CHAPTER 3: MATERIALS AND METHODS

3.1 Collecting of plant material

Several field trips were conducted to representative areas in southern Africa to collect plant material and to study the plants in their natural habitat. A representative area is usually the centre of diversity of the genus, and hence, Namibia was chosen as the major sampling area (Fig. 3.1). Limpopo and KwaZulu-Natal provinces in South Africa were also targeted for having the southern distribution limits of certain species. Voucher specimens of the collected material were deposited in the A.P. Goossens Herbarium (PUC), Potchefstroom, South Africa. Duplicates of specimens collected in South Africa were deposited in the National Herbarium, Pretoria (PRE), South Africa and duplicates collected in Namibia in the National Herbarium (WIND), Windhoek, Namibia. Photographs of plant habitat, habit, flowers and fruits were taken with a Canon EOS 350D and a 100 m Sigma macrolens.

Fresh plant material was collected in 70% ethanol as well as in 4% paraformaldehyde (pH 7.2) for use in morphological, anatomical and palynological studies. Young leaves were placed in 50 ml centrifuge tubes with 10 g of silica gel for later DNA extraction.

3.2 Morphology

3.2.1 Leaves

Micrographs of the leaves were taken with a Canon 450D fitted with a 100 mm Sigma macro lens. Ten specimens per species were subjected to measurements (length and breadth of the leaves as well as the length of the petioles). Three leaves and petioles were measured per specimen. Terminology for the shape of the leaf follows Hickey & King (2000).

3.2.2 Flowers

Micrographs of the flowers were taken at 30x, 40x and 50x magnification with a Nikon Digital Camera DXM 1200 F fitted on a Nikon SMZ 1500 stereomicroscope. Flowers collected in 4% paraformaldehyde as well as flowers from herbarium specimens were dissected. Flowers from herbarium specimens were rehydrated for 10 min in boiling water. Three flowers per species (one each from Namibia, Botswana and South Africa, where available) were dissected and the length of the following parts measured: peduncle, pedicle, flower, upper and lower portions of the flower, stamens and ovary. The type of inflorescence and the terminology for the shape of the upper and the lower part of the flower follows Hickey & King (2000).

3.2.3 Anthocarp

Micrographs of the anthocarps were taken at 10x, 20x and 30x magnification with a Nikon Digital Camera DXM 1200 F fitted on a Nikon SMZ 1500 stereomicroscope. The length and width of three anthocarps each of ten herbarium specimens per species were measured. Terminology follows Hickey & King (2000).

3.3 Light microscopy

Stem, leaf and anthocarp material was fixed in 4% aqueous paraformaldehyde. Fixed material was then washed three times in 0.05 M cacodylate buffer for 15 min per treatment followed by three rinses with distilled water for 15 min each. The material was dehydrated in an ethanol series of 50%, 70%, 90% and twice in 100% ethanol for 15 min each followed by 15 min in 100% resin (L.R. White™ Wirsam/London Resin Company). This was followed by two changes in resin for 60 min each and then left over night at 20 ºC before being imbedded and polymerised overnight at 65 °C. Embedded material was cut with a Reichert-Jung Ultracut E microtome into sections of 1.4 µ. Sections were also stained for pectinaceous substances in 0.02% ruthenium red in water for 10 min, and for lipids in Sudan black B in 70% ethanol for 30 min. Micrographs were taken with a Nikon Digital Sight camera fitted on a Nikon Eclipse 80i microscope at 40x, 60x and 100x magnification and a Nikon Digital Camera DXM 1200 F fitted on a Nikon Eclipse E 800 at 40x, 60x and 100x magnification.

Sections of anthocarps, leaves and stems were made from mature plant material. Sections of stems were made from the centre of an internode towards the nodes. Anthocarps and leaves were sectioned from the centre towards the apexes.

3.4 Scanning Electron Microscope (SEM)

Leaf and anthocarp material stored in 70% ethanol was dehydrated successively in 90% ethanol and twice in 100% ethanol for 10 min per treatment before critical point drying. The material was then mounted on specimen stubs and sputter-coated with gold/palladium. Leaf and anthocarp material collected from herbarium specimens were directly mounted on specimen stubs and sputter-coated with gold/palladium. In all cases specimens were examined and micrographs were taken with a FEI Quanta 200 environmental scanning electron microscope (ESEM).

3.4.1 Critical point drying

The scanning electron microscope operates in high vacuum, requiring the study material to be dry. Critical point drying is an established method for dehydrating biological material before examination with the SEM. During critical point drying the 100% ethanol within the samples is replaced with liquid CO_2. The CO_2 is then released by heating the chamber of the critical point dryer to the critical point for CO_2 (31º C), which turns the CO_2 into vapor with no surface tension.

3.4.2 X-ray micro-analysis

The elemental composition of the crystals occurring in the leaves and anthocarps was determined on a FEI Quanta 200 ESEM with an integrated Oxford Inca X-sight EDS system operating at 15 kV.

3.5 Palynology

3.5.1 Scanning Electron Microscopy

Pollen was acetolized according to the method of Erdtman (1969) and Coetzee (1975) with slight modifications. Pollen from herbarium material and material collected in 70% ethanol was centrifuged at 5 000 rpm for 10 min in glacial acetic acid. The glacial acetic acid was replaced with an acetolysis mixture (acetic anhydride and sulphuric acid, 9:1) and heated to 96 °C in a water bath for 20 min, after which it was centrifuged for 10 min at 5 000 rpm. This was followed by two washes in distilled water and centrifugation for 10 min at 5 000 rpm. The pollen was then washed in an ethanol series of 50%, 70% and 96% and centrifuged at 5 000 rpm for 10 min each. A drop of 96% ethanol/pollen mixture was placed on specimen stubs and sputter-coated with gold/palladium. Specimens were examined and micrographs taken with a FEI Quanta 200 ESEM or a JEOL JSM 840 SEM using Orion version 6.60.4 to take the micrographs. A minimum of eight pollen grains per species were measured to obtain the diameter of the grain, the diameter of the pores and the length of the spinules.

3.5.2 Transmission Electron Microscope

Anthers fixed in 4% aqueous paraformaldehyde were washed three times in 0.05 M cacodylate buffer for 15 min each followed by three rinses with distilled water for 15 min each. The material was then immersed in 2% uranyl acetate (pH 2) for 30 min followed by three rinses with distilled water for 15 min each. The material was dehydrated in an ethanol series of 50%, 70%, 90% and twice in 100% ethanol for 15 min each followed by 15 min in 100% resin (L.R. White™ Wirsam/London Resin Company). This was followed by two changes in resin for 60 min each and then left over night at 20 °C before being imbedded and polymerised overnight at 65 °C. Embedded material was cut with a Reichert-Jung Ultracut E microtome into sections of 180 nm which were then contrasted with 2% uranyl acetate (pH 2) for 4 min and lead citrate for 1 min Sections were examined and micrographs taken with a Philips CM10 transmission electron microscope.

3.5.3 Light microscopy

Pollen grains were prepared as in section 3.5.2 and embedded material was cut with a Reichert-Jung Ultracut E microtome into sections of 1.4 µm and stained with aqueous 0.5% toluidine blue in 1% boraxs and 0.1% aqueous neufuchsin for 15 sec. Micrographs were taken with a Nikon Digital Camera DXM 1200 F fitted on a Nikon Eclipse E 800 at 40x, 60x magnification.

Terminology used for the palynological descriptions follows Punt *et al*. (2007).

3.6 Molecular work

3.6.1 DNA extraction

Leaf tissue (1 mm^2) was homogenised with the TissueLyzer (Qiagen) to a fine powder. DNA was extracted using a CTAB (Cetyl trimethyl ammonium bromide) method of Saghai-Maroof *et al.* (1984) with modifications. The powdered leaf tissue was incubated with 500 µl of Buffer PL1 (CTAB-based; NucleoSpin Plant II Kit, Separations) with 2 µl of proteinase K (10 mg/ml; Sigma-Aldrich) at 60 °C overnight. Thereafter the suspension was extracted with chloroform:iso-amylalcohol (24:1). The phases were separated by centrifuging at 20 000 rcf (4 °C) for 10 min and transferred to a new tube. This was repeated twice. DNA was precipitated with ⅔ volume of ice cold isopropanol and incubated overnight at –20 °C. DNA was pelleted at 16 100 rcf (4 °C) for 20 min. The pellets were washed with 200 µl of 70% ethanol twice and dried at 55 °C for 15 min. The DNA was re-suspended in miliQH_2O.

3.6.2 Polymerase Chain Reaction (PCR) Amplification and sequencing

PCR amplification and sequencing were done using six primers (ITS4F, ITS5a, Nyct_ndhF1F, Nyct_ndhF13R, Nyct_ndhF972F and Nyct_ndhF22R). Twenty nanogrammes of DNA template were added in a reaction volume of 20 µl containing 1x KAPA ReadyMix (Kapa Biotech/Lasec) and 4 pmol of each primer (ITS4F and ITS5a). PCR was performed using a Verity (Applied Biosystems) with the following

cycling conditions: 95 °C for 5 min followed by 30 cycles at 95 °C for 30 sec, 50 °C for 60 sec and 72 °C for 60 sec, and a final extension at 72 °C for 10 min.

Forty nanogrammes of DNA template were added to a reaction volume of 20 µl containing 1x KAPA HiFi ReadyMix (Kapa Biotech/Lasec) and 4 pmol of each primers (Nyct_ndhF1F, Nyct_ndhF13R, Nyct_ndhF972F and Nyct_ndhF22R). PCR was performed using a Verity (Applied Biosystems) with the following cycling conditions: 95 °C for 5 min followed by 35 cycles at 98 °C for 30 sec, 55 °C for 45 sec and 68 °C for 50 sec, and a final extension at 68 °C for 2 min. The cycling conditions for primers Nyct_ndhF1F and Nyct_ndhF22R were as follows: 95 °C for 2 min followed by 35 cycles at 98 °C for 20 sec, 55 °C for 15 sec and 68 °C for 66 sec, and a final extension at 68 °C for 2 min.

Post-PCR purification was done using the NucleoFast Purification System (Separations). Sequencing was performed with BigDye Terminator V1.3 (Applied Biosystems) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystems). Sequences were analysed and trimmed using Sequencing Analysis V5.3.1 (Applied Biosystems).

3.6.3 Sequence alignment

Sequence verification and alignment were done with CLC DNA Workbence 6 (CLC bio, Aarhus, Denmark), using the following settings during alignment: gap open cost (10), gap extension cost (1) and end gap cost (as any other). Base calling was done by manual verification and ambiguous bases corrected by visual inspection.

3.6.4 Phylogenetic analyses

Both ITS and *ndh*F matrices were analysed separately and in combination using MEGA version 5 (Tamura *et al*., 2011). Missing data and gaps were eliminated. A distance method, Neighbor-Joining (Saitou & Nei, 1987), as well as a model based approach, Maximum Likelihood (Felsenstein, 1981) were used. The congruency index (I_{cong}) and the P-value for the two matrices were calculated (De Vienne *et al*., 2007) before the matrices were combined. Neighbor-Joining was performed using the Jukes-

Cantor model (Jukes & Cantor, 1969). The substitution models for use with the Maximum Likelihood were calculated with the Bayesian Information Criterion available within Mega 5 and evaluated using the Akaike Information Criterion. The Tamura 3-Parameter + G Model (Tamura, 1992) were used for the ITS dataset, the General Time Reversal Model + G (Tavaré, 1986) for the *ndh*F dataset and the Hasegawa-Kishino-Yano + G Model (Hasegawa *et al*., 1985) for the combined matrices. The heuristic search model used for the Maximum Likelihood was the Close-Neighbor-Interchange (Nei & Kumar, 2000). Bootstrap analysis (1000 replicates) (Felsenstein, 1985) was performed to determine internal support. A bootstrap percentage of 80 – 100% is considered a high bootstrap support, a bootstrap support of 50 – 80% as moderate and a bootstrap support of less than 50% as weak. The bootstrap consensus trees of the neighbor-joining and maximum likelihoods are reported.

3.6.5 Outgroup and type specimen taxa

Five species (*Pisoniella arboresens* (Lag. & Rodr.) Standl., *Acleisanthes lanceolata* (Wooton) R.A.Levin, *Acleisanthes longiflora* A.Gray, *Mirabilis jalapa* L. and *Mirabilis multiflora* (Torr.) A.Gray) were used as outgroups and their ITS and *ndhF* sequences were obtained from National Center for Biotechnology Information (NCBI) in Bethesda, Maryland, United States of America.

The type specimen of *Commicarpus* (*Commicarpus scandens* (L.) Standl.) was included to verify that the sequences were of *Commicarpus* origin. These ITS and *ndhF* sequences were also obtained from NCBI. The type specimen of *Boerhavia* (*Boerhavia erecta* L.) was not included as there are only three, unpublished sequences (and therefore unvalidated) of a *B*. *erecta* available on NCBI.

3.7 Distribution maps, habitat information and uses

Distribution patterns and habitat information for the *Boerhavia* and *Commicarpus* species were obtained from herbarium specimens from the following herbaria (acronyms according to Holmgren *et al*., 1990): BLFU, BOL, GRA, J, KMG, KSAN, NH, NMB, NU, PRE, PRU, PUC, UCBG, UNIN, WIND and ZULU, as well as

collections and observations made during field trips. Plant localities were mapped using ArcView 9.2 (ESRI, 2006). Information on the uses of the species was gathered from label information on herbarium specimens and a broad literature survey of various articles and books.

3.8 Red list assessment

Red list assessments of the indigenous South African species were done by Raimondo *et al*. (2009). However, the Namibian endemics, namely *Boerhavia deserticola*, *Commicarpus decipiens, Commicarpus fallacissimus* (Heimerl) Heimerl ex. Obermeyer, Schweickerdt & Verdoorn and *Commicarpus squarrosus* (Heimerl) Standl. had to be assessed according to the International Union for Conservation of Nature (IUCN) (2001) criteria.

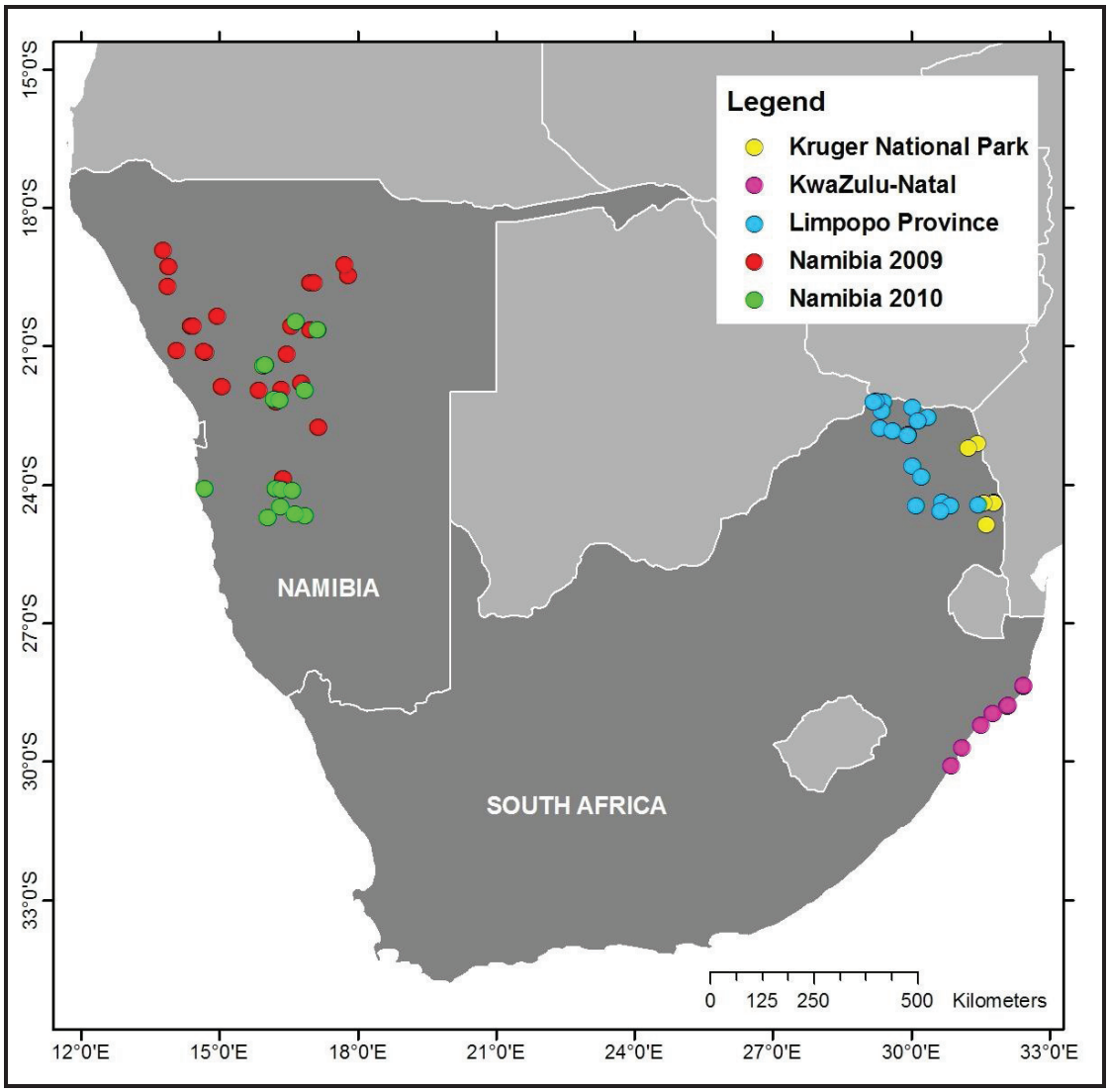

Figure 3.1: Localities where specimens were collected during five field trips to representative areas containing southern distribution limits and endemic species of *Boerhavia* and/or *Commicarpus*.