THE SYSTEMATICS OF THE GENUS *GARULEUM* CASS. (ASTERACEAE)

by

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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Bloemfontein

South Africa

2013

List of contents

Acknowledgements

I would like to acknowledge the following persons and institutions for their contribution in the completion of this study.

- To my study leader Dr Mariette Jackson, my sincere thanks you for the invaluable guidance, support and encouragement during this study.
- To my co-study leader Dr Lize Joubert, thank you for all the patient advice, guidance and support.
- I would like to thank both my study leaders for providing me with the best opportunities and education. Thank you for your high standard of excellence and dedication to hard work, which I aspire to emulate in my life.
- Thank you to the South African National Biodiversity Institution for their grant enabling this study.
- All the curators of the herbaria mentioned in Chapter 3, thank you for allowing me to examine your collections on loan.
- Thank you to all my colleagues at the Plant Sciences Department, University of the Free State, for your good humoured encouragement and support.
- Ms Anet Kotze thank you for the beautiful illustrations of *Garuleum*.
- Dr Anofi Ashafa thank you for providing fresh specimens of *Garuleum woodii*.
- Mr Ralph Vincent Clark, thank you for providing important distribution information on *Garuleum tanacetifolium*.
- Thank you to all the companions during the field work for your patience and hard work.
- Bill and Alison Brown at Glen Avon, thank you for allowing us access to do field work on your farm.
- Richard and Kitty Viljoen of Asante Sana private game reserve, thank you for allowing us access to do field work on the reserve.
- Thank you to my family for your support and endless encouragement during the course of my studies. Thank you for instilling in me a love and respect for nature.

List of Abbreviations

	A
AIC	Akaike information criterion
	B
B	Botswana
BI	Bayesian inference
Bp	Base pairs
BLAST	BLAST
BRAHMS	Botanical Research and Herbarium Management System
	C
CI	Consistency Index
cpDNA	Chloroplast DNA
ct	Capitate trichome
CTAB	Cetyl trimethylammonium bromide
	D
DNA	Deoxyribonucleic acid
	E
E	Equatorial diameter
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic acid
	F
FS	Free State
	G
G	Gauteng
gc	Guard cell
GIS	Geographic information system
	I
IAA	Isoamylalcohol

ILD	Likelihood heterogeneity test
IPNI	International plant names index
ITS	Internal transcribed spacer
	K
KZN	Kwazulu-Natal
	L
L	Limpopo
LE	Lesotho
	M
MCMC	Markov Chain Monte Carlo
Mp	Maximum parsimony
MP	Mpumalanga
MZ	Mozambique
	N
NA	Namibia
NaCl	sodium chloride
NC	Northern Cape
NCBI	National Centre for Biotechnology Information
ndhF	NADH dehydrogenase F
ng	Nanograms
	P
P	Polar axis
PCR	Polymerase chain reaction
PP	Posterior probability
PSC	Phylogeny Species Concept

	R
RNase A	Ribonuclease A
rbcL	Ribulose-1.5-bisphosphate
RI	Retention index
	S
S	Swaziland
SEM	Scanning electron microscope
sl	Stomatal ledge
st	Simple trichome
	T
TBR	Tree bisection and reconnection
TEM	Transmission electron microscopy
Tris-HCl	Tris(hydroxymethyl) aminomethane
	U
UV	Ultraviolet light
	W
WC	Western Cape
	Z
ZIM	Zimbabwe

List of tables and figures

Chapter 1

Introduction

Garuleum Cass. is a small genus in the tribe Calenduleae, of the family Asteraceae (Compositae) (Bremer, 1994). The Asteraceae is the largest family of vascular plants, and is distributed over all continents of the world excluding Antarctica. The family consists of an estimated 30 000 species, of which about 24 000 species have been formally described (Funk et al., 2009).

There are currently 1600 − 1700 recognized angiosperm genera of which more or less 10 % belong to the Asteraceae. With an estimated 250 000 – 350 000 angiosperm species, one out of every eight to twelve belong to the Asteraceae (Funk et al., 2009).

The Asteraceae has numerous economical uses (Table 1.1). The entire family is an important source of pollen for the production of honey. Species like *Chrysanthemoides cinerariifolium* Vis. and *Chrysanthemoides coccineum* Wild. produce the secondary metabolite pyrethrin, which is used as a natural insecticide (Boussaada et al., 2008). A few other species have pharmacological and phytochemical properties, which may be important in the medical- and technical industries. Selected species are also used for traditional purposes in different cultures. Many Asteraceae species are used as ornamentals, potted plants, bedding plants and cut flowers. (Simpson, 2009) The majority of Asteraceae species have a restricted distribution, but there are a few species of thistles, dandelions and goldenrods, which benefit from disturbance and are conscidered weeds (Funk et al., 2005).

The tribe Calenduleae is geographically centred in southern Africa and only one genus, namely *Calendula* L., is found in the Northern Hemisphere, mainly in the Mediterranean.

Some of the species in the Calenduleae produce secondary compounds useful in chemistry as chemotaxonomical markers. *Dimorphotheca* Vaill. ex Moench contains cyanoglycosides, for example linamarin. A unique fatty oil, dimorphecolic acid, is produced from Calenduleae achenes, which has commercial potential in the technical industry (Nordenstam and Källersjö, 2009).

Table 1.1. Economically important species and their different uses, within the Asteraceae. Adapted from Simpson (2009).

Use	**Common name**	**Species**
Edible crops	Artichoke	*Cynara scolymus* L.
	Chicory	*Cichorium intybus* L
	Lettuce	*Lactuca sativa* L.
	Cardoon	*Cynara cardunculus* L.
	Burdock	*Arctium lappa* L.
	Yacon	*Polymnia sonchifolia* Poepp & Endl.
	Jerusalem artichoke	*Helianthus tuberosus* L.
Bio-fuel	Sunflowers	*Helianthus annuus* L.
Seed oils	Sunflowers	*Helianthus annuus* L.
	Safflowers	*Corthamus tinctorius* L.
	Niger	*Guizotia abyssinica* Cass.
Beverages	Chamomile tea	Different species. Example: *Marticaria reticutita* L.
	Chicory	*Cichorium intybus* L.
	Absinthe in liqueur	*Artemisia pontica* L.
Sweeteners	Chicory root	*Cichorium intybus* L.
	Dandelion	*Taraxacum officinale* Webb.
	Jerusalem artichoke	*Helianthus tuberosus* L.
	Salisfy	*Tragopogon porrifolius* L.
	Stevia	*Stevia rebaudiana* Bertoni.
Spices	Tarragon	*Artemisia dracunculus* L.
	Bolivian coriander	*Porophyllum ruderale* (Jacq.) Cass.
	Chrysanthemum leaves	*Chrysanthemum* sp.
Dyes	Safflowers	*Corthamus tinctorius* L.
Rubber	Guayule	*Parthenium argentatum* A.Gray.

Many species of Calenduleae contain diterpenes (Nordenstam and Källersjö, 2009). A few *Dimorthotheca* species are sold as ornamental plants under the name *Osteospermum* L. *Dimorphotheca* causes hydrocyanic poisoning in sheep and *Chrysanthemoides monilifera* (L.) T. Norl. is a noxious weed in Australia (Nordenstam and Källersjö, 2009). Due to the variety of secondary metabolites they contain, the Calenduleae have been widely used in traditional medicine in Africa, China and Europe.

The genus *Garuleum* is endemic to southern Africa and consists of eight species with two subspecies. Species of *Garuleum* are found in all the South African provinces except for the North West Province and Limpopo. One species of *Garuleum* also occur in Namibia. Some of the species appear to be rare and under collected. Most of the examined specimens collected contain little data on the distribution and ecology of the species.

Garuleum bipinnatum (Thunb.) Less. (Fig. 1.1), has been used traditionally by the early Cape colony settlers as a remedy against snake bites. Europeans in the Transvaal used it as an ingredient in a brandy extract, which was used for treatment of haemorrhoids (Watt and Beyer-Brandwijk, 1962). In a phytochemical study performed by Timmerman (2004), several compounds from the isopimarane-type diterpenoid class were extracted from *G. bipinnatum*. There is no recorded information on uses for the remaining species in the genus, but this may be due to the limited ethnobotanical research preformed on the genus.

Garuleum was last revised by Tyco Norlindh in 1977, as part of a greater revision of the tribe Calenduleae. This revision was based purely on morphological data and the analysis thereof predates the current phylogenetic approach to systematics in general. The advantage of a classification based on phylogenetic systematics is that it not only provides a classification which is helpful for identification, but it can also be used to predict evolutionary trends and properties of the studied group. A disadvantage associated with traditional classifications is that species are delimited based on characters believed to be important by the researcher, thus relying on biased assessments and methods.

Figure 1.1 Photographs of *Garuleum bipinnatum*. In (a) plant habit; (b) an inflorescence; (c) a dorsal view of the capitulum and involucres of bracts; and (d) a stem with leaves. The photographs were taken in the Eastern Cape near the formerly known Andries Vosloo Kudu reserve, which now forms part of the Great Fish River reserve (November 2011).

Phylogenetic classifications are constructed through the application of empirical methods, thus not relying on the authority or intuition of the researcher (Wiley et al., 1991). There is consequently a need for phylogenetic investigation in the Calenduleae and its genera.

The aim of the study was to provide a taxonomic revision based on the molecular phylogenetic analysis of the eight *Garuleum* species described in Plants of southern Africa: an annotated checklist (Germishuizen and Meyer, 2003). The taxonomic revision will includes an identification key to the species, a revision of type diagnoses, clarification and designation of type specimens, compilation of morphological and micromorphological descriptions, distribution maps and ecological data on all *Garuleum* species. The study further aims to obtain a well resolved phylogeny for the genus *Garuleum*, using the nuclear gene region ITS and the chloroplast intergenic spacers *trn*T–*trn*F and *psb*A–*trn*H. And attempt to resolve the evolutionary history of *Garuleum* from the phylogeny obtained for *Garuleum*, which was then compared to observations made from the morphological descriptions of the species.

Chapter 2

Introduction and historical review

2.1 Taxonomy

2.1.1 Position of *Garuleum* and classification of the Asteraceae

The first natural classification of the Asteraceae was proposed by Alexandre Henri Gabriel de Cassini. He based his classification on characters obtained from stamens, styles, achenes, pappus and corollas of the flowers. Between 1816 and 1819 he produced classifications with different numbers of tribes, but his final classification divided the Asteraceae into 20 tribes (Table 2.1) (Cassini, 1819a, 1819b). Most of these tribes are still recognized today and most of the diagnostic characters used by Cassini to define the tribes are still used today (Bonifacino et al., 2009).

In 1832 Christian Friedrich Lessing classified the Asteraceae into 8 tribes and 45 subfamilies (Table 2.1). However, the characters he used for his classification were not sufficiently informative and led to unnatural groups. For his 1836 classification A.P. De Candolle used the same characters as Lessing, but his classification divided the family into 9 tribes (Table 2.1).

The next significant work on the classification of the Asteraceae was done by George Bentham (1873a) (Table 2.1), which remained in use until 1975. Bentham based his classification for the most part on the same characters that Cassini used for his classification, which devided the Asteraceae into 20 tribes (1819a, 1819b). Bentham reduced the number of tribes to only 13 (Bentham, 1873a), but these mostly agree with the 20 tribes of Cassini (1819a, 1819b). Bentham (1873a) selected these characters without any prior knowledge of Cassini's classification. According to Bentham (1873b) the most primitive tribe in the Asteraceae was the Heliantheae.

Both Carlquist (1976) and Wagenitz (1976) independently concluded that the Asteraceae could be divided into two subfamilies, namely Cichorioideae and Asteroideae (Table 2.1).

Table 2.1 Classifications of the Asteraceae from 1819−1976. Subfamilies, tribes and subtribes in bold are referred to in the text. Some of the names are used in different context in the different classifications, because there were no clear definitions or rules for naming tribes, subtribes, subfamilies and families in the early years.

Cassini (1819)	**Lessing (1832)**		**De Candolle (1836-1838)**	**Bentham (1873)**	**Carlquist (1978) &Wagenitz (1976)**	
Tribes:20	**Tribes**	**Subtribes:45**	**Tribes: 9**	**Tribes: 13**	**Subfamily**	**Tribes: 13**
Adénostyleae	Asteroideae	**Astereae**	**Asteroideae**	Anthemideae	Asteroideae	Astereae
Ambrosieae		Buccharideae	Cichoraceae	Arctotideae		**Calenduleae**
Anthémidees		Buphthalmeae	Cynareae	Asteroideae		Eupatorieae
Arctotideae		Ecilipteae	Eupatoriaceae	**Calendulaceae**		Helenieae
Astereae		Inuleae	Mutisiaceae	Cichoriaceae		Heliantheae
Calenduleae		Tarchonantheae	Nassauviaceae	Cynaroideae		Inuleae
Carduineae	Circhoraceae	Hieracieae	Senecionideae	Eupatoriaceae		Senecioneae
Carlineae		Hyoserideae	Vernoniaceae	Helenioideae	Cichorioideae	Arctotideae
Centaurieae		Hypochoerideae	Veroniaceae	Helianthoideae		Cardueae
Echinopseae		Lactuceae		Inuloideae		Echinopeae
Eupatorieae		Lampsaneae		Mutisiaceae		Liabeae
Héliantheae		Scolymeae		Seneciodeae		Mutiseae
Inuleae		Scorzonereae		Veroniaceae		Vernonieae
Lactuceae	Cynareae	Arctotideae				
Mutisieae		**Calenduleae**				
Nassauvieae		Cardopateae				
Sénécioneae		Carduineae				
Tagétineae		Centaurieae				
Tussilagineae		Echinosideae				
Veronieae		**Othonnineae**				
		Xeranthemeae				
	Eupatoriaceae	Alomieae				
		Agerateae				

Table 2.1 continue

Cassini (1819)	Lessing (1832)		De Candolle (1836-1838)	Bentham (1873)	Carlquist (1978) &Wagenitz (1976)	
Tribes:20	**Tribes**	**Subtribes:45**	**Tribes: 9**	**Tribes: 13**	**Subfamily**	**Tribes: 13**
		Tussilagnieae				
	Mutisiaceae	Facelideae				
		Lerieae				
		Mutisieae				
	Nassauviaceae	Nassauvieae				
		Trixideae				
	Senecionideae	Ambrosieae				
		Artemisieae				
		Belhanieae				
		Chrysanthemineae				
		Flaverieae				
		Gnaphalieae				
		Heliantheae				
		Helenieae				
		Senecioneae				
		Tagetineae				
	Veroniaceae	Elephatopodeae				
		Liabeae				
		Pectideae				
		Rolandreae				
		Trichospineae				
		Veronieae				

Both authors' views were based on the micromorphology of flowers, vegetative anatomy, palynology, carpology, embryology and phytochemical data (Carlquist, 1976; Wagenitz, 1976).

2.1.2. Classification of the Calenduleae and *Garuleum*

The tribe Calenduleae was first described by Alexandre Henri Gabriel de Cassini (1819c). The outgrowths and lack of pappus on fruit were the character states he emphasized as diagnostic for the tribe. He recognized nine genera in the tribe, but only four of these are still recognized today namely *Osteospermum* L., *Garuleum*, *Gibbaria* Cass. and *Calendula* L. The rest of the genera were later synonymized with either *Chrysanthemoide*s Fabr. or *Dimorphotheca* (Nordenstam and Källersjö, 2009). *Garuleum* was first described by Cassini (1819c), based on *G. viscosum* Cass. [=*G. pinnatifidum* Cass.].

The Calenduleae was reduced to a small subtribe in the tribe Cynareae by Lessing (1832) and included three genera namely *Calendula*, *Oligocarpus* Less. and *Tripteris* Less. *Osteospermum* was transferred to the subtribe Othonninae in the tribe Cynareae and *Dimorphotheca* was moved to the subtribe Chrysantheminae in the tribe Senecionideae (Lessing, 1832). Lessing (1832) placed *Garuleum* in the subtribe Astereae and *Gibbaria* in a group of inadequately known genera (Table 2.1). Lessing's arrangement of the Calenduleae has been superseded by a more defendable hypothesis, but he contributed taxonomically by adding the new genera *Oligocarpus* and *Tripteris* to the subtribe.

The unnatural arrangement of the Calenduleae by Lessing was largely followed by De Candolle (1836–1838), who used the same tribes, but dropped the sub-tribal classification of Lessing (1832). De Candolle (1838) placed *Dimorphotheca* in Senecionideae and *Garuleum* in the Asteroideae (Table 2.1). He added new genera, however none are still recognized today. De Candolle (1836) was the first to restrict *Calendula* as a strictly northern hemisphere genus.

Bentham (1873a) retained many of Lessing (1832) and De Candolle's (1836) tribes, but reinstated others and recognized Calenduleae as a tribe. Bentham, however, added three genera, *Dipterocome* Fisch. & Mey., *Eriachaenium* Sch.Bip. and *Ruckeria* DC. to the tribe, which have been superseded by a more defendable hypothesis. *Eriachaenium* and *Dipterocome* were included in the Calenduleae by Norlindh (1943), but he excluded *Eriachaenium* from the tribe (Norlindh, 1977). *Ruckeria* was synonymised with *Euryops* (Cass.) Cass., and placed in the Senecioneae (Nordenstam, 1968). *Dipterocome* was later also excluded from the Calenduleae (Nordenstam, 1994a).

Much of the systematic knowledge of the Calenduleae was contributed by Norlindh (1948−1977). He made generic level changes like synonymising *Blaxium* with *Osteospermum* instead of *Dimorphotheca*, sinking *Tripteris* and *Oligocarpus* into *Osteospermum* and re-establishing *Chrysanthemoides*, *Gibbaria* and *Castalis* Cass. Many of his generic arrangements were changed recently by Nordenstam (1994 a, 1994 b, 1996), who placed *Blaxium* as a synonym of *Dimorphotheca*, also recognized *Tripteris* and *Oligocarpus* as two separate genera.

2.2. Phylogeny of Asteraceae.

Systematics has changed from being based on only morphological data, to the combination of morphological and molecular data. Phylogenetic analysis has helped researchers to obtain a better understanding of the evolutionary history of flowering plants. Phylogenies provide a classification that is more reliable in portraying the relationships among species (Heywood, 2009). Phylogenies can provide information on how certain traits evolved and how others were lost in different species. This may help in clarifying why certain species are more widely distributed and adapted to a variety of habitats while others are more habitat specific. Phylogenies may also help the researcher to predict how species may evolve in the future and how adaptable they may be to changing conditions (Baum and Smith, 2012). For phylogenetic studies to provide the most congruent results, data from multiple gene regions, such as nuclear and chloroplast DNA, need to be combined (Schaal et al., 1998).

The first major attempt at a phylogenetic classification of the Asteraceae, was the study of Jansen and Palmer (1987), which was based on chloroplast DNA. Through

restriction site mapping, this study showed that a 22-kb inversion was present in most Asteraceae. The 22-kb inversion was lacking in the Barnadesiinae (Mutiseae), indicating an ancient evolutionary split in the Asteraceae. This study of resulted in a significant change in Asteraceae classification, by showing that the evolutionary hypotheses regarding Heliantheae as the most primitive tribe in the Asteraceae, were not a defendable hypothesis (Table 2.2).

In the phylogeny of Jansen and Palmer (1987), the Heliantheae tribe was placed in a derived position on the tree and the most basal branch was represented by a part of the Mutisieae tribe, which was not monophyletic (Jansen and Palmer, 1987). The phylogeny also showed that the classification system in which Vernonieae and Eupatorieae were closely related were unsupported and that these tribes were placed on separate parts of the tree (Funk et al., 2009). Studies following this initial research also made use of restriction site mapping of chloroplast DNA (Jansen et al., 1988, 1990, 1991).

Phylogenetic studies that made significant contributions to the classification of the Asteraceae include a study by Jansen et al. (1990) which provided strong support for the monophyly of the subfamily Asterioideae. The subfamily included the tribes Anthemideae, Astereae, Calenduleae, Coreopsideae, cladistic classfication of the family based on a parsimony analysis from morphological data (Table 2.2).

This classification was an invaluable data source of the Asteraceae for students at that time. From 1987 to 1995 phylogenetic studies at higher taxonomic levels in the Asteraceae were mostly based on restriction site mapping or amplification of the chloroplast DNA gene regions ribulose-1,5-bisphosphate carboxylase (rbcL) or NADH dehydrogenase F (ndhF) (Kim and Jansen 1995). Over time more gene regions became available for analysis, varying in resolution at different taxonomic ranks and varying in effectiveness between different taxa. In 2002 a revision of the phylogeny of the Asteraceae, based on a combination of nine chloroplast markers,

Table 2.2 Classifications of Asteraceae based on cladistic analysis or molecular analysis. Calenduleae are shown in bold. Only the tribes of the Asteroideae subfamily and not the tribes of the Cardoideae, Cichorioideae and Mutisioideae are shown by Funk et al. (2009).

Jansen and Palmer (1987)		**Bremer (1994)**		**Panero & Funk (2002)**		**Funk et al. (2009)**	
Subfamily	**Tribes:**	**Subfamily**	**Tribes:**	**Subfamily**	**Tribes:**	**Subfamily**	**Tribes:**
Asteroideae	Anthemideae Astereae **Calenduleae** Cotuleae Helenieae Heliantheae Inuleae Senecioneae Tageteae Ursinieae	Asteroideae	Anthemideae Astereae **Calenduleae** Eupatorieae Gnaphalieae Helenieae Heliantheae Inuleae Plucheeae Senecioneae	Asteroideae	Anthemideae Astereae Athoroismeae Bahleae **Calenduleae** Chaenactideae Coreopsideae Eupatorieae Gnaphalieae Helenieae Heliantheae Inuleae Madieae Millerieae Neurolaeneae Perityleae Plucheeae Polymnideae Senecioneae Tagetesa	Asteroideae	Anthemideae Astereae Athhroismeae Bahieae **Calenduleae** Chaenactideae Coreopsideae Eupatorieae Feddeeae Gnaphalieae Helenieae Heliantheae Inuleae Madieae Millerieae Neurolaeneae Perytileae Polymnieae Senecioneae Tageteae
Barnadesioideae	Barnadesieae	Barnadesioideae	Barnadesieae	Barnadesioideae	Barnadesieae	Cardoideae	
Cichorioideae	Arctoteae Cardueae Cichorieae Echinopeae Eupatorieae Mutiseae Nassauviinae Liabeae Vernonieae	Cichorioideae	Arctoteae Cardueae Liabeae Lactuceae Mutiseae Vernonieae	Carduoideae	Cardueae Dicomeae Tarchonantheae	Cichorioideae Mutisioideae	

Table 2.2 continue

Jansen and Palmer (1987)		Bremer (1994)		Panero & Funk (2002)		Funk et al. (2009)	
Subfamily	**Tribes:**	**Subfamily**	**Tribes:**	**Subfamily**	**Tribes:**	**Subfamily**	**Tribes:**
				Cichorioideae	Arctoteae Cichorieae Guedelleaea Liabeae Vernanieae		
				Corymbioideae	Corymbieae		
				Gochnatioideae	Gochnatleae		
				Gymnarrhenoideae	Gymnarrheeeae		
				Hecastocleioideae	Hecastocleideae		
				Mutisiodeae	Mutisieae		
				Pertyoideae	Pertyeae		
				Stifftia group	Mutisleae		

divided the family into 11 subfamilies and 36 tribes (Table 2.2) (Panero and Funk, 2002).

The most recent classification of the Asteraceae is based on combined morphological and molecular data. This classification divides the family into four subfamilies and 43 tribes (Table 2.2), and is constantly being revised as new phylogenies are produced for different taxa in need of revision (Funk et al., 2009). A revised super meta tree of the Phylogeny of the Compositae is available from the International Compositae Alliance (www.compositae.org).

The use of molecular data in phylogenetic analysis has resulted in a better resolved position of Calenduleae within the family. New genera have been included in the Calenduleae, and the most recent revision of the tribe recognizes twelve genera namely *Calendula, Chrysanthemoides, Dimorphotheca*, *Garuleum*, *Gibbaria, Inuloides* B.Nord, *Monoculus* B.Nord, *Nephrotheca* B.Nord and Källersjö, *Norlindhia* B.Nord, *Oligocarpus, Osteospermum* and *Tripteris* (Nordenstam, 2007). Further changes within the tribe seem to be inevitable when the phylogeny of the paraphylytic genera *Osteospermum* and *Chrysanthemoide*s are resolved. In the past the position of *Garuleum* within the Calenduleae was uncertain, but the recent classifications based on molecular data have led to a more stable classification of this tribe.

2.2.1 Popular gene regions used in Phylogeny

2.2.1.1 The chloroplast region *trn*T–*trn*F

The use of the non-coding intergeneric chloroplast region *trn*T–*trn*F in evolutionary studies of the angiosperms was first proposed by Taberlet et al. (1991). Intergeneric chloroplast regions are used in phylogenetic studies, due to their high rate of nucleotide substitution (Gielly and Taberlet, 1994). Chloroplast DNA regions are not used independently of nuclear DNA, because their evolutionary rate is less than that of nuclear DNA, which means they may not provide a well resolved phylogeny. Chloroplast DNA is uniparentally inherited and is not informative in cases where relationships need to be resolved in taxa that evolved through hybridization or allopolyploidy (Chapman et al., 2007).

The *trn*T–*trn*F region consists of three regions the *trn*T–*trn*L spacer, *trn*L intron and *trn*L–*trn*F spacer (Fig. 2.1). The most popular of the three regions is the trnL–*trn*F spacer, which has become the most regularly used non-coding region of cpDNA in phylogenetic studies at species and generic level (Barker et al., 2009). The wide use of this region is due to universal primers designed by Taberlet et al. (1991) and its easy amplification.

The trnL intron region is also easily amplified, but does not provide as much variability as the two spacers (Shaw et al., 2005). It has catalytic properties and forms secondary structures. The use of the trnL intron is more suited for studies at higher taxonomic levels (Taberlet et al., 1991).

The use of *trn*T–*trn*L spacer region has not been very popular, because amplification of this region has proven to be difficult. The original primer set of *trn*T–*trn*L, were unsuccessful in amplification of this region in several taxa (Shaw et al., 2005). This problem was overcome by the design of a new universal forward primer (Cronn et al., 2002).

A study by Bayer and Starr (1998) using *trn*L–*trn*F and the *trn*L intron was successful in resolving the phylogeny of the Asteraceae at tribal level. This study also showed that these regions gave as much resolution as the rbcL and ndhF chloroplast regions, which are longer regions and more difficult to amplify in comparison to *trn*L–*trn*F and the *trn*L intron.

The *trn*L–*trn*F region has been used successfully in combination with the nuclear Internal Transcribed Spacer (ITS) region, to prove the monophyly of Nannoglottis Maxim. (Asteraceae) (Liu et al., 2002).The use of the *trn*L–*trn*F region in combination with ITS and *psb*A–trnH to determine the phylogeny of section Jacobeae in the tribe Senecioneae (Asteraceae), was unsuccessful. These markers proved to be insufficiently variable for a well resolved phylogeny of Jacobeae (Pelser et al., 2003).

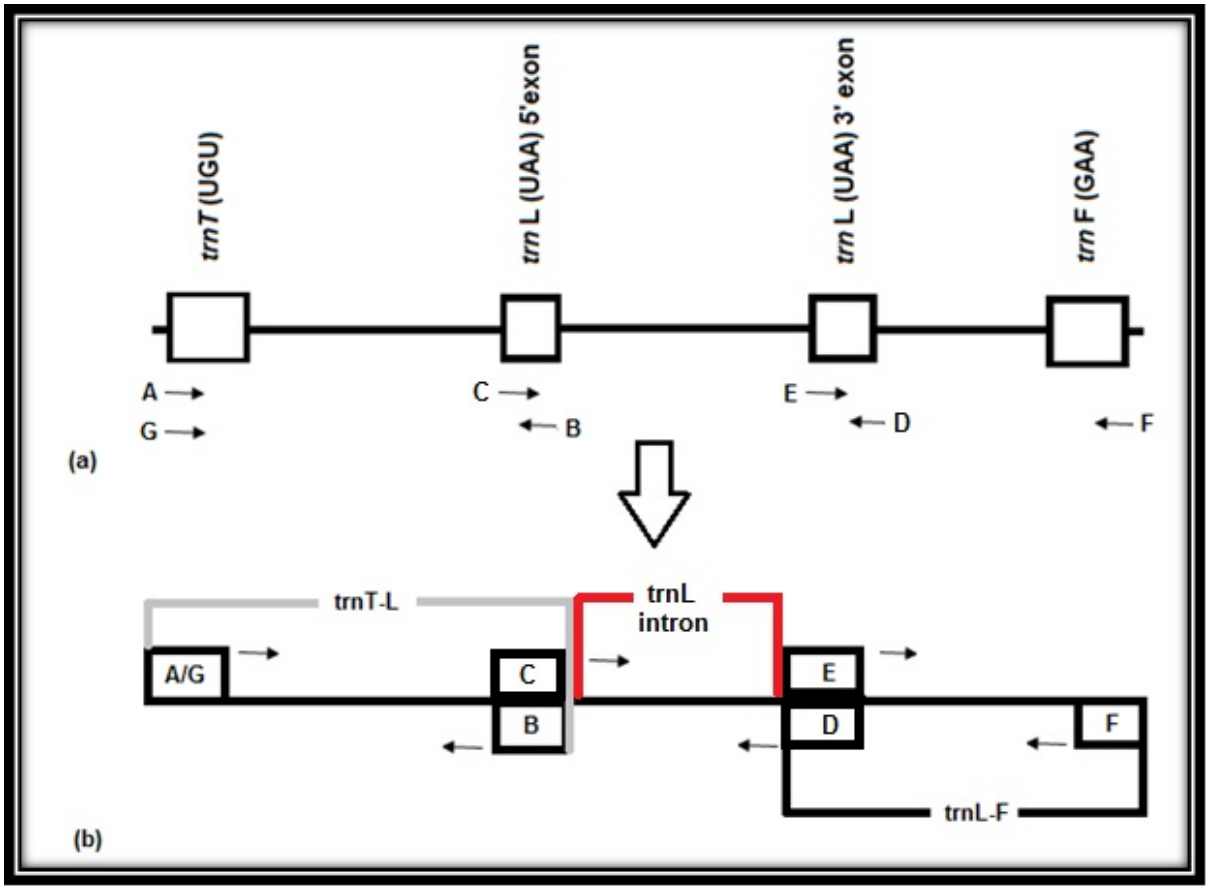

Figure 2.1 Schematic representation of the non-coding cp DNA *trn*T–*trn*F region. In (a), the *trn*T–*trn*F region, adapted from Taberlet et al. (1991) is presented. In (b) the gene region *trn*T–*trn*L can be amplified with primers A and B or alternatively primer A can be replaced with the more effective primer G designed by Cronn et al. (2002). The *trn*L–*trn*F gene region can be amplified by primers E and F, primers A–F were designed by Taberlet et al. (1991). Primers C and D are used to amplify the *trn*L intron region.

The *trn*L–*trn*F region has also been used in combination with the ndhF region to delimit a new genus in the Calenduleae, *Nephrotheca* Nord. (Nordenstam et al., 2006).

The *trn*T–*trn*L spacer is reported to be much more variable than the *trn*L intron and *trn*L–*trn*F spacer (Cronn et al., 2002; Borsch et al., 2003; Shaw et al., 2005). Borsch, et al. (2003) found that the size of the different *trn*T–*trn*F regions in angiosperms range between 467−1411 base pairs (bp) for the *trn*T–*trn*L spacer, 324−615 bp for the *trn*L intron and 164−441 bp for the *trn*L–*trn*F spacer. The variation in the base pair lengths in the spacer regions is clearly indicative of why the region is successful at providing well resolved phylogenies at different taxonomic levels. When the base pair length amplified for a region is short the resolution it provides will be insufficient at lower taxonomic levels.

2.2.1.2 The ITS nuclear DNA region.

The utility of the nuclear DNA region ITS, for phylogenetic studies in the Asteraceae, was first described by Baldwin (1992). Since ITS was first utilized in phylogenetics, it has become the most popular region used in phylogenetic studies (Goertzen et al., 2003).

The ITS region is found in nuclear ribosomal DNA (nrDNA). This region consists of three coding subunits, the 18S, 5.8S and 26S which are separated by two spacers ITS-1 and ITS-2 (Fig. 2.2).

The 5.8S subunit has a highly conserved evolutionary sequence which made the design of internal primers possible (Baldwin et al., 1995). The ITS region can be easily amplified using the universal primers described by Blattner (1999). These primer sets make it possible to amplify the whole ITS region using primers A and B. If the DNA is badly degraded it is possible to amplify the region in two separate amplicons using primers A and C for ITS1 and primers B and D for ITS2. The complete ITS region in angiosperms is approximately 500–700bp in length (Alvarez and Wendel, 2003).

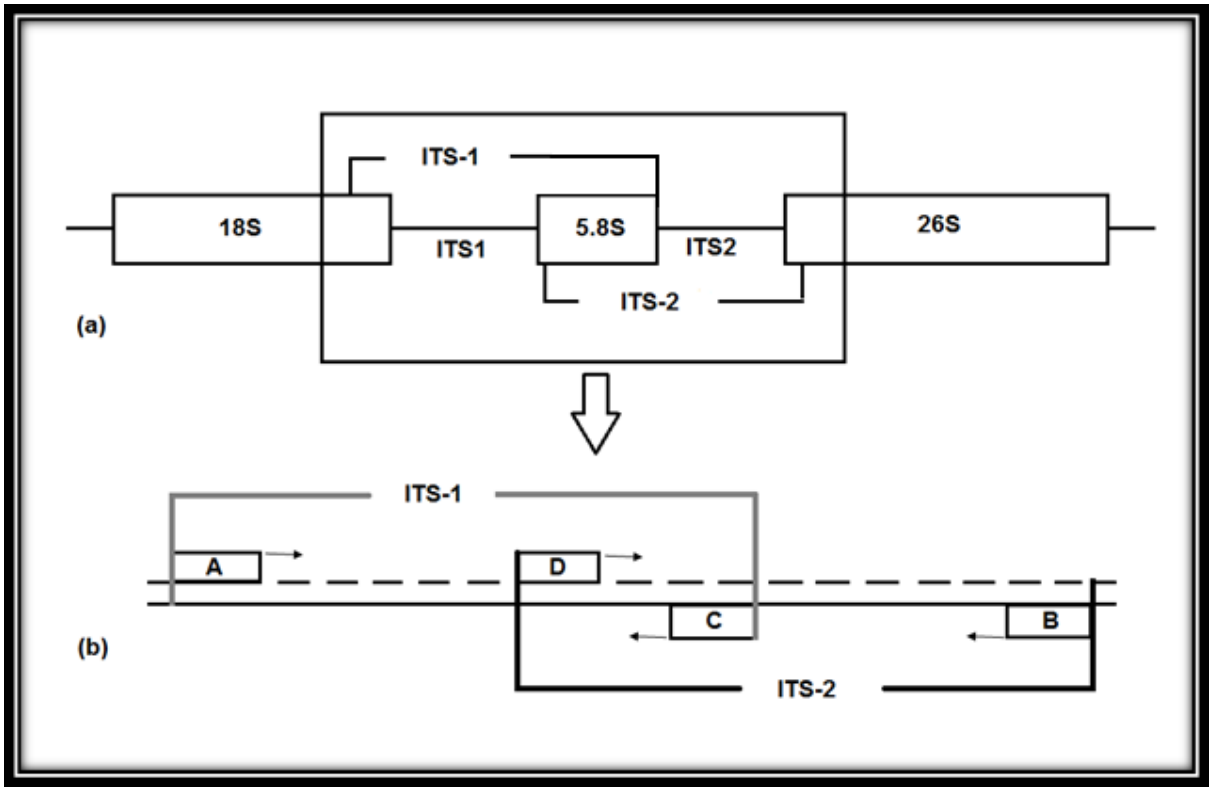

Figure 2.2 Schematic representation of the ITS region. In (a) the three coding regions 18S, 5.8S and 26S, are indicated with the ITS1 and ITS2 spacer regions among them. In (b) the complete ITS region can be amplified using primers A and B. ITS-1 and ITS-2 can be amplified separately, with primers A and C, B and D respectively. This figure was adapted from Blattner (1999).

The advantages associated with using ITS in a phylogeny include its biparental inheritance, making it useful in revealing cases of past reticulation or hybrid speciation. The universality of the primers designed by Blattner (1999) simplify the use of this gene region in plants and fungi. ITS is also highly repeated within the nuclear DNA of the plant genome, with repeats in the size range of 10 kb. They are present in thousands of copies organized in tandem repeats at chromosomal loci or multiple loci. This allows easier isolation than most low-copy number nuclear loci. The small size and high copy number of the target DNA makes it easy to amplify and permits the use of herbarium material. ITS is subject to concerted evolution which, when carried to completion, removes variation within genomes and leave species- and clade-specific characterstates for phylogenetic reconstruction (Alvarez and Wendel, 2003).

Problems associated with the use of ITS include a high possibility of erroneous assessments and phylogenetic incongruence. This is because in nrDNA, character evolution could be responsible for comparisons of paralogs (genes which have been duplicated and evolved a new function) (Mort et al., 2007). If two paralogous copies of ITS exist in the same genome, concerted evolution has occurred, which leads to homogenization. Homogenization can result in loss of all but one differing copy sequence, which may cause misleading results. Homogenization is similar to lineage sorting, which means the gene tree and species tree are different from each other (Mort et al., 2007).

Chimeric nuclear sequences may be the result of recombination arrays if incomplete homogenization occurred, leading to incorrect separating hypotheses of gene evolution. Another potential problem encountered in ITS phylogenetics is the presence of pseudogenes (non-functional copy of a functional gene) which evolve at different rates than their functional counterparts. The difference in evolution may be sufficient to lead to long-branch artifacts which produce a confusing phylogeny (Alvarez and Wendel, 2003; Mort et al., 2007). More problems associated with ITS include base substitution in the secondary structures of mature ITS RNA, caused by evolutionary constraints which result in non-independent characters. The existence of secondary structures and rDNA arrays may lead to difficulty in amplification of ITS.

Homoplasy (character state shared, but not from a common ancestor) is increased due to rapid evolution, leading to conflicting results. The universal nature of the primers of ITS may give rise to a potential problem of contamination, due to the amplification of a contaminant from unsterile practices in the DNA sample (Mort et al., 2007).

Given the problems that may arise with the use of ITS, it is more advantageous to corroborate any phylogenetic inference derived from ITS with independent sources of evidence like morphological data or chloroplast DNA (cpDNA) (McKenzie et al., 2006). Even with all the warnings associated with the use of the ITS region, it is still the most manageable nuclear region for molecular systematics at species and genus level (Barker et al., 2009; Mort et al., 2007).

ITS has been used extensively in constructing the phylogeny of the Asteraceae. The ITS region has been successfully used in combination with the noncoding *trn*L–*trn*F intergeneric region to indicate that the genus *Nannoglottis* Maxim. (Asteraceae) is monophyletic (Liu et al., 2002). The use of the ITS region in the construction of a phylogeny for the subtribe Arctotidinae (Asteraceae: Arctotideae), showed that the genus *Arctotis* L. and *Haplocarpha* Less. are polyphyletic and supported the retention of *Dymondia* Compton and the resurrection of *Landtia* Less. (McKenzie et al., 2006). The ITS region has been successfully used in combination with morphological data to show that three taxa formerly treated as *Senecio scapiflorus* l'Her., belong to the genus *Bolandia* Cron. (Manning and Cron, 2010).

2.2.1.3 The *psb*A–*trn*H chloroplast DNA region.

The *psb*A–*trn*H intergenic region was first proposed for the use of phylogenetic studies by Sang et al. (1997). This region is useful for phylogenetic studies at lower taxonomic levels and has proven to be more variable than some of the other cpDNA regions, such as *trn*L–*trn*F spacer and matK (Sang et al., 1997). Even though this region has been shown to be highly variable (Hamilton et al., 2003), it is a relatively short region in the Asteraceae and is usually used in combination with other regions such as *trn*L–*trn*F, as it may not provide a well resolved phylogeny when used on its own. The *psb*A–*trn*H region is a rapidly evolving region with a small inversion, which may lead to problematic alignments if not recognized (Dong et al., 2012).

The average size of *psb*A–*trn*H is 465 bp (Fig. 2.3), but it can range between 198−1077 bp (Shaw et al., 2005). The short length of the *psb*A–*trn*H spacer can also be an advantage, because the whole region can be easily sequenced with the use of only a forward or reverse primer in most taxa (Shaw et al., 2005). Primers designed for optimal results for this region include the *trn*H^{GUG} primer (Tate and Simpson, 2009) and the *psb*A primer (Sang et al., 1997).

The *psb*A–*trn*H region was suggested as a possible candidate for DNA barcoding because it was possible to amplify this region for eight genera from seven different plant families (Kress et al., 2005). One of which was *Solidago* L. (Asteraceae). This study also showed this region has good length, priming sites and is very variable (Kress et al., 2005). The use of the t *psb*A–*trn*H region and ITS region in the construction of a phylogeny for the subtribe Arctotidinae (Asteraceae: Arctotideae), showed that the genus *Arctotis* L. and *Haplocarpha* Less. are polyphyletic and supported the retention of *Dymondia* Compton and the resurrection of *Landtia* Less. to generic-level (McKenzie et al., 2006). Kress and Erickson (2007), showed that the *psb*A–*trn*H spacer region provides more accurate identification of species, when the sequences are compared to those found in Genbank, than rbcL.

The three gene regions *trn*T–trnF, ITS and *psb*A–*trn*H are very popular for use in phylogenetic studies today. Many more gene regions exist and most publications on how to choose a gene region for a study advise researches to first do a trial. The aim of such a trail is to establish if the chosen gene regions provide enough variation for a well resolved phylogeny (Shaw et al., 2005). The gene regions that should be used for each study differ, because certain gene regions provide more variation in certain taxa, than in others.

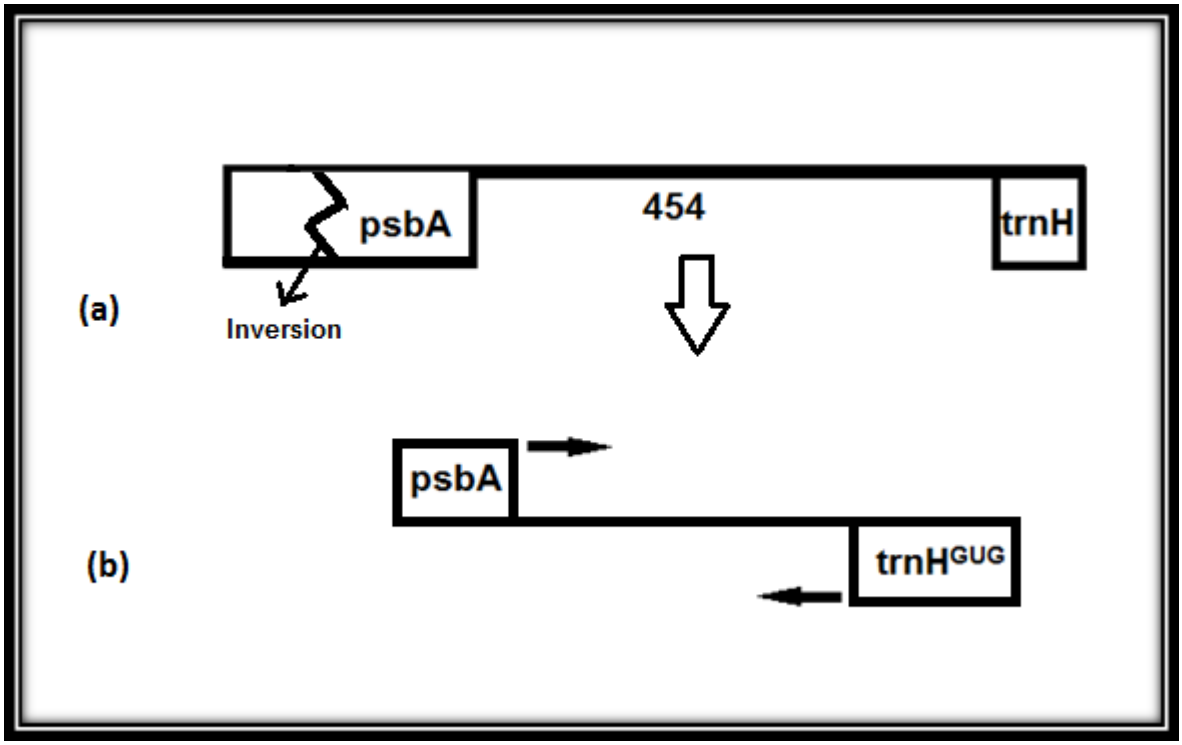

Figure 2.3. Schematic representation of the *psb*A–*trn*H intergenic region**.** In (a) the *psb*A–*trn*H region with the small inversion in the *psb*A region. In (b) the *psb*A and *trn*H^{GUG} primers are used to amplify this region.

Chapter 3

Materials and methods

3.1 Taxonomic treatment of genus and species

All *Garuleum* species are indigenous to the southern Africa sub-region consisting of Botswana, Lesotho. Namibia, Swaziland and the nine provinces of South Africa (Fig. 3.1).

Fieldwork was conducted in the Eastern Cape, Free State and Northern Cape. Fresh plant material was collected for *G. bipinnatum*, *G. tanacetifolium* and *G. pinnatifidum.* Herbarium vouchers were collected and observations of the habitat as well as vegetative and floral characteristics of the species were made. Herbarium specimens on loan from the major herbaria of Europe and southern African were studied (Table 3.1). Scans of type specimens were examined on JSTOR plant Sciences' electronic database (www.plants.jstor.org). Diagnoses literature was consulted and synonyms were declared. Original publication details were obtained by consulting the International Plant Names Index (IPNI) at (www.ipni.org).

Literature used to describe the species includes:

Descriptions of leaf shape follow: Lawrence (1951) and Systematics association committee for descriptive biological terminology (1962). Basic leaf shapes illustrated in Fig. 3.3 b.

Herbarium acronyms are given as in Holmgren et al.(1990).
Authors of plant names are given as in Brummitt and Powell (1992).

Data from herbarium specimens was captured in the Botanical Research and Herbarium Management System (BRAHMS) database. Distribution maps for the different species were compiled using geographic information system (GIS) software, DIVA-GIS, (www.diva-gis.org). Georeferencing was done using Google Earth (http.earth.google.com) for specimens where no GPS coordinates were indicated. All specimens used to generate species maps are available in addendum I.

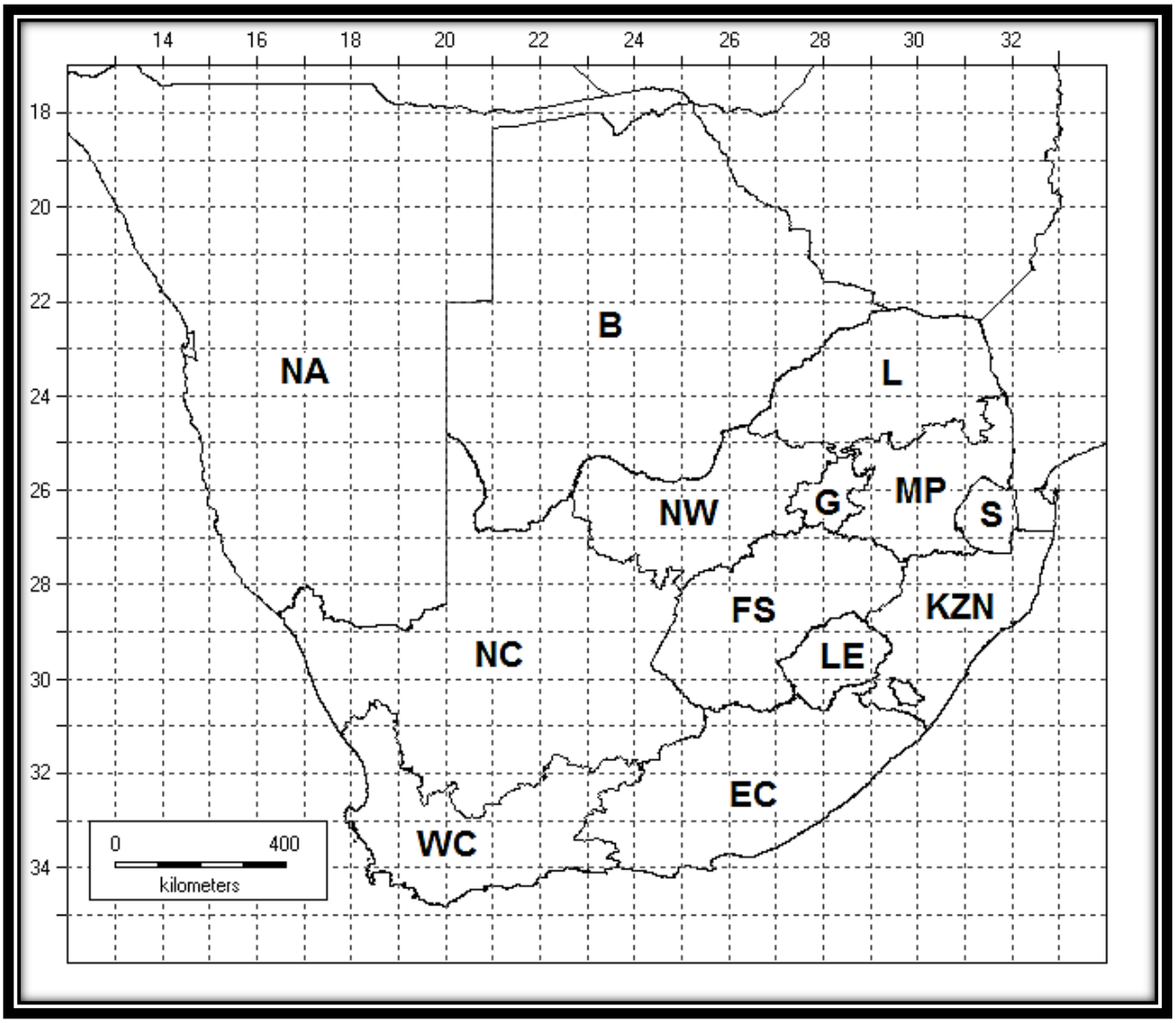

Figure 3.1 A map of countries and provinces in southern Africa. Abbreviations indicated as follow; B: Botswana, LE: Lesotho, NA: Namibia, S: Swaziland. Provinces of South Africa: EC: Eastern Cape, FS: Free State, G: Gauteng, KZN: Kwazulu-Natal, L: Limpopo, MP: Mpumalanga, NC: Northern Cape, NW: North West, WC: Western Cape. Scale bar = 400 km.

Table 3.1 List of herbaria that provided specimens or data for the study.

Abbreviation	Herbarium
BLFU	Geo Potts Herbarium, Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa.
BOL	Bolus Herbarium, University of Cape Town, Rondebosch, South Africa.
G	Herbarium Conservatoire et Jardin botaniques de la Ville de Genève, Genève, Switzerland. (G-DC).
GRA	Herbarium, Botanical Research Institute, Grahamstown, South Africa.
K	Herbarium, Royal Botanic Gardens, Kew, Richmond, England.
L	National Herbarium, Leiden, Nederland.
NBG	Compton Herbarium, National Botanical Gardens of South Africa, Claremont, South Africa.
NH	Natal Herbarium, Botanical Research Unit, Durban, South Africa.
PRE	National Herbarium, National Biodiversity Institute, Pretoria, South Africa.
Z	Herbarium, Institut für Systematische Botanik, Universität Zürich, Zürich, Switzerland.

3.2 Micromorphology of pollen

Flowers were collected from herbarium vouchers and kept for 48 hours in 95 % (v/v) ethanol. Specimens used to examine pollen micromorphology shown in table 3.2.

The flowers were dissected, pollen removed and prepared for scanning electron microscopy (SEM). The pollen was washed into centrifuge tubes with glacial acetic acid and prepared according to the acetolysis method of Erdtman (1960) and Hesse and Waha (1989). The pollen was further prepared for SEM investigation according to the method of Reitsma (1969) by rinsing the acetolysed pollen in acetic acid, washing twice with water, followed by 95 % (v/v) ethanol, mounting on stubs, air-drying and sputter coating with gold. Pollen samples were examined and photographed using a Jeol Winsem 6400 SEM at 10 kV and working distance of 17 mm.

The remainder of acetolysed pollen material was mounted in glycerine jelly and sealed with paraffin wax which was used for light microscopy studies. Samples were examined using an Olympus AX70 photo microscope.

Pollen grains were measured and the length of the polar axis (P) and the length of the equatorial diameter (E) were determined. These measurements were used to determine the P/E ratio of the pollen. The number of spines, spine length and also the number and width of colpi were determined. These measurements were done for at least 5 pollen grains of each specimen and the standard deviation determined for all the measurements. The measurements done for all the pollen grains are shown in Figure 3.2.

3.3 Leaf epidermal surfaces

Fresh leaves were collected and preserved in 3 % (v/v) phosphate-buffered glutaraldehyde. Leaves collected with permission from herbarium vouchers were rehydrated for 48 hours in 3 % (v/v) phosphate-buffered glutaraldehyde.

For epidermal surface studies leaf samples were cut into pieces of 5 x 5 mm sections, and dehydrated in an 30 %, 50 %, 70 %, 95 %, 100 % (v/v) ethanol series.

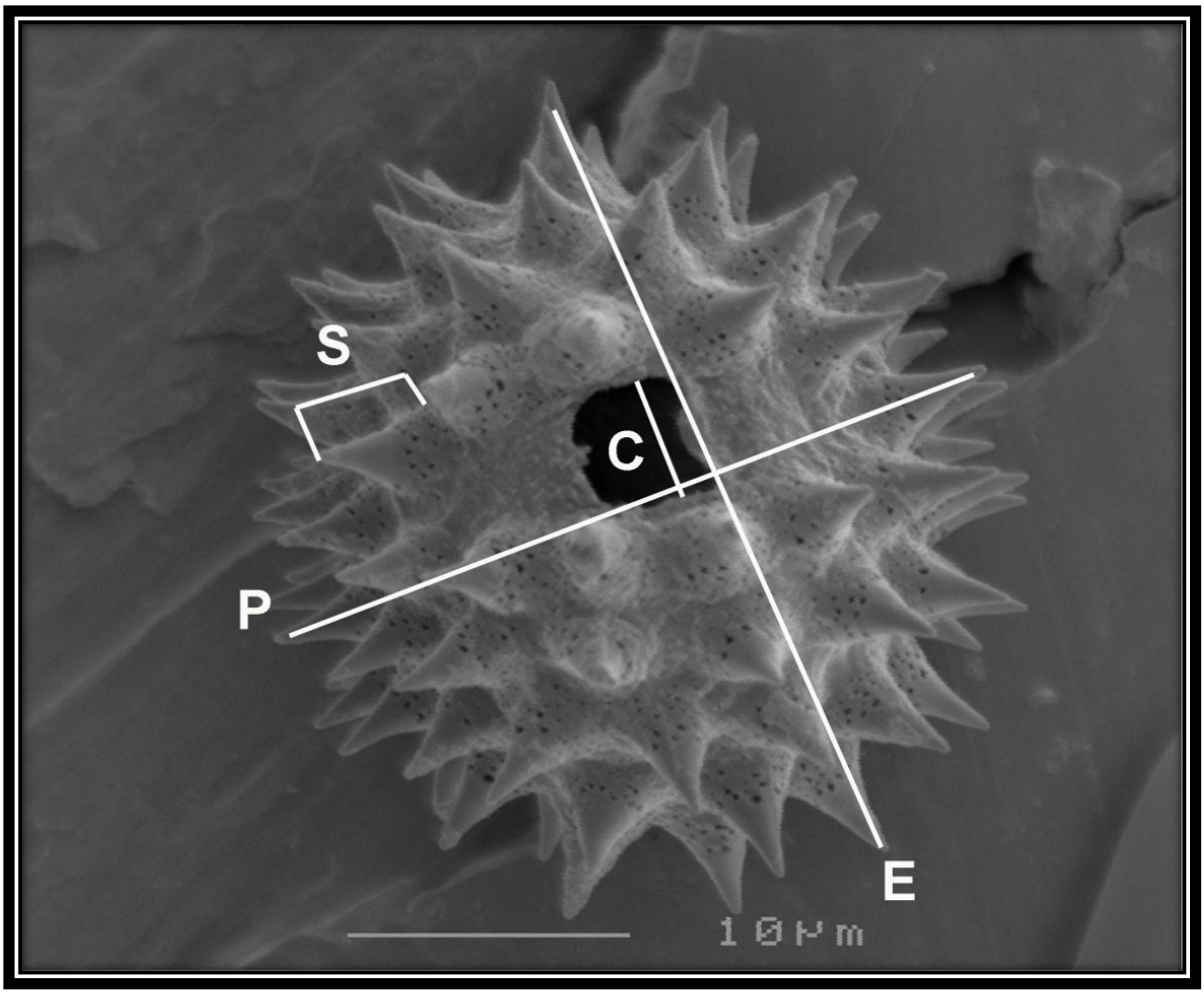

Figure 3.2 Measurements determined for the pollen grains of each of the *Garuleum* species. Legend: (P) polar axis, (E) equatorial axis, (S) spine, (C) colpus. Scale bar = 10 µm. Specimen: *Pegler 1199* (BOL).

Table 3.2. Specimens examined for pollen micromorphology.

Specimens used to examine pollen micromorphology				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
GRA	*Garuleum album*	P.B. Phillipson	4326	22/06/1995
Z	*Garuleum bipinnatum*	R.D.A. Bayliss	2845	11/05/1965
BOL	*Garuleum latifolium*	J.M. Wood	160	unknown
BLFU	*Garuleum pinnatifidum*	J. van Zyl	3	10/03/2011
Z	*Garuleum schinzii*	P. MacOwan	1889	unknown
BOL	*Garuleum sonchifolium*	A. Pegler	1199	09/06/1905
Z	*Garuleum tanacetifolium*	J.M. Wood	1382	unknown
Z	*Garuleum woodii*	A. Rehmann	6792	00/00/1875

The dehydrated leaf samples were dried in a critical point drier, mounted on stubs with epoxy glue and painted with silver paint in the corners to increase conductivity. Samples were sputter coated with gold and studied with the Jeol Winsem 6400 scanning electron microscope at 10 kV and a working distance of 17 mm.

Leaf terminology used for classification is shown in Figure 3.3 a. Specimens used to examine leaf epidermal surfaces are shown in table 3.3.

3.4 Achene pericarp surfaces

Dry achenes were collected from herbarium vouchers and fresh specimens. The achenes were mounted on stubs with epoxy glue and painted with silver paint on the side. Specimens were sputter coated with gold and studied using a Jeol Winsem 6400 scanning microscope at 10 kV and a working distance of 25 mm. Specimens examined for achene pericarp surfaces are shown in table 3.4.

3.5 Floral morphology

When possible, fresh flowers were dissected in the field using a field microscope. Fresh flowers were also collected and preserved in 3 % (v/v) phosphate-buffered glutaraldehyde. Flowers collected from herbarium vouchers were rehydrated in 3 % (v/v) phosphate-buffered glutaraldehyde for 48 hours. SEM studies were performed on ray and disc florets for each species. These were dehydrated in an ethanol series as described in section 3.3 of this chapter. Dehydrated specimens were critical point dried and mounted on stubs with epoxy glue. The corners of the dried specimens were painted with silver paint, after which the specimens were sputter coated with gold. The specimens were examined and photographed using a Jeol Winsem 6400 scanning electron microscope at 10 kV and a working distance of 25 mm.

For light microscopy the dried flowers for all species were rehydrated by heating them over a open flame in a diluted soap solution for a short period of time.

The rehydrated flowers were dissected and examined under an Olympus SZ-40 stereomicroscope. Dissected flowers were mounted on paper with herbarium glue and stored for future use.

The flower terminology is shown in Figure. 3.4. Specimens used to examine floral micromorphology are shown in table 3.5.

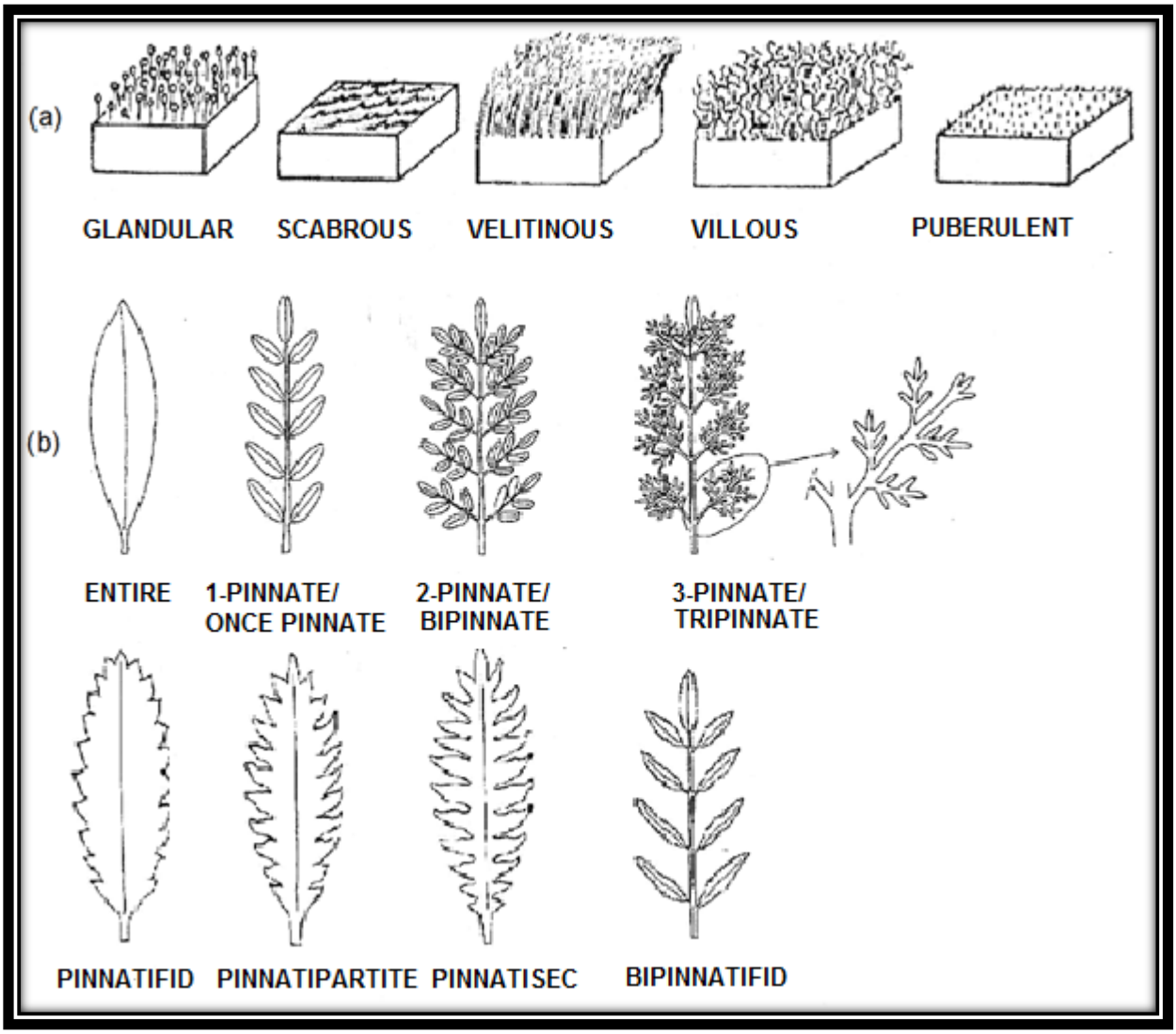

Figure 3.3 Leaf terminology used in study. In (a) terminology associated with leaf surface, in (b) terminology used to describe leaf division and branching. Adapted from Beentje (2010) and Radford et al. (1976).

Table 3.3. Specimens examined for leaf epidermal surfaces.

Specimens used to examine leaf epidermal surfaces				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
GRA	*Garuleum album*	P.B. Phillipson	4326	22/06/1995
BOL	*Garuleum album*	A. Pegler	1569	18/01/1910
BOL	*Garuleum album*	H. Bolus	8718	00/01/1895
Z	*Garuleum bipinnatum*	R.D.A. Bayliss	2845	11/05/1965
BLFU	*Garuleum bipinnatum*	J. van Zyl	9	26/11/2011
BLFU	*Garuleum bipinnatum*	J. van Zyl	14	26/11/2011
Z	*Garuleum latifolium*	J.M. Wood	299	00/04/1884
BOL	*Garuleum latifolium*	E.E. Galpin	21496	22/01/1913
L	*Garuleum latifolium*	A.G.H. Rudatis	1896	unknown
BLFU	*Garuleum pinnatifidum*	J. van Zyl	2	10/03/2011
BLFU	*Garuleum pinnatifidum*	J. van Zyl	3	10/03/2011
BLFU	*Garuleum pinnatifidum*	J. van Zyl	4	10/03/2011
Z	*Garuleum schinzii*	P. MacOwan	1889	unknown
GRA	*Garuleum schinzii*	C.A. Mannheimer	2882	15/02/2004
BOL	*Garuleum schinzii*	H.H. W. Pearson	7935	00/00/1912
BOL	*Garuleum sonchifolium*	A. Pegler	1199	09/06/1905
BOL	*Garuleum sonchifolium*	E. Esterhysen	27845	00/07/1958
BOL	*Garuleum sonchifolium*	C. Goulmis	s.n.	00/04/1944
Z	*Garuleum tanacetifolium*	J.M. Wood	1382	unknown
BLFU	*Garuleum tanacetifolium*	J. van Zyl	16	30/11/2011
BLFU	*Garuleum tanacetifolium*	J. van Zyl	22	30/11/2011
BLFU	*Garuleum woodii*	Ashafa	s.n.	00/00/2011
BOL	*Garuleum woodii*	J.M. Wood	4860	06/12/1892
Z	*Garuleum woodii*	A. Rehmann	6792	00/00/1875

Table 3.4. Specimens used to examine achene pericarp surfaces.

Specimens used to examine achene pericarp surfaces				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
GRA	*Garuleum album*	P.B. Phillipson	4326	22/06/1995
BLFU	*Garuleum bipinnatum*	J. van Zyl	9	26/11/2011
Z	*Garuleum latifolium*	J.M. Wood	299	00/04/1884
BLFU	*Garuleum pinnatifidum*	J. van Zyl	2	10/03/2011
L	*Garuleum schinzii*	R. Marloth	2043	00/07/1894
Z	*Garuleum sonchifolium*	P. MacOwan	2015	unknown
BLFU	*Garuleum tanacetifolium*	J. van Zyl	17	30/11/2011
Z	*Garuleum woodii*	A. Rehmann	6792	00/00/1875

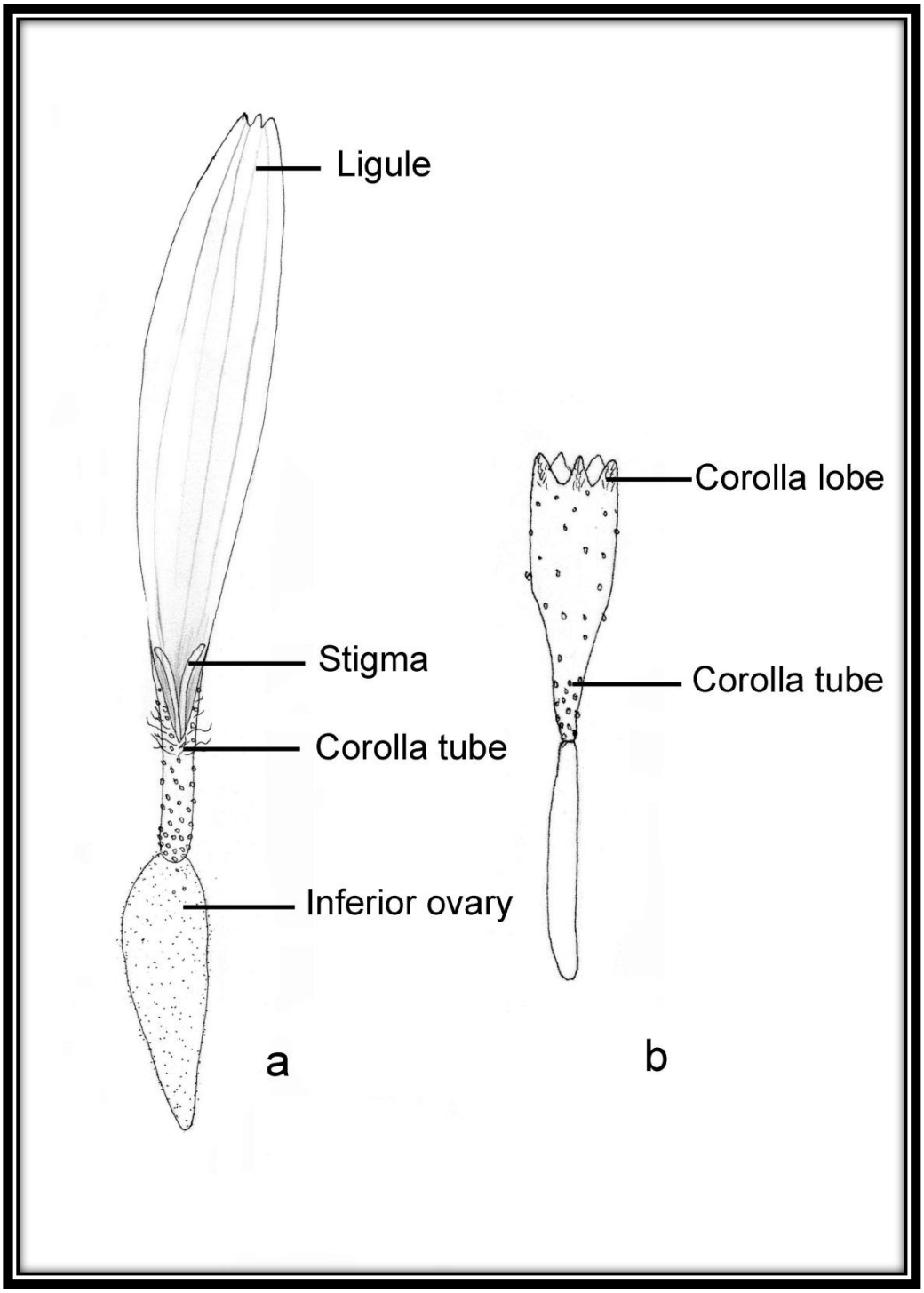

Figure 3.4 Flower terminology used in this study. In (a) ray floret terminology, in (b) disc floret terminology is shown.

Table 3.5. Specimens used to examine floral micromorphology.

Specimens used to examine floral micromorphology				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
GRA	*Garuleum album*	P.B. Phillipson	4326	22/06/1995
BLFU	*Garuleum bipinnatum*	J. van Zyl	9	26/11/2011
Z	*Garuleum latifolium*	J.M. Wood	299	00/04/1884
BLFU	*Garuleum pinnatifidum*	J. van Zyl	4	10/03/2011
GRA	*Garuleum schinzii*	C.A. Mannheimer	2882	15/02/2004
BOL	*Garuleum sonchifolium*	A. Pegler	1199	09/06/1905
Z	*Garuleum tanacetifolium*	P. MacOwan	748	00/12/1881
BLFU	*Garuleum woodii*	Ashafa	s.n.	00/00/2011

3.6 Phylogeny of *Garuleum*.

3.6.1 DNA extraction and purification

Fresh leaf material of *G. bipinnatum, G. pinnatifidum* and *G. tanacetifolium* was harvested in the field and dried in silica gel. For species that were not obtained in the field, small amounts of material were taken with permission from herbarium vouchers. All specimens used for DNA extraction are indicated in Addendum III. Voucher specimens successfully amplified and sequenced are indicated in Table 3.6.

DNA extraction was based on a modified cetyl-trimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). Leaf tissue was ground into a fine powder with a Qiagen® TissueLyzer. The fine powder was weighed to 0.7 g and 750 µL CTAB extraction buffer [2 % (w/v) CTAB, 100 mM Tris-hydroxymethyl aminomethane (Tris-HCl) (pH 8.0), 20 mM Ethylenediaminetetraacetic acid (EDTA), 1.4 M sodium chloride (NaCl), and 0.2 % (v/v) 2-mercapto-ethanol] were added. With the first precipitation step, after 500 µl of ice-cold 70 % (v/v) ethanol was added, the incubation was done overnight at 4 ºC, after which it was left for 40 minutes at room temperature. The final precipitation step was done for three days at -20 ºC.

Some DNA samples needed further purification and this was done using a FavorPrep™ PCR/Gel Purification kit (Favorgen Biotech Corporation) according to the manufacturer's specifications. DNA was eluted in 40 µl elution buffer.

The quality and quantity of the purified DNA was confirmed by separating 5 µl of the DNA on a 1 % (w/v) agarose gel containing 0.5 µg.ml-1 ethidium bromide (EtBr) in 0.5 x TAE [20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.28 % (v/v) acetic acid]. Before separation the DNA was dissolved in 0.015 % (w/v) bromophenol blue, 2.5 % (w/v) ficoll and resolved at 12 $V.cm^{-1}$ using 0.5 x TAE as running buffer.

The DNA was visualized under ultraviolet light (UV) illumination and photographed using a Bio-Rad gel documentation system.

Table 3.6 List of specimens used and sequenced for each gene region, Genbank accession numbers will be available when results are published.

Specimens used for each gene region				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
ITS				
GRA	*Garuleum album 1*	PB. Phillipson	4326	22/06/1995
BLFU	*Garuleum bipinnatum 1*	J. van Zyl	15	26/11/2011
GRA	*Garuleum bipinnatum 2*	D. Shearing	K207	08/06/1987
G	*Garuleum latifolium 1*	H. Rudatis	1864	22/01/1913
BLFU	*Garuleum pinnatifidum 1*	J. van Zyl	1	03/03/2011
BLFU	*Garuleum pinnatifidum 2*	CA. Beck	3222	00/00/1924
GRA	*Garuleum schinzii 1*	C. Mannheimer	2882	15/02/2004
Z	*Garuleum schinzii 2*	K. Dinter	4810	02/08/1925
BOL	*Garuleum sonchifolium 1*	W. Marais	526	22/09/1954
BOL	*Garuleum sonchifolium 2*	E. Esterhysen	27845	00/07/1958
BLFU	*Garuleum tanacetifolium 1*	J. van Zyl	18	30/11/2011
BLFU	*Garuleum tanacetifolium 2*	J. van Zyl	22	30/11/2011
BLFU	*Garuleum woodii 1*	RD. Stam	420	10/03/1970
BLFU	*Garuleum woodii 2*	Ashafa	s.n.	00/00/2011
***trn*L–*trn*F**				
GRA	*Garuleum album 1*	PB. Phillipson	4326	22/06/1995
BLFU	*Garuleum bipinnatum 1*	J. van Zyl	15	26/11/2011
GRA	*Garuleum bipinnatum 2*	D. Shearing	K207	08/06/1987
G	*Garuleum latifolium 1*	H. Rudatis	1864	22/01/1913
BLFU	*Garuleum pinnatifidum 1*	J. van Zyl	1	03/03/2011
BLFU	*Garuleum pinnatifidum 2*	CA. Beck	3222	00/00/1924
GRA	*Garuleum schinzii 1*	C. Mannheimer	2882	15/02/2004
Z	*Garuleum schinzii 2*	K. Dinter	4810	02/08/1925
BOL	*Garuleum sonchifolium 1*	W. Marais	526	22/09/1954
BOL	*Garuleum sonchifolium 2*	E. Esterhysen	27845	00/07/1958
BLFU	*Garuleum tanacetifolium 1*	J. van Zyl	18	30/11/2011
BLFU	*Garuleum tanacetifolium 2*	J. van Zyl	22	30/11/2011
BLFU	*Garuleum woodii 1*	RD. Stam	420	10/03/1970
BLFU	*Garuleum woodii 2*	Ashafa	s.n.	00/00/2011
***psb*A–*trn*H**				
GRA	*Garuleum album 1*	PB. Phillipson	4326	22/06/1995
GRA	*Garuleum bipinnatum 1*	D. Shearing	K207	08/06/1987
BLFU	*Garuleum bipinnatum 2*	J. van Zyl	15	26/11/2011
G	*Garuleum latifolium 1*	H. Rudatis	1864	22/01/1913
BLFU	*Garuleum pinnatifidum 1*	J. van Zyl	1	03/03/2011
BLFU	*Garuleum pinnatifidum 2*	CA. Beck	3222	00/00/1924

Table 3.6 continue

Specimens used for each gene region				
Herbarium	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
***psb*A–trnH**				
Z	*Garuleum schinzii 1*	K. Dinter	4810	02/08/1925
GRA	*Garuleum schinzii 2*	C. Mannheimer	2882	15/02/2004
BOL	*Garuleum sonchifolium 1*	W. Marais	526	22/09/1954
BLFU	*Garuleum tanacetifolium 1*	J. van Zyl	18	30/11/2011
BLFU	*Garuleum tanacetifolium 2*	J. van Zyl	22	30/11/2011
BLFU	*Garuleum woodii 1*	RD. Stam	420	10/03/1970

3.6.2 Amplification of nuclear and chloroplast genes

The complete nuclear ITS gene region was amplified using Polymerase Chain Reaction (PCR), with four primers respectively (Table 3.7) as described by Blattner (1999).

The entire ITS region was amplified using primers A and B. In cases where the DNA was too degraded to amplify the whole ITS region, the two ITS regions were amplified separately using primers A and C for ITS1 and primers B and D for ITS2.

Ten nanograms (ng) DNA of each sample were amplified using 2 x Kapa readymix (Kapa Biosystems) with 10 µM of the respected forward and reverse primers. The amplification regime was as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 sec, 57 °C for 30 sec and 72 °C for 1 min. A final cycle followed at 72 °C for 7 min. The annealing temperature varied according to each primer set and its different melting temperature (tm).

In instances when amplification with Kapa readymix was unsuccessful, the DNA was amplified using Emerald AMP®MAX HS PCR Master mix (Takara Bio Inc) each reaction consisted of 2 x Emerald master mix and 50 µM for each of the respective forward or reverse primers. The amplification regime was as follows: 30 cycles of 98 °C for 10 sec, 60 °C for 30 sec, 72 °C for 1 min.

The chloroplast DNA regions *trn*T–*trn*L, *trn*L–trnF and *psb*A–*trn*H, were similarly amplified with 2 x Kapa ready mix (Kapa Biosystems), as described above. In instances where amplification was unsuccessful, 2 x Emerald master mix (Takara Bio Inc), was used as described above. Primers used for the amplification of the chloroplast DNA regions are shown in Table 3.7. Annealing temperature was determined according to the different tm values of the different primer sets as indicated in Table 3.7.

Five micro litres of the PCR products were separated on a 1 % (w/v) agarose gel (as described in section 3.6.1 of this chapter).

Table 3.7 Nucleotide sequences of the primers used.

Reference	Gene region	Primer name	Primer sequence	Primer tm	Forward/ Reverse
Blattner (1999)	ITS	ITS-A	5'-GGAAGGAGAAGTCGTAACAAGG-3'	55°C	Forward
Blattner (1999)	ITS	ITS-B	5'-CTTTTCCTCCGCTTATTGATATG-3'	51°C	Reverse
Blattner (1999)	ITS	ITS-C	5'-CTTTTCCTCCGCTTATTGATATG-3'	60°C	Reverse
Blattner (1999)	ITS	ITS-D	5'-CTCTCGGCAACGGATATCTCG-3'	64°C	Forward
Shaw, et al (2005)	*trn*T–*trn*L	*trn*T(UGU)	5'-CAAATGCGATGCTCTAACCT-3'	58°C	Forward
Taberlet, et al (1991)	*trn*T–*trn*L	B	5'-TCTACCGATTTCGCCATATC-3'	58°C	Reverse
Taberlet, et al (1991)	*trn*L–*trn*F	E	5'-GGTTCAAGTCCCTCTATCCC-3'	62°C	Forward
Taberlet, et al (1991)	*trn*L–*trn*F	F	5'- ATTTGAACTGGTGACACGAG-3'	58°C	Reverse
Shaw, et al (2005)	*psb*A–*trn*H	*psb*A	5'-GTTATGCATGAACGTAATGCTC-3'	58°C	Forward
Shaw, et al (2005)	*psb*A–trnH	*trn*H(GUG)	5'-CGCGCATGGTGGATTCACAATCC-3'	66°C	Reverse

3.6.3 Purification and sequencing

The remainder of the PCR products were purified using a PCR/Gel Purification kit (Favorgen Biotech Corporation) according to the manufacturer's specifications. The purified fragments were eluted in 40 µl. The quality and quantity of the purified product was confirmed by separating 5 µl of the product on a 1 % (w/v) agarose gel (as described in section 3.6.1 of this chapter).

Ten ng of the PCR product was sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's specifications. Each fragment was sequenced with either the forward or reverse primer for each gene region respectively.

The sequenced products were purified by adding 5 µl of 125 mM EDTA and 60 µl 100 % (v/v) ethanol. The solution was mixed and incubated at room temperature for 15 min. Samples were centrifuged at 12 000 rpm for 15 min at 4 °C. The supernatant was removed and the pellet washed with 70 % (v/v) ethanol. The tubes were centrifuged for 5 min at 12 000 rpm at 4 °C and the pellet air-dried overnight in the dark. The nucleotide composition of each sample was determined by using the Applied Biosystems 3130*x*l Genetic Analyser.

3.6.4 Sequence editing and alignment

Sequences were edited using the following programs Chromas Lite v.2.01 and Bio-Edit v.7.1.5.0. Editting included base calling, insertion or removal of nucleotides and combining the sequences in the cases where sequences were obtained with both the forward and reverse primers respectively. Alignment of the different species for each of the gene regions was done using the online version of MAFFT v.6.956b.

3.6.5 Determination of which to outgroups use for tree construction

Very little phylogenetic data is available on the Calenduleae. To determine the correct outgroups need for this study is difficult. A phylogenetic showing *Garuleum* as sister to all other genera within the Calenduleae is published in the Calenduleae chapter of the book; Compositae: Systematic, evolution and biogeography of Compositae. In the phylogenetic tree in the Calenduleae chapter it showed that not all the genera within the Calenduleae are monophyletic. The genus Calendula was shown as monophyletic in this tree, and sequences were available on Genbank for this genus.

Not being certain if *Calendula* is too closely related to the genus *Garuleum*, the decision was made to also use the sequences from a monophyletic genus from the tribe Gnaphalieae, which is sister tribe to the Calenduleae, according to the super meta tree generated by Funk et al., in 2005. *Stoebe* was a monophyletic genus within the tribe Gnaphalieae, which had sequences available on Genbank for the different gene regions. Steirodiscus was also used as an outgroup since it belongs to the tribe Senecioneae, which was once thought to be the tribe closest related to the Calenduleae. And the necessary sequences for *Steirodiscus* were available on Genbank.

3.6.6 Tree construction

3.6.6.1 Maximum parsimony trees

Maximum parsimony (Mp) analysis were conducted in PAUP* 4.0b 10 (Swofford, 2003). Trees were constructed for the ITS, *trn*L–*trn*F and *psb*A–*trn*H gene regions. A combined tree was constructed for ITS and *trn*L–*trn*F, after the p-value was determined. Gaps were treated as missing data and all characters were weighted equally. A heuristic search was used to obtain the most parsimonious trees, with 100 random addition-sequence replicates and branch swapping set to tree bisection and reconnection (TBR). Due to a lack of memory for the computer, trees held for each step were set at 1000. Nonparametric bootstrapping was conducted to obtain clade support, and set at 1000 replicates with 100 random addition-sequence replicates and TBR branch swapping.

3.6.6.2 Bayesian inference analysis

The nucleotide substitution models needed for the Bayesian inference (BI) analysis were determined for each of the gene regions using the Akaike information criterion (AIC) function in jModeltest version 2.1.1. (Guindon and Gascuel 2003; Darriba et al. 2012). The nucleotide substitution models were indicated to be SYM+G model for ITS, the F81+G model for *trn*L–*trn*F, and the TIM3+G model for *psb*A–*trn*H. The analysis was done using the new parallel version of MrBayes version 3.1.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The search, starting from a random tree and using four Markov Chain Monte Carlo (MCMC) chains, consisted of 3,000,000 generations sampling every 500 generations. The

average standard deviation of split frequencies (<0.01) was used to assess the convergence of the two runs. Burning of 500 was done prior to analysis. Posterior probability (PP) values of the clades were incorporated into most parsimonious tree chosen from the PAUP analysis, where PP values match with the PAUP trees. Only *Steirodiscus* was used as outgroup in the Bayesian analysis, while *Stoebe* and *Calendula* was run with the ingroup.

Chapter 4

Micromorphology of *Garuleum* leaf and achene epidermal surfaces.

4.1 Introduction

Primary sculpture of the epidermal surfaces of leaves and achenes may be of diagnostic value if epidermal cell shapes can be distinguished. The secondary sculpture, including striations and cuticular folds on the epidermal surface may also be of diagnostic value in certain species. Micro-papillae are an example of taxonomically informative secondary sculpture which is characteristic of the Brassicaceae (Barthlott, 1981). Tertiary sculpture, including wax and wax platelets is of diagnostic value at family level and higher. Rosette-like clustering of wax platelets, of *Cercis* L. is particularly typical for the Cesalpinioideae (Barthlott, 1981). The genus *Pseudophoenix* H.Wendel (Arecaceae) is characterized by large wax plates along cell boundaries of leaves (Barthlott, 1981). Not all characters have the same diagnostic value for all taxa and should be carefully evaluated when choosing characters for a specific study (Theobald et al., 1950).

Achene surface studies have been widely used for classification at generic and species-level in the Asteraceae (Bremer, 1994). Research by Inceer et al. (2012) showed that fruit surface characters are diagnostically useful at interspecific level in the genus *Tripleurospermum* Sch.Bip. (Asteraceae). Achene size, shape and epidermal surface characters were used to separate *Achillea phrygia* Boiss.& Balansa (Asteraceae) and *Achillea gypsicola* Hub.Mor. (Asteraceae) (Ackin and Ackin, 2010). Abid and Ali (2010) used micromorphological characters obtained from achenes to distinguish between nine genera in the Senecioneae and to distinguish species within the studied genera.

In addition to primary, secondary and tertiary sculpture, one of the most important micromorphological characters used in systematic studies of Angiosperm leaf surfaces are trichomes. Trichomes are outgrowths on the epidermal surface with diverse form and function and only a small number of angiosperms are totally devoid of trichomes. In addition trichomes are easy to observe and have variable surface patterns which may be taxonomically informative (Theobald et al., 1950).

Metcalfe and Chalk (1950) noted that size, length and density of trichomes are variable due to environmental conditions and are of less value taxonomically. They did, however, find that trichome type is informative in genus and species level studies.

In trichome description, the indumentum (overall surface appearance) should be established, and thereafter the morphology of the individual trichomes. Trichomes are divided into seven types: papillae, simple, two to five armed, stellate, dendritic, specialized trichomes and scales (Theobald et al., 1950). The trichome complement should be established after individual trichome morphology has been determined, after which the histological descriptions of the trichomes should be considered. The histological characteristics include aspects such as glandular or non-glandular functions, multicellular or unicellular structure, presence of surface formations such as straitions, warts, papillae and presence of constricted or swollen parts of the cells (Theobald et al., 1950).

Trichome characters have been used widely in species and genus delimitation in the Asteraceae. *Artemisia absinthium* L. can be identified by its simple and capitate trichomes on its leaf surface (Westerkamp and Demmelmayer, 1997). *Achillea phrygia* and *Achillea gypsicola* (Asteraceae) are identified by having long simple crispate trichomes and capitate trichomes on abaxial and adaxial leaf surfaces in both species. The diffrence in concentration between the different trichomes of the *Achillea* species can distinguish between the two species (Ackin and Ackin, 2010).

Other important micromorphological characters, to be considerd for systematic classification are stomatal type and structure. The shape and arrangement of subsidiary cells are also important for classification (Wilkinson, 1950). Different types of stomata include actinocytic, allelocytic, amphistomatic, amphiscyclic, anisocytic. (Wilkinson, 1950).

In a study to determine the importance of leaf surface characters in the Asteraceae, *Vernonia amygdalina* Delile. and *Vernonia cinerea* Less. could be delimited from each other by the presence of anisocytic stomata (Adedeji and Jewoola, 2008).

In a study by Sajo and Menezes (1994), it was found that Vernonia psilophylla D.C. and V. sessilifolia Less. could be distinguished from V. linearis Spreng. using stomata as a taxonomic character. All three species had anomocytic stomata, but V. psilophylla and V. sessifolia were amphistomatic, while V. linearis only had stomata on the abaxial epidermis of the leaf.

The size of stomata are viewed by some reseachers as an too variable a character for diagnostic purposes (Metcalfe and Chalk, 1950), but others find it has diagnostic importance (Stace, 1966). Related species usually have stomata of the same size. The range of stomatal size and fequences is thought to be important for descriptions, but has been unsuccesfullly used as a diagnostic character (Metcalfe, 1960).

In this study the leaf and achene surfaces of the eigth *Garuleum* species will be observed to determine if any of these prove to be useful in species-level classification of the genus.

4.2 Materials and methods

All material and methods in this chapter are described in 3.3 and 3.4 (Chapter 3).

4.3 Results

4.3.1 Leaf micromorphology

Garuleum album

The adaxial epidermis consists of isodiametric, tetra- to hexagonal cells with convex periclinal walls. Anticlinal walls are curved or straight and slightly sunken (Fig. 4.1.1 b). Long, simple, crispate trichomes and multicellular, capitate, glandular trichomes are present (Fig. 4.1.1 a–c). The simple trichomes are unicellular and 150–250 µm long, capitate, glandular trichomes are 30–35 µm long with a basal diameter of 10−40 µm and a head diameter of 30–45 µm. The cuticle is slightly striated.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal cells and is densely covered by long, simple, crispate trichomes which are 150–250 µm long (Fig. 4.1.2 a, b). Capitate, glandular trichomes are sparsely arranged, 30–35 µm long with a basal diameter of 10–40 µm and a head diameter of 30–45 µm (Fig. 4.1.2 c). The cuticle is smooth to slightly striated.

The leaves are amphistomatic. On the adaxial surface there are 19 stomata per 1 mm^2, with guard cells 30−60 µm long, 30–40 µm wide and stomatal pores which are 10 µm long and covered by a stomatal ledges of 5–6 µm wide (Fig. 4.1.1 d). Subsidiary cells are covered by cuticular striations radiating from the stomatal pore. On the abaxial epidermis there are 113 stomata per 1 mm^2, with guard cells ±15 µm long, and 15–23 µm wide and the stomatal pores 12–18 µm long and covered by stomatal ledges of 6–8 µm wide (Fig. 4.1.2 d).

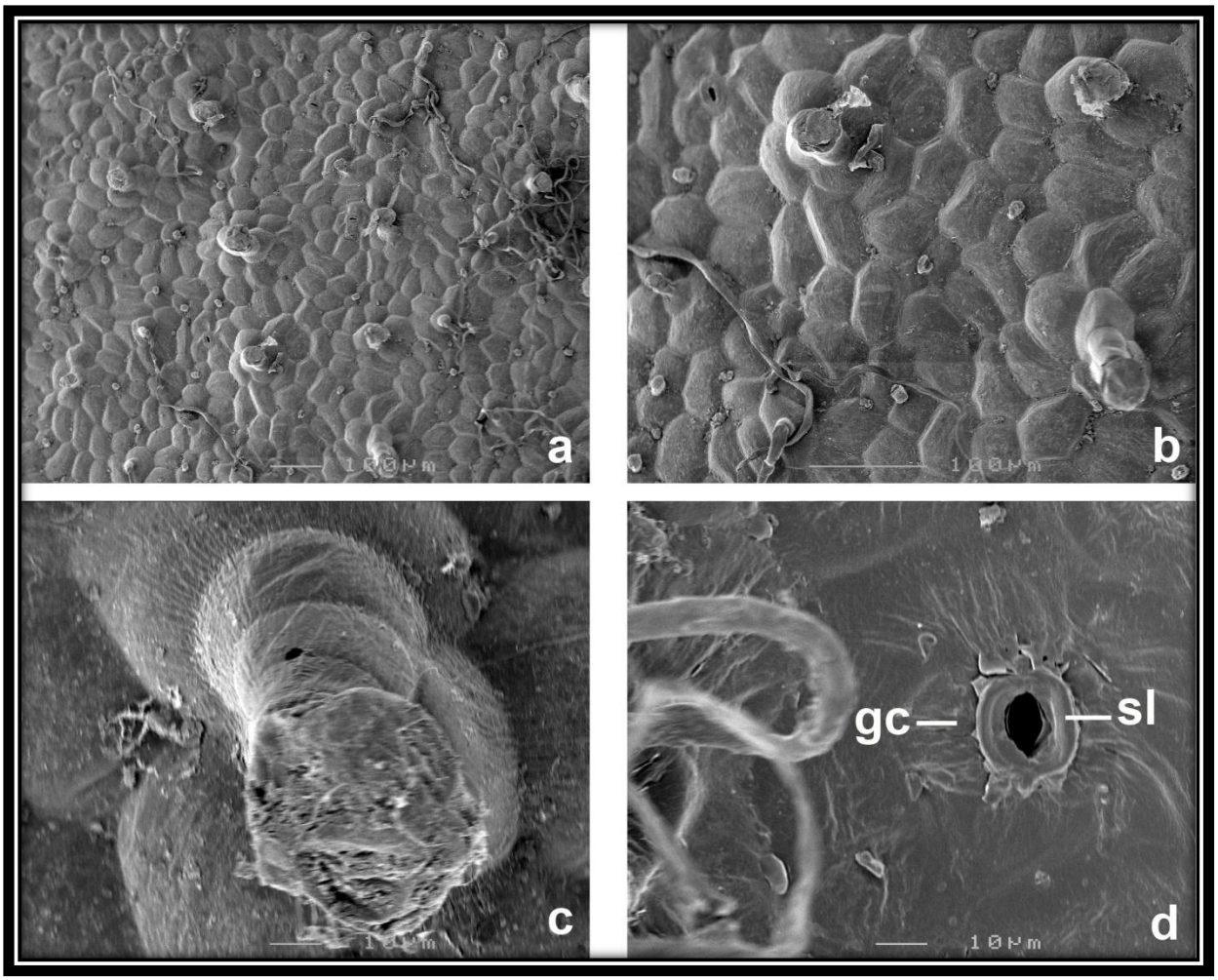

Figure 4.1.1 Adaxial leaf epidermis of *Garuleum album*. In (a) epidermis covered with simple crispate trichomes and capitate, glandular trichomes; (b) periclinal walls convex, anticlinal walls curved or straight; (c) capitate, glandular trichome with cuticular striations radiating from the trichome base; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm; (c, d) = 10 µm. Specimens: (a–c) *Bolus 8718* (BOL), (d) *Phillipson 4326* (GRA).

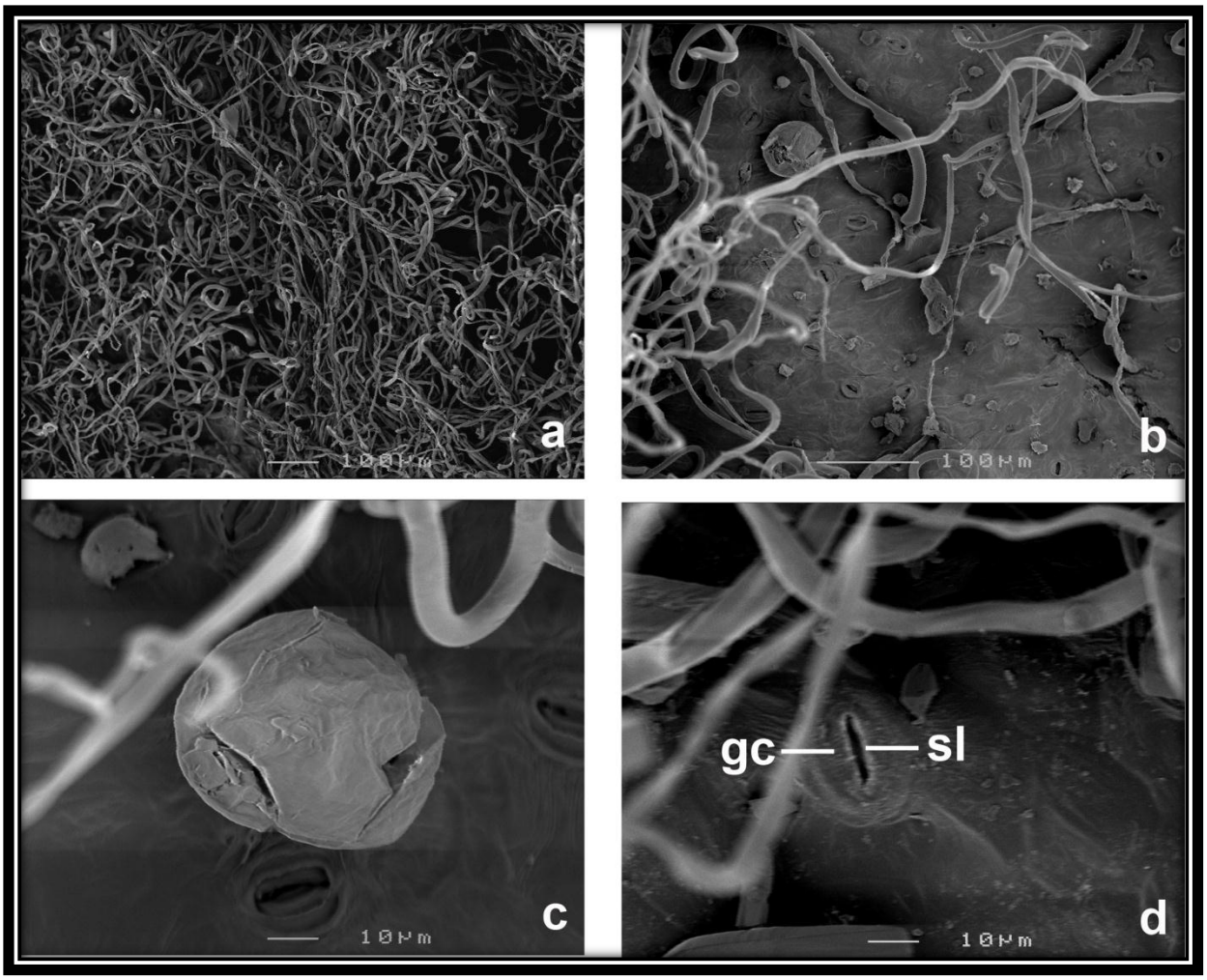

Figure 4.1.2 Abaxial leaf epidermis of *Garuleum album*. In (a, b) epidermis covered with densely arranged, simple, crispate trichomes; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a) *Phillipson 4326* (GRA), (b, c) *Pegler 1569* (BOL), (d) *Bolus 8718* (BOL).

Garuleum bipinnatum

The adaxial epidermis consists of elongated epidermal cells with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.2.1 b). Short, simple trichomes and capitate trichomes are present on the adaxial epidermal surface (Fig. 4.2.1 a−c). The simple trichomes are multicellular and 11−22 µm long while for the capitate, glandular trichomes the basal diameter could not be determined but the head diameter is 53−84 µm. The cuticle is smooth to slightly striated.

The abaxial epidermis consists of elongated cells with tabular periclinal walls and curved or straight anticlinal walls and is covered with capitate trichomes and very sparsely arranged short simple trichomes (Fig. 4.2.2 a, b). The simple trichomes are 35–40 µm long. Capitate, glandular trichomes have a head diameter of 50–100 µm (Fig. 4.2.2 c). The cuticle is smooth to slightly striated.

The leaves are amphistomatic. On the adaxial surface there are 116 stomata per 1 mm^2, with guard cells 28 µm long and 17–19 µm wide and stomatal pores of 7–16 µm long, covered by stomatal ledges of ±3.3 µm wide (Fig. 4.2.1 d). The guard cells are covered by a striated cuticle with striations parallel to the stomatal pore. On the abaxial epidermis there are 69 stomata per 1 mm^2, with guard cells of 27.75–36.63 µm long and 15.56–16.75 µm wide, while the stomatal pores are 10–14.43 µm long and covered by stomatal ledges of ±2.22 µm wide (Fig. 4.2.2 d).

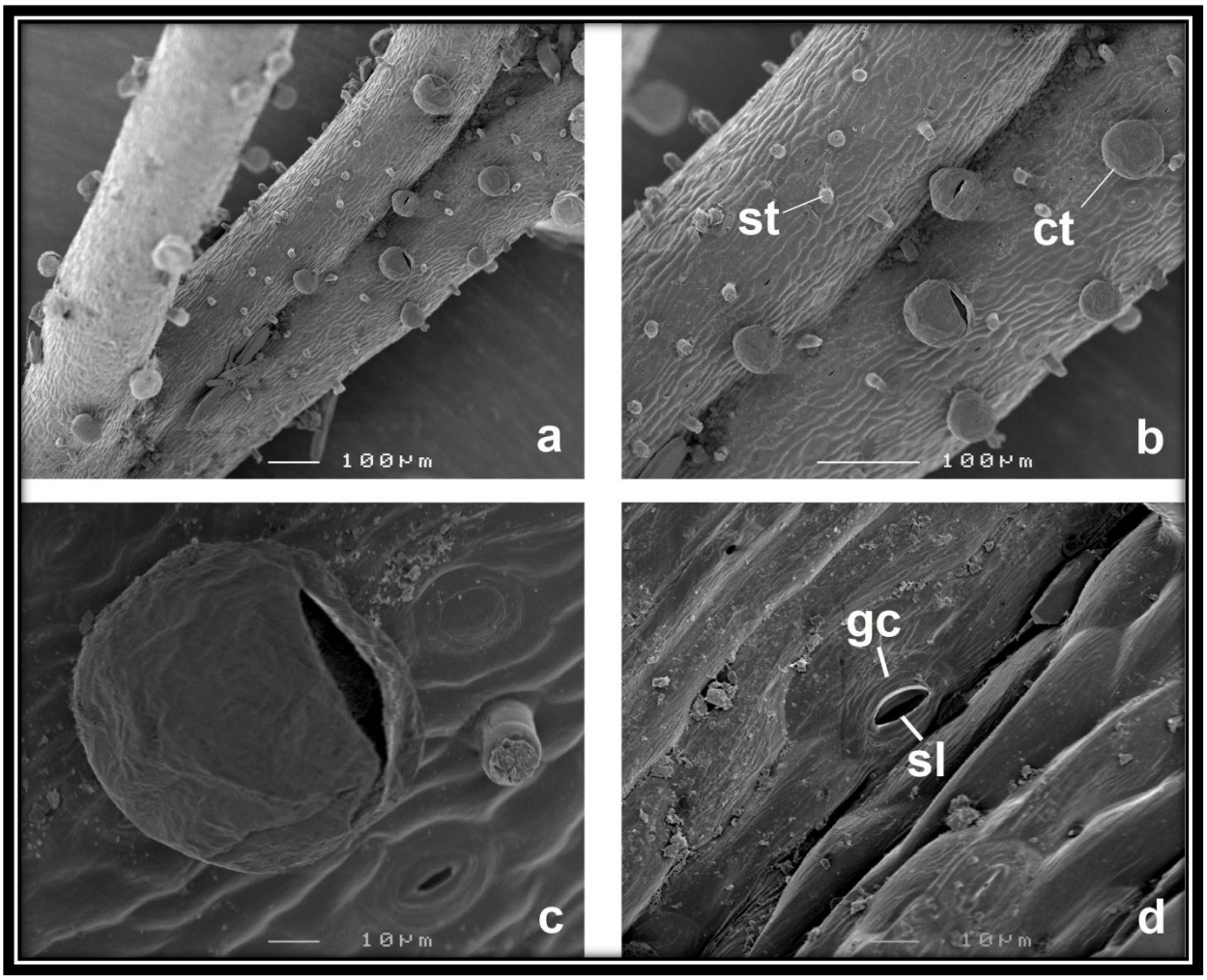

Figure 4.2.1 Adaxial leaf epidermis of *Garuleum bipinnatum.* In (a) epidermis covered with multicellular simple trichomes, capitate, glandular trichomes; (b) periclinal walls convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: Ct = capitate trichome, gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Van Zyl 9* (BLFU), (c) *Van Zyl 14* (BLFU), (d*) Shearing K 207* (GRA).

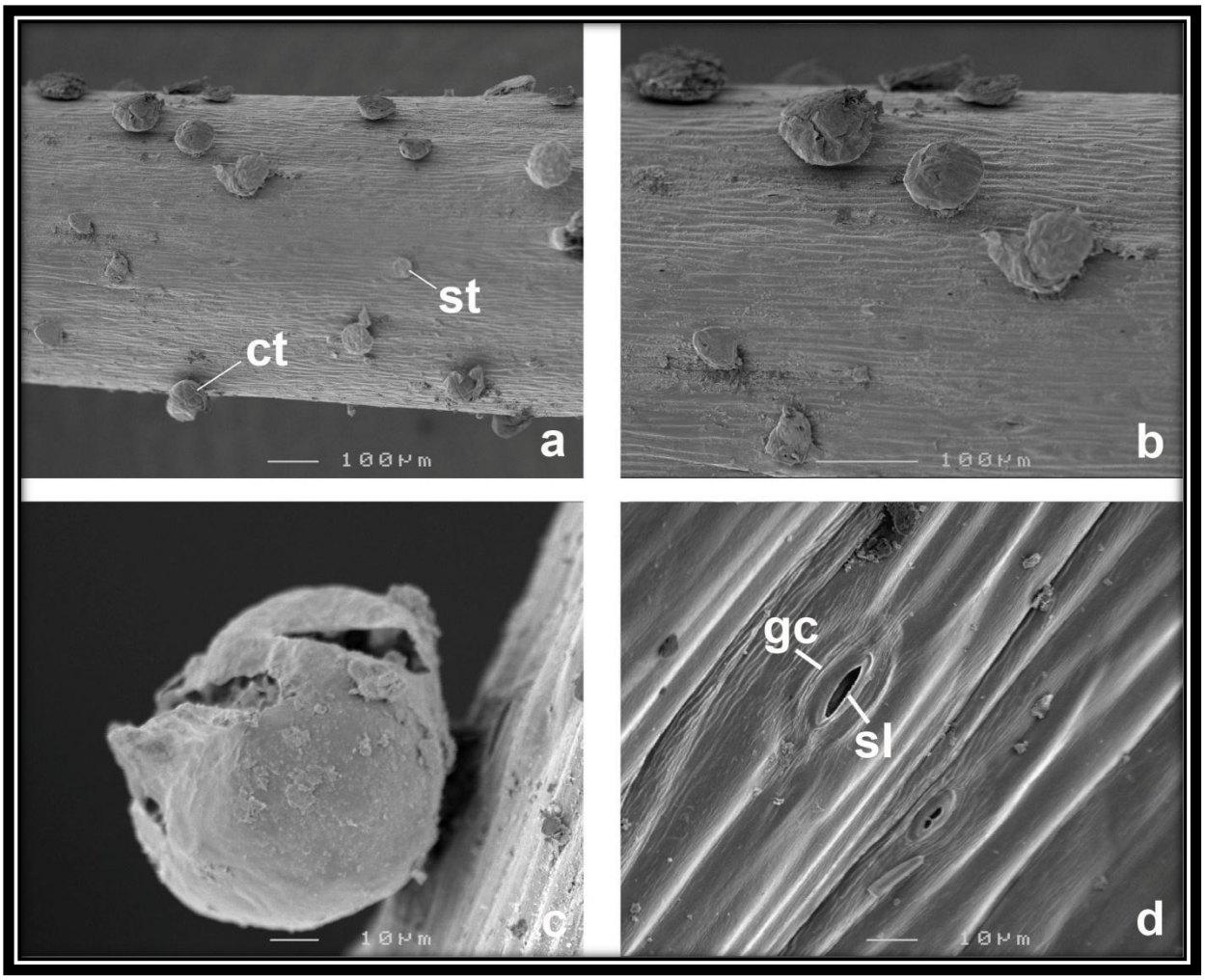

Figure 4.2.2 Abaxial leaf epidermis of *Garuleum bipinnatum.* In (a, b) epidermis covered with short simple trichomes and capitate, glandular trichomes; (c) capitate, glandular trichome; (d) stoma. Legend: ct = capitate trichome, gc = guard cell, sl = stomatal ledge, st = simple trichome. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Van Zyl 9* (BLFU), (c) *Van Zyl 14* (BLFU), (d) *Shearing K 207* (GRA).

Garuleum latifolium

The adaxial epidermis consists of concave periclinal walls and curved or straight anticlinal walls (Fig. 4.3.1 b). Cell shape is undetermined due to folds in the cuticule. Capitate, glandular trichomes are present on the adaxial epidermal surface (Fig. 4.3.1 a–c). These are 36.6–100 µm long with a basal diameter of 16.6–36 µm and a head diameter of 24.5–41 µm. The cuticle is smooth to slightly striated.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells with tabular periclinal walls and curved or straight anticlinal walls (Fig. 4.3.2 a, b). Cell shape is undetermined due to folds in the cuticule. Capitate, glandular trichomes cover the epidermis (Fig. 4.3.2 c). These are 38.8–47 µm long with a basal diameter 14.4–31.75 µm and a head diameter of 28.9–39 µm. The cuticle is folded.

The leaves are amphistomatic. On the adaxial surface there are are 55 stomata per 1 mm^2, with guard cells 18.8–26.7 µm long and 9.6–16.7 µm wide and stomatal pores 5.6–14.6 µm long, covered by stomatal ledges of 1.6–2.9 µm wide (Fig. 4.3.1 d). On the abaxial epidermis there are 272 stomata per 1 mm^2, with guard cells17–30 µm long and 9–17.5 µm wide; and the stomatal pores 5.6−10 µm long and covered by stomatal ledges of 1.6–2.5 µm wide (Fig. 4.3.2 d).

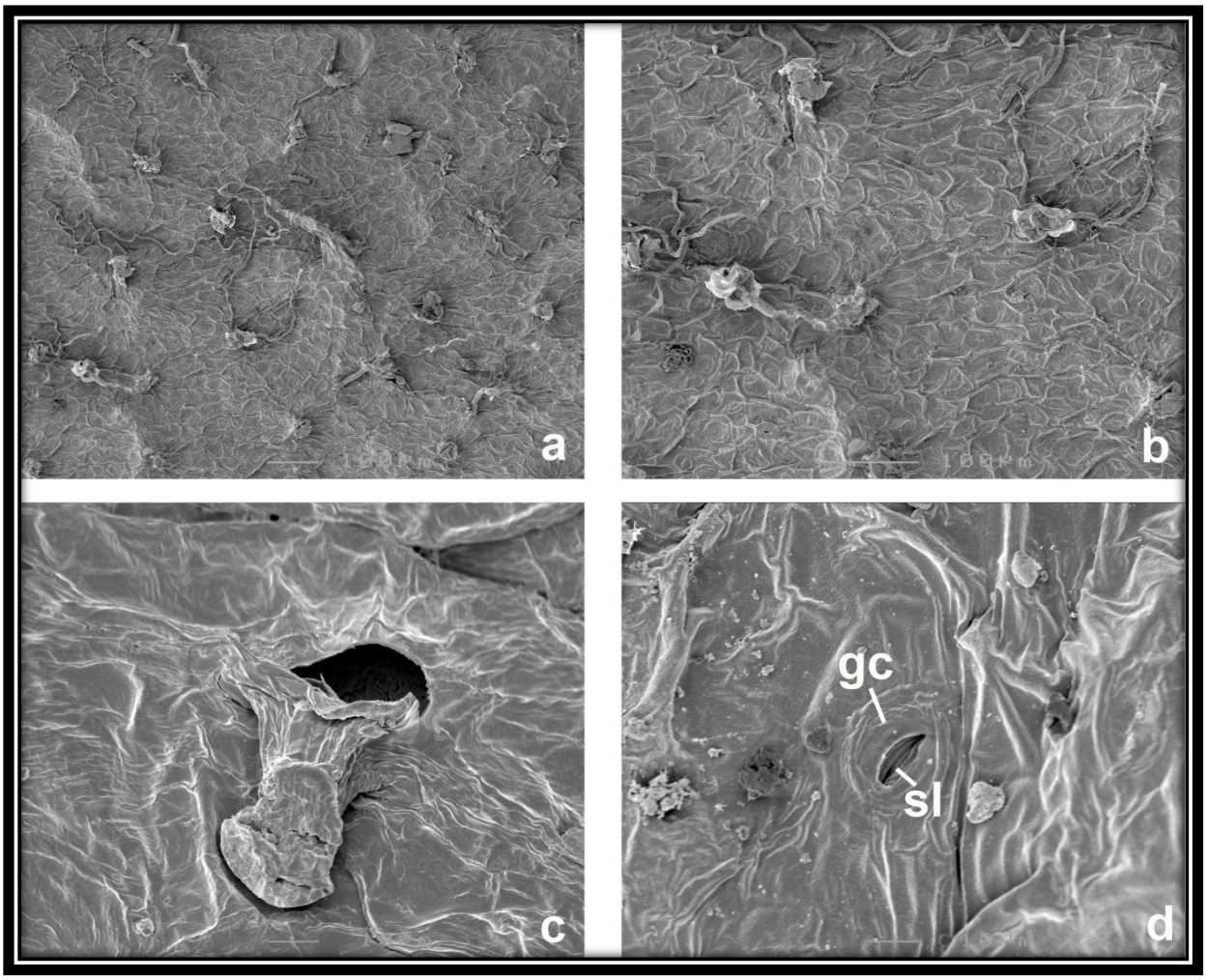

Figure 4.3.1 Adaxial leaf epidermis of *Garuleum latifolium.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls concave, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Wood* 299 (Z), (c) *Rudatis 1864* (L), (d*) Galpin 21496* (BOL).

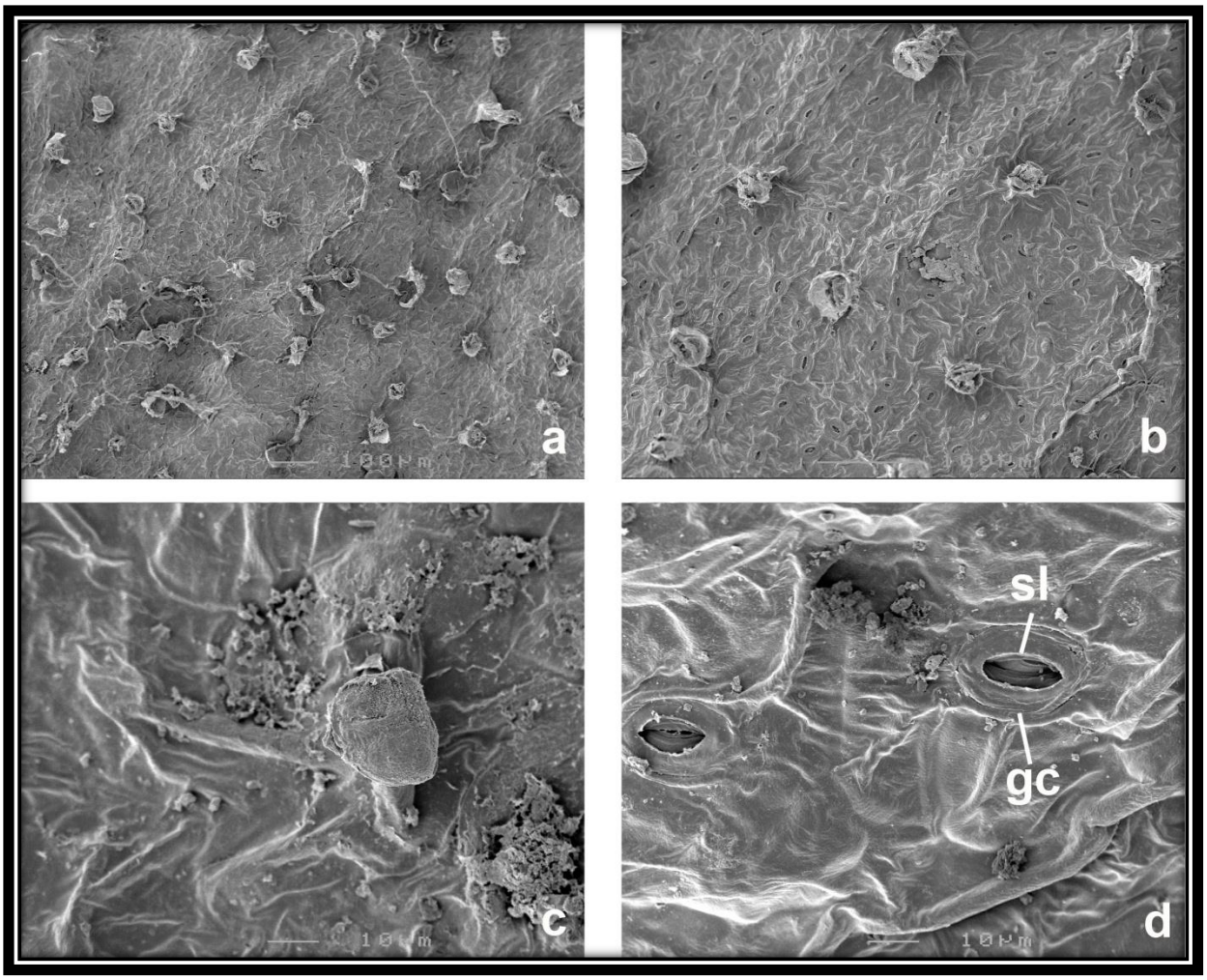

Figure 4.3.2. Abaxial leaf epidermis of *Garuleum latifolium.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls tabular, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Wood 299* (Z), (c, d) *Galpin 21496* (BOL).

Garuleum pinnatifidum

The adaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells with convex periclinal walls and wavy anticlinal walls, which are slightly sunken (Fig. 4.4.1 b). Long simple trichomes and multicellular capitate, glandular trichomes are present on the epidermal surface (Fig. 4.4.1 a–c). Simple trichomes are unicellular and 172–394 µm long. The capitate, glandular trichomes are 20–30 µm long with a basal diameter of 18–25 µm and a head diameter of 22–34 µm. The cuticle is smooth.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells with periclinal walls which are convex and anticlinal walls which are curved or wavy and slightly sunken (Fig. 4.4.2 a, b). The epidermis is covered with long simple trichomes which are sparsely arranged and evenly arranged capitate trichomes (Fig. 4.4.2 c). The capitate, glandular trichomes are 16–20 µm long with a basal diameter of 13–20 µm and a head diameter of 27–31 µm. The cuticle is smooth.

The leaves are amphistomatic. On the adaxial surface there are 130 stomata per 1 mm^2, with guard cells 23–30 µm long and 17–21 µm wide and stomatal pores of 10–16 µm long, covered by stomatal ledges of 2.5–4 µm wide (Fig. 4.4.1 d). Guard cells are covered by a striated cuticle with striations radiating from the stomata ledge. On the abaxial epidermis there are 137 stomata per 1 mm^2, with guard cells 20–25 µm long and 15–20 µm wide, with stomatal pores of 10–12 µm long and covered by stomatal ledges of 2–4 µm wide (Fig. 4.4.2 d). Cuticular striations over guard cells are parallel to the stomatal ledge.

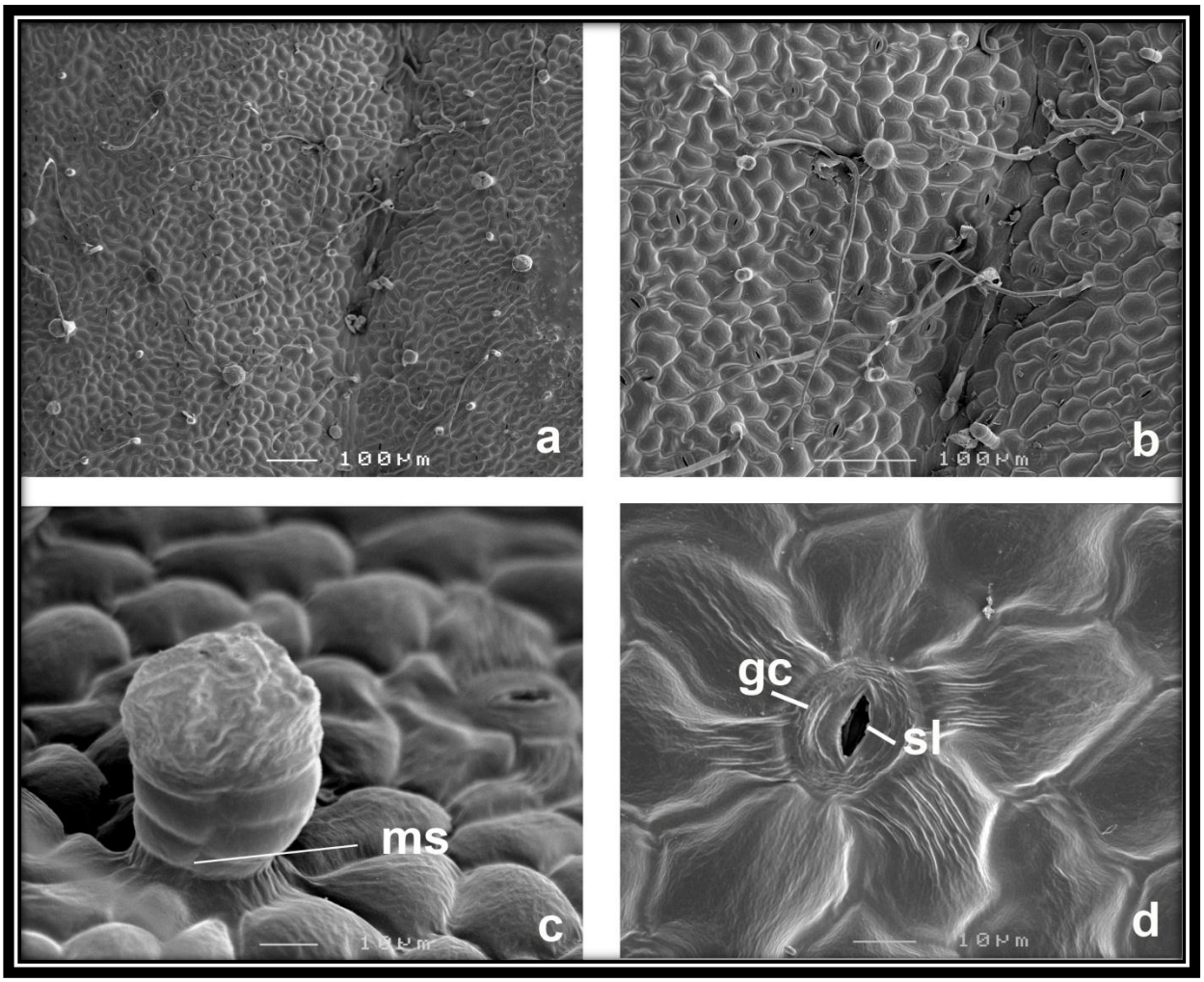

Figure 4.4.1 Adaxial leaf epidermis of *Garuleum pinnatifidum.* In (a) epidermis covered with long simple trichomes and capitate, glandular trichomes; (b) periclinal walls concave, anticlinal walls curved or straight; (c) capitate, glandular trichome with a multicellular stalk; (d) stoma. Legend: gc = guard cell, ms = multicellular stalk, sl = stomatal ledgeScale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b, d*) Van Zyl 2* (BLFU), (c) *Van Zyl 4* (BLFU).

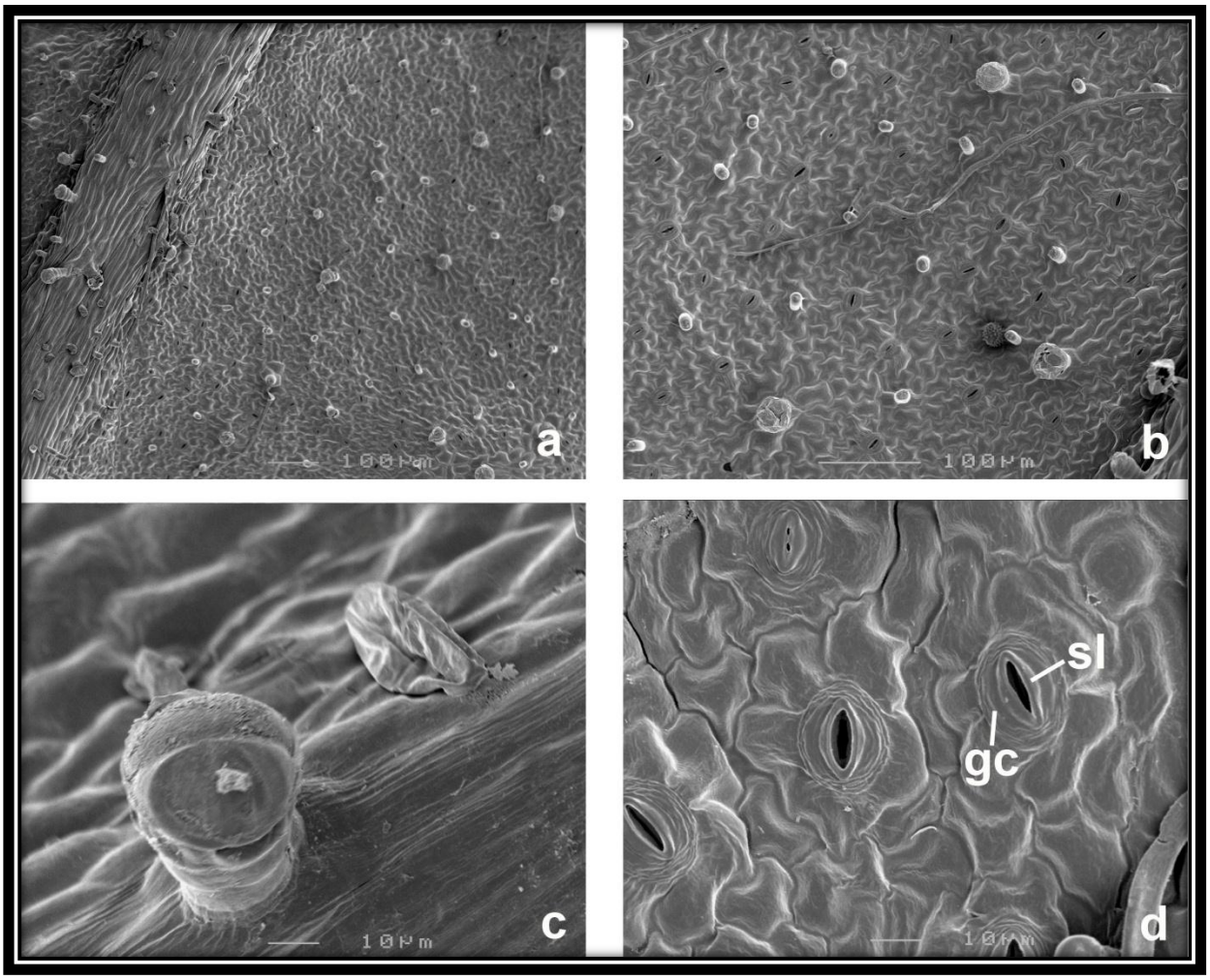

Figure 4.4.2 Abaxial leaf epidermis of *Garuleum pinnatifidum.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls tabular, anticlinal walls curved or wavy; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Van Zyl 2* (BLFU), (c) *Van Zyl 3* (BLFU), (d) *Van Zyl 4* (BLFU).

Garuleum schinzii

The adaxial epidermal cells have tabular periclinal walls and curved or straight anticlinal walls (Fig. 4.5.1 b). Epidermal cell shape was indiscernible. Capitate, glandular trichomes are present on the adaxial epidermal surface (Fig. 4.5.1 a–c). These are 30–75 µm long with a basal diameter of 25–63 µm with a head diameter of 30–35 µm. The cuticle is slightly striated.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells and multicellular, multiseriate simple trichomes and capitate trichomes (Fig. 4.5.2 a, b, c). Capitate, glandular trichomes are 80–130 µm long with a basal diameter of 50–80 µm and a head diameter of 35–40 µm. The simple trichomes are 216–333 µm long. The cuticle is folded and slightly striated.

The leaves are amphistomatic. On the adaxial surface there are 204 stomata per 1 mm^2, with guard cells 21–28 µm long and 17–22 µm wide and stomatal pores of 9–16 µm long, covered by stomatal ledges of 2–2.5 µm wide (Fig. 4.5.1 d). On the abaxial epidermis there are 156 stomata per 1 mm^2, 20–25 µm long and 15–18 µm wide. The stomatal pores are 11–12 µm long and covered with stomatal ledges of 2 µm wide (Fig. 4.5.2 d).

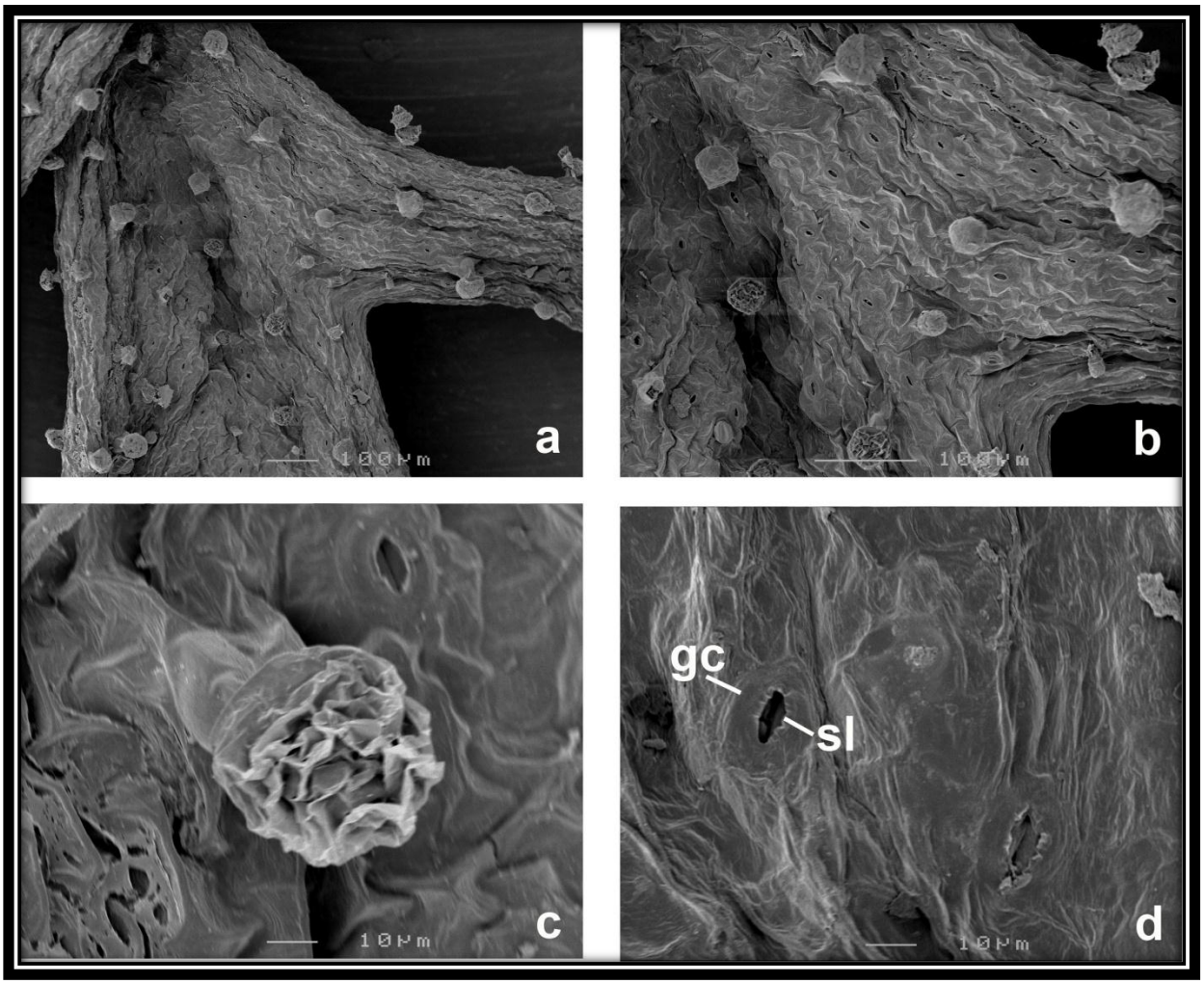

Figure 4.5.1 Adaxial leaf epidermis of *Garuleum schinzii.* In (a) epidermis covered with long simple trichomes and capitate, glandular trichomes; (b) periclinal walls concave, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a–c) *Mannheimer 2882* (GRA), (d) *Pearson 7935* (BOL).

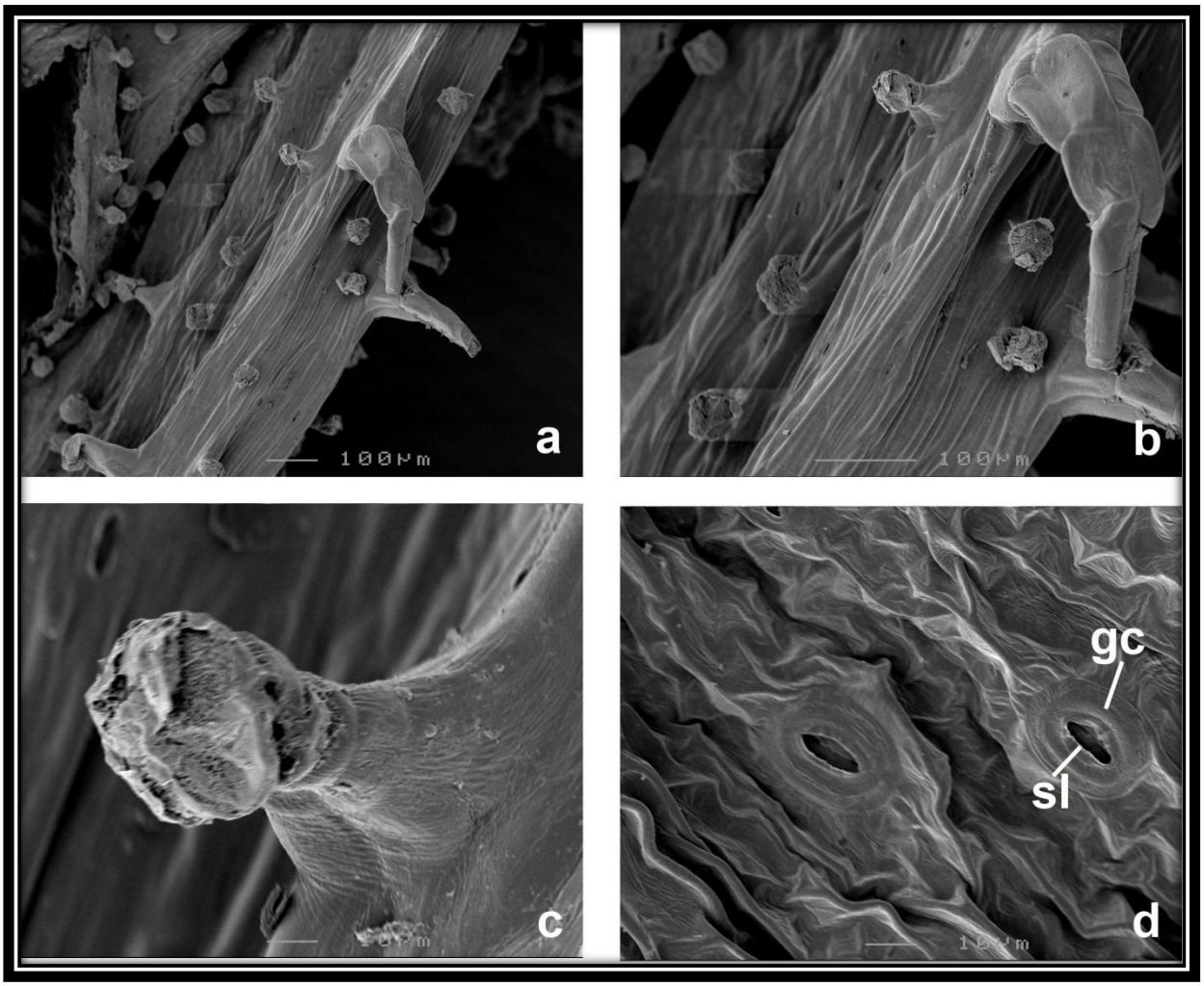

Figure 4.5.2 Abaxial leaf epidermis of *Garuleum schinzii.* In (a, b) epidermis covered with simple, multicellular trichomes and capitate, glandular trichomes; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cell, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a–c) *Pearson 7935* (BOL), (d) *Mannheimer 2882* (GRA).

Garuleum sonchifolium

The adaxial epidermis consists of isodiametric, tetra- to hexagonal cells with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.6.1 b). Capitate, glandular trichomes are present on the epidermal surface and are 57–80 µm long with a basal diameter of 19–35 µm with a head diameter of 30–40 µm (Fig. 4.6.1 a–c). The cuticle is smooth to slightly striated.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.6.2 a, b). The epidermis is covered with multicellular, multiseriate simple trichomes and capitate, glandular trichomes. Simple trichomes are 116 µm long, while capitate trichomes are 53–80 µm long with a basal diameter of 14–38 µm and a head diameter of 27–41 µm (Fig. 4.6.2 c). The cuticle is smooth to slightly striated.

The leaves are amphistomatic. On the adaxial surface there are 36 stomata per 1 mm^2, with guard cells 21−30 µm long and 22–28 µm wide and stomatal pores of 10–14 µm long, coverd with stomatal ledges 3–4 µm wide (Fig. 4.6.1 d). On the abaxial epidermis there are 145 stomata per 1 mm^2, with guard cells 25–30 µm long and 18–20 µm wide and the stomatal pores 13–15 µm long, covered by stomatal ledges of 2–4 µm wide (Fig. 4.6.2 d). Cuticular striations over guard cells are parallel to the stomatal ledge.

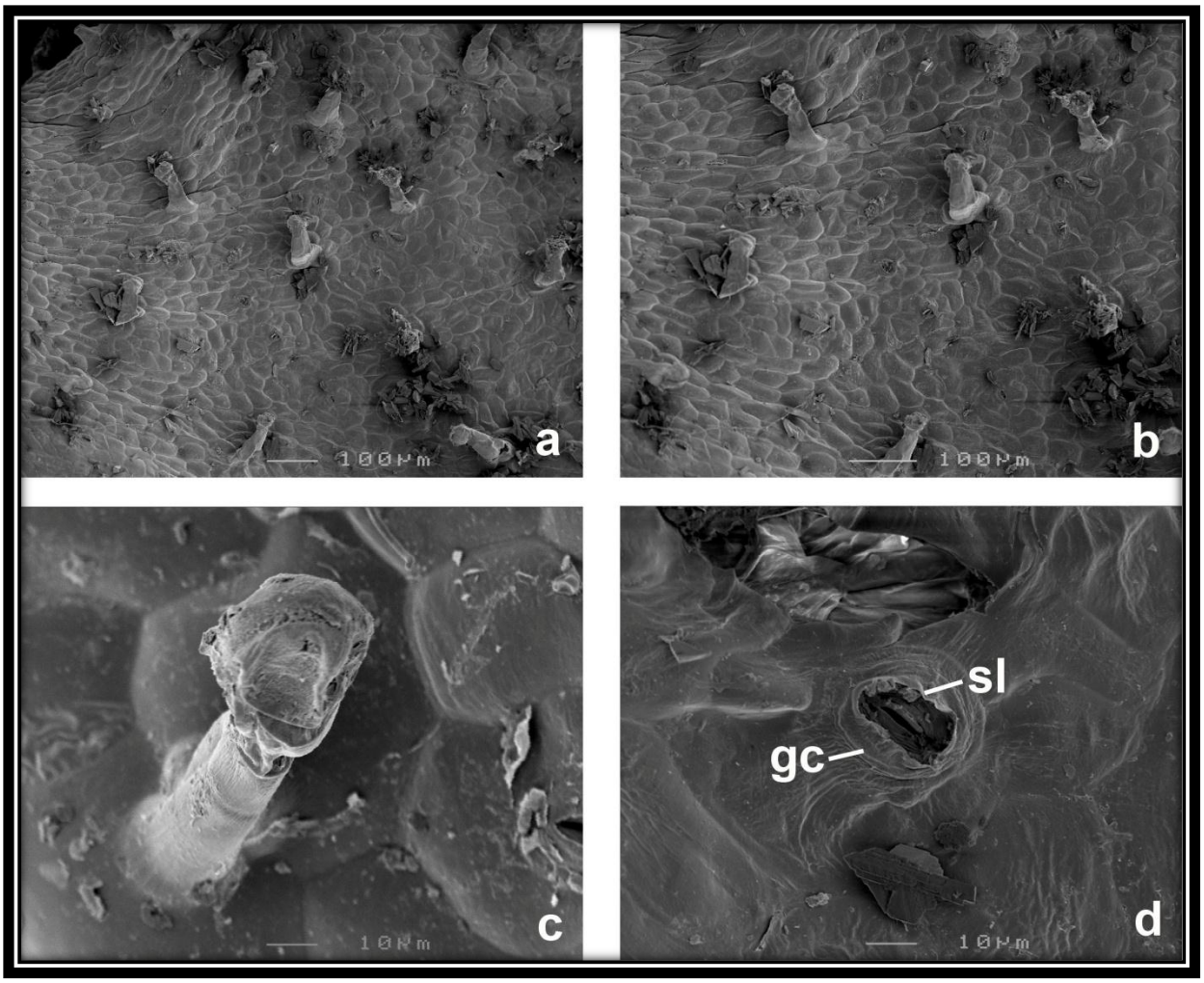

Figure 4.6.1 Adaxial leaf surface of *Garuleum sonchifolium.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b, d) *Pegler 1199* (BOL), (c) *Goulimis 45763* (BOL).

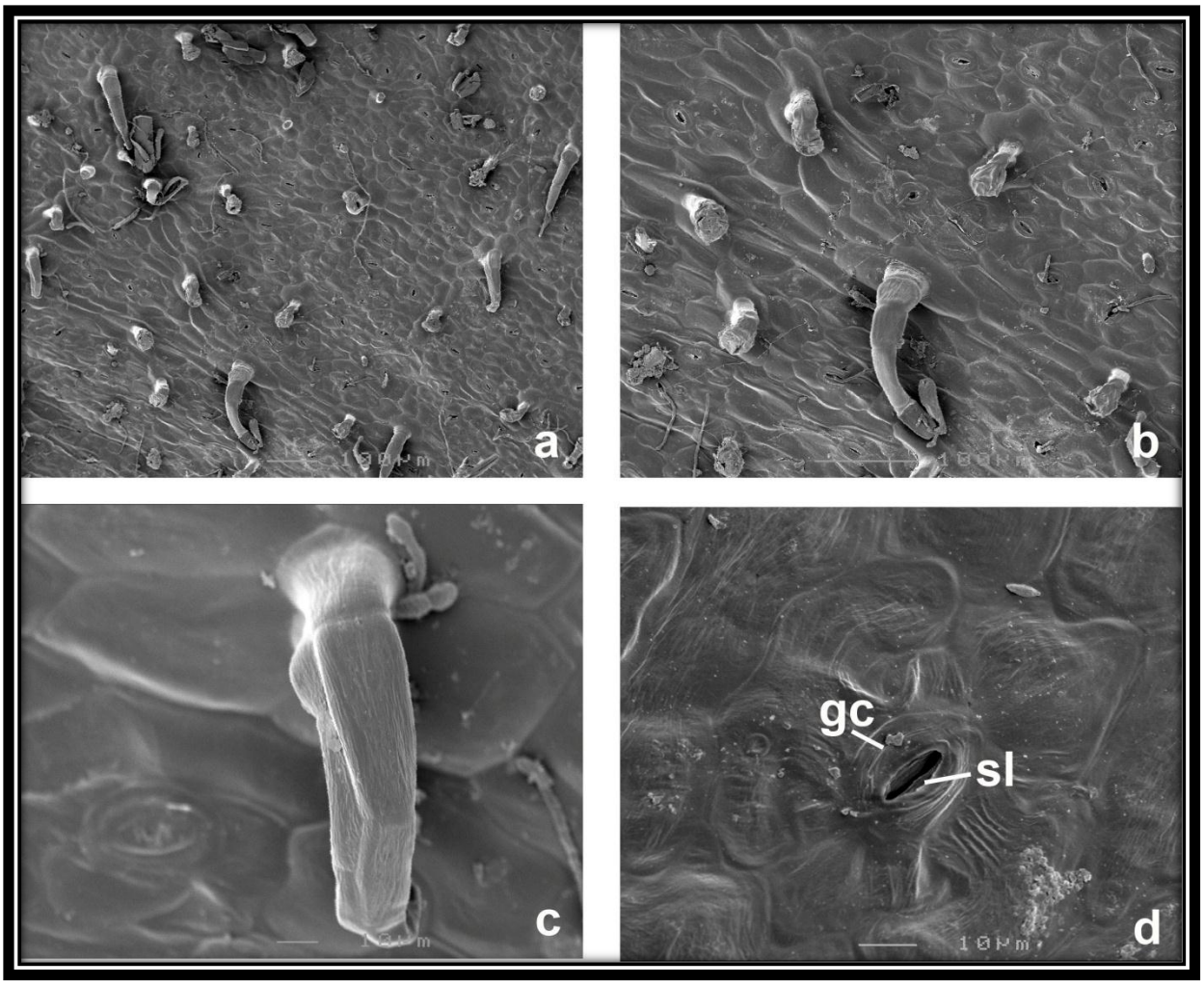

Figure 4.6.2 Abaxial leaf surface of *Garuleum sonchifolium.* In (a, b) epidermis covered with simple, multicellular trichomes and capitate, glandular trichomes, periclinal walls convex, anticlinal walls curved or straight; (c) simple trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a–c) *Goulimis 45763* (BOL), (d) *Esterhysen 27845* (BOL).

Garuleum tanacetifolium

The adaxial epidermis consists of isodiametric, tetra- to hexagonal epidermal cells with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.7.1 b). Capitate, glandular trichomes with striations around their bases are present on the epidermal surface (Fig 4.7.1 a–c). These are 25–100 µm long with a basal diameter of 30–35 µm with a head diameter of 45–50 µm. The cuticule is striated with striations parallel and continuous over several cells.

The abaxial epidermis consists of elongated cells with convex periclinal walls and curved or straight anticlinal walls. The surface is covered with capitate, glandular trichomes (Fig. 4.7.2 a, b). Around the bases of capitate trichomes the cuticle is striated with striations radiating from the trichome base (Fig. 4.7.2 c). Capitate trichomes are 40 µm long with a basal diameter of 20–25 µm with a head diameter of 32–40 µm. The cuticle is smooth.

The leaves are amphistomatic. On the adaxial surface there are 52 stomata per 1 mm^2, with guard cells 26–34 µm long and 20–25 µm wide and the stomatal pores 11–15 µm long, covered by stomatal ledges 3 µm wide (Fig. 4.7.1 d). On the abaxial epidermis there are 74 stomata per 1 mm^2, with guard cells 15–30 µm long and 8–22 µm wide, with the stomatal pores 6–15 µm long, covered by stomatal ledges of 1–2 µm wide (Fig. 4.7.2 d). The cuticle covering the guard cells is smooth.

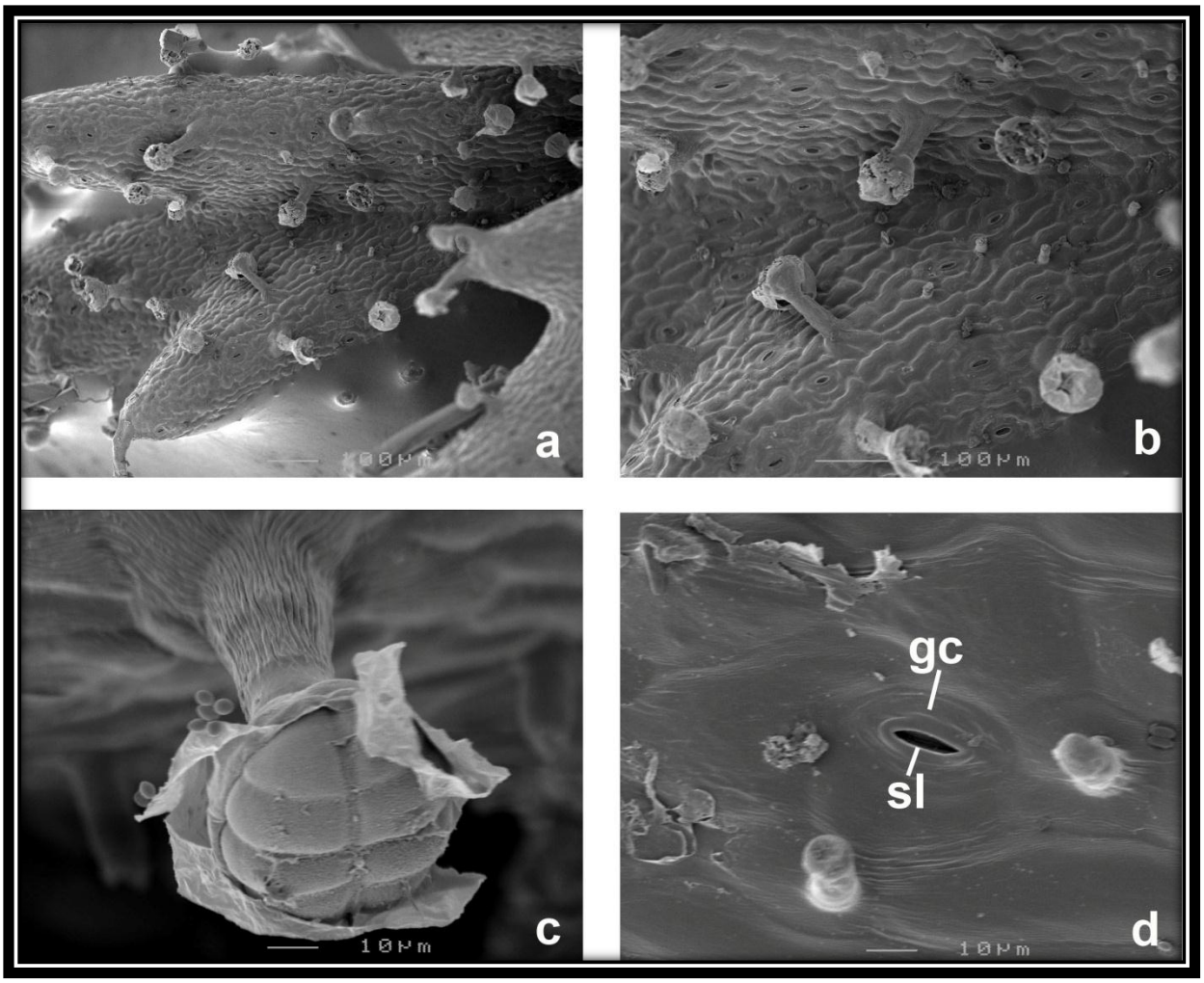

Figure 4.7.1 Adaxial leaf epidermis of *Garuleum tanacetifolium.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls are convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b, d) *Van Zyl 16* (BLFU), (c) *Van Zyl 22* (BLFU).

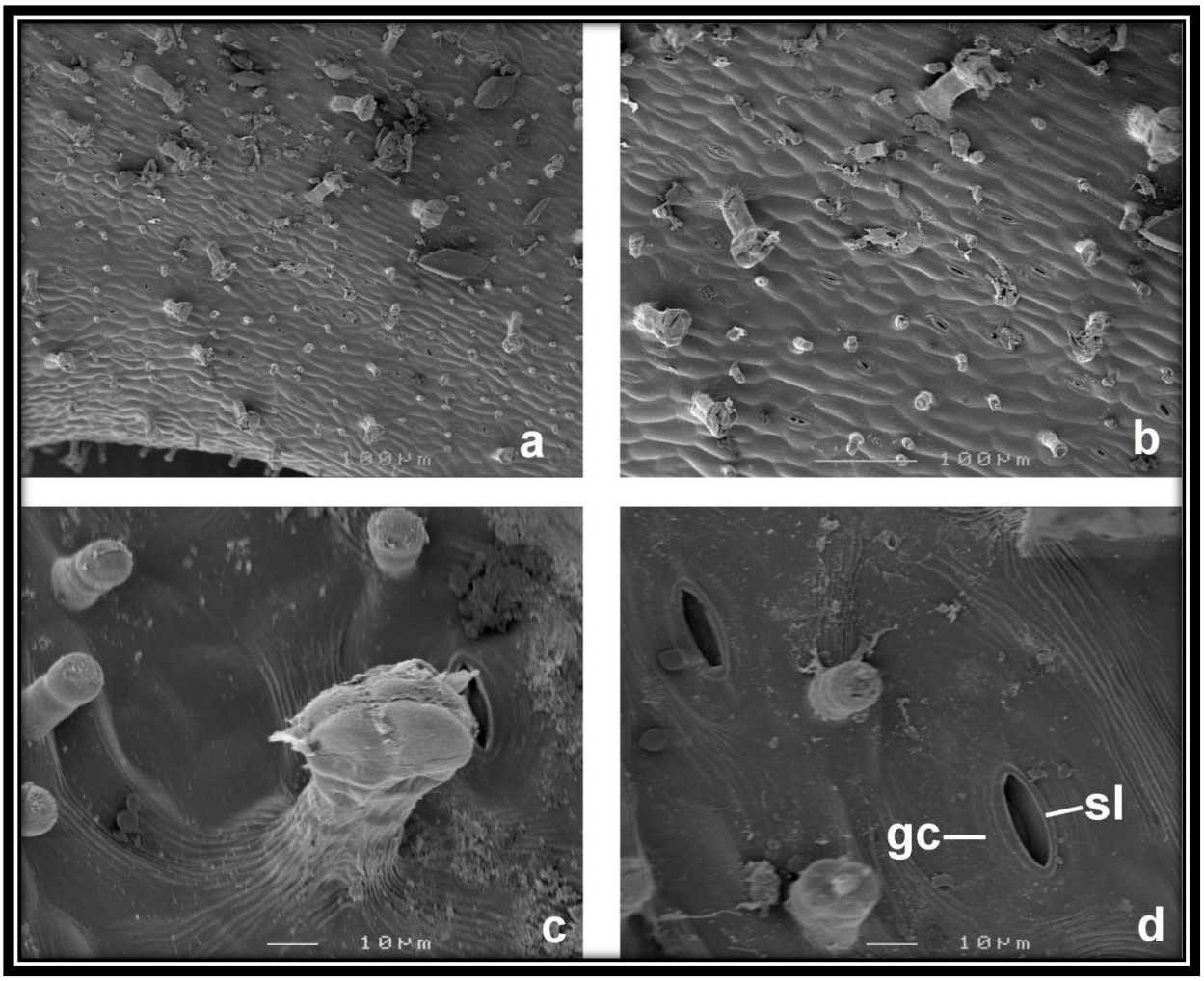

Figure 4.7.2 Abaxial leaf surface of *Garuleum tanacetifolium.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a–c) *Van Zyl 22* (BLFU), (d) *Van Zyl 16* (BLFU).

Garuleum woodii

The adaxial epidermis consists of isodiametric, tetra- to hexagonal cells with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.8.1 b). Capitate, glandular trichomes are present on the epidermal surface (Fig. 4.8.1 a–c). These are 64–150 µm long with a basal diameter of 24–46 µm and a head diameter of 48–74 µm. The cuticle is slightly striated.

The abaxial epidermis consists of isodiametric, tetra- to hexagonal cells, with convex periclinal walls and curved or straight anticlinal walls (Fig. 4.8.2 a, b). Capitate, glandular trichomes cover the epidermis (Fig. 4.8.2 c). These are 55–125 µm long with a basal diameter of 34–46 µm and a head diameter of 50 µm. The cuticle is smooth.

The leaves are amphistomatic. On the adaxial surface there are 215 stomata per 1 mm^2, with guard cells 28–32 µm long and 16–25 µm wide with stomatal pores of 10–14 µm long, covered by stomatal ledges 2–3 µm wide (Fig. 4.8.1 d). On the abaxial epidermis there are 194 stomata per 1 mm^2, with guard cells 22–33 µm long and 13–20 µm wide; and stomatal pores of 5–13 µm long, covered by stomatal ledges of 1–2 µm wide (Fig. 4.8.2 d).

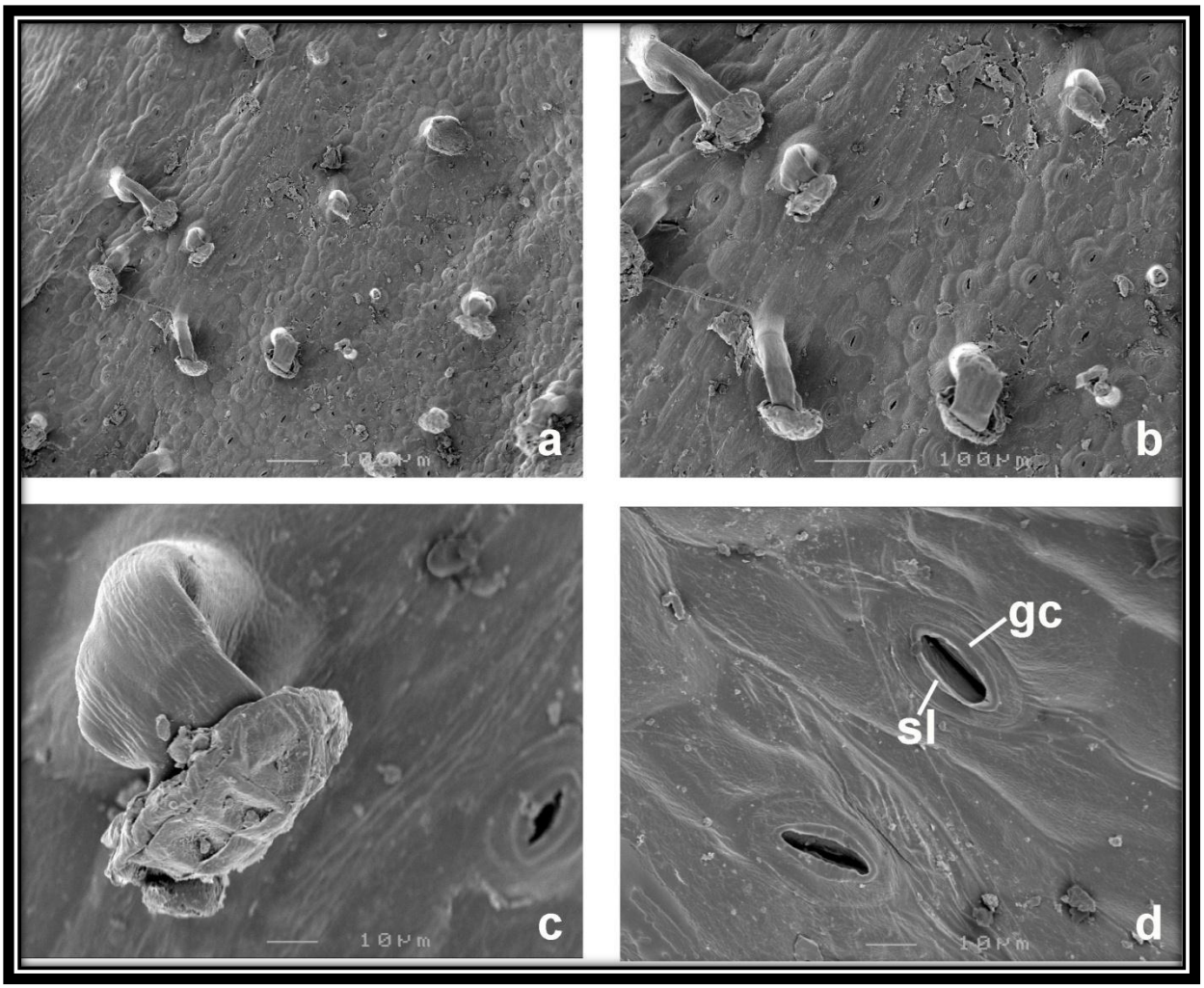

Figure 4.8.1 Adaxial leaf epidermis of *Garuleum woodii.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls are convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b, d) *Ashafa s.n.* (BLFU), (c) *Wood 4860* (BOL).

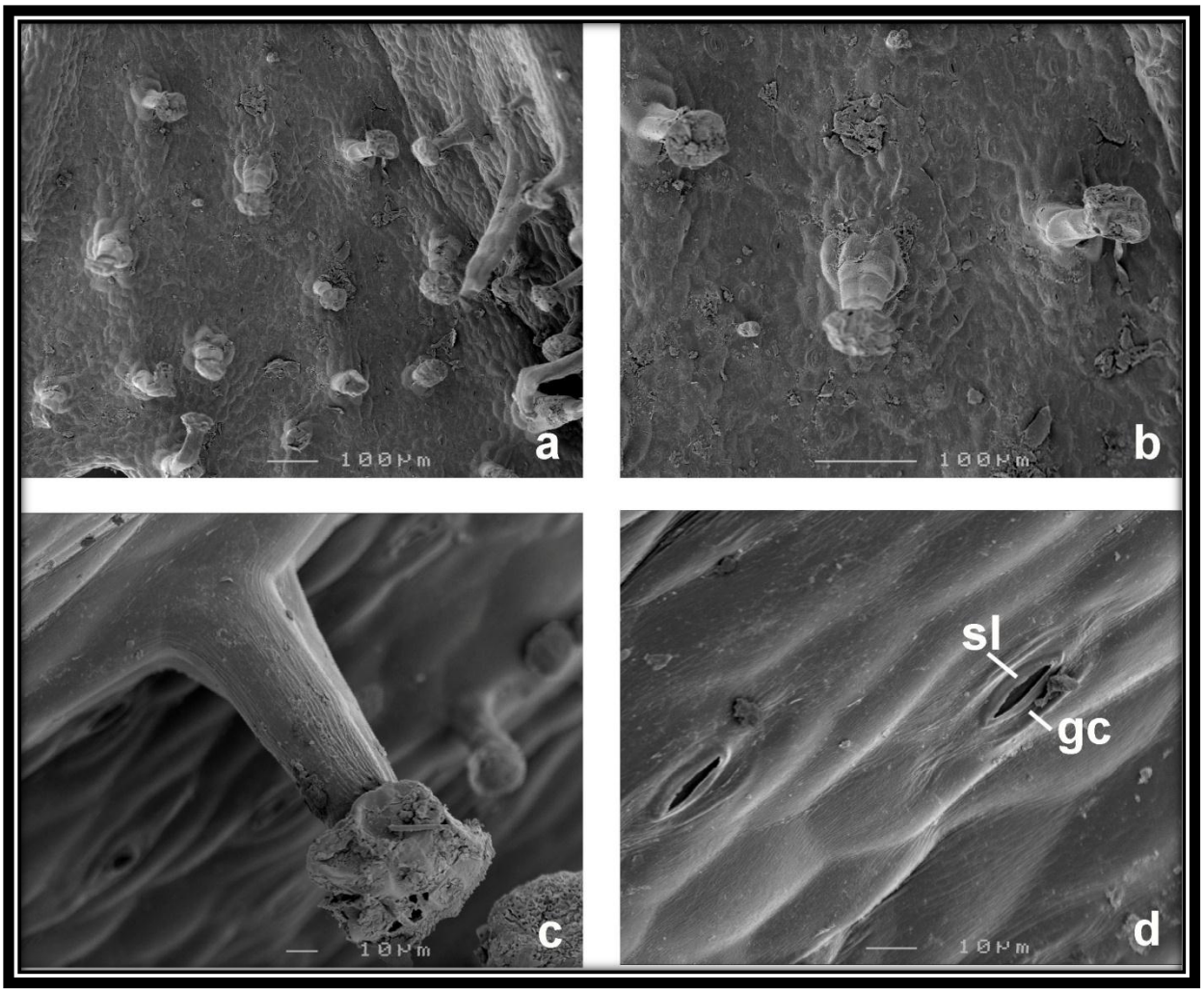

Figure 4.8.2 Abaxial leaf epidermis of *Garuleum woodii.* In (a) epidermis covered with capitate, glandular trichomes; (b) periclinal walls convex, anticlinal walls curved or straight; (c) capitate, glandular trichome; (d) stoma. Legend: gc = guard cells, sl = stomatal ledge. Scale bars: (a, b) = 100 µm, (c, d) = 10 µm. Specimens: (a, b) *Ashafa s.n.* (BLFU), (c, d) *Wood 4860* (BOL).

4.3.2 Achene surface micro morphology

Garuleum album

Only ray florets produce fertile achenes. The achenes are obovate and the epidermal surface is smooth with inconspicuous longitudinal ridges (Fig. 4.9 a–c). The epidermal cells are isodiametric and tetra- to hexagonal. Periclinal walls are tabular and the anticlinal walls are straight and level with the epidermal surface. The cuticle is smooth to slightly granular (Fig. 4.9 c).

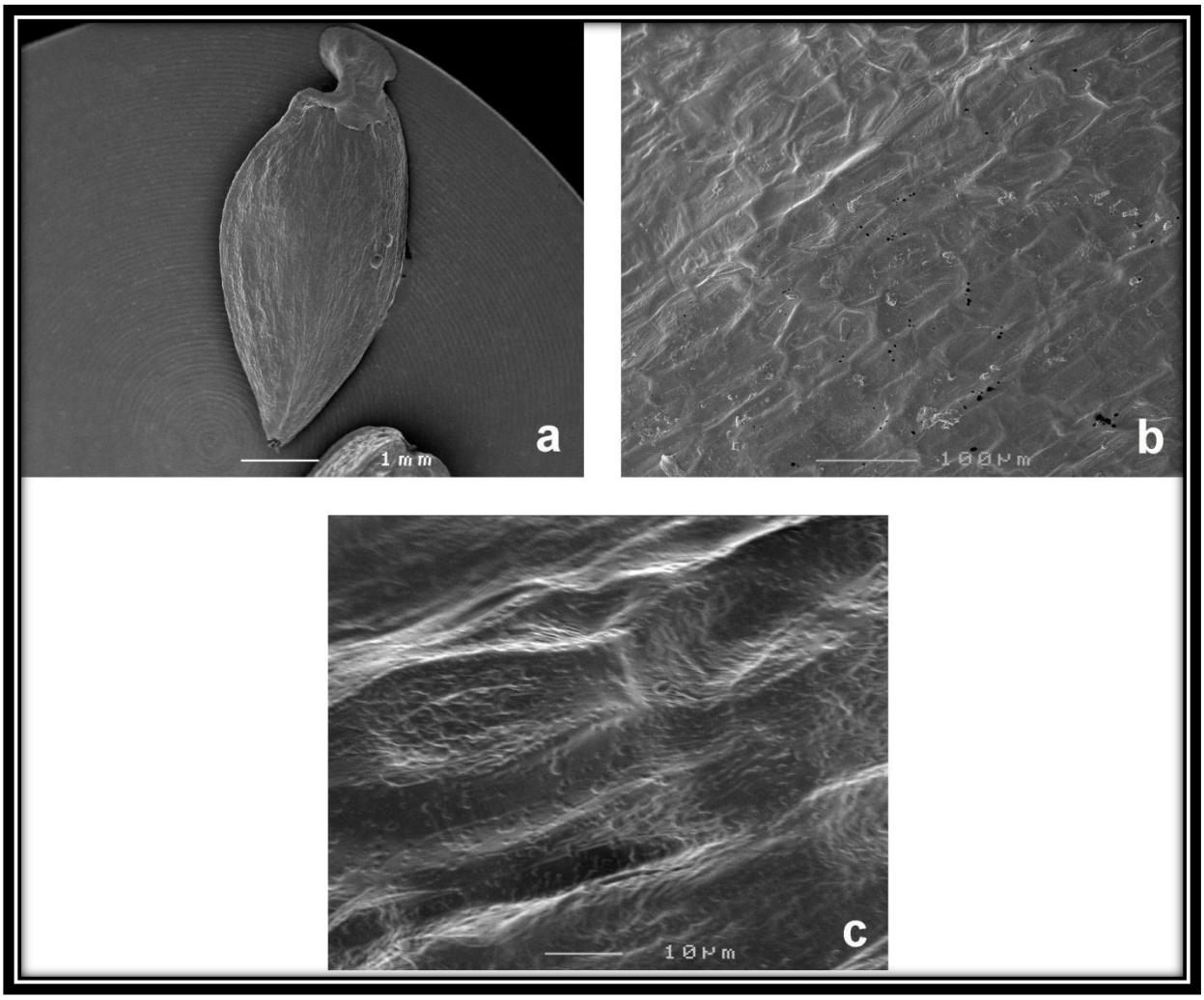

Figure 4.9 Ray floret achene of *Garuleum album.* In (a) achene shape; (b) epidermal cells with tabular periclinal walls; (c) granular cuticle covering epidermal cells. Scale bars: (a) = 1 mm, (b) = 100 µm, (c) = 10 µm. Specimen: (a–c) *Phillipson 4326* (GRA).

Garuleum bipinnatum

Both the ray and disc florets produce fertile achenes.

The ray floret achenes are obovoid to obcordate (heartshaped). The epidermal surface of the achene has deep grooves radiating from the centre to the margins of the achene (Fig. 4.10.1 a, b). The epidermal cells are isodiametric and tetra- to hexagonal. Periclinal walls are convex, while the anticlinal walls are curved or straight and sunk into the epidermal surface. Simple trichomes of 50–100 µm long are sparsely distributed over the achene surface (Fig. 4.10.1 b).

The disc floret achenes are obovate to obcordate (heartshaped), while the epidermal surface is smooth. The epidermal surface of the achene consist of isodiametric and tetra- to hexagonal cells (Fig. 4.10.2 a, b). Periclinal walls are convex or concave, while the anticlinal walls are curved or straight and sunken below the epidermal surface. The cuticle is granular and uneven (Fig. 4.10.2 c).

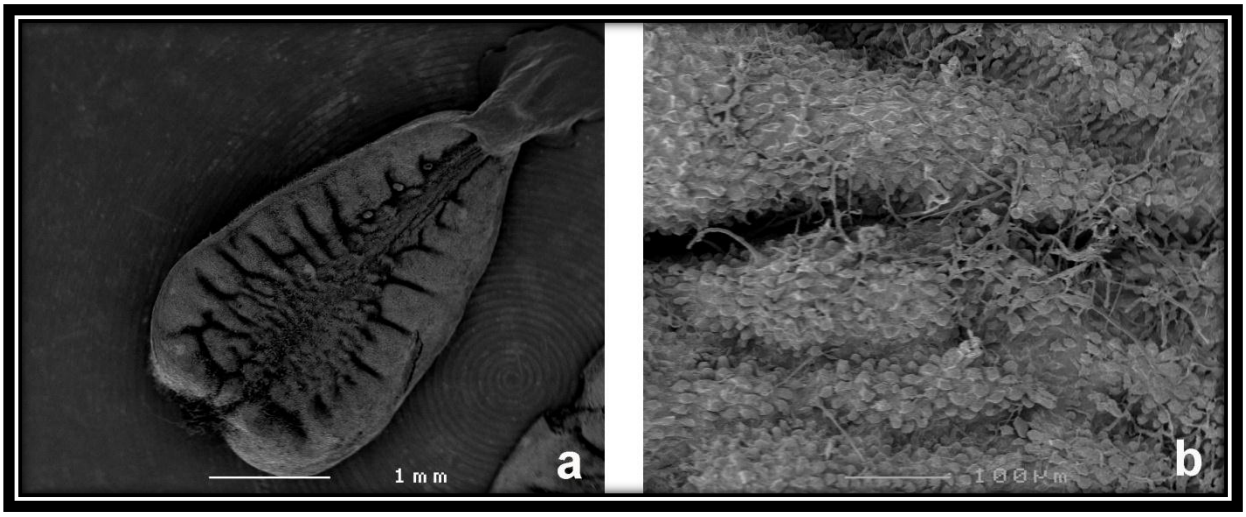

Figure 4.10.1 Ray floret achene of *Garuleum bipinnatum.* In (a) achene shape and epidermal grooves; (b) folded epidermal surface consisting of isodiametric, tetra- to hexagonal cells. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–b) *Van Zyl 9* (BLFU).

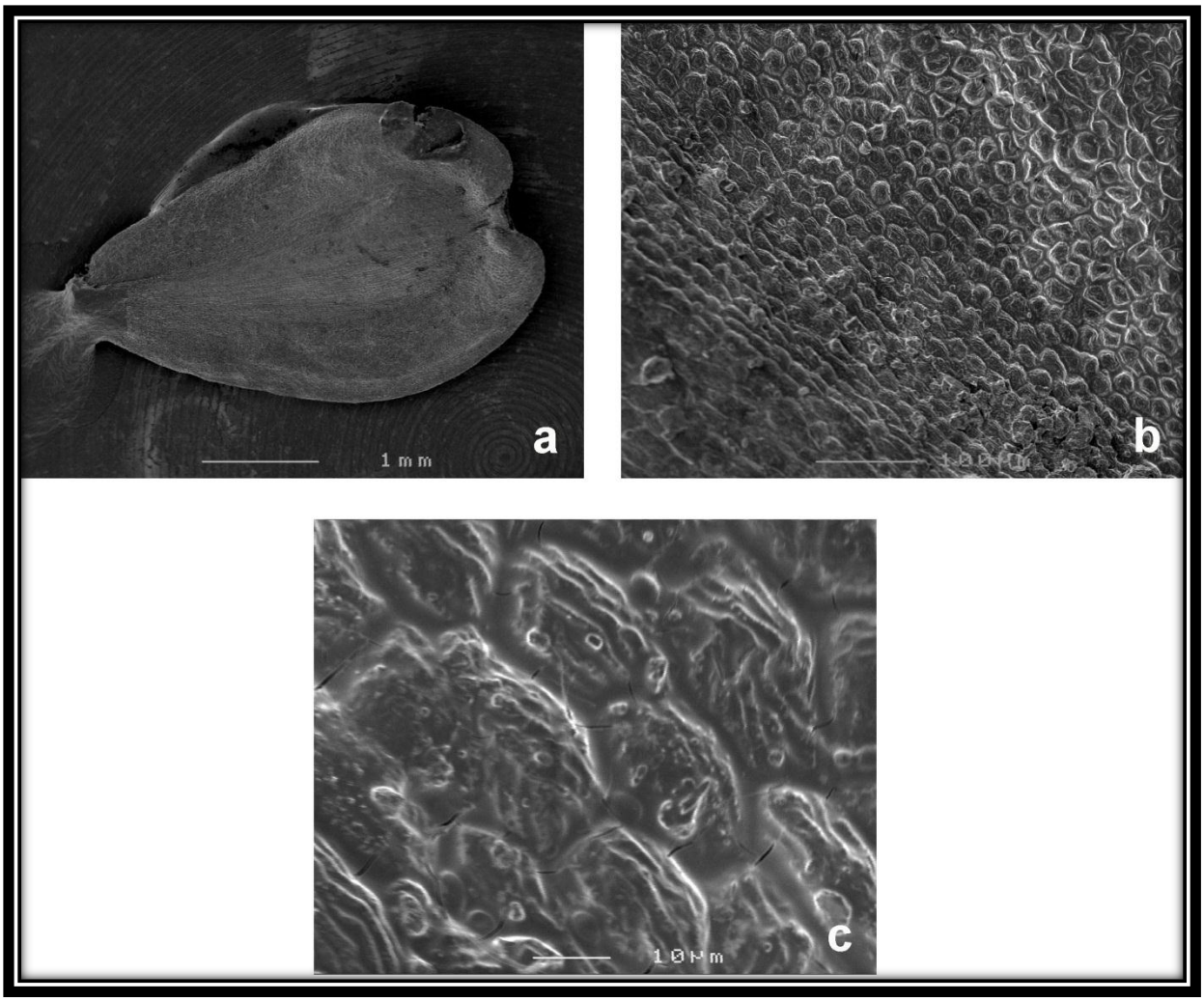

Figure 4.10.2 Disc floret achene of *Garuleum bipinnatum.* In (a) achene shape; (b) epidermal surface consisting of isodiametric, tetra- to hexagonal cells; (c) granular, uneven cuticle. Scale bars: (a) = 1 mm, (b) = 100 µm, (c) = 10 µm. Specimen: (a–c) *Van Zyl 9* (BLFU).

Garuleum latifolium

Only ray florets produce fertile achenes. The achenes are obovate and the margins slightly thickened. The achene surface has narrow longitudinal ridges along its length (Fig. 4.11 a). Epidermal surfaces of the achene are covered with wart-like protuberances. Protuberances are 20–80 x 34–42 µm in size and consist of deformed cells (Fig. 4.11 b). Cells between the protuberances are obscured by nodular, cuticular striations, which radiate from protuberances. Striations are straight to slightly wavy, parallel and continuous over intercellular boundaries (Fig. 4.11 c).

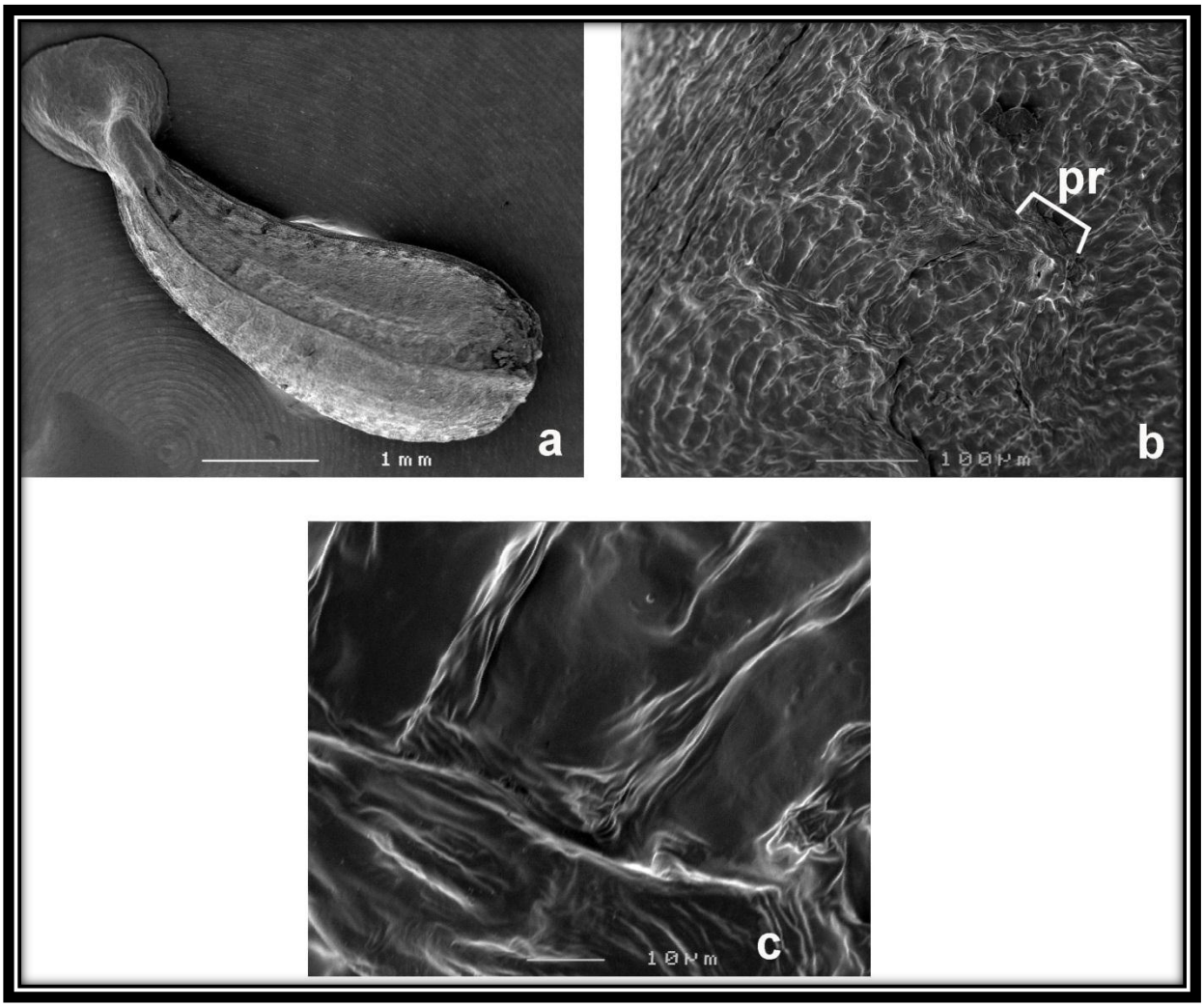

Figure 4.11 Ray floret achene of *Garuleum latifolium.* In (a) achene shape; (b) epidermal surface of achene, covered with wart-like protuberances and nodular striations; (c) striations continuous over intercellular boundaries. Legend: Pr = protuberance. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–c) *Wood 299* (BLFU).

Garuleum pinnatifidum

Only ray florets produce fertile achenes. The achenes are obovoid with raised longitudinal ridges (Fig. 4.12 a). The achene surface is rough with grooves and unevenly spaced wart-like protuberances of 16–28 x 22–42 µm (Fig. 4.12 b). The epidermal cell shape is not visible and is obscured by a nodular cuticle (Fig. 4.12 b).

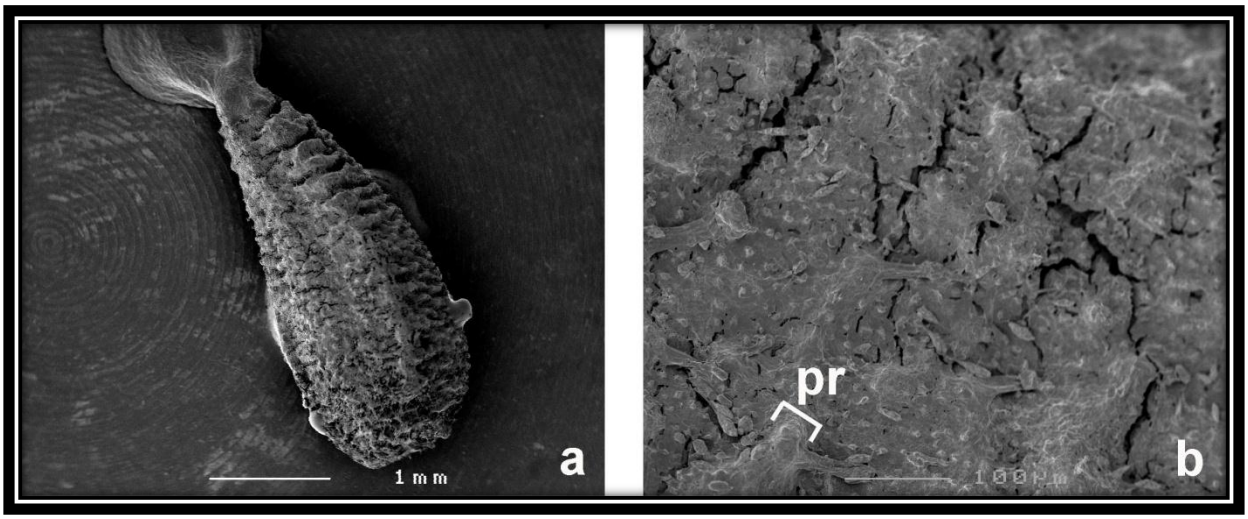

Figure 4.12 Ray floret achene of *Garuleum pinnatifidum.* In (a) achene shape and longitudinal ridges; (b) epidermal surface covered by nodular cuticle and unevenly spaced protuberances. Legend: Pr = protuberance. Scale bars: (a) = 1 mm, (b) = 100 µm (c) = 10 µm. Specimen: (a–b) *Van Zyl 2* (BLFU).

Garuleum schinzii

Uncertainty exists if ray florets or disc florets produce fertile achenes. The achenes are obovoid with rough, grooved surfaces (Fig. 4.13 a). The epidermal surface is characterised by raised longitudinal ridges and deep grooves perpendicular to the ridges. The epidermal surface consists of isodiametric, tetra- to hexagonal cells (Fig. 4.13 b). Periclinal walls are convex, while the anticlinal walls are straight or curved and slightly sunken. The raised longitudinal ridges are covered with simple trichomes, 28–85 µm long, and with a basal diameter of 11–20 µm.

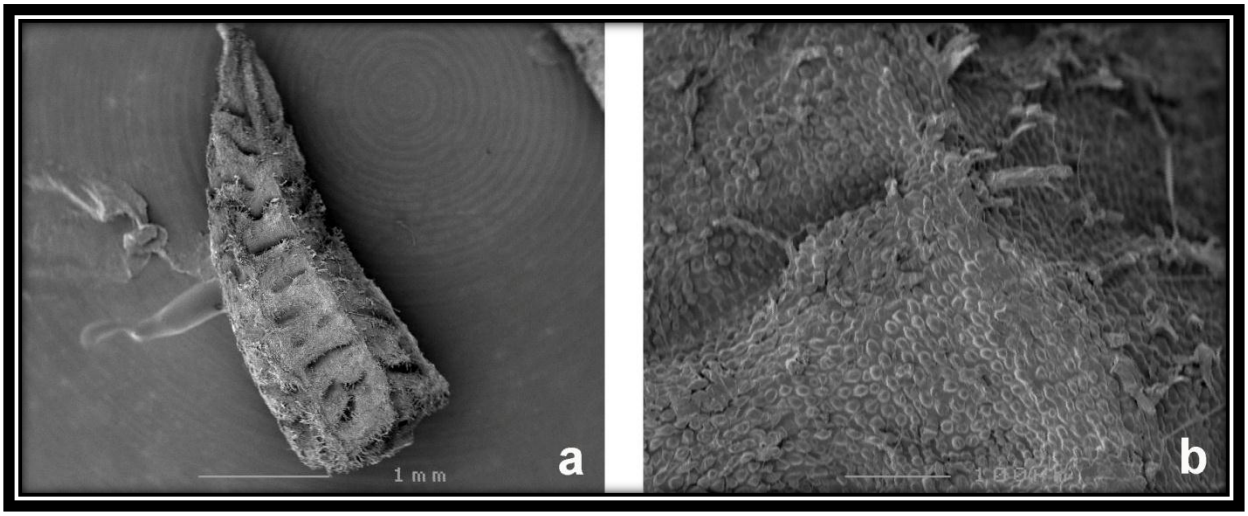

Figure 4.13 Achene of *Garuleum schinzii.* In (a) achene shape; (b) epidermal surface consisting of isodiametric, tetra- to hexagonal cells and simple trichomes on raised longitudinal ridges. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–b) *Marloth 2043* (L).

Garuleum sonchifolium

Only ray florets produce fertile achenes (Fig. 4.14 a). The achenes are obovoid and the margins slightly thickened. The epidermal surface is covered with wart-like protuberances (Fig. 4.14 b). Protuberances are 57–85 x 71–85 µm in size and consist of deformed epidermal cells. Cell shape between the protuberances is obscured by nodular cuticular striations, which radiate from protuberances.

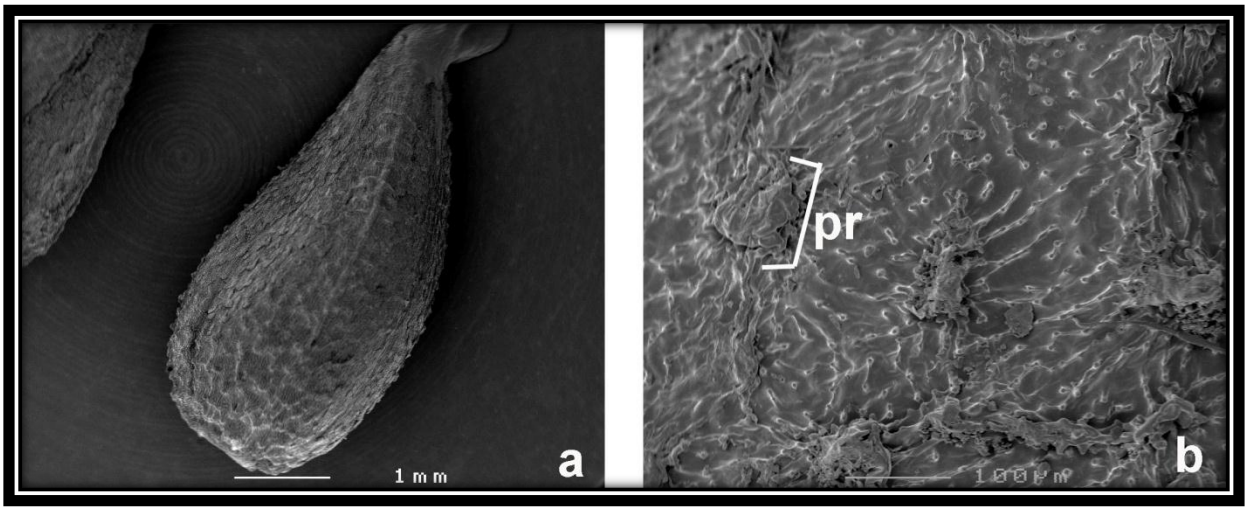

Figure 4.14 Ray floret achene of *Garuleum sonchifolium.* In (a) achene shape; (b) epidermal surface, covered with wart-like protuberances and nodular striations. Legend: Pr = protuberance. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–b) *MacOwan 2015* (Z).

Garuleum tanacetifolium

Only ray florets produce fertile achenes. The achenes are oblong and the general appearance is rough, characterised by alternating longitudinal ridges and grooves. Wart-like protuberances are distributed over the ridge surfaces. Epidermal cell shape is not clearly distinguishable (Fig. 4.15 a, b).

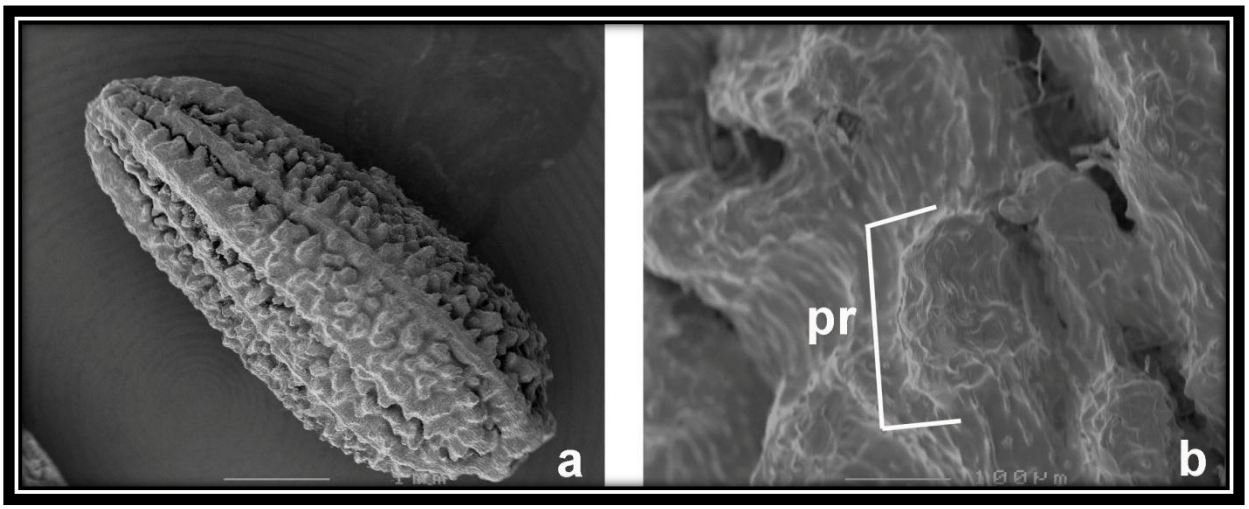

Figure 4.15 Ray floret achene of *Garuleum tanacetifolium.* In (a) achene shape; (b) achene surface, covered with wart-like protuberances and nodular striations. Legend: Pr = protuberance. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–b) *Van Zyl 17* (BLFU).

Garuleum woodii

Only ray florets produce fertile achenes (Fig. 4.16 a). The achenes are narrowly obovate while the margins are slightly thickened and grooved. The achene surface is smooth with grooves on one side of the achene. Periclinal walls of epidermal cells are tabular but cell shape was not discernible (Fig 4.16 b).

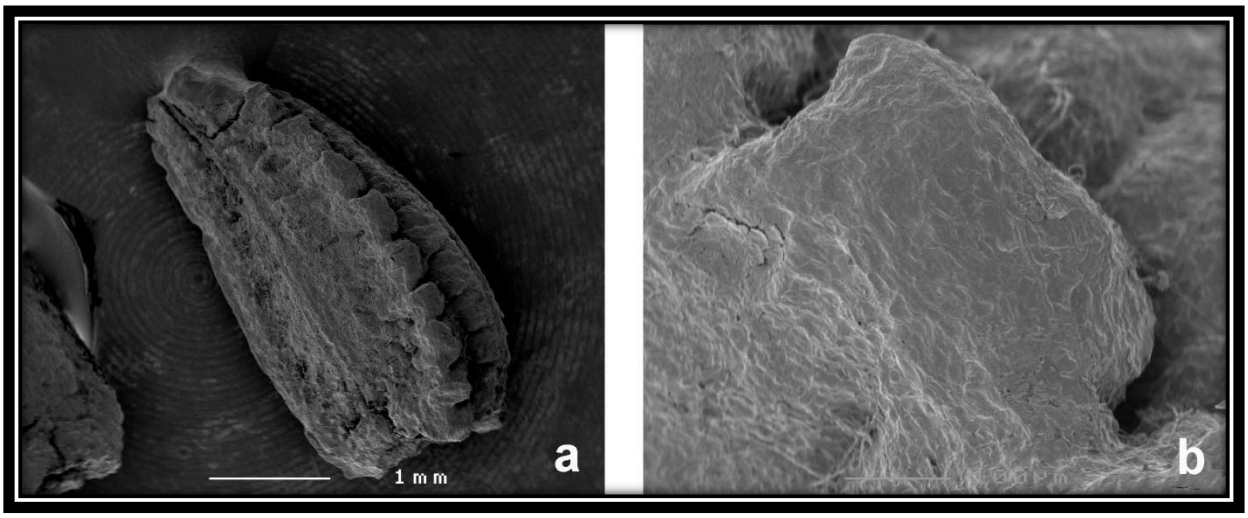

Figure 4.16 Ray floret achene of *Garuleum woodii.* In (a) achene shape; (b) cell shape indiscernible with tabular periclinal cell walls. Scale bars: (a) = 1 mm, (b) = 100 µm. Specimen: (a–b) *Rehmann 6792* (Z).

4.4 Discussion

The leaf epidermal surfaces do not have many characters which can be used to effectively distinguish between the eight *Garuleum* species. Stomatal size may be used to delimit species or species groups in *Garuleum*. The stomatal size on the adaxial leaf surfaces of *Garuleum bipinnatum*, *G. pinnatifidum*, *G. schinzii, G. sonchifolium, G. tanacetifolium* and *G. woodii* are in the same size range (16–28 µm wide). In *G. album* the stomatal size is (30–40 µm wide) and in *G. latifolium* (9–17 µm wide).

The number of stomata present on the adaxial and abaxial surfaces was compared for the *Garuleum* species (Table 4.1). The change in stomatal number from adaxial to abaxial leaf surface was useful in dividing the eight species into three groups as indicated in Table 4.1. Though the use of size and number of stomata is not effective in distinguishing between all eight species, it can be used to narrow down the number of possible species for a certain specimen.

The leaves of all *Garuleum* species are amphistomatic and have anomocytic stomata, therefore these characters are not useful for species delimitation in the genus *Garuleum*.

In the case of the genus *Garuleum*, there was no specific type of trichome which could delimit the different *Garuleum* species. Small differences in the trichomes may be used to distinguish between certain species (Table 4.2). *Garuleum album* is easily distinguished from the rest of the *Garuleum* species, because it is the only species with long simple trichomes on the abaxial surface of its leaves. *Garuleum bipinnatum* is the only species with large short stalked capitate trichomes and short simple trichomes, on both leaf surfaces. The trichome morphology of the rest of the *Garuleum* species is very similar, and cannot clearly distinguish between the different species.

The achene shape, size, cuticular striations and outgrowths on the epidermis are useful for species-level identification in *Garuleum* (Table 4.3). There is some similarity in the shape and appearance of achenes of G*. latifolium* and *G. sonchifolium*. These two species are still distinguishable from each other by the size of their wart-like protuberances on the achenes.

Table 4.1 Comparison between the number of stomata present on the adaxial and abaxial leaf epidermal surfaces of the eight *Garuleum* species.

Garuleum species	**Stomatal count difference between adaxial and abaxial leaf surface.**
Garuleum album	Increase in stomatal count from adaxial to abaxial surface
Garuleum bipinnatum	Decrease in stomatal count from adaxial to abaxial surface
Garuleum latifolium	Increase in stomatal count from adaxial to abaxial surface
Garuleum pinnatifidum	Stomatal count ± the same for both surfaces
Garuleum schinzii	Decrease in stomatal count from adaxial to abaxial surface
Garuleum sonchifolium	Increase in stomatal count from adaxial to abaxial surface
Garuleum tanacetifolium	Stomatal count ± the same for both surfaces
Garuleum woodii	Stomatal count ± the same for both surfaces

Table 4.2 Comparison of trichome types found on the adaxial and abaxial leaf surface for all eight *Garuleum* species.

Trichome types on adaxial leaf epidermis	**Trichome types on abaxial leaf epidermis**	**Adaxial cuticular striations**	**Abaxial cuticular striations**	**Adaxial stomata nr**	**Abaxial stomata nr**
Long simple, capitate	long simple , capitate	Slightly striated	Smooth to slightly striated	19 per 1 mm^2	113 per 1 mm^2
simple short, capitate	short simple, capitate	Smooth to slightly striated	Smooth to slightly striated	116 per 1 mm^2	69 per 1 mm^2
capitate	capitate	Smooth to slightly striated	Folded	55 per 1 mm^2	272 per 1 mm^2
simple, capitate	simple, capitate	Smooth	Smooth	130 per 1 mm^2	137 per 1 mm^2
capitate	simple, capitate	Slightly striated	Folded and slightly striated	204 per 1 mm^2	156 per 1 mm^2
capitate	simple, capitate	Smooth to slightly striated	Smooth to slightly striated	36 per 1 mm^2	145 per 1 mm^2
capitate	capitate	Parallel striations continuous over several cells	Smooth	52 per 1 mm^2	74 per 1 mm^2
capitate	capitate	Slightly striated	Smooth	215 per 1 mm^2	194 per 1 mm^2

Table 4.3 Characters and states investigated for the achenes of the eight different *Garuleum* species.

***Garuleum* species**	**Ray-/ disc floret achene**	**Achene size**	**Achene shape**	**Periclinal walls**	**Anticlinal walls**	**Epidermal surface**	**Cuticular striations**	**Outgrowths or trichomes**
Garuleum album	Ray	±4.4 x 2.3 mm	obovate	Tabular	Straigth, level	Smooth	Smooth to granular	None
Garuleum bipinnatum	Ray	±3.4 x 2.2 mm	obovoid to obcordate	Convex	Curved or straigth, sunk	Deep grooves	None	Simple trichomes
Garuleum bipinnatum	Disc	±4.4 x 2.3 mm	obovoid to obcordate	Convex or concave	Curved or straigth, sunk	Smooth	Granular, uneven	None
Garuleum latifolium	Ray	±4.3 x 1.6 mm	obovate	Not visible	Not visible	Parallel longitudinal ridges	Straight to wavy striations	Wart-like protuberances
Garuleum pinnatifidum	Ray	±3.55 x 1.5 mm	obovoid	Not visible	Not visible	Rough grooves	Nodular striations	Wart-like protuberances
Garuleum schinzii	Ray/Disc undetermined	±3.1 x 1.2 mm	obovoid	Convex	Curved or straigth, sunk	Deep grooves	None	Simple trichomes
Garuleum sonchifolium	Ray	±5.1 x 2.3 mm	obovoid	Not visible	Not visible	Margins slightly raised	Nodular striations	Wart-like protuberances
Garuleum tanacetifolium	Ray	±5.2 x 2.2 mm	oblong	Not visible	Not visible	Rough alternating longitudinal ridges and grooves		Wart-like protuberances
Garuleum woodii	Ray	±3.7 x 1.9 mm	Narrowly obovate	Tabular	Not visible	Smooth, margin grooved	Not visible	None

Garuleum bipinnatum produces two fruit types. This phenomenon is called heterocarpy. *Garuleum schinzii* may also have the ability to reproduce through heterocarpy. There was only one herbarium voucher for sampling, and it would not have been possible to verify if heterocarpy is present in *G. schinzii* without destroying the voucher. Only ray florets produced achenes in the rest of the *Garuleum* species. Heterocarpy has been described in a number of Asteraceae tribes, including Calenduleae, Cicihoriae, Inuleae, Heliantheae and Senecioneae (Burtt, 1977).

Heterocarpy is associated with differential germination responses and differential dispersal mechanisms (Tanowitz et al., 1987). The ray floret achenes have a thick pericarp, enabling them to stay dormant in the seed bed for long periods. The disc floret achenes are morphologically adapted to be lighter, disperse further and easier than ray florets, to find new habitats where the species can grow. The disc florets need to germinate fast, because they cannot stay dormant for a long period (Tanowitz, et al., 1987). Due to these different dormancy periods disc and ray floret achene genotypes are separated in time and space.

The differential germination response of heterocarpy of *G. bipinnatum*, clearly helps with more effective distribution of the species, because this species has the widest distribution of all the *Garuleum* species. *Garuleum schinzii* also has a wide distribution. Heterocarpy may provide these species the ability to survive in a greater range of habitat conditions, to be more habitat tolerant and disperse easier between fragmented microhabitats which are suitable for the species.

In species-level identification, achene micromorphology is very useful for *Garuleum*. It provides many useful characters for identification. Leaf micromorphology may be useful in some instances, but does not provide a lot of useful characters for identification, and may produce incorrect identifications if characters used are influenced by the surrounding habitat.

Chapter 5

Micromorphology of *Garuleum* flowers.

5.1. Introduction

Floral morphology has always been a very important diagnostic character in Asteraceae classification. The flower colour, shape, size, anther shape and appendages, as well as stigma shape are all of diagnostic value (Bremer 1994).

The presence and distribution of glandular and non-glandular trichomes also provide diagnostic characters used in many taxonomic studies on Asteraceae. The glandular trichomes on flowers are believed to produce volatiles which help with attraction of pollinators. The position of the simple trichomes is also thought to assist in attracting pollinators to the flowers (Martin and Glover, 2007).

Baagöe (1977) advises that sufficient caution has to be taken when using micromorphological characters of flowers, to ensure that the characters used did not evolve several times in parallel, leading to incorrect classifications. Micromorphological characters evolving in parallel have occurred in the Calenduleae. Ligule micromorphological characters alone are not sufficient to make major taxonomic conclusions. Such characters may reflect and substantiate results obtained from more reliable characters (Baagöe, 1977).

The aim of this section is to evaluate the diagnostic value of floral micromorphology in the *Garuleum* species.

5.2 Materials and methods

All material and methods in this chapter is as described in 3.5 (Chapter 3).

5.3 Results:

Garuleum album

The base of the ray floret corolla tube is densely covered with capitate trichomes (Fig. 5.1.1 a–c), which have a length of 115.4–153.8 µm, a head diameter of 53.8–76.9 µm and a basal diameter of 46.1–69.3 µm. Towards its upper part, the ray floret corolla tube is covered by both capitate and multicellular, simple trichomes. The ligule base of the ray floret is densely covered by simple trichomes 84.6–246.1µm in length (Fig. 5.1.1 d, e). The ray floret stigma is bi-lobed (Fig. 5.1.1 f).

Simple trichomes are sparsely arranged around the base of the disc floret corolla tube and become more densely arranged towards the apex of the corolla tube and on the corolla lobes (Fig. 5.1.2 a–c). The apices of trichomes on the corolla lobes are acute, whereas apices of trichomes on the corolla tube are obtuse. The trichomes are multicellular, multiseriate and 133–277 µm in length (Fig. 5.1.2 d).

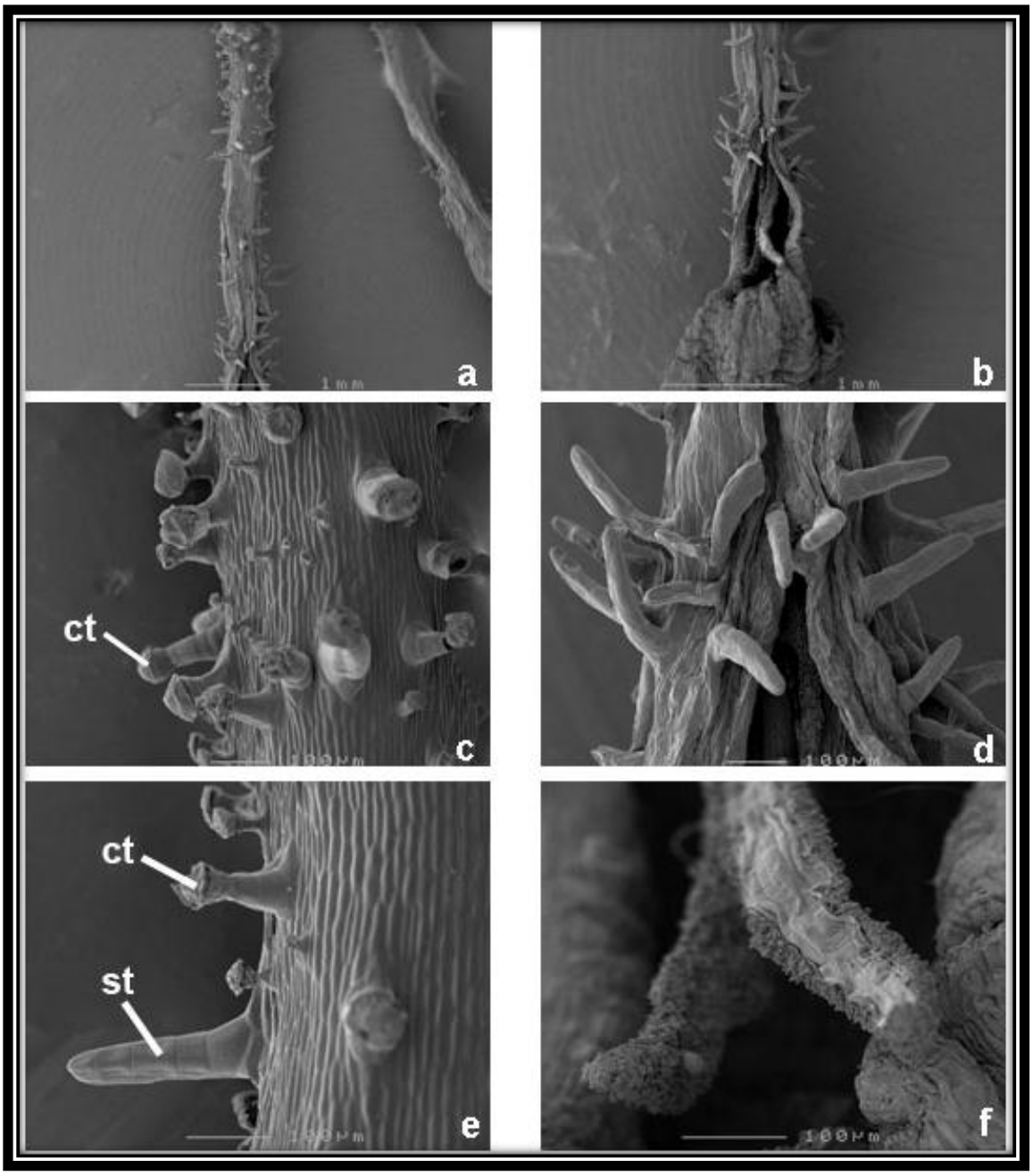

Figure 5.1.1 *Garuleum album* ray floret. In (a) corolla tube; (b) ligule; (c) corolla tube base covered with capitate trichomes; (d) ligule base covered with simple trichomes; (e) corolla tube, middle section, covered with simple and capitate trichomes; (f) bilobed stigma. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a, b) = 1 mm, (c–f) = 100 µm. Specimen: (a–f) *Phillipson 4326* (GRA).

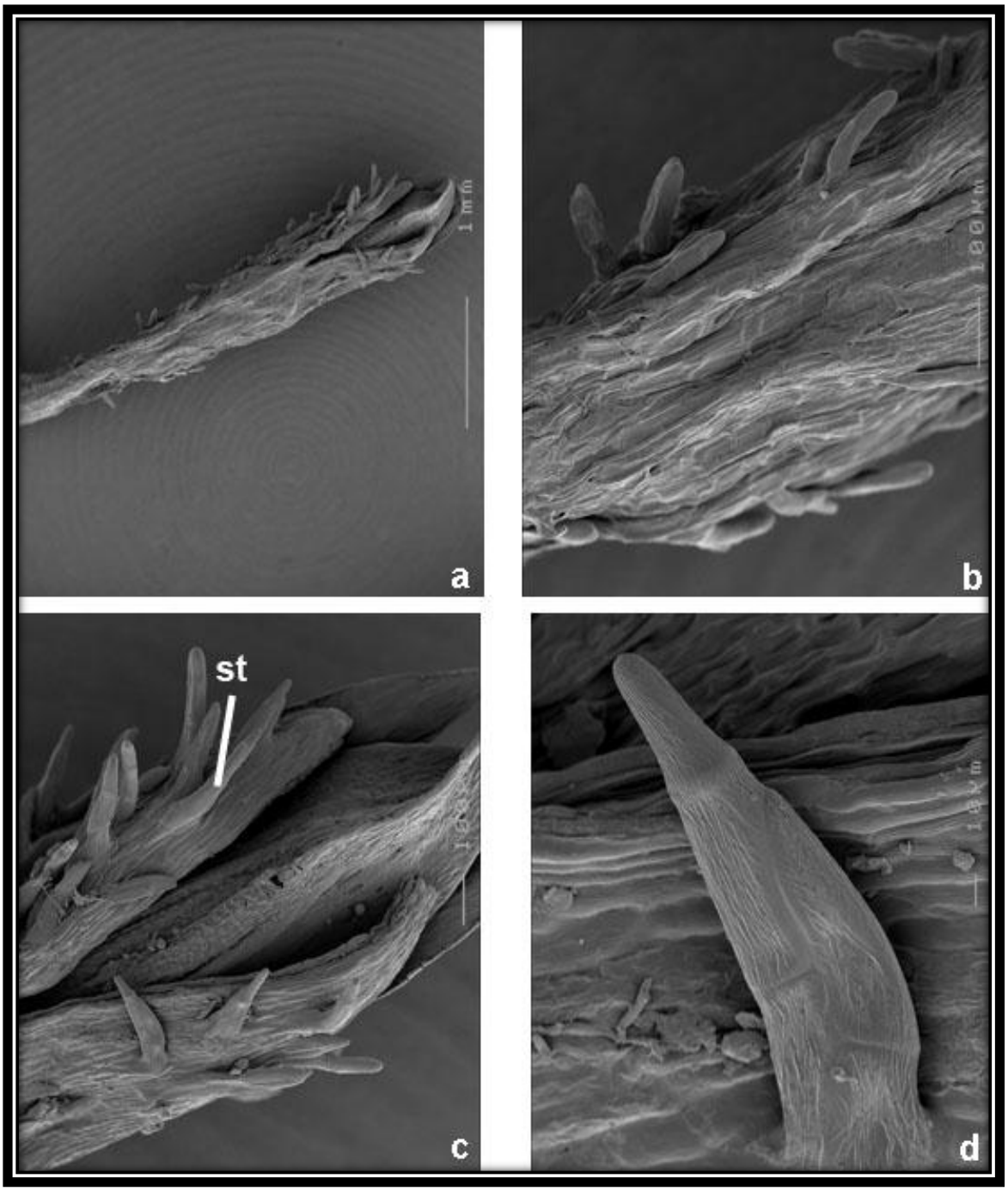

Figure 5.1.2 *Garuleum album* disc floret. In (a) external view of corolla tube; (b) middle section of corolla tube covered with simple trichomes; (c) corolla lobes covered with simple trichomes; (d) simple trichomes on disc floret. . Legend: st = simple trichome. Scale bars: (a) = 1 mm, (b, c) = 100 µm, (d) = 10 µm. Specimen: (a–d) *Phillipson 4326* (GRA).

Garuleum bipinnatum

The ray floret corolla tube is covered with capitate trichomes, which are more concentrated at the base of the tube and around the base of the ligule (Fig. 5.2.1 a–d). The capitate trichomes at the base of the corolla tube are 40–92 µm long with a head diameter of 36–64 µm and a basal diameter of 20–52 µm (Fig 5.2.1 e). The capitate trichomes at the ligule base are longer, being 78–128 µm long with a head diameter of 28–71 µm and a basal diameter of 50–57 µm (Fig 5.2.1 f).

The disc floret corola tube and lobes are evenly covered by capitate trichomes (Fig. 5.2.2 a, b, d). The middle of the corolla tube and the corolla lobes also has a small number of simple trichomes (Fig. 5.2.2 c, d). The capitate trichomes are 61–92 µm long with a head diameter of 46–61 µm and a basal diameter of 38−61 µm. Simple trichomes are 84–110 µm long (Fig. 5.2.2 d).

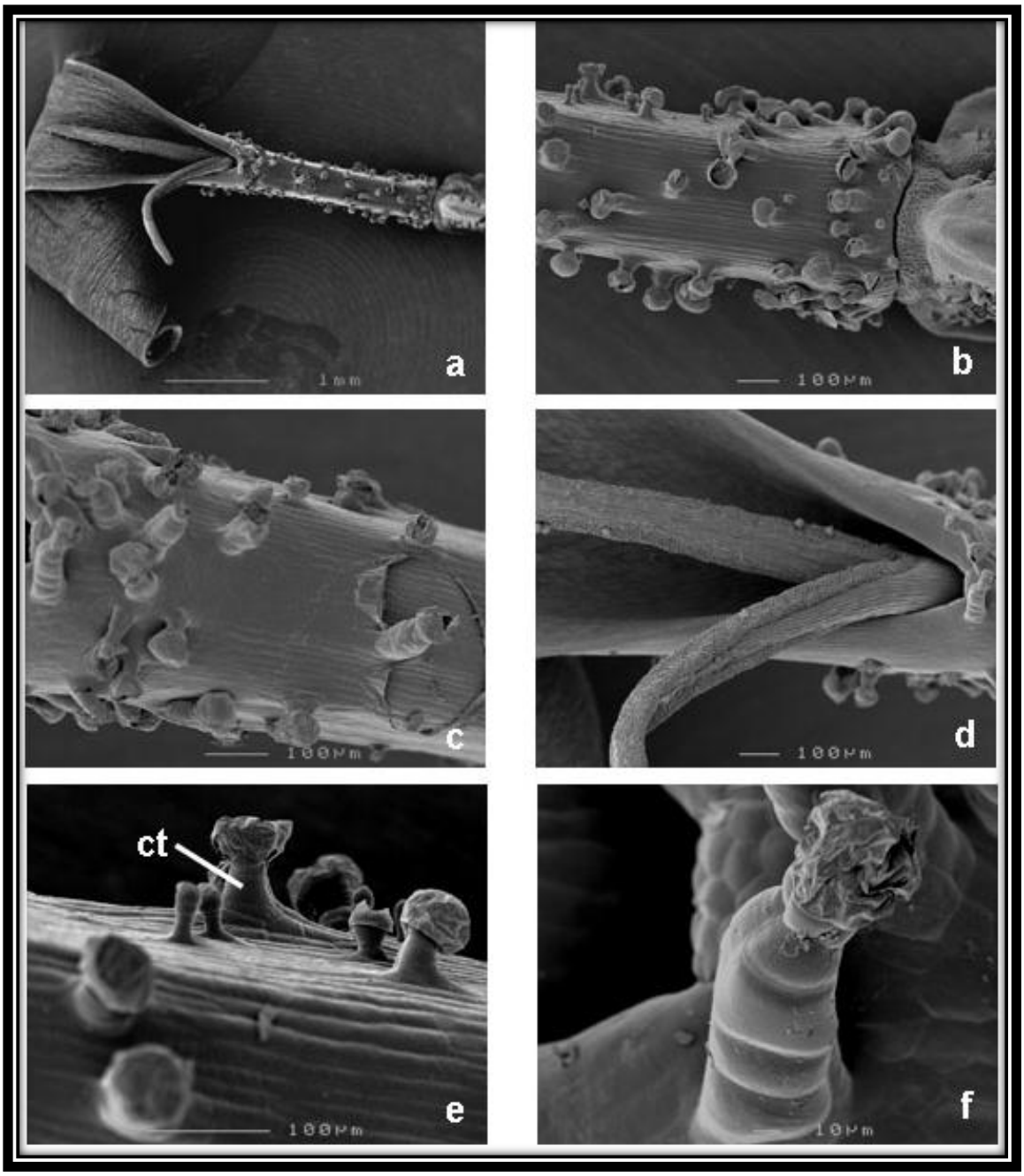

Figure 5.2.1 *Garuleum bipinnatum* ray floret. In (a) corolla and bilobed stigma; (b) corolla tube base covered with capitate trichomes; (c) corolla tube middle covered with capitate trichomes; (d) ligule base covered with capitate trichomes; (e) capitate trichomes on ray floret tube base; (f) capitate trichomes on ray floret ligule base. Legend: ct = capitate trichomes. Scale bars: (a) = 1 µm, (b–e) =100 µm (f) = 10 µm. Specimen: (a–f) *Van Zyl 9* (BLFU).

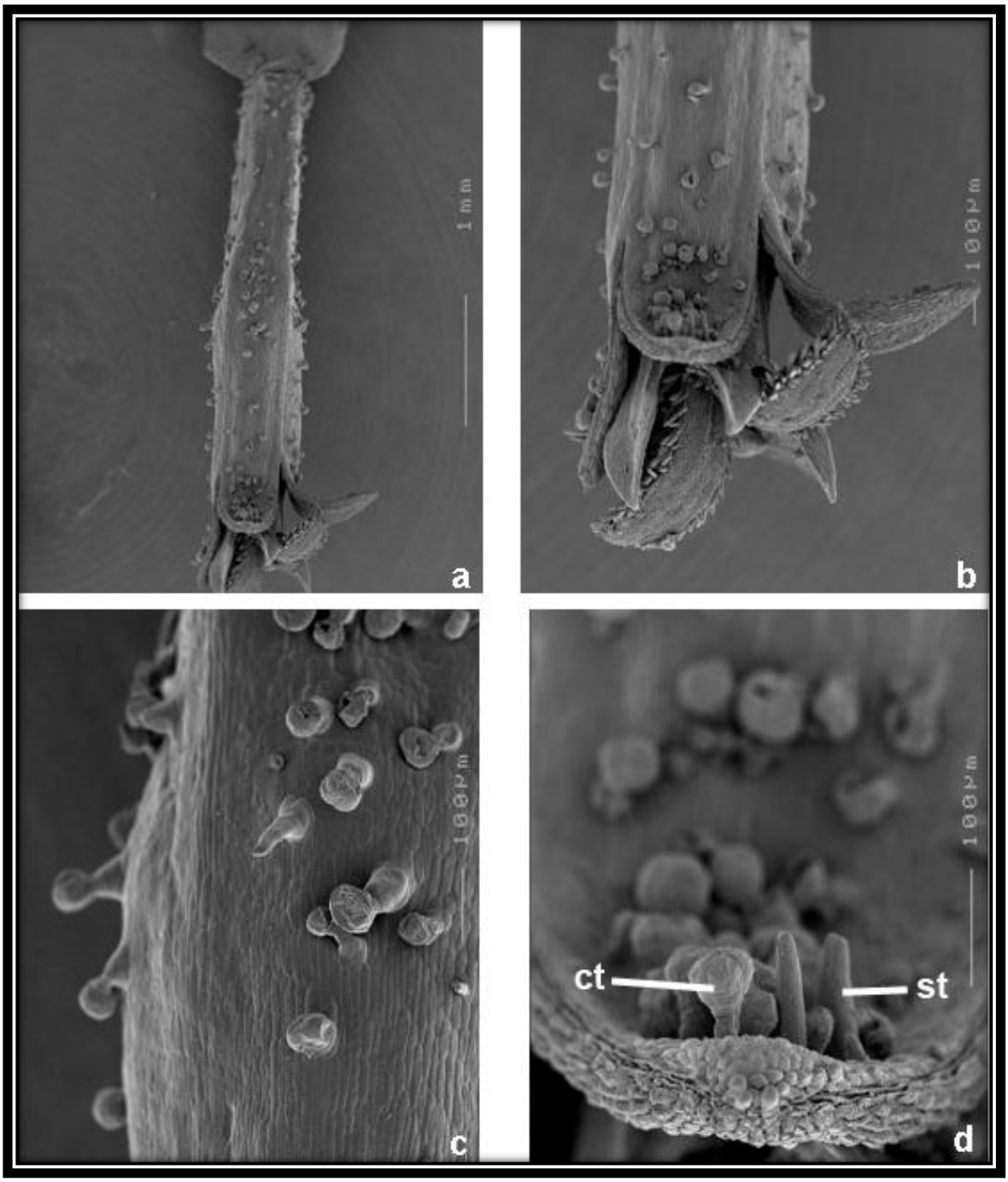

Figure 5.2.2 *Garuleum bipinnatum* disc floret. In (a) corolla; (b) corolla lobes covered with simple and capitate trichomes; (c) corolla tube middle covered with capitate and simple trichomes; (d) capitate and simple trichomes on corolla lobe apex. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–d) =100 µm Specimen: (a–d) *Van Zyl 9* (BLFU)

Garuleum latifolium

The ray floret corolla tube is covered with capitate tricomes which are densely arranged around the base of the corolla tube, gradually becoming more sparsely arranged towards the corolla tube apex (Fig. 5.3.1 a–c, e). The ligule base of the ray floret is covered by densely arranged simple, multicellular, multiseriate trichomes and sparsely arranged capitate trichomes (Fig. 5.3.1 d, f). The capitate trichomes are 30–50 µm long with a head diameter of 30–37.5 µm and a basal diameter of 15–32 µm. The simple trichomes are 100–450 µm in length.

The disc floret corolla tube is evenly covered by capitate trichomes (Fig. 5.3.2 a, c). The corolla lobes of the disc floret also have simple, mutlicellular, multiseriate trichomes and stomata (Fig. 5.3.2 b, d). The capitate trichomes are 30–80 µm long, with a head diameter of 30–60 µm and a basal diameter of 20–50 µm. The simple trichomes are 166–333 µm long. Stomata are 23 µm long and 16.6 µm wide, with the stomatal pore 11.6 µm long and the stomatal ledge 1.6 µm wide (Fig. 5.3.2. d).

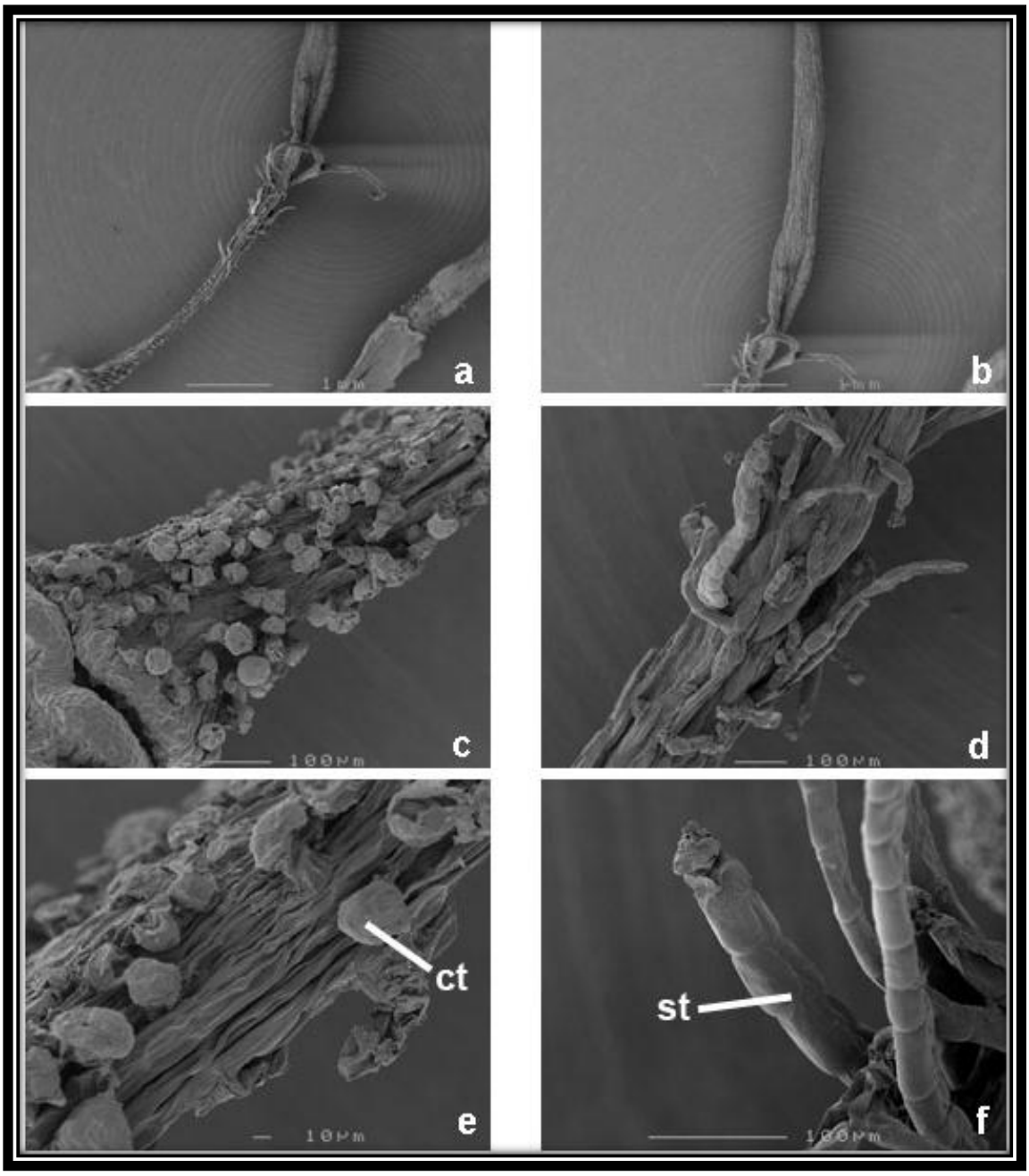

Figure 5.3.1 *Garuleum latifolium* ray floret. In (a, b) corolla; (c) corolla tube base covered with capitate trichomes; (d) ligule base covered with simple trichomes; (e) capitate trichomes; (f) simple trichomes. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a–b) = 1 mm, (e) = 10 µm (c–f) =100 µm. Specimen: (a–f) *Wood 299* (Z).

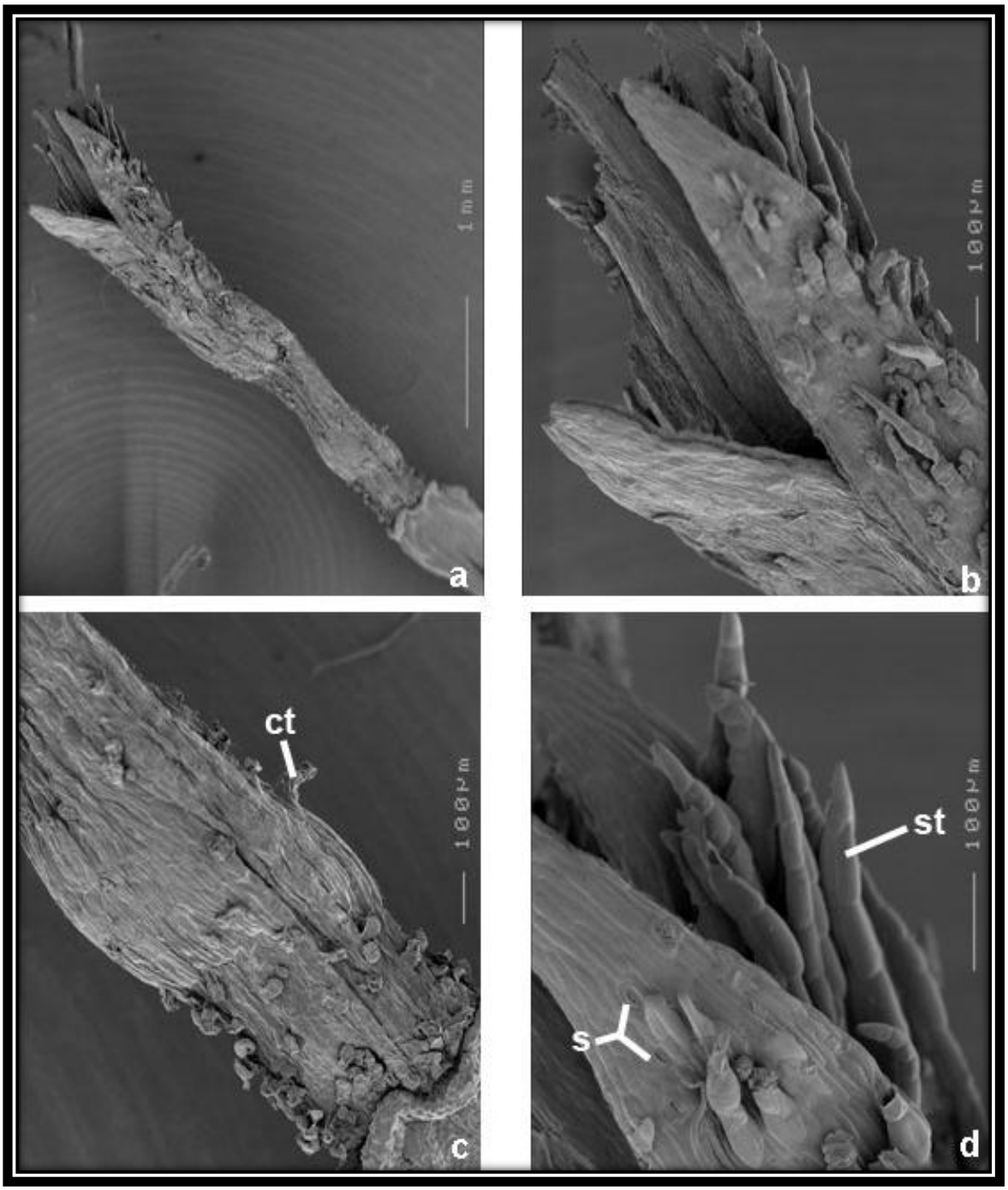

Figure 5.3.2 *Garuleum latifolium* disc floret. In (a) corolla; (b) corolla lobes covered with simple trichomes and capitate trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with capitate and simple trichomes and stomata. Legend: ct = capitate trichome, s = stomata, st = simple trichome. Scale bars: (a) = 1 mm, (b–d) = 100 µm Specimen: (a–d) *Wood 299* (Z).

Garuleum pinnatifidum

The ray floret corolla tube is evenly and densely covered with capitate trichomes (Fig. 5.4.1 a–c). The capitate trichomes at the base of the ray floret corolla tube are 25–75 µm long with a head diameter of 35–60 µm and a basal diameter of 62–75 µm (Fig. 5.4.1 e). The capitate trichomes around the ligule base of the ray floret are 50–137.5 µm long, with a head diameter of 37.5–62.5 µm and a basal diameter of 25–50 µm (Fig. 5.4.1 d).

The whole of the disc floret corolla tube is covered by capitate trichomes (Fig. 5.4.2 a, c). The highest concentration of capitate trichomes is at the base of the corolla tube, where there are also a few simple, multicellular, multiseriate trichomes (Fig. 5.4.2 a). The corolla lobes of the disc floret also have simple trichomes and stomata (Fig. 5.4.2 b, d). The capitate trichomes are 28–59 µm long with a head diameter of 15–34 µm and a basal diameter of 15–21 µm. The simple trichomes are 100–222 µm long.

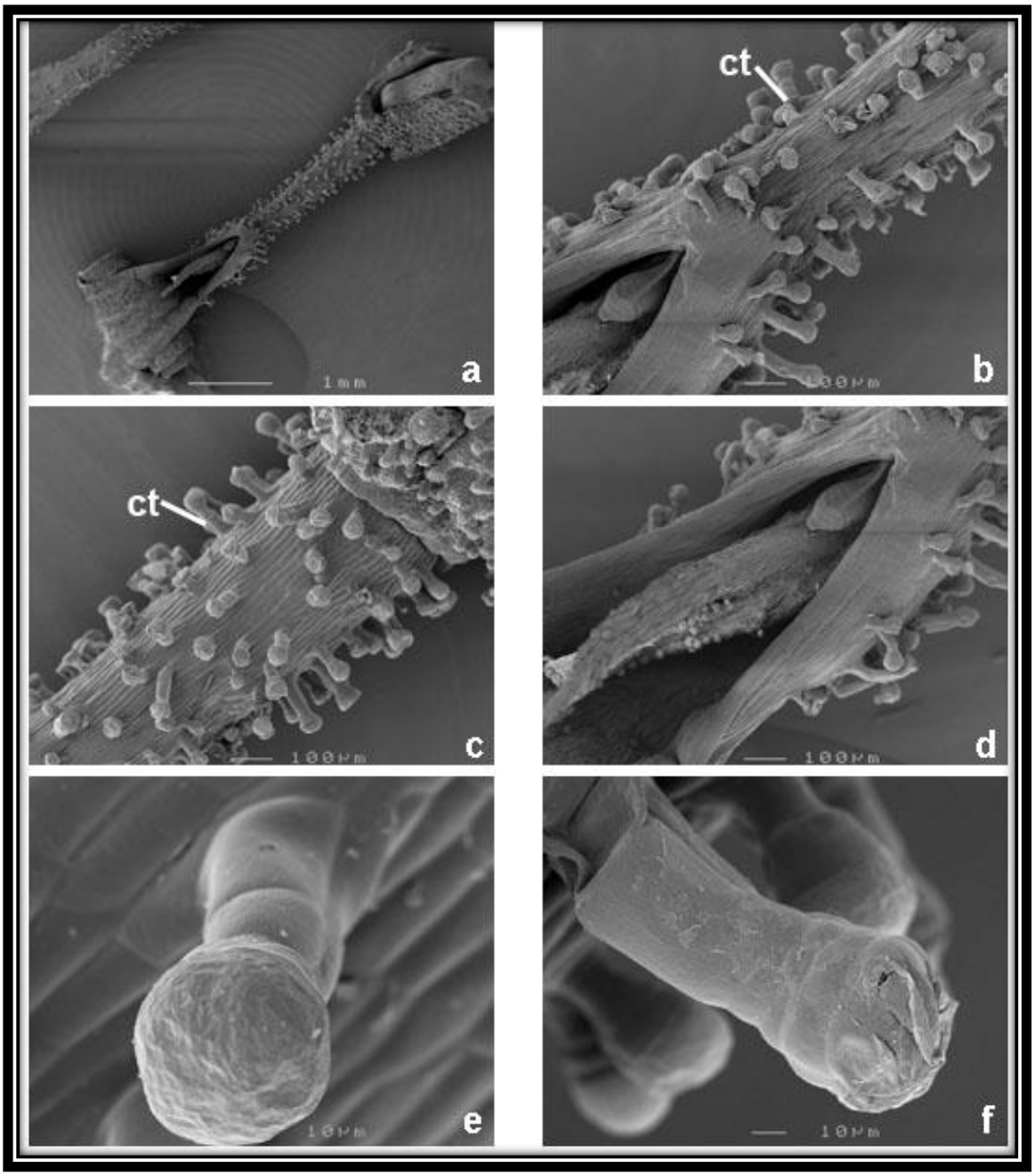

Figure 5.4.1 *Garuleum pinnatifidum* ray floret. In (a) corolla; (b) ligule base covered with capitate trichomes; (c) corolla tube base covered with capitate trichomes; (d) ligule base covered with capitate trichomes; (e) capitate trichome on corolla tube base; (f) capitate trichome on ligule. Legend: ct = capitate trichome. Scale bars: (a) = 1 mm, (b–d) = 100 µm (e–f) = 10 µm. Specimen: (a–f) *Van Zyl 4* (BLFU).

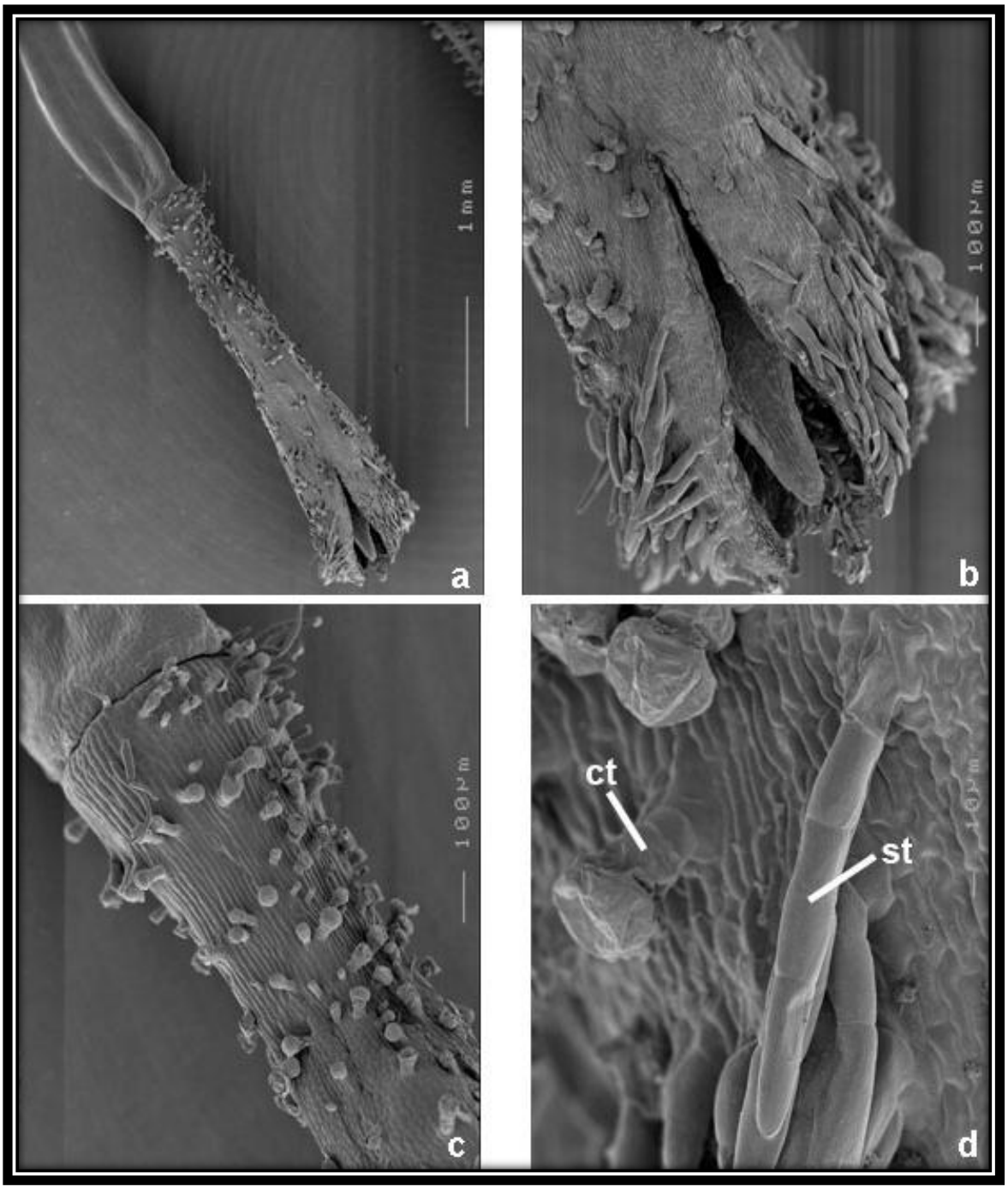

Figure 5.4.2 *Garuleum pinnatifidum* disc floret. In (a) corolla and inferior ovary; (b) corolla lobes covered with simple and capitate trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with capitate and simple trichomes. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–c) = 100 µm, (d) = 10 µm. Specimen: (a–d) *Van Zyl 4* (BLFU).

Garuleum schinzii

The base of the ray floret corolla tube is covered with capitate trichomes (Fig. 5.5.1 a, c, e). The capitate trichomes range from 25–38 µm long with a head diameter of 23–34 µm and a basal diameter of 10–19 µm. The ligule base has a high concentration of simple trichomes and capitate trichomes (Fig. 5.5.1 d, f). Simple trichomes are 133–186 µm long. Capitate trichomes at the ligule base are 6–113 µm long with a head diameter of 26–33 µm and a basal diameter of 26–46 µm.

The disc floret is covered by capitate trichomes from the base of the corolla tube to the lobes (Fig. 5.5.2 a, c). The lobes of the floret are covered with simple trichomes (Fig. 5.5.2 b, d). The capitate trichomes are 21–69 µm long with a head diameter of 14–38 µm and a basal diameter of 10–39 µm. The simple trichomes are 93–141 µm long.

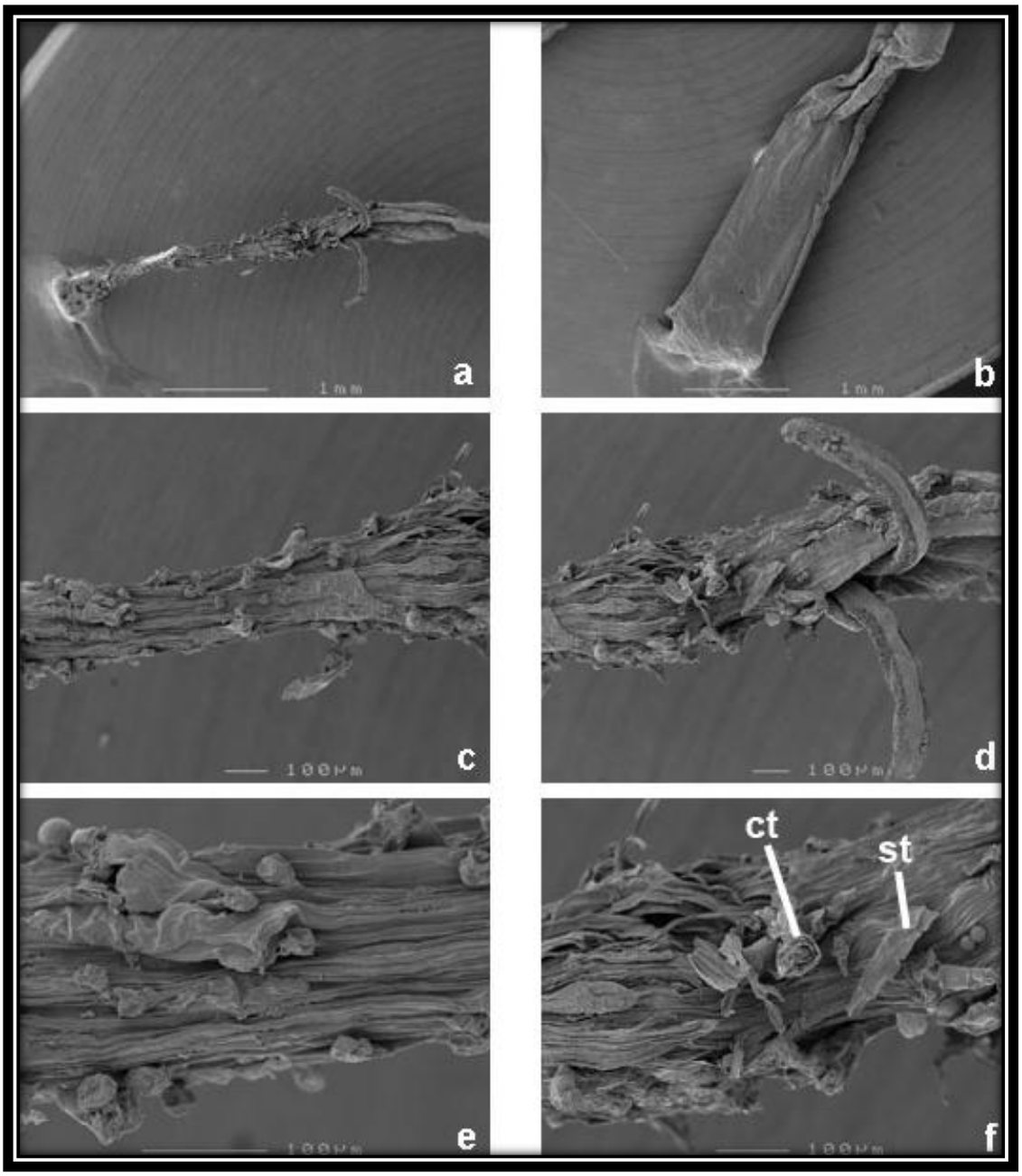

Figure 5.5.1 *Garuleum schinzii* ray floret. In (a) corolla; (b) ligule; (c) corolla tube base covered with capitate trichomes; (d) ligule base covered with capitate trichomes and simple trichomes; (e) capitate trichomes on corolla tube base; (f) capitate and simple trichomes on ligule base. Legend: ct = capitate trichomes, st = simple trichomes. Scale bars: (a–b) = 1 mm, (c–f) = 100 µm. Specimen: (a–f) *Mannheimer 2882* (GRA).

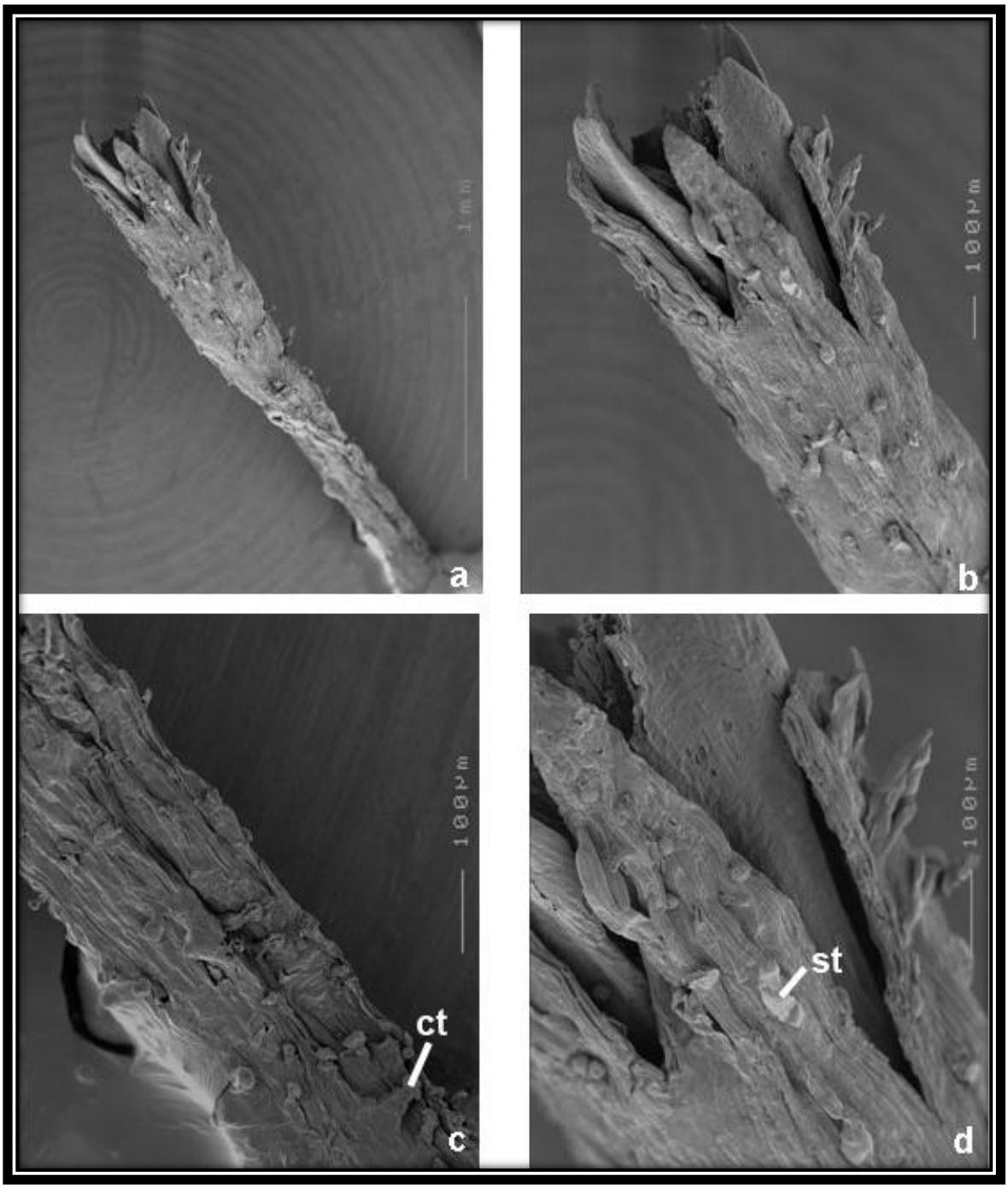

Figure 5.5.2 *Garuleum schinzii* disc floret. In (a) corolla; (b) corolla lobes covered with simple trichomes and capitate trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with capitate and simple trichomes. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–d) = 100 µm. Specimen: (a–d) *Mannheimer 2882* (GRA).

Garuleum sonchifolium

The base of the ray floret corolla tube is covered with capitate and simple trichomes (Fig. 5.6.1 a, b). The capitate trichomes are 115.4–153.8 µm long with a head diameter of 53.8–76.9 µm and a basal diameter of 46.1–69.3 µm. The ligule base has a high concentration of simple trichomes which are 60–210 µm long (Fig. 5.6.1 c, d).

The disc floret has capitate trichomes, densely arranged on the base of the corolla tube, becoming sparser towards the middle of the corolla tube (Fig. 5.6.2 a, c). The corolla lobes of the floret are sparsely covered with simple trichomes (Fig. 5.6.2 b, d). The capitate trichomes are 61.5–77 µm long with a head diameter of 30–46 µm and a basal diameter of 23–31 µm. The simple trichomes are 83–300 µm long.

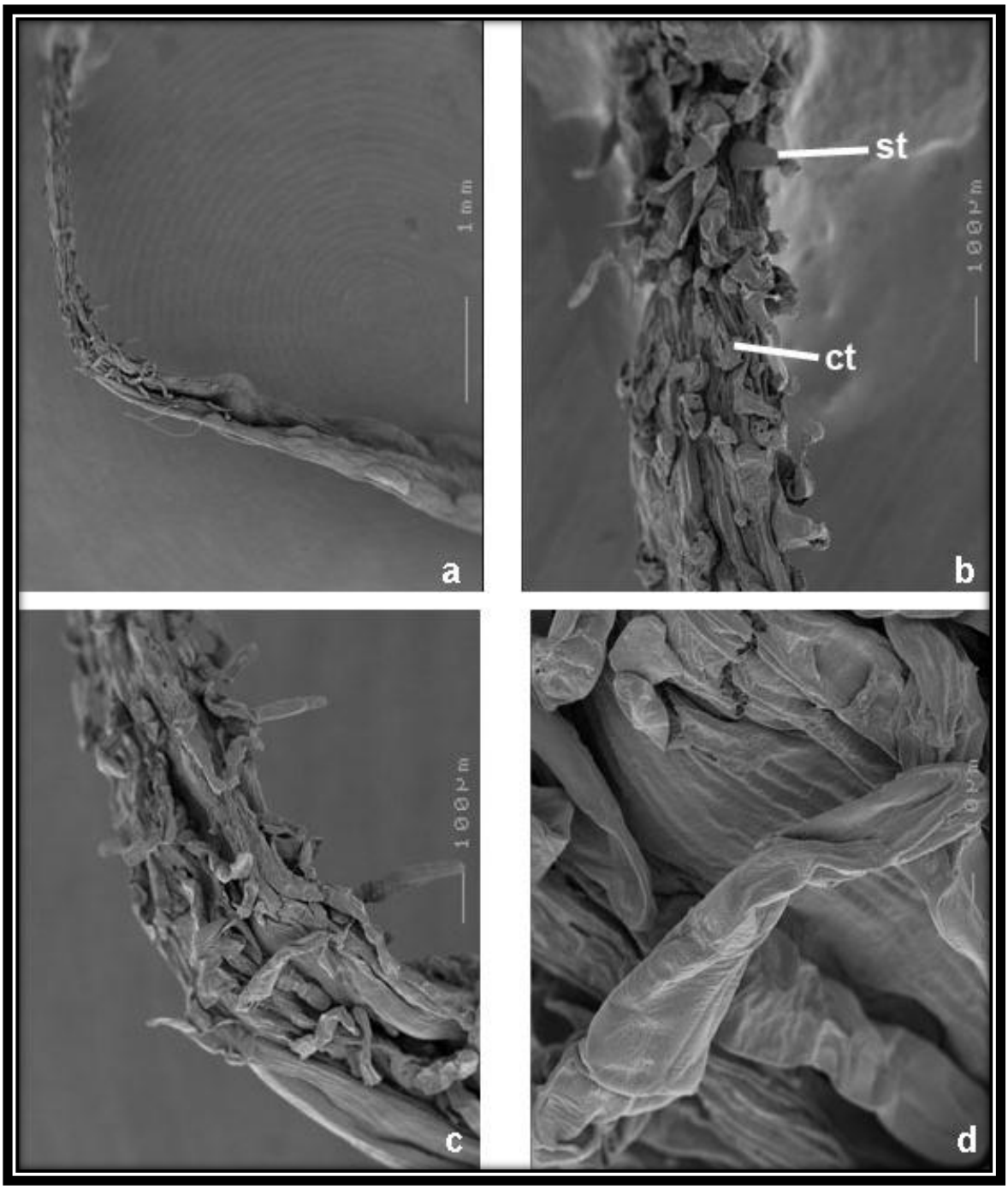

Figure 5.6.1 *Garuleum sonchifolium* ray floret. In (a) corolla; (b) corolla tube base covered with capitate trichomes and simple trichomes; (c) ligule base covered with capitate and simple trichomes; (d) simple trichomes on ligule base. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–d) = 100 µm. Specimen: (a–d) *Pegler 1199* (BOL).

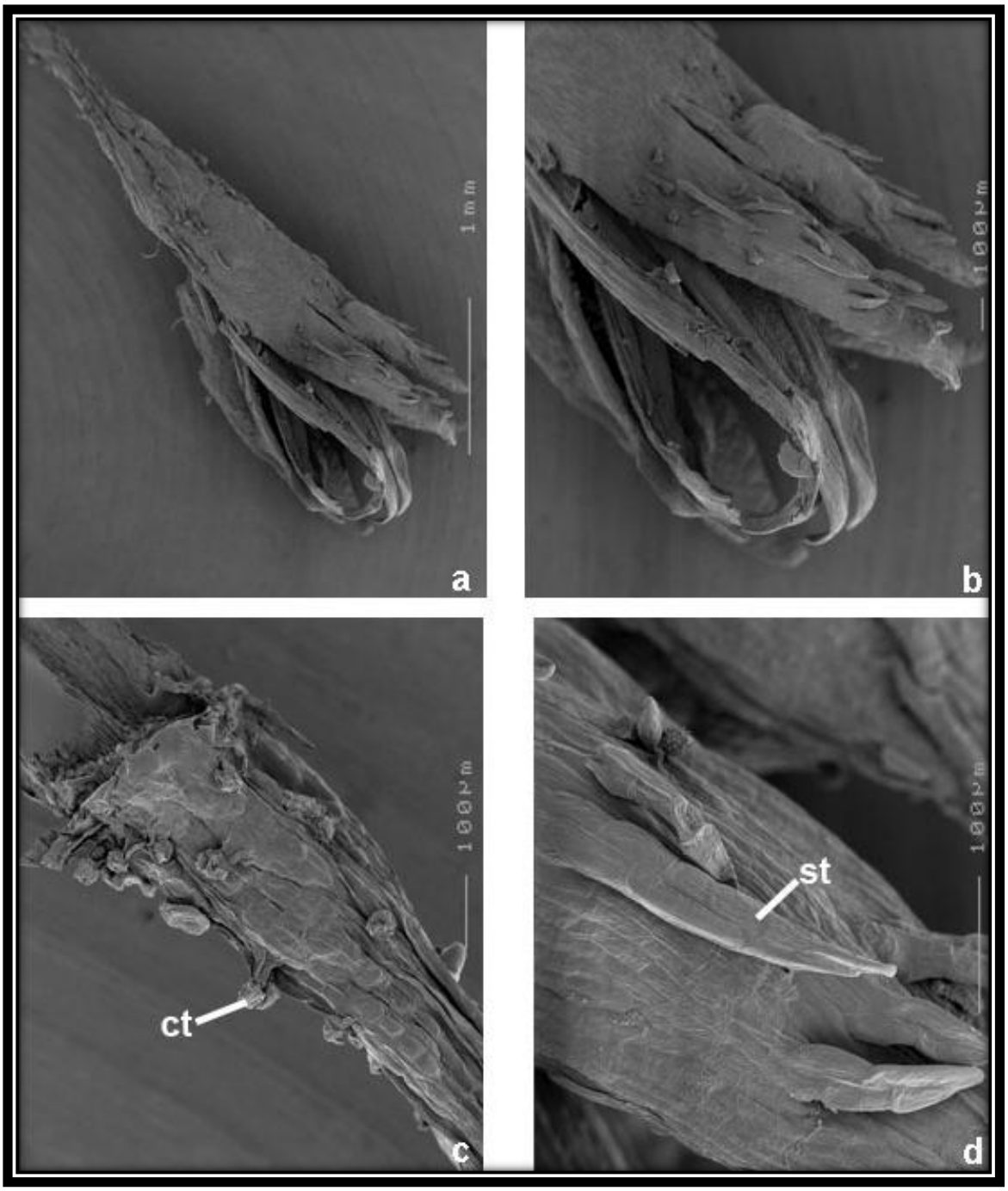

Figure 5.6.2 *Garuleum sonchifolium* disc floret. In (a) corolla; (b) corolla lobes covered with simple trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with simple trichomes. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–d) = 100 µm. Specimen: (a–d) *Pegler 1199* (BOL).

Garuleum tanacetifolium

The base of the ray floret corolla tube is covered with capitate trichomes (Fig. 5.7.1 a, b, c, e). The capitate trichomes are 32–48 µm long with a head diameter of 32–40 µm and a basal diameter of 12–16 µm. The ligule base has a high concentration of multicellular, multiseriate, simple trichomes and capitate trichomes (Fig. 5.7.1 d, f). The simple trichomes are 125–412 µm long and capitate trichomes at the ligule base are 87–100 µm long with a head diameter of 50–62 µm and a basal diameter of 25–50 µm.

The disc floret has capitate trichomes and simple trichomes on the base and the middle of the corolla tube (Fig. 5.7.2 a, c, d). The highest concentration of capitate trichomes is at the base of the disc floret corolla tube. The lobes of the floret are covered with a few simple trichomes (Fig. 5.7.2 b). The capitate trichomes are 30–68 µm long with a head diameter of 16–30 µm and a basal diameter of 12–22 µm. The multicellular, simple trichomes are 60–110 µm long.

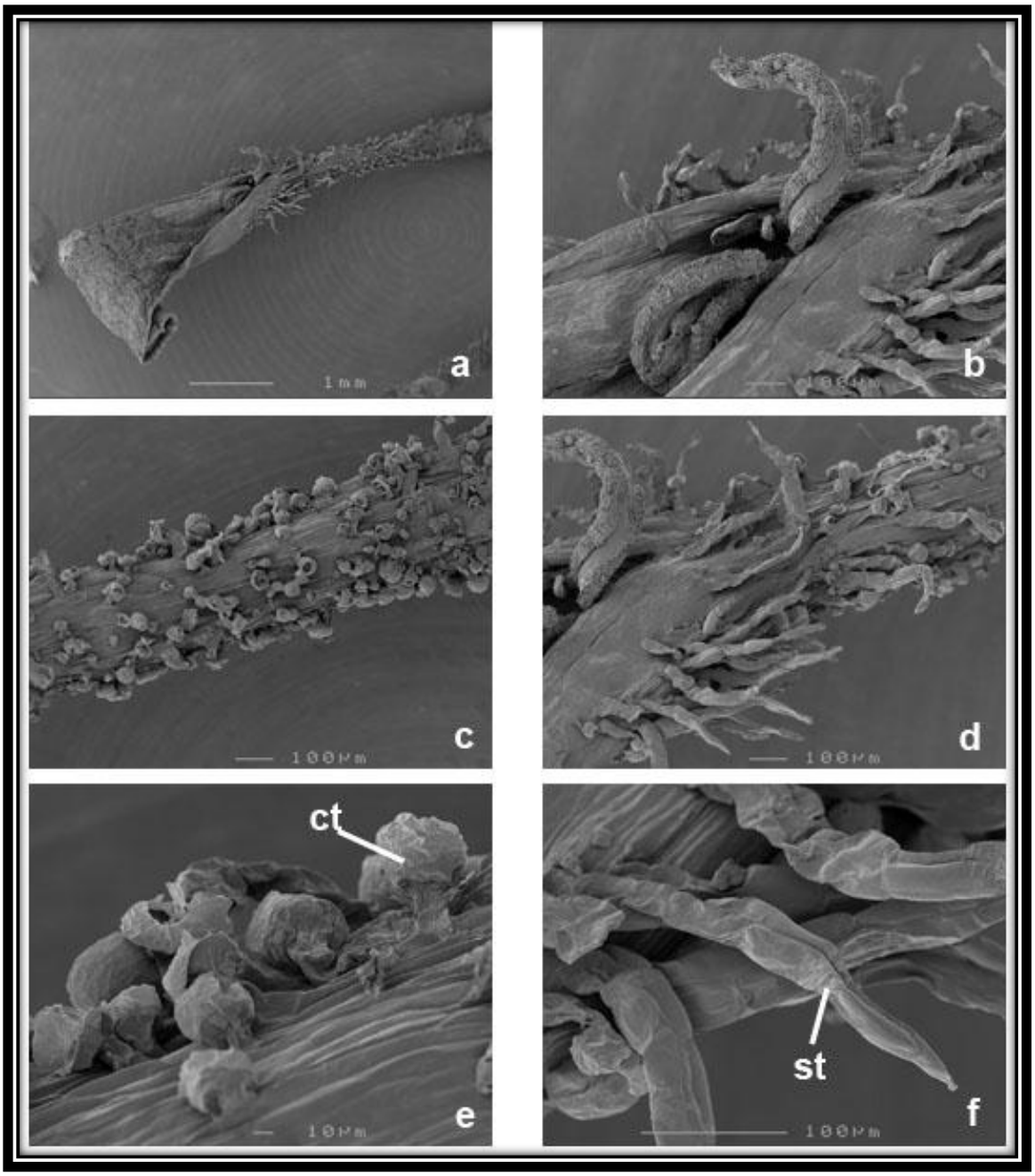

Figure 5.7.1 *Garuleum tanacetifolium* ray floret. In (a) corolla; (c) corolla tube base covered with capitate trichomes; (b, d) ligule base covered with capitate trichomes and simple trichomes; (e) capitate trichomes on corolla tube base; (f) capitate and simple trichomes on ligule base. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b, c, d, f) = 100 µm, (e) = 10 µm. Specimen: (a–f) *MacOwan 748* (Z).

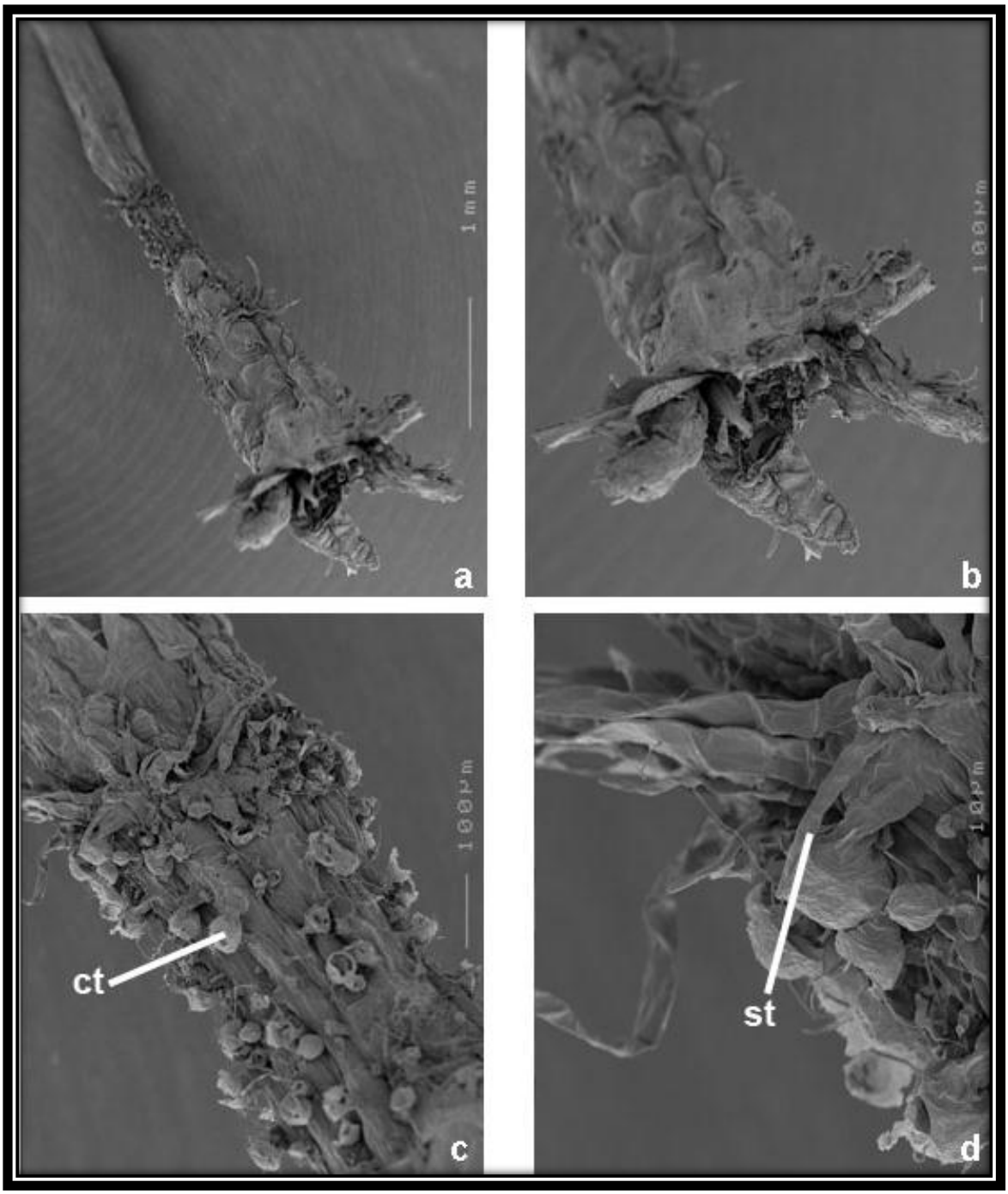

Figure 5.7.2 *Garuleum tanacetifolium* disc floret. In (a) corolla; (b) corolla lobes covered with simple trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with simple trichomes. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b, c) = 100 µm, (d) = 10 µm. Specimen: (a–d) *MacOwan 748* (Z).

Garuleum woodii

The base and middle of the ray floret corolla tube are covered with capitate trichomes (Fig. 5.8.1 a–e). The capitate trichomes are 81–111 µm long with a head diameter of 37–51 µm and a basal diameter of 22–48 µm. The ligule base has a high concentration of multicellular, multiseriate, simple trichomes (Fig. 5.8.1 d, f). The simple trichomes are 188–300 µm long.

The disc floret has capitate trichomes on the base of the corolla tube (Fig. 5.8.2 a, b). The capitate trichomes are 43–103 µm long with a head diameter of 28−40 µm and a basal diameter of 28–38 µm. The middle of the corolla tube and the corolla lobes are covered by simple and capitate trichomes (Fig. 5.8.2 c, d). The capitate trichomes on the lobes are 100–112 µm long with a head diameter of 62–64 µm and a basal diameter of 34−37 µm. The simple trichomes are 186−512 µm long.

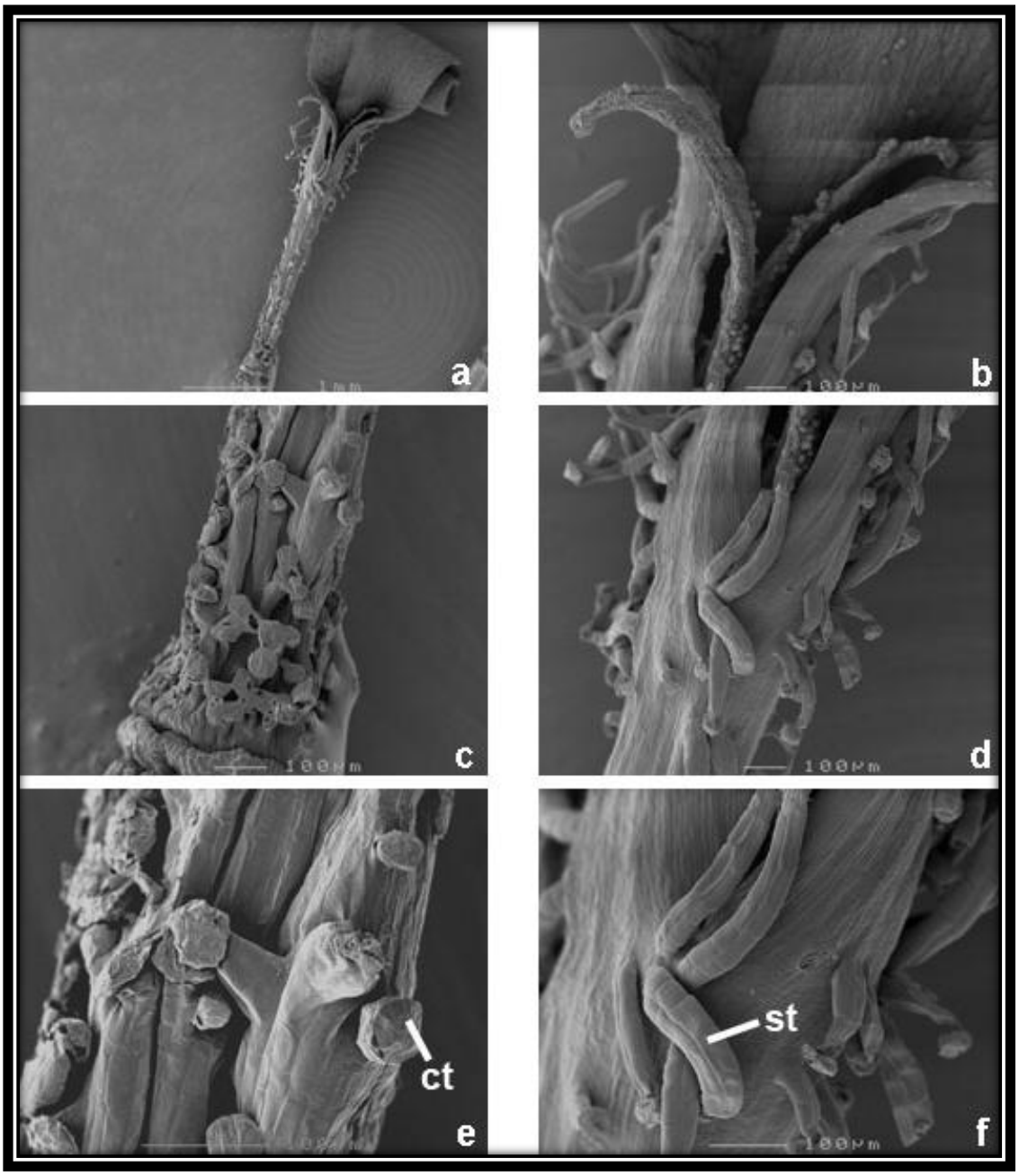

Figure 5.8.1 *Garuleum woodii* ray floret. In (a) corolla; (c) corolla tube base covered with capitate trichomes; (b, d) ligule base covered with capitate trichomes and simple trichomes; (e) capitate trichomes on corolla tube base; (f) capitate and simple trichomes on ligule base. Legend: ct = capitate trichome, st = simple trichome. Scale bars: (a) = 1 mm, (b–f) = 100 µm. Specimen: (a–f) *Ashafa s.n.* (BLFU).

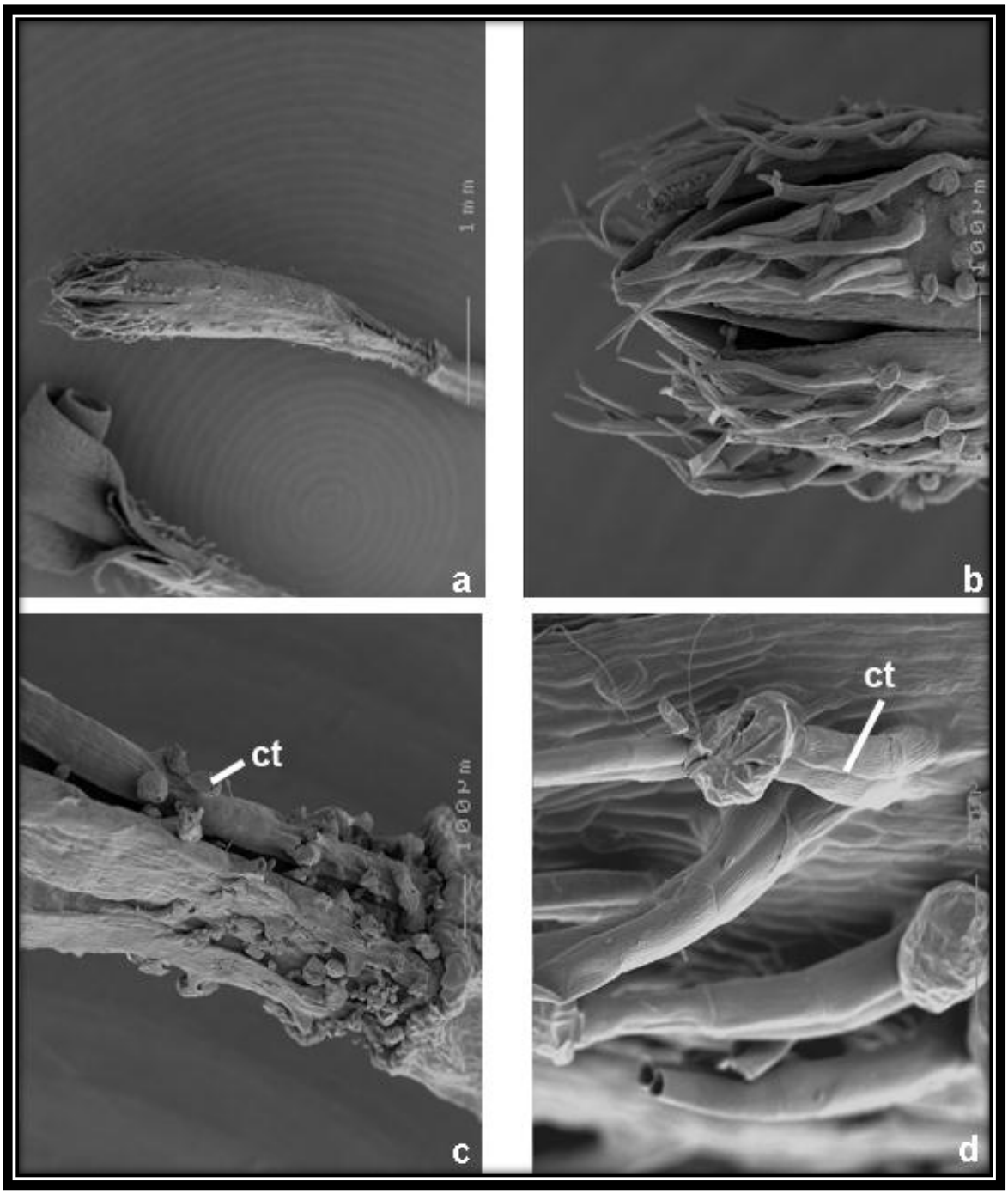

Figure 5.8.2 *Garuleum woodii* disc floret. In (a) corolla; (b) corolla lobes covered with simple trichomes and capitate trichomes; (c) corolla tube base covered with capitate trichomes; (d) corolla lobe covered with capitate trichomes. Legend: ct = capitate trichome, st = simple trichomes. Scale bars: (a) = 1 mm, (b–d) = 100 µm. Specimen: (a–d) *Ashafa s.n.* (BLFU).

5.4 Ligule surfaces of the eight *Garuleum* species

The ligule surface texture of all eight *Garuleum* species is similar. Epidermal cells on the adaxial ligule surfaces are elongate with periclinal walls forming a longitudinal ridge along the middle of each cell, and anticlinal walls deeply sunken. Cuticular striations are arranged perpendicular to the longitudinal axes of epidermal cells. The striations are straight and parallel, resulting in a barred appearance. Epidermal cells of *G. bipinnatum*, *G. latifolium*, *G. sonchifolium* and *G. woodii* appear to be straight, whereas epidermal cells of *G. album*, *G. pinnatifidum, G. schinzii* and *G. tanacetifolium* appear to be wavy (Fig. 5.9.1 a–d; Fig. 5.9.2 a–d). However, this could be an artefact of desiccation.

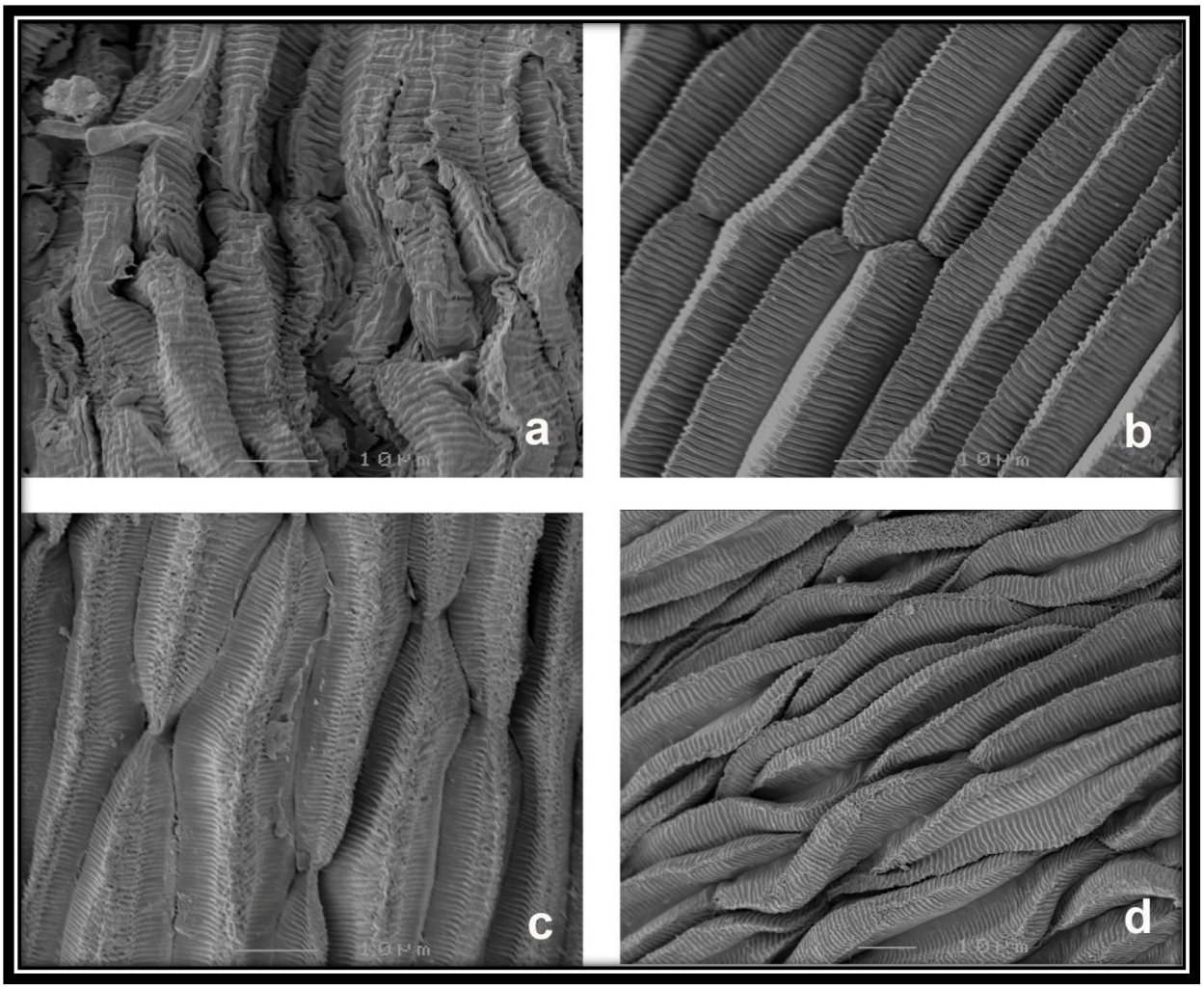

Figure 5.9.1 SEM micrographs of ligule surfaces of (a) *Garuleum album*; (b) *Garuleum bipinnatum*; (c) *Garuleum latifolium;* (d) *Garuleum pinnatifidum*. Scale bars: (a–d) = 10 µm. Specimens: (a) *Phillipson 4326* (GRA); (b) *Van Zyl 9* (BLFU); (c) *Wood 299* (Z); (d) *Van Zyl 4* (BLFU).

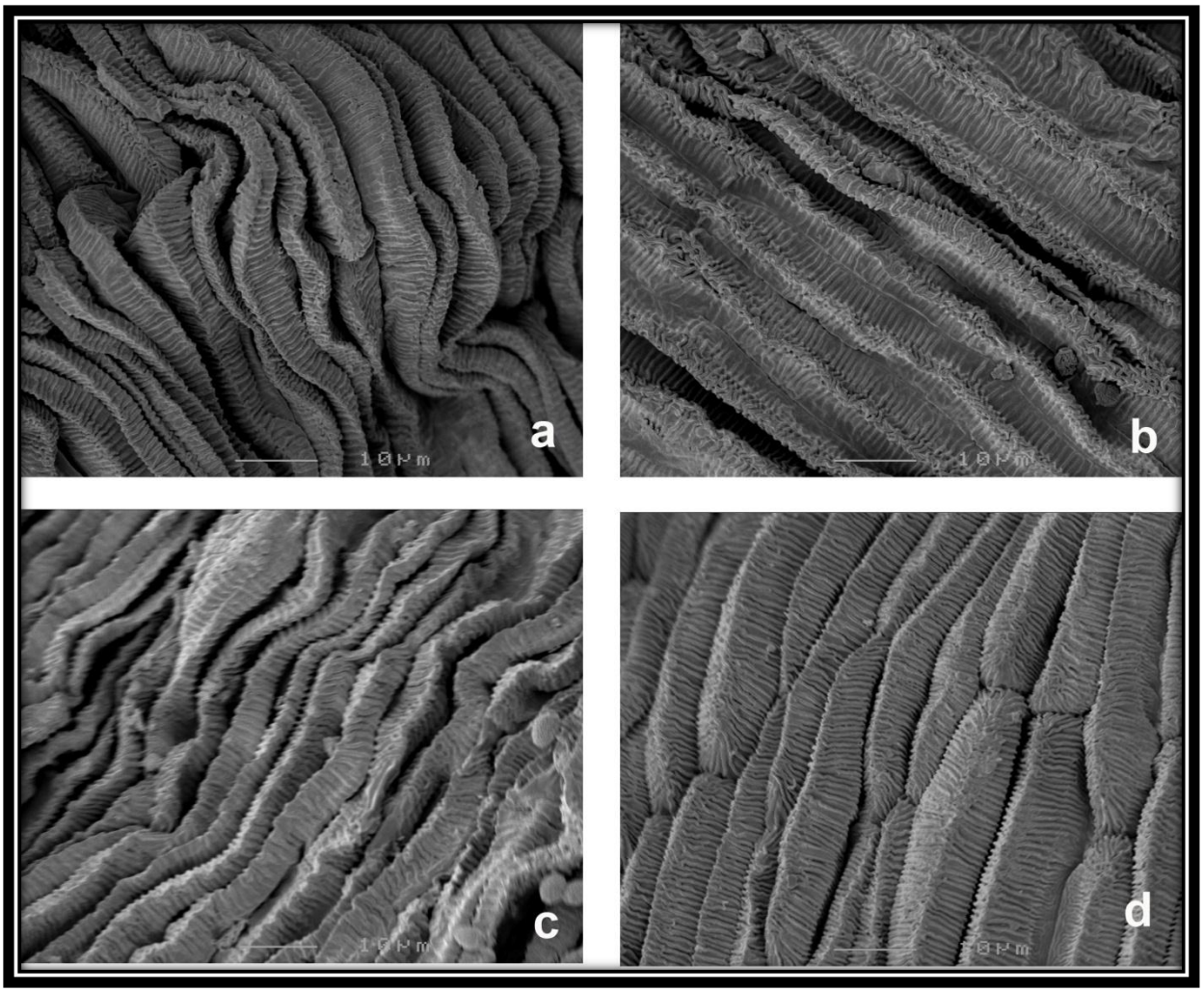

Figure 5.9.2 SEM micrographs of ligule surfaces of (a) *Garuleum schinzii*; (b) *Garuleum sonchifolium*; (c) *Garuleum tanacetifolium;* (d) *Garuleum woodii*. Scale bars: (a–d) = 10 µm. Specimens: (a) *Mannheimer 2882* (GRA); (b) *Pegler 1199* (BOL); (c) *MacOwan 748* (Z); (d) *Ashafa s.n.* (BLFU).

5.5 Discussion

The different species of *Garuleum* can be distinguished from each other using the combined trichome complement of the corolla tube bases and ligule bases of the ray florets and the corolla tube bases and corolla lobe apices of the disc florets (Table 5.1). Different combinations of of simple, capitate and simple and capitate trichomes on the various parts of the ray and disc floret corollas differentiate between *G. album*, *G. latifolium*, *G. schinzii*, *G. sonchifolium*, *G. tanacetifolium* and *G. woodii*. *Garuleum bipinnatum* and *G. pinnatifidum* are similar in the trichome complement of all parts of the ray and disc florets, except for the middle of the corolla tube of the disc floret which is covered by only capitate trichomes in *G. pinnatifidum,* but has both capitate and simple trichomes in *G. bipinnatum*.

When comparing trichome characters shared in the ray florets, *G. album* and *G. latifolium* form a group, while *G. bipinnatum* and *G. pinnatifidum* form a second group. The rest of the species share different combinations and positions of simple and capitate trichomes. These groupings are not supported by the distribution of trichomes on the disc florets.

In the disc florets *G. latifolium*, *G. pinnatifidum*, *G. schinzii* and *G. woodii* group together with the same trichome types. *Garuleum sonchifolium* and *G. tanacetifolium* share the same trichomes. Only in *G. album* and *G. bipinnatum* do the trichome types and positions on disc florets vary enough not to match that of the other species.

Trichome complement of the ray and disc florets has not been incorporated in many other studies in the Asteraceae. This may be due to the variability of trichome complement which makes it an unfavourable phylogenetic and diagnostic character. The variability of trichome complement in intra-taxon and intra-population level in disc florets are clearly indicated in the study of Carpenter (1999) who did a comparison of trichome morphology on the disc florets of *Encelia* Adans. (Asteraceae).The variability of trichome complement at intra-population level for *Garuleum* is not clear because only one specimen per species was used in this study.

Striations on the ligule surface were tested to see if it can distinguish between the different species. Two groups were formed, those with straight striations, *G. bipinnatum*, *G. latifolium*, *G.sonchifolium* and *G.woodii,* and a second group with wavy striations, *G. album*, *G.pinnatifidum*, *G. schinzii* and *G. tanacetifolium*. These groupings are not supported by other more reliable characters, and may be incorrect due to artefacts from drying. The ligules may also be at different stages of development, which affects the surface patterns that can be seen at that stage of development (Thomas et al., 2009). Ligule micromorphological characters are not stable enough for taxonomic use in this genus.

For this study the micromorphological characters used from the ray and disc florets, did prove to be variable enough for species-level classification, but the accuracy of this character will have to be confirmed by repetitive studies with specimens from different populations for each species.

Table 5.1. A comparison between the different trichome types and their positions on the ray and disc florets for the eight *Garuleum* species.

Ray florets	**Trichome types**		
***Garuleum* species**	**corolla tube base**	**corolla tube middle**	**ligule base**
Garuleum album	capitate	capitate and simple	simple
Garuleum bipinnatum	capitate	capitate	capitate
Garuleum latifolium	capitate	capitate and simple	simple
Garuleum pinnatifidum	capitate	capitate	capitate
Garuleum schinzii	capitate and simple	capitate	capitate and simple
Garuleum sonchifolium	capitate and simple	capitate and simple	capitate and simple
Garuleum tanacetifolium	capitate	capitate	simple
Garuleum woodii	capitate	capitate	capitate and simple
Disc florets	**Trichome types**		
***Garuleum* species**	**corolla tube base**	**corolla tube middle**	**corolla lobe**
Garuleum album	simple	simple	simple
Garuleum bipinnatum	capitate	capitate and simple	capitate and simple
Garuleum latifolium	capitate	capitate	capitate and simple
Garuleum pinnatifidum	capitate	capitate	capitate and simple
Garuleum schinzii	capitate	capitate	capitate and simple
Garuleum sonchifolium	capitate	capitate and simple	simple
Garuleum tanacetifolium	capitate	capitate and simple	simple
Garuleum woodii	capitate	capitate	capitate and simple

Chapter 6

Micromorphology of *Garuleum* pollen grains

6.1 Introduction

Early palynological studies utilized only light microscopy and showed that the pollen of most taxa in the Asteraceae were very similar in appearance (Skvarla et al., 1977). The utilization of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) data in taxonomic studies has promoted the discovery of new useful characters and correct interpretation of such characters in the Asteraceae (Lane, 1985). The main advantages associated with using SEM in addition to light microscopy are that it provides a greater magnification range. The ability to show the pollen surface independent of underlying structural components provides more focus and depth. Samples can also be prepared with relative ease (Walker and Doyle, 1975). In addition to surface micromorphology, pollen samples can also be fractured to reveal the pollen wall structure (Cerceau Larrival and Roland Heydacker, 1972).

The pollen morphology of the Asteraceae was first investigated by Wodehouse (1926, 1928a, 1928b, 1928c, 1929a, 1929b, 1929c, 1930, 1931), who focused on pollen surface patterns. He recognized three surface patterns: unadorned or smooth (pisilate), conspicous spines (exinate) or consisting of depressions and ridges (lophate). He also showed that Asteraceae pollen was tricolpate with three germination apetures, each consisting of a furrow and germinal pore (Skvarla et al., 1977).

Skvarla and Turner (1966) distinguished between three types of exine patterns in the Asteraceae: Helianthoid pattern, Anthemoid pattern and the Senecioid pattern. They observed the Helianthoid pattern (caveate exines with internal formations) in the tribes Astereae, Calenduleae, Eupatorieae, Helenieae, Heliantheae, Inuleae and Senecioneae and less commonly in the Anthemideae (Skvarla et al., 1977). The Senecioid pattern (internal formations absent), has been noted for the Ambrosieae, Inuleae and the Senecioneae. The Anthemoid pattern (thick, long series of basal columellae alternating with internal tecta, a reduced endexine and thick foot layer) was observed in the Mutisieae and Veronieae.

The characters of Asteraceae pollen have been shown to be very variable (Blackmore et al., 2009) and may show a high degree of homoplasy. Pollen characters are taxonomically useful at tribal level in the Asteraceae (Wortley et al., 2008). Useful characters used at or above generic-level include the surface sculpture of the pollen grain, exine structure, size, shape and apertures of the pollen grain (Blackmore et al., 2009).

Blackmore et al. (2009) described pollen characters for the Asteraceae tribes following the classification of Funk et al. (2005). The Calenduleae description on page 128 was as follows: “Prolate or spheroidal, triangular or round in polar view, elliptic or round in equatorial view, tricolporate, colpi separate, narrow to medium, lolongate or circular, tectum microperforate, mesoaperture present sometimes, echinate, spines few to many, average to dense, distributed evenly, long pointed to conical pointed, 1–4 µm, swollen perforated base, tectum not raised, non-lophate, infratetum single-layered, outer infratectum columellate, internal tectum solid, single, indistict, internal foramina ubiquitos, spine comellae free hanging, fully or (occasionally) partially caveate, cavea shallow to deep, exine is thicker than foot layer”.

The pollen of the tribe Calenduleae was first known through the work of Stix (1960), who used only light microscopy and in the process neglected to observe imporant diagnostic characters in *Osteospermum* pollen which are visible when using SEM. The first comprehensive study of the pollen of the Calenduleae was preformed by Praglowski and Grafström (1980). Praglowski and Grafström (1980) described five pollen types in Calenduleae: *Calendula*-type, *Diptercome*-type, *Garuleum*-type, *Gibbaria*-type and *Osteospermum*-type. *Diptercome* was later removed from Calenduleae (Nordenstam, 1994a).

The *Calendula*-type pollen is characterized by relatively large grains (±50 µm equatorial diameter), with the ora lolongate (longitudinally elongated endoaperture) and unconstricted, the solid zone of the pollen wall consisting of a large number of slender spines (100–130). The *Garuleum*-type is a smaller pollen grain (±34 µm in equatorial diameter). The pollen wall is covered with spines (100–120), and the internal structure of the pollen wall consists of an undelying and upper solid zone with one or two minute holes. Individual spines are closely situated at the base to each other, while the ora is lolongate and thin.

The *Gibbaria*-type pollen have the smallest grain size (±29 µm in equatorial diameter), with lolongate ora and the pollen wall covered with spines (±70). The internal structure of the pollen wall consists of an upper solid zone and underlying zone, with the upper solid zone exhibiting one or two minute holes in the spines. The *Osteospermum*-type pollen grain size is slightly smaller than that of the *Garuleum*-type (±32 µm in equatorial diameter) and have slightly constricted lalongate (transversely elongated endoaperture) ora with pointed ends. The pollen wall of the *Osteospermum*-type pollen grain is covered in spines (65–80), while the internal structure of the pollen wall consists of an upper solid zone and underlying zone, with the upper solid zone exhibiting one minute hole in each the spine (Praglowski and Grafström, 1980).

No classification based solely on pollen characters exists to date. Instead pollen characters have been found to be congruent with phylogenetic trees based on molecular studies, and provide evidence to support phylogenetic relationships among genera, not included in the molecular studies (Blackmore et al., 2009).

6.2 Materials and methods

All material and methods in this chapter are described in 3.2 (Chapter 3).

6.3 Results

All species of *Garuleum* possess pollen grains that are tetracolporate, with the exine densely covered with spines (Fig. 6.1 a–d; Fig. 6.2 a–d). Spines of all eight species have minute perforations on their bases and the ora (germinating pores) are lolongate (Fig. 6.3). The polar axis/equatorial diameter (P/E) ratio of all eight species range between 0.88–0.99 and their spine lengths between 3–5 µm (Table 6.1). *Garuleum latifolium* had a slightly larger spine length than the rest of the *Garuleum* species.

Table 6.1 Pollen measurement results for eight *Garuleum* species.

Species	Specimen	Polar axis length (µm)	Equatorial axis length (µm)	Spine length (µm)	P/E value
Garuleum album	*Phillipson 4326* (GRA)	30.75 ±4.99 (28.8–35.91)	33.59 ±5.87 (25–38.02)	3.95 ±0.38 (3.5–4.22)	0.92
Garuleum bipinnatum	*Bayliss 2845* (Z)	27.25 ±4.87 (21.17–30.98)	31.06 ±6 (24.28–35.91)	3.52 ±0.27 (3.5–3.57)	0.88
Garuleum latifolium	*Wood 160* (BOL)	30.99 ±4.26 (24.7–36.61)	34.73 ±4.7 (26.47–38.02)	4.42 ±0.44 (4.22–5.2)	0.89
Garuleum pinnatifidum	*Van Zyl 3* (BLFU)	30.14 ±1.26 (28.87–31.69)	34.2 ±2.26 (31.69–37.32)	3.79 ±0.39 (3.5–4.22)	0.88
Garuleum schinzii	*MacOwan 1889* (Z)	30.54 ±3.69 (24.58–34.5)	32.89 ±5.34 (23.63–37.32)	3.52 ±0.045 (3.5–3.6)	0.92
Garuleum sonchifolium	*Pegler 1199* (BOL)	30.51 ±2.46 (26.52–33.09)	33 ±3.2 (29.13–35.21)	3.58 ±0.18 (3.5–3.91)	0.92
Garuleum tanacetifolium	*MacOwan 1382* (Z)	31.5 ±4.43 (24.44–36.61)	34.37 ±3.2 (28.88–37.32)	3.57 ±0.15 (3.5–3.85)	0.92
Garuleum woodii	*Rehman 6792* (Z)	29.19 ±1.7 (26.95–31.69)	33 ±2.57 (28.46–34.5)	3.7 ±0.5 (3.5–4.61)	0.88

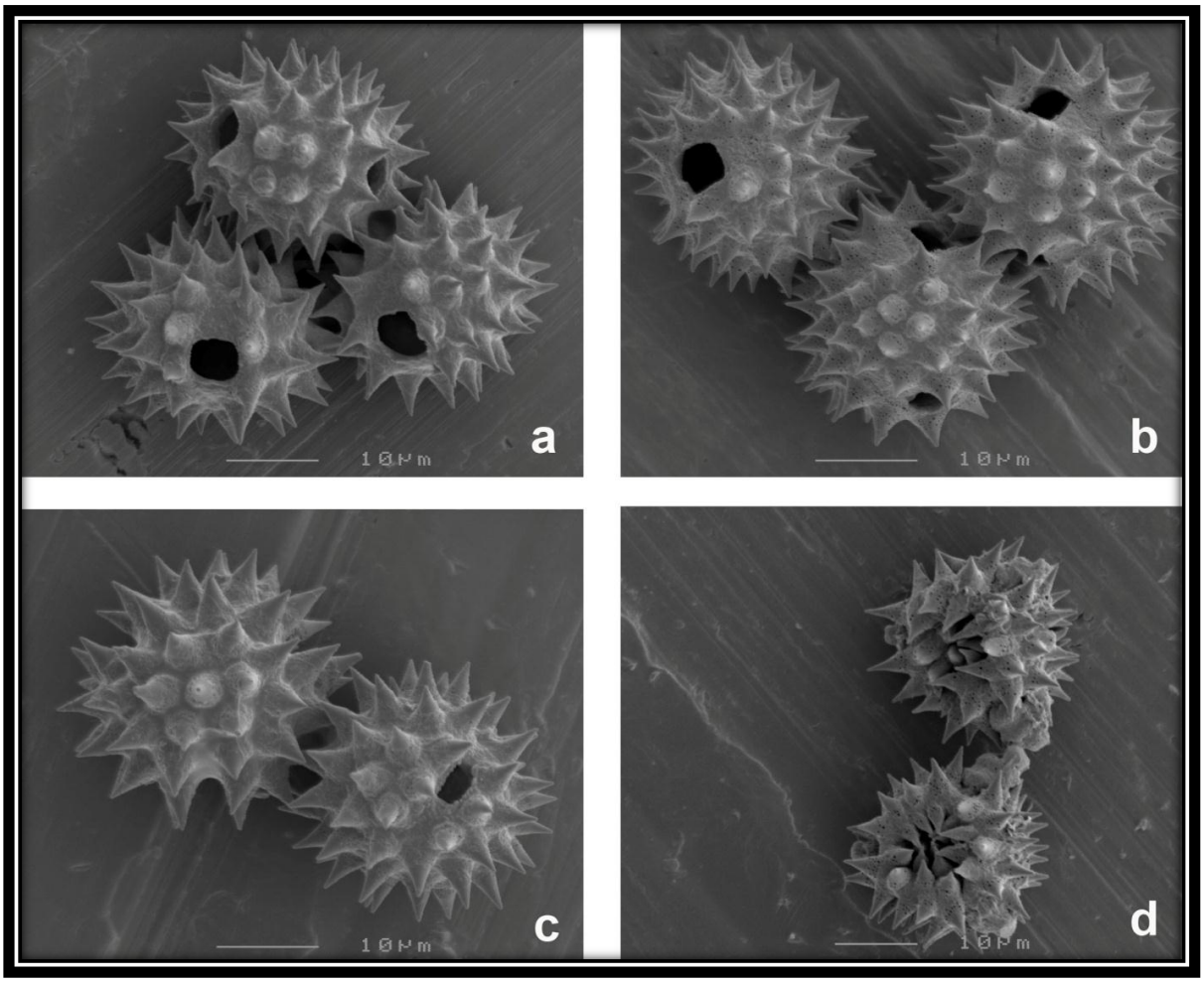

Figure 6.1 SEM micrographs of *Garuleum* pollen (a) *Garuleum album*; (b) *Garuleum bipinnatum*; (c) *Garuleum latifolium*; (d) *Garuleum pinnatifidum*, in which the pollen grain interior walls collapsed during preparation of sample. Scale bars: (a–d) = 10 µm. Specimens: (a) *Phillipson 4326* (GRA), (b) *Bayliss 2845* (Z), (c) *Wood 160* (BOL), (d) *Van Zyl 3* (BLFU).

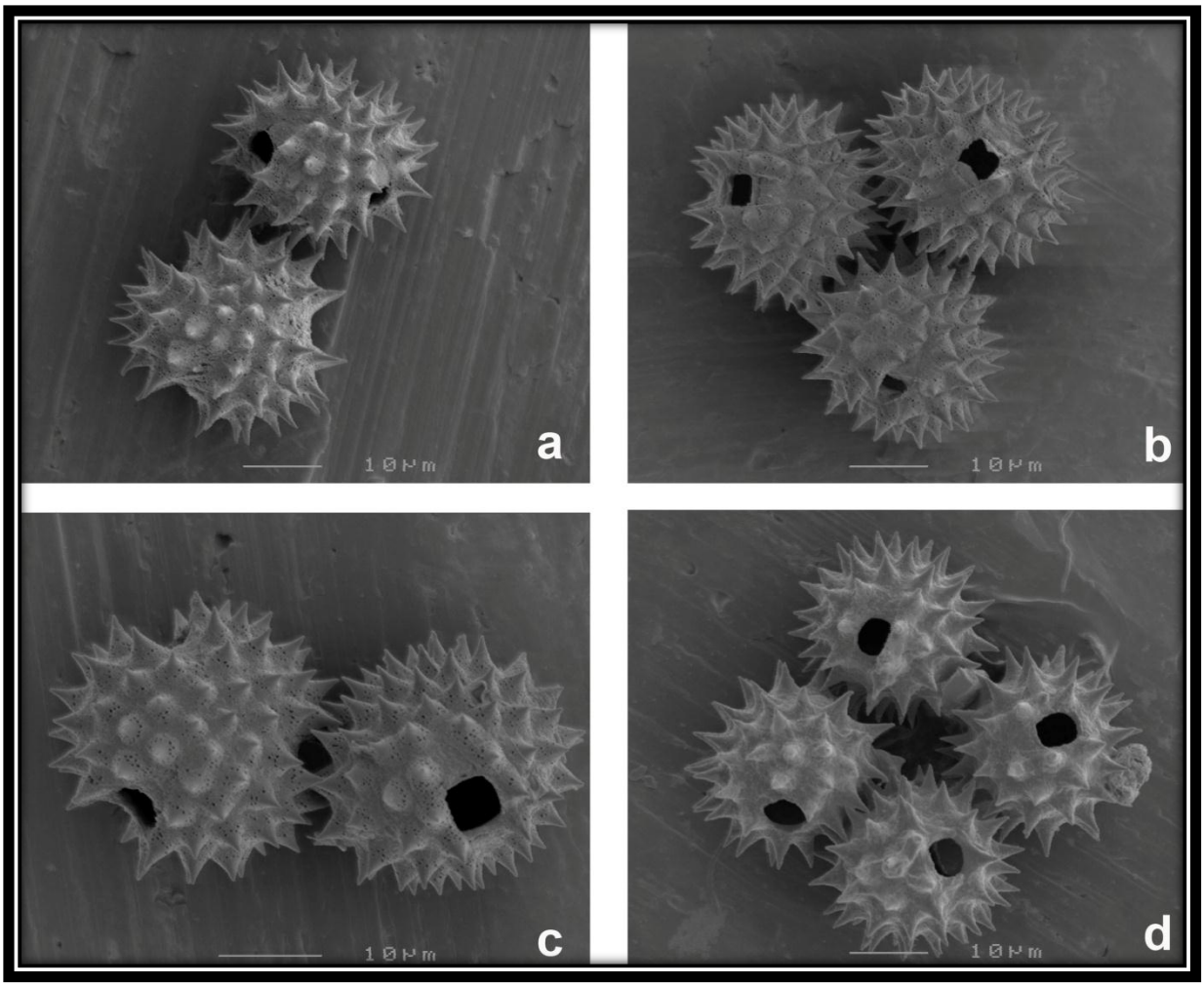

Figure 6.2 SEM micrographs of *Garuleum* pollen (a) *Garuleum schinzii*; (b) *Garuleum sonchifolium*; (c) *Garuleum tanacetifolium*; (d) *Garuleum woodii*. Scale bars: (a–d) = 10 µm. Specimens: (a) *MacOwan 1889* (Z), (b) *Pegler 1199* (BOL), (c) *MacOwan 1382* (Z), (d) *Rehman 6792* (Z).

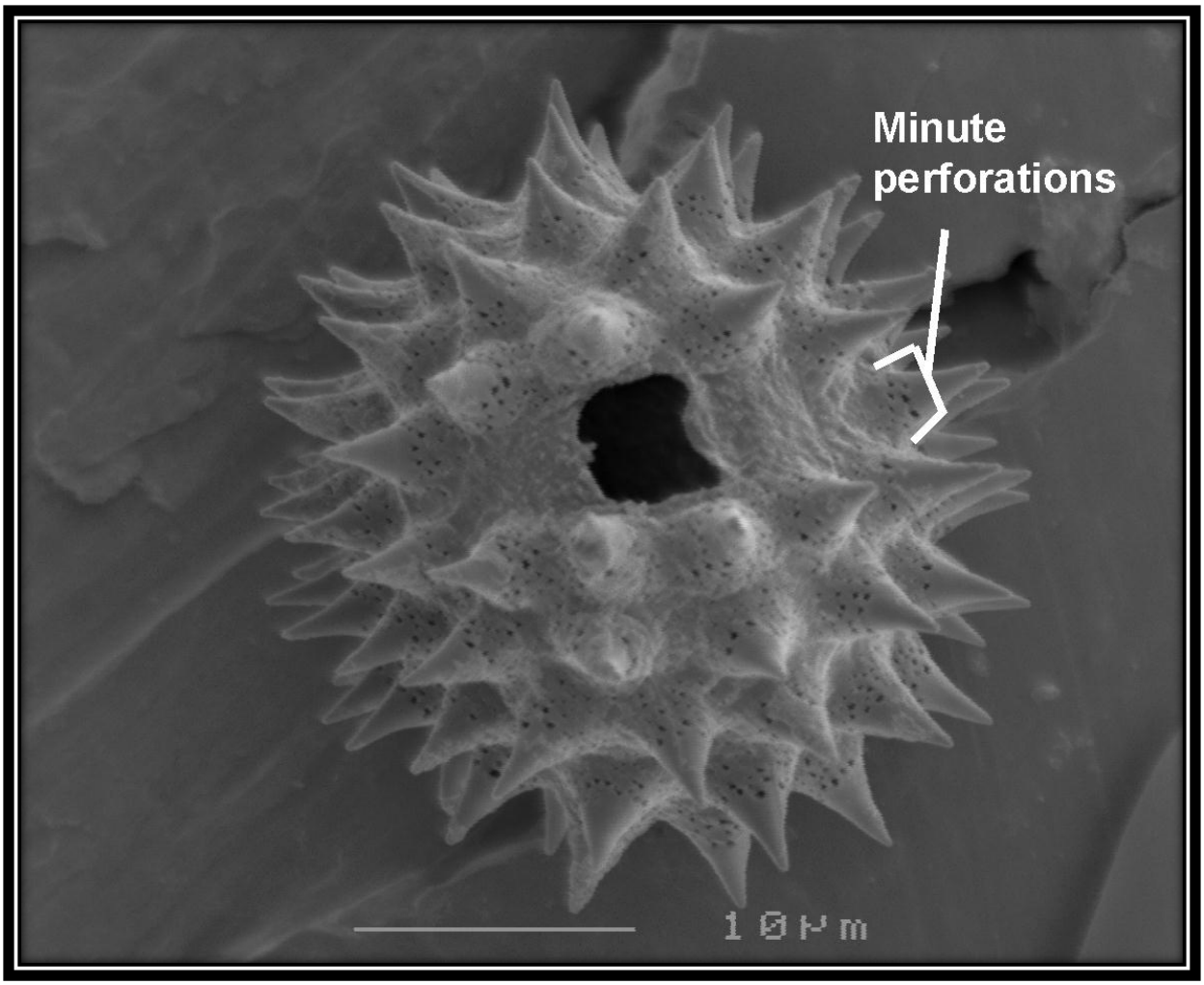

Figure 6.3 SEM micrograph of a pollen grain of *Garuleum sonchifolium*. This indicates the minute perforations found on the base of the spines of *Garuleum* pollen. Scale bar = 10 µm. Specimen: *Pegler 1199* (BOL).

6.4 Discussion

Comparison of the results of the present study with those of Praglowski and Grafström (1980), showed that the pollen of all eight species belong to the *Garuleum*-type. The equatorial diameter is 35 µm or less in all species, while the spines are 1–4 µm in length, the ora are lolongate and spines densely covering the pollen grain. The P/E ratio ranges from 0.88 to 1.14, which means the pollen is spheroidal. The characteristics conform to the characteristics described for the Calenduleae by Blackmore et al. (2009).

The characters used in this study did not provide enough variability to distinguish the eight *Garuleum* species. The spine length average for *G. latifolium* was a little higher than the rest of the species, which could be a character useful in distinguishing this species. In future studies the pollen may be fractured to include internal pollen wall structures and characteristics, which may provide more variability to distinguish between species. This is the first survey of *Garuleum* pollen which includes all species and shows that all of the species are characterised by the *Garuleum*-type pollen. In addition this pollen-type is unique to *Garuleum* (Praglowski and Grafström, 1980) and supports the monophyly of the genus.

Chapter 7:

Taxonomic treatment

7.1 Generic description of *Garuleum*.

Garuleum Cass. in Plassan, Bulletin des Sciences la Société Philomatique Paris: 172 (1819c).

Type: *Garuleum viscosum* Cass. *nom illegit*. [*Garuleum pinnatifidum* (L'Hêr) DC.]

A perennial or annual, aromatic shrub, 0.6–2 m tall. Woody rootstock occasionally present. **Stems** branching from base, erect, herbaceous, semi-succulent or woody; epidermis glandular, velutinous, scabrous or pubescent. **Leaves** alternate, densely arranged, sessile; base clasping stem; blade ovate, pinnatipartite, bipinnately-parted or tripinnately-parted; size and degree of lobing decreases towards the stem apex; margins serrated; adaxial surface glandular, green; abaxial surface velutinous or glandular, whitish-green or green; apex cassidate; stipules occassionally present. **Inflorescences** capitula, radiate, 4–18 mm in diameter, solitary or arranged in lax corymbs; peduncles present or rarely absent, velutinous, minutely velutinous or glandular; involucre campanulate, involucral bracts, green, herbaceous, velutinous, apices recurved, arranged imbricately in series of 3 rows surrounding the capitula, all bract of equal size or the outer row of bracts larger than the two inner rows. Pappus absent. **Ray florets** sessile; corolla mauve, purple or white, trigomes present on corolla tube; ligule with 3 apical teeth; pistillate, ovary fertile inferior, style 2.8–7 mm long, stigma bi−lobed, collar of sweeping hair extending below point of furcation. **Disc florets** sessile; bisexual or functionally male, pentamerous; corolla yellow, tubular, trigomes present, concentrated on the corolla lobe apices; stamens fertile, inserted 1–2 mm from base of corolla tube, filaments 1–1.5 mm long, anthers 1–3 mm long, lanceolate, monodelphously fused; ovary inferior, style apically bilobed, collar extending below point of furcation. **Achenes** develop from ray florets of all species and disc florets of one species (possibly in a second spesies); ray floret achenes brown, light brown or dark brown, obovoid, obcordate, oblong or cyllindrical, surfaces smooth, folded or warty; disc floret achenes light brown, obovoid to obcordate, surfaces smooth.

7.1.1 Diagnostic characteristics

Aromatic shrublet. Leaves alternate, sessile, pinnatipartite, bipinnately-parted or tripinnately-parted. Capitula radiate, with mauve, purple or white ray florets surrounding yellow disc florets. Involucre bracts are green, herbaceous, arranged in three rows. Pappus absent. The style is apically deeply bilobed, supapical collar of sweeping hairs extending below point of furcation. No papille are present on achenes.

7.1.2 Distribution and ecology

Garuleum is widely distributed over Southern Africa including Lesotho, Namibia and South Africa, where it grows in the Eastern Cape, Free State, Kwazulu-Natal, Mpumalanga, Northern Cape and Western Cape. The highest concentration of species is found in the Eastern Cape, Free State, Kwazulu-Natal and Western Cape (Fig. 7.1.1). *Garuleum* grows in a wide variety of habitats ranging from semi-desert to karoo in the north-western parts of the region and savannah and afromontane forest vegetation in the central and eastern parts of the region. The genus grows at moderate to high altitudes, on moderate slopes, in full-sun or semi-shade. This genus is associated with a variety of geological formations, including cave sandstone, dolerite, granite and shale. Associated soil types include well drained, sandy stony loam, clay, gravel and worn shale and granite. *Garuleum* species populations are localized, but abundant where suitable habitat is found. Most species flower throughout the year, usually after rains.

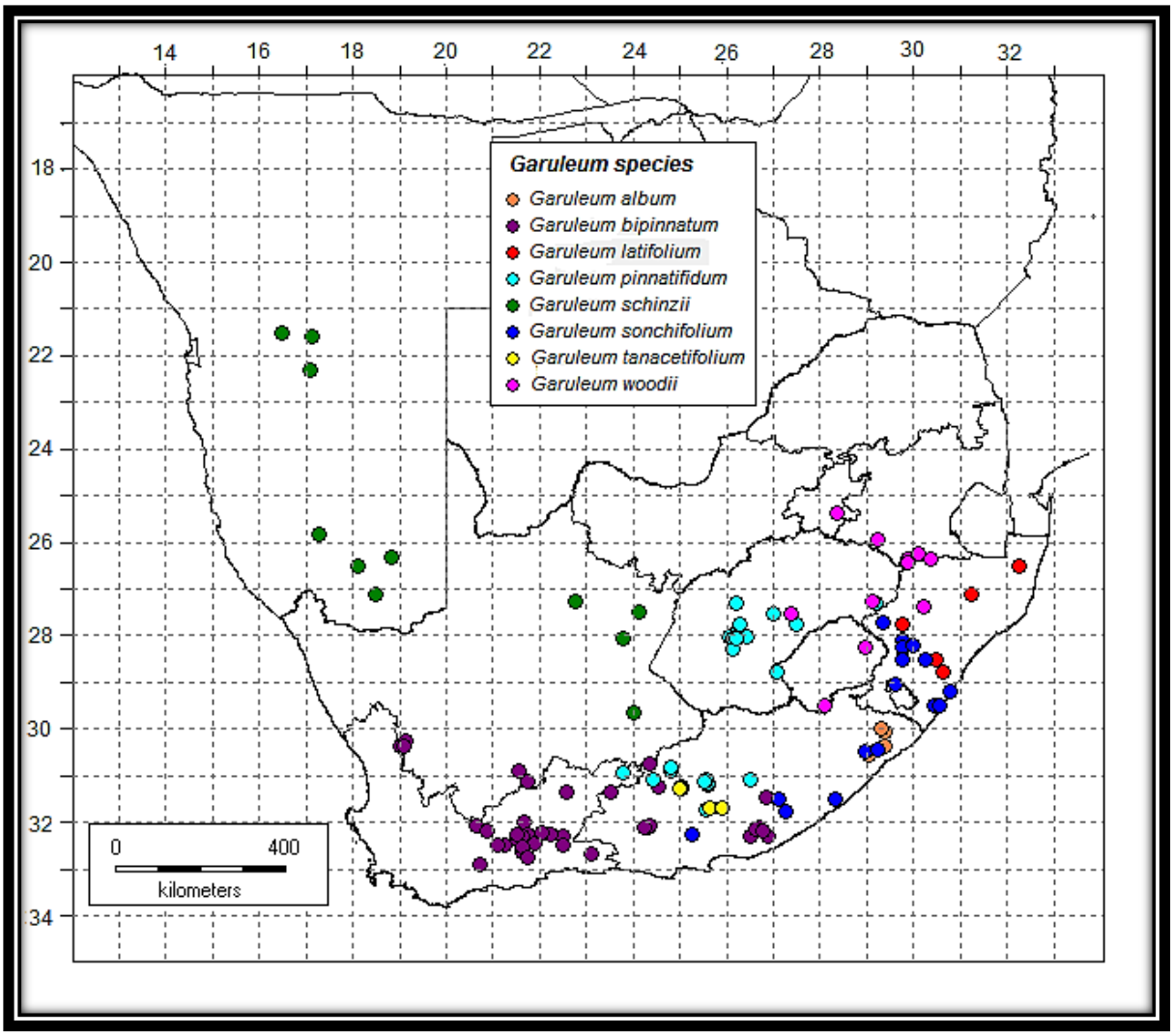

Figure 7.1.1 Known geographical distribution of the genus *Garuleum* in southern Africa. Scale bar = 400 km.

7.2 Key to the species of *Garuleum*:

1a Leaves pinnatipartite to bipinnately parted

2a Leaves pinnatipartite

3a Abaxial leaf surface velutinous, white…….. ***G. album***

3b Abaxial leaf surface glandular, green

4a Capitula surrounded by three rows of bracts, all of equal size …….***G. latifolium***

4b Capitula surrounded by three rows of bracts, first row larger than the second and third row ……..***G. sonchifolium***

2b Leaves bipinnately parted

5a Margins of leaf lobes bluntly serrated……...***G. pinnatifidum***

5b Margins of leaf lobes entire……***G. schinzii***

1b Leaves tripinnately parted

6a Peduncle absent……***G. tanacetifolium***

6b Peduncle present

7a Leaf margins crenate, disc florets functionally male, only ray florets produce achenes……***G. woodii***

7b Leaf margins serrated, disc florets bisexual, ray and disc florets produce achenes……***G. bipinnatum***

7.3 Description of species.

7.3.1 ***Garuleum album*** S.Moore in Timmens and S.Moore, Journal of Botany, British and Foreign, 16: 133 (1878).

Type: South Africa, Kaffaria, Shawburg, Griqualand East [Eastern Cape Province]. *Baur, R. 226* (K, holotype (K000273562, K000273563 scan!)).

An aromatic shrub, up to 1 m tall. **Stem** base 30–50 mm wide; herbaceous to semi-succulent; young stems velutinous; older stems glandular with longitudinal grooves. **Leaves** sessile; base clasping stem; blades 45–97 x 21–50 mm, ovate, pinnatipartite; margins serrated, adaxial surfaces glandular, green; abaxial surfaces velutinous, white-green; apices cassidate; stipules occassionally present. **Inflorescences** capitula, 10–18 mm wide, peduncles 45–120 mm long, velutinous; involucral bracts 4–8 x 2–5 mm, velitinous, apices recurved, arranged imbricately in a series of 3 rows surrounding capitula, outer and inner rows similar in size. **Ray floret** corolla mauve to purple, tube base glandular, tube apex villous, ligulate; ligule 10–15 mm long; style 5–7 mm long, covered with sweeping hair, stylar lobes 2–3 mm long. **Disc floret** corolla 5–6 mm long, tube ±4 mm long, lobes ±2 mm long, trigomes sparsely arranged on corolla, concentrated on corolla lobe apices. Stamens inserted ±2 mm from corolla tube base, filaments ±1.5 mm long, anthers 2–2.5 mm long. Pistil with style ±5 mm long, stylar lobes ±1 mm long, hairy. **Achenes** develop from ray florets, brown, obovoid, smooth, 4.4 x 2.3 mm. Figure 7.2.1.

Diagnostic characters

Leaves pinnatipartite. Adaxial leaf surfaces glandular green, abaxial leaf surfaces velutinous white. Peduncles and bracts velutinous. Bracts 4–8 x 2–5 mm in size, apices recurved.

Distribution and habitat

Garuleum album grows in the northern parts of the Eastern Cape (previously Transkei) at altitudes of 610–700 m (Fig. 7.2.2). This species inhabits shrub-lands, growing on gentle slopes, in full-sun in well-drained weathered shale or loam soil.

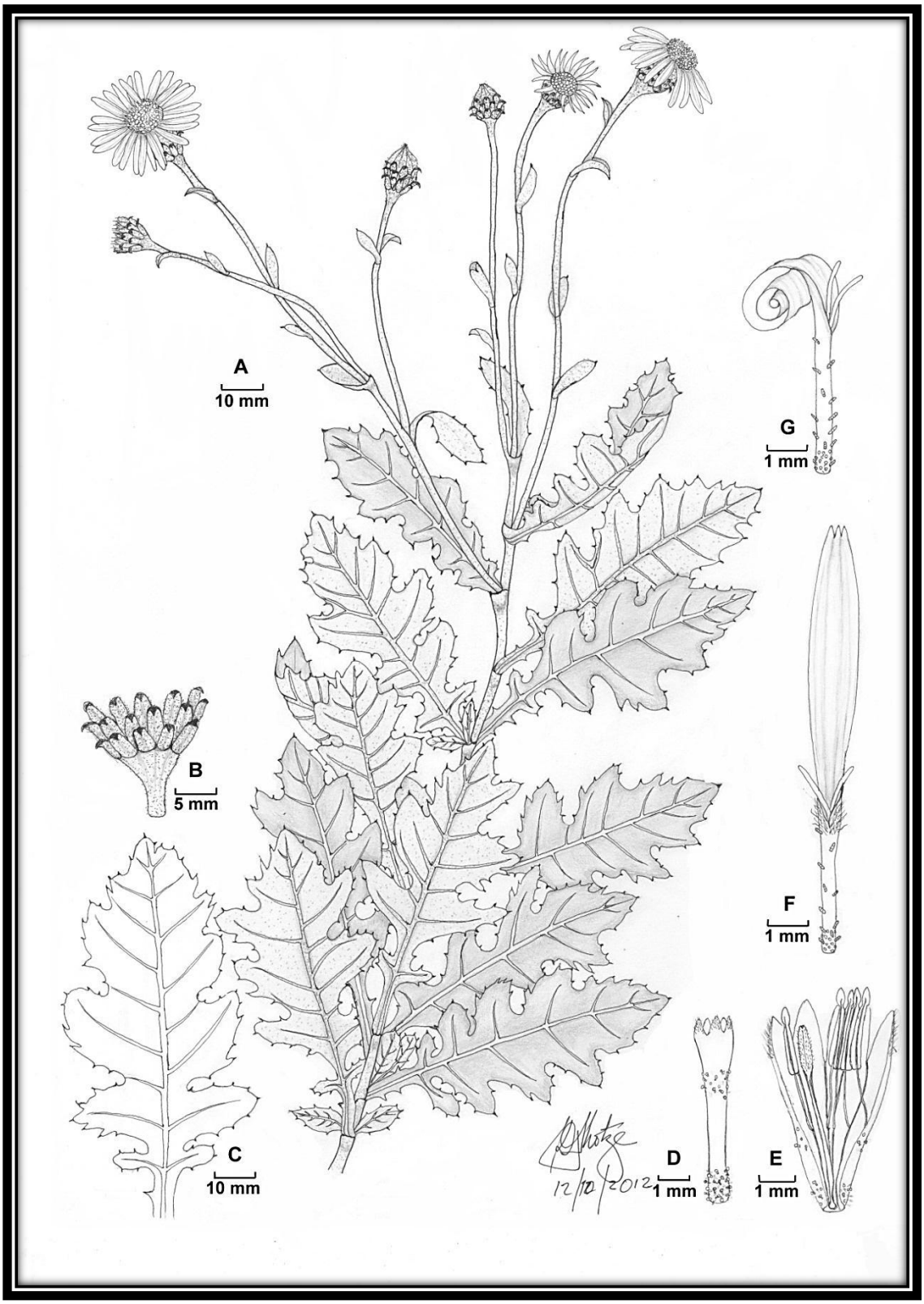

Figure 7.2.1 Illustration of *Garuleum album.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) disc floret; (E) dissected disc floret; (F–G) ray floret. Specimens: (A) Combination of *Wopula 153* (NH) and *Styles 3072* (NH), (B–C) *Wopula 153* (NH), (D–G) *Phillipson 4326* (GRA).

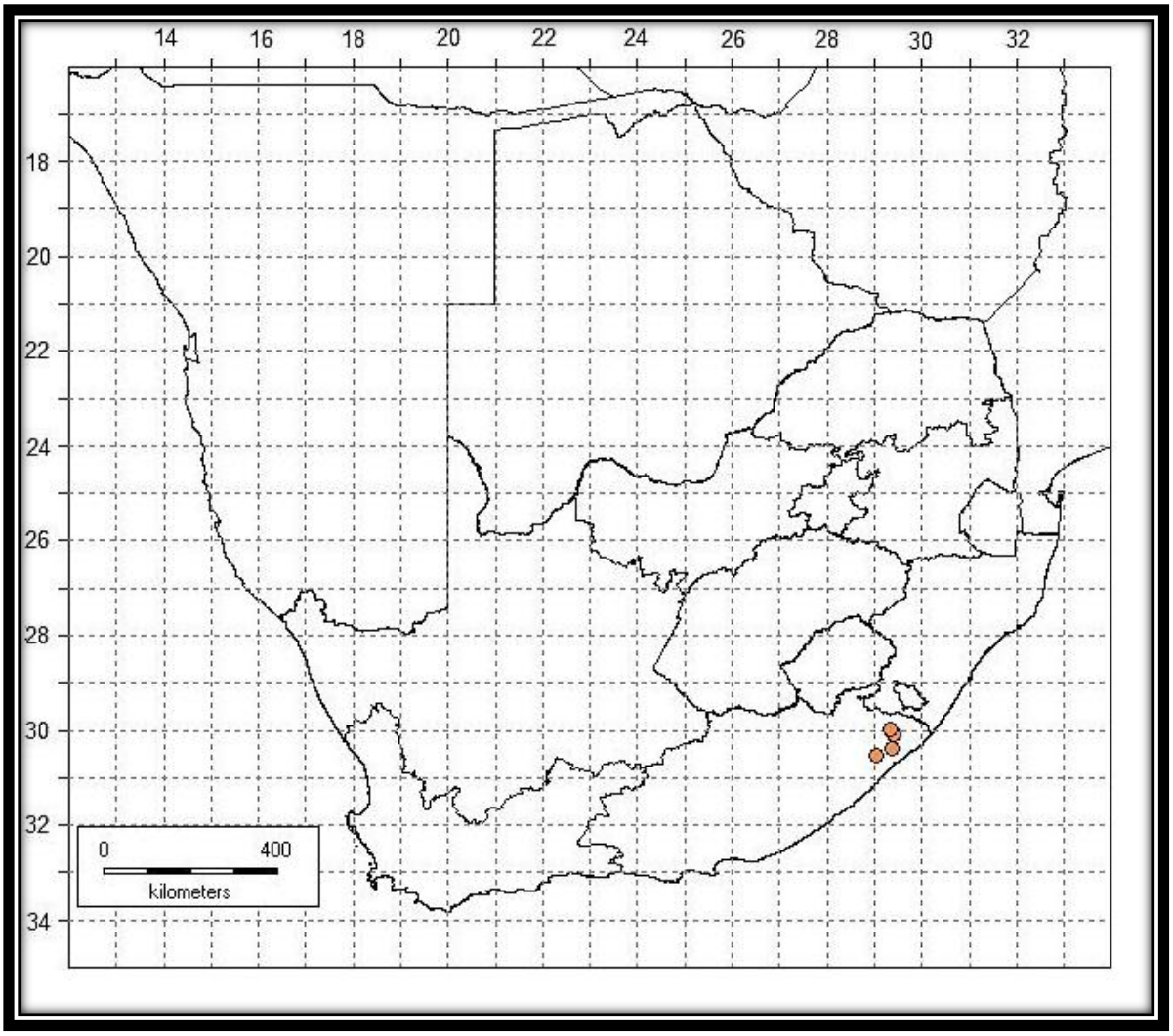

Figure 7.2.2 Known geographical distribution of *Garuleum album*.

Representative specimens

— 30º58'58'' S, 29º19'13'' E: South Africa, Eastern Cape, Tabankulu, Oala above road to Gomo forest, 15 March 1995, *Wopula, L.D.*, 153 (NH).

— 31º04'25'' S, 29º24'05'' E: South Africa, Eastern Cape, Tabankulu, on road from Tabankulu to Lusikisiki, 22 June 1995, *Phillipson, P.*, *Dold, T., Cloete, E. 4326* (GRA).

— 31º22'30'' S, 29º22'30'' E: South Africa, Eastern Cape, [between] Umtata [and] et St Johns, January 1896, *Bolus, H. BH 10160* (BOL).

— 31º32'30'' S, 29º02'72'' E: South Africa, Eastern Cape, [between] Umtata [and] et St Johns, Mlegana, 18 January 1910, *Pegler, A. 1569* (BOL).

— 31º32'30'' S, 29º02'72'' E: South Africa, Eastern Cape, Port St. John's, between Port St John's and Umtata, 9 June 1941, *Kannemeyer, D.V. s.n.* (NH).

— 31º32'30'' S, 29º02'72'' E: South Africa, Eastern Cape, Umtata, near Khoweni forest, Libode area, 28 April 2006, *Styles, D. 3072* (NH).

7.3.2 ***Garuleum bipinnatum*** (Thunb.) Less., Synopsis Generum Compositarum Earumque Dispositionis Novae Tentamen Monographiis Multarum Capensium Interjectis: 194 (1832) ; DC.: 309 (1836). Basionym: *Osteospermum bipinnatum* Thunb., Prodromus Plantarum Capensium: 167 (1800); 717 (1823); Baill.: 300 (1886). [Note1]

Type: South Africa Cape, Bonæ Spei, Hantam, *Thunberg s.n.* (UPS, lectotype).

= *Dimorphotheca multifida* DC., Prodromus Systematis Naturalis Regni Vegetabilis, 6: 73 (1838) (Synonymy according to Norlindh (1977)). [Note2]

Type: South Africa, Fisch River [Eastern Cape Province], *Drege 5120* (P, lectotype (P028626, scan!) here designated; P, isolectotype (P028627, scan!); G, isolectotype (scan!)). South Africa, Albany, *Ecklon and Zeyher s.n.* (G, syntype (scan!)).

An aromatic shrub, up to 1.5 m tall. Woody rootstock present. **Stem** base 30−150 mm wide; branching from base, glandular. **Leaves** sessile; bases semi-stem clasping; blades 18–45 x 6–28 mm, ovate, tri-pinnate, lobes awl-shaped; adaxial and abaxial surfaces semi-glandular, green; apices cassidate; stipules absent. **Inflorescences** capitula, 6−14 mm in diameter; peduncles 45−120 mm long, semi−velutinous; involucral bracts 4−8 x 1−2 mm, glandular, apices not recurved, arranged imbricately in a series of 3 rows surrounding capitula, outer and inner rows same size. **Ray floret** corolla mauve, purple or white; tube base glandular, ligulate; ligule 14−18 mm long; style 5−6 mm long, stylar lobes 1−2 mm long. **Disc floret** corolla 5−5.5 mm long, tube ±2.5 mm long, lobes 0.7 mm long, covered with glandular trigomes, concentrated at tube base and on corolla lobe apices. Stamens inserted 1−2.5 mm from corolla tube base, filaments ±1 mm long, anthers ±2 mm long. Pistil with style 4−5.5 mm long, stylar lobes ±1 mm long, glandular. **Achenes** develop from ray and disc florets; ray floret achenes light brown to beige, obovoid to obcordate, smooth surface ±3.4 x 2.2 mm; disc florets achenes light brown, obovoid to obcordate, surface folded, ±4.4 x 2.3 mm. Figure 7.3.1.

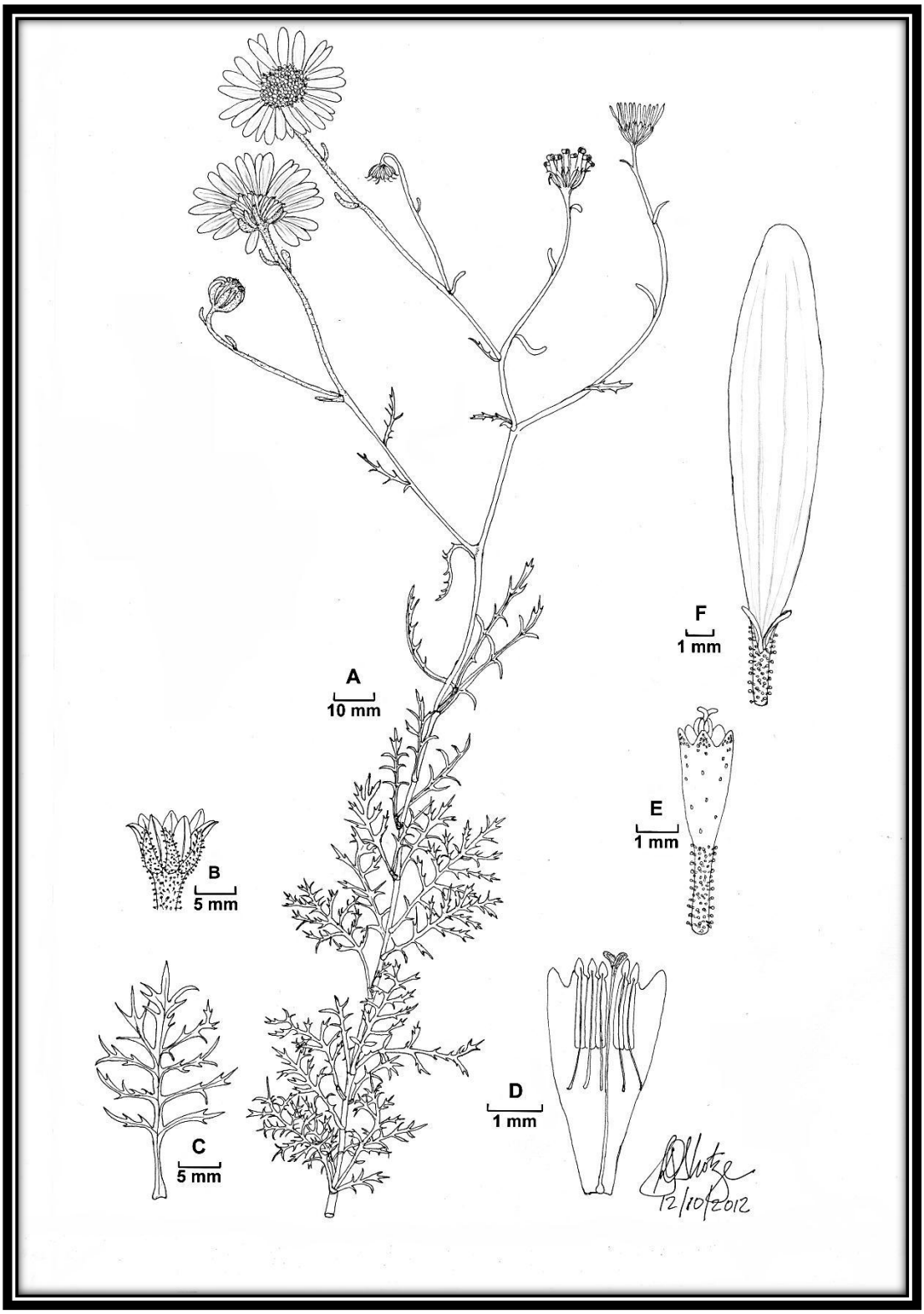

Figure 7.3.1 Illustration of *Garuleum bipinnatum.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) dissected disc floret; (E) disc floret; (F) ray floret. Specimens: (A) Combination of *Roux 4141* (NBG) and *Compton 11124* (BOL), (B–C) *Compton 11124* (BOL), (D–F) *Bayliss 4326* (NBG).

Notes

Note 1: No type specimens were designated in the publications of Thunberg (1800) or Lessing (1823), for *Osteospermum bipinnatum* and *Garuleum bipinnatum* respectively. Thunberg (1832) mentions a specimen in his publication of Flora Capensis, and since this is the first reference to a specimen, this is regarded as the holotype.

Note 2: Norlindh (1977) synonymised *Dimorphotheca multifida* with *Garuleum bipinnatum*, after thoroughly comparing the two species.

Diagnostic characters

Tri-pinnate leaves with awl-shaped lobes. Adaxial and abaxial leaf surfaces semi-glandular. Peduncles semi-velutinous. Bracts are glandular and 4−8 x 1−2 mm in size. Bract apices are not recurved. Both ray and disc florets produce achenes.

Distribution and Habitat

Garuleum bipinnatum is found in the Little Karoo, centred in the Western Cape and Eastern Cape with some specimens found in the southern parts of the Northern Cape. This species grows at altitudes ranging from 400−1350 m (Fig. 7.3.2). *Garuleum bipinnatum* grows in grazed lands, near road sides, on moderate slopes in full-sun. The species is associated with a wide variety of soils ranging from well-drained sand, sandy stony loam and clay to gravel, overlying dolerite and weathered ecca shale.

Vernacular names

Slanghoutjie, Gifhoutjie or Kowerbos in Afrikaans (Watt and Breyer-Brandwijk, 1932).

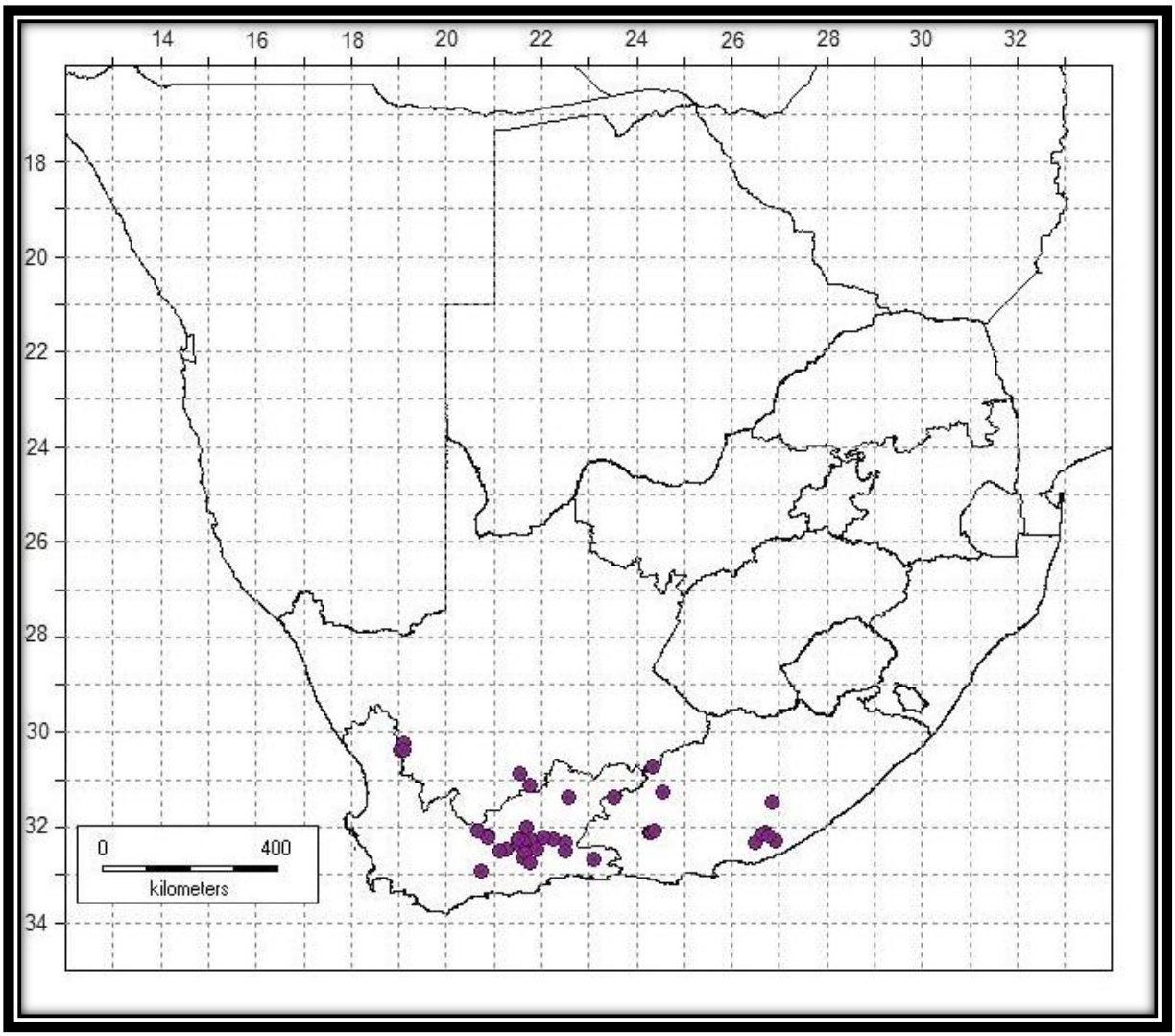

Figure 7.3.2 Known geographical distribution of *Garuleum bipinnatum*.

Representative specimens

—32º28'49" S, 26º50'70" E: South Africa, Eastern Cape, Grahamstown, between Fort Beaufort and Grahamstown, 18 July 2001, *Koekemoer, M. 2077* (PRE).

—31º15' S, 19º07' E: South Africa, Northern Cape, Nieuwoudville Reserve, dolorite kopies north end, 15 June 1983, *Perry & Snijman, D. 2119* (NBG).

—33º29' S, 22º30' E: South Africa, Western Cape, Oudtshoorn, Doornkraal, De Rust, 19 October 1970, *Dahlstrand, K.A. 9426* (NBG).

—33º04'12" S, 20º39'51" E: South Africa, Western Cape, Laingsburg, road to Sutherland Farm: Josephskraal, 30 August 2006, *Roux, J.P. 4141* (NBG).

—33º17'05" S, 21º37'16" E: South Africa, Western Cape, Gamkapoort Nature Reserve, W of Dwyka river, S slope of 2nd highest ridge, 6 September 1983, *Laidler, D.F. 650* (NBG).

— 33º07' S. 24º15' E: South Africa, Eastern Cape, Steytlerville, 3 October 1971, *Bayliss, R.D.A.*, 4889 (GRA)

7.3.3 ***Garuleum latifolium*** Harv., in Harv. and Sond., Flora Capensis, 3: 92 (1865); Zahlbr.: 312 (1910); Hilliard.: 516 (1977).[Note1]

Type: South Africa, Natal, Tongaat river, *Gerrard 167* (TCD, holotype; K, isotype (scan!)).

= *Osteospermum calendulaceum* Harv., Flora Capensis 3: 440 (1865), *nom illegit non* L.f. (1781). (synonymy according to Hilliard (1977)).

Type: South Africa, Natal, *Gueinzius s.n.* (UPS, holotype).

An aromatic shrub, up to 2 m tall. **Stem** base 30–70 mm wide, herbaceous, semi glandular. **Leaves** sessile; base clasping stem; blades 35–75 x 8–40 mm, broadly ovate, pinnatipartite; margins coarsely toothed; adaxial and abaxial surfaces glandular, green; apices setaceous; stipules absent. **Inflorescences** capitula, 6−15 mm wide; peduncles 90–111 mm long, semi-velutinous; involucral bracts 4−6 mm x 1–3 mm, semi-glandular and semi velutinous, apices not recurved, arranged imbricately in series of 3 rows surrounding capitula, outer and inner rows same size. **Ray floret** corolla mauve to purple, tube base densely glandular, tube apex villous, ligulate; ligule 9–12 mm long; style 5−6 mm long, stylar lobes 1−2 mm long. **Disc floret** corolla 4.5–5 mm long, tube 1.5–2 mm long, stylar lobes ±1.5 mm long, glandular trigomes cover corolla tube base and corolla lobe apices. Stamens inserted 1.5–2 mm from corolla tube base, filaments 1–1.5 mm long, anthers 2.5−3 mm long. Pistil with style 4–6 mm long, stylar lobes 1–2 mm long. **Achenes** develop from ray florets; brown, obovoid, margins slightly thickened, surface slightly folded with longitudinal ridges present along middle of achene, 4.3 x 1.6 mm. Figure 7.4.1.

Notes

Note 1: Harvey (1865) published both *Garuleum latifolium* Harv. and *Osteospermum calendulaceum* Harv. in Flora Capensis. Since *G. latifolium* appears first in this publication it is considered to be the accepted name, while *O. calendulaceum* Harv. is a later synonym. Since the name *O. calendulaceum* L.f. was already used by Linnaeus in a different context, the *O. calendulace*um Harv. is also a later homonym, and consequently an illegitimate name.

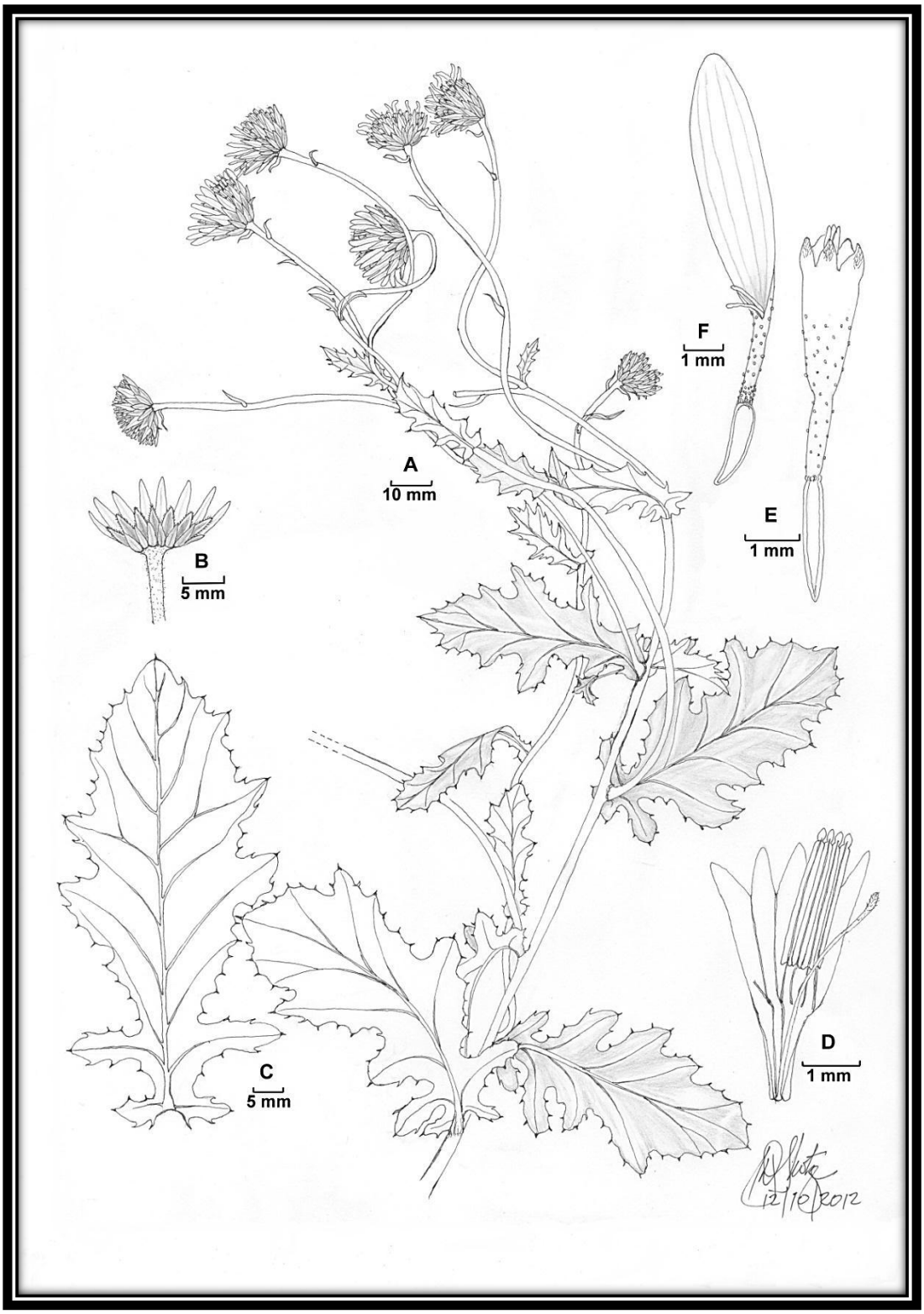

Figure 7.4.1 Illustration of *Garuleum latifolium.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) dissected disc floret; (E) disc floret; (F) ray floret. Specimens: (A) Combination of *Ward 12972* (GRA) and *Wood 299* (Z), (B–C) *Ward 12972* (GRA), (D–F) *Wood 299* (Z).

Diagnostic characters

Leaves are pinnatipartite. Adaxial and abaxial leaf surface are glandular green. All three rows of bracts are the same size 4–6 x 1–3 mm. Bracts are semi-glandular and semi-velutinous at their bases. Stipules are absent.

Distribution and habitat

Garuleum latifolium is found in Kwazulu-Natal and grows at altitudes of 540–1200 m (Fig. 7.4.2). This species is localized in thicket margins and in woodlands, in partial shade and on a gentle slope. The substrate in which *G. latifolium* grows is sandy loam and organic matter, where there is Natal Group sandstone.

Representative specimens

—29º30' S, 30º30' E: South Africa, Kwazulu-Natal, Pietermaritzburg, Camperdown, 11 February 1908, *Franks, M*. *10860* (NH).

—29º46' S, 30º39' E: South Africa, Kwazulu-Natal, Durban, Hammersdale area. Hector eskom substation site, 29 March 1995, *Ward, C.J. 12972* (NH).

— Coordinates unknown: South Africa, Zululand Mkuze, August 1932, *Galpin, E.E. 21496* (BOL).

—Coordinates unknown: South Africa, Natal, February 1884, Wood, J. M. 160 (BOL).

— Coordinates unknown: South Africa, Kwazulu-Natal, Alexandra district, 22 January 1913, *Rudatis, A.G.H. 1864* (L).

— Coordinates unknown: South Africa, Kwazulu-Natal, Nkandla, Mahlabatini, April 1933, *Gerstner, J. s.n.* (NH).

— Coordinates unknown: South Africa, Kwazulu-Natal, April 1884, *Wood, J.M.*, 299 (Z).

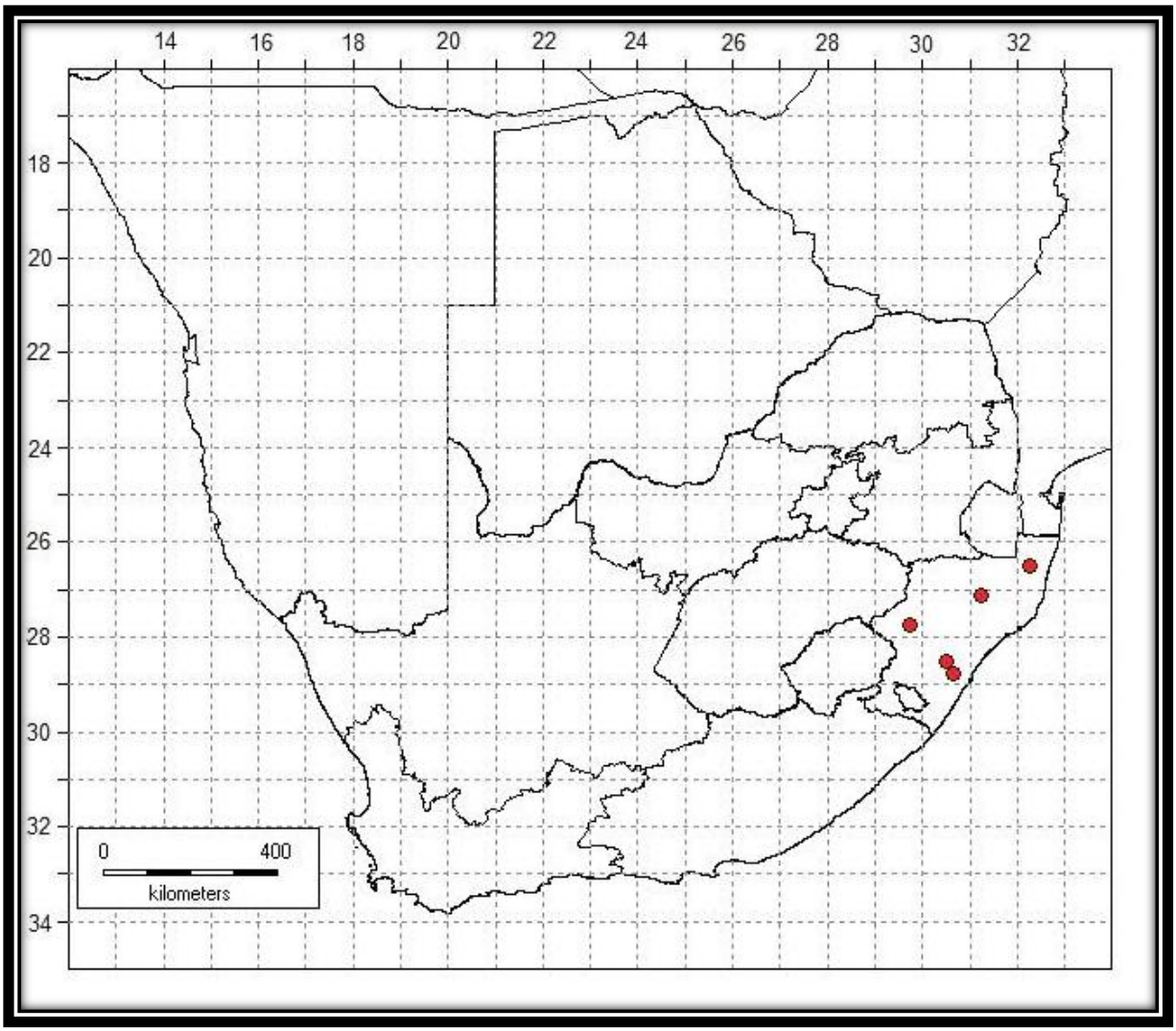

Figure 7.4.2 Known geographical distribution of *Garuleum latifolium*.

7.3.4 ***Garuleum pinnatifidum*** (L'Hér.) DC., Prodromus Systematis Naturalis Regni Vegetabilis 5: 309 (1836); DC: 468 (1837). Basionym: *Osteospermum pinnatifidum* L'Hér., Stirpes novae aut minus cognitae: 11 (1785); Thunb.: 167 (1800), 717 (1823).

Type: L'Heritier, Plate VI (1836), (iconotype specimen, here designated).

= *Garuleum viscosum* Cass. in Plassan, Bulletin des Sciences la Société Philomatique Paris 172 (1819); et in Dict. Sc. Nat. 18. 163 (1819c) *nom. illegit*. [Note1] (Synonymy according to De Candolle (1836) and implied by Cassini in 1819).

No type designated by authors.

= *Chrysanthemoides caerulea* (Jacq.) Moench., Methodus Plantas Horti Botanici et Agri Marburgensis: 584 (1794). **= *Garuleum caeruleum*** (Jacq.) Aiton. in Steudel, Nomenclator Botanicus, seu: Synonymia Plantarum Universalis, eunumerans, Ordine Alphabetico Nomina Atque Synonyma 2: 236 (1841). Basionym: *Osteospermum caeruleum* Jacq., Collectaneae ad botanicum Miscellanea, I: 78 (1787). (Synonymy according to De Candolle (1836)).

Type: Jacquin, (1787), (iconotype specimen)

An aromatic shrub, up to 1 m tall. **Stem** base 20–40mm wide, minutely velutinous and glandular. **Leaves** sessile; base clasping stem, blades 22–50 x 10–30 mm, broadly ovate, bipinnate, margins toothed, lobes blunt serratures; adaxial and abaxial surfaces minutely velutinous and glandular, green; apices broadly acuminate; stipules absent. **Inflorescences** capitula, 4–12 mm wide; peduncles 50–130 mm long, glandular; involucral bracts 3–6 x 1–3 mm, semi-glandular, apices not recurved, arranged imbricately in series of 3 rows surrounding capitula, outer and inner rows same size. **Ray floret** corolla mauve, purple to white; tube evenly glandular, ligulate; ligule 9–12 mm long; style 2.8–4.5 mm long, stylar lobes 0.8–1.5 mm long.

Disc floret corolla 4–5 mm long, tube base 1.5−2 mm long, stylar lobes ±1 mm long, glandular trigomes cover the base and the corolla lobe apices. Stamens inserted 1–1.5 mm from corolla tube base, filaments ±1 mm long, anthers 2–2.5 mm long. Pistil with style 4–4.5 long, stylar lobes ±1 mm long, glandular. **Achenes** develop from ray florets; beige, brown to obovoid, surfaces folded or grooved with raised longitudinal ridges, 3.55 x 1.5 mm. Figure 7.5.1.

Notes

Note 1: Cassini (1819c) transferred *O. pinnatifidum* to *G. viscosum*, this was *nom. illegit*, because the specific epithet should not have been changed by Cassini. The correct name should have been *G. pinnatifidum*.

Diagnostic characters

Leaves are bipinnate and have blunt serratures on leaf margins. The adaxial and abaxial leaf surfaces are glandular, green. The bracts are semi-glandular. Apices are not recurved.

Distribution and habitat

Garuleum pinnatifidum is found in the Free State and the Eastern Cape, with some specimens found in Mpumalanga and Western Cape. This species grows at altitudes ranging from 790–1524 m (Fig. 7.5.2). *Garuleum pinnatifidum* grows in full-sun or in semi-shade on slopes. It is associated with well-drained or moist sandy rocky soil, loam and weathered shale. This species grows under trees or shrubs including *Lycium* L. and *Olea europaea* L. subsp*. africana* (Mill.) P.S. Green.

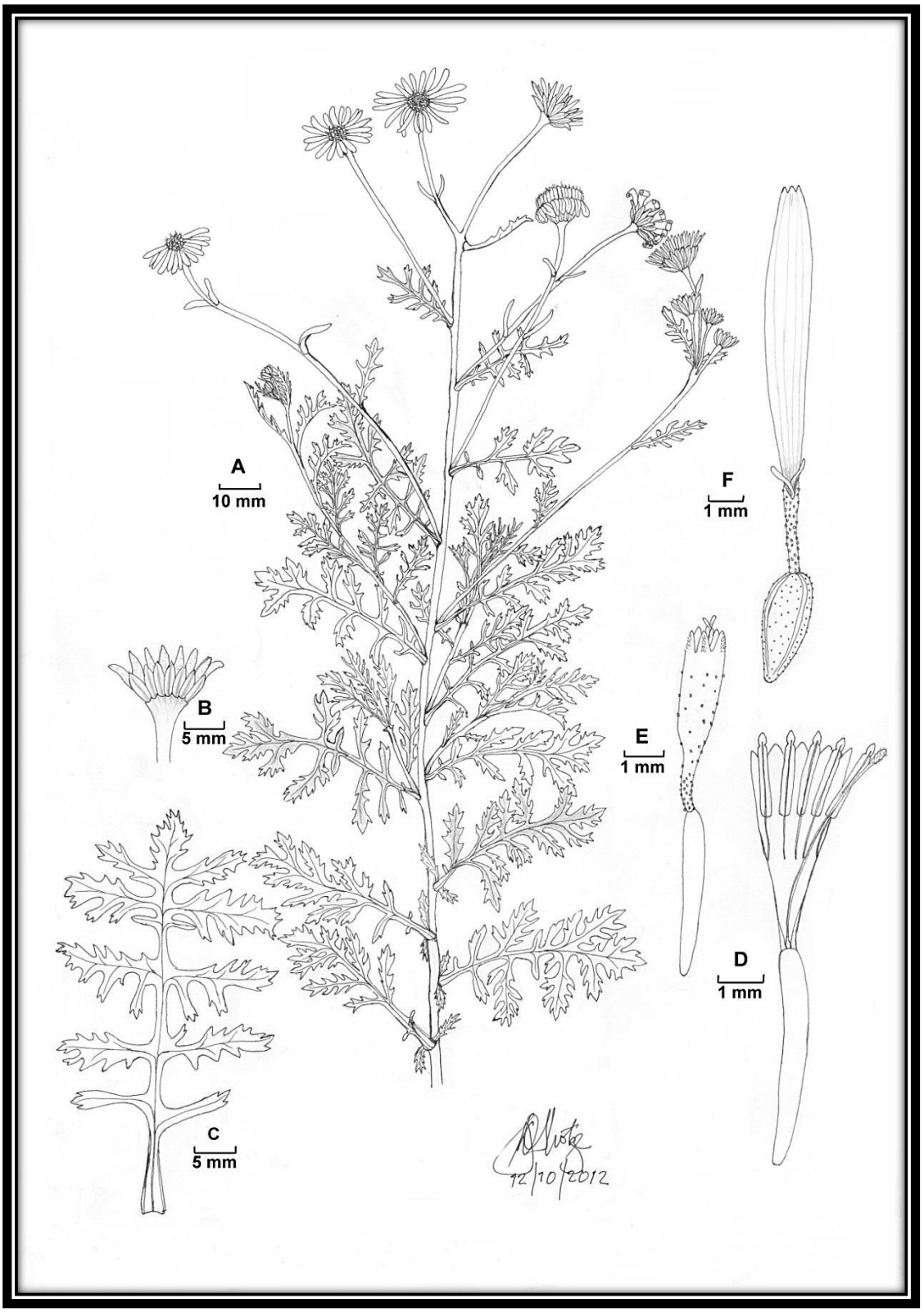

Figure 7.5.1 Illustration of *Garuleum pinnatifidum.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) dissected disc floret; (E) disc floret; (F) ray floret. Specimens: (A) combination of *Rossouw 61* (BLFU) and *Müller 285* (NBG), (B–C) *Rossouw 61* (BLFU), (D–F*) Potts 500* (BLFU).

Representative specimens

—27º25'00" S, 29º53'01" E: South Africa, Kwazulu-Natal, Charlestown, near Charles Town Drakensberg, 10 January 1894, *Wood, J.M. 5185* (BOL).

—28º16'84" S, 26º12'02" E: South Africa, Free State, Bloemfontein, U.F.S. botanical gardens, 3 March 2011, *Van Zyl, J. 1* (BLFU).

—28º16'84" S, 29º12'02" E: South Africa, Free State, Bloemfontein, U.F.S. botanical gardens, 28 May 1968, *Müller, D.B.* 285 (NBG).

— 28º45' S, 26º17' E: South Africa, Free State, Glen, Near Modderriver, 22 November 1979, Rossouw, R., 61 (BLFU).

—29º01'40" S, 26º02'24" E: South Africa, Free State, Bloemfontein, Poundisford on kopie [hill], December 1915, *Potts, G*. *500* (BLFU).

—32º06'19" S, 24º25'49" E: South Africa, Eastern Cape, Graaff-Reinet, Oudeberg, 30 March 1866, *Bolus, H. BH 4* (BOL).

—32º04'33" S, 25º34'48" E: South Africa, Eastern Cape, Somerset East, Visch rivier [Fisch river], June, *McOwan, P. 1633* (Z).

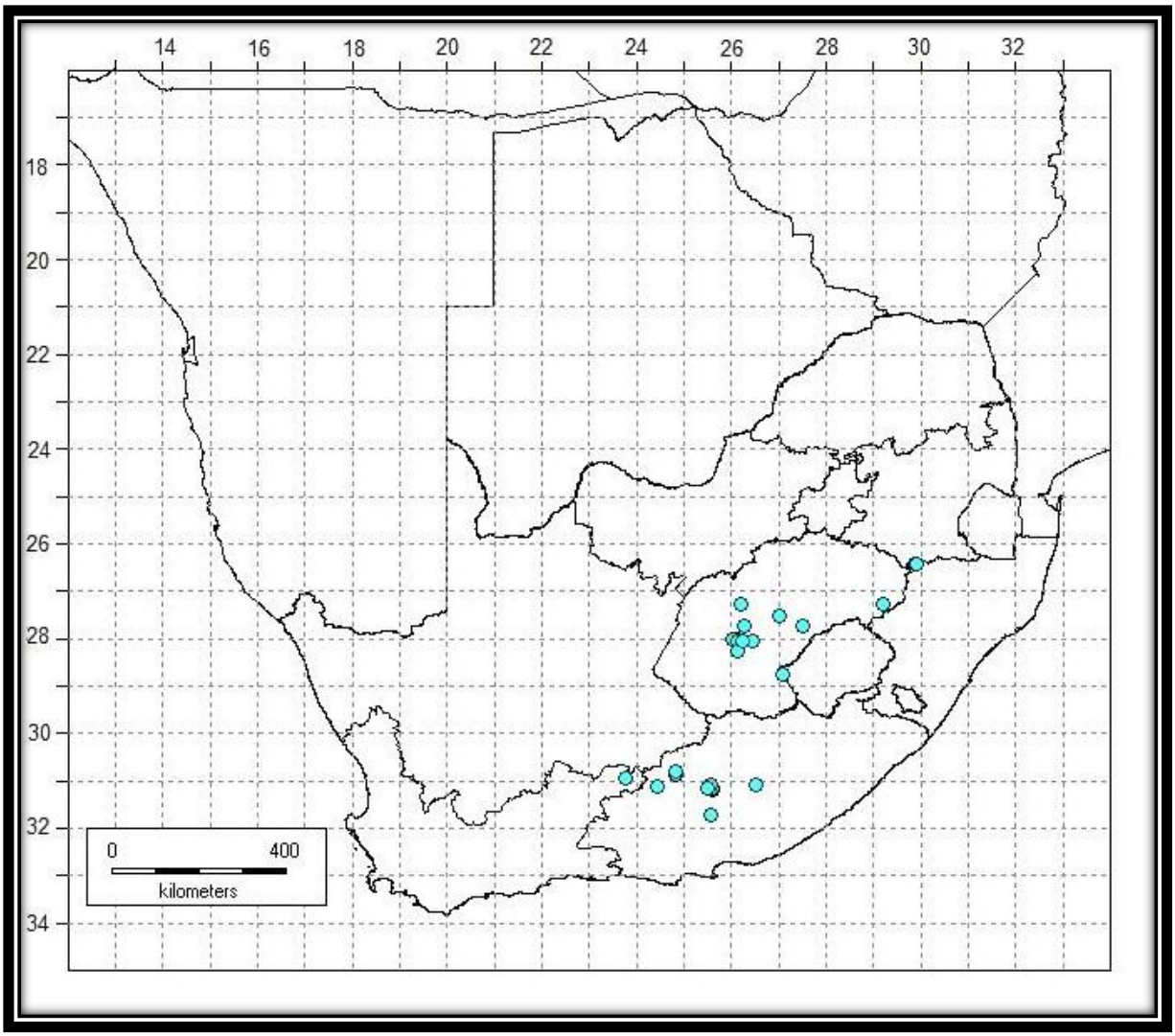

Figure 7.5.2 Known geographical distribution of *Garuleum pinnatifidum*.

7.3.5 ***Garuleum schinzii*** O.Hoffm. in Autran., Bulletin de l'Herbier Boissier 1: 74 (1893); München: 157 (1950), 73 (1984).

Type: Namibia, Tiras, *Schinz 698* (B, lectotype; Z!, isolectotype). Namibia, Auasberge, *Dinter 4645* (HBG, syntype (scan!); B, HUH, K, Z, isosyntype (scans!)).

= *Garuleum schinzii* subsp*. schinzii* Prodromus: einer flora von Südwestafrika 139: 71 (1967). (Synonymy here designated).

Type: Namibia, Tiras, *Schinz, 698* (B, holotype; Z!, isotype) here designated.

= *Garuleum schinzii* subsp*. crinitum* (Dinter) Merxm., Prodromus: einer flora von Südwestafrika 139: 71 (1967). Basionym**:** *Garuleum crinitum* Dinter., in Feddes Repertorium 30: 184 (1932). (Synonymy here designated).

Type: Namibia, Auasberge, *Dinter 4645* (HBG, holotype (scan!); B, HUH, K, Z, isotype (scans!)).

= *Garuleum bipinnatum* Dinter., Feddes Repertorium 17: 308 (1921). *nom. illeg*.[Note1]

No type specimen ever designated.

An aromatic shrub, growing up to 1 m tall. **Stem** base 20–50 mm wide; herbaceous, velutinous and glandular. **Leaves** sessile; base clasping stem; blades 40–90 x 10–40 mm, broadly ovate, bipinnate, adaxial and abaxial surfaces glandular, green; margins pectinate; apices simple; stipules present. **Inflorescences** capitula, 8–13 mm wide; peduncles 50–130 mm long, glandular; involucral bracts 5–9 x 1–1.5 mm wide, arranged imbricately in series of 3 rows surrounding capitula, outer and inner rows same size. **Ray floret** corolla mauve, purple to white; tube base and apex densely glandular, ligulate; ligule 11–12 mm long; style 4–4.5 mm long, stylar lobes ±1 mm long, glandular and velitinous. **Disc floret** corolla 4–5 mm long, tube 1.5–2 mm, stylar lobes ±0.5 mm, trigomes cover the base, lobe apices semi-velutinous. Stamens inserted 1.5–2 mm from corolla tube base, filaments ±1 mm long, anthers ±2 mm long. Pistil with style 4–5 mm long, stylar lobes ±1 mm long. **Achenes** develop from ray florets; light brown, cylindrical, surface is folded and grooved with raised ridges, pubescent or glandular, ±3.1 x 1.2 mm. Figure 7.6.1.

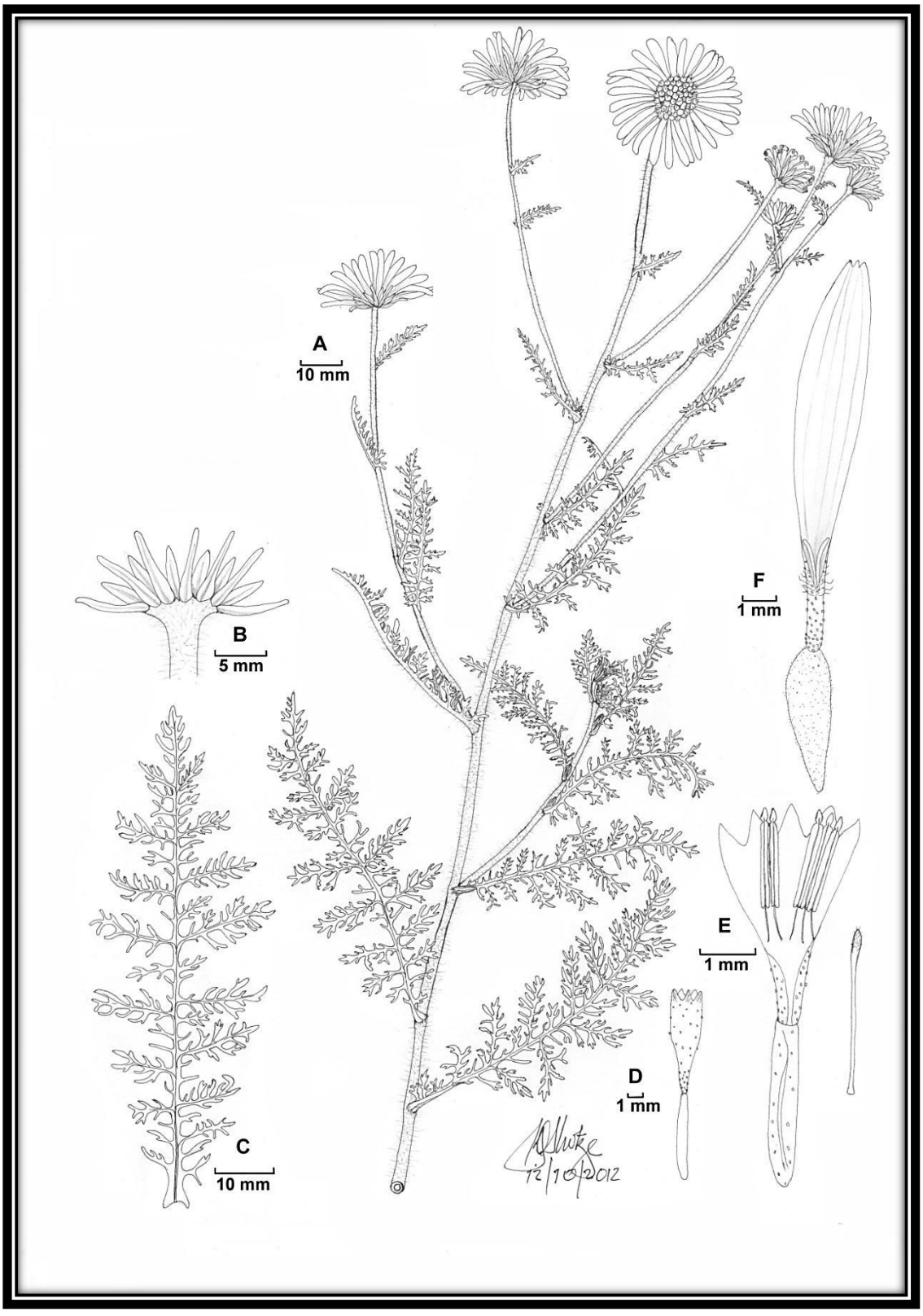

Figure 7.6.1 Illustration of *Garuleum schinzii.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) dissected disc floret; (E) disc floret, (F) ray floret. Specimens: (A) combination of *Mannheimer 2882* (GRA) and *Littlewood s.n.* (NBG). (B–F) *Mannheimer 2882* (GRA).

Notes

Note 1: Dinter's (1921) name, *Garuleum bipinnatium,* is a *nom. illegit* because this name was already published in a different context by Lessing (1832).

Note 2: The subspecies status of *G. schinzii* subsp. *schinzii* and *G. schinzii* subsp. *crinitum*, is no longer recognised. This is because their separation was based on a difference in ray floret colour and this phenomenon occurs in other species of *Garuleum* and is thus not sufficient for separation of subspecies. The two subspecies are not separated geographically and thus cannot retain their subspecies status (Fig. 7.6.2). *Garuleum schinzii* subsp*. schinzii* occured in the Northern Cape and Namibia while *Garuleum schinzii subsp. crinitum* was restricted to Namibia.

Diagnostic characters

Leaves fine and bipinnate. Leaf margins are pectinate (entire). Stems and bracts glandular, bracts 5–9 x 1–1.5 mm. Bract apices not recurved. Stipules present.

Distribution and habitat

Garuleum schinzii is found in the Northern Cape and Namibia (Fig. 7.6.2). This species grows at altitudes ranging from 1400−1716 m, in well-drained soil sandy to rocky loam, and weathered granite. *Garuleum schinzii* grows on the foot hills and rocky slopes of granite koppies. It is associated with *Acacia mellifera* Benth. in Highland savannah, often growing near roadsides in semi-shade.[Note2]

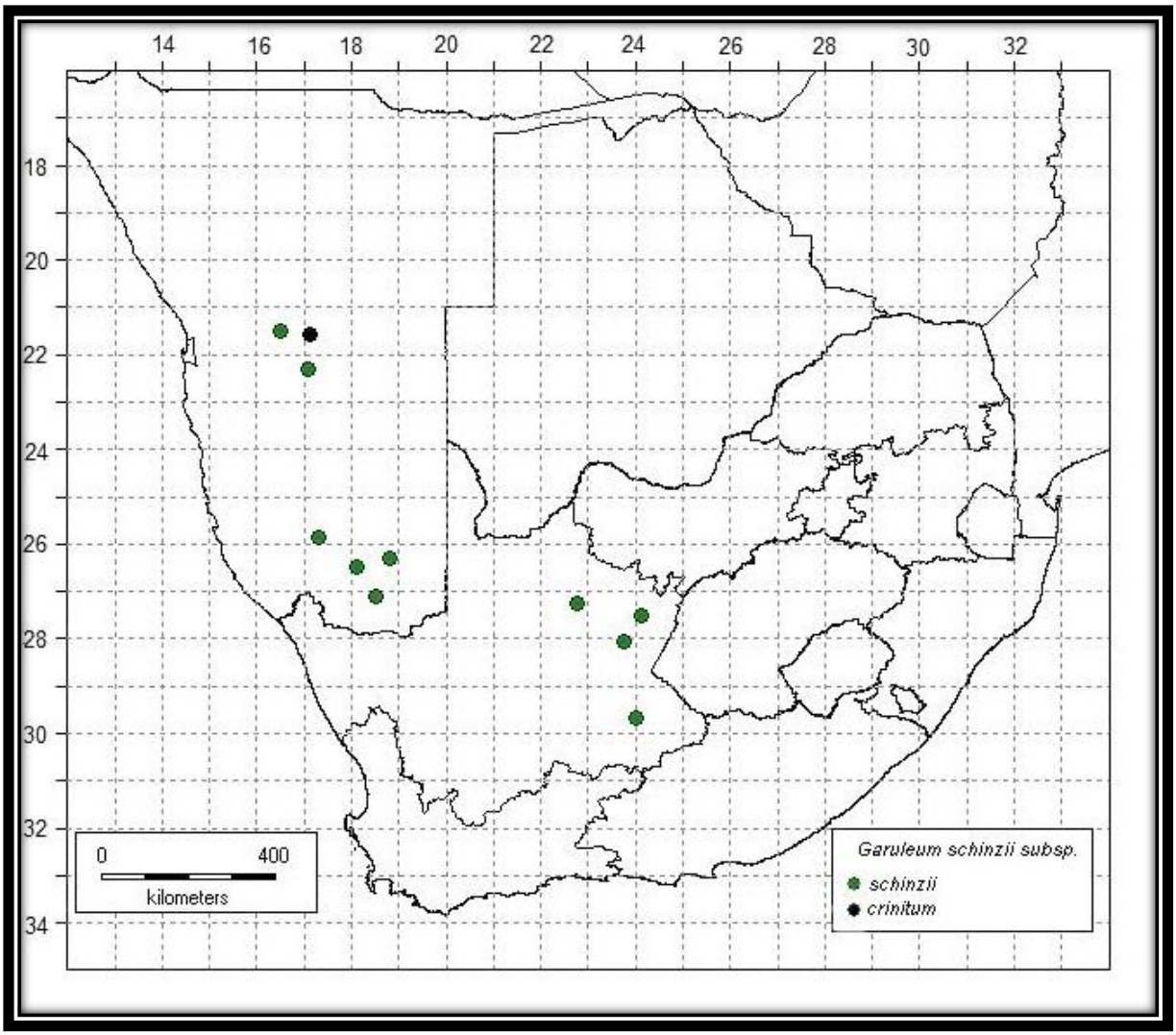

Figure 7.6.2 Known geographical distribution of *Garuleum schinzii*.

Representative specimens

—22º34'47" S, 17º07'37" E: Namibia, Khomas, Avis Dam area, 15 February 2004, *Mannheimer, C.A. 2882* (GRA).

—26º50'34" S, 17º17'25" E: Namibia, Karas, 2 August 1925, *Dinter, K. 4810* (Z).

— 27º30' S, 18º07' E: Namibia, Grunau, Grabwasser, May 1961, Littlewood, R.C., s.n. (NBG).

—28º07' S, 18º30' E: Namibia, Keetmanshoop, 85 km N of Karasburg, 8 July 1954, *Schelpe, A.S.L. 149* (BOL).

—28º15' S, 22º45' E: South Africa, Northern Cape, Glen Lyon, Floradale, April 1940, *Esterhuysen, E. 2325* (BOL).

—28º30' S, 24º07' E: South Africa, Northern Cape, Kimberley, Schmidts Drift, March 1938, *Wilman, M. 5369* (BOL).

—30º39'33" S, 24º00'46" E: South Africa, Northern Cape, De Aar, 152 km from De Aar, 1928, *Pole-Evans, I.B. 2310* (BOL).

7.3.6 ***Garuleum sonchifolium*** (DC.) Norl., Studies in the Calenduleae 1: 425 (1943). Basionym: *Osteospermum sonchifolium* DC., Prodromus Systematis Naturalis Regni Vegetabilis 6: 465 (1838); Hilliard: 517 (1977).

Type: South Africa, between Omtata and Omsamvubo (Port. St. John's District), Eastern Cape, *Drège s.n.* (G, holotype (scan!); MO, isotype (scan!)).

An aromatic shrub, up to 2 m tall. **Stem** base 30–50 mm wide; herbaceouse glandular and pubescent. **Leaves** sessile; base clasping stem, blades 35–75 x 13–25 mm, broadly ovate, pinnatipartite; margins coarsely toothed; adaxial and abaxial surfaces glandular and pubescent, green; apices setaceous; stipules present. **Inflorescences** capitula, 7–12 mm wide, peduncles 90–111 mm long, semi−velutinous; glandular; involucral bracts 5–10 mm x 3–4 mm, outer row longer and narrower than the two inner rows, semi-glandular, apices not recurved, arranged imbricately in series of 3 rows surrounding the capitula. **Ray floret** corolla mauve to purple, tube glandular and villous, trichomes concentrated around tube base and apex, ligulate; ligule 12–13 mm long; style 4.5–6 mm long, stylar lobes ±1.5 mm long. **Disc floret** corolla 5–6 mm long, tube ±2 mm long, stylar lobes ±1.5 mm long, trichomes concentrated at tube base and lobe apices. Stamens inserted 1−1.5 mm from corolla tube base, filaments ±1.5 mm long, anthers 2.5–3 mm long. Pistil with style 4.5–7 mm long, stylar lobes 1–2 mm long. **Achenes** develop from ray florets; brown, obovoid, margins slightly thickened, surfaces warty, ±5.1 x 2.3 mm. Figure 7.7.1.

Diagnostic characters

Leaves pinnatipartite, margins coarsely toothed. Abaxial leaf surfaces without velutinous cover. Involucre of bracts with the outer row longer and wider than the bracts in the inner two rows, 5–10 mm x 3–4 mm. Stipules are present.

Distribution and habitat

Garuleum sonchifolium is found in Kwazulu-Natal and the Eastern Cape. It grows at altitudes ranging from 1000–1860 m (Fig. 7.7.2). This species grows in forest margins and in open places near roadsides.

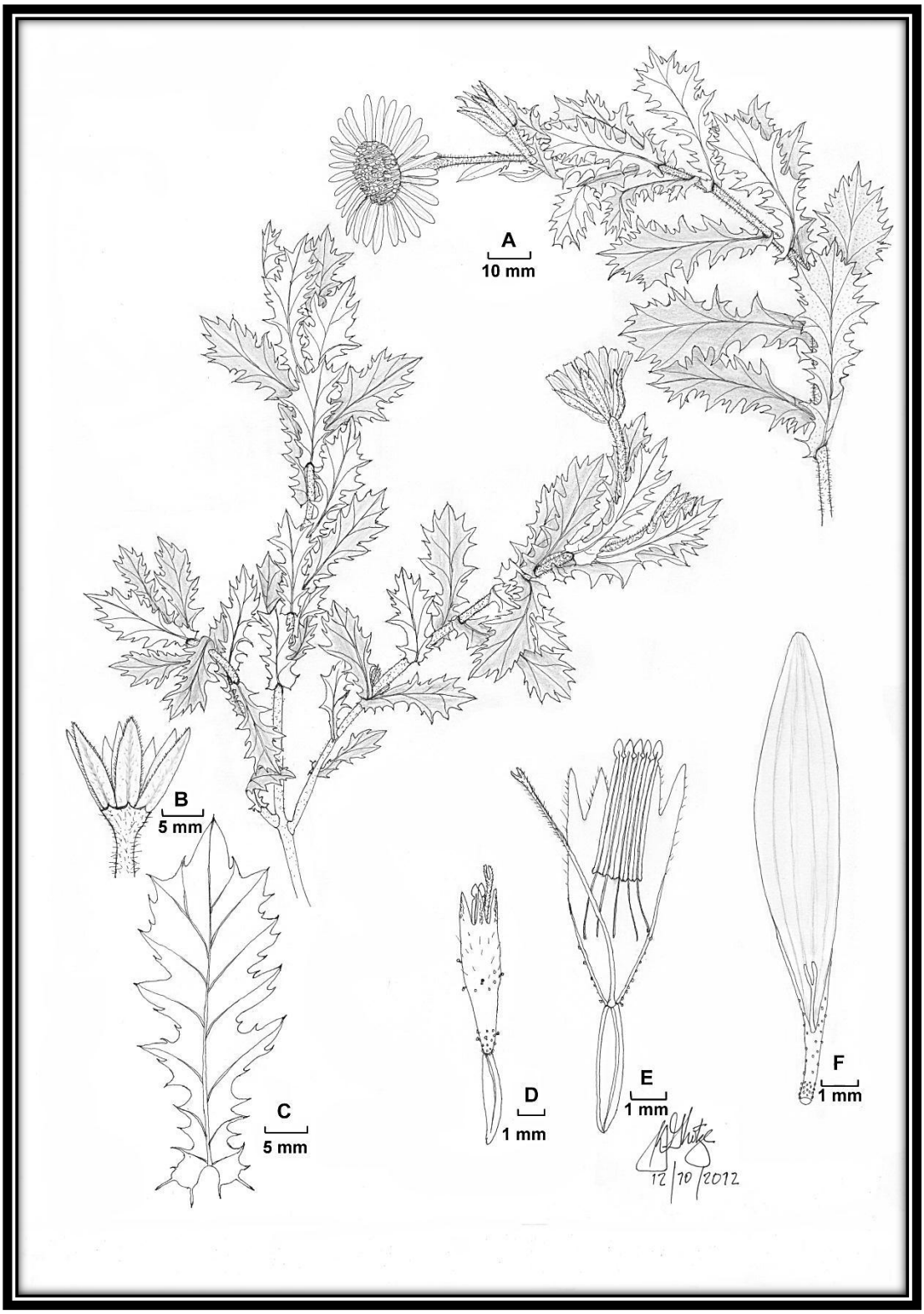

Figure 7.7.1 Illustration of *Garuleum sonchifolium.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) dissected disc floret; (E) disc floret; (F) ray floret. Specimens: (A) combination of *Batten 1 Plate 54* (NBG) and *MacOwan 2015* (GRA) (B–C) *Batten 1 Plate 54* (NBG); (D–F) *Schlechter 6835* (Z).

Vernacular names

Udwetya in Xhosa. Recorded on specimen label collected by *Van Eeden B369* (PRE).

Voucher specimens

—28º43'50" S, 29º21'04" E: South Africa, Free State, Bergville, Mnweni area Drakensberg slopes, N aspect, July 1958, *Esterhuysen, E. 27845* (BOL).

—29º07' S, 29º45' E: South Africa, Kwazulu-Natal, 15 km S of Estcourt at Glenbella, 26 March 1988, *Green, D.* 521 (NH).

—29º12'38"S, 30º00'10"E: South Africa, Kwazulu-Natal, Mooi River, 1895, *Schlechter, R.* 6835 (L).

—30º01'49" S, 29º36'12" E: South Africa, Eastern Cape, Malowe, Griqualand East, February 1885, *Tyson, W.* 1047 (Z).

—32º46'10" S, 27º16'00" E: South Africa, Eastern Cape, King William's town, Isedenga forest, 9 March 1964, *Balten, A.U.* 1 Plate 54 (NBG).

—33º15' S, 25º15' E: South Africa, Eastern Cape, Weza, Zuurberg, 3 March 1974, *Hillard, O. M.* 5465 (NBG).

—Coordinates unknown: South Africa, Buffelsberg, November, MacOwan, P., 2015 (GRA), (NH), (Z-000061155, Z-000078517).

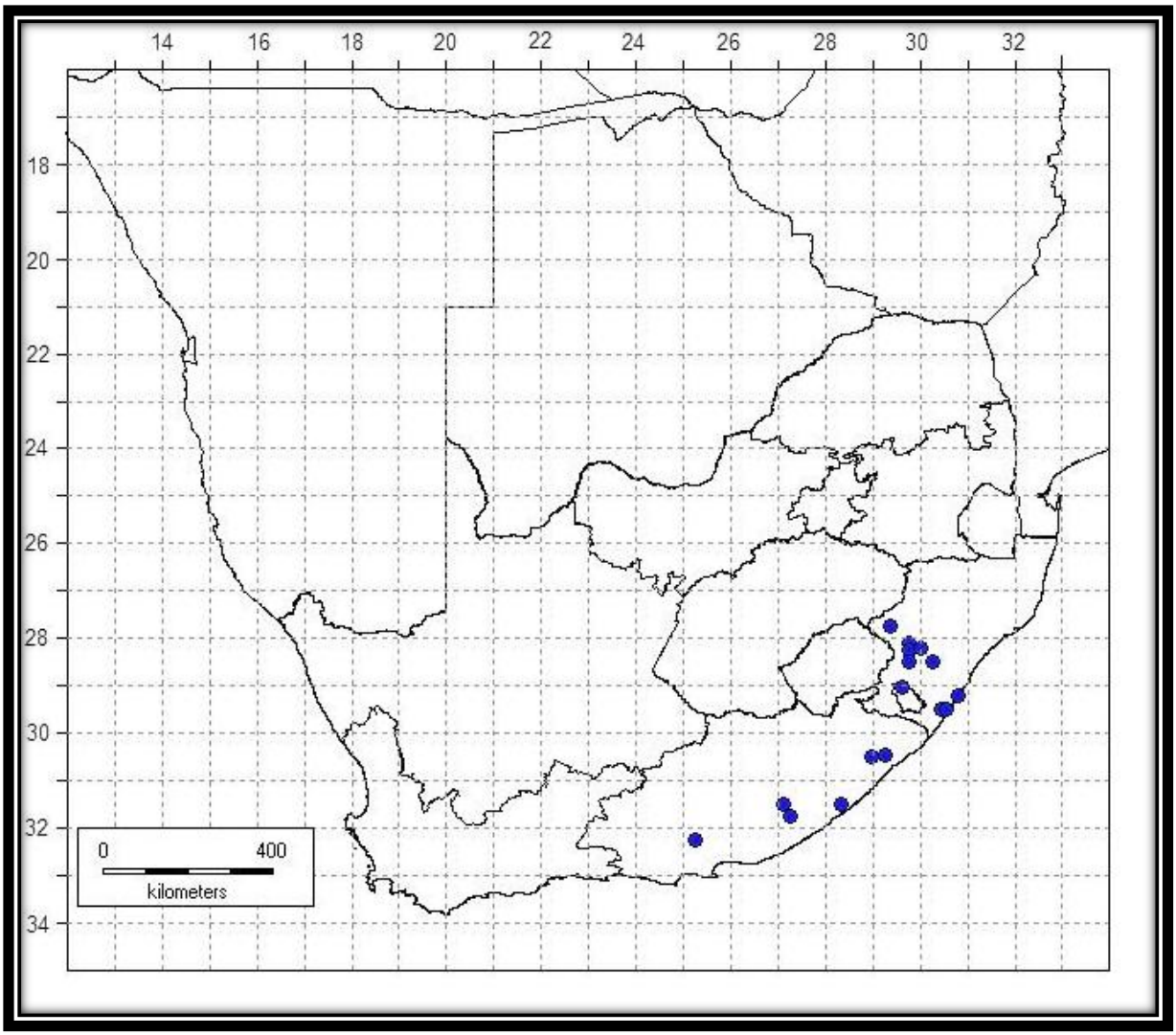

Figure 7.7.2 Known geographical distribution of *Garuleum sonchifolium*.

7.3.7 ***Garuleum tanacetifolium*** (MacOwan) Norl., Studies in the Calenduleae 1: 425 (1943). Basionym: *Osteospermum tanacetifolium* MacOwan in Hooker's Icones Plantarum 19: t. 1839 (1889).

Type: South Africa, Somerset East, Boschberg, *MacOwan 1382* (NYBG, holotype (scan!); GRA, isotype (scan!), BOL, isotype, K, isotype (K000273573 , K000273574, scan!)).

An aromatic shrub, up to 2 m tall. **Stem** base 30–60 mm wide, herbaceous, scabrous to pubescent. **Leaves** densely arranged, sessile; base clasping stem; blades 22–30 x 10–12 mm, broadly ovate, tri−pinnate; margins acutely incised; adaxial and abaxial surfaces scabrous to pubescent, green; apices setaceous; stipules present. **Inflorescences** capitula, 10–12 mm wide; peduncles absent; involucral bracts 5–10 x 3–4 mm, semi-glandular, apices not recurved, arranged imbricately in series of 3 rows surrounding the capitula, outer and inner rows same size. **Ray floret** corolla mauve, purple to white; tube base densely glandular, apex villous, ligulate; ligule ±12 mm long; style ±5 mm long, stylar lobes ±1 mm long. **Disc floret** corolla ±4.5 mm long, tube ±1.5 mm, lobes ±1 mm, recurved, trichomes cover corolla evenly. Stamens inserted ±1.5 mm from corolla tube base, filaments ±1.5 mm long, anthers ±2.5 mm long. Pistil with style 4.5–7 mm long, stylar lobes 1–2 mm long, glandular to villous. **Achenes** develop from ray florets; light brown, oblong, margins slightly thickened, surface warty, ±5.2 x 2.2 mm. Figure 7.8.1.

Diagnostic characters

Leaves are tri-pinnate from distal half of leaf, densely arranged, alternating, margins acutely inscised, stipules present. Adaxial and abaxial surface is scabrous and pubescent, green. Involucre bracts are glandular and 5–10 x 3–4 mm.

Distribution and habitat

Garuleum tanacetifolium is found in the Eastern Cape at altitudes ranging from 1675–1731 m (Fig. 7.8.2). This species grows on mountain slopes on the E or NE facing slopes in well-drained rocky soil or weathered shale. Associated species include *Helichrysum* Mill. sp.

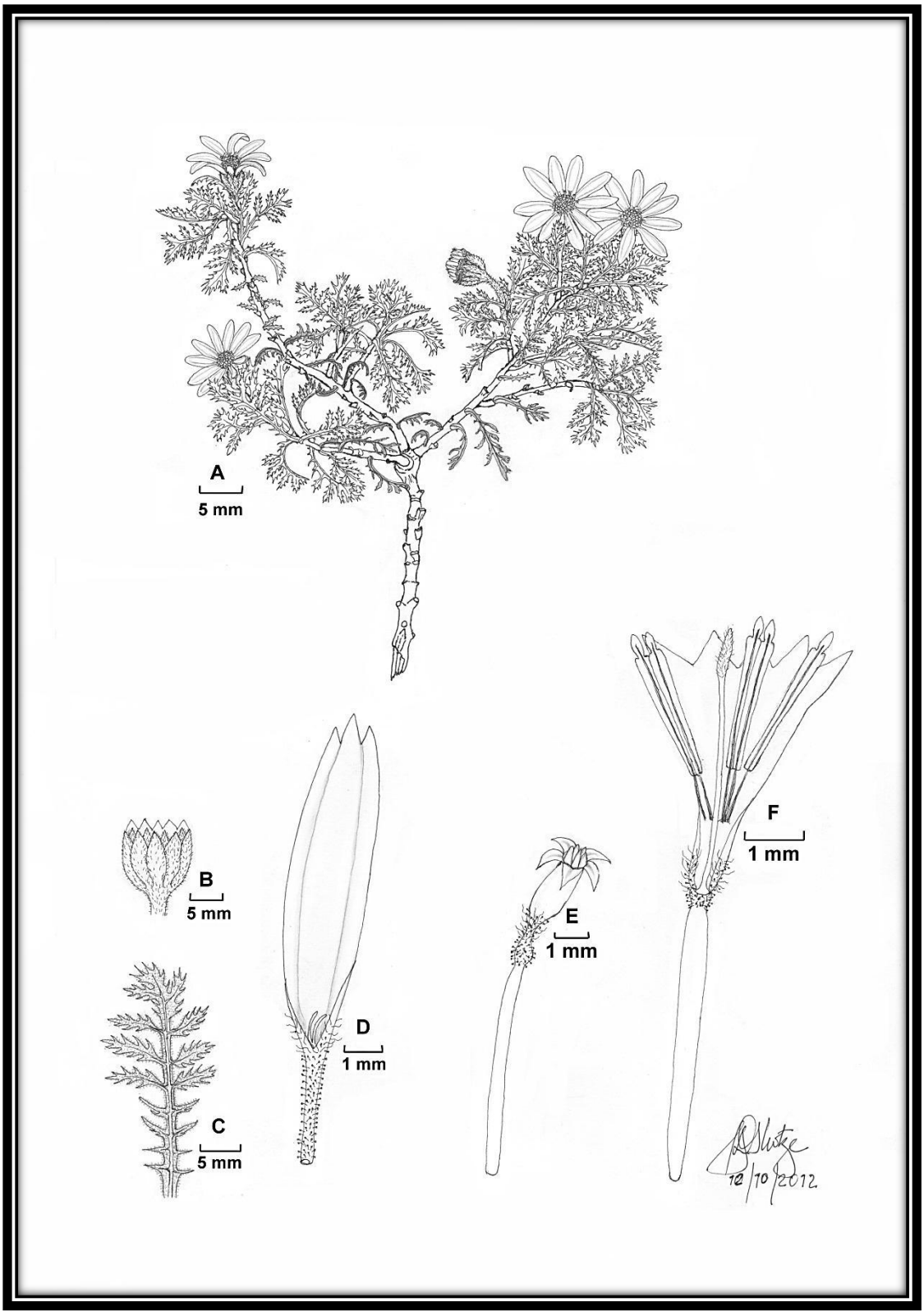

Figure 7.8.1 Illustration of *Garuleum tanacetifolium.* In (A) plant habit; (B) involucre of bracts; (C) leaf; (D) ray floret; (E) disc floret; (F) dissected disc floret. Specimens: (A–F) *MacOwan, P. s.n.* (Z).

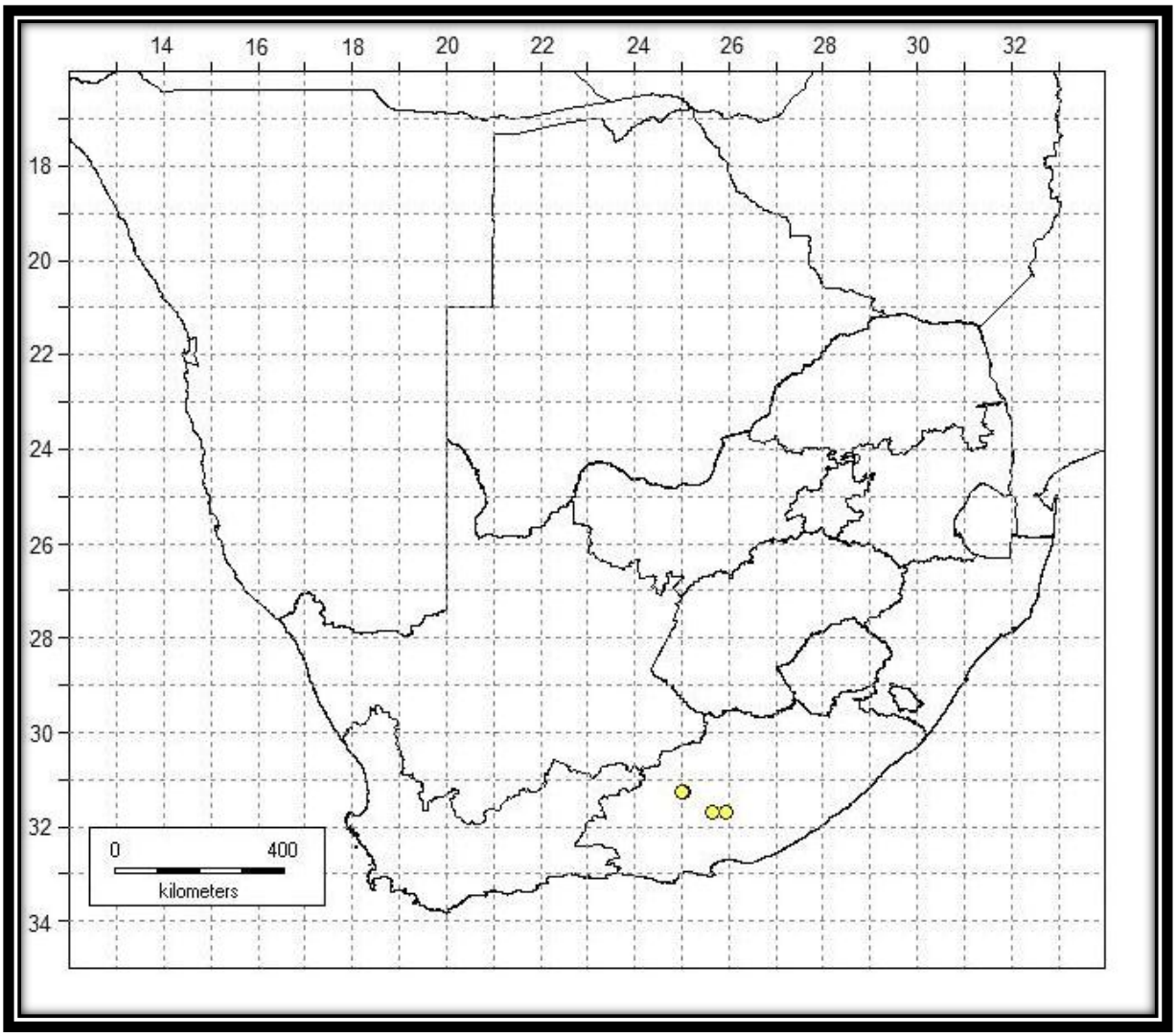

Figure 7.8.2 Known geographical distribution of *Garuleum tanacetifolium*.

Representative specimens

—32º15'33'' S, 25º01'19'' E: South Africa, Eastern Cape, Graaff-Reinet, Asante Sana, Suurkloof, 30 November 2011, *Van Zyl, J. 17* (BLFU).

—32º15'40'' S, 25º00'67'' E: South Africa, Eastern Cape, Graaff-Reinet, Asante Sana, Suurkloof, 30 November 2011, *Van Zyl, J. 21* (BLFU).

—32º41'45'' S, 25º38'23'' E: South Africa, Eastern Cape, Boschberg, December 1881, *McOwan, P. 748* (Z).

7.3.8 ***Garuleum woodii*** Schinz., in Autran, Bulletin de l'Herbier Boissier 3: 440 (1895); Hilliard: 518 (1977) Retief and Herman (1997).

Type: South Africa, Drakensberg [Kwazulu-Natal Province], near cold stream, *Wood 4496* (K, lectotype (scan!) here designated; B, HUH, NH, GH (scan!), NBG, PRE, isolectotypes). South Africa, Charlestown [Kwazulu-Natal Province], valley of Buffalo river, *Wood 4840* (Z, syntype (scan!); BOL!, B, BM, NH, K (scan!), NBG, P, PRE, isosyntype).

An aromatic shrub, up to 600 mm tall. Woody rootstock present. **Stem** base 20–50 mm wide, well-branched, woody, glandular. **Leaves** sessile; base clasping stem; blades 25–42 x 6–20 mm; tri-pinnate, elliptic, margins crenate; adaxial and abaxial surfaces are scabrous and glandular, apices broadly acuminate, stipules present. **Inflorescences** capitula, 8–14 mm wide, peduncles 90−100 mm long, glandular, involucral bracts 4–6 x 2 mm, scabrous and glandular, apices not recurved, arranged imbricately in series of 3 rows surrounding the capitula, outer and inner rows same size. **Ray floret** corolla mauve to purple; tube base densely glandular, tube apex villous**,** ligulate; ligule 11–12 mm long; style 4–4.5 mm long, stylar lobes ±1 mm long. **Disc floret** corolla 4.5–5.5 mm long, tube ±2 mm long, lobes ±1 mm, minutely glandular cover over whole floret and large concentration of long trichomes on corolla lobes. Stamens inserted 1–1.5 mm from corolla tube base, filaments ±1 mm long, anthers ±3 mm long. Pistil with style 4–5mm in long, stylar lobes ±1 mm long, glandular and villous. **Achenes** develop from ray florets; light brown, cylindrical, margins slightly thickened, surface grooved with a prominent ridge on one side of achene with turned u-shaped grooves, 3.7 x 1.9 mm. Figure 7.9.1.

Diagnostic characters

Basal stems woody. Leaves tri-pinnate from distal half of leaf, elliptic outline smaller than that of *G. pinnatifidum*. Leaf margins crenate. Apices not recurved. Stipules present. Involucre bracts glandular and 4–6 x 2 mm long.

Distribution and habitat

Garuleum woodii is found the Free State, Kwazulu-Natal, Lesotho and Mpumalanga. This species grows at altitudes ranging from 1524–2200 m, in rocky soil overlying sandstone (Fig. 7.9.2.).

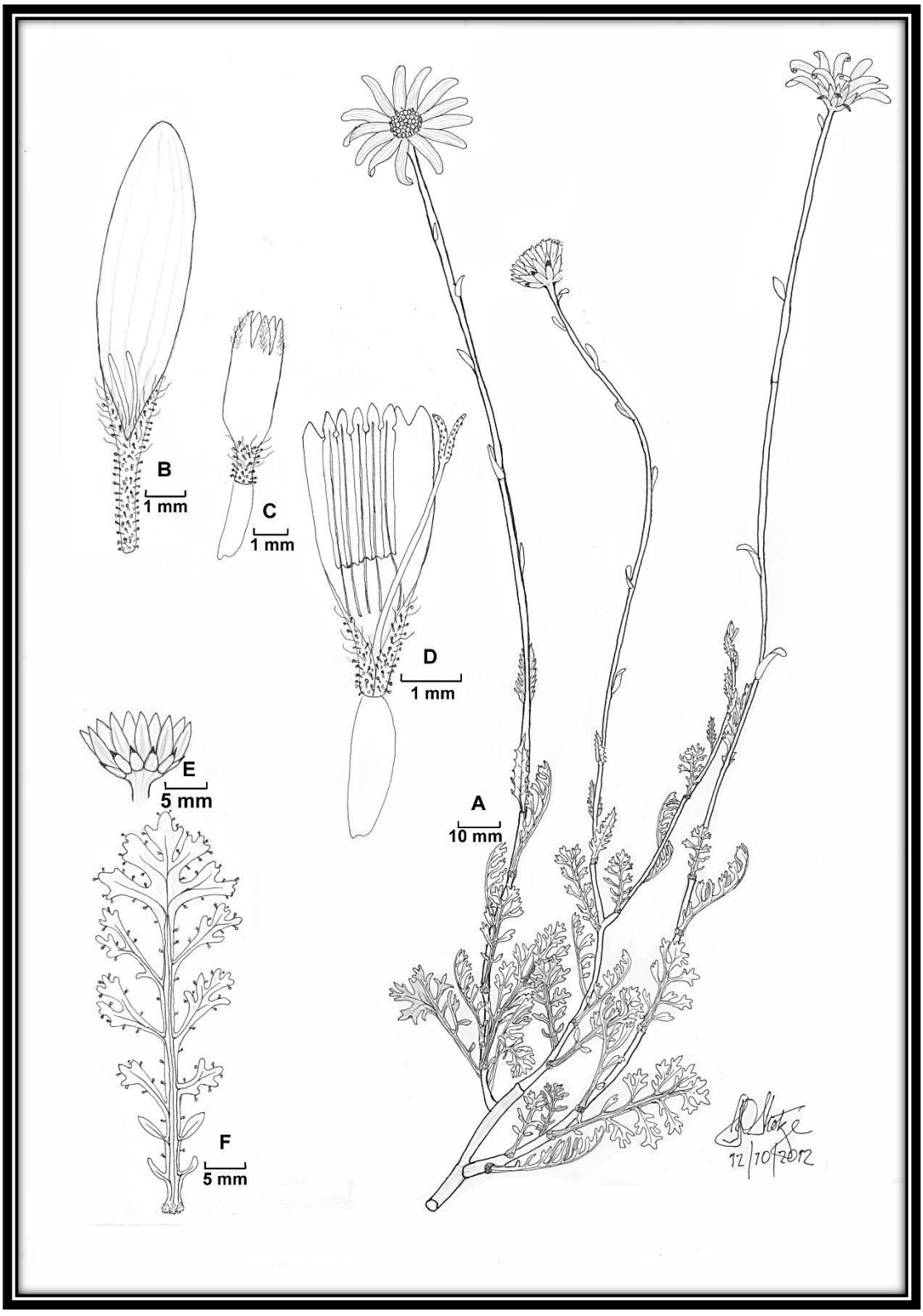

Figure 7.9.1 Illustration of *Garuleum woodii.* In (A) plant habit; (B) ray floret; (C) disc floret, (D) dissected disc floret; (E) involucre of bracts; (F) leaf. Specimens: (A) combination of *Wood 4840* (BOL) and *Rehmann 6792*; (B–D) *Galpin s.n.* (BOL); (E–F) *Wood 4840* (BOL).

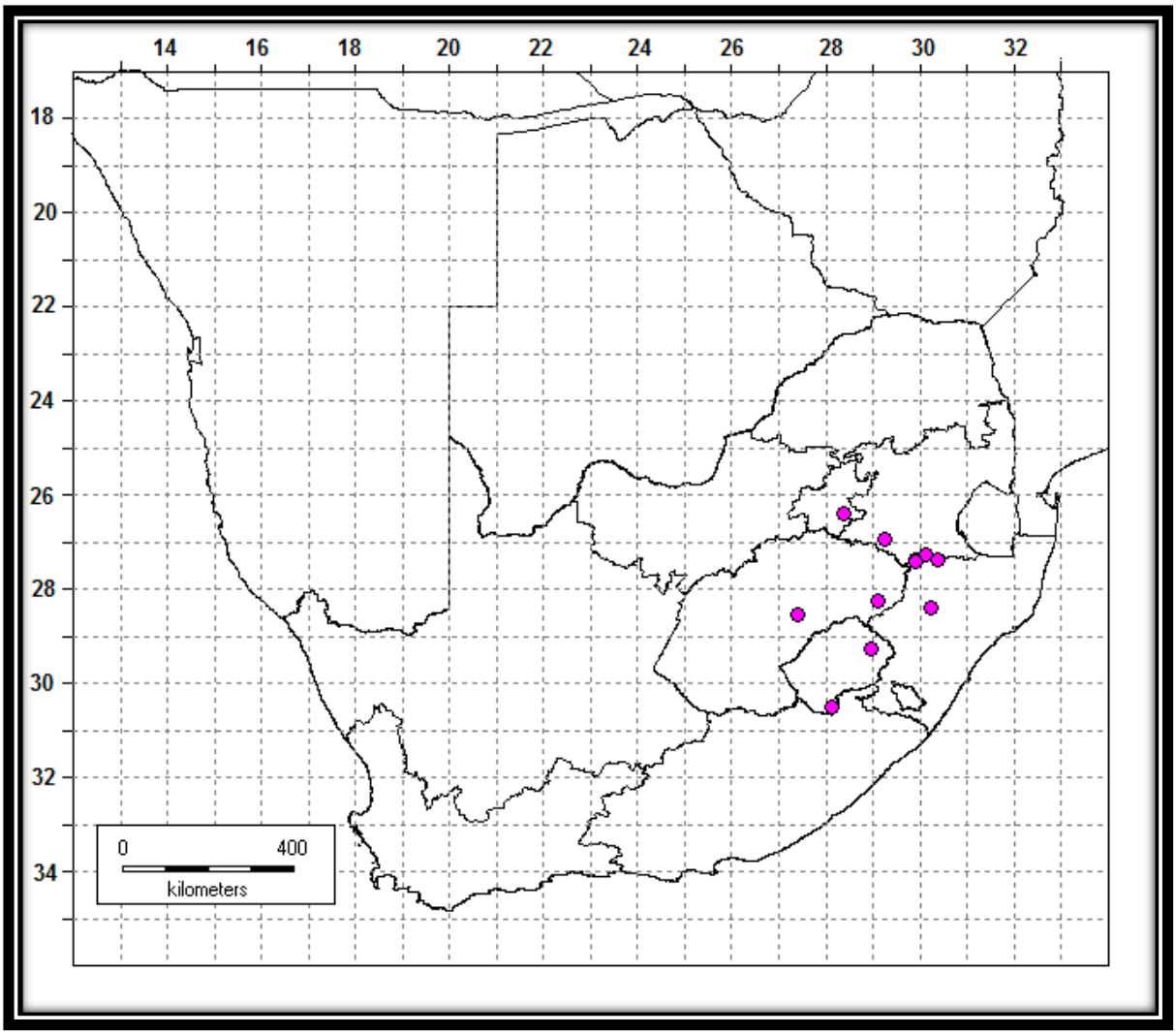

Figure.7.9.2 Known geographical distribution of *Garuleum woodii*.

Representative specimens

— 26º56'51'' S, 29º14'49'' E: South Africa, Mpumalanga, Standarton, 1875, *Rehmann, A.*, 6792 (Z) (Z-000061161, Z-000078524)

— 27º21'38'' S, 29º53'41'' E: South Africa, Mpumalanga, Volksrust, 8 January 1932, *Galpin, E.E.*, *s.n*. (BOL)

—27º15' S, 30º07' E: South Africa, Kwazulu-Natal, Vryheid, Wakkerstroom, *Thode, J. s.n.* (NH).

—27º25'07'' S, 29º53'33'' E: South Africa, Kwazulu-Natal, Charlestown, Valley of buffalo river, 6 December 1892, *Wood, J.M. 4840* (BOL).

—28º15' S, 29º07' E: South Africa, Free State, Harrismith, Loskop, 10 March 1970, *Stam, R.D.* 420 (BLFU).

—29º15'56' S, 28º57'37' E: Lesotho, Mokhotlong, 27 February 1949, *Compton, R.H*. *21539* (NBG).

Chapter 8

Phylogeny of *Garuleum*

8.1 Introduction

There has always been a debate on just how to define a species. This debate has led to the proposal of several species concepts for the construction of classifications. In the past, species were defined using morphological data, which is still being used today. In recent studies, morphology-based taxonomies are being tested using DNA sequence data (Jansen and Palmer, 1987). The reason for incorporating DNA sequence data with morphological-based classifications is to determine the evolutionary history of a species. It also leads to a better defined monophyletic (derived from a single ancestor) group and provides more characters with which to define the group, than that provided by traditional morphological data alone. DNA sequence data has become a valuable tool in reconstructing phylogenies and delimiting species (Howis, 2007).

In the plant cell there are three potential DNA sequence data sources, namely nuclear, mitochondrial and chloroplast DNA. When choosing gene regions for the phylogenetic study, it is important to choose regions that amplify and sequence well for the particular taxa. The gene regions should also provide sufficient resolution among the species when a phylogeny is constructed (Shaw et al., 2005).

Nuclear DNA mutate faster than chloroplast DNA (Albach and Chase, 2004). This provides a fast changing character-set which is more informative at lower taxonomic levels. The rapid evolution of nuclear DNA also makes it very valuable in phylogenetic studies where the chloroplast regions provided little or no variation (Jeandroz et al., 1997).

Most of the phylogenetic studies done on the tribe Calenduleae have not been at species-level, but at genus level (Nordenstam and Källersjö, 2009).

For this reason, there is little data available on which gene regions will possibly give the best resolution for *Garuleum*. The majority of phylogenetic studies done on the Asteraceae in the past incorporate the nuclear ribosomal gene region ITS and it has been successfully used at species-level studies for many taxa (Gao et al., 2010). There are certain problems associated with the use of ITS and it is therefore advisable to be used in combination with other gene regions and morphological characterstics (Alvarez and Wendel, 2003). Such gene regions include the chloroplast intergeneric region *trn*T–*trn*F and *psb*A–*trn*H. These gene regions have provided both well resolved and poorly resolved phylogenies in studies at different taxonomic levels in the Asteraceae (Shaw et al., 2005).

This study aims to elucidate the evolutionary history of the genus *Garuleum*, by obtaining a well resolved phylogeny using the nuclear ribosomal gene region ITS and chloroplast regions *trn*T–*trn*F and *psb*A–*trn*H.

8.2 Materials and methods

All material and methods in this chapter is as described in 3.6 (Chapter 3).

8.3 Results

8.3.1 DNA extraction and PCR amplification

The DNA extracted from fresh specimens, were of good quality. After separation on an agarose gel, a clear intact genomic DNA band larger than 20 000 bp in length was visible for each specimen. The DNA extracted from the herbarium specimens was badly degraded. After separation on an agarose gel, no intact DNA was visible, only a smear.

Amplification of the gene regions was not possible for all the herbarium specimens, due to the DNA being too badly degraded. The gene region *trn*T–*trn*L proved to be very difficult to amplify and the length of the sequences varied in many of the samples. The amplified fragments for each of the other gene regions were the correct size for all the *Garuleum* species; namely: ITS (± 750 bp), *trn*L–*trn*F (±475 bp), *trn*T–*trn*L and *psb*A –*trn*H (± 450 bp). The entire ITS region was successfully amplified for all the specimens.

8.3.2 DNA sequencing and nucleotide alignments

Sequences were viewed using Chromas Lite (Version 2.1) and the electropherograms varied from bad quality electropherograms with multiple peaks per nucleotide base (Fig. 8.1 a) to good quality with good clear single peaks per nucleotide base (Fig. 8.1 b). All the sequences obtained for the region were of very bad quality and could not be use to edit poor quality sequences into useable sequences. The gene region *trn*T–*trn*L especially had very noisy electropherograms where the peaks were not clearly defined and the sequence length varied for the different species after multiple sequencing attempts.

To determine the identity of the amplified fragments for each of the *Garuleum* species, the Basic Local Alignment Search Tool (BLAST) from National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. No specimens were available on Genbank for the chosen gene regions of *Garuleum* itself, but homology was shown with other species in the Asteraceae family. Results for the search are shown in Table 8.1. E-values equal or close to zero, show the sequence in Genbank most homologous to each of the query sequences.

Nucleotide sequences for all *Garuleum* species were aligned for each gene region respectively. The alignment for the ITS region was good with only a few small gaps present. The alignment for the *trn*L–*trn*F and *psb*A–*trn*H regions respectively had more gaps than the ITS region, but the alignments were still acceptable. The alignment of the nucleotide sequences for the *trn*T–*trn*L region did not show regions with continuous high homology among the species and had numerous large gaps throughout the alignment for the available species. The length of the sequences also varied from 440—550 bp in length for *trn*T–*trn*L. The difficulty experienced in amplifying, sequencing and aligning the *trn*T–*trn*L region, led to the exclusion of this region from the study. Alignments of the different gene regions can be seen in addendum II.

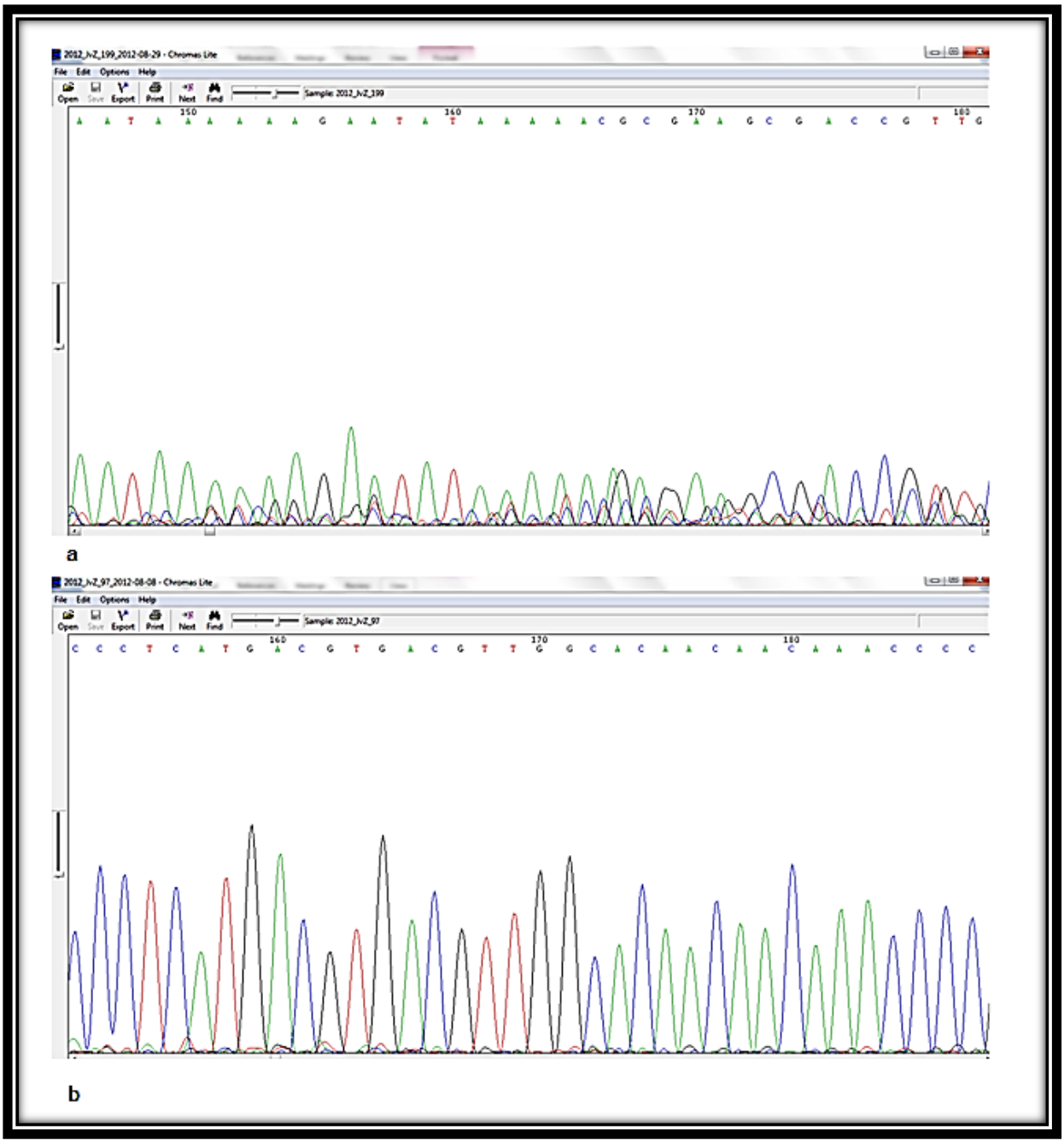

Figure 8.1 Examples of electropherograms for *Garuleum pinnatifidum*. In (a), an electropherogram shows badly defined multiple peaks per nucleotide base pair for gene region *trn*T–*trn*L. In (b), a good quality electropherogram with single peaks per nucleotide base pair for gene region ITS.

Table 8.1 BLAST results for the gene regions used in this phylogeny.

ITS			
Garuleum species	**Accession nr**	**Blast result**	**E-value**
Garuleum album	FJ861498.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum bipinnatum	FJ861475.1	*Osteospermum corymbosum* (Asteraceae) ITS	0
Garuleum latifolium	FJ861501.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum pinnatifidum	FJ861498.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum schinzii	FJ861498.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum sonchifolium	FJ861501.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum tanacetifolium	FJ861501.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
Garuleum woodii	FJ861498.1	*Chrysanthemoides monilifera subsp. canescens* (Asteraceae) ITS	0
***trn*L-*trn*F**			
Garuleum species	**Accession nr**	**Blast result**	**E-value**
Garuleum album	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	3.00E-119
Garuleum bipinnatum	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	6.00E-122
Garuleum latifolium	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	8.00E-131
Garuleum pinnatifidum	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	1.00E-124
Garuleum schinzii	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	2.00E-112
Garuleum sonchifolium	GU817982.1	*Capelio caledonica* isolate (Asteraceae) *trn*L-*trn*F	1.00E-123
Garuleum tanacetifolium	EF538094.2	*Chersodoma jodopappa* (Asteraceae) *trn*L-*trn*F	7.00E-127
Garuleum woodii	EF538094.2	*Chersodoma jodopappa* (Asteraceae) *trn*L-*trn*F	5.00E-133
***psb*A-*trn*H**			
Garuleum species	**Accession nr**	**Blast result**	**E-value**
Garuleum album	AY215581.1	*Neurolaena lobata* (Asteraceae) *psb*A-*trn*H	2.00E-68
Garuleum bipinnatum	AY215581.1	*Neurolaena lobata* (Asteraceae) psbA-trnH	4.00E-130
Garuleum latifolium	AY215581.1	*Neurolaena lobata* (Asteraceae) psbA-trnH	8.00E-87
Garuleum pinnatifidum	GU014471.1	*Eupatorium truncatum* (Asteraceae) psbA-trnH	7.00E-123
Garuleum schinzii	AY215581.1	*Neurolaena lobata* (Asteraceae) psbA-trnH	1.00E-134
Garuleum sonchifolium	EU549769.1	*Guizotia abyssinica* (Asteraceae) chloroplast Complete genome	1.00E-89
Garuleum tanacetifolium	AY215581.1	*Neurolaena lobata* (Asteraceae) psbA-trnH	3.00E-151
Garuleum woodii	FM173169.1	*Metalasia densa* (Asteraceae) psbA-trnH	2.00E-107
***trn*T-*trn*L**			
Garuleum species	**Accession nr**	**Blast result**	**E-value**
Garuleum album	AY215581.1	*Neurolaena lobata* (Asteraceae) *trn*T-*trn*L	5.00E-134
Garuleum bipinnatum	HM002826.1	*Centaurea cyanus* (Asteraceae) *trn*T-*trn*L	9.00E-87
Garuleum latifolium	HM002826.1	*Centaurea cyanus* (Asteraceae) *trn*T-*trn*L	6.00E-164
Garuleum pinnatifidum	AY215893.1	*Bahia absinthifolia* (Asteraceae) *trn*T-*trn*L	5.00E-89
Garuleum schinzii	FJ775954.1	*Centaurea aetolica* (Asteraceae) *trn*T-*trn*L	3.00E-81
Garuleum sonchifolium	GU120098.1	*Parthenium argentatum* (Asteraceae) chloroplast sequence	2.00E-123
Garuleum tanacetifolium	FJ775958.1	*Centaurea argecillensis* (Asteraceae) *trn*T-*trn*L	2.00E-128
Garuleum woodii	EU661113.1	*Cousinia abolinii* (Asteraceae) *trn*T-*trn*L	5.00E-61

8.3.3 Construction of trees

8.3.3.1 ITS gene tree

A most parsimonious tree was constructed for the ITS gene region. This gene region provided 746 characters of which 123 were informative. Uniformative characters were excluded from the analysis. Only bootstrap values above 70 % are indicated on the tree. The consistency index (CI) value was 0.786 and the retention index (RI) value was 0.544. A most parsimonious tree chosen and the bootstrap values obtained and consensus tree combined on one tree (Fig. 8.2).

In the most parsimonious tree *G. latifolium* and two groupings were observed. Clade 1; consisting of *G. album*, *G. sonchifolium* and *G. woodii.* And clade 2; consisting of *G. bipinnatum*, *G. pinnatifidum*, *G. schinzii* and *G. tanacetifolium*. The posterior probability (PP) values obtained in the BI-analysis were also indicated on the most parsinonious tree obtained from PAUP parsimony analysis (Fig. 8.2). For this gene region three outgroups were included namely *Steirodiscus capillaceus*, *Stoebe aethiopica* and *Calendula officinalis*. The sequence data for this outgroups were obtained from Genbank and their accession numbers are shown in Table 8.2. These outgroups were able to root the tree.

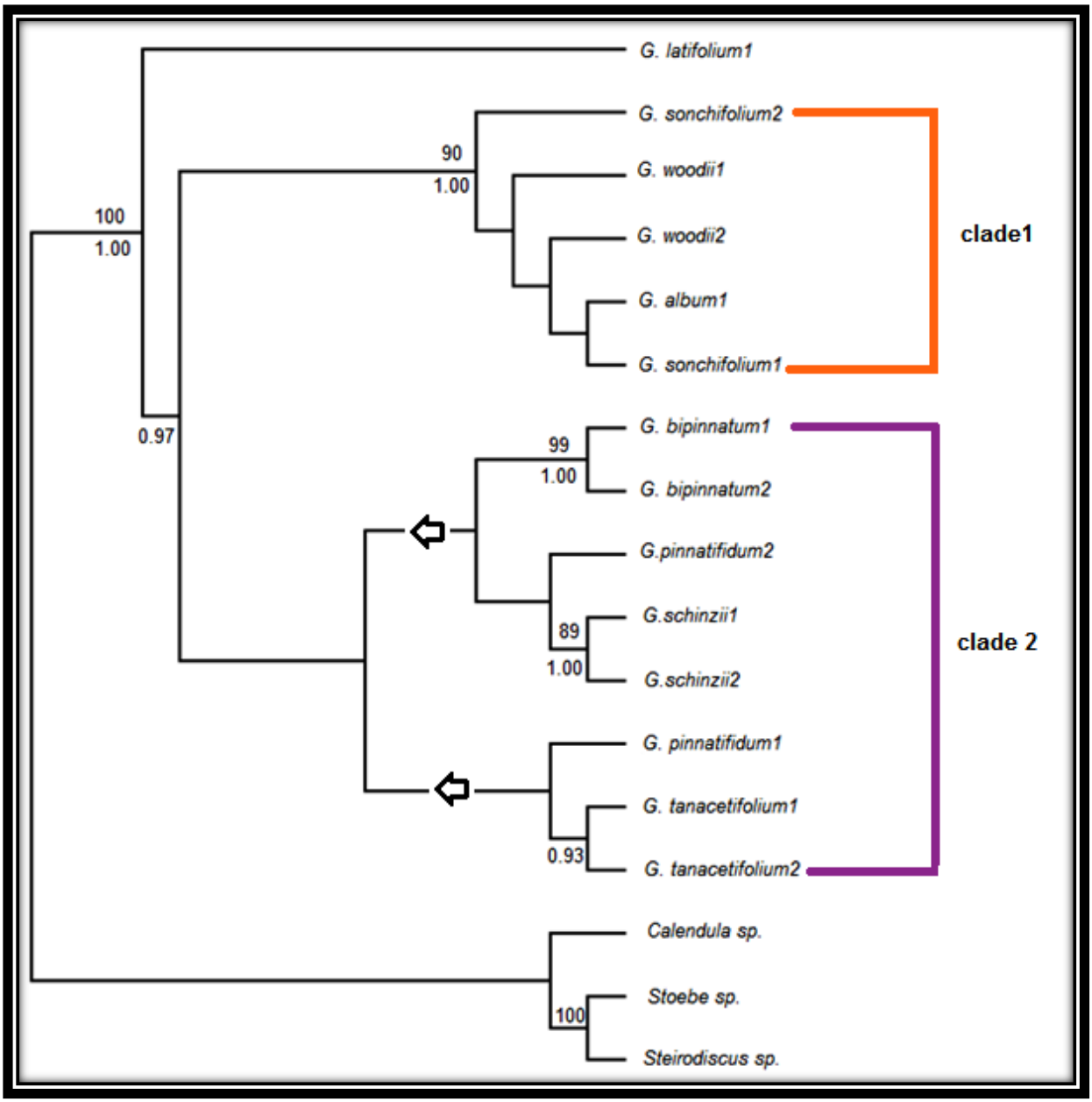

Figure 8.2 A most parsimonous tree obtained for the ITS region with PAUP parsimony analysis. Bootstrap values are indicated above the branch and PP-values below the branch. Branches without values indicate a bootstrap and PP-value lower than 70 %. Species showing a 1 or 2 behind the name indicate where two specimens were used for each species. Uninformative characters were excluded from the analysis. Trees generated were 119, tree length was 598. The CI value was 0.786 and the RI value was 0.544. Strict consensus tree generated, arrows indicate were a branch collapses on the consensus tree.

Table 8.2 The outgroups obtained from Genbank for the different gene regions.

ITS gene region tree	
Accession nr	**Species**
GU818718.1	*Steirodiscus capillaceus*
JF893878.1	*Stoebe aethiopica*
JN315941.1	*Calendula officinalis*
***trn*L–*trn*F gene region tree**	
GU818094.1	*Steirodiscus capillaceus*
AF100508.1	*Stoebe aethiopica*
JN315917.1	*Calendula officinalis*
***psb*A–*trn*H gene region tree**	
GU818475.1	*Steirodiscus capillaceus*
FM173177.1	*Stoebe aethiopica*
GU818348	*Calendula arvensis*

8.3.3.2 *trn*L–*trn*F gene tree

A parsimony analysis of this gene region was done and a most parsimoniousl tree was chosen. On this tree, bootstrap values above 70 % were allowed (Fig. 8.3). The relationships shown in the most parsimonious tree can be considered a polytomy, for there are no support values to validate their accuracy. The characters provided by this region were 493 of which 44 characters were informative. Uninformative characters were excluded from the analysis. This gene region had a CI-value of 0.722 and a RI-value of 0.528. A consensus tree was combined with most parsimonious tree (Fig. 8.3). For the BI-analysis, PP-values were also indicated on the most parsimonious tree obtained with PAUP parsimony analysis (Fig. 8.3). Outgroups included for this tree were *Steirodiscus capillaceus*, *Stoebe aethiopica* and *Calendula officinalis* (Table 8.2). *G. woodii1* is shown to be more closely related to *Calendula officinalis*, while *Steirodiscus capillaceus* and *Stoebe aethiopica* are more closely related to the rest of the *Garuleum* species. Theses outgroups may not be able to effectively root this tree due to homoplasy.

8.3.3.3 *psb*A–*trn*H gene tree

A parsimony analysis of this gene region was done and a most parsimonious tree was chosen. On this tree, bootstrap values above 70 % were allowed (Fig. 8.4). The characters provided by this region were 532 and 83 characters were informative.Uninformative characters were excluded from the analysis. The PP-values obtained from the BI analysis are indicated on the most parsimonious tree constructed with PAUP parsimony analysis. The consensus tree is combined with the most parsimonious tree (Fig. 8.4).

The most parsimonious tree grouped *G. latifolium* with the *Steirodiscus* species, but the maximum likelihood analysis of the Baysian analysis groups *G. latifolium* with the other *Garuleum* species. This tree provided no resolution for species-level classification. Outgroups included for construction of the tree was *Calendula arvensis, Steirodiscus capillaceus* and *Stoebe aethiopica* (Table. 8.2).

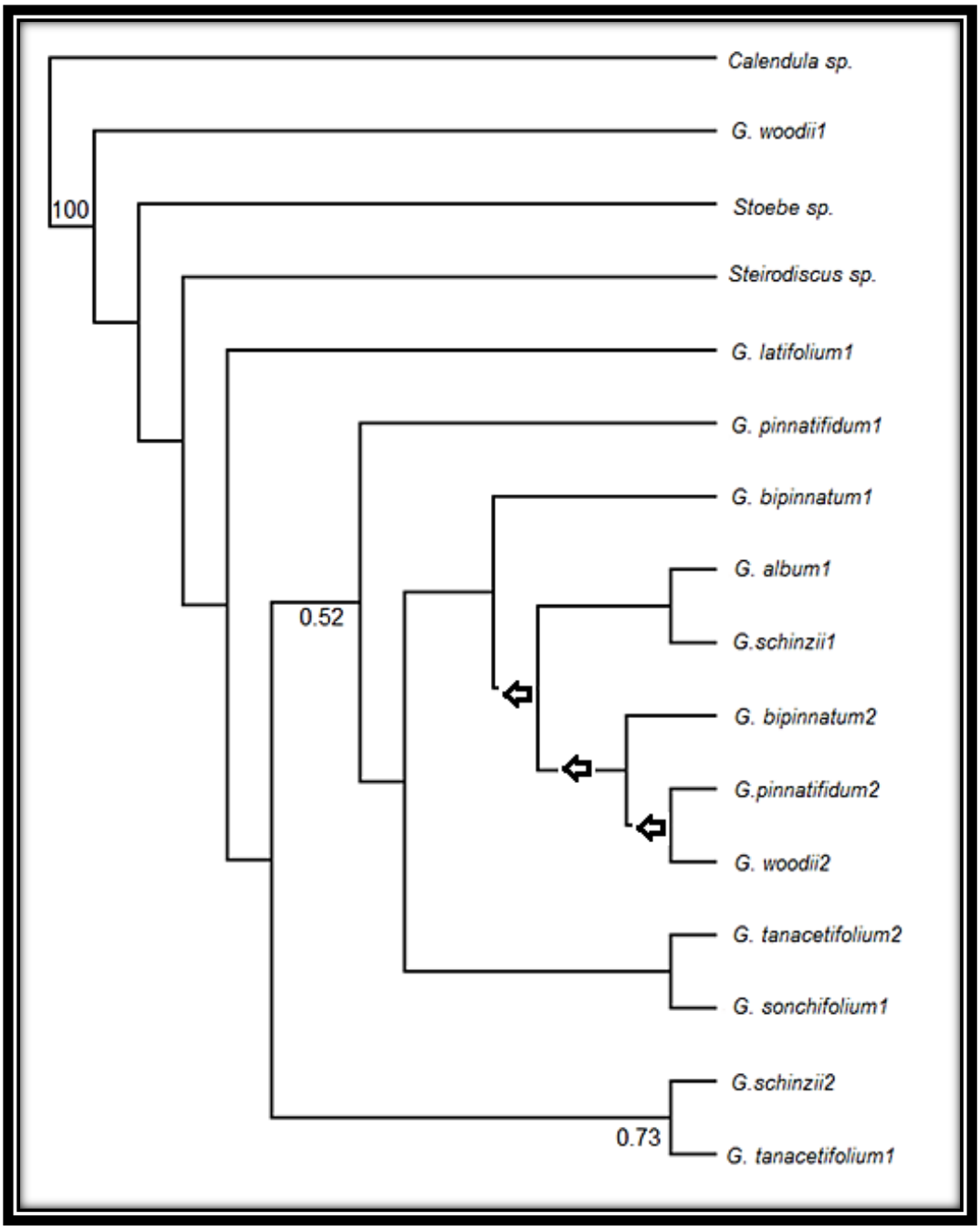

Figure 8.3 Most parsimonious tree obtained for *trn*L–*trn*F region obtained with PAUP parsimony analysis. Bootstrap values are indicated above the branches and PP-values below the branches. Branches without values indicate a bootstrap and PP-value lower than 70 %. Species showing a 1 or 2 behind the name indicate where two specimens were used for each species. Uninformative characters were excluded from the analysis. Trees generated were 1000, tree length was 90. The CI value was 0.722 and the RI value was 0.528. Strict consensus tree generated, arrows indicate were a branch collapses on the consensus tree.

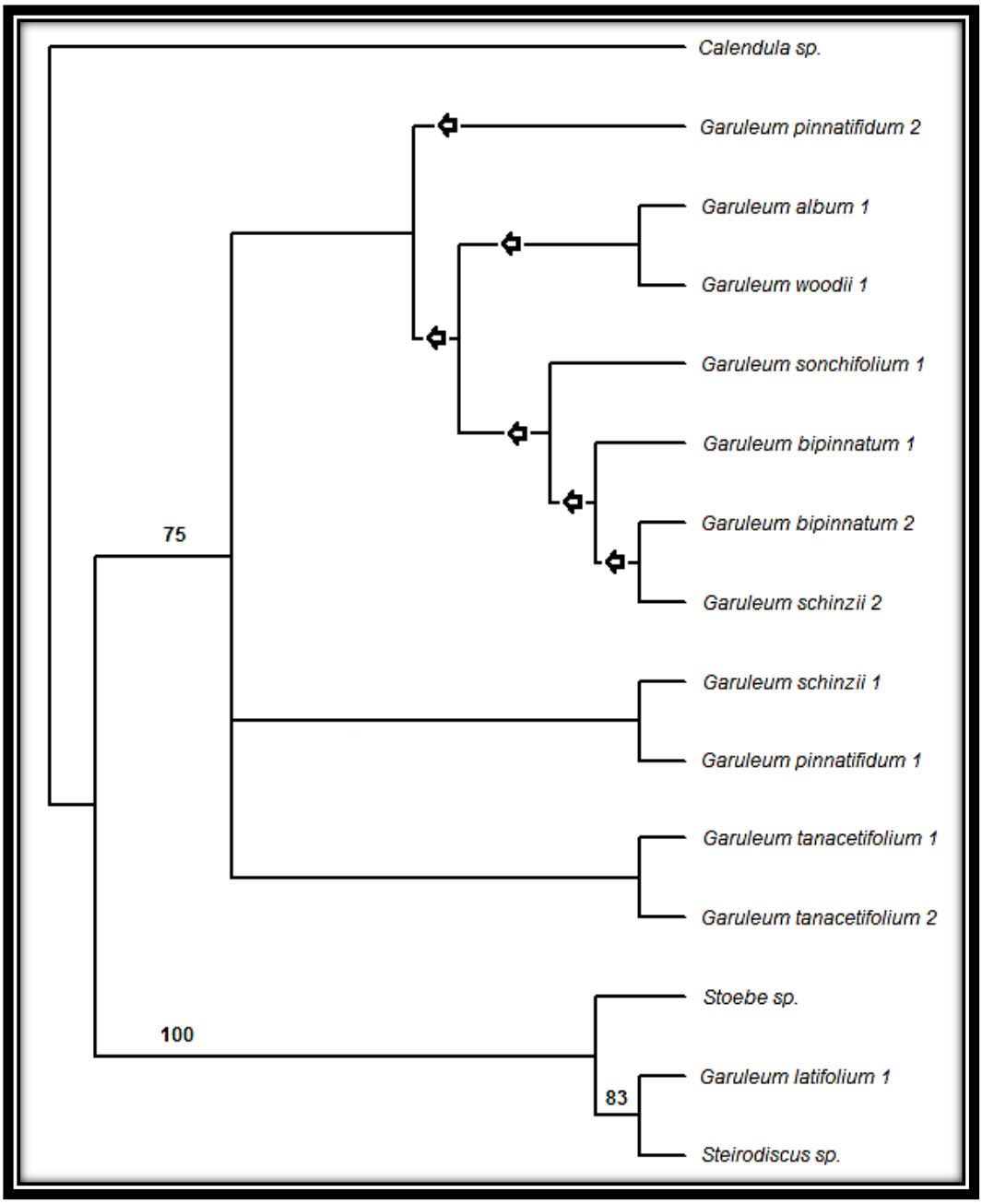

Figure 8.4 Most parsimonious tree for *psb*A–*trn*H region obtained with PAUP analysis. Bootstrap values are indicated above the branches and PP-values below the branches. Branches without values indicate a bootstrap and PP-value lower than 70 %. Species showing a 1 or 2 behind the name indicate where two specimens were used for each species. Uninformative characters were excluded from the analysis. Trees generated were 558, tree length was 183. The CI value was 0.650 and the RI value was 0.475. Baysian analysis included *G. latifolium* in the ingroup clade. Strict consensus tree generated, arrows indicate were a branch collapses on the consensus tree.

8.3.3.4 Combination of data

The data sets of the three genes were compared and only datasets with no strong incongruence (bootstrap values higher than 80%) was combined. There was no strong incongruence between ITS and *trn*L–*trn*F, but strong incongruence was present for *psb*A–*trn*H. Therefor only ITS and *trn*L–*trn*F was combined.

The combined tree obtained with PAUP for the ITS and *trn*L–*trn*F data sets are shown in Figure 8.5. The characters provided by this region were 1283 and 167 characters were informative.Uninformative characters were excluded from the analysis.The PP-values obtained for the combined data tree in the BI-analysis is indicated on the same most parsimonious tree (Fig.8.5).

Two clades (A+B) could be distinguished in this tree. Clade A consists of *G. album, G. schinzii, G. sonchifolium* and *G. woodii.* Clade B consists of *G. bipinnatum, G. pinnatifidum* and *G. tanacetifolium*.

8.4 Discussion

For the combination of data sets, three schools of thought exist. The first are, researchers who believe you should always combine your data and the second are those who believe you should never combine data sets. The third are those who first test if the data sets may be combined and only combine if the test confirms a combination. Data sets are combined because it increases the phylogenetic accuracy of the tree (Bull et al., 1993; Cunningham, 1997) and represents the evolution of the species, and not just a single gene. The reliability of incogruence test to determine if datasets may be combined has been proved to be of variable reliance (Yoder et al., 2001; Barker and Lutzoni., 2002). In this study data sets were studied separately and combined if if no clade with strong incongruence were found.

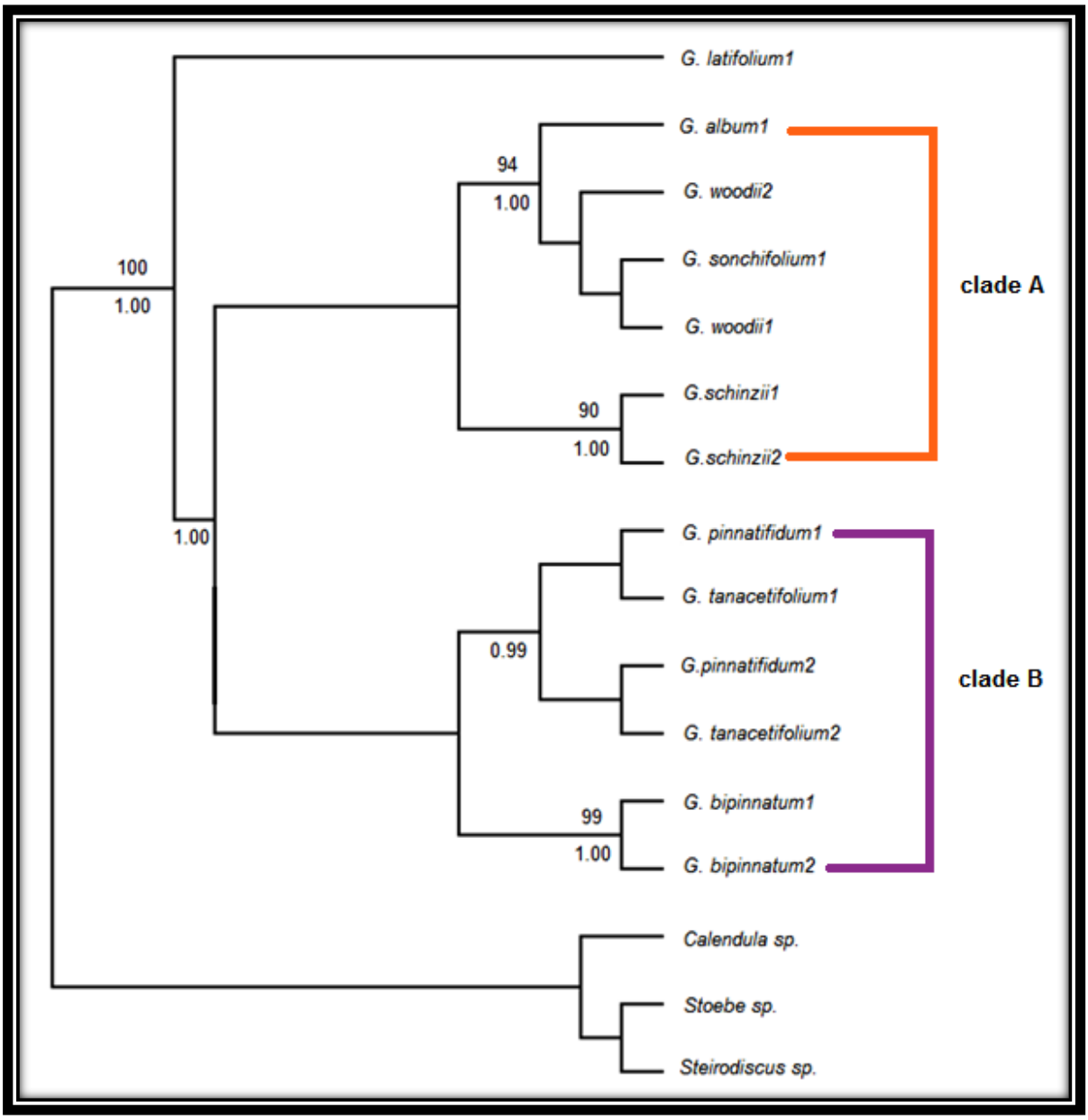

Figure 8.5 Most parsimonious tree for combined ITS and *trn*L–*trn*F regions obtained with PAUP analysis. Bootstrap values are indicated on top of branches and PP-values below the branches. Branches without values indicate a bootstrap and PP-value lower than 70 %. Species showing a 1 or 2 behind the name indicate where two specimens were used for each species. Uninformative characters were excluded from the analysis. Trees generated were 6, tree length was 428. The CI value was 0.621 and the RI value was 0.500.

The tree obtained from the ITS gene region (Fig. 8.2), was effectively rooted by the three chosen outgroups. This tree indicates that *G. latifolium* was possibly the first species to diverge from the ancient ancestor group from which *Garuleum* originated. The bootstrap value for *G. latifolium* was 100 %, but the PP-value obtained from the BI analysis was 1.00 providing sufficient support that this might have occurred. Two clades (1+2) sister to *G. latifolium* diverged from a common ancestor group. Clade 1 includes *G. album*, *G. sonchifolium*, and *G. woodii* and is well supported with a bootstrap value of 90 % and PP-value of 1.00. Clade 2 consists of *G. bipinnatum*, *G. pinnatifidum*, *G. schinzii* and *G. tanacetifolium* but it lacks the support of bootstrap and PP-values. In clade 1 (Fig. 8.2), the two *G. sonchifolium* specimens do not group together on the tree, instead *G. sonchifolium1* groups with *G. album1*. This may be due to homoplasy. These two species may have evolved the same characters to adapt to similar environments. The CI-value obtained for this analysis was 0.786, which means approximately 22 % of the characters used in this analysis are influenced by homoplasy, which could affect the accuracy of the relationships portrayed on the tree.

Even though the internal nodes are not very well supported in the phylogeny obtained for the ITS region, this region provided the most informative characters for a well resolved phylogeny.

The phylogeny did show some resolution for the relationships in the genus *Garuleum*. The only major difference between the ITS tree and the combined tree, is the position of *G. schinzii*. The rest of the relationships portrayed in the two clades of both trees, are very similar. This is due to the combined tree portraying the evolution of the species and the ITS tree shows the evolution of the single gene ITS. The species tree and a gene tree can differ in topology. Gene trees are constructed from gene replication events. A gene is replicated and passed on to its offspring; sometimes the copy of the gene is imperfect to the original copy, causing the branching points on the gene tree. A species tree is the combination of many gene trees and the branching points of a species tree are speciation events (Maddison, 1997). The incongruence between the two trees may also be due to nuclear DNA having a faster mutation rate than chloroplast DNA (Albach and Chase, 2004).

The tree constructed (Fig. 8.3) for the *trn*L–*trn*F region, did not portray any well supported relationships among the *Garuleum* species. Very few informative characters were obtained for the analysis of this region. The outgroups chosen for this study were determined by choosing monophyletic genera from within the Calenduleae and from the two tribes closest related to the Calenduleae.The outgroups were ineffective at properly rooting the tree, this may be due to homoplasy. Since no other phylogenetic studies were previously done on *Garuleum*, there were no outgroups that could be used which would work with certainty. The bootstrap values were all below 70 % and the PP-values were below 0.75, meaning that none of the relationships portrayed in this tree is well supported. The tree topology is a polytomy, thus providing no resolution at species-level for *Garuleum*. This region is usually more effective at higher taxonomic ranks, than at species-level (Taberlet et al., 1991).

The tree obtained from the chloroplast region *psb*A–*trn*H (Fig. 8.4), also did not provide enough variable characters for a well resolved phylogeny. The length of this region is probably too short to provide resolution at species-level. The species *G. latifolium* group together with the outgroup Steirodiscus. The results of the maximum likelihood analysis showed that *G. latifolium* should instead be grouped together with the other *Garuleum* species. And that the *Garuleum* genus is monophyletic.

The data of the gene regions ITS and *trn*L–*trn*F, were combined (Fig. 8.5). Gene region *psb*A–*trn*H was excluded since there was strong incogruence between the results for this gene region and that of the other two gene regions. The combined tree shows *G. latifolium* to be the first to diverge from the most recent common ancestor. This tree also shows two clades (A+B) diverging from an ancestral group which is sister to *G. latifolium*. Clade A, includes *G. album*, *G. schinzii*, *G. sonchifolium* and *G. woodii*. Clade B, includes *G. bipinnatum*, *G. pinnatifidum* and *G. tanacetifolium*.

In clade A, *G. album*, *G. sonchifolium* and *G. woodii* are indicated to be closely related and the relationship is well supported by a bootstrap value of 94 % and PP-value of 1.00. *Garuleum schinzii* is a sister group to the above mentioned group, but a low bootstrap value (lower than 70 %) and PP-value below 0.70 does not support this relationship. In clade B, *G. pinnatifidum* and *G. tanacetifolium* are shown to be closely related. This relationship is only supported by a PP-value of 0.99, but the bootstrap value was below 70 %. The relatedness of *G. bipinnatum* to *G. pinnatifidum* and *G. tanacetifolium* is not well supported by values from the bootstrap and BI-analysis.

Not all the relationships portrayed by these trees for the different species are well supported. This might be due to insufficient sampling for specific species and due to a lack of fresh material, since the sequence quality from the herbarium specimens were of poor quality. A broad survey on the effectiveness of chloroplast and ITS genes to resolve species-level phylogenies in the plant family has shown that more than half of recent studies obtain poorly resolved phylogenies (Hughes et al., 2005). The use of more variable non-coding chloroplast genes has provided some studies with more resolution (Shaw et al., 2005), but this increases the volume of DNA sequences and do not guarantee better resolution at species-level studies (Highes et al., 2005). More herbarium specimens from which could to be sampled, and more specimens per species might have led to a better resolved phylogeny. This genus has not been revised in recent years, which means few specimens are currently available of *Garuleum* and most of these were collected over 100 years ago. The information on the areas of the species' locations and its preferred habitat is very outdated, thus making it very difficult to collect fresh material in the field. Badly degraded DNA extracted from these old herbarium specimens, may also have led to unresolved relationships of species.

Even though all the trees were not completely resolved and did not indicate a well-supported species relationship, it was clear to see that the genus *Garuleum* is a monophyletic group. The chloroplast regions in combination and used separately did not provide enough resolution to reconstruct the phylogeny or delimit the species for *Garuleum*. The ITS region provided some resolution at species-level classification The ITS and *trn*L–*trn*F gene regions in comination provided a well resolved phylogeny for the genus *Garuleum*. This combined phylogeny provides the first insight into the evolutionary history of the genus.

Chapter 9

General discussion and conclusion

Systematics is the study of the evolutionary history of living organisms. The evolutionary history obtained for a specific organism is dependent on the amount of data and techniques which were available to the scientist at the time of investigation. As more data and better techniques are developed for systematics, the evolutionary history of a group may become better resolved. To obtain the most accurate account of the evolutionary history of the genus *Garuleum*, the results obtained from all the previous chapters are combined and a possible evolutionary history interpreted. These results include the phylogenetic, morphological characteristics and the distribution data of the different species. The micromorphological data of the leaves, achenes, flowers and pollen will also be taken into consideration.

The phylogeny obtained from the combined nuclear and chloroplast gene regions (Fig. 8.5.) indicated that *Garuleum* is monophyletic. The monophyly of *Garuleum* was also supported by the pollen micromorphology of the different species. It was shown that all Garuleum species exhibit the *Garuleum*-type pollen, which was similar for all the species to the description of the *Garuleum*-type pollen described by Praglowski and Grafstöm in 1980 in their study of Calenduleae pollen. The *Garuleum*-type pollen is ±34 µm in diameter and covered with 100-120 spines which are closely situated at the base from each other. This phylogeny also indicated that *G. latifolium* was the first species to diverge from the most recent common ancestor of all *Garuleum* species. Garuleum latifolium is sister to a group formed by two clades (A and B) which diverged from a common ancestor (Fig. 8.9). The first clade (A) consists of *G. album*, *G. schinzii*, *G. sonchifolium* and *G. woodii*. The second clade (B) consists of *G. bipinnatum*, *G. pinnatifidum* and *G. tanacetifolium*.

Distribution data of the *Garuleum* species indicated that some of the species may be altitude specific. The distribution of the *Garuleum* species is shown in figure 9.1. The disjunct distribution of *G. pinnatifidum* is an artefact of under collection of specimens in the southern parts of the Free Sate and Northern parts of the Eastern Cape. The altitude ranges in which each of the *Garuleum* species occur are shown in table 9.1.

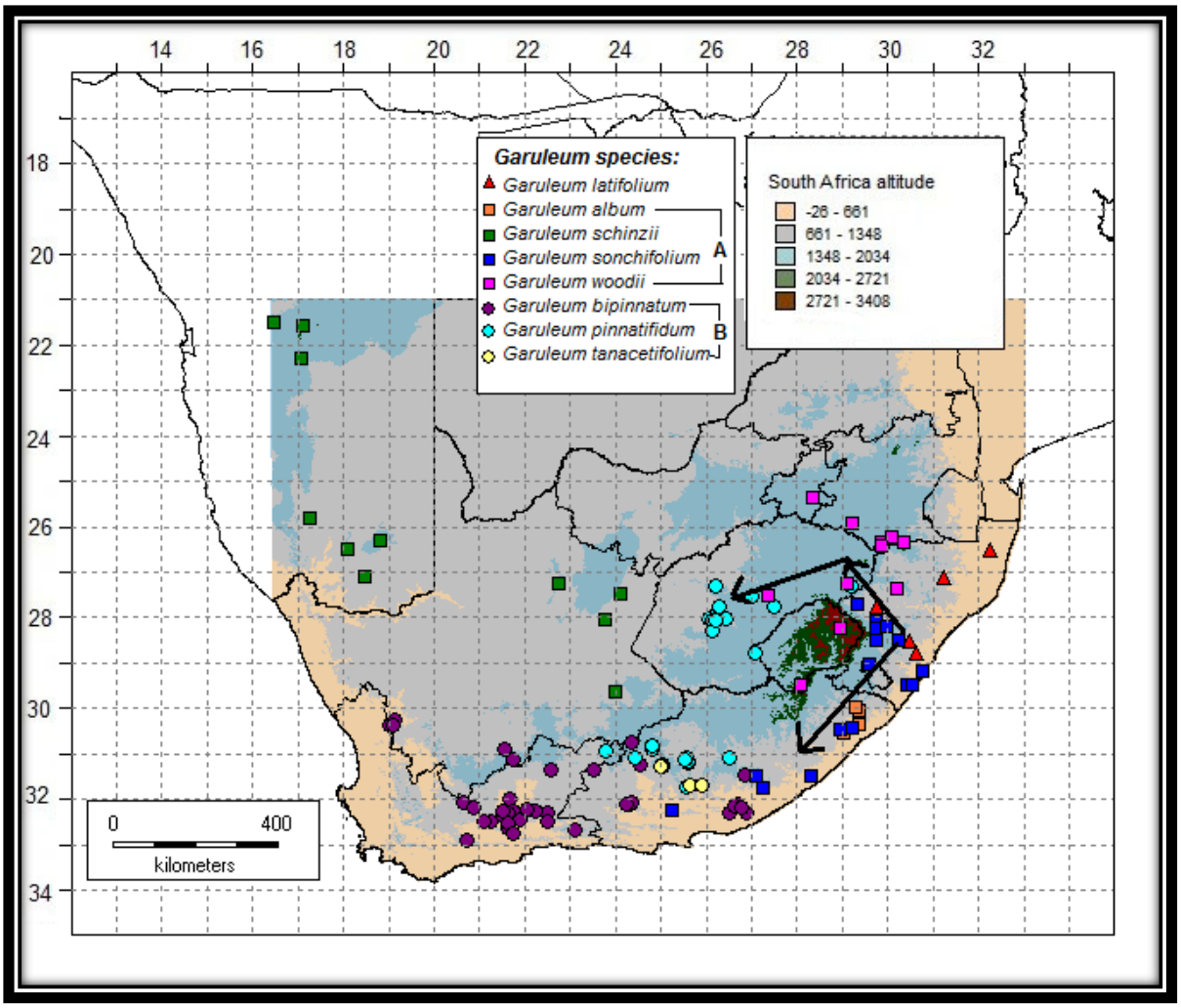

Figure 9.1 Distribution map of the different *Garuleum* species indicating the altitudinal range of each species. The species belonging to clade A of the combined nuclear and chloroplast gene tree are indicated with squares and species belonging to clade B are indicated with circles. *Garuleum latifolium* is indicated with a triangle. Arrows indicate possible origin and migration followed by speciation in *Garuleum*.

Table 9.1 The different altitude ranges at which the *Garuleum* species occur.

***Garuleum* species**	**Clade**	**Altitude ranges (m above sea level)**	
Garuleum album	A	610–710 m	Foothill
Garuleum bipinnatum	B	400–1350 m	Foothill to montane
Garuleum latifolium	*****	540–1200 m	Foothill to montane
Garuleum pinnatifidum	B	790–1524 m	Foothill to montane
Garuleum schinzii	A	1400–1716 m	Montane
Garuleum sonchifolium	A	1000–1860 m	Montane
Garuleum tanacetifolium	B	1675–1731 m	Subalpine
Garuleum woodii	A	1524–2200 m	Subalpine

Different climate gradients are associated with low and high altitudes and may have been a factor in speciation for some of the *Garuleum* species. UV-B radiation experienced by plants increases, atmospheric pressure decreases, the air temperature decreases and the growing season shortens at higher altitudes (Körner, 2007). At high altitudes in the Drakensberg species have to adapt to very low temperatures, snow in the winter, frost until late October and strong winds. The south-east facing slopes are reported to be colder than north facing slopes. The south facing slopes are also moister in the winter due to a reduction in solar radiation experienced by these slopes. The Drakensberg is frequented with thunder storms and mist in the summer (Hilliard & Burtt, 1987). These conditions are similar at Boschberg, the Sneeuberge (Clark et al., 2009, 2011) and Platberg (Brand et al., 2008). Two phenomena associated with an increase in altitude are a decrease in competition and a decrease in the species pool size (Bruun et al., 2006).

A study on *Dendrosenecio* (Hauman ex Hedberg) B.Nord. (Asteraceae), which involved the construction of a phylogeny from chloroplast DNA, shows how altitude effects diversification of genera (Know and Palmer 1995). This phylogeny allowed the researchers to reconstruct the geographic and altitudinal radiation of the giant senecios into every part of Eastern Africa. They hypothesized that early diversification involved geographical movement from high altitudes among tall mountains and then into lower habitats. The importance of altitude as an environmental variable with a selective force on species diversification can also be seen in a study performed on *Mimulus cardinalis* Douglas ex Benth. and *M. lewisii* Pursh (Phrymaceae) (Angert and Schemske, 2005). Angert and Schemske (2005) showed that different phenotypes are necessary at low versus high altitudes as suggested by speciation at different elevation ranges by *M. cardinalis* and *M. lewisii*.

In clade A (Fig 8.8.), *G. album* is restricted to foothills altitudes, while *G. woodii* is restricted to the subalpine altitudes. *Garuleum schinzii* grows at montane altitudes while *G. sonchifolium* grows at low and montane altitudes (Fig. 9.1.). The difference in altitude may have resulted in the species undergoing allopatric speciation in the case of *G. woodii*, for this species had to adapt to the different environmental factors associated with subalpine altitudes and was thus separated geographically from the species growing at lower altitudes.

Mountains can act as barriers, which can be considered to have the same isolation effect as islands in the ocean (MacArthur and Wilson, 1967). The higher altitude at which *G. woodii* grows, would also mean less competition with other *Garuleum* species. In clade B, *G. bipinnatum* grows at foothill to montane altitudes, while *G. tanacetifolium* grows at subalpine altitudes and *G. pinnatifidum* grows at foothill to montane altitudes.

Another possible selective force for speciation in *Garuleum* is the amount of water available to the plant. In a study on *Melianthus* L. (Melianthaceae), the researchers found that *Melianthus* most likely adapted from moist habitats to survive in more arid habitats (Linder et al., 2006). The researchers of the *Melianthus* study almost misinterpreted their data, because the moisture requirement for *M. major* was misrepresented by the rainfall pattern of the region where the species grows. This species, although it grew in dry regions, was found to always grow near ground water or in streams, which meant it required a lot of moisture to grow (Linder et al., 2006). The transition from mesic to arid habitats has also been reported for several other taxa in South Africa (Linder and Mann, 1998; Verboom et al., 2003).

The annual precipitation for the different regions of South Africa is shown in figure 9.2. The combined data from figure 9.1 and figure 9.2 indicates that all the species in clade A, except *G. schinzii*, grow in regions where the annual precipitation is higher than 600 mm. This is also true for *G. latifolium*. The species of clade B and *G. schinzii* all grow in regions with an annual precipitation lower than 600 mm. Since *Garuleum* is not associated with seepage areas or marshy habitats the rainfall pattern will closely reflect the moisture requirements of the *Garuleum* species growing in a particular area.

The possibility that speciation was driven by water availability is supported by the leaf morphology of the different *Garuleum* species. In clade A, *G. schinzii* and *G. woodii* have compound leaves, while the rest of the species in clade A have simple leaves. *Garuleum latifolium* also has simple leaves. Water can be used more effectively by plants which have developed compound leaves (Dudley, 1996). The compound leaves have a reduced surface which reduces the amount of water lost through transpiration (Givnish, 1988).

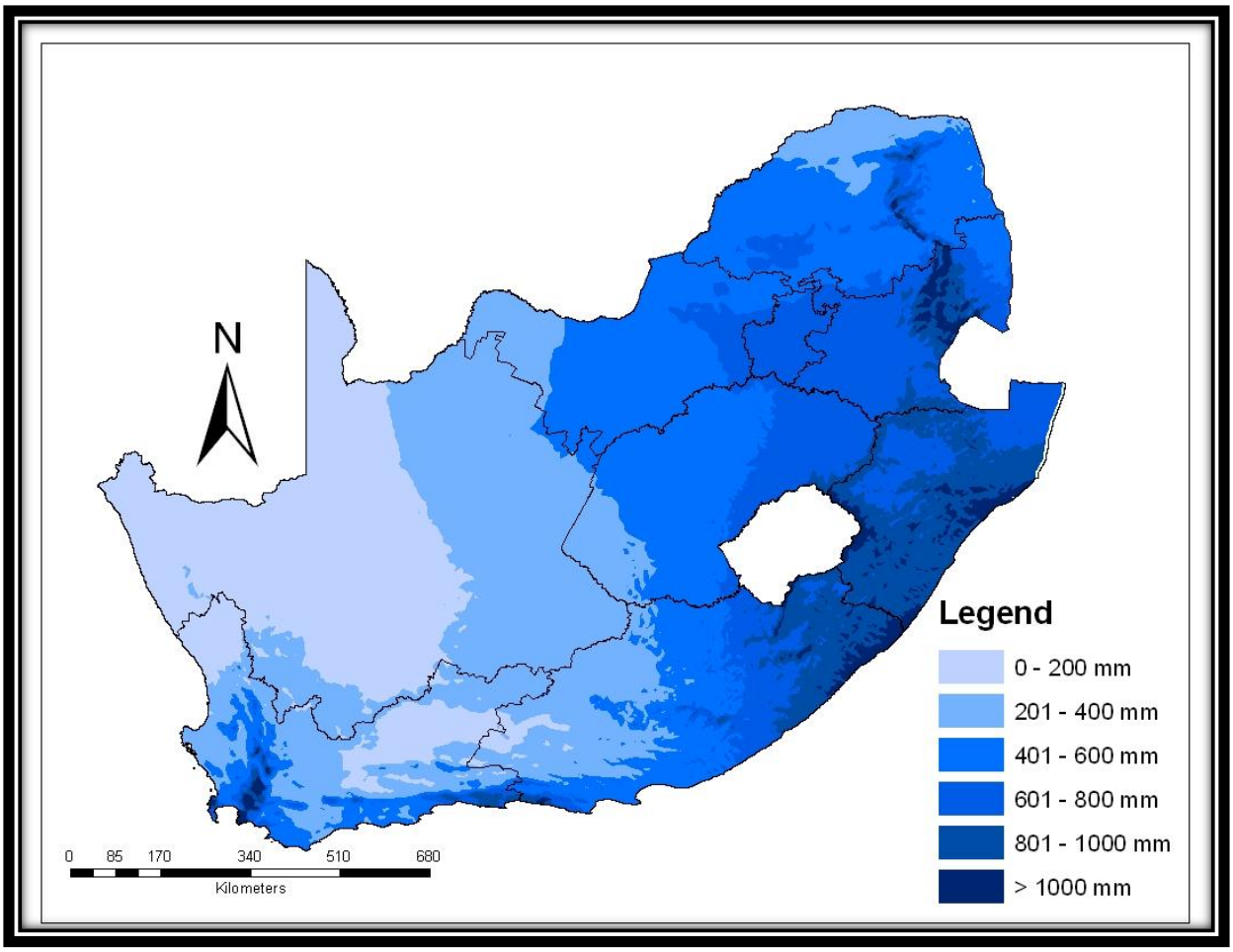

Figure 9.2 The annual precipitation of South Africa. Scale bar = 680 km. Map obtained from the Geography Department of the University of the Free State.

In the case of *G. woodii,* even though it grows in a region where the annual precipitation is higher than 600 mm, the species may have had to develop compound leaves to limit water loss, because it grows on rocky slopes or cliffs in weathered cave sandstone which has little water retention capabilities (Pooley, 2003). The large simple leaf blades of *G. album*, *G. latifolium* and *G. sonchifolium* are a characteristic associated with plants that grow in habitats where water is abundant and wind damage is minimal (Stern et al., 2008).

All the species in clade B (Fig. 8.9.) and *G. schinzii* have developed compound leaves and are found in regions where the annual precipitation is less than 600 mm, which support the necessity for these species to have this adaptation. Compound leaves evolved independently in clade A and clade B, which is consequently an example of convergence.

The micromorphology of the achene surfaces of the different species of *Garuleum* indicated that there is little variation between *G. latifolium* and *G. sonchifolium* (clade A). The differences between the two species were that the achene size and protuberances found on the surface of *G. sonchifolium* are larger than that of *G. latifolium*. The micromorphology of the achene surface is highly variable among all the *Garuleum* species. The achenes of *G. album* are obovoid and their surface smooth. *Garuleum pinnatifidum* had folded longitudinal ridges in the middle of the achene. *Garuleum schinzii* and *G. woodii* has cylindrical achenes with a folded and grooved surface, while *G. woodii* also had a prominent ridge on one side of the achene. *Garuleum tanacetifolium* has oblong achenes with slightly thickened margins and a warty surface. In clade B, *G. bipinnatum* produces two types of achenes, ray and disc floret achenes (heterocarpy). Both types of achenes are obovoid to obcordate and the ray floret surface is smooth while the disc floret surface is folded. In all the other *Garuleum* species only the ray florets produce achenes (homocarpy).Uncertainty exist about the production of achenes in *G. schinzii* due to the availability of only one herbarium specimen with achenes and thus not being able to confirm the possibility of heterocarpy without destroying the specimen.

Heterocarpy is an adaptive response associated with open unpredictable, arid environments with frequently disturbed habitats (conditions in habitat can change rapidly for example flash floods, fires, rain in the desert) (Cruz-Mazo et al., 2009). Heterocarpic plants reproduce under a variety of conditions and are not subject to a predictable development pattern (Ruiz De Clavijo, 2005). In order for the species to avoid population extinction, the species needs the ability to exploit new habitats fast (Valen, 1971). Heterocarpy is an advantageous character because it provides the plant with two reproductive strategies. The disc floret achenes are small with a wide dispersal area and cannot remain dormant for long periods of time. The ray floret achenes do not disperse far from the parent plant. The dormant ray achenes with their greater size and harder pericarp stay viable for long periods of time separating the genomes in space and time (Tanowitz et al., 1987). The shift from homocarpy to heterocarpy was reported in other genera in the Asteraceae, such as *Doronicum* L. and *Scorzoneroides* Moench. (Álvarez Fernández et al., 2001, Cruz-Mazo et al., 2009).

Garuleum bipinnatum possibly developed heterocarpy to be able to survive better in the frequently disturbed Great Karroo and Little Karroo, where this species grows. The advantage of having the adaptation of heterocarpy, can be seen when comparing the wide distribution of *G. bipinnatum* to that of other *Garuleum* species which reproduce through homocarpy. All homocarpic *Garuleum* species have smaller distribution ranges. The reason for suggesting that *G. schinzii* may also be heterocarpic, is because it also grows in the Great Karroo as well as the Namib Desert which are frequently disturbed, arid environments and this adaptation will be an advantage for the survival of this species. *Garuleum schinzii* also has a very wide distribution over Southern Africa which is similar to the distribution to the heterocarpic *G. bipinnatum*.

The morphological and micromorphological characters shared by *G. latifolium, G. album* and *G. sonchifolium* from clade A, in combination with the shared habitat preferences of these species, would suggest that these shared characters may be the plesiomorphic characters of *Garuleum*. Since the distribution ranges of these species converge in Kwazulu-Natal and northern Eastern Cape, speciation in this genus may have been initiated in this area.

If the current distribution of *G. latifolium* represents the previous distribution of the common ancestor of the genus, then the topology of the Drakensberg may have influenced the path of distribution and speciation of *Garuleum*. With the Drakensberg acting as a barrier, distribution of *Garuleum* to the western parts of the country, could be either southward along the coast or northward in the direction of Harrismith and then westward to the interior of South Africa. These migration routes are indicated by arrows in figure 9.2.

The speciation pattern of *Garuleum* species was probably due to the selective forces of both altitude and water availability. The moisture requirements and altitudinal range of the different species correlate with the distribution of the different *Garuleum* species throughout Southern Africa. The species *G. album*, *G. sonchifolium*, *G. latifolium* are species that are limited to low or medium altitudes and they would have spread to the western parts of South Africa along the coast. *Garuleum woodii* grows at high altitudes and is adapted to survive in more arid regions, which enabled this species to spread to the interior parts of Southern Africa. Where it developed homoplasious characters shared with *G. pinnatifidum*. *Garuleum pinnatifidum* is well adapted to the drier conditions and low to medium altitudinal ranges associated with the interior of Southern Africa. The low altitudinal range of *G. bipinnatum* and ability to reproduce through heterocarpy allowed for this species to have a wide distribution throughout the Western Cape. The speciation route can only be hypothesized, for the phylogeny obtained for *Garuleum* was not resolved enough to provide the precise route of speciation.

In the phylogeny obtained from the combined nuclear and chloroplast genes the position of *G. schinzii* are not very stable.The position of this species is only supported by a PP value of 0.76; which is below the general accepted value of 0.80. The position of *G. schinzii* also does not have any support from the bootstrap value obtained from the Mp analysis. This coupled with not sharing a habitat with an annual precipitation above 600 mm, like the rest of the species in clade A, make the position of this species in clade A uncertain. Two species that are geographically very close to each other have a better chance to be closely related (Irwin, 2002). Since *G. schinzii* is geographically closer to species in clade B this may support a phylogenetic relationship similar to that shown in the ITS tree (Fig. 8.2).

In conclusion the genus *Garuleum* is monophyletic. The position of *G. latifolium* and *G. schinzii* is still uncertain in the phylogeny obtained from the combined ITS and *trn*L–trnF gene regions. If *G. latifolium* is accepted as the first species to have diverged from the most recent common ancestor, then the pleisiomorphic state of this genus may have been adaptions to moist, stable environments with summer rainfall and homocarpy. The species may then have transitioned to be able to exploit and survive in more arid regions.

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Summary

The aim of this study is to provide a taxonomic revision and molecular phylogenetic analysis of the eight *Garuleum* Cass. (Asteraceae) species of southern Africa. The taxonomic revision resulted in the construction of an identification key to the species, a revision of all type literature and nomenclature, clarification and designation of type specimens, compilation of morphological and micromorphological descriptions, distribution maps and ecological data for all *Garuleum* species. The molecular phylogenetic investigation resulted in the first complete phylogeny for the genus *Garuleum*, based on the nuclear gene region ITS and the chloroplast intergenic spacers *trn*T–*trn*F and *psb*A–*trn*H.

All available type specimens related to *Garuleum* in southern Africa were studied. Where holotype specimens could not be located; lectotypes were designated from available isotypes or syntypes. In cases where only syntypes had been given by authors of species names, lectotypes were designated. Additional herbarium specimens on loan from several South African and European herbaria were investigated and additional material was collected in the field. These specimens were used to compile morphological descriptions and distribution maps. Micromorphological investigation of leaf surfaces, fruit, flowers and pollen was also completed.

Garuleum species are aromatic, herbaceous shrubs with the inflorescence arranged in a capitulum composed of white or mauve coloured ray florets and yellow disc florets. The abaxial and adaxial leaf surfaces, leaf structure and involucral bract sizes are important for species identification.

Garuleum is widely distributed over southern Africa, with the majority of the species found in the Eastern Cape. Some species, such as *G. album,* have restricted distribution ranges while others, such as *G. bipinnatum,* are very common and widely distributed. The annual precipitation of a specific region, in addition to altitude are possibly important factors influencing the biogeography and evolution of the eight species. Heterocarpy has evolved in *G. bipinnatum,* providing this species with an ability to survive in frequently disturbed areas and to distribute widely. *Garuleum*

schinzii also has a wide distribution and may have the ability to reproduce through heterocarpy. The rest of the *Garuleum* species have more restricted distribution ranges and only reproduce through homocarpy.

The micromorphological characters of the leaves of *Garuleum* are not taxonomically informative, due to their intraspecific variability. Micromorphological analysis of the flowers of the different species indicated that trichome complement on ray- and disc floret corollas may be taxonomically important. The micromorphology of the epidermal surfaces of achenes from different species is informative for species identification. The micromorphology of the pollen is not sufficiently variable for species level identification, but does support the monophyly of the genus.

The phylogeny obtained from the combined nuclear and chloroplast regions revealed that *G. latifolium* may belong to the earliest diverging lineage within the genus. The rest of the species are divided into two sister clades. Clade A consists of *G. album*, *G. sonchifolium, G. schinzii* and *G. woodii*. Clade B consists of *G. bipinnatum*, *G. pinnatifidum* and *G. tanacetifolium*. The position of *G. schinzii* in clade A is poorly supported, while the morphology and geographic proximity of this species to the species in clade B, suggests that *G. schinzii* belongs in clade B. This phylogeny, in combination with geographical, micromorphological and morphological data provided insight into a possible point of origin for the genus in Kwazulu-Natal or the northern parts of the Eastern Cape. The topography of the Drakensberg may have influenced the distribution routes of the different species, forcing distribution either along the coast or into the interior of Southern Africa by acting as a barrier.

Keywords: Asteraceae, *Garuleum*, Calenduleae, ITS, palynology, phylogeny, systematics, taxonomy, *psb*A–*trn*H, *trn*T–*trn*L.

Opsomming

Die doel van hierdie studie is die samestelling van ‘n taksonomiese hersiening en molekulêre filogenetiese analise van die agt *Garuleum* Cass. (Asteraceae) spesies van Suider Afrika. Die taksonomiese hersiening het tot die konstruksie van ‘n identifikasiesleutel vir die spesies, ‘n hersiening van alle tipe literatuur en nomenklatuur, opklaring en aanwysing van tipe eksemplare, samestelling van morfologiese en mikromorfologiese beskrywings, verspreidingskaarte en ekologiese data oor al die *Garuleum* spesies gelei. Die molekulêre filogenetiese ondersoek het tot die eerste volledige filogenie vir die genus *Garuleum*, gelei en is gebaseer op die die kerngeenstreek ITS en chloroplast intergeniese spasiëerder geenstreke *trn*T–*trn*F en *psb*A–*trn*H.

Alle beskikbare tipe-eksemplare met betrekking tot *Garuleum* in Suider Afrika is bestudeer. Waar geen holotipe eksemplare gevind is nie, is geskikte iso- of sintipes tot lektotipes verklaar. In gevalle waar slegs sintipes aangewys is deur outeurs van spesiename is lektotipes verklaar. Addisionele herbariumeksemplare op lening vanaf verskeie Suid Afrikaanse en Europese herbaria is bestudeer en addisionele materiaal is in die veld versamel. Hierdie eksemplare is gebruik vir die saamstel van morfologiese beskrywings en verspreidingskaarte. Mikromorfologiese studies van die blaar oppervlaktes, vrugte, blomme en stuifmeel is ook voltooi.

Garuleum spesies is aromatiese, kruidagtige struike, met bloeiwyses wat in ‘n hofie gerangskik is en bestaan uit wit tot perskleurige lintblomme en geel buisblomme. Die boonste en onderste blaaroppervlaktes, blaarvorm en die groottes van die skutblare is belangrik vir spesie identifikasie.

Garuleum het ‘n wye verspreiding oor Suider Afrika, maar die meerderheid van die spesies kom in die Oos-Kaap voor. Sommige spesies, soos *G. album,* het ‘n beperkte verspreiding terwyl ander spesies, soos *G. bipinnatum,* meer algemeen en wydverspreid voorkom. Die jaarlikse gemiddelde reënval van ‘n spesifieke gebied asook hoogte bo seevlak mag dalk belangrike faktore wees wat die biogeografie en evolusie van die agt spesies beïnvloed het. Heterokarpie is deur *G. bipinnatum*

ontwikkel en verleen aan hierdie spesie die vermoë om in dikwels-versteurde omgewings te oorleef en wyd te verprei. *Garuleum schinzii* het ook ‘n wye verspreiding en mag ook die vermoë hê om twee soorte sade te produseer. Die res van die *Garuleum* spesies het meer beperkte verspeidings en voortplanting geskied slegs deur homokarpie.

Die mikromorfologiese kenmerke van die blare is nie van taksonomiese belang nie, as gevolg van die hoë mate van intraspesifieke variasie in die kenmerke. Mikromorfologiese analise van die blomme van die verskillende spesies dui daarop dat trigoomkompliment of die lint- en buisblom kroonblare taksonomies belangrik mag wees. Die mikromorfologie van die epidermale oppervlakke van dopvruggies van die verskillende spesies is informatief vir spesie identifikasie. Die mikromorfologie van die stuifmeel is nie variëerbaar genoeg vir spesie identifikasie nie, maar ondersteun wel die monofilie van die genus.

Die filogenie verkry vanaf die gekombineerde kern- en chloroplast geenstreke het onthul dat *G. latifolium* moontlik behoort aan die eerste stamlyn wat van die gemeenskaplike voorouer gedivergeer het. Die res van die spesies word verdeel in twee suster klades. Klade A wat bestaan uit *G. album*, *G. sonchifolium*, *G. schinzii* en *G. woodii*. Klade B wat bestaan uit *G. bipinnatum*, *G. pinnatifidum* en *G. tanacetifolium*. Die posisie van *G. schinzii* in klade A word swak ondersteun, terwyl die morfologie en geografiese nabyheid van hierdie spesie aan die spesies in klade B voorstel dat *G. schinzii* in klade B hoort. Die filogenie, in kombinasie met die geografiese, mikromorfologiese en morfologiese data, verskaf insig oor ‘n moontlike punt van oorsprong vir die genus in Kwazulu-Natal of in die noordelike gedeeltes van die Oos-Kaap. Die topografie van die Drakensberge mag die verspreidingsroetes beinvloed het van die verskillende spesies langs die kus en na die binneland toe deur as ‘n verspering op te tree.

Sleutelwoorde: Asteraceae, Calenduleae, filogenie, *Garuleum*, ITS, palinologie, *psb*A–*trn*H, sistematiek, taksonomie, *trn*T–*trn*L.

Addendum I

Herbarium specimens of *Garuleum* species examined.

G. album

— 30º58'58" S, 29º19'13" E: South Africa, Eastern Cape, Tabankulu, Oala above road to Gomo forest, 15 March 1995, *Wopula, L.D.*, 153 (NH)

— 31º04'25" S, 29º24'05" E: South Africa, Eastern Cape, Tabankulu, On road from Tabankulu to Lusikisiki, 22 June 1995, *Phillipson, P.*, *Dold, T; Cloete, E.* 4326 (GRA)

— 31º22'30" S, 29º22'30" E: South Africa, Eastern Cape, (between) Umtata et (and) Port St Johns, January 1896, *Bolus, H.*, BH 8718 (BOL); *Bolus, H.*, BH 10160 (BOL);

— 31º32'30" S, 29º02'72" E: South Africa, Eastern Cape, Umtata et St Johns, Mlegana, 18 January 1910, *Pegler, A.*, 1569 (BOL); South Africa, Eastern Cape, Port St Johns, Between Port St Johns and Umtata, 9 June 1941, *Kannemeyer, D.V.*, (NH); South Africa, Eastern Cape, Umtata, Near Khoweni forest, Libode area, 28 April 2006, *Styles, D.*, 3072 (NH)

G. bipinnatum

— 31º15' S, 19º07' E: South Africa, Northern Cape, Nieuwoudville reserve, Dolorite koppies north end, 15 June 1983, *Perry*, *Snijman, D.* 2119 (NBG); 25 July 1983, *Perry*, *Snijman, D.* 2171 (NBG)

— 31º22'45" S, 19º06'28" E: South Africa, Northern Cape, Nieuwoudville, 10 km NW of Nieuwoudville, July 1930, *Lavis, M.*, *s.n.* (BOL)

— 31º23'20" S, 19º01'36" E: South Africa, Western Cape, Van Rhyns dorp, Van Rhyns Pass, 25 July 1941, *Compton, R.H.*, 11124 (BOL)

— 31º44'22" S, 24º20'13" E: South Africa, Eastern Cape, Graaff-Reinet, Sneeuberg, in valley, May 1889, *Bolus, H.*, (Z); *McOwan, P.*, 945 (Z)

— 31º53'01" S, 21º32'49" E: South Africa, Northern Cape, Fraserburg, 11 February 1930, *Nel, G.C.*, 15830 (NBG)

— 32º07' S, 21º45' E: South Africa, Northern Cape, Fraserburg; Layton, opposite perdeberg kraal, 20 July 1992, *Shearing, D.*, A 68 (NBG)

— 32º15'08" S, 24º32'26" E: South Africa, Eastern Cape, Graaff-Reinet, April 1890, *Bolus, H.*, 849 (BOL)

— 32º20'52" S, 23º30'18" E: South Africa, Western Cape, Oorlogspoort, 7 May 1990, *Powrie, L.*, 965 (GRA)

— 32º22'12" S, 22º34'06" E: South Africa, Western Cape, Commonage Beaufort Wes, Top of Dam Koppie, 8 June 1987, *Shearing, D.*, K 207 (GRA)

— 32º28'49" S, 26º50'70" E: South Africa, Eastern Cape, Grahamstown, Between Fort Beaufort & Grahamstown, 18 July 2001, *Koekemoer, M.*, 2077 (PRE)

— 32º59'57" S, 21º41'09" E: South Africa, Western Cape, Prince Albert, Prince Albert road, May 1920, *Marloth, R.*, 63 (NBG)

— 33º04'12" S, 20º39'51" E: South Africa, Western Cape, Laingsburg, Road to Sutherland Farm: Joseph's kraal, 30 August 2006, *Roux, J.P.*, 4141 (NBG)

— 33º04' S, 24º21' E: South Africa, Eastern Cape, Jansenville, Teasdale, 10 March 1955, *Hoffman, T.*, 574 (GRA)

— 33º06' S, 24º18' E: South Africa, Eastern Cape, Teasdale, Klipplaat, 2 May 1985, *Hoffman, T.*, 773 (GRA); Andries Vosloo Kudu reserve, 40 km N.E. from Grahamstown, 3 April 1980, *Palmer, A.R.*, 405 (GRA)

— 33º06' S, 26º42' E: South Africa, Eastern Cape, Andries Vosloo Kudu reserve, 40 km N.E. of Grahamstown, 3 August 1981, *Palmer, A.R.*, 1003 (GRA)

— 33º07' S. 24º15' E: South Africa, Eastern Cape, Steytlerville, 3 October 1971, *Bayliss, R.D.A.*, 4889 (GRA)

— 33º08'37" S, 26º37'71" E: South Africa, Eastern Cape, Grahamstown, Near Andries Vosloo park, 26 November 2011, *Van Zyl, J.*, 9, 10, 11, 12, 13, 15, 16 (BLFU)

— 33º11'43" S, 20º51'35" E: South Africa, Western Cape, Laingsburg, 24 January 1941, *Bond, P.*, 831 (NBG)

— 33º11'48" S, 20º51'48" E: South Africa, Western Cape, Laingsburg, Ngaapkop, 27 September 1926, *Compton, R.H.*, 3092 (BOL)

— 33º13'31" S, 22º01'48" E: South Africa, Western Cape, Prince Albert, 11 May 1965, *Bayliss, R.D.A.*, 2845 (Z)

— 33º15' S, 21º30' E: South Africa, Southern Cape, Gamka-poort Nature reserve, Witpoort NE boundary veld near main gate, 4 November 1982, *Cattell, P*, *Cattel, J.* 220 (PRE)

— 33º15' S, 21º45' E: South Africa, Southern Cape, Gamkapoort Nature reserve, P47 near workshop complex, 13 May 1989, *Erasmus, R.*, 194 (NBG)

— 33º15' S, 22º15' E: South Africa, Western Cape, Prince Albert, Boschluiskloof, 16 July 1954, *Lewis, G.J.*, 68695 (PRE)

— 33º17'05" S, 21º37'16" E: South Africa, Southern Cape, Gamkapoort Nature reserve, W. of Dwyka river, S. slope of 2nd highest ridge, 6 September 1983, *Laidler, D.F.*, 650 (NBG)

— 33º18'04" S, 26º53'58" E: South Africa, Eastern Cape, Grahamstown, Near Frasers camp, 30 November 1950, *Barker, W.F.*, 6973 (NBG)

— 33º18'14" S, 26º30'07" E: South Africa, Eastern Cape, Grahamstown, on ecca shale hummocks on road to Ballinafad, 24 July 1981, *Brink, E.*, 727 (GRA)

— 33º18'53" S, 22º30'04" E: South Africa, Western Cape, Prince Albert, Eikenkraal; between Prince Albert and Klaarsdorp, 27 September 1935, *Leipoldt, C.L.*, *s.n.* (BOL)

— 33º22'06" S, 21º29'53" E: South Africa, Western Cape, Prince Albert, Bosluiskloof Pass, 16 July 1954, *Rycroft, H.B.*, 1618 (NBG)

— 33º26'43" S, 21º53'20" E: South Africa, Western Cape, Oudtshoorn, Kuilsriver road to Calitzdorp on Shale hills, 25 September 2004, *Goldblatt, P.*, *Porter, L.J.* 12557 (NBG)

— 33º28'43" S, 21º15'42" E: South Africa, Western Cape, Ladismith, Near NW foot of town's berg, 16 July 1967, *Levyns, M.R.*, 11604 (BOL)

— 33º29' S, 21º07' E: South Africa, Western Cape, Ladismith, South of Ladismith along road, 26 August 1988, *Bohnen, P.*, 8891 (NBG)

— 33º29' S, 21º07' E: South Africa, Southern Cape, Noukloof nature reserve, 1.9km from dam on circular drive, 6 July 1982, *Laidler, D.*, 59 (PRE)

— 33º29' S, 22º30' E: South Africa, Western Cape, De Rust, Doornkraal, 7.5 km E from De Rust, 19 October 1970, *Dahlstrand, K.A.*, 1461 (PRE)

— 33º29' S, 22º30' E: South Africa, Western Cape, De Rust, Doornkraal, 29 September 1968, *Dahlstrand, K.A.*, 1503 (GRA); 19 October 1970, *Dahlstrand, K.A.*, 9426 (NBG)

— 33º30'59" S, 21º38'41" E: South Africa, Western Cape, Huis river pass, Steap rocky hillside, 31 July 1955, *Van Niekerk, G.*, 539 (BOL)

— 33º32'14" S, 21º41'07" E: South Africa, Western Cape, Calitzdorp, Gamka Mtu, Tierkloof, 9 June 1975, *Boshoff, A.F.*, P 175 (NBG)

— 33º37'30" S, 21º37'30" E: South Africa, Southern Cape, Gamka mountain reserve, Site behind reservoir, 17 May 1982, *Cattell, P.*, *Cattell, J.* 19 (NBG)

— 33º40'18" S, 23º06'05" E: South Africa, Western Cape, Uniondale, Vet vlei, July 1928, *Markotter, E.J.*, 8864 (NBG)

— 33º45' S, 21º45' E: South Africa, Southern Cape, Farm Kleinfontein, 8 August 1984, *Laidler, D.F.*, 576 (PRE)

— 33º54'25" S, 20º43'06" E: South Africa, Western Cape, Barrydale, Muintjieskraal, September 1913, *Muir, J.*, 1037 (NH)

— Coordinates unknown: South Africa, May 1928, *Dyer, R.A.*, 1536 (PRE)

— Coordinates unknown: South Africa, April, *Burke, J.*, *s.n*. (L)

— Coordinates unknown: South Africa, *Bolus, H.*, *s.n*. (Z) (Z-000078511, Z-000078512)

— Coordinates unknown: South Africa, *Ecklon, C.F.*, *s.n*. (L) (L0834238, L0834242)

— Coordinates unknown: South Africa, *McOwan, P.*, 945 (Z)

— Coordinates unknown: South Africa, *Unknown*, 232 (L)

— Coordinates unknown: South Africa, *Unknown*, *s.n*. (L) (L0834239, L0834240)

G. latifolium

— 28º45' S, 29º45' E: South Africa, Kwazulu-Natal, Weenen Nature reserve, 2 February 1988, *Huse, J.*, 19.1 (NH)

— 29º30' S, 30º30' E: South Africa, Kwazulu-Natal, Pietermaritzburg, Camperdown, 11 February 1908, *Franks, M*, 10860 (NH)

— 29º46' S, 30º39' E: South Africa, Kwazulu-Natal, Durban, Hammersdale area. Hector Eskom substation site, 29 March 1995, *Ward, C.J.*, 12972 (NH)

— Coordinates unknown: South Africa, Kwazulu-Natal, February 1884, *Wood, J.M.*, 160 (BOL)

— Coordinates unknown: South Africa, Kwazulu-Natal, April 1884, *Wood, J.M.*, 299 (Z)

— Coordinates unknown: South Africa, Kwazulu-Natal, 14 April 1891, *Wood, J.M.*, 4466 (NH)

— Coordinates unknown: South Africa, Kwazulu-Natal, *Wood, J.M.*, *s.n.* (Z)

— Coordinates unknown: South Africa, Kwazulu-Natal, Zululand, Mkuze, August 1932, *Galpin, E.E.*, 21496 (BOL); Alexandra district, 22 January 1913, *Rudatis, A.G.H.*, 1864 (L)

— Coordinates unknown: South Africa, Kwazulu-Natal, Nkandla, Mahlabatini, April 1933, *Gerstner, J. Rev.*, (NH)

G. pinnatifidum

— 27º25'07'' S, 29º53'33'' E: South Africa, Kwazulu-Natal, Charlestown, Near Charlestown Drakensberg, 10 January 1894, *Wood, J.M.*, 5185 (BOL), (Z)

— 28º16'84'' S, 29º12'02'' E: South Africa, Free State, Bloemfontein, U.F.S Botanical gardens, 28 May 1968, *Müller, D.B.*, 285 (NBG); 3 March 2011, *Van Zyl, J.*, 1 (BLFU)

— 28º45' S, 26º17' E: South Africa, Free State, Glen, Near Modderriver, 22 November 1979, *Rossouw, R.*, 61 (BLFU)

— 28º32'02" S, 27º01'00" E: South Africa, Free State, Winburg, 20 January 1936, *Gillet, M.C.*, 1110 (BOL)

— 28º45' S, 27º30' E: South Africa, Free State, Bloemfontein, Mequatling; Clocalan, 23 November 1969, *Van Zinderen Bakker, E.M.*, 179 (BLFU)

— 29º00' S, 26º07' E: South Africa, Free State, Bloemfontein, Grant's hill near top, 13 April 1917, *Greys college herbarium collector*, 2646 (BLFU)

— 29º01'40" S, 26º02'24" E: South Africa, Free State, Bloemfontein, Poundisford on Kopie, December 1915, *Potts, G*, 500 (BLFU)

29º02'19" S, 26º25'96" E: South Africa, Free State, Maselspoort, 18 February 2003, *Venter, H.J.*, 9897 (BLFU)

— 29º03'02" S, 26º12'79" E: South Africa, Free State, Bloemfontein, Free State Botanical gardens, 10 March 2011, *Van Zyl, J.*, 2 (BLFU)

— 29º03'07" S, 26º12'75" E: South Africa, Free State, Bloemfontein, Free State Botanical gardens, 10 March 2011, *Van Zyl, J.*, 3 (BLFU)

— 29º03'17" S, 26º12'78" E: South Africa, Free State, Bloemfontein, Free State Botanical gardens, 10 March 2011, *Van Zyl, J.*, 4 (BLFU)

— 29º03'31" S, 26º07'27" E: South Africa, Free State, Bloemfontein, 1875, *Rehmann, A.*, 3781, 3782 (Z)

— 29º07' S, 26º07' E: South Africa, Free State, Bloemfontein, Bayswater farm, 1924, *Beck, C.A.*, 3222 (BLFU)

— 29º16'13" S, 26º08'29" E: South Africa, Free State, Kaalspruit, *Rogers, F.A.*, 18494 (Z)

— 29º46'19" S, 27º04'33" E: South Africa, Free State, Wepener, 29 January 1945, *Acocks, J.P.H.*, 11169 (L)

— 31º49' S, 24º49' E: South Africa, Eastern Cape, Middelburg, Blaauwater, Sneeuberg, 4 December 1926, *Gill, A.*, 57 (BOL)

— 31º53'33" S, 24º48'33" E: South Africa, Northern Cape, Richmond, Leopard's vlei, December 1917, *Bolus, H.*, 14097 (BOL)

— 31º56'59" S, 23º46'01" E: South Africa, Eastern Cape, Murraysburg, *Tyson, W.*, 143 (BOL); "The Cave", October 1879, *Tyson, W.*, 248 (BOL)

— 32º04'33" S, 25º34'48" E: South Africa, Eastern Cape, Somerset East, Visch rivier (Fish river), July 1888, *McOwan, P.*, 929 (BOL) (Z); June, *McOwan, P.*, 1633 (Z) (Z-000078520, ZT-00014705)

— 32º06'19" S, 24º25'49" E: South Africa, Eastern Cape, Graaff-Reinet, Oudeberg, 30 March 1866, *Bolus, H.*, BH 4 (BOL)

— 32º08'28" S, 25º30'39" E: South Africa, Eastern Cape, Cradock, 1861, *Cooper, T.*, 485 (Z) (Z-000078520, Z-000078522)

— 32º10'08'' S, 25º36'50'' E: South Africa, Eastern Cape, Cradock, January 1901, *Kensit, L.*, 9275 (BOL)

— 32º11'35'' S, 25º36'37'' E: South Africa, Eastern Cape, Cradock, South of Cradock, 1 December 1950, *Hall, H.*, 254 (NBG)

— Coordinates unknown: South Africa, Mpumalanga, Baberton ?, 1912, *Rogers, F.A.*, *s.n.* (BOL)

— Coordinates unknown: South Africa, 11 May 1990, *Palmer, A.R.*, 2921 (GRA)

— Coordinates unknown: South Africa, *Herb. Reinwardt, C.G.C.*, 3919 (L)

— Coordinates unknown: South Africa, *Herb, Splitgerder, F.L.*, *s.n.* (L)

— Coordinates unknown: South Africa, *Zeyher, C.L.P.*, 102 (L)

— Coordinates unknown: South Africa, *Unknown*, *s.n*. (L) (L0834224, L0834225, L0834226, L0834227, L0834229)

G. schinzii

— 22º30' S, 16º30' E: Namibia, Otjimbingwe, Khomas, 9 July 1950, *Nortier, P.*, *s.n.* (BOL)

— 22º34'47'' S, 17º07'37'' E: Namibia, Khomas, Avis dam area, 15 February 2004, *Mannheimer, C.A.*, 2882 (GRA)

— 22º35'01'' S, 17º06'38'' E: Namibia, Windhuk berg area, Avis, 13 June 1963, *Seydel, R.*, 3555 (L)

— 23º18'05'' S, 17º04'31'' E: Namibia, Rehoboth, Buellspoort, 26 March 1945, *Compton, R.H.*, 2305 (BOL), (GRA)

— 26º50'34'' S, 17º17'25'' E: Namibia, Karas, 2 August 1925, *Dinter, K.*, 4810 (Z) (Z-000061153, Z-000078513)

— 27º30' S, 18º07' E: Namibia, Grunau, Grabwasser, May 1961, *Littlewood, R.C.*, *s.n.* (NBG)

— 27º18'49'' S, 18º49'23'' E: Namibia, Nanuda + Knei Kluft, Great Karasberg, 1912, *Pearson, H.H.W.*, 7935 (BOL)

— 28º07' S, 18º30' E: Namibia, Keetmans hoop, 145 km N from Karasburg, 8 July 1954, *Schelpe, A.S.L.*, 149 (BOL)

— 28º15' S, 22º45' E: South Africa, Northern Cape, Glen Lyon, Floradale, April 1940, *Esterhuysen, E.*, 2325 (BOL)

— 28º30' S, 24º07' E: South Africa, Northern Cape, Kimberley, Schmidts Drift, March 1938, *Wilman, M.*, 5369 (BOL)

— 29º03'31'' S, 23º46'11'' E: South Africa, Northern Cape, Douglas, 1896, *McOwan, P.*, 1889 (Z)

— 30º39'33'' S, 24º00'46'' E: South Africa, Northern Cape, De Aar, 237.5 km from De Aar, 1928, *Pole-Evans, I.B.*, 2310 (BOL)

— Coordinates unknown: July 1894, *Marloth, R.*, 2043 (L)

— Coordinates unknown: 21 April 1885, *Schinz, H.*, 698 (Z)

— Coordinates unknown: 28 September 1963, *Seydel, R.*, 3643 (L)

— Coordinates unknown: 1898, *Dinter, K.*, 7 (Z)

— Coordinates unknown: *Dinter, K.*, 346 (Z)

G. sonchifolium

— 28º43'50'' S, 29º21'04'' E: South Africa, Free State, Bergville, *Mnweni* area Drakensberg slopes N aspect, July 1958, *Esterhuysen, E.*, 27845 (BOL)

— 29º07' S, 29º45' E: South Africa, Kwazulu-Natal, Estcourt, 15km S Estcourt at Glenbella, 26 March 1988, *Green, D.*, 521 (NH)

— 29º07' S, 29º45' E: South Africa, Kwazulu-Natal, Estcourt, 16 April 1967, *Wrigth, F.B.*, 164 (NH)

— 29º12'38'' S, 30º00'10'' E: South Africa, Kwazulu-Natal, Mooi River, 22 February 1895, *Schlechter, R.*, 6835 (L), (Z) (Z-000061142, Z-000061143, ZT-00014702)

— 29º15' S, 29º45' E: South Africa, Kwazulu-Natal, Nothingham Rd., March 1939, *McClean, A.P.D.*, 922 (NH)

— 29º30' S, 29º45' E: South Africa, Kwazulu-Natal, Sevenfontein, 21 April 1905, *Wood, J.M.*, 10455 (NH)

— 29º30' S, 30º15' E: South Africa, Kwazulu-Natal, Hilton Rd., March 1930, *Ford, S.*, (NH)

— 30º01'49'' S, 29º36'12'' E: South Africa, Eastern Cape, Malowe, Griqualand East, February 1885, *Tyson, W.*, 1047 (BOL), (Z) (Z-000078505, Z-000078506, ZT-00014703)

— 30º01'49'' S, 29º36'12'' E: South Africa, Eastern Cape, Malowe, Griqualand East, February 1886, *Tyson, W.*, 1471 (Z)

— 30º01'49'' S, 29º36'12'' E: South Africa, Eastern Cape, Malowe, Griqualand East Malowe mountains, February 1885, *Tyson, W.*, 2080 (Z)

— 30º12'03'' S, 30º47'01'' E: South Africa, Kwazulu-Natal, Umkomaas, Near Umkomaas, 24 March 1892, *Wood, J.M.*, *s.n*. (Z)

— 30º30'00'' S, 29º93'33'' E: South Africa, Eastern Cape, Malowe, Clydesville, February 1885, *Tyson, W.*, 2080 (BOL)

— 30º86'67'' S, 29º15'00'' E: South Africa, Eastern Cape, Cedarville, Hoenyani, October 1920, *Bandert, E.*, 8 (BOL)

— 31º29'14" S, 28º58'34" E: South Africa, Eastern Cape, Nyandeni, Libode, Transkei, 12 May 1954, *Barker, W.F.*, 8237 (NBG)

— 32º29' S, 27º07' E: South Africa, Eastern Cape, Stutterheim, 21.25 km from Stutterheim on Keiskammahoek, 22 September 1954, *Marais, W.*, 526 (BOL)

— 32º29' S, 27º07' E: South Africa, Eastern Cape, Stutterheim, Keiskammahoek, April 1944, *Goulimis, C.*, *s.n*. (BOL)

— 32º30'31" S, 28º18'48" E: South Africa, Eastern Cape, Kentani, Edge of forest, 9 June 1905, *Pegler, A.*, 1199 (BOL)

— 33º15' S, 25º15' E: South Africa, Eastern Cape, Weza, Zuurberg, 3 March 1974, *Hillard, O.M.*, 5465 (NBG)

— 32º46'10" S, 27º16'00" E: South Africa, Eastern Cape, King William's town, Isedenga forest, 9 March 1964, *Balten, A.U.*, 1 Plate 54 (NBG)

— Coordinates unknown: South Africa, Buffelsberg, November, *MacOwan, P.*, 2015 (GRA), (NH), (Z) (Z-000061155, Z-000078517)

— Coordinates unknown: November 1901, *Herb. Alleizette, A.C d'*, 3919 (L)

— Coordinates unknown: 1860, *Cooper, T.*, 227 (NH), (Z)

— Coordinates unknown: June 1914, *Rogers, .F.A.*, 1284 (Z)

— Coordinates unknown: *Schlechter, R*., 6206 (Z)

— Coordinates unknown: *Unknown*, *s.n*. (L)

G. tanacetifolium

— 32º15'40" S, 25º00'67" E: South Africa, Eastern Cape, Graaff-Reinet, Asante Sana Suurkloof, 30 November 2011, *Van Zyl, J.*, 21, 22 (BLFU)

— 32º15'33" S, 25º01'19" E: South Africa, Eastern Cape, Graaff -Reinet, Asante Sana Suurkloof, 30 November 2011, *Van Zyl, J.*, 17, 18, 19, 20 (BLFU)

— 32º41'45" S, 25º38'23" E: South Africa, Eastern Cape, Boschberg, December 1881, *McOwan, P.*, 748 (Z)

— 32º42'04" S, 25º55'13" E: South Africa, Eastern Cape, Somerset East, Kagaberg, *MacOwan, P.*, 1382 (BOL), (Z) (Z-000061156, Z-000078519, ZT-00035531)

G. woodii

— 26º56'51" S, 29º14'49" E: South Africa, Mpumalanga, Standarton, 1875, *Rehmann, A.*, 6792 (Z) (Z-000061161, Z-000078524)

— 27º15' S, 30º07' E: South Africa, Kwazulu-Natal, Vryheid, Wakkerstroom, *Thode, J*, *s.n*. (NH)

— 27º21'38'' S, 29º53'41'' E: South Africa, Mpumalanga, Volksrust, 8 January 1932, *Galpin, E.E.*, *s.n*. (BOL)

— 27º25'07'' S, 29º53'33'' E: South Africa, Kwazulu-Natal, Charlestown, Valley of buffalo river, 6 December 1892, *Wood, J.M.*, 4840 (BOL)

— 27º25'07'' S, 29º53'33'' E: South Africa, Kwazulu-Natal, Charlestown, 10 January 1894, *Wood, J.M.*, 5185 (Z)

— 28º15' S, 29º07' E: South Africa, Free State, Harrismith, Loskop, 10 March 1970, *Stam, R.D.*, 420 (BLFU)

— 28º22'30'' S, 29º73'00'' E: South Africa, Free State, Harrismith, Platberg, 14 December 1976, *Jacobsz, M.L.*, 3054 (BLFU)

— 28º31'19'' S, 27º23'01'' E: South Africa, Free State, Ficksburg, Moolmans hoek peak, 30 October 1934, *Galpin, E.E.*, *s.n*. (BOL)

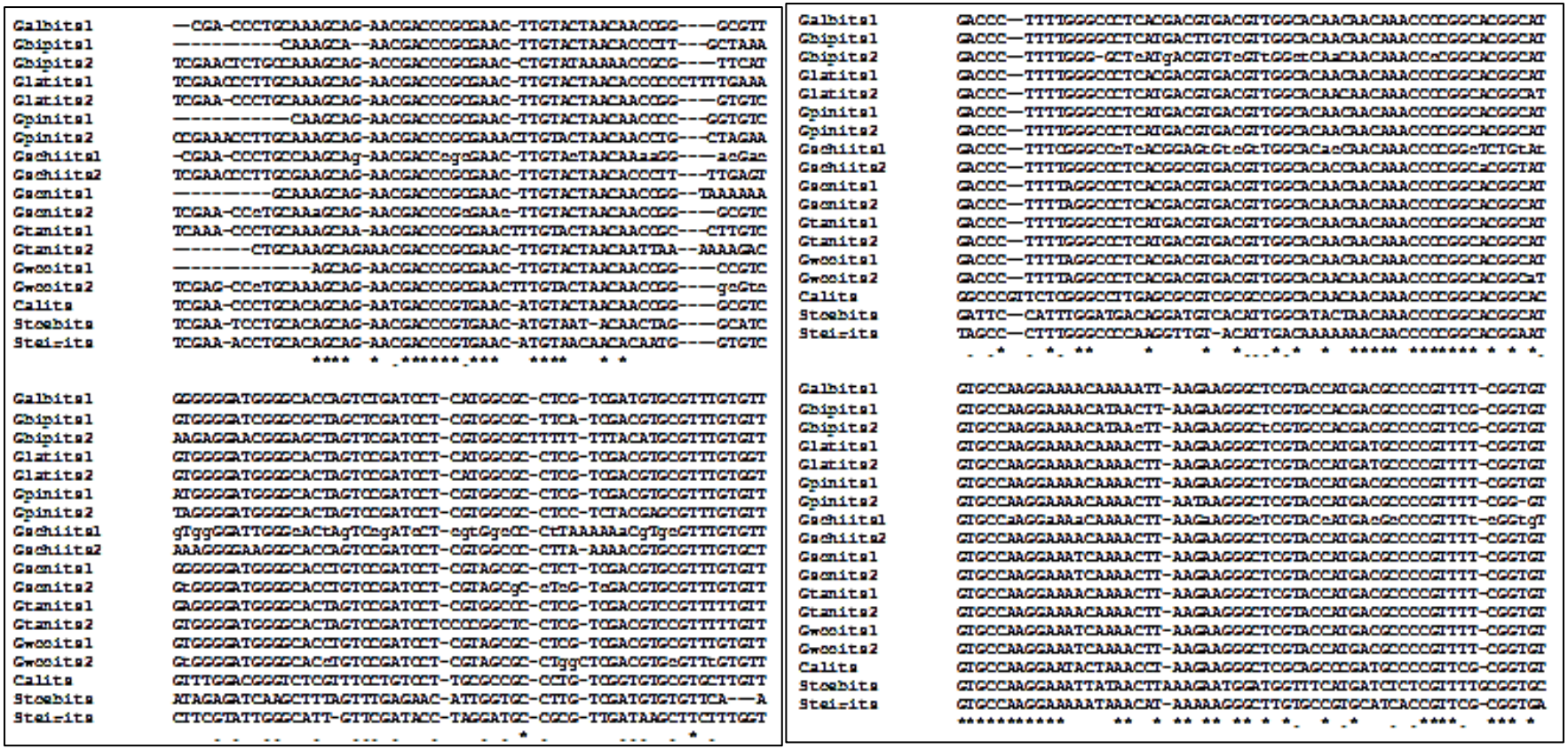
— 29º15'56'' S, 28º57'37'' E: Lesotho, Mokhotlong, 27 February 1949, *Compton, R.H*, 21539 (NBG)

— 30º30' S, 28º07' E: South Africa, Kwazulu-natal, Buffalo river, Waterfall, 15 March 1904, *Galpin, E.E.*, 6660 (BOL)

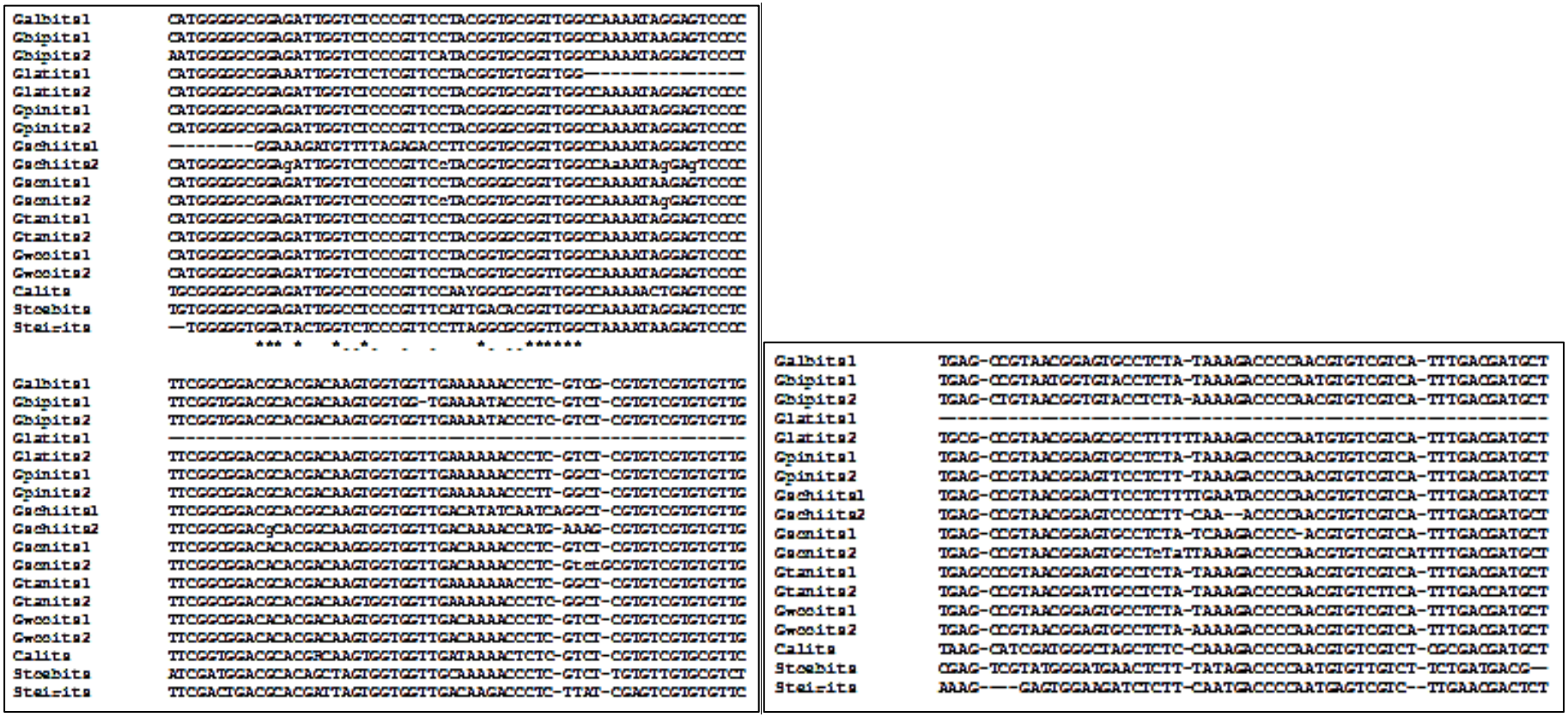
Addendum II

MAFFT alignments of nuclear and chloroplast DNA regions

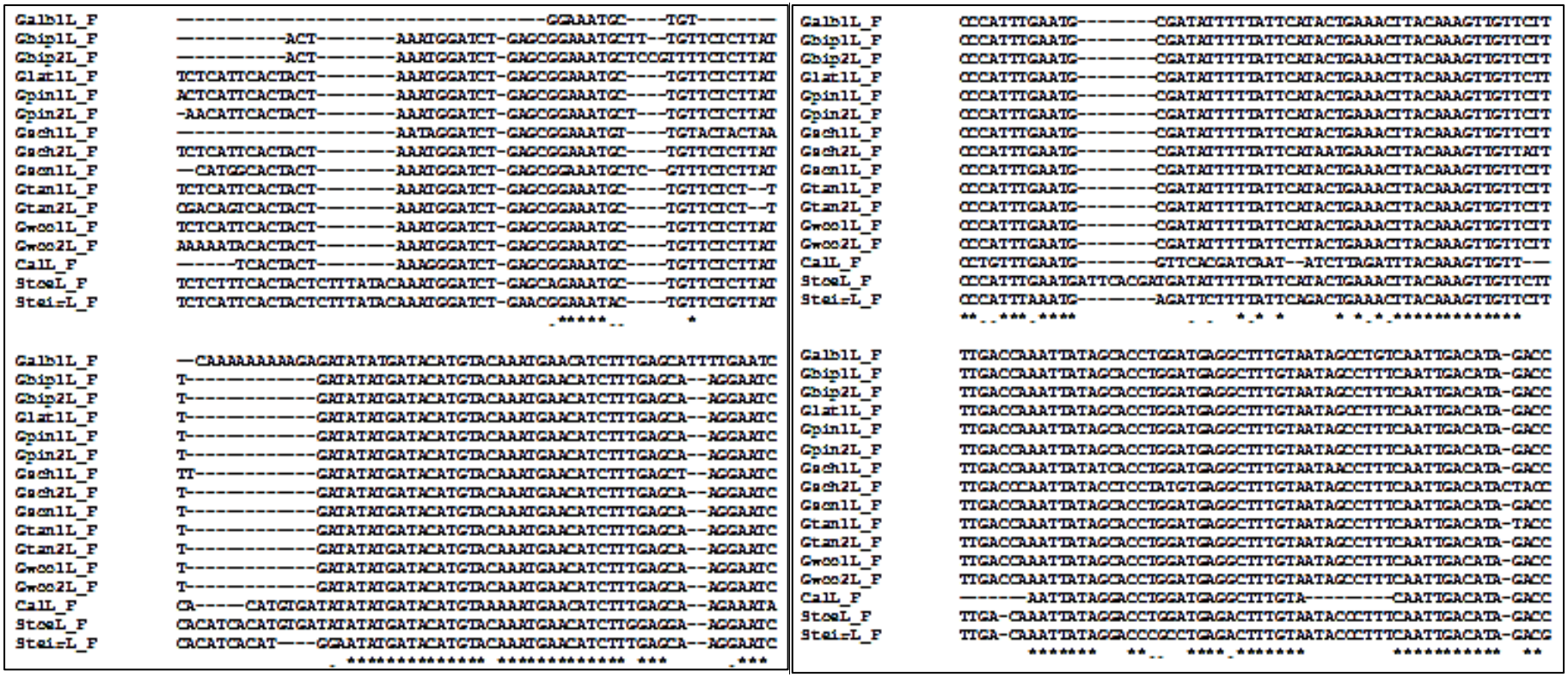
Alignment of the sequences for the gene region ITS, as obtained from online version of MAFFT.




Galbita1
Gbipita1
Gbipita2
Glatita1
Glatita2
Gpinita1
Gpinita2
Gsehiita1
Gsehiita2
Gsenita1
Gsenita2
Gtsnita1
Gtsnita2
Gweeita1
Gweeita2
Calita
Stoebita
Stei-ita

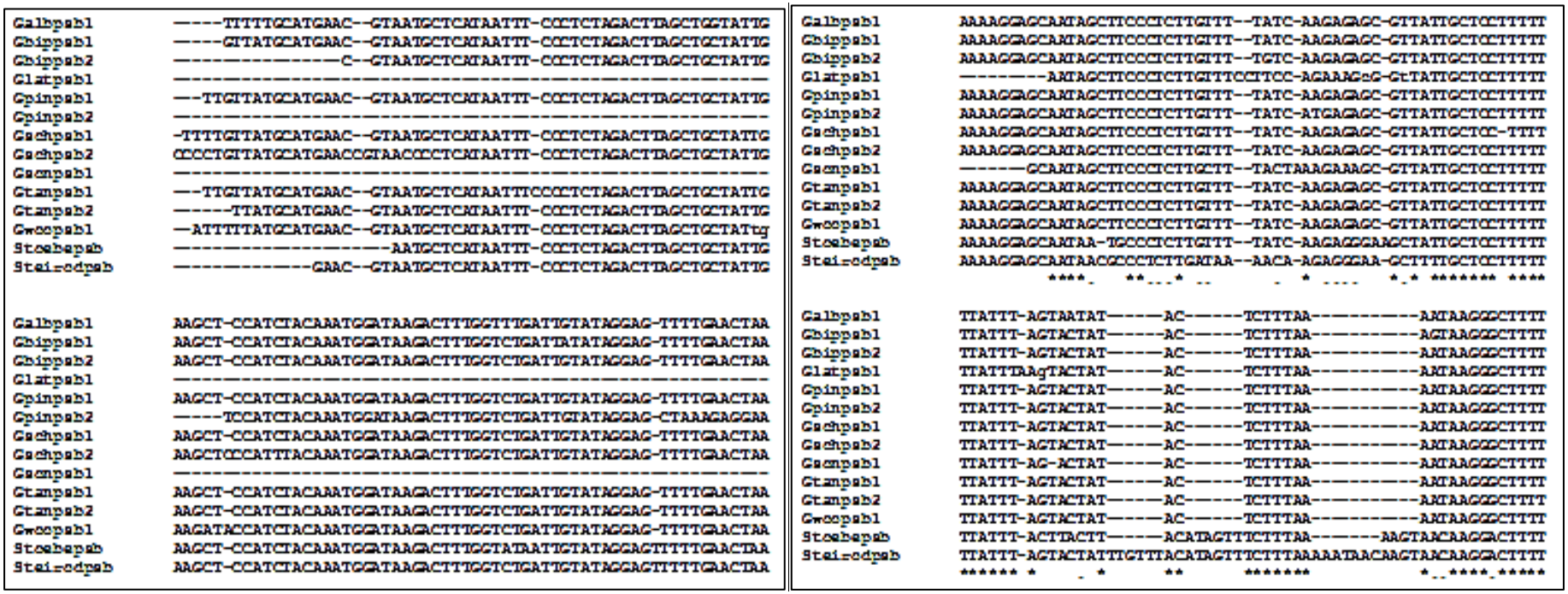


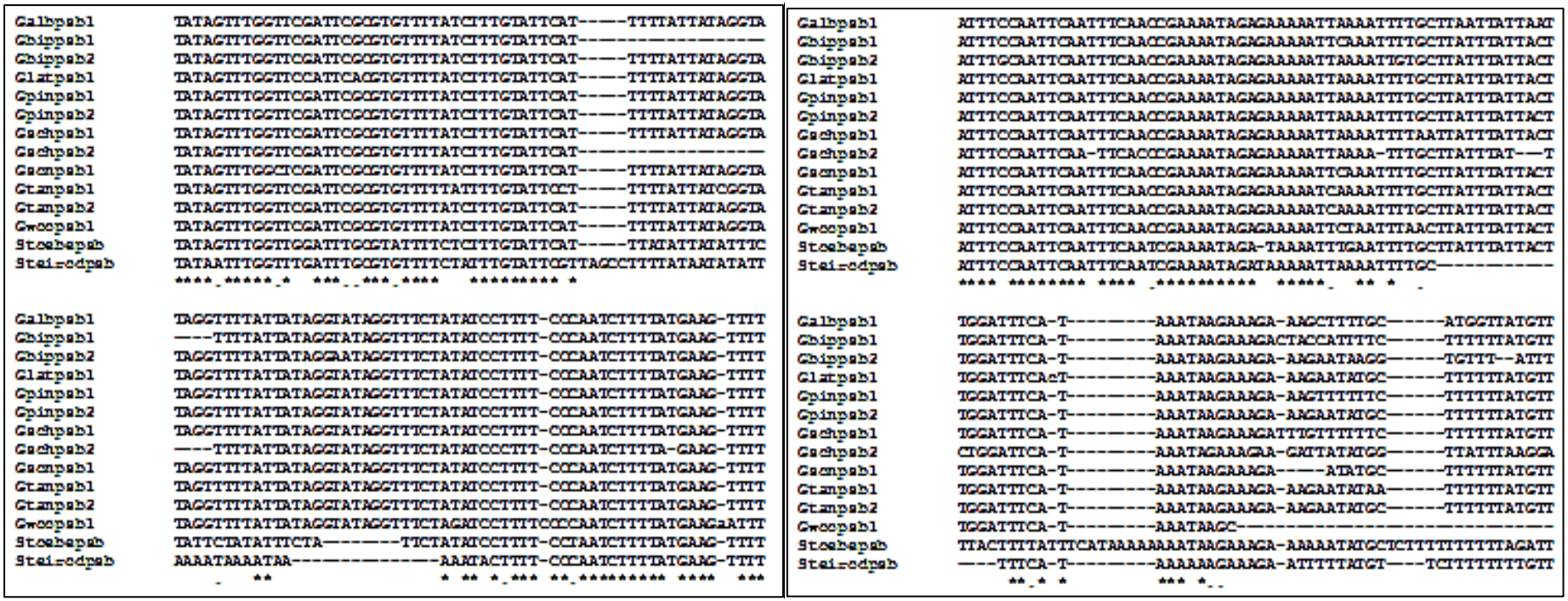
Alignment of the gene region trnL-F, as obtained from the online version of MAFFT.


Galb1L_F
Gbip1L_F
Gbip2L_F
Glat1L_F
Gpin1L_F
Gpin2L_F
Gsch1L_F
Gsch2L_F
Gscn1L_F
Gtan1L_F
Gtan2L_F
Gwoo1L_F
Gwoo2L_F
CalL_F
StoaL_F
Stei=L_F


GalblL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GbiplL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
Gbip2L_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GlatlL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GpinlL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
Gpin2L_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GschlL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCTGGATATCTCAC
Gsch2L_F CCCTTTCTCTAGTAAAAGAAAA-TGAGG----------
GsenlL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GtanlL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
Gtan2L_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
GwoolL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
Gwoo2L_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATCGTCGGGATAGCTCAG
CalL_F CACGTTCTTTAGTAAAATGAAAATGAGGATGAGACATCAGGAATAGTCGGGATAGCTCA-
StooL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCAGGAATAGTCGGGAT-----
SteiaL_F CACGTTCTCTAGTAAAATGAAAATGAGGATGAGACATCGGGAATAGTTGGGATAGCTCAG

Alignment of the gene region *psb*A-trnH, as obtained from the online version of MAFFT.




Galbpsb1 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gbippsb1 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT----------
Gbippsb2 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Glatpsb1 TATAGTTTGGTTCCATTCACGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gpinpsb1 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gpinpsb2 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gschpsb1 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gschpsb2 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT----------
Gscnpsb1 TATAGTTTGGCTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gtampsb1 TATAGTTTGGTTCGATTCGCGTGTTTTTATTTTGTATTCCT-----TTTTATTATCGGTA
Gtampsb2 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Gwccpsb1 TATAGTTTGGTTCGATTCGCGTGTTTTATCTTTGTATTCAT-----TTTTATTATAGGTA
Stoehapsb TATAGTTTGGTTGGATTTGCGTATTTTCTCTTTGTATTCAT-----TTATATTATATTTC
Stei-odpsb TATAATTTGGTTTGATTTGCGTGTTTTCTATTTGTATTCGTTAGCCTTTTATAATATATT
**** ***** * *** ** *** **** ********* *

Galbpsb1 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gbippsb1 ---TTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gbippsb2 TAGGTTTTATTATAGGAATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Glatpsb1 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gpinpsb1 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gpinpsb2 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gschpsb1 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gschpsb2 ---TTTTATTATAGGTATAGGTTTCTATATCCCTTT-CCCAATCTTTTA-GAAG-TTTT
Gscnpsb1 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gtampsb1 TAGTTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gtampsb2 TAGGTTTTATTATAGGTATAGGTTTCTATATCCTTTT-CCCAATCTTTTATGAAG-TTTT
Gwccpsb1 TAGGTTTTATTATAGGTATAGGTTTCTAGATCCTTTTCCCCAATCTTTTATGAAGaATTT
Stoehapsb TATTCTATATTTCTA-------TTCTATATCCTTTT-CCTAATCTTTTATGAAG-TTTT
Stei-odpsb AAAATAAAATAA-------------AAATACTTTT-CCCAATCTTTTATGAAG-TTTT
- ** * ** *_*** **_********* **** ***

Galbpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTAAAATTTTGCTTAATTATTAAT
Gbippsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTCAAATTTTGCTTATTTATTACT
Gbippsb2 ATTTGCAATTCAATTTCAACCGAAAATAGAGAAAAATTAAAATTGTGCTTATTTATTACT
Glatpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTAAAATTTTGCTTATTTATTACT
Gpinpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTAAAATTTTGCTTATTTATTACT
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Gschpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTAAAATTTTAATTATTTATTACT
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Gscnpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTCAAATTTTGCTTATTTATTACT
Gtampsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATCAAAATTTTGCTTATTTATTACT
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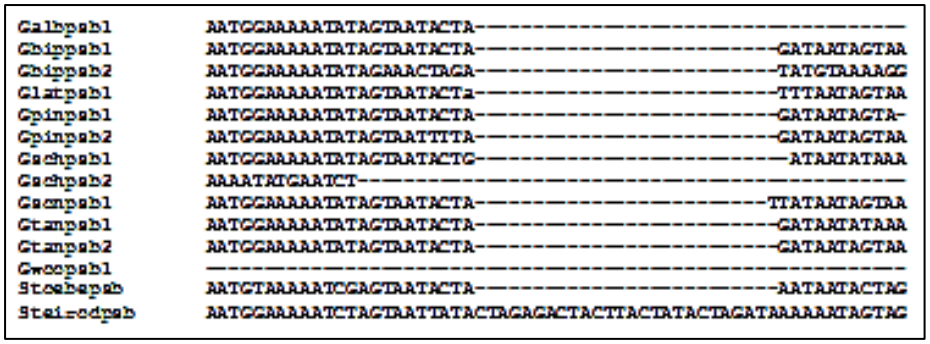
Gwccpsb1 ATTTCCAATTCAATTTCAACCGAAAATAGAGAAAAATTCTAATTTAACTTATTTATTACT
Stoehapsb ATTTCCAATTCAATTTCAATCGAAAATAGA-TAAAATTTGAATTTTGCTTATTTATTACT
Stei-odpsb ATTTCCAATTCAATTTCAATCGAAAATAGATAAAAATTAAAATTTTGC----------
**** ******** **** _********** _***** _ ** * _

Galbpsb1 TGGATTTCA-T---------AAATAAGAAAGA-AAGCTTTTGC------ATGGTTATGTT
Gbippsb1 TGGATTTCA-T---------AAATAAGAAAGACTACCATTTTC------TTTTTTATGTT
Gbippsb2 TGGATTTCA-T---------AAATAAGAAAGA-AAGAATAAGG-----TGTTT--ATTT
Glatpsb1 TGGATTTCAcT---------AAATAAGAAAGA-AAGAATATGC------TTTTTTATGTT
Gpinpsb1 TGGATTTCA-T---------AAATAAGAAAGA-AAGTTTTTTC------TTTTTTATGTT
Gpinpsb2 TGGATTTCA-T---------AAATAAGAAAGA-AAGAATATGC------TTTTTTATGTT
Gschpsb1 TGGATTTCA-T---------AAATAAGAAAGATTTGTTTTTTC------TTTTTTATGTT
Gschpsb2 CTGGATTCA-T---------AAATAGAAAGAA-GATTATATGG------TTATTTAAGGA
Gscnpsb1 TGGATTTCA-T---------AAATAAGAAAGA-----ATATGC------TTTTTTATGTT
Gtampsb1 TGGATTTCA-T---------AAATAAGAAAGA-AAGAATATAA------TTTTTTATGTT
Gtampsb2 TGGATTTCA-T---------AAATAAGAAAGA-AAGAATATGC------TTTTTTATGTT
Gwccpsb1 TGGATTTCA-T---------AAATAAGC----------
Stoehapsb TTACTTTTATTTCATAAAAAAAAATAAGAAAGA-AAAAATATGCTCTTTTTTTTTTTAGATT
Stei-odpsb ---TTTCA-T---------AAAAAAGAAAGA-ATTTTTATGT----TCTTTTTTTTGTT
_* * * *_-


GalbpsbI AATGGAAAAATATAGTAATACTA----------
GbippsbI AATGGAAAAATATAGTAATACTA----------GATANTAGTAA
GbippsbI AATGGAAAAATATAGAAACTAGA----------TATGTAAAAGG
GlatpsbI AATGGAAAAATATAGTAATACTa----------TTTANTAGTAA
GpinpsbI AATGGAAAAATATAGTAATACTA----------GATANTAGTA-
GpinpsbI AATGGAAAAATATAGTAATTTTA----------GATANTAGTAA
GschpsbI AATGGAAAAATATAGTAATACTG----------ATANTATAAA
GschpsbI AAAATNTGAATCT----------
GsmpsbI AATGGAAAAATATAGTAATACTA----------TTATANTAGTAA
GtmpsbI AATGGAAAAATATAGTAATACTA----------GATANTATAAA
GtmpsbI AATGGAAAAATATAGTAATACTA----------GATANTAGTAA
GwoopsbI ----------
StoshpsbA AATGTAAAAATCGAGTAATACTA----------AATANTACTAG
StsiscdpsbA AATGGAAAAATCTAGTAATTATACTAGAGACTACTTACTATACTAGATAAAAANTAGTAG

Alignment of the gene region *psb*A-trnH, as obtained from the online version of MAFFT.


galb1T_L
gbipT_L
glatT_L
gpinT_L
gschT_L
gsenT_L
gtanT-L
gweoT_L
stei=_L

Addendum III:
Specimens used for DNA extraction.

Specimens used for DNA extraction					
Barcode	**Herbarium**	***Garuleum* spesies**	**Collector**	**Collector nr.**	**Date collected**
45724	BOL	*Garuleum album*	H. Bolus	BH 10160	00/00/1896
	GRA	*Garuleum album*	PB. Phillipson	4326	22/06/1995
	BLFU	*Garuleum bipinnatum*	J. van Zyl	9	26/11/2011
	BLFU	*Garuleum bipinnatum*	J. van Zyl	10	26/11/2011
	BLFU	*Garuleum bipinnatum*	J. van Zyl	15	26/11/2011
	BLFU	*Garuleum bipinnatum*	J .van Zyl	16	26/11/2011
G00302972	G	*Garuleum bipinnatum*	RDA. Bayliss	8692	11/05/1965
	GRA	*Garuleum bipinnatum*	D. Shearing	K207	08/06/1987
G00302979	G	*Garuleum latifolium*	F. Wylie	s.n.	11/09/1917
G00302999	G	*Garuleum latifolium*	H. Rudatis	1864	22/01/1913
45739	BOL	*Garuleum latifolium*	JM. Wood	160	00/02/1884
	BLFU	*Garuleum pinnatifidum*	J. van Zyl	1	03/03/2011
	BLFU	*Garuleum pinnatifidum*	J. van Zyl	2	10/03/2011
	BLFU	*Garuleum pinnatifidum*	J .van Zyl	3	10/03/2011
5346	BLFU	*Garuleum pinnatifidum*	CA. Beck	3222	00/00/1924
G00302987	G	*Garuleum schinzii*	R. Seydel	3643	28/09/1963
	GRA	*Garuleum schinzii*	C. Mannheimer	2882	15/02/2004
45758	BOL	*Garuleum schinzii*	HHW .Pearson	7935	00/00/1912
	Z	*Garuleum schinzii*	K. Dinter	4810	02/08/1925
Z-000078516	Z	*Garuleum sonchifolium*	FA. Rodgers	1284	00/06/1914
Z-000061144	Z	*Garuleum sonchifolium*	R. Schlechter	6206	–
Z-00061145	Z	*Garuleum sonchifolium*	W. Tyson	2080	00/02/1885
45764	BOL	*Garuleum sonchifolium*	W. Marais	526	22/09/1954
45762	BOL	*Garuleum sonchifolium*	E. Esterhysen	27845	00/07/1958
ZT-00035532	Z	*Garuleum tanacetifolium*	P. MacOwan	748	00/12/1881
45771	BOL	*Garuleum tanacetifolium*	P. MacOwan	1382	00/00/1839
	BLFU	*Garuleum tanacetifolium*	J. van Zyl	18	30/11/2011
	BLFU	*Garuleum tanacetifolium*	J. van Zyl	22	30/11/2011
45776	BOL	*Garuleum woodii*	JM. Wood	4840	06/12/1892
	BLFU	*Garuleum woodii*	RD. Stam	420	10/03/1970
	BLFU	*Garuleum woodii*	Ashafa	s.n.	00/00/2011