14 x 2 mm, creamy-white, stalked glandular trichomes covering entire corolla tube; lobes 4–5 x 3–4 mm, glabrous; bosses yellow, puberulent trichomes running down the entire length inside corolla tube. **Stamens** inserted ± 2 mm from corolla tube base; anthers white, 2 mm long; filaments 3–5 mm long, cream. **Ovaries** pyriform-tubular, 2–3 x 2 mm. **Style** orange-brown, 5–6 mm long. **Stigma** 1 x 1 mm. **Capsule** 10–11 mm long, golden-yellow or light green, smooth. **Seeds** orbicular, 3 mm long, orange, puberulous (Figure 7.3.7 A–D).

Figure 7.3.7 Photographs of *C. galpinii* **in:** (A; B) erect, multi-stemmed herb; (C; D) leaves linear, glabrous with narrowly acute apices and entire leaf margins; bracts lanceolate and glabrous; buds creamy-white; (E, F) habitat, growing on gentle hill slopes under full sun conditions with few trees in the surrounding area. **Photos:** Dr. L. Joubert.

Diagnostic features

Southern African *Crabbea* species similar to *C. galpinii*: *C. pedunculata.*

Crabbea galpinii is one of two species with a fibrous root system, along with *C. pedunculata*. Leaves for this species are linear to narrowly lanceolate or narrowly oblanceolate, with a leaf length:width ratio of 1:0.11 and leaf apices are narrowly acute. The corolla is creamy-white, with stalked trichomes with globose heads moderately covering the exterior surface of the corolla. The capsule surface is smooth and seeds have an orbicular shape.

Crabbea pedunculata leaves are oblanceolate or elliptic-ovate, rarely oblong, with a leaf length:width ratio of 1:0.31 and leaf apices broadly acute to obtuse. The corolla is light pinkish-white, with stalked trichomes with globose heads absent on the exterior surface of the corolla. The capsule surface is colliculate and seeds have an ovate shape.

Ecology, distribution and habitat

Crabbea galpinii has a narrow distribution range, covering an area from Nelspruit (Mpumalanga) up to Hhelehhele, Manzini (Swaziland) and down to north-eastern KwaZulu-Natal (Figure 7.3.8). Like other *Crabbea* species in southern Africa, *C. galpinii* is localised in small populations. This species grows in an altitudinal range between 100–1200 m. The surrounding environment is highly developed due to urbanisation and agricultural activities. Therefore, more effort should be made to determine the conservation status of the species*. Crabbea galpinii* herbarium specimens are limited, and in many cases provide minimal habitat and/or ecological information. *Crabbea galpinii* is habitat specific, growing on hills slopes with granite outcrops, stony soils, under full sun conditions (Figure 7.3.7). Lowveld savannah and mesic highveld grassland bioregions support *C. galpinii* (Figure 7.3.7 E–F)*.* Flowering season occurs from September to December.

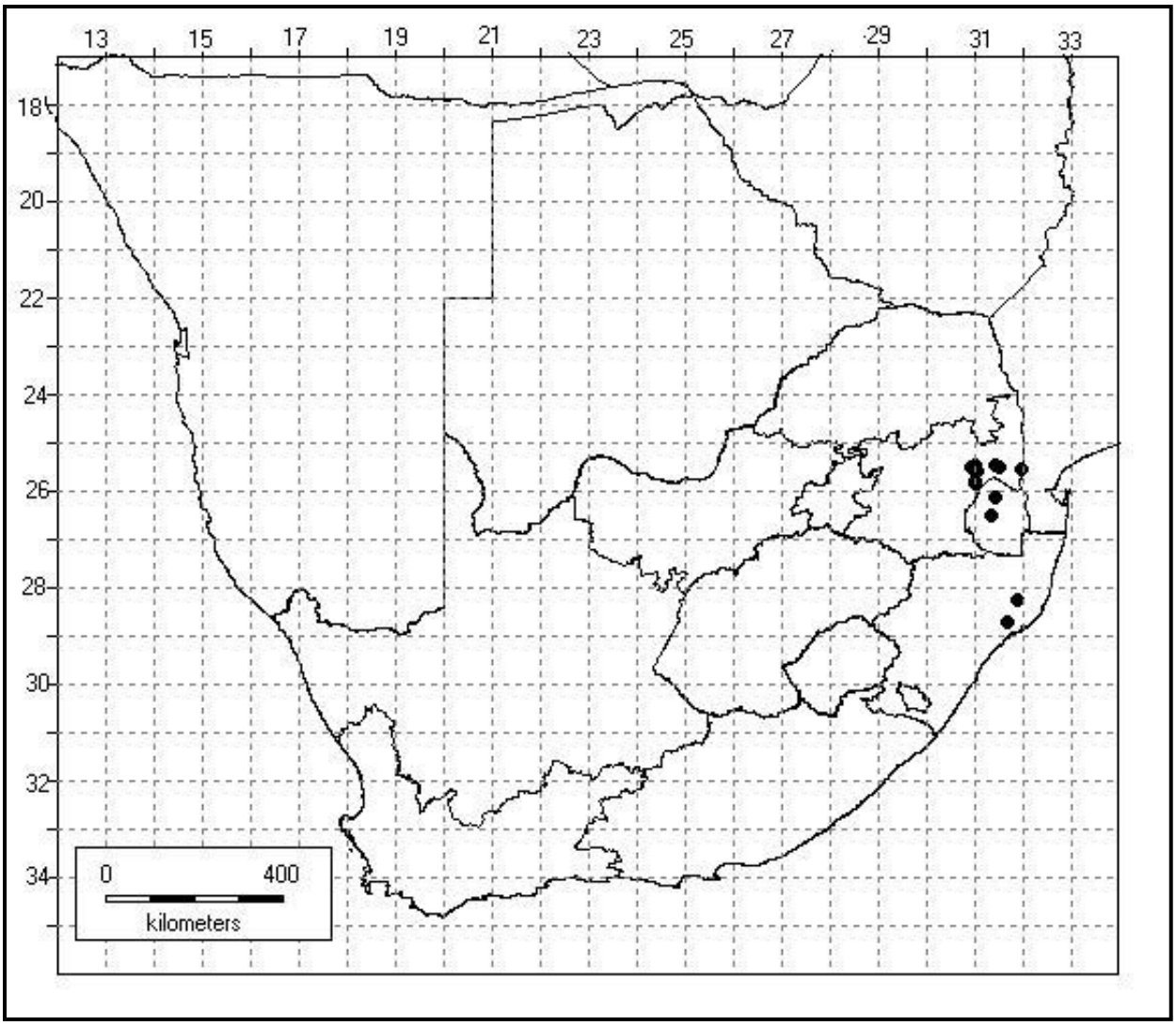

Figure 7.3.8 Known distribution of *C. galpinii* in southern Africa.

Notes

Note 1:

Buys (1982) proposed that *C. galpinii* should be regarded as a subspecies of *C. nana* - *C. nana* subsp. *galpinii* (C.B. Clarke) Voorendyk. However, IPNI (2015) does not recognise *C. nana* subsp. *galpinii* (www.ipni.org). Thus, this classification of *C. galpinii* as a subspecies of *C. nana* is rejected as the findings were never validly published.

Representative specimens

—25°30'55" S, 31°30'57" E: Mpumalanga, Nelspruit**,** Nelspruit Highlands Small Holdings, 22 November 2015, *de Gouveia, A. 171* (BLFU).

—25°32'57" S, 31°58'35" E: Mpumalanga, Nelspruit, Renosterkop, next to path, 20 November 2015, *de Gouveia, A. 169C* (BLFU).

—25°49'17" S, 31°02'20" E: Mpumalanga, Barberton, Moody's Estate, 21 November 2015, *de Gouveia, A.* 170 (BLFU).

7.5.5 *Crabbea ovalifolia* Ficalho and Hiern, in The transactions of the Linnean Society of London 2: 24–25 (1881); Retief and Herman: 220 (1997); Welman: 97 (2003). [Note 1]

Type: Angola, Ninda River, *Serpa Pinto 21* (LISU-scan! holotype [LISU59523ANG]).

Description

Decumbent or procumbent. **Roots** tuberous, (17) 34–75 mm long. **Stems** above ground, horizontally orientated, multi-stemmed (25) 43–690 (705) mm long; leathery, scabrous, puberulous or glabrous; immature stems reddish-brown or greenish-brown; mature stems reddish-brown. **Leaves** evenly distributed along stem; length:width ratio 1:0.50; axils glabrous or puberulous; petiole light to dark green or brown, 2–18 mm long; blades elliptic-obovate or elliptic-ovate, (19) 30–97 x 5–38 mm; leathery, puberulous, rarely glabrous adaxially and abaxially; apex broadly acute or obtuse; venation 2–6 secondary veins on either side of primary vein, green, glabrous or puberulous, occasionally velutinous adaxially, green, lime or yellow, glabrous or puberulous abaxially; margins entire or crenulate, occasionally denticulate, puberulous. **Inflorescences** along stem length, peduncle 2–15 mm long, dark brown, puberulous. **Bracts** lanceolate, rarely ovate, 13–31 x 2–10 mm, hirsute. **Buds** 4–9 mm long, puberulent, creamy-white. **Sepals** 8–10 mm x 0.5 mm, pilose. **Corolla** 16 x 2 mm, creamy-white, puberulent across entire corolla tube; lobes 4–8 x 4–6 mm, weakly puberulent; bosses golden-yellow, puberulent trichomes concentrated below bosses inside corolla mouth. **Stamens** inserted ± 4–6 mm from corolla tube base; anthers cream, 2 mm long; filaments 3–6 mm long, creamy yellow. **Ovaries** pyriform-tubular, 2–3 x 1–2 mm. **Style** reddish-orange, 3–7 mm long. **Stigma** 1 x 1 mm. **Capsule** 6–9 mm long, golden-yellow or light green, smooth. **Seeds** ovate, 2 mm long, orange (Figure 7.3.9 A–E).

Figure 7.3.9 Photographs of *C. ovalifolia* **in:** (A) decumbent or procumbent herb with elliptic-ovate leaves; (B–C) bracts lanceolate with acute apices; (D–E) corolla tube funnel-shaped, creamy-white with raised, paired, golden-yellow bosses; (F) habitat, growing in woodlands, on flat terrain under semi-shade conditions. **Photos:** Dr. L. Joubert.

Diagnostic features

Southern African *Crabbea* species similar to *C. ovalifolia*: *C. angustifolia* and *C. cirsioides*.

Crabbea ovalifolia is a decumbent and/or procumbent *Crabbea* species. Leave shape is elliptic-obovate or elliptic-ovate with a leaf length:width ratio of 1:0.50. Leaf apices are broadly acute to obtuse. The exterior surface of the corolla tube is covered by puberulent trichomes. Bosses on the lower corolla lobes are golden-yellow. Seeds are ovate.

Crabbea angustifolia has linear or narrowly lanceolate leaves with a leaf:width ratio of 1:0.09. Leaf apices are narrowly acute. The exterior surface of the corolla is covered by puberulent trichomes that are concentrated on upper half of corolla tube, while the lower half is glabrous. Bosses on the lower corolla lobes are yellow. Seeds are orbicular.

Crabbea cirsioides has lanceolate to broadly lanceolate, elliptic, occasionally oblanceolate or oblong leaves with a leaf length:width ratio of 1:0.32. The exterior surface of the corolla is covered by puberulent trichomes that are concentrated on upper half of corolla tube, while the lower half is glabrous.Bosses on the lower corolla lobes are yellow. Seeds are elliptic.

Ecology, distribution and habitat

Crabbea ovalifolia is distributed mainly in the north central interior of South Africa: Gauteng, southern Limpopo, Mpumalanga and eastern North West (Figure 7.3.10). This species grows at an altitudinal range of 180–1400 m. Moisture availability is seasonal for *C. ovalifolia* habitats, occurring largely in summer. *Crabbea ovalifolia* can be found in the open bushveld, grasslands and woodlands, on flat terrains and hills growing in dark, red, sandy, well-drained soils, under full sun or semi-shade conditions. Central bushveld and lowveld savannah and dry highveld and mesic highveld grassland bioregions largely support *C. ovalifolia* habitat (Figures 7.3.9 F). Flowering occurs from November to March.

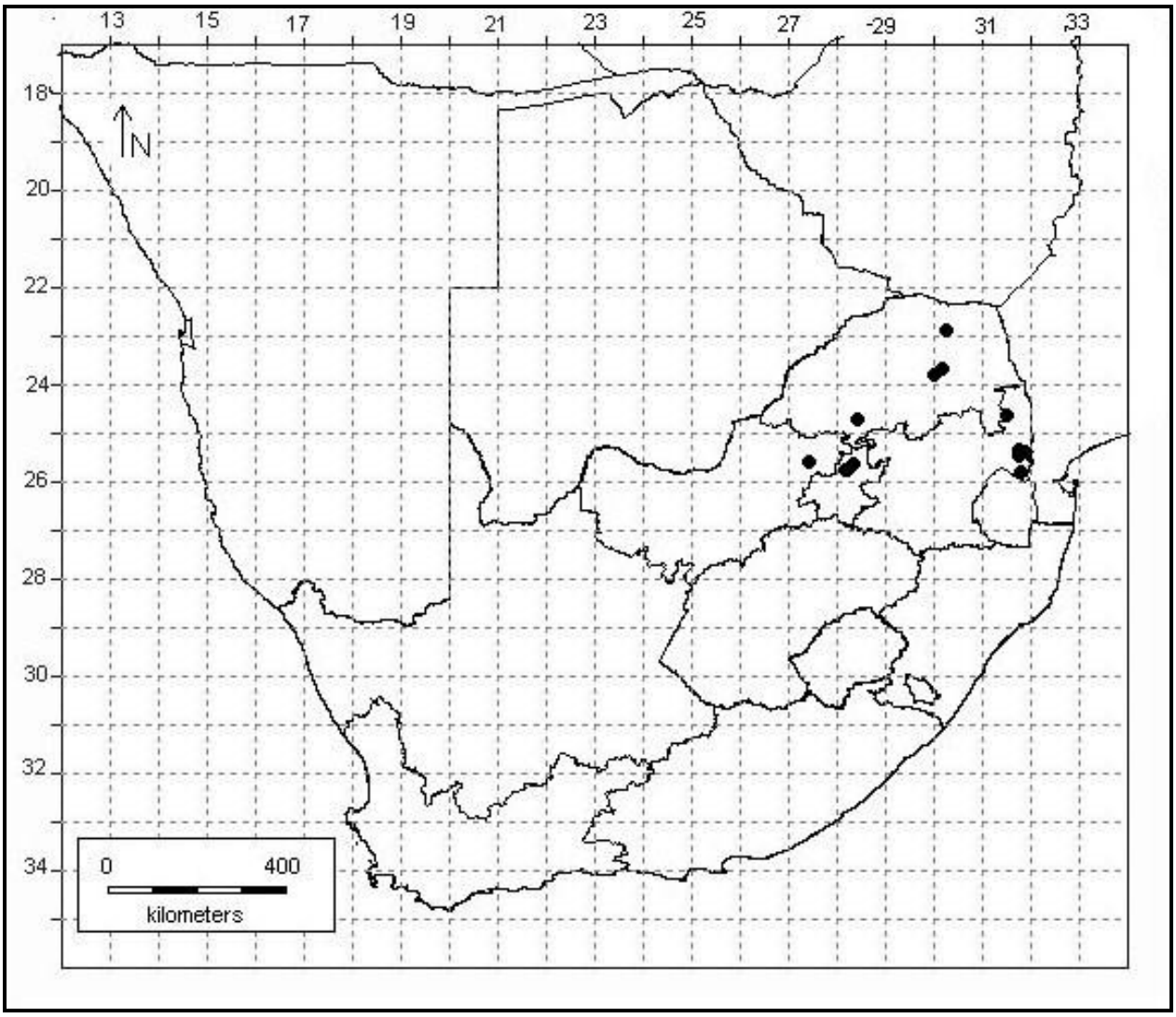

Figure 7.3.10 Known distribution range of *C. ovalifolia* in southern Africa.

Notes

Note 1:

Vollesen (2015) classified *C. ovalifolia* as a synonym of *C. cirsioides*. The synonomy of *C. ovalifolia* under *C. cirsioides* is supported by the absence of a prominent peduncle: “inflorescences subsessile or with peduncle to 0.5(1) cm long.”

Representative specimens

—25°26'00" S, 31°46'30" E: Mpumalanga, Marloth Park, N4 turnoff to Marloth Park, about 200 m adjacent to cement track, east of the road, 27 March 2015, *de Gouveia*, *A*. *134* (BLFU);

—25°38'34" S, 28°21'41" E: Gauteng, Pretoria, Roodeplaatdam Nature Reserve, 27 December 1979, *van Rooyen, N. 2224* (PRE [PRE0663133-0]).

—25°41'30" S, 28°12'18" E: Gauteng, Pretoria, Trigaardspoort, 6 February 1938, *Repton, J.E. 1220* (PRE [PRE0126704-0]).

7.5.6 *Crabbea pedunculata* N.E.Br, in C.B.Clarke, Flora Capensis, 5 (1): 40 (1901); Ross: 324 (1972). [Note 1]

Type: South Africa, Inanda [KwaZulu-Natal], *Wood, J.M. 365* (K-scan! lectotype designated by Vollesen (2015) [K000394681]; *Wood, J.M. 365* (K-scans! syntype two specimens on one sheet [K000394682; K000394683]); *Wood, J.M. 365* (SAM-scan! syntype two sheets [SAM0018959-1; SAM0018959-2]); without precise locality, *Sanderson, J. 466* (K-scan! syntype designated by Vollesen (2015) [K000394684]; TCD-scan! syntype designated here [*s.n.*]); Krantz Kloof [KwaZulu-Natal], *Schlechter, F.R. 3210* (B†, syntype).

Description

Erect. **Roots** fibrous. **Stems** above ground, vertically orientated, multi-stemmed, 11–127 (400) mm long; herbaceous, glabrous; immature stems greenish-brown; mature stems reddish-brown or green. **Leaves** evenly distributed along stem; length:width ratio 1:0.31; axils glabrous or weakly puberulous; petiole light to dark green or brown, 2–15 mm long; blades oblanceolate or elliptic-ovate, rarely oblong, 29–14 x 10–29 mm; herbaceous, glabrous, rarely puberulous adaxially and abaxially; apex broadly acute or obtuse; venation 3–7 secondary veins on either side of primary vein, green or lime, glabrous adaxially; green or lime, glabrous, rarely slightly puberulous abaxially; Margins entire, rarely slightly crenulate, glabrous, rarely slightly puberulent. **Inflorescences** along stem length, peduncle 6–112 mm long, dark brown, rarely green, puberulous or glabrous. **Bracts** lanceolate, 15–34 x 6–15 mm; glabrous to weakly puberulous. **Buds** 9–12 mm long puberulent, creamy-white. **Sepals** 11–13 x 1 mm, pilose. **Corolla** 17 x 3 mm, light pinkish-white, puberulent across entire corolla tube; lobes 4–6 x 4–7 mm, weakly puberulent; bosses yellow, puberulent trichomes running down the entire length inside corolla tube. **Stamens** inserted ± 6–7 mm from corolla tube base; anthers creamy-white, 1–2 mm long; filaments 3–5 mm long, cream. **Ovaries** pyriform-tubular, 4 x 1–2 mm. **Style** orange, 7–10 mm long. **Stigma** 1–2 x 1–2 mm. **Capsule** 10–11 mm long, golden-yellow or light green, colliculate. **Seeds** ovate, 2–3 mm long, orange (Figure 7.3.11 A–E).

Figure 7.3.11 Photographs of *C. pedunculata* **in:** (A–B) erect herb with oblanceolate leaves; (C–D) corolla funnel-shaped, light pink with raised, paired, yellow bosses; (E) bracts lanceolate, with acute apices; (F) habitat, growing on hill slopes, in moderately dense vegetation areas. **Photos:** Dr. L. Joubert.

Diagnostic features

Southern African *Crabbea* species similar to *C. pedunculata*: *C. galpinii* and *C. velutina*.

Crabbea pedunculata, along with *C. galpinii*, has fibrous roots, herbaceous stems and leaves. Leaf shape is oblanceolate or elliptic-ovate and rarely oblong, with a leaf length:width ratio of 1:0.31. Leaf apices are broadly acute or obtuse. The corolla is light pinkish-white. Stalked trichomes with globes heads are absent from the exterior corolla surface. Capsules are colliculate and seeds are ovate.

Crabbea galpinii leaves are linear to narrowly lanceolate or narrowly oblanceolate, with a leaf length:width ratio of 1:0.11 and leaf apices are narrowly acute. The corolla is creamy-white, with stalked trichomes with globose heads moderately covering the exterior surface of the corolla. The capsule surface is smooth and seeds have an orbicular shape.

Crabbea velutina can be easily distinguished from *C. pedunculata* by having tuberous roots, leathery stems and leaves. The corolla tube is white, with puberulent tichomes distributed across entire exterior surface of the corolla tube. Capsules are smooth and seeds are elliptic.

Ecology, distribution and habitat

The distribution range of *C. pedunculata* is along eastern South Africa, from Mpumalanga down to the Eastern Cape (Figure 7.3.12). The species grows at an altitudinal range between 10–1600 m. *Crabbea pedunculata* is habitat specific and also localised into small populations, as in the case of *C. galpinii*. This species grows on humus-rich, well-drained sandy spoils on semi-shade hill slopes with relatively dense vegetation growth. *Crabbea pedunculata* commonly grows in areas subject to seasonal fires. Sub-escarpment and mesic highveld grassland and sub-escarpment and lowveld savannah bioregions support *C. pedunculata* habitat (Figure 7.3.11 F). *Crabbea acaulis* and *C. cirsioides* are commonly found in the vicinity of *C. pedunculata*. Flowering season starts in September and ends in December.

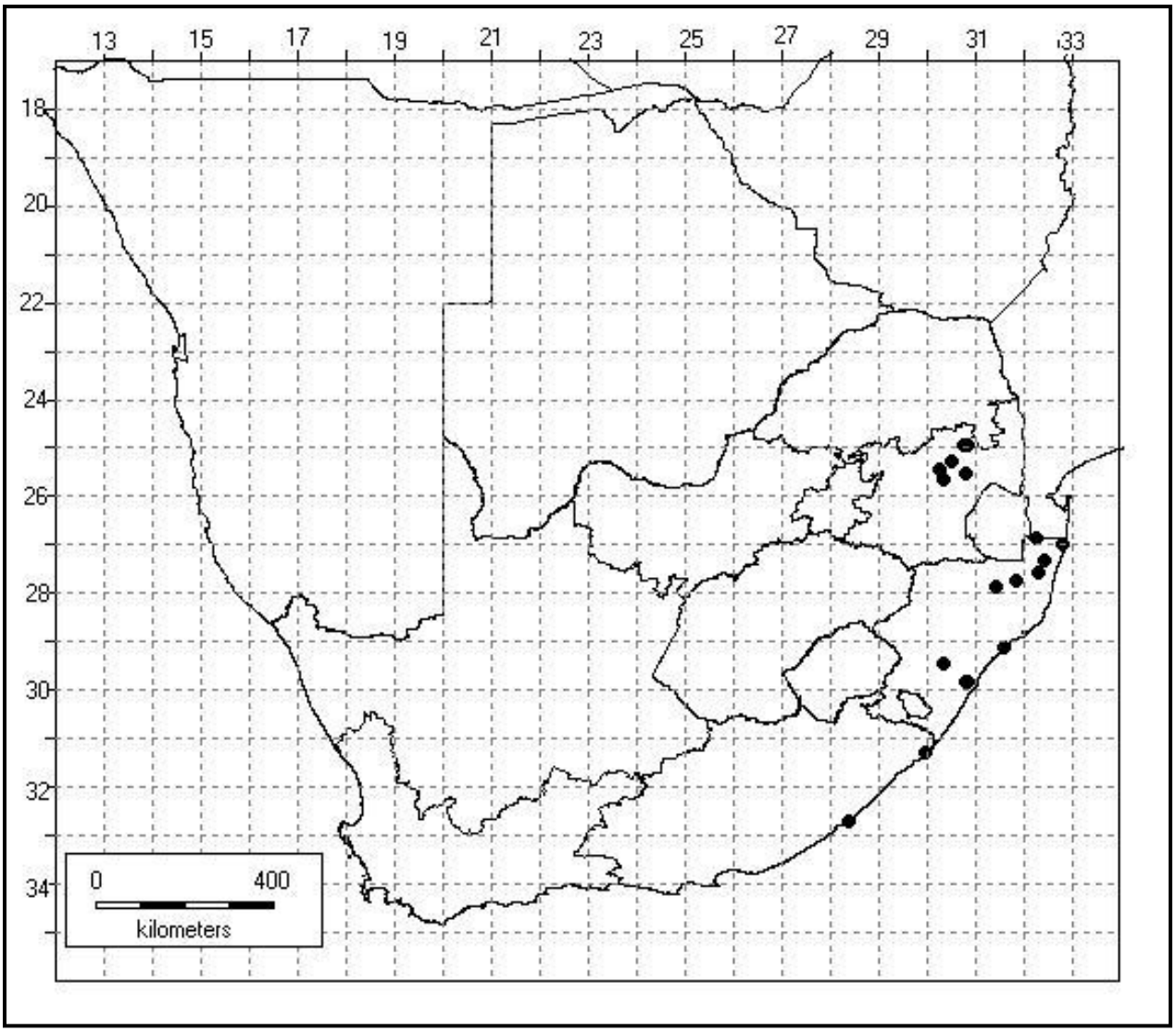

Figure 7.3.12 Known distribution of *C. pedunculata* in southern Africa.

Note 1:

Buys (1982) proposed that *C. pedunculata* should be regarded as a synonym of *C. nana* subsp. *nana* (Nees) Voorendyk. However, IPNI (2015) does not recognise *C. nana* subsp. *nana* (www.ipni.org). Thus, this classification of *C. pedunculata* as a synonym of *C. nana* subsp. *nana* is rejected, as the findings were never validly published.

Representative specimens

—25°16'43" S, 30°30'28" E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Helichrysum Trail (Upper Forest Trail), near stream, N.W. of Andrew's Falls, 23 March 2015, *de Gouveia*, *A*. *94* (BLFU).

—25°25'16" S, 30°15'26" E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, Welgedacht Farm, past rock pool and opposite chalet nr. 2 en route to waterfall, 22 March 2015, *de Gouveia*, *A*. *81* (BLFU).

—25°25'21'' S, 30°15'29'' E: Mpumalanga, Lydenburg, Verlorenkloof Nature Reserve, Welgedacht Farm, S.W. face of mountain slope on waterfall trail, at forest margin, 26 November 2015, *de Gouveia*, *A*. *177* (BLFU).

7.5.7 *Crabbea velutina* S.Moore, in The Journal of Botany, British and foreign, 32: 135 (1894); Burkill and C.B.Clarke, 5:119 (1899–1900); Retief and Herman: 220 (1997); Welman: 97 (2003); Vollesen, 8(6): 12 (2015).

Type: Tropical Africa. *Gregory, J.W. s.n.* (BM-scan! lectotype here designated [BM000645011]); locality unknown. *Taylor, W.E. s.n.* (BM-scan! syntypedesignated by Vollesen (2015) [BM000645012]). Note 1

= *Crabbea reticulata* C.B.Clarke, in Burkill and C.B.Clarke, Flora of Tropical Africa, 5: 119 (1899–1900). Note 2

Type: British East Africa, Ukamba, 4 000 ft [Kenya], *Scott-Elliot, G.F. 2309* (K-scan! lectotype designated here [K000394696]; BM-scan! isolectotype designated here BM000645007); German East Africa, Karagwe, 4 000–5 000 ft [Tanzania], *Scott-Elliot, G.F.8147* (K-scan! syntype designated by Vollesen (2015) [K000394695]; BM-scan! isosyntype designated by Vollesen (2015) [BM000645006]).

Description

Erect. **Roots** tuberous, 36–112 mm long. **Stems** above ground, vertically orientated, muti-stemmed, (20) 36–185 (300) mm long; leathery, scabrous, velutinous; immature stems dark brown, occasionally light green; mature stems reddish-brown. **Leaves** evenly distributed along stem; length:width ratio 1:0.32; axils velutinous or arachnoid; petioles brownish-orange, 3–22 mm long; blades oblanceolate or elliptic-ovate, occasionally lanceolate, (24) 33–147 (168) x 10–29 mm; leathery, velutinous or puberulous adaxially and abaxially; apex broadly acute or obtuse; venation 5–10 secondary veins on either side of primary vein, green or lime, velutinous or pilose adaxially, green or lime, velutinous or pilose; margins entire or slightly crenulate, puberulent. **Inflorescences** along stem length, peduncle 11–122 (190) mm long, orange brown, velutinous. **Bracts** lanceolate or ovate, 13–25 x 5–12 mm, hispid. **Buds** 8–10 mm long, puberulent, creamy-white. **Sepals** 6–12 x1 mm, pilose to velutinous. **Corolla** 18 x 2 mm, white, puberulent across entire corolla tube; lobes 6–8 x 5 mm,

weakly puberulent; bosses yellow, puberulent trichomes concentrated below bosses inside corolla mouth. **Stamens** inserted ± 3 mm from corolla tube base; anthers creamy-white, 2 mm long; filaments 3–4 mm long, cream. **Ovaries** ovoid, 2–3 x 2 mm. **Style** orange, 6–9 mm long. **Stigma** 1 x 1 mm. **Capsule** 9–11 mm long, golden-yellow, smooth. **Seeds** elliptic, 2 mm long, orange (Figure 7. 3.13 A–E).

Diagnostic features

Southern African *Crabbea* species similar to *C. velutina*: *C. pedunculata*.

Crabbea velutina has an erect growth form. Stems are leathery, scabrous and velutinous. Roots are tuberous. Leaves are leathery, velutinous or puberulous. Bracts are hispid. The corolla is white. Ovaries are ovoid. Capsules are smooth and seeds are elliptic.

Crabbea pedunculata has herbaceous, glabrous stems. The roots are fibrous. Leaves are herbaceous, glabrous and rarely puberulous. Bracts are glabrous to weakly puberulous. The corolla tube is light pinkish-white. The ovaries are pyriform-tubular. Capsules are colliculate and seeds are ovate.

Ecology, distribution and habitat

Of all southern African *Crabbea*, *C. velutina* has the largest distribution range, spanning from Limpopo, central and eastern southern Africa (Figure 7.3.14), central Africa and along the eastern interior of Africa up to Ethiopia. This species grows in an altitude range from 200–1 500 m above sea level. *Crabbea velutina* grows on hill slopes, on stony, loamy soils, rock crevices in semi-shade conditions in relatively dense, overgrown vegetation habitats. Surrounding geology includes granite, gneiss and schists. *Crabbea velutina* habitat can be found in the Lowveld, mopane and central bushveld bioregions, all forming part of the Savanna Biome (Figure 7.3.13 F). species include *Terminalia prunioides* M.A. Lawson, *Adansonia digitata* L.*, Combretum* Loefl., *Cussonia* Thunb., *Dichrostachys* Wight & Arn. and *Acacia* Mill. The peak flowering season for *C. velutina* is predominantly during the summer months starting from November to February. Flowering does extend into March and April and sometimes around June, July in the more tropical areas.

Figure 7.3.13 Photographs of *C. velutina* **in:** (A–B) erect herbs with oblanceolate leaves; (C–E) corolla tubes funnel-shaped, white, with raised, paired, yellow bosses; bract lanceolate, hispid with acute apices; (F) habitat, growing in woodlands under semi-shade conditions. **Photos:** Dr. L. Joubert.

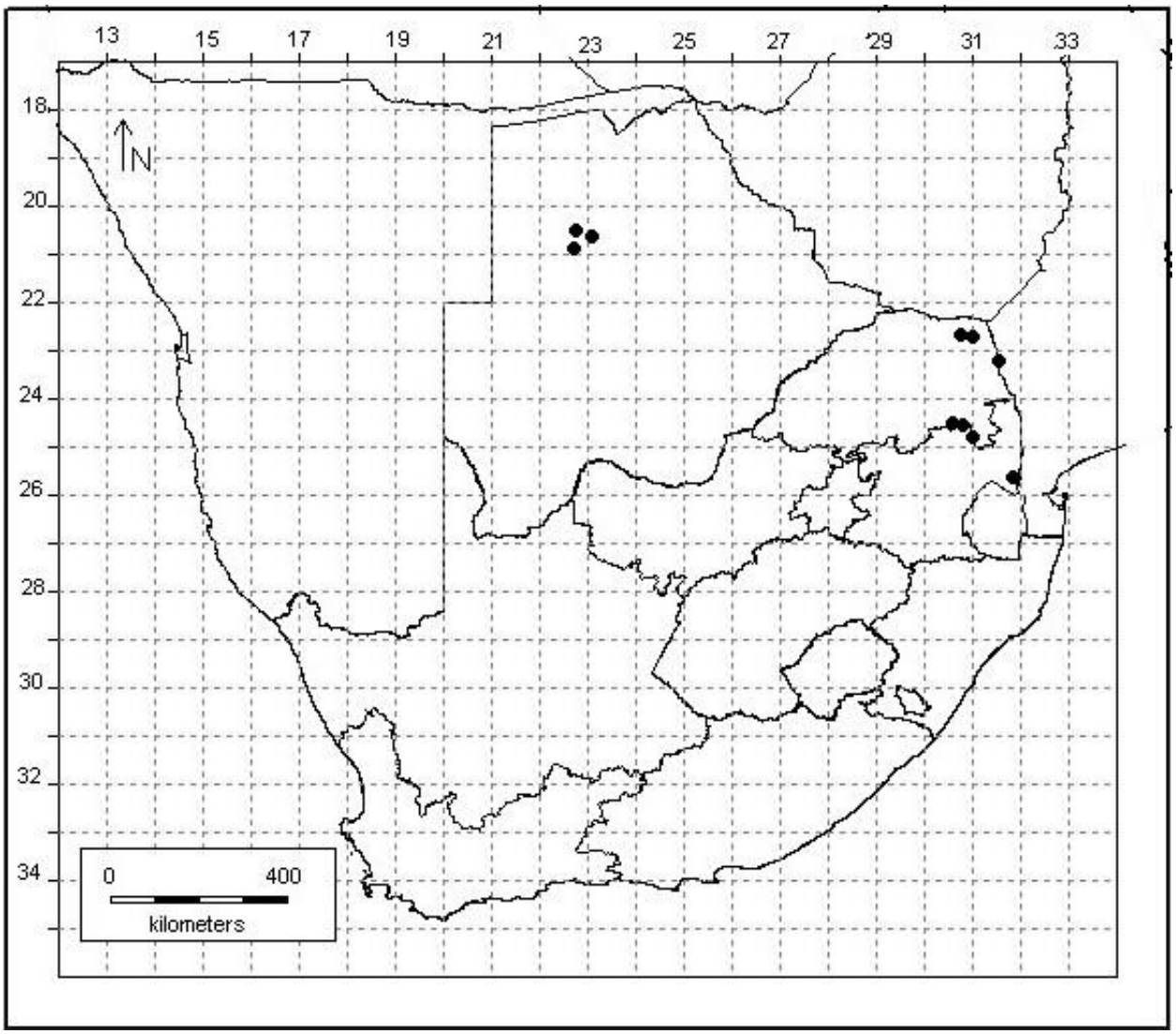

Figure 7.3.14 Known distribution of *C. velutina* in southern Africa.

Vernacular name(s) and ethnobotanical use(s)

Crabbea velutina is used to treat headaches among certain Zambian nationals (Fowler, 2007). In the Bule Hora District, Southern Oromia, Ethiopia, *C. velutina* is used to treat excessive drooling by chopping the leaves and roots together, making a solution and pouring one cup of the solution through the nostrils (Eshete *et al*., 2016). This species is believed to increase the chances of accumulating wealth and prosperity (Arnold *et al*., 2002).

Notes

Note 1:

JStor Plant Sciences (2016) (www.plants.jstor.org) incorrectly labelled both syntypes of *C. velutina* as "not a type specimen." Analysis of the original publication reveals that both syntypes are located at BM Herbarium under the barcodes BM000645011 and BM000645012. From these two syntypes, the specimen first mentioned by Moore (1894) is here designated as the lectotype (BM000645011).

Note 2:

Crabbea reticulata was synonymised under *C. velutina* by Buys (1982). Welman (2003) maintained the classification of *C. reticulata* under *C. velutina*, following Buys (1982). Vollesen (2015) also regards *C. reticulata* as a synonym of *C. velutina*.

Representative specimens

—24°30'48'' S, 30°50'14'' E: Mpumalanga, Swadini, Blyde Canyon Reserve, outside camp, next to road en route to dam, 19 November 2015, *de Gouveia*, *A*. *164* (BLFU).

—24°31'40'' S, 30°47'13'' E: Mpumalanga, Swadini, Blyde Canyon Reserve, next to walk, close to dam, 19 November 2015, *de Gouveia*, *A*. *163* (BLFU).

—25°52'25" S, 31°54'26" E: Limpopo, Komatiepoort, Lebombo Mountains, 24 February 1953, *van der Schijff, H.P*. *2306* (PRE [PRE0126763-0]).

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

In order to achieve the most informative account of the evolutionary history of the southern African *Crabbea* species, a combination of taxonomically important characters was investigated separately and later integrated. Characters sets used in this study that were of systematic importance for *Crabbea* included leaf anatomy (cystoliths), leaf micromorphology, pollen micromorphology, macromorphology and DNA sequences

8.1 An integrative tree based on molecular, anatomical and morphological data for the southern African *Crabbea* species and biogeographical implications.

The best phylogenetic results will not necessarily be achieved by including and comparing more data but rather by choosing the characters suited to give the best resolution in the group under investigation (Stuessy, 2009). Following the approach of Stuessy (2009), in an attempt to overcome the limitations imposed by the absence of ITS sequence data, anatomical and morphological data was used to increase the resolution of the phylogenetic tree (Figure 8.1). The 20 selected characters could be easily used to group, identify, classify and/or delimit the southern African *Crabbea* species (Tables 6.3; 6.4). Eight of the 20 characters (Table 6.3) can be mapped adjacent to the phylogeny to emphasize the congruence between molecular, anatomical and morphological data (Figure 8.1; Table 8.1). However, for simplicity purposes, four main characters (Chapters 3–5; 7), one from each chapter, was placed next to the phylogeny (Chapter 6) (Figure 8.1).

Growth form proved to be one of the characters that best complement the tree topology (Figure 8.1: A 1-8; Table 8.1: Clades A–D). The erect-growing *Crabbea* species, *C. galpinii*, *C. pedunculata* (Clade B) and *C. velutina* (Clade A) form two distinct clades. *Crabbea angustifolia*, *C. cirsioides* [= *C. nana*] and *C. ovalifolia* are procumbent,

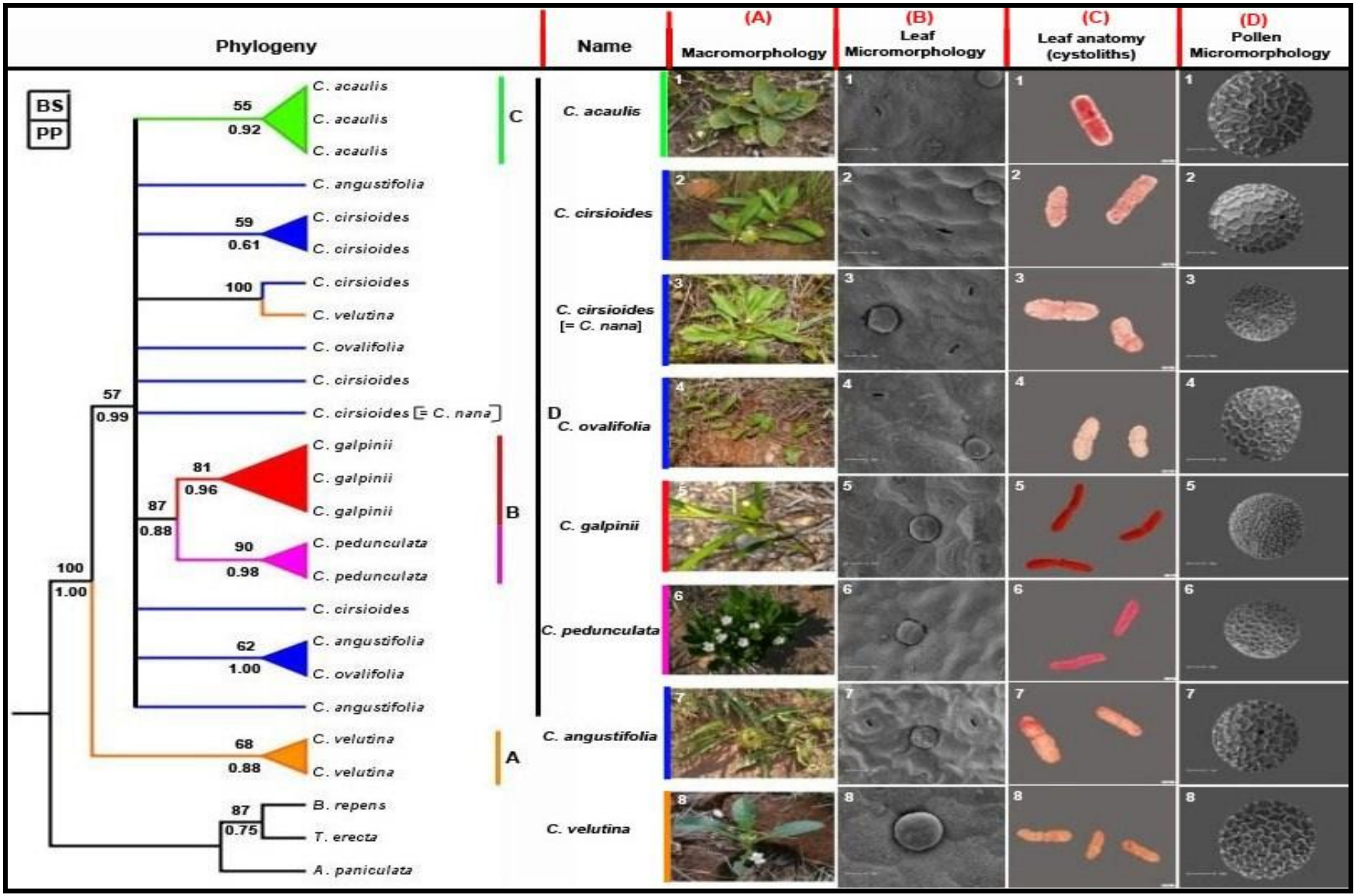

Figure 8.1 Integrated phylogeny, anatomy and morphology tree of the southern African *Crabbea* species.

Table 8.1 The eight macromorphological, anatomical, leaf and pollen micromorphological characters showing congruence with the phylogeny.

Clade name		**(A)** ***C. velutina***	**(B)** ***C. galpinii*** ***C. pedunculata***	**(C)** ***C. acaulis***	**{Species with unresolved phylogenetic position within *Crabbea*}** ***C. angustifolia*** ***C. cirsioides*** **[= *C. nana*]** ***C. ovalifolia***
Character set					
Macro-morphology	Growth form	Erect	Erect	Rosette	Decumbent/procumbent
	Roots	Tuberous	Fibrous	Tuberous	Tuberous
	Stem texture	Leathery	Herbaceous	Leathery	Leathery
	Stem position	Above ground	Above ground	Below ground	Above ground
	Stem orientation	Vertical	Vertical	Vertical	Horizontal
Anatomy	Adaxial leaf cystolith attachment width	< 50 µm	< 50 µm	> 50 µm	> 50 µm
Leaf micro-morphology	Stomatal distribution	Hypostomatic	Hypostomatic	Amphistomatic	Amphistomatic
Pollen micro-morphology	Pollen grain gemma width	< 2 µm	> 2 µm	< 2 µm	< 2 µm

decumbent species and are unplaced within the larger Clade D. The basal rosette is diagnostic of *C. acaulis*, which forms a distinct clade (Clade C). With stomatal features being under genetic control (Payne, 1979; Croxdale, 2000) and the occurrence of both amphistomatic and hypostomatic leaves among Acanthaceae taxa (Solereder, 1908; Metcalfe and Chalk, 1950a), a close relationship is observed between growth form and stomatal distribution on the phylogenetic tree (Figure 8.1; Table 8.1). *Crabbea galpinii*, *C. pedunculata* and *C. velutina* have an erect growth from, coupled with hypostomatic leaves (Figure 8.1: A 5, 6, 8; B 5, 6, 8; Table 8.1; Clades A, B). The occurrence of the latter character state may be driven by environmental pressures in an attempt to reduce water loss via transpiration as the leaves are more exposed to the surrounding atmosphere. Amphistomatic leaves are diagnostic of the lower growing (rosette, procumbent and decumbent) southern African *Crabbea* species, namely *C. acaulis,* C. *angustifolia, C. cirsioides* [=*C. nana*] and *C. ovalifolia* (Figure 8.1: A 1–4, 7; B 1–4, 7; Table 8.1; Clade C). Importantly, the stomatal distribution across the leaf surfaces remained constant over the sampled geographical range of each *Crabbea* species. This confirms the strong genetic influence on stomatal distribution among the southern African *Crabbea* species, and consequently the phylogenetic implications and importance of this character.

In addition to defence (da Costa *et al*., 2009), cystoliths may also function in scattering light, thus, reducing photo-inhibition by shifting light from upper photo-saturated to lower photo-limited mesophyll cells (Gal *et al*., 2012). With reference to growth form and stomatal distribution, another notable relationship is observed between the two above-mentioned characters and average cystolith attachment width on the adaxial leaf surface (Figure 8.1; Table 8.1) among the southern African *Crabbea* species. The erect-growing *Crabbea* species (*C. galpinii*, *C. pedunculata* and *C. velutina*), display an average adaxial leaf cystolith attachment width of less than 50 µm. Conversely, *C. acaulis,* C. *angustifolia, C. cirsioidess* [= *C. nana*] and *C. ovalifolia,* which are lower-growing species, have an average cystolith attachment width of more than 50 µm, (Figure 8.1 A–C, 1–8; Table 8.1). This suggests that the erect-growing species, having a greater average leaf-sun exposure, need to reflect less sunlight to the photo-limited mesophyll tissue on the lower leaf epidermis, for effective photosynthetic activity. The

opposite effect would occur on the lower-growing species, which needs to reflect more sunlight to the lower, photo-limited tissue by having broader cystoliths.

Factors influencing pollen grain size have been effectively explained by various authors (Bell, 1959; Reitsma, 1969; Walker and Doyle, 1975; Fægri *et al.*, 1989; Hesse and Waha, 1989); however, a relationship between the presence of muri and lumina to gemmae width has been recorded. The investigated *Crabbea* species showed two main pollen types: pollen with muri and lumina and pollen lacking muri and lumina. *Crabbea* species in the former group constitute *C. acaulis*, *C. angustifolia*, *C. cirsioides* [= *C. nana*], *C. ovalifolia* and *C. velutina* which all have an average gemma width of less than 2 µm (Figure 8.1 D 1–4, 7; Table 8.1). The latter group, *C. galpinii* and *C. pedunculata*, has an average gemma width of more than 2 µm (Figure 8.1 D 5, 6; Table 8.1). The larger gemmae size among *C. galpinii* and *C. pedunculata* may be attributed to more available space over the pollen grain for gemmae distribution, when energy is only invested in gemmae synthesis and not muri formation as well. The opposite condition occurs in the former group where energy must be invested for both gemmae and murus production, resulting in smaller, average gemmae.

Van As *et al.* (2012) report that the eastern part of South Africa receives more rainfall than the western parts. It has been confirmed that the southern African *Crabbea* species are predominantly concentrated along the eastern part of southern Africa (Figure 7.2). Two different root types have been observed among the investigated species, namely fibrous (*C. galpinii* and *C. pedunculata*) (Figure 8.1; Clade B; Table 8.1) and tuberous roots (*C. acaulis, C. angustifolia*, *C. cirsioides* [= *C. nana*] and *C. ovalifolia* and *C. velutina*) (Figure 8.1; Clades A, C; Table 8.1). However, the two root types (tuberous or fibrous) are present among the different *Crabbea* species, irrespective of location, as in the case of *Crabbea acaulis*, *C. cirsioides* and *C. pedunculata*, which can be found growing in very similar, neighbouring habitats. *Crabbea acaulis* and *C. cirsioides* grow in open plains, under full sun conditions, between rocky outcrops, either close to water bodies or away from water sources. Irrespective of the presence of water, both species consistently display a tuberous root system. *Crabbea pedunculata* grows in relatively dense vegetation and semi-shade

conditions, on hill slopes, close to water bodies, in humus-rich, well drained soils. The close association of *C. pedunculata* with water bodies suggests that constant presence of water results in the species developing fibrous roots. Root characteristics for each investigated *Crabbea* species remains constant over the respective geographic range, implying that root features are under strong genetic control.

Corolla morphology across the southern African *Crabbea* is relatively uniform; therefore, suggesting that the corolla morphology is more significant at genus rather than species level. This will be confirmed once the entire genus has received taxonomic work. In all the investigated species, the flowers are zygomorphic, funnel-shaped and bisexual. The exterior of each *Crabbea* corolla is weakly striate and puberulous. *Crabbea galpinii* is the only southern African *Crabbea* species in which the exterior of the corolla is covered by stalked glandular trichomes. Additionally, all of the investigated specimens are 2-lipped, with the upper lip being 2-lobed and the lower lip being 3-lobed. Paired, raised, yellow, bosses are also seen on the lower corolla lip in all the investigated species. The corolla lobe colour is predominantly creamy-white, but a light pink corolla tube may occasionally be found in *C. acaulis* and is consistently present in *C. pedunculata.* The light corolla colour, with the yellow bosses and the funnel shape of the corolla tube suggests moth pollination (Stuessy, 2009). *Barleria* (± 300 sp.) (Balkwill and Balkwill, 1997) is more species rich than *Crabbea* (16 sp.) (Thulin, 2007). The contributing factor to the species richness of *Barleria* may be attributed to the large variation in corolla colour (e.g. white, pink, purple, red, blue, yellow) and appearance (e.g. showy, sub-actinomorphic to zygomorphic), due to co-evolution with a variety of pollinators (Balkwill and Balkwill, 1997).

As the phylogenetic tree in this study only represents southern African *Crabbea* (Figure 8.1), the monophyly of *Crabbea* will only be confirmed once all sequences of *Crabbea* and *Lasiocladus* species and an increased number of *Barleria* species have been sequenced and phylogenetically analysed (McDade *et al*., 2008). *Crabbea* species for which sequences are still lacking are those from the taxonomic revisions of Thulin (2007) (Somalia) and Vollesen (2015) (southern tropical Africa (excluding Angola)).

Findings from both publications (Thulin, 2007; Vollesen, 2015) were based on alpha taxonomy only.

8.2 Significance of macromorphological, anatomical and micromorphological characters in identifying southern African *Crabbea* species

Four character sets were analysed individually to determine the significance of each character set at genus and species level. These character sets include macromorphology, leaf anatomy (cystoliths), leaf micromorphology and pollen micromorphology. To establish the taxonomic significance of each character set (i.e. can all southern African *Crabbea* species be delimited), identification keys were constructed (Chapter 3 - 3.5; Chapter 4 - 4.5; Chapter 5 - 5.5; Chapter 7 - 7.4) (Table 8.2).

Of all four identification keys, the macromorphological identification key proved to be the most informative at species level for the southern African *Crabbea* species, as all except *C. cirsioides* and *C. nana* could be keyed-out successfully. Seven groupings were successfully achieved. The effective identification of each species is a result of combining an array of morphological characters (root, stem, leaf, bract, corolla, bosses etc.) instead of one or two. Root appearance, stem and leaf texture, together, proved most useful in splitting the investigated *Crabbea* species into two distinct groups. In addition to root appearance, stem and leaf texture, growth form and leaf shape can be readily used to further delimit the species from one another. Macromorphological character states that are not useful in delimiting the various *Crabbea* species are corolla shape, the androecium and the gynoecium. Significantly, *C. galpinii* and *C. pedunculata* were placed in a group distinct from *C. nana* (Table 8.2).

The leaf micromorphology identification key scored the second best result of the four identification keys, obtaining six groups. Stomatal distribution across both leaf surfaces was the most significant feature to split the southern African *Crabbea* species into two main groups. Further divisions were based on cuticular, trichome and stomatal features.

Table 8.2 Comparison of the significance of macromorphology, leaf anatomy (cystoliths), leaf micromorphology and pollen micromorphology character sets. The groupings obtained for the southern African *Crabbea* species are also indicated. The layout is obtained from the generated identification key from each respective chapter.

	Macromorphology		Pollen micromorphology		Leaf anatomy (cystoliths)		Leaf micromorphology	
	Chapter 7		Chapter 5		Chapter 4		Chapter 3	
	Character state	Species	Character state	Species	Character state	Species	Character state	Species
Groupings	Roots tuberous; stems + leaves leathery	*C. acaulis*	Murus and lumen present; gemma width < 2 µm	*C. acaulis*	Adaxial leaf cystolith attachment width > 50µm	*C. acaulis*	Amphistomatic leaves	*C. acaulis*
		C. angustifolia		*C. cirsioides* [= *C. nana*]		*C. angustifolia*		*C. cirsioides* [= *C. nana*]
		C. cirsioides [= *C. nana*]		*C. ovalifolia*		*C. cirsioides* [= *C. nana*]		*C. angustifolia*
		C. ovalifolia		*C. angustifolia*		*C. ovalifolia*		*C. ovalifolia*
		C. velutina		*C. velutina*	Adaxial leaf cystolith attachment width < 50 µm	*C. galpinii*	Hypostomatic leaves	*C. galpinii*
	Roots fibrous; stems + leaves herbaceous	*C. galpinii*	Murus and lumen absent; gemma width > 2 µm	*C. galpinii*		*C. pedunculata*		*C. pedunculata*
		C. pedunculata		*C. pedunculata*		*C. velutina*		*C. velutina*

Importantly, *C. nana* was placed in a different group from that of *C. galpinii* and *C. pedunculata* (Table 8.2). Stomatal measurements were not regarded as important identification features as these characters showed a size range overlap. Additionally, external factors such as environmental conditions, preservation and preparation methods could influence stomatal measurements.

Four different groups were achieved using the pollen micromorphology identification key, placing this identification key in third place. This was attained by combining pollen grain size, murus, lumen and gemmae features. The presence or absence of muri and lumina in conjunction with gemmae width were the most informative characters in delimiting the southern African *Crabbea* into two groups (Table 8.2). As with the two previous identification keys, *C. nana* is positioned in a group separate from that of *C. galpinii* and *C. pedunculata.*

Tied in third place, together with the pollen micromorphology identification key, is the cystolith identification key. Four groups were also obtained. Adaxial leaf cystolith attachment width was the most informative character state to divide the investigated species into two groups. Cystolith measurements were mainly used to further separate the *Crabbea* species from each other. Therefore, cystolith appearance proved least useful for *Crabbea* species level identification. Instead, *Crabbea* cystolith appearance seems to be more significant at genus level and up. As with the three above mentioned identification keys, *C. galpinii* and *C. pedunculata* are found in a group distinct from that of *C. nana* (Table 8.2).

8.3 The taxonomic position of the southern African *Crabbea* species

The taxonomic data generated from this study provides a different classification system than that of Buys (1982), Welman (2003) and Vollesen (2015). However, this study does show some congruency with respect to Buys (1982); Welman (2003) and Vollesen (2015). *Crabbea* species for which the classification remained unaltered between the four taxonomic studies is *C. velutina*. With reference to molecular, anatomical, macro- and micromorphological data, the nomenclature, the type material and distribution range

of the species confirm the position of *C. velutina*. Molecular data suggests that *C. velutina* is placed in a well supported clade, separate from the remaining southern African *Crabbea* species. (Figure 8.1; Clade A). This species has the most northern distribution range of all the southern African *Crabbea* species (Figure 7.3.14). *Crabbea velutina* is the only southern African *Crabbea* species with the strong velvety indumentum over the leaf surface. Leaf anatomy, leaf micromorphology and pollen micromorphology identification keys all keyed out *C. velutina*.

The difference in the classification of the southern African species of *Crabbea* may possibly be attributed to the quantity and quality of herbarium vouchers available for each species under investigation. This may have been limiting factor to the three previous taxonomic revisions (Buys, 1982; Welman, 2003; Vollesen, 2015) (Table 8.3).

Buys (1982) used growth form, leaf shape, bract indumentum and flowering period to distinguish between the two subspecies of *C. nana* (Table 8.4). Vollesen (2015) referred to peduncle length to separate *C. cirsioides* from *C. nana* (Table 8.5). Results of the present study show that, morphologically, *Crabbea galpinii* and *C. pedunculata* can be distinguished from each other on the basis of leaf shape, leaf length:width ratio, leaf apex, corolla colour, corolla trichomes, capsule surface, seed shape and habitat (Table 8.6). These characters remain constant over the sampled geographic range (Figures 7.3.3; 7.3.12). In addition molecular, anatomical and micromorphological data support the recognition to the two distinct species, *C. galpinii* and *C. pedunculata*. Different habitat preferences are also observed for the two species (Table 8.6).

The combined molecular and morphological tree shows that the two species are sister species and form a distinct, well supported clade (Figure 8.1; Clade B). These two species show an overlap in distribution range (Figures 7.3.3; 7.3.12), also suggesting a close relationship between the two.

Table 8.3 Comparison of the classification of *Crabbea* in southern Africa by Buys (1982), Welman (2003) and Vollesen (2015) to the current classification.

Buys (1982)		**Welman (2003)**	**Vollesen (2015)**	***Crabbea* in southern Africa; current classification**
***Crabbea* species**	***Crabbea* subspecies**	***Crabbea* species**	***Crabbea* species**	***Crabbea* species**
C. acaulis		***C. acaulis***	**Not included in revision**	***C. acaulis***
C. angustifolia *= C. undulatifolia*		***C. angustifolia*** *= C. undulatifolia*	***C. cirsioides*** *= C. angustifolia** *= C. hirsuta** *= C. nana sensu* Burkill and Clarke (1899–1899) and Clarke (1901) *= C. ovalifolia** *= C. robusta* *= C. undulatifolia* *= Ruellia cirsioides*	***C. angustifolia*** *= C. undulatifolia*
C. hirsuta *= C. cirsioides* *= C. robusta*		***C. hirsuta*** *= C. cirsioides* *= C. robusta*		***C. cirsioides*** *= C. hirsuta* *= C. robusta* *= C. nana*
C. ovalifolia		***C. ovalifolia***		***C. ovalifolia***
C. nana	***C. nana* subsp. *galpinii*** *= C. galpinii*	***C. galpinii***	***C. nana*** *= C. galpinii* *= C. pedunculata* *= R. nana*	***C. galpinii***
	C. nana* subsp. *nana *= C. pedunculata*	***C. nana*** *= C. pedunculata*		***C. pedunculata***
C. velutina *= C. reticulata*		***C. velutina*** *= C. reticulata*	***C. velutina*** *= C. reticulata*	***C. velutina*** *= C. reticulata*

* In the taxonomic treatment of Vollesen (2015), three of the *Crabbea* species recognized in taxonomic treatments of the genus in southern African, namely *C. angustifolia, C. hirsuta* and *C. ovalifolia,* are subsumed under *C. cirsioides.*

Table 8.4 Features used by Buys (1982) to delimit *C. nana* subsp. *galpinii* and *C. nana* subsp. *nana*.

Character	***C. nana* subsp. *galpinii***	***C. nana* subsp. *nana*** = *C. pedunculata*
Growth form	Erect	Erect, occasionally prostrate
Leaf shape	Linear	Obovate to small-obovate
Bract indumentum	Margins without spines	Margins with or without spines
Flowering season	August to December	November to March

Table 8.5 Feature used by Vollesen (2015) to distinguish *C. cirsioides* from *C. nana*.

Character	***C. cirsioides*** = *C. angustifolia** = *C. hirsuta** = *C. nana sensu* Burkill and Clarke (1899–1899) and Clarke (1901) = *C. ovalifolia** = *C. robusta* = *C. undulatifolia* = *R. cirsioides*	***C. nana*** = *C. galpinii* = *C. pedunculata* = *R. nana*
Inforescences	Subsessile or with peduncle to 0.5 (1) cm long	With peduncle 1–5 (15) cm long

Table 8.6 Morphological and ecological differences between *C. galpinii* and *C. pedunculata*.

Character	*C. galpinii*	*C. pedunculata*
Leaf shape	Linear, occasionally narrowly lanceolate or narrowly oblanceolate.	Oblanceolate, elliptic-ovate or oblong
Leaf length:width ratio	1:0.09	1:0.31
Leaf apex	Narrowly acute	Broadly acute to obtuse
Corolla colour	Whitish-pink	Creamy-white
Corolla trichomes	Stalked, glandular trichomes present	Stalked glandular trichomes absent
Capsule surface	Not colliculate	Colliculate
Seed shape	Orbicular	Ovate
Habitat	Hill slopes with granite outcrops; full sun; stony soils	Hill slopes with relatively dense vegetation growth; semi-shade; humus-rich, well-drained sandy soils

Crabbea galpinii and *C. pedunculata* are no longer regarded as subspecies of *C. nana*, because in all four identification keys (Table 8.2) both species were placed in their own distinct group separate from *C. nana*. Additionally, the characters that were used in Table 8.2 are comparable and of equal weight in terms of delimiting species in one of two main groups. However, characters used (Table 8.2) to delimit *C. cirsioides* from *C. nana* could not clearly recognise the two species as separate species. Instead, an anatomical and morphological overlap was observed. In addition, molecular data does not support the recognition of *C. nana* as distinct from *C. circioides.* Both species were validly published by Nees von Esenbeck (1841) but *C. cirsioides* (= *R. cirsioides*) was listed before *C. nana* (= *R. nana*), therefore, the name *C. nana* is no longer an accepted name but rather a synonym of *C. cirsioides*.

In conclusion, anatomical, macromorphological, micromorphological and molecular data were used in the systematic study of *Crabbea* in southern Africa. Identification keys were constructed using anatomy, macromorphology and micromorphology in an attempt to recognize and identify all the southern African species of *Crabbea*. Each key allowed recognition of major species groups. In some cases, more than one species was grouped together - indicating that no further character could be used to delimit the certain *Crabbea* species and/or there is a great similarity among species of the group.

This study revised the southern African species of *Crabbea*, only. The suite of macromorphological characters used is useful enough to identify each *Crabbea* species under study. Live specimens were collected for all the southern African species of *Crabbea* and were used in conjunction with herbarium vouchers. As a result, a robust *Crabbea* morphological template is established/generated and can be readily used to study the remaining *Crabbea* species outside southern Africa.

Prior to this study, molecular data for *Crabbea* was severely lacking. During the course of this study a significant number of chloroplast DNA sequences (*rps*16 and *trn*L-*trn*F) were successfully generated for each southern African *Crabbea* species from multiple populations. The molecular data could resolve the evolutionary relationships and species dynamics to some extent.

This study effectively clarified and resolved the nomenclature of the southern African *Crabbea* species. *Crabbea* in southern Africa is represented by seven species, of which three are endemic to the region. *Crabbea acaulis* and *C. cirsioides* have the broadest distribution of all *Crabbea* species in South Africa. However, *C. galpinii* has the smallest distribution range. It was also found that, within the distribution range of *C. galpinii,* the area is highly transformed through agricultural activities and urbanisation, indicating that more research should be done to determine whether this species has a conservation status of least concern, vulnerable or endangered.

8.4 Biogeography and taxonomic relationships in the Barlerieae and future work

Acanthaceae *sensu lato* established about 82 million years ago (mya) during the Cretaceous Period and Barlerieae first appeared around the Oligocene, about 31 mya, and experienced prominent lineage diversification around the mid Miocene Period, about 15 mya (Tripp and McDade, 2014). McDade *et al* (2008) concluded that the BAWN (Barlerieae, Andrographideae, Whitfielidieae and *Neuracanthus*) Clade shares a complex history of biogeographic events, particularly in the Old World (OWD) where the four lineages most likely originated.

Unlike other OWD Acanthaceae lineages such as Acantheae (McDade *et* al., 2005), Justicieae, Isoglossinae (Kiel *et al*., 2006), *Ruellia* (Tripp, 2007), *Tetramerium* Nees (Daniel *et al*., 2008) and Thunbergioideae (Borg *et al.*, 2008), Barlerieae diversified very little after the tribe had dispersed to the New World (NWD) (Balkwill and Balkwill, 1997; McDade *et al*., 2008). *Barleria* is largely found in the OWD, spanning from the tropical and subtropical regions of the Far East, to southern Asia, Madagascar and a significant portion of Africa. *Crabbea*, *Acanthostelma, Golaea* and *Lasiocladus* are also largely OWD lineages (McDade *et al*., 2008). However, Benoist (1911) and Wasshausen and Wood (2004) found that NWD *Lophostachys* could be subsumed under *Lepidagathis*, which is largely an OWD genus, with some NWD species. Along with *Barleria*, some species of *Lepidagathis* can be found on Madagascar, while *Lasiocladus* is restricted to Madagascar. Benoist (1967) and Onjalalaina and Darbyshire (2016) record the close

affinity between *Crabbea* and the associated genera in Madagascar. However, biogeography, taxonomy and phylogeny of this taxonomic group remain open for further systematic studies, despite research that has been done.

The primary aim of any taxonomic study is to revise the taxa under investigation and generate clear and descriptive species and genus boundaries. However, a taxonomic account does not only clarify the issues and/or problems initially identified within a particular taxonomic group. Instead, a taxonomic revision reveals areas in need of further research. That being said, the following areas have been identified for further research in *Crabbea* and allied genera:

Firstly, for a proper circumscription of the entire genus and each species complex, especially those that have a broad distribution range (*C. cirsioides*, *C. ovalifolia* and *C. velutina*), a single taxonomic revision encompassing the entire genus is recommended. Once the entire genus has received taxonomic treatment, it is highly advisable that genera closely associated with *Crabbea* should be revised and compared to *Crabbea* to effectively determine whether *Crabbea* should be expanded or retained as is. Associated genera that should be revised, are those from Madagascar, mentioned by Onjalalaina and Darbyshire (2016).

Secondly, in order to establish the significance of the various character sets (leaf micromorphology, cystoliths and pollen micromorphology) and their associated character states at genus and species level, all the *Crabbea* species must be examined. Once the taxonomic significance of the various character sets has been identified for *Crabbea*, genera closely allied to *Crabbea* should receive the same treatment.

Thirdly, the collection of sequences from the remaining *Crabbea* species and closely related genera is urgently needed (McDade *et al*., 2008; Onjalalaina and Darbyshire, 2016). The optimisation of amplification and sequencing of ITS from *Crabbea* still needs considerable attention. Once ITS sequences have been generated for *Crabbea*, the techniques used obtain ITS should serve as a basis for the remaining genera closely associated with *Crabbea*. The availability of ITS sequences will improve the resolution of genus and species boundaries and provide insight on the evolutionary dynamics

within Barlerieae. The molecular work of McDade *et al*. (2008) can serve as a basis for further molecular work within Barlerieae, especially the gene regions that have not been included in this study (*trn*S-*trn*G and *trn*T-*trn*L). Next generation sequencing (NGS) can be considered to help resolve the phylogeny within Barlerieae. Using NGS, accurate sequences of the entire Barlerieae genome will be quickly generated at a cost-effective manner. Thus, reducing time and money spend in an attempt to optimize and sequence one gene region at a time.

Lastly, the biogeography of *Crabbea* and allied genera has not received proper investigation. This will be achieved upon completion of complete taxonomic revision of the entire *Crabbea* and associated genera; by studying herbarium vouchers and making notes on Barlerieae hotspots across the world and obtaining a well resolved phylogeny.

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APPENDIX 1

List of herbarium specimens, on loan and scans, examined during the course of this study

Crabbea acaulis

South Africa

—24°43'48" S, 30°30'34" E: Limpopo, Sekhukhuneland, 20 January 2005, *Maserumule, M.K*. *273* (PRE [PRE0843219-0]).

—24°52'32" S, 30°45'34" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *126* (BLFU).

—24°52’50” S, 30°45’38” E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, turn off to Vaalhoek Rd, about 34 km before Graskop, 18 November 2015, *de Gouveia*, *A*. *159* (BLFU); *de Gouveia*, *A*. *160* (BLFU).

—24°52'51" S, 30°45'39" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *118* (BLFU); *de Gouveia*, *A*. *119* (BLFU).

—24°52'53" S, 30°45'37" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *121* (BLFU); *de Gouveia*, *A*. *122* (BLFU); *de Gouveia*, *A*. *123* (BLFU).

—25°13'07" S, 30°40'29" E: Mpumalanga, Sabie, unnamed dirt road, south of Sabie, 26 March 2015, *de Gouveia*, *A*. *133* (BLFU).

—25°18’20” S, 30°29’59” E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Kransview Road, 24 November 2015, *de Gouveia*, *A*. *180* (BLFU).

—25°25'26'' S, 30°15'30'' E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, past rock pool and opposite chalet nr. 2, on foothill of mountain slope, near fence, 22 March 2015, *de Gouveia*, *A*. *76* (BLFU).

—25°25'33'' S, 30°15'32'' E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, past rock pool and opposite chalet nr. 2, on foothill of mountain slope, near fence, 22 March 2015, *de Gouveia*, *A*. *77* (BLFU); *de Gouveia*, *A*. *78* (BLFU); *de Gouveia*, *A*. *79* (BLFU); *de Gouveia*, *A*. *80* (BLFU).

—25°32'04'' S, 30°07'14'' E: Mpumalanga, Belfast, Elandskloof Farm, 14 March 2016, *Steyn, H.M*. *2089* (BLFU).

—26°11'41'' S, 28°03'19'' E: Gauteng, Doornfontein Johannesburg, *Gilfillian, D.F. 6245* (GRA [GRA0002729-0]); *Gilfillian, D.F. 6245* (PRE [PRE0125197-0]).

—26°12'14'' S, 28°04'21'' E: Gauteng, Jeppestown Ridges, near Johannesburg, *Gilfillian in Herb. Galpin. 6245* (K, holotype [K000394693]).

—26°20'01'' S, 26°09'59'' E: North West, Lichtenburg, Hakboslaagte, 26 April 1948, *Kinges, H.G*. *1710* (PRE [PRE0125205-0]).

—26°46'33'' S, 29°59'31'' E: Mpumalanga, Amersfoort, N11, turn off into Familiehoek Road, between Ermelo and Amersfoort, 28 March 2015, *de Gouveia*, *A*. *149* (BLFU); *de Gouveia*, *A*. *150* (BLFU); *de Gouveia*, *A*. *151* (BLFU); *de Gouveia*, *A*. *152* (BLFU); *de Gouveia*, *A*. *153* (BLFU); *de Gouveia*, *A*. *154* (BLFU); *de Gouveia*, *A*. *181* (BLFU).

—26°50'28'' S, 30°31'29'' E: Mpumalanga, Piet Retief, Iswepe locality, 6 March 1949, *Sidey, J.L. 1619* (PRE [PRE0125207-0]).

—27°01'35'' S, 29°51'27'' E: Mpumalanga, Amersfoort, Uitspanning, Platberg Farm, 4 February 1986, *Turner, B.J. 793* (PRE [PRE0683225-0]).

—27°11'38'' S, 28°51'14'' E: Free State, Frankfurt, 9 km north of Cornelia on road to Anniesgift, 28 January 1988, *Crosby, M.J.A.W. 604* (PRE [PRE0723125-0]).

—27°15'13" S, 28°29'36" E: Free State, Frankfurt, East of Petrus Steyn, Farm Modus Vivendi (Mrs. van Wyk), 25 January 1988, *Crosby, M.J.A.W. 490* (PRE [PRE0722978-0]).

—28°15'50" S, 28°22'00 E: Free State, Bethlehem, Vicinity of boundary between farms Poorte and Trekpad, about 6 miles [east] south-east of Bethlehem on road to Clarens, 13 February 1967, *Scheepers, J.C. 1363* (PRE [PRE0125214-0]).

—28°49'59" S, 26°30'10" E: Free State, Brandfort, Landboukollege, Glen, 23 April 1971, *van der Berg, J.A. 3903* (PRE [PRE0125216-0]).

—29°00'33" S, 29°54'32" E: KwaZulu-Natal, Harrismith, 4 miles east of Estcourt, 19 March 1970, *Strey, R.G*. *9747* (PRE [PRE0123920-0]).

—29°00’10” S, 29°52’29” E: KwaZulu-Natal, Estcourt, Bushman's River, 01 February 1945, *Grass, G.J*. *23* (NU [NU0039884]).

—29°10’56” S, 26°10’52” E: Free State, Bloemfontein, near Kimberly rail-way line, January 1935, *Moraik, J. 4872* (BLFU).

—29°45’22” S, 29°30’09” E: KwaZulu-Natal, Himeville, 17 January 1944, *Thiewel, W.O*. *74* (NU [NU0039882]).

—29°47’18” S, 29°46’13” E: KwaZulu-Natal, Bulwer, Sunset Farm, 24 February 1990, *Vos, W*. *41* (NU [NU0039883]).

—31°42'26" S, 27°03'09" E: Eastern Cape, Lady Fere, February 1919, *Pegler, A.M. 1684* (PRE [PRE0123931-0]).

Crabbea angustifolia

Botswana

—25°43’23” S, 25°17’17” E: Betschuanaland, near Mafikeng [North West Province], *Bolus, F. 6413* (BOL, neotype [BOL138566]).

South Africa

—23°55'08'' S, 29°24'03'' E: Limpopo, Polokwane, 25 January 1954, *Meeuse, A.J. 9150* (PRE [PRE0126695-0]).

—24°12'06'' S, 28°57'31'' E: Limpopo, Mokopane, January 1909, *Leendertz, R. 6561* (PRE [PRE0125144-0]).

—24°10'30'' S, 29°21'40'' E: Limpopo, Polokwane, between Zebediela and Marabastad, NW of Chuniespoort, 20 January 1955, *Meeuse, A.D.J. 9589* (PRE [PRE0126714-0]).

—24°50'57” S, 28°34'08'' E: Limpopo, Bela-Bela, 13 km from Bela-Bela on Nylstroom road, 18 January 1988, *Immelman, K.L. 131* (PRE [PRE0732035-0]).

—23°32'22'' S, 29°54'08'' E: Limpopo, Morebeng, Dwars River Farm, 372 KT, 18 February 1986, *Krynauw, S. 1116* (PRE [PRE0690824-0]).

—25°41’34” S, 29°35’15” E: Mpumalanga, Middleburg, Along Highway 555, about 60 km SW of Steelpoort, *Daniel, T.F., Balkwill, K.* and *Butterwick, M*. *9370* (BR [BR938362]).

—25°06'12'' S, 30°26'05'' E: Mpumalanga, Lydenburg, December 1895, *Wilms, F. 1205* (PRE [PRE0123942-0]).

—25°26’28” S, 29°18’48” E: Mpumalanga, Groblersdal, Loskop Dam Nature Reserve, *Steyn, H.M. 2294* (BLFU).

—25°36'56'' S, 29°18'39'' E: North West, Brits, "Beestekraal" Game Reserve,11 February 1989, *Barker, N.P. 535* (PRE [PRE0732028-0]).

—25°39’43” S, 28°13’22” E: Gauteng, Pretoria, *Phipps, V*. *7* (NU [NU0039887]); 9 January 1913, *Theiler, A.C. 9320* (PRE [PRE0740033-0]).

—25°46’15’’ S, 27°47’16’’ E: North West, Magaliesberg, *Burke, J. 405* (K, holotype (K000394685); (PRE, isotype [PRE0126663-0]).

—25°47'53" S, 29°22'44" E: Mpumalanga, Middleburg, R555, turn off to Olifants River Lodge, ± 2 km from turnoff, 21 March 2015, *de Gouveia, A. 74* (BLFU); *de Gouveia, A. 75* (BLFU).

—25°50'42" S, 27°24'47" E: North West, Rustenburg, Hamerkop Private Nature Reserve, *Burrows, J.E.* and *Burrows, S.E. 14638* (BLFU).

—25°39'48" S, 27°11'17" E: North West, Rustenburg, April 1916, *Rogers, F.A.* 18718 (PRE [PRE0125146-0]).

—25°44'14" S, 28°12'25" E: Gauteng, Pretoria, Prinshof Veld Reclamation Camp, 13 February 1935, *Liebenberg, L.C.C. 3391* (PRE [PRE0125153-0]).

—25°44'49" S, 27°13'14" E: North West, Rustenburg, Rustenburg Nature Reserve, 5 February 1971, *Jacobsen, N.H.G. 1752* (PRE [PRE 0125165-0]).

—25°38'40" S, 29°40'18" E: Mpumalanga, Middelburg, About 5 km from Middelburg on Belfast road, at train bridge, next to river, 1 April 1997, *Meyer, J.J. 1161A* (PRE [PRE0562938-0]).

—26°42'29" S, 26°59'07" E: North West, Potchefstroom, 18 October 1944, *Louw, W.J. 1299* (PRE [PRE0126681-0]).

—25°55'53" S, 25°36'12" E: North West, Mahikeng, 12 February 1982, *Gubb, A.A. 238/82* (PRE [PRE0789078-0]).

—26°30'49" S, 29°57'11" E: Mpumalanga, Ermelo, Nooitgedagt Farm, 5 February 1927, *Henrici, M.G.A.1558* (PRE [PRE0126683-0]).

—28°16'53" S, 27°09'37" E: Free State, Winburg, Willem Pretorius Game Reserve, below rest camp, 9 April 1962, *Leistner, O.A. 2970* (PRE [PRE0125168-0]).

Swaziland

—26°30'09" S, 31°25'45" E: Mbabane, Manzini, Tulwane, 7 February 1964, *Compton, R.H*. *31934* (PRE [PRE0701214-0]).

Crabbea cirsioides

South Africa

—23°28'43'' S, 30°56'09" E: Limpopo, Morebeng, Elim-Molima Road north of Soekmekaar, 9 February 1976, *Brenan, J.P.M*. *14177* (PRE [PRE0514904-0]).

—24°42'34'' S, 28°22'41'' E: Limpopo, Nylstroom, Schuinzkloof Farm, 5 March 1987, *Westfall, R.H*. *2250* (BR [BR16150935]).

—24°17'14" S, 29°15'47" E: Limpopo, Bela-Bela, Towoomba Pasture Research Station, 3 February 1926, *Codd, L.E.W*. *3656* (PRE [PRE0126806-0]).

—24°29'28" S, 29°24'41" E: Limpopo, Zebediela, January 1909, *Leendertz, R*. *6570* (PRE [PRE0123936-0]).

—24°33'58" S, 27°25'17" E: Limpopo, Thabazimbi, Zimthabi Resort, 7 January 1984, *Immelman, K.L*. *400* (PRE [PRE0634827-0]).

—24°52'32" S, 30°45'34" E: Mpumalanga, Pilgrim's Rest, next to gravel road leading out of Pilgrim's Rest, before bridge, 25 March 2015, *de Gouveia*, *A*. *124* (BLFU); *de Gouveia*, *A*. *125* (BLFU); 18 Novembeer 2015, *de Gouveia*, *A*.,*161* (BLFU); *de Gouveia*, *A*. 1*62* (BLFU).

—24°29'58" S, 28°43'31" E: Limpopo, Naboomspruit District, Mosdene, *Galpin, E.E. 273* (PRE [PRE0126795-0]).

—24°54'09" S, 30°45'18" E: Mpumalanga, Pilgrim's Rest, on town hill, 20 February, *Galpin E.E. 14366* (PRE [PRE0126815-0]).

—25°00'02" S, 31°35'59" E: Mpumalanga, Skukuza, Kruger National Park, Nkhulu Exclosures, 3 December 2010, *van Coller, H., Siebert, H.* and *Siebert, F*. *4259* (PRE [PRE0858109-0]); 6 February 1962, *Schlieben, H-J.E*. *9394* (PRE [PRE 0126794-0]).

—25°07'04'' S, 30°45'51'' E: Mpumalanga, Sabie, R37 to Mashinging, opposite pine plantations, about 5 km south of Sabie, 26 March 2015, *de Gouveia*, *A*. *127* (BLFU); *de Gouveia*, *A*. *128* (BLFU); *de Gouveia*, *A*. *129* (BLFU).

—25°57'83'' S, 26°38'80'' E: North West, Groot Marico, Rooderand Farm, 20 January 1970, *Carter, J. 917* (PRE [PRE0126720-0]).

—25°04'57'' S, 30°28'05'' E: Mpumalanga, Lydenburg, January 1895, *Wilms, F.*, *1206* (PRE [PRE0126819-0]).

—25°13'07'' S, 30°40'29'' E: Mpumalanga, Sabie, unnamed dirt road, south of Sabie, 26 March 2015, *de Gouveia*, *A*. *130* (BLFU); *de Gouveia*, *A*. *131* (BLFU); *de Gouveia*, *A*. *132* (BLFU).

—25°10'53'' S, 29°23'59'' E: Limpopo, Groblersdal, next to golf course between fence and 400 yards from road, 1 February 1978, *Venter, S*. *2987* (PRE [PRE0528599-0]).

—25°09'33'' S, 29°23'51'' E: Limpopo, Groblersdal, Mantrombi, *du Toit, G.J*. *2266* (PRE [PRE0581044-0]).

—25°18'20'' S, 30°29'59'' E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Kransview Road, 24 November 2015, *de Gouveia*, *A*. *175* (BLFU).

—25°20'02'' S, 30°29'15'' E: Buffelskloof Nature Reserve, along Klipspringer Road, below Breakfast Rock, 22 March 2015, *de Gouveia*, *A*. *90* (BLFU).

—25°25'28'' S, 30°15'26'' E: Mpumalanga, Verlorenkloof Private Nature Reserve, Welgedacht Farm, path leading to fence, after rock pool, near chalet nr. 2, 22 March 2015, *de Gouveia*, *A*. *85* (BLFU); *de Gouveia*, *A*. *178* (BLFU).

—25°25'31'' S, 30°15'26'' E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, Welgedacht Farm, before rock dam, next to path near car park and chalet nr. 2, 22 March 2015, *de Gouveia*, *A*. *87* (BLFU); *de Gouveia*, *A*. *88* (BLFU); *de Gouveia*, *A*. *89* (BLFU).

—25°28’54’’ S, 29°19’27’’E: Mpumalanga, Groblersdal, Loskop Dam Nature Reserve, 6 March 2016, *Steyn, H.M. 2288* (BLFU).

—25°34’33’’ S, 31°01’45’’ E: Mpumalanga, Nelspruit, Nelspruit Highlands Small Holdings, 21 November 2015, *de Gouveia*, *A*. *172* (BLFU).

—25°39'10" S, 28°15'00’’ E: Gauteng, Pretoria, Doornpoort Extension, fields next to watercourse where drainage line crosses Amandelboom Road, 3 January 2004, *Bester, S.P. 4621* (PRE [PRE0755738-0]).

—25°26'03" S, 28°08'25" E: Gauteng, Pretoria, near Pretoria soutpan [salt pan], 6 February 1929, *Leeman, A.C*. *27276* (PRE [PRE0126809-0]).

—25°47'58" S, 30°47'07" E: Mpumalanga, Barberton, R38, between Barbeton and Badwater, 28 March 2015, *de Gouveia*, *A*. *142* (BLFU); *de Gouveia*, *A*. *143* (BLFU); *de Gouveia*, *A*. *144* (BLFU).

—25°54'22" S, 26°08'70" E: North West, Zeerust, January 1912, *Leendertz, R. 11521* (PRE [PRE0125198-0]).

—25°39'24" S, 28°20'08" E: Gauteng, Pretoria, Roodeplaat, 21 March 1906, *Leendertz, R*. *8615* (PRE [PRE0635202-0]); February 1916, *Beyer, H.G*. *59340* (PRE [PRE0800882-0]).

—25°39'50" S, 28°15'00" E: Gauteng, Pretoria, 21 January 1913, *Theiler, A.C*. *26673* (PRE [PRE0126808-0]).

—25°45'58" S, 31°02'26" E: Mpumalanga, Barberton, February 1932, *Holt, W.E*. *165* (PRE [PRE0126824-0]).

—26°06'10" S, 31°16'00’’ E: Mbabane, Malandzela area, road to Maphallaleni, 14 km from Nyokane (tar road), 26 January 1994, *Hobson, S.R*. *2120* (PRE [PRE0808999-0])

—26°29'13" S, 28°18'37" E: Gauteng, Johannesburg, Rooikop, 10 April 1925, *Smuts, J.C. 1654* (PRE [PRE0126804-0]).

—26°32'02" S, 31°11'71" E: Mbabane, 17 March 1959, *Compton, R.H*. *28651* (PRE [PRE0701797-0]).

—26°44'51" S, 30°00'10" E: Mpumalanga, Ermelo, Familiehoek Road between Ermelo and Uitspanning, next to dirt road and fence, about 20 m from Vaal River Bridge, 28 March 2015, *de Gouveia*, *A*. *146* (BLFU); *de Gouveia*, *A*. *179* (BLFU).

—26°59'18'' S, 27°15'10'' E: Free State, Vredefort, Spitskop, 29 March 1983, *du Preez, P.J. 28* (BLFU).

—26°57'21'' S, 29°12'44" E: Mpumalanga, Standerton, January 1912, *Leendertz, R*. *11082* (PRE [PRE0125200-0]).

—27°08'11" S, 31°59'32" E: 17 KwaZulu-Natal, Ingwavuma, January 1963, *Strey, R.G*. *4679* (PRE [PRE0126743-0]).

—27°39'14'' S, 27°11'47'' E: Free State, Kroonstad, West of Vals River, January 1929, *Pont, J.W*. *4021* (BLFU).

—27°26'18" S, 29°52'02" E: KwaZulu-Natal, Charlestown, beginning of Langsneck Pass, 5 km past Charlestown, March 1937, *Arnold, T.H*. *203* (PRE [PRE0126724-0]).

—27°40'56" S, 30°17'39" E: KwaZulu-Natal, Utrecht, January 1916, *Wahl, A*. *15531* (PRE [PRE 0126731-0]).

—28°04'41" S, 32°08'32" E: KwaZulu-Natal, Hlabisa, Hluhluwe Game Reserve, 8 April 1959, *Guy, R.D*. *48* (PRE [PRE0125186-0]); 30 November 1953, *Ward, C.J. 1879* (PRE [PRE0125169-0]).

—28°21'43" S, 29°22'00" E: Free State, Harrismith, Near van Reenen's Pass, October 1927, *Pont, J.W*. *197* (PRE [PRE0126769-0]).

—28°44'05'' S, 29°20'45'' E: KwaZulu-Natal, Bergville, April 1975, *van Rensburg, G.A*. *4* (NU [NU0039902]).

—28°41'13'' S, 31°42'21'' E: KwaZulu-Natal, Nogeya, Lower Umfolozi, Umhlatuzi Valley, 31 May 1967, *Venter, H.J.T*. *3714* (BLFU).

—28°53’18’’ S, 31°2654’’ E: KwaZulu-Natal, Eshowe, 28 February 1937, *Gerstner, J*. *2259* (NU [NU0039917]).

—28°55’24’’ S, 27°17’17’’ E: Free State, Excelsior, Korannaberg, 15 April 1987, *du Preez, P.J. 823* (BLFU); *du Preez, P.J. 824* (BLFU).

—29°06’37’’ S, 26°13’27’’ E: Free State, Bloemfontein, top of Naval Hill, 00 March 1917, *Potts, G*. *2576* (BLFU).

—29°35’59’’ S, 30°20’42’’ E: KwaZulu-Natal, Pietermaritzburg, Villiers Drive, 13 February 1988, *Kennedy, H*. *236* (BR [BR0031774]).

—29°52’21’’ S, 30°30’09’’ E: KwaZulu-Natal, Eston, January 1944, *Schuter, R.L*. *15* (NU [NU0039904]).

—29°54’21 S, 30°56’15’’ E: KwaZulu-Natal, Durban, 10 February 2002, *Styles, D*. *496* (NU [NU0009708-0]).

—30°24'26" S, 29°54'43’’ E: KwaZulu-Natal, Ndlovini Village, about 15 km N.E. of Harding, 13 February 2008, *Ngwenya, A.M*. *3170* (PRE [PRE0858150-0]).

—30°21'15" S, 28°48'37" E: Eastern Cape, Matatiele, January 1884, *Tyson, W*. *1633* (PRE [PRE0126751-0]).

—31°44’23’’ S, 28°56’30’’ E: Eastern Cape, Umtata, Road between Port St. Johns and Umtata, 26 January 2016, *Steyn, H.M*. *2287* (BLFU).

—31°48'46" S, 28°44'58" E: Eastern Cape, Mqanduli, 00 *s.n.*1900, *Pegler, A.M*. *555* (PRE [PRE0126750-0]).

—31°57'57" S, 26°49'41" E: Eastern Cape, Queenstown, 38 km E. by S.E. of Queenstown, 21 February 1955, *Acocks, J.P.H*. *17937* (PRE [PRE0123930-0]).

—32°30'23" S, 28°00'50" E: Eastern Cape, Tambukiland, rising banks of the Zwarte-Key River, *Ecklon, C.F. s.n.* (GZU, lectotype [GZU000261868], syntype [GZU000249783]).

—32°30'25'' S, 28°00'28'' E: Eastern Cape, Tambukiland, *Ecklon, C.F.* and *Zeyher, C.L.P. s.n.* (S [S09-2553]).

—32°49'30'' S, 26°51'30'' E: Eastern Cape, Alice, Near Tukulu Farm, S.E. of town, 7 January 1984, *Phillipson, P.B*. *807* (PRE [PRE0726066-0]).

—32°88'95'' S, 27°41'18'' E: Kwazulu-Natal, Ukomaas, *Wood, J.M*. *8141* (PRE [PRE0126768-0]).

—32°57'45'' S, 26°45'04'' E: Eastern Cape, Fort Beaufort to the Kat River, foot of Chumi Mountains, *Ecklon, C.F. s.n.* (GZU, holotype [GZU000249781]).

—33°19'39'' S, 26°32'48'' E: Eastern Cape, Grahamstown, Belmont Valley, 7 December 1926, *Britten, L.L*. *5483A* (PRE [PRE0123923-0]).

—Coordinates: unkown, Natal [KwaZulu-Natal], *Williamson, J. s.n.*(K, holotype [K000394688], isotype [K000394690]).

Swaziland

—25°45'00'' S, 31°25'00'' E: [Mbabane], Horo, *Galpin, E.E. 1265* (K, holotype [K000394692]; PRE, isotype [PRE0125220-0]).

—Coordinates: unknown *Ecklon, C.F. s.n.* (S [S09-2556]).

—Coordinates: unknown *Ecklon, C.F. s.n.* (S [S09-2557]).

Crabbea galpinii

South Africa

—25°28'14'' S, 31°25'25'' E: Mpumalanga, Khandizwe, 6 December 1976, *Nel, P.J*. *6041* (PRE [PRE0635203-0]).

—25°30'55" S, 31°30'57" E: Mpumalanga, Nelspruit**,** Nelspruit Highlands Small Holdings, 22 November 2015, *de Gouveia, A. 171* (BLFU).

—25°31’01’’ S, 30°54’54’’ E: Mpumalanga, Nelspruit, October 1917, *Breyer, H.G*. *17696* (PRE [PRE0125194-0]).

—25°32’37’’ S, 30°58’24’’ E: Mpumalanga, Nelspruit, 28 Renosterkop, Uitkyk Road, 23 November 1982, *Onderstall, J. 953* (PRE [PRE0631681-0]).

—25°32’57” S, 31°58’35” E: Mpumalanga, Nelspruit, Renosterkop, next to path, 20 November 2015, *de Gouveia, A. 169C* (BLFU).

—25°49’17” S, 31°02’20” E: Mpumalanga, Barberton, Moody's Estate, 21 November 2015, *de Gouveia, A.* 170 (BLFU).

—25°33'08" S, 30°59'47’’ E: Mpumalanga, Nelspruit, Renosterkop 28, South of Nelspruit, 8 December 1980, *Kluge, J.P. 2324* (PRE [PRE0611211-0]).

—25°47'04" S, 31°02'01" E: Mpumalanga, Barberton, *Galpin, E.E. 1148* (K, holotype [K000394679]; PRE, isotype [PRE0125191-0]).

—25°47'04" S, 31°01'40" E: Mpumalanga, Barberton, 27 September 1930, *Liebenberg, L.C.C. 2648* (PRE [PRE0125193-0]).

—25°46'35" S, 31°00'35" E: Mpumalanga, Barberton, December 1916, *Pott-Leendertz, R. 5663* (PRE [PRE0125195-0]).

—28°50'14" S, 31°54'24" E: KwaZulu-Natal, Nogeya, Umhlatuzi Valley, 31 May 1967, *Venter, H.J.T. 3714* (PRE [PRE0125188-0]).

Swaziland

—26°05’05’’ S, 31°16’01’’ E: Hhohho, Nyokane, 26 January 1994, *Hobson, S.R*., *2120* (PRE [PRE0808999-0]).

—26°30'14" S, 31°24'00" E: Mbabane, Manzini, Hhelehhele, 12 September 2002, *Dlamini, G.M. 2690* (PRE [PRE0718342-0]).

Crabbea ovalifolia

Angola

—14°51'15'' S, 21°22'37'' E: [Moxico Province], [Lumbala], Ninda River, *Serpa Pinto 21* (LISU, holotype [LISU59523ANG]).

South Africa

—22°51'17'' S, 30°14'29'' E: Limpopo, Mazwimba, 22 May 1982, *van Wyk, A.E*. 5653 (PRE [PRE0631682-0]).

—24°34'48'' S, 31°20'21'' E: Mpumalanga, Gazabkulu, Manyeleti Reserve, 31 December 1991, *Fabian, A. 1419* (PRE [PRE0823269-0]).

—23°38'11'' S, 30°12'27'' E: Limpopo, Duiwelskloof, 7 km from Duiwelskloof on road to Polokwane, 20 March 1980, *Germishuizen, G*. *1334* (PRE [PRE0559977-0]).

—23°54’06'' S, 30°04'08'' E: Limpopo, Letaba, old road about opposite where Politsi Tzaneen Road joins tar road, 17 February 1958, *Scheepers, J.C.* 120 (PRE [PRE0126797-0]).

—24°24'44'' S, 30°04'55'' E: Mpumalanga, Thokwane, 16 km from Chukaja on road to Thokwane,18 January 1953, *van der Schijff, H.P*. *1795* (PRE [PRE0125180-0]).

—24°42'56'' S, 28°23'53'' E: Limpopo, Modimolle, January 1902, *Galpin, E.E*. *6511*(PRE [PRE0126710-0]).

—25°00’12’’ S, 31°35’42’’ E: Mpumalanga, Skukuza, 16 km from Skukuza, 18 January 1953, *van der Schijff, H.P. 1795* (PRE [PRE0125180-0]).

—25°21’35” S, 31°53’36’’ E: Mpumalanga, Komatipoort, Crocodile Bridge, 9 November 1954, *van der Schijff, H.P. 3531* (PRE [PRE0125177-0]).

—25°26'00'' S, 31°46'30'' E: Mpumalanga, Marloth Park, N4 turnoff to Marloth Park, about 200 m adjacent to cement track, east of the road, 27 March 2015, *de Gouveia*, *A*. *134* (BLFU); *de Gouveia*, *A*. *135* (BLFU); *de Gouveia*, *A*. *136* (BLFU); *de Gouveia*, *A*.

137 (BLFU); *de Gouveia*, *A*. *138* (BLFU); *de Gouveia*, *A*. *139* (BLFU); *de Gouveia*, *A*. *140* (BLFU); 22 November 2015, *de Gouveia*, *A*. *173* (BLFU).

—25°26’18’’ S, 31°46’44’’ E: Mpumalanga, Marloth Park, N4 turnoff to Marloth Park, between N4 and railway, next to bridge, 22 November 2015, *de Gouveia*, *A*. *174* (BLFU).

—25°21'00'' S, 31°46'00'' E: Mpumalanga, Seekoeigat, November 1952, *van der Schijff, H.P. 1207* (PRE [PRE0125177-0]); 6 January 1955, *van der Schijff, H.P. 4156* (PRE [PRE0125179-0]).

—25°21'38'' S, 31°46'35'' E: Mpumalanga, Marloth Park, 9 November 1954, *van der Schijff, H.P*. *1207* (PRE [PRE0125177-0]); 14 November 1996, *van der Schijff, H.P*. *4156* (PRE [PRE0125179-0]).

—25°47’06’’ S, 31°47’39’’ E: Mpumalanga, Nkomazi West, Mahushe Shongwe Game Reserve, 14 November 1996, *Estes, L.*, 14 (PRE [PRE575436-0]).

—25°35'59'' S, 27°24'17'' E: North West, Rustenburg, Beestekraal Farm, 7 March 1985, *Welman, W.G.* 566 (PRE [PRE0745588-0]).

—25°38'34'' S, 28°21'41'' E: Gauteng, Pretoria, Roodeplaatdam Nature Reserve, 27 December 1979, *van Rooyen, N. 2224* (PRE [PRE0663133-0]).

—25°38'29'' S, 28°12'51'' E: Gauteng, Pretoria, 23 March 1929, *Mogg, A.O.D. 40342* (PRE [PRE0126780-0]).

—25°41'30'' S, 28°12'18'' E: Gauteng, Pretoria, Trigaardspoort, 6 February 1938, *Repton, J.E. 1220* (PRE [PRE0126704-0]).

Crabbea pedunculata

South Africa

—24°54'09'' S, 30°45'18'' E: Mpumalanga, Pilgrim's Rest, December 1919, *Rogers, F.A.* 20738 (PRE [PRE0125170-0]).

—24°55'31'' S, 30°50'13'' E: Mpumalanga, Graskop, 3 December 1937, *Galpin, E.E. 14575* (PRE [PRE0125173-0]).

—25°16'42'' S, 30°30'25'' E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, western section of Upper Forest Trail, 23 March 2015, *de Gouveia*, *A*. *98* (BLFU); *de Gouveia*, *A*. *99* (BLFU).

—25°16'43'' S, 30°30'28'' E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Helichrysum Trail (Upper Forest Trail), near stream, N.W. of Andrew's Falls, 23 March 2015, *de Gouveia*, *A*. *94* (BLFU); *de Gouveia*, *A*. *95* (BLFU); *de Gouveia*, *A*. *96* (BLFU); *de Gouveia*, *A*. *97* (BLFU); *de Gouveia*, *A*. *176* (BLFU).

—25°25’21’’ S, 30°15’29’’ E: Mpumalanga, Lydenburg, Verlorenkloof Nature Reserve, Welgedacht Farm, S.W. face of mountain slope on waterfall trail, at forest margin, 26 November 2015, *de Gouveia*, *A*. *177* (BLFU).

—25°25'15'' S, 30°15'28'' E: Mpumlanga, Lydenburg, Verlorenkloof Private Nature Reserve. Welgedacht Farm, 40 km directly S.W. of Lydenburg on road R36, 28 November 2008, *Bester, S.P*. *8669* (PRE [PRE0851588-0]).

—25°25'16'' S, 30°15'26'' E: Mpumalanga, Lydenburg, Verlorenkloof Private Nature Reserve, Welgedacht Farm, past rock pool and opposite chalet nr. 2 en route to waterfall, 22 March 2015, *de Gouveia*, *A*. *81* (BLFU); *de Gouveia*, *A*. *82* (BLFU); *de Gouveia*, *A*. *83* (BLFU); *de Gouveia*, *A*. *84* (BLFU).

—25°16'43'' S, 30°30'28'' E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Helichrysum Trail (Upper Forest Trail), near stream, N.W. of Andrew's Falls, 23 March 2015, *de Gouveia*, *A*. *94* (BLFU)

—25°25’21’’ S, 30°15’29’’ E: Mpumalanga, Lydenburg, Verlorenkloof Nature Reserve, Welgedacht Farm, S.W. face of mountain slope on waterfall trail, at forest margin, 26 November 2015, *de Gouveia*, *A*. *177* (BLFU).

—25°16'43'' S, 30°30'60'' E: Mpumalanga, Lydenburg, Buffelskloof Nature Reserve, Helichrysum Trail, 21 September 2003, *Burrows, J.E. 8174* (PRE [PRE0757380-0]).

—25°38’19’’ S, 30°19'34’’ E: Mpumalanga, Waterval Boven, November 1915, *Rogers, F.A*. *18509* (PRE [PRE0125172-0]).

—26°56’08’’ S, 32°48’10’’ E: KwaZulu-Natal, KwaMazambane, beside the road from Kosi Estuary to Kosi Lake Campsite, 18 November 1982, *Balkwill, K*. *584* (NU [NU0039876]).

—27°11'39'' S, 32°42'16'' E: KwaZulu-Natal, Umbombo, Vasi Swamp, October 1972, *Stephen, J.J.F. 1183* (PRE [PRE0125189-0]).

—29°59'11'' S, 30°51'57'' E: KwaZulu-Natal, Mpangazi, Sand Peaks, 10 January 1964, *Strey, R.G*. *5074* (PRE [PRE0125187-0]).

—29°07’03’’ S, 31°35’46’’ E: KwaZulu-Natal, Mtunzini District, Amatigulu Nature Reserve, 6 December 2015, *Steyn, H.M*. *2203* (BLFU).

—29°21’20’’ S, 30°31’20’’ E: KwaZulu-Natal, New Hanover, Buccleugh, Newington Farm, December 1948, *Hunttey, K.D*. *372* (NU [NU0039918]).

—27°33’421’’ S, 32°05’01’’ E: KwaZulu-Natal, Ubombo, October 1972, *Van Graan, S*. and *Schwabe*, *1183* (PRE [PRE0125189-0]).

—27°11’50’’ S, 31°42’20’’ E: KwaZulu-Natal, Ngome, Vasi Swamp, December 1999, *Edwards, T*. and *Potgieter, C*. *1690* (NU [NU0039893]); KwaZulu-Natal, Ngome, Vasi Swamp, November 2006, *Edwards, T*. *3321* (NU [NU0018275-0]).

—29°50’34’’ S, 30°50’02’’ E: KwaZulu-Natal, Pinetown, 7 June 1932, *Galpin, E.E*. *12085* (PRE [PRE0125184-0]).

—29°41''25'' S, 30°56'70'' E: KwaZulu-Natal, Inanda, *Wood, J.M. 365* (K, lectotype [K000394681]); (K, syntype [two specimens on one sheet] [K000394682; K000394683]); (SAM, syntype [two sheets] [SAM0018959-1; SAM0018959-2]).

—31°37'32'' S, 29°32'12'' E: Eastern Cape, Port St. Johns, 5 December 1985, *Shackleton, C.M*. *342* (PRE [PRE0725389-0]).

—32°40’46’’ S, 28°21’49’’ E: Eastern Cape, Kei Mouth, 23 December 2016, *Steyn, H.M*. *2265* (BLFU).

—Coordinates unknown: Without precise locality, *Sanderson, J. 466* (K, syntype [K000394684]); *Sanderson, J. 466* (TCD, syntype [*s.n.*]).

Crabbea velutina

Botswana

—20°15'43'' S, 22°54'20'' E: Maun, Ngwanaekau Hills, 13 March 1969, *Buerger, A.D*. *1148* (PRE [PRE0126756-0]).

—20°30'00’’ S, 22°45'00’’ E: Maun, Ngamiland, Mwakupan, around camp, 13 March 1969, *de Hoogh, R.J. 170* (PRE [PRE0858135-0]).

—20°37’20’’ S, 23°05’30’’ E: Maun, on the N-facing slope of one of the Kgwebe Hills, 26 December 1977, *Smith, P.A. 2163* (NU [NU0039890]).

Kenya

—01°42’13’’ S, 30°58’37’’ E: German East Africa [Tanzania], Karagwe, *Scott-Elliot, G.F. 8147* (K, syntype [K000394695]; BM, isosyntype [BM000645006]).

South Africa

—23°49'02’’ S, 31°21'16” E: Limpopo, Phalaborwa, Kruger National Park, at the foot of Gondegonde, on the southern side, 15 January 1994, *Zambatis, G. 174* (PRE [PRE0797402-0]).

—22°41'32'' S, 31°01'00'' E: Limpopo, Punda Maria, Dzandweni Hill, 11.5 m. S.E. of Punda Maria,15 March 1949, *Codd, L.E.W*. *5313* (PRE [PRE0126761-0]).

—22°37'43'' S, 30°38'04'' E: Limpopo, Mutale, 3 February 1981, *van Rooyen, N*. 3138 (PRE [PRE0611806-0]).

—24°28'46'' S, 30°36'11'' E: Limpopo, Abel Erasmus Pass, en route to J.G. Strydom Tunnel, next to tar road, 25 March 2015, *de Gouveia*, *A*. *110* (BLFU); *de Gouveia*, *A*. *111* (BLFU); *de Gouveia*, *A*. *112* (BLFU); *de Gouveia*, *A*. *113* (BLFU); *de Gouveia*, *A*. *114* (BLFU); *de Gouveia*, *A*. *115* (BLFU); *de Gouveia*, *A*. *157* (BLFU).

—24°30'48'' S, 30°50'14'' E: Mpumalanga, Swadini, Blyde Canyon Reserve, outside camp, next to road en route to dam, 19 November 2015, *de Gouveia*, *A*. *164* (BLFU).

—24°31'40'' S, 30°47'13'' E: Mpumalanga, Swadini, Blyde Canyon Reserve, next to walk, close to dam, 19 November 2015, *de Gouveia*, *A*. *163* (BLFU).

—24°26'57'' S, 30°36'28'' E: Limpopo, Abel Erasmus Pass, northern side of J.G. Strydom Tunnel, 7 December 1979, *Retief, E*. *122* (PRE [PRE0611581-0]).

—25°52'25'' S, 31°54'26'' E: Limpopo, Komatiepoort, Lebombo Mountains, 24 February 1953, *van der Schijff, H.P*. *2306* (PRE [PRE0126763-0]).

—Coordinates unknown: Tropical Africa. *Gregory, J.W. s.n.* (BM, lectotype [BM000645011]).

—Coordinates unknown: British East Africa [Kenya], Ukamba, *Scott-Elliot, G.F. 2309* (K, lectotype [K000394696]; BM, isolectotype [BM000645007]).

—Coordinates unknown: Locality unkown, *Taylor, W.E. s.n.* (BM, syntype [BM000645012]).